UNIVERSITÉ DE STRASBOURG

ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE

Laboratoire d'innovation thérapeutique, UMR 7200, Faculté de Pharmacie

THÈSE présentée par :

Léa SAAB

soutenue le : **04 Juillet 2013**

pour obtenir le grade de : **Docteur de l'université de Strasbourg**

Discipline/ Spécialité : Pharmacologie-Toxicologie

Development of a cellular predictive model of inflammation associated idiosyncratic drug-induced hepatotoxicity and investigation of its underlying cellular and molecular mechanisms

Hépatotoxicité idiosyncrasique liée à un stress inflammatoire: modèle de prédiction et mécanismes cellulaires et moléculaires

THÈSE dirigée par :

Pr. Geneviève UBEAUD-SEQUIER	PH-PU, université de Strasbourg

RAPPORTEURS :

Pr. George HOUIN	Professeur, université de Toulouse
Pr. Jean-Yves JOUZEAU	Professeur, université de Lorraine

AUTRES MEMBRES DU JURY :

Pr. Jean Marc JELTSCH	Professeur, université de Strasbourg
Pr. Jean-Pierre GIES	Professeur, université de Strasbourg
Dr. Christiane GUILLOUZO	Docteur, université de Rennes 1

I have always dreamt of becoming a successful university professor! Back then it was just a dream but now the dream has started to become reality. This thesis constitutes the cornerstone of my career as a professor. As much as I am proud of this achievement I am grateful for all those who contributed in realizing it.

I would like to thank my director Genevieve Ubeaud Sequier for welcoming me in her laboratory and granting me the opportunity to accomplish my ultimate dream of becoming a university professor. I thank her for her help, guidance and advices but most importantly I thank her for her lovely spirit, for being extremely kind, caring and understanding all through my doctoral years.

I am extremely grateful to my supervisor Dr. Jean Peluso without whom accomplishing this thesis would not have been possible. Thank you Dr. Peluso for teaching me all what I know today from the basics of cellular biology to advanced in vitro research techniques. A bigger thank you for being there for me at all moments on the professional and personal level, for embracing me and treating me as a member of your family, for making me feel at home when miles away from my family.

I would like to thank every single member of my thesis committee: Pr. Georges Houin, Pr. Jean-Yves Jouzeau, Pr. Jean-Marc Jeltsch, Pr. Jean-Pierre Gies and Dr. Christiane Guillouzo for their time, efforts and helpful comments. Thank you for your valuable contribution to the achievement of this thesis despite the endless duties and loaded schedules of every one of you.
Particularly, I would like to express my most sincere gratitude to Dr. Christiane Guillouzo for being my idol in scientific research. I thank you Dr. Guillouzo for your generous help and advices, for never hesitating to share with me your valuable knowledge and priceless expertise. You influenced me in so many lovely ways that will shape my scientific career forever.

I would also like to thank Pr. Jean-Pierre Gies for his helpful advices, for his interesting proposition to integrate molecular biology in my thesis work and most importantly for welcoming me in his laboratory and granting me the opportunity to accomplish all the experiments related to molecular biology.

I would like to thank Dr. Christian Muller for his continuous help, support, advices and most importantly for his sense of humor and for the agreeable atmosphere he generates in the laboratory.

I would like to thank all the BTS students and 5th year pharmacy trainees who worked with me during my doctoral years. Particularly, thank you Natacha Sawkhee for being there whenever I needed you and most importantly for helping me in cell culture when I was abroad.

I would like to thank my friends Israa Dandache and Romain Hany. Thank you Israa for encouraging me to come to France and to pursue my PhD at the University of Strasbourg. A bigger thank you for being an amazing friend on all aspects and for your generous help all along my doctoral years. Thank you Romain for your generous help in western blot at the most critical final stages of my thesis.

I would like to thank Dr. Christophe Chesne and every member of his company, Biopredic, for their generous advices, help and collaboration. Thank you for granting me the opportunity to visit your company and perform several experiments with your employees. It was a very fruitful experience on the professional and personal level.

Last but never the least I would like to thank my family and fiancé for their tenderness, support and endless love. I dedicate this thesis to my mom who devoted her life to make me what I am today. I owe you mom all my achievements and success.

$O_2^{-\bullet}$ superoxide anion
5-HT-2A 5-hydroxytryptamine receptor 2A
5-NT 5-nucleotidase

A

ABC ATP binding cassette
ADME absorption, distribution, metabolism and excretion
ADRs adverse drug reactions
AhR aryl hydrocarbon receptor
AIH auto-immune hepatitis
ALF acute liver failure
ALL acute lymphoblastic leukemia
ALP alkaline phosphatase
ALT alanine aminotransferase
AML acute myeloid leukemia
ANOVA analysis of variance
AP alkaline phosphatase
AP-1 activator protein 1
Apaf1 apoptotic protease activating factor 1
APAP acetaminophen
APC antigen presenting cell
APS ammonium persulfate
A-SMase acidic sphingomyelinase
AST aspartate aminotransferase
ATP adenosine triphosphate
AU arbitary units

B

BA bile acids
Bad Bcl-2 associated death promoter
Bak Bcl-2 homologous antagonist/killer
Bax Bcl-2 associated X protein
Bcl-2 B-cell lymphoma 2
Bcl-xL B-cell lymphoma-extra large
BCRP breast cancer resistance protein
BEE basolateral early endosome
Bid BH3 interacting-domain death agonist
BODIPY 493/503 SE, 4,4-Difluoro-1,3,5,7-Tetramethyl-4-Bora-3a,4a-Diaza-*s*Indacene-8-Propionic Acid, Succinimidyl Ester
BQ-123 selective endothelin receptor antagonist
BSA bovine serum albumin
BSEP bile salt export pump
BSP bromosulfophthalein

C

Ca^{2+} calcium
cAMP cyclic adenosine monophosphate
CAR constitutive androstane receptor
CCK cholecystokinin
CCL21 Chemokine (C-C motif) ligand 21
CDF 5,6-carboxy-2',7'dichlorofluorescein
CDFDA 5,6-carboxy-2',7'dichlorofluorescein diacetate
cGMP cyclic guanosine monophosphate
CML chronic myeloid leukemia
CS-PG chondroitin sulphate proteoglycans
CTGF connective tissue growth factor
CYP450,**P450** cytochrome P450enzymes
Cytochrome c cytochrome complex

D

DBD DNA-binding domain
DHE dihydroethidium
DILI drug-induced liver injury
DISC death-inducing signaling complex
DMEM dulbecco's modified eagle medium
DMSO dimethyl sulfoxide
DNA deoxyribonucleic acid

E

ECM extracellular matrix
EGFR epidermal growth factor receptor
ELISA enzyme linked immunosorbent assay
ESC embryonic stem cells

F

FasL Fas ligand
FDA Food and Drug Administration
FITC Fluorescein isothiocyanate
FXR farnesoid X receptor

G

GA golgi apparatus
GISTs Gastro-intestinal stromal tumors
GIT gastrointestinal tract
GSH reduced glutathione
GST glutathione-S-transferase

H

H202 hydrogen peroxide
HBSS hanks' balanced salts solution
HC hepatic cells
HIV human immunodeficiency virus
HL Hodgkin's lymphoma
HMG-CoA
5-hydroxy-3-methylglutaryl- coenzyme A
HNF1*α* hepatic nuclear factor 1 alpha
HPC hepatic progenitor cells
HRP horseradish peroxidase
HSC hepatic stellate cells
HSC hepatic stem cells
HS-PG heparan sulfate proteoglycans

I

ICAM-1 intracellular adhesion molecule 1
ICP intrahepatic cholestasis of pregnancy
IDILI idiosyncratic drug-induced liver injury
IF immunofluorescence
IL interleukin
iPSC induced pluripotent stem cells
ITAC IFN-gamma-inducible Tcell alpha chemoattractant

J

JAK Janus kinase
JNK c-jun N-terminal kinase

K

KC Kupffer cells
KC neutrophil chemokines

L

LBD ligand-binding domain
LDH lactate dehydrogenase
LPS Lipopolysaccharide
LSEC liver sinusoidal endothelial cells
LXR liver X receptor

M

Mac-1 macrophage 1 antigen
MAPC multipotent adult progenitor cells
MAPK mitogen-activated protein kinases
MATE multidrug and toxin extrusion transporter
MCP-1 monocyte chemotactic peptide
M-CSF macrophage colony-stimulating factor
MDR multidrug resistance protein
MDS Myelodysplastic Syndrome
MEM minimum essential medium
MF-HSC myofibroblastic hepatic stellate cells
MHC II major histocompatibility complex
MIG-1 monokine-induced by gamma interferone
MIP macrophage inflammatory proteins
MPT mitochondrial permeability transition
mRNA messenger ribonucleic acid
MRP multidrug resistance-associated protein
MSC mesenchymal stem cells
MTX methotrexate

N

Na+ sodium
NAFLD nonalcoholic fatty liver disease
NF nitrofurantoin
NF-IL6 nuclear factor-IL6
NF-Kβ nuclear factor kappa-light-chain-enhancer of activated B cells
NHL non-hodgkin's lymphoma
NO nitric oxide
NO_2 nitrogen dioxide
NPC non-parenchymal cells
Nrf2 nuclear factor-erythoroid 2 p45-related factor 2
NSAID nonsteroidal anti-inflammatory drug
N-SMase neutral sphingomyelinase
NTCP Na+-taurocholate cotransporting polypeptide

O

O2 oxygen gas
OA organic anions
OAT organic anion transporter
OATP organic anion transporting polypeptide
OCT organic cation transporter
OSM Oncostatin M
OST organic solute transporter

P

P2Y2 purinergic G protein-coupled receptors
PAGE polyacrylamid gel electrophoresis
PBS phosphate buffered saline
PC parenchymal cells
PFIC2 progressive familial intrahepatic cholestasis type 2
P-gp P-glycoprotein
p-i concept pharmacological interaction of drugs with *i*mmune receptors concept
PI propidium iodide
PKA protein kinase A
PPAR peroxisome proliferator-activated receptor
PSC Pluripotent stem cells
PVDF polyvinylidene difluoride
PXE pseudoxanthoma elasticum
PXR pregnane X receptor

R

RANTES (CCL5) Regulated on activation, normal T cell expressed and secreted
RER rough endoplasmic reticulum
RIP-1 receptor interacting protein1
RIPA radioimmunoprecipitation assay
RNA ribonucleic acid
RNS reactive nitrogen species
ROS reactive oxygen species
RPM rounds per minutes
RT room temperature
RXR retinoid X receptor

S

SAC subapical compartment
SCH sandwich-cultured primary hepatocytes
SEM standard error of the mean
SDS sodium dodecyl sulfate
SER smooth endoplasmic reticulum
STAT Signal Transducer and Activator of Transcription
SULT sulfotransferases

T

TBS tris buffered saline
TC taurocholate
TCR T-cell receptor
TEMED tetramethylethylenediamine tetraacetic acid
TGF-1 transforming growth factor-1
TGF-β transforming growth factor β connective tissue growth factor
TGN trans-Golgi network
TMAs tissue microarrays
TMB 3,3',5,5'-tetramethylbenzidine
TNF-α tumor necrosis factor
TNFR-1 tumor necrosis factor receptor 1
TRADD TNF-R-associated death domain
TRAF-2 TNF-R associated factor 2
TRAIL TNF-related apoptosis-inducing ligand

U

UGT uridine diphosphate glucuronosyltransferase
ULN or 2N upper limit of normal
UTP Uridine-5'-triphosphate
VCAM-1 vascular cell adhesion molecule 1
VDR vitamin D receptor
WB western blot
XMTS xenobiotic metabolizing and transporter systems
XREs xenobiotic response elements
α-SMA α-smooth muscle actin
γ-GT γ-glutamyltranspeptid

Chapter 1
Introduction

1. Introduction

Over the past decades, numerous drugs have been withdrawn from the market or have received black box warning due to prominent hepatotoxic adverse effects (Li, 2004). The liver may be considered as a primary target for drug induced toxicity mainly due to its anatomical and functional interposition between the site of absorption (the gastro-intestinal tract) and the systemic circulation on one hand and its crucial role in drug metabolism and elimination on the other hand (Russman et al. 2009). This central role rendered it a primary target for adverse drug reactions (Holt and Ju, 2010). Adverse drug reactions are divided into two main subtypes: Intrinsic and idiosyncratic (Roth and Ganey, 2010). While intrinsic hepatotoxicity commonly obeys the fundamental rules of toxicology by acting in a dose dependent manner with remarkable intra and interspecies consistency; idiosyncratic hepatotoxicity proved to be predominantly host-dependent, unpredictable and unaffected by the chemical or biological characteristics of the drug (Roth and Ganey, 2010). It is thought to be the result of individual hypersensitivity to certain drug adverse drug reactions, which is often related to genetic and/or environmental susceptibility (Deng et al. 2009). This type of liver toxicity contradicted the famous statement of the great-great-great grandfather of toxicology, Paracelsus: “all compounds are toxic, it is only the dose that distinguishes a remedy from a poison;” by exhibiting no clear relation to drug dose and occurring at normal therapeutic doses that are generally safe to others with an inconsistent temporal relation to drug exposure (Roth and Ganey, 2009). Despite the fact that idiosyncratic adverse drug reactions (IADRs) occur in a minority of patients (< 1%) it accounts for approximately 13% of all acute liver failure cases and represents the main reason for post-marketing drug withdrawal (Shaw et al. 2010). The latter can be attributed partly to the lack of efficient predictive *in vitro* and *in vivo* models that are able to detect potentially idiosyncratic drugs before they reach the market; and partly to the ambiguity of the mechanisms underlying idiosyncratic hepatotoxicity in susceptible individuals (Dambach et al. 2005). Therefore, the development of efficiently predictive models of idiosyncratic hepatotoxicity, with the capacity to screen out potentially idiosyncratic drugs during the early phases of drug development will be of valuable benefit to pharmaceutical companies as well as for human health in general. Recent studies suggested that integrating the fields of pharmacogenomics, toxicogenomics, proteomics, and metabonomics in toxicity studies will ameliorate the detection of idiosyncratically hepatotoxic agents while providing accurate insights on their mode of action (Kaplowitz, 2005). Greater knowledge of the key mechanisms underlying idiosyncratic hepatotoxicity and the discovery of more sensitive biomarkers, associated to toxic responses, will with no doubt help prevent the detrimental effects of idiosyncratic adverse drug reactions (Kiyosawa and al. 2009).

1.1 Basic anatomy and physiology of the liver

The liver is considered as the largest internal organ of the body, weighing between 1.4-1.6 kg and accounting for approximately 2 to 3% of average body weight (Abdel-Misih and Bloomston, 2010). It is located in the upper right quadrant of the abdominal cavity underneath the right hemidiaphragm and at the right of the stomach (Abdel-Misih and Bloomston, 2010). Enveloped in a thin capsule (Glisson's capsule) and protected by the rib cage, it maintains its position by the

help of peritoneal reflections known as ligamentous attachments (Abdel-Misih and Bloomston, 2010). The liver is divided by fissures into four main lobes of distinct size and shape namely the right (the biggest lobe), left, quadrate and caudate lobes (LeCluyse et al. 2012). The whole liver consists of numerous functional units, usually visualized as a six-side structure, known as the 'classical lobule' (LeCluyse et al. 2012). The polygon-like structure of the classical lobule in addition to the 3D architechture of the liver are shown in Figure 1.1A and B. Each corner of the polygonal lobule is made up of portal triads, which contain branches of the portal vein, bile duct, and hepatic artery (Bioulac-Sage et al. 2007; Khan et al. 2007). Along the central axis of each lobule runs the central vein, from which hepatic cords radiate towards the portal triad (Vollmar and Menger, 2009). These cords, separated by sinusoids, occupy the mass of the lobule (Rodés et al. 2007). Plates of parenchymal cells (hepatocytes) spread out from the central vein to the perimeter of the lobule, defining the basic functional unit of the liver, namely the acinus (LeCluyse et al. 2012). The latter contains the essential cellular and physiological features that define the unique architecture of the liver tissue thus serving as a microcosm of the major hepatic microenvironment (LeCluyse et al. 2012). The liver acinus is separated into three distinct zones: zone 1 is the periportal region; zone 2 is the midlobular region; and zone 3 is the pericentral region (Rappaport, 1977; Ito and McCuskey, 2007). Blood enters the liver at the portal triads, precisely from the portal veins and hepatic arteries, flows through the sinusoidal microvasculature enclosed by the plates of parenchymal cells, to finally leave the liver through the central vein (Vollmar and Menger, 2009). The particular arrangement of cells along the microvasculature and the directionality of flow through the lobular units, allowed the existence of various chemical gradients and microenvironments (LeCluyse et al. 2012); leading to variation in several mechanisms related to cell maturation, matrix chemistry, solute concentrations, oxygen tension, gene expression and xenobiotic clearance across the acinus (LeCluyse et al. 2012; Turner et al. 2011; Wang et al. 2011). An example of the variations in the zonal expression of specific hepatic genes is shown in Figure 1.2 using antibodies against cytochrome P450 (CYP3A4). As is the case with the majority of cytochrome P450 enzymes, the highest levels of CYP3A4 expression are located in zone 3 (pericentral) (LeCluyse et al. 2012). The positional difference in expression partially contribute to the zonal pattern of toxicity elucidated *in vivo* upon exposure to a variety of bioactivated compounds, such as carbon tetrachloride, bromobenzene, chloroform and acetaminophen (Moon et al. 2010). Specifically, midlobular (zone 2) necrosis is observed in rodents exposed to natural and synthetic substances, like cocaine, phytol and germander (Mackie et al. 2009), whereas zone 1 specific toxicity may be observed with other hepatotoxins such as allyl alcohol and phosphorus.

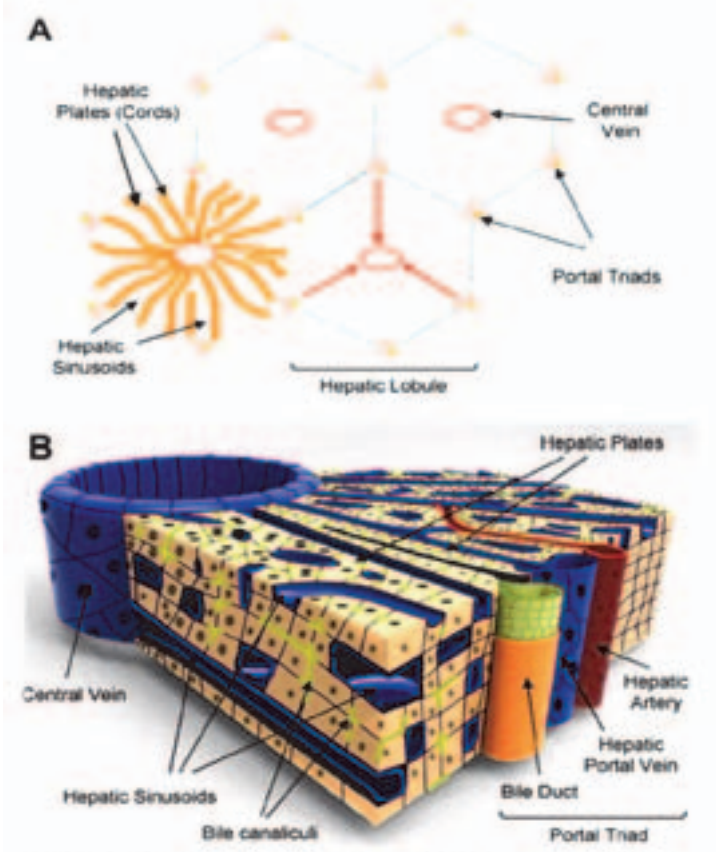


Figure 1.1. Representation of histotypic liver microstructure. (A) Illustration of the hepatic lobule and acinus substructure demonstrating the relative direction of blood flow from portal triads towards the central veins (red arrows). (B) Diagram illustrating the three-dimensional architecture of the liver between a portal triad and the central vein. The networks of bile canaliculi (yellow-green) run parallel and counter to the blood flow through the sinusoids. (*Adapted from LeCluyse et al. 2012).*

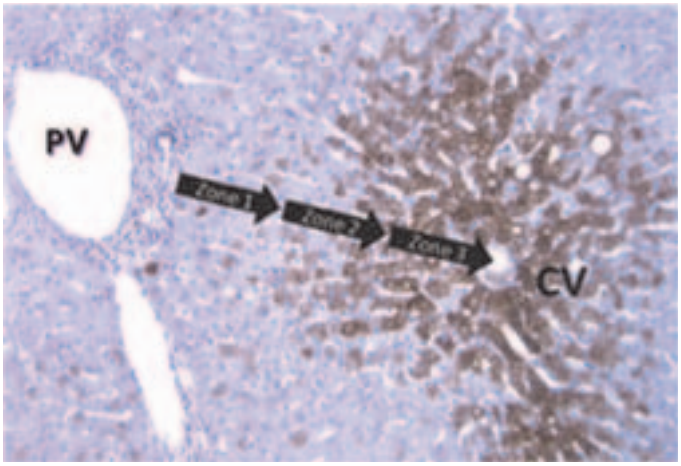


Figure 1.2. Structural and functional zonation of the liver. Immunostaining of human liver tissue with antibodies against CYP3A4 (brown stain) showing the differential expression of CYP enzymes across the zones of the liver microstructure. The greatest expression of CYP enzymes is predominantly in pericentral hepatocytes (zone 3) with a distinct boundary or gradient at the mid-lobular region (zone 2) PV: Portal Vein; CV: Central Vein. (*Adapted from LeCluyse et al. 2012).*

1.2 Major cell types of the liver

The liver is made up of two broad categories of cells: parenchymal cells and non-parenchymal cells (NPC) (LeCluyse et al. 2012). The parenchymal fraction is constituted of hepatocytes whereas the non-parenchymal fraction comprises hepatic stellate cells (HSC), Kupffer cells (KC), bile duct epithelial cells (cholangiocytes), liver sinusoidal endothelial cells (LSEC) and pit cells (intrahepatic lymphocytes or nature killer cells) (LeCluyse et al. 2012). NPC are known to contribute efficiently to the regulation of hepatic growth and function (Kmiec, 2001); including the production of growth factors and other mediators of transport and metabolism. NPC are often considered as the primary targets of certain hepatotoxins, or as important mediators of certain physiological and pathological response to other cells (Parola and Pinzani, 2009).

1.2.1 Hepatocytes

Hepatocytes, which represent 80% of liver's volume and 60% of the total hepatic cell population, perform most of the liver's physiological functions (LeCluyse et al. 2012). They are extremely susceptible to injury due to their extensive involvement in the biotransformation and elimination of a wide range of exogenous and endogenous compounds; in addition to their role in the production and transport of bile acids and their presence amongst numerous immune cells (Malhi et al. 2010). Based on their zonal location in the liver, hepatocytes display evident functional, morphologic, and biochemical heterogeneity (Turner et al. 2011). Their cell size is known to increase from Zone 1 to Zone 3, escorted by characteristic zonal modifications in the morphological features of the mitochondria, endoplasmic reticulum, lipid vesicles and glycogen granules (LeCluyse et al. 2012). Normally, hepatocytes are cuboidally-shaped and may contain one or more nuclei with protruding nucleoli (LeCluyse et al. 2012). The presence of two nuclei is a normal feature that aids hepatocytes in managing their laborious roles in drug detoxification, endogenous metabolism and ATP production (Sigal et al. 1999). Functionally hepatocytes are rich in several cellular components, which aid them to perform their numerous laborious tasks. For example the abundantly present mitochondria provide hepatocytes with high amounts of energy and essential enzymes necessary for various vital processes like citric acid cycle and fatty acids oxidation (LeCluyse et al. 2012). Furthermore, the extensive presence of both the rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER) in the cytoplasm of hepatocytes confers a wide variety of important functions (LeCluyse et al. 2012). The abundant presence of RER, where ribosomes are found, is correlated with the hepatocyte's secretory nature on one hand and its extensive involvement in protein synthesis and glycogenesis on the other hand (LeCluyse et al. 2012). SER are extensively rich in microsomes, where various P450 enzymes especially monooxygenases are abundantly present, hence they are significantly correlated with drug detoxification and bilirubin conjugation (LeCluyse et al. 2012). Moreover SER are involved in the hepatic synthesis of cholesterol and primary bile acids (LeCluyse et al. 2012). Hepatocytes exhibit a characteristic polarity, possessing distinct poles with different sinusoidal and canalicular plasma membrane domains that are separated by tight junctions (LeCluyse et al. 2012). The marked differences in the structure, composition, and function of these membrane domains are essential for the hepatocyte's role in the uptake, metabolism, and

biliary elimination of both endogenous and exogenous substrates (Montfoort et al. 2003). In a healthy liver, hepatocytes play crucial roles in the efficient transport of numerous endobiotics and exobiotics from blood into bile. Hepatobiliary transport is concerned, on one hand, with the production and secretion of bile components, which are required for fat absorption in the gut (Rodés et al. 2007); and on the other hand with the detoxification of both endogenous and exogenous compounds. It is noteworthy that the hepatic production and transport of bile depends predominantly on the correct localization of transport proteins at their respective basolateral or canalicular pole and on their coordinated activity (Klaassen and Aleksunes, 2010). Drug-induced perturbation of these transport mechanisms is one of the causes of intrahepatic cholestasis, which leads to toxic drug accumulation in both the liver and plasma.

1.2.2 Liver sinusoidal endothelial cells (LSEC)

LSEC, like most vascular endothelial cells, are thin and elongated in shape; they line the walls of hepatic sinusoids possessing a relatively large number of pinocytotic vesicles, which suggests significant endocytotic activity (Perri and Shah, 2005). LSEC plasma membrane is characterized by relatively small pores (50–200 nm in diameter), known as fenestrations, allowing the free diffusion of many substances, but not particles between the blood and the hepatocytic basolateral membrane (Cogger et al. 2010). The enhanced intercellular permeability and surface fenestrae, in addition to the lack of tight intracellular adhesions and strong basement membrane between the LSEC and parenchyma favor the exposure of hepatocytes to soluble components in the circulating blood on one hand (Perri and Shah, 2005); and enhance the passive transport of many endogenous and exogenous substrates on the other hand (Braet and Wisse, 2002). The facilitated contact of hepatocytes with the blood provides them with continuous O2 supply and allows efficient clearance of drugs and other xenobiotics (LeCluyse et al. 2012). LSEC are important members of the reticuloendothelial system (RES); functionally, they contribute significantly to the maintenance of hepatic homeostasis through three vital roles (LeCluyse et al. 2012). Firstly, they represent a “selective sieve” allowing the entrance of specific substances to hepatocytes while prohibiting others (LeCluyse et al. 2012). Secondly, they act as a “scavenger system”, eliminating macromolecular waste products from the blood; which is favored by their potent endocytic capacity for colloids and many other ligands (DeLeve, 2007). Third, LSEC play an important role in mediating immune tolerance to neo-antigens formed during drug metabolism and in enhancing hepatic immunity to foreign pathogens (Perri and Shah, 2005; DeLeve, 2007). LSEC also function as antigen-presenting cells (APC) and are considered active secretors of cytokines, eicosanoids, endothelin-1 (ET-1), nitric oxide, and some extracellular matrix (ECM) components (DeLeve et al. 2004; Deleve et al. 2008). Similar to hepatocytes, LSEC are considered as a target for some types of xenobiotic-induced hepatotoxicity since they proved to exhibit a significant role in the metabolism and elimination of drugs and other xenobiotics (Deaciuc et al. 2001; Xie et al. 2010). Particularly, LSEC proved to be a primary target for acetaminophen-induced cytotoxicity in the absence of hepatocytes, implying that these cells are fully capable of biotransforming acetaminophen into its reactive metabolite (Ito et al. 2003; Xie et al. 2010). Moreover, LSEC also elucidated prominent phase II enzyme activity further

supporting their involvement in drug metabolism (Utesch and Oesch, 1992). Despite the fact that the metabolic activity of LSEC is ~1/10th that of hepatocytes; their role in hepatic metabolism and clearance of compounds deserve to be thoroughly investigated (Schrenk et al. 1991; Sacerdoti et al. 2003; Wu et al. 2008).

1.2.3 Hepatic stellate cells (HSC)

HSC, known as perisinusoidal cells, Ito cells or fat-storing cells, dwell in the space of Disse in the liver. The space of Disse represents the perisinusoidal space between the basolateral surface of hepatocytes and the anti-luminal side of sinusoidal endothelial cells (Asahina et al. 2009). In a healthy adult liver, HSC display numerous dendrite-like extensions, which wrap around the sinusoids; this will allow the "embracement" of endothelial cells and the facilitation of intercellular communication by means of soluble mediators and cytokines (Friedman, 2008). HSC are extensively involved in regulating sinusoid contractility, storing vitamin A and controlling the production and turnover of the extracellular matrix (ECM) (LeCluyse et al. 2012). After attaining maturity, HSC produce a wide variety of substances including various types of fibrillar and network collagens, big amounts of elastin and both chondroitin sulphate proteoglycans (CS-PG) and heparan sulfate proteoglycans (HS-PG) and (Wang et al. 2010; Parola and Pinzani, 2009). Furthermore, mature HSC are also able to secrete several important cytokines and growth factors (PDGF and IGF-I and II) essential for intercellular communication in both healthy and injured liver (Ramadori et al. 2008; Asahina et al. 2009). During liver injury, HSC exhibit a noticeable decrease in the levels of vitamin A and in the expression of α-smooth muscle actin (α-SMA); this indicates the activation of HSC and their phenotypic shift to myofibroblastic hepatic stellate cells (MF-HSC) phenotype (Friedman, 2008). In its activated state, MF-HSC become involved in the production of transforming growth factor β (TGF-β) and the expression of connective tissue growth factor (CTGF), thus regulating hepatocyte growth and promoting fibrogenesis (Ramadori et al. 2008; Parola and Pinzani, 2009). HSC play a potent role in hepatic inflammation predominately through inducing the recruitment of leukocytes to the liver and through the release of a wide variety of pro-inflammatory mediators such as: macrophage colony-stimulating factor (M-CSF), monocyte chemotactic peptide (MCP)-1, CCL21, RANTES, IL-8, IL-6 and IL-10 (Ramadori et al. 2008). Moreover, highly activated HSC are typically involved in the onset and progression of cirrhosis, which is characterized by a progressive increase in deposition of ECM proteins and scar tissue formation all through the liver (Wandzioch et al. 2004).

1.2.4 Kupffer cells (KC)

Kupffer cells (KC) represent the largest population of resident macrophages in the body and exhibit prominent endocytic and phagocytic capacity (Jaeschke, 2007). They have mesenchymal origins and possess long cytoplasmic extensions that facilitate direct cell-to-cell contact with hepatocytes despite their localization within the sinusoidal microvasculature on the luminal side of endothelial cells (LeCluyse et al. 2012). KC are extensively involved in the efficient elimination of cellular debris, soluble bacterial products, endotoxins and other macromolecular

complexes through endosomal and lysosomal processing pathways (Roberts et al. 2007). They are known to modulate the turnover of hepatocytes and other cell types by secreting several factors that are able to induce apoptosis (Hoebe et al. 2001). KC are characterized by a highly heterogeneous morphology and bio-capacity; they exhibit better phagocytosis in the periportal area due to their large size while displaying enhanced production of cytokines and inflammatory response in the centrilobular area (Roberts et al. 2007). Kupffer cells exhibit potent immune-regulatory roles; they activate cytotoxic T-cells during inflammation and infection and suppress their proliferation and prolonged activation after the infectious agent is eliminated (Kolios et al. 2006). KC are extensively involved in the endocytosis of foreign particles and bacterial endotoxins, which results in their activation (Kolios et al. 2006). Subsequently, activated kuppfer cells produce and release numerous modulators of cell signaling pathways, such as ROS, eicosanoids, leukotrienes, prostaglandins, TNF-α, TGF-α, IL-1, IL-6 and others (Kolios et al. 2006). KCs demonstrated contradictory roles in several cases of chemical-induced liver injury whereby they enhanced liver regeneration or promoted hepatic injury depending on the severity of the stimulating agent. KC proved capable of altering the metabolic activity of hepatocytes through producing several cytokines (e.g. IL-1, IL-6, TNFμ) that stimulate the expression of acute phase proteins on one hand; and induce the down-regulation of genes implicated in the metabolism and clearance of xenobiotics on the other hand (Hoebe et al. 2001). KC-produced pro-inflammatory cytokines are tightly correlated with potent and complete suppression of cytochrome P450, Uridine 5′-diphospho (UDP)-glucuronosyl transferase systems and hepatic transporter expression (Sunman et al. 2004; Wu et al. 2006; Higuchi et al. 2007; Morgan, 2009). Based on these metabolic characteristics, the integration of KC along with hepatocytes in the establishment of cellular predictive models of drug-induced liver injury may reproduce more accurately the physiological response of the liver to drug exposure.

1.2.5 Cholangiocytes

Cholangiocytes or intrahepatic bile duct cells constitute approximately 5% of the liver cell population. They display a heterogeneous morphology with respect to their location; they are cuboidal in shape as they line up the epithelium of the small interlobular bile duct but become gradually columnar and mucus secreting in larger bile ducts approaching the porta hepatis (transverse fissure of the liver) and the extrahepatic ducts. As is the case with morphology, the cholangiocyte population is also considered heterogeneous in its secretion and expression patterns; in addition to its response to various stimuli such as growth factors, cytokines, bile acids, injury or toxins (Marzioni et al. 2002; Bogert and LaRusso, 2007; Glaser et al. 2006). Functionally, cholangiocytes contribute efficiently to the regulation of localized hepatic immune responses either through releasing cytokines and other mediators that influence invading inflammatory cells or through expression of adhesion molecules on the cell surface that mediate direct interaction with immune cells (Fava et al. 2005; Glaser et al. 2009). They play an important role in the regulation of ductal bile secretion as well as in the absorption and secretion of water, organic anions and cations, lipids and electrolytes (Tietz and LaRusso, 2006). This cholangiocyte fluid/electrolyte secretion is enhanced by several hormones and locally acting

mediators such secretin, acetylcholine, and ATP (LeCluyse et al. 2012). Hepatic cholangiocytes are involved in bile secretion through the release of bicarbonate in both the canaliculi and the bile ducts generating bile-salt independent flow (Tietz and LaRusso, 2006; Xia et al. 2006).

1.2.6 Hepatic progenitor cells (HPC)

HPC are bi-potent stem cells that dwell in human and animal liver, in a compartment encompassed within the canals of Hering, possessing the ability to differentiate into both hepatocytic and cholangiocytic lineages (Gaudio et al. 2009; Turner et al. 2011). In healthy adult liver, HPC are characterized by their small quiescent shape, elongated or vesicular nuclei, tiny nucleoli and scarce cytoplasm (LeCluyse et al. 2012). Under normal conditions, they exhibit a relatively low proliferation rate and constitute a reserve compartment, which is only activated following mature epithelial cell injury or loss (Zhang et al. 2008). In case of severe injury to epithelial cells or inhibition of their replication, HPC are activated and spread out from the periportal to the pericentral zone; giving rise to mature hepatocytes and/or cholangiocytes (Vig et al. 2006; Santoni-Rugiu et al. 2005). The stem cell population is supported by the HPC niche, which represents the cellular and extracellular microenvironment that comprises various types of cells like LSEC, HC, cholangiocytes, KC, Pit cells (hepatic natural killer cells) and other inflammatory cells (Moore and Lemischka, 2006). This peculiar microenvironment and interaction with specific cell types is considered as an underlying key mechanism of stem cells' maturation and self-renewal capacities (LeCluyse et al. 2012). In fact, several types of signalling and adhesion molecules within the niche influence stem cell quiescence and provide proliferation- or maturation-inducing signals when numerous progenitor cells are required to generate mature cell lineages (LeCluyse et al. 2012). However, an important limiting factor for the activation and proliferation of HPC is the occurrence of a significant loss of mature cell mass (50% loss of mature hepatocytes) regardless of the primary cause or stimulus (Bird et al. 2008; Katoonizadeh et al. 2006). One of the main mechanism underlying liver disease and carcinogenesis is targeting HPC and their niche leading to the disruption of various molecular pathways related to liver regeneration and cellular maturation (Katoonizadeh et al. 2006). The potent activation of HPC proved to inhibit the replication of mature hepatocytes in long-term chronic liver disease and extensive chemical exposure; implying that HPC activation and proliferation is tightly correlated with the extent of fibrosis (Libbrecht et al. 2000). The explicit role of the HPC compartment in promoting hepatic injury or conferring hepatoprotection in response to chemical- or drug-induced injury remains ambiguous to date; however sufficient evidence was provided regarding the efficient contribution of HPC to hepatotoxic responses for them to be worthy of being included in future strategies of toxicity tests (LeCluyse et al. 2012).

1.3 Hepatocellular architecture and cell polarity

The vast majority of epithelial cells typically possess one canalicular and one basolateral (blood-facing) domain on opposing epithelial membranes (LeCluyse et al. 2012). Conversely, hepatocytes display two basolateral domains that interface with the sinusoidal microvasculature on opposite sides of parenchymal plates (Figure 1.3) (LeCluyse et al. 2012). This configuration

forms relatively peculiar cell architecture amongst epithelial cells; in that the canalicular domain lies halfway between the lateral domains of opposing hepatocytes, and hence is entirely enclosed within the hepatic plates (LeCluyse et al. 2012). The canalicular domains arise between two adjacent cells as intracellular channels that are later on characterized by tight intermediate gap junctions, well-formed microvilli and marked desmosomes clearly delineating its boundaries (Khan et al. 2007). The belt-structure canaliculi, located at the periphery of adjacent hepatocytes, interconnect to form a complex network of small tubular compartments all along the cell plates of the liver lobule (LeCluyse et al. 2012). This network ends at the portal triad to become interconnected with bile ductules through the canals of Hering, allowing drainage into the common bile duct and the gall bladder (Rodés et al. 2007). Both the canalicular and sinusoidal domains exhibit pole-specific membrane protein expression; in addition to disparate cytochemical, immunological and biochemical characteristics, that are fundamental in sustaining normal hepatic function (Chapman and Eddy, 1989). The biosynthesis of hepatic transport proteins begins in the endoplasmic reticulum to be later on modified in the Golgi apparatus and eventually sorted at the trans-Golgi network (TGN) (Wang and boyer, 2004). From the TGN, they travel through the post-Golgi biosynthetic pathway to reach the canalicular membrane domain (Kipp and Arias, 2000). After reaching the plasma membrane, a protein has two fates: retention or internalization (endocytosis) (Bonilha et al. 1997). After endocytosis, some proteins may be transported, by a process known as transcytosis, to the opposite membrane domain of hepatocytes (Wang and boyer, 2004). Earlier studies have demonstrated that proteins designated for the hepatic sinusoidal membrane traffic from the Golgi complex to their destination directly (Matter et al. 1994). Conversely, the majority of canalicular proteins traffic by an indirect route; beginning by the sinusoidal membrane, and then traversing through the basolateral early endosomes by transcytosis to reach a subapical compartment, before finally arriving to the canalicular domain (Matter et al. 1994). These canalicular proteins comprise single transmembrane proteins such as dipeptidyl peptidase IV, aminopeptidase N, and polymeric immunoglobulin A receptor; in addition to glycosyl- phosphatidylinositol (GPI)-anchored proteins like 5-nucleotidase (5-NT) (Wang and boyer, 2004). Until recently, the indirect transcytotic pathway represented the only route through which canalicular proteins reach their final destination in hepatocytes (Wang and boyer, 2004). However, mounting evidence has proved the latter wrong since two canalicular adenosine triphosphate (ATP)–binding cassette transporters, namely the multidrug resistance protein 1 (Mdr1) and the bile salt export pump (Bsep), exhibited direct trafficking from the Golgi to the canalicular domain (Wang and boyer 2004).

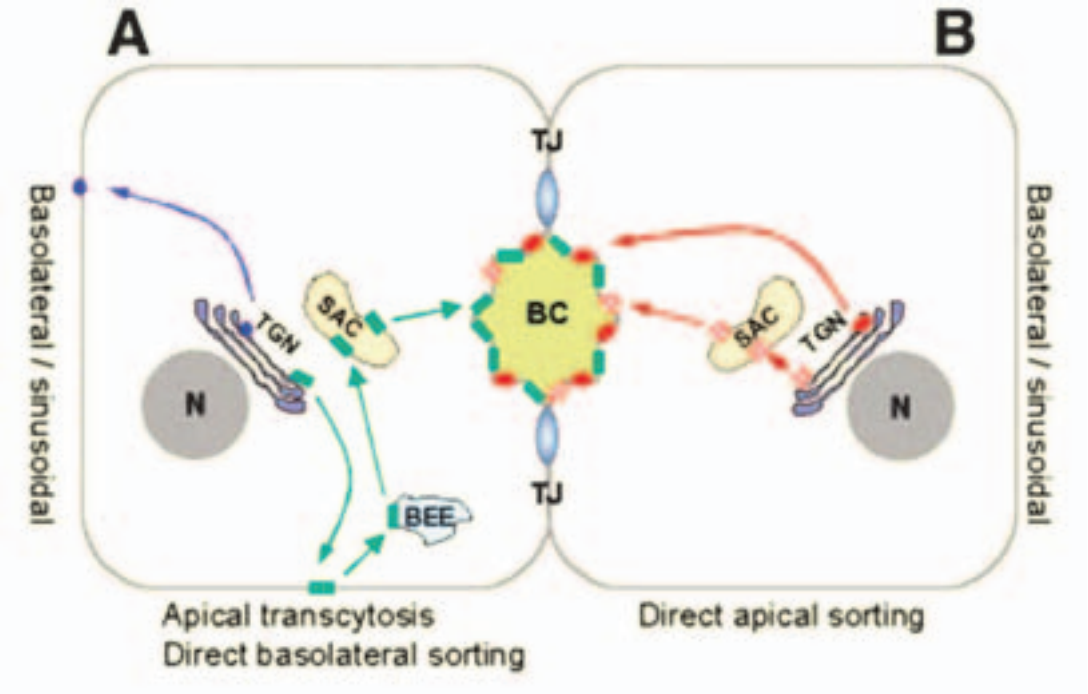


Figure 1.3. The post-Golgi biosynthetic trafficking in hepatocytes. A hepatocyte couplet is shown with the basolateral and apical membranes separated by tight junctions (TJ). (A) Sinusoidal membrane proteins (blue circles) take a direct route (blue arrows) from the TGN (trans-Golgi network) to the basolateral membrane. Most canalicular proteins (green patches) are targeted initially to the basolateral membrane and then undergo transcytosis (green arrows) through the basolateral early endosome (BEE) and a subapical compartment (SAC) before reaching the canalicular membrane. (B) In contrast, Mdr1 (solid red patch) traffics directly (red arrow) from the TGN to the canalicular membrane. Bsep (hatched red patch) may traffic first to a subapical compartment (also termed as SAC) and then to the canalicular membrane.BC: Bile Canaliculus. *(Adapted from Wang and Boyer 2004).*

1.4 Major hepatic functions

1.4.1 Metabolism

The liver performs a crucial role in the metabolism of various vital compounds such as carbohydrates, proteins and lipids (Shaffer and Myers, 2005). Furthermore, it is extensively involved in the regulation of critical homeostatic functions such as endocrine activity (synthesis, activation and catabolism of hormones) and haemostasis (production of the majority of coagulation factors and inhibitors) (Shaffer and Myers, 2005). The liver maintains a constant adequate level of sugar in the body by storing excess glucose in the form of glycogen during hyperglycemia (Postic et al. 2004). Nevertheless, in times of need, or hypoglycemia, the liver undergoes glycogenolysis, breaking down glycogen to glucose or gluconeogenesis to form glucose from non-carbohydrate sources such as amino acids in order to restore glucose homeostasis (Postic et al. 2004). The liver is involved in the synthesis of the majority of plasma proteins, namely albumin and most of the globulins with the exception of gamma globulins (Shaffer and Myers, 2005). Albumin acts as a carrier for a vast array of drugs and endogenous hydrophobic compounds, like unconjugated bilirubin, through the plasma providing most of its

oncotic pressure. Globulins comprise the coagulation factors V, VII, IX and X in addition to fibrinogen and prothrombin (factor II) (Shaffer and Myers, 2005). Some of these factors (II, VII, IX and X) are thought to be vitamin K-dependent; the latter is a fat-soluble vitamin implying that its bioavailability or absorption requires adequate bile salts concentration (Shaffer and Myers, 2005). It is noteworthy that the plasmatic concentration of these factors decreases in certain conditions such as fat malabsorption and heptocellular disease (Shaffer and Myers, 2005). During fat malabsorption, the decreased concentration of coagulation factors may be associated with prolonged cholestasis which results in depressed bile salts secretion and hence reduced fat emulsification and absorption of vitamin K (Shaffer and Myers, 2005). Conversely, in hepatocellular disease, this deficiency is thought to be vitamin K-independent (Shaffer and Myers, 2005). The liver is also considered to be the principle site of most amino acid catabolism and interconversions (Shaffer and Myers, 2005). The catabolism of amino acids to urea consumes prominent amounts of ammonia, a product of nitrogen metabolism and a potential neurotoxin, hence detoxifying it and protecting the body from its harmful effects (Shaffer and Myers, 2005). Fatty acids undergo hepatic esterification to triglycerides, which are packaged in the liver along with cholesterol, phospholipids and one apoprotein into a lipoprotein (Shaffer and Myers, 2005). Then lipoprotein is released into the circulation either for direct utilization or for storage in adipocytes (Shaffer and Myers, 2005). The majority of cholesterol synthesis takes place in the liver with bile salts being the major products of its catabolism (Shaffer and Myers, 2005).

1.4.2 Bile formation

Bile is predominantly an aqueous solution, equally osmotic with respect to plasma, including less than 5% of solids (Shaffer and Myers, 2005). In addition to providing the main excretory route for cholesterol, lipid waste products and toxic drug metabolites; bile is thought to be crucial for the efficient emulsification and absorption of dietary fats and fat-soluble vitamins (vitamins A, D, E, and K) (Iqbal and Hussain, 2010). Bile salts, that represent the majority of biliary organic solutes, are exclusively synthesized in the liver from cholesterol and constitute the major driving force behind bile formation (Meier and Stieger, 2000). The active transport of bile salts into the canaliculi provides an osmotic gradient across the hepatocyte, leading to the translocation of solutes and water into bile in order to sustain equal osmolarity (Meier and Stieger, 2000). After being subsequently released from the liver, bile is concentrated and stored in the gallbladder under fasting conditions (Shaffer and Myers, 2005). However, during eating the small intestine releases cholecystokinin (CCK) resulting in a cholinergic discharge; the latter induces the gallbladder to contract and the sphincter of Oddi to relax favoring the evacuation of bile into the duodenum (Shaffer and Myers, 2005). Bile salts enhance fat absorption in the duodenum by acting as biologic detergents (Iqbal and Hussain, 2010). Subsequently, they are absorbed by active transport in the ileum to return via the portal vein to the liver where they are readily taken up and secreted once again into the duodenum; this cycle is known as the enterohepatic circulation (Shaffer and Myers, 2005). The human liver secretes around 500 mL of bile daily, eliminating potentially toxic products from the body and providing the essential biological

detergents for fat solubilization and digestion (Meier and Stieger, 2000). The hepatocytic membrane is functionally divided into the following two regions: 1) The Basolateral (sinusoidal) region, which represents 85% of the total surface area, with its basal portion facing the sinusoidal space and its lateral surfaces joining adjacent hepatocytes. 2) The smaller apical (canalicular) region, which includes about 15% of the surface area, and consists of groove-like clefts between neighboring liver cells (Meier and Stieger, 2000). Tight junctions separate the apical from the basolateral hepatocyte membrane, thus limiting the free exchange of ions, organic solutes, and water with the extracellular space (Meier and Stieger, 2000). Uptake transports are commonly localized on the basolateral domain next to the portal blood vessels, whereas export transporters settle on the canalicular domain where bile forms (Meier and Stieger, 2000). Solutes must either traverse the transcellular hepatocytic pathway or move through the intercellular junctional complexes (paracellular pathway) to reach the canaliculus (Meier and Stieger, 2000).The majority of organic solutes such as bile salts and bilirubin follow the transcellular pathway to the canalicular domain, to be much more concentrated in the canalicular bile than in the serum (Shaffer and Myers, 2005). Such active transport necessitates the production of energy through ATP hydrolysis, which engages the coupling of cellular transport to the movement of other ions ("secondary active transport") (Shaffer and Myers, 2005). Once arrived to the apical membrane, bile salts, reduced glutathione (GSH) and other negatively charged organic ions are prevented from diffusing back to the liver by intercellular "tight" junctional complexes (Zsembery et al. 2000). Only water and some electrolytes (through solvent drag) are able to diffuse back through the paracellular route between cells. Canalicular bile formation consists of three main components: 1) The "bile salt-dependent bile flow," which is defined as the active transport of bile salts and GSH; 2) The "bile salt-independent bile flow," which represents the apical bile salt-independent secretion of bicarbonate; and 3) a ductular component created in the bile channels (bile ducts), and controlled by hormones such as secretin and neuropeptides. Eventually bile is secreted from the liver to be concentrated in the gallbladder (5-10 folds) (Zsembery et al. 2000).

1.4.3 Xenobiotic metabolism and elimination

In terms of both anatomy and microstructure, the liver is perfectly designed to be the principle drug clearance organ (Lee, 2003). Most foreign exogenous substances, including therapeutic drugs, enter the body through being absorbed from the gastrointestinal tract (GIT) to subsequently enter the liver via the portal vein aided by the flow of venous blood from the GIT to the liver (Piñeiro-Carrero and Piñeiro, 2004). In addition to intestinal drug metabolizing enzymes and drug transporters, the liver constitutes a cogent barrier that limits the entrance of xenobiotics into the blood circulation (Malarkey et al. 2005). The process through which a portion of an absorbed drug is metabolized by the liver is termed 'firstpass clearance' (Shen et al. 2007). The firstpass cleared portion of a drug added to its GIT-absorbed portion defines its 'bioavailability' or the fraction of an orally administered drug that reaches the systemic circulation as intact drug (Shen et al.1997). For a number of drugs, like glyceryl trinitrate and lidocaine, the degree of first-pass clearance is so potent that oral administration becomes

prominently ineffective (Wilkinson, 2005). The liver extensively contributes to three different phases of drug metabolism, named phase I, phase II and phase III (Liddle and Stedman, 2008). Phase I metabolism involves the formation of more polar metabolites through the introduction of functional groups into drug molecules by oxidation, reduction or hydrolysis (Liddle and Stedman, 2008). This is followed by phase II metabolism, which implicates the conjugation of active metabolites with hydrophilic groups, such as glucuronide, glutathione, sulfate, acetyl coenzyme A and amino acid, leading to more water-soluble products and thus enhancing their excretion into urine or bile (Liddle and Stedman, 2008). Phase III metabolism refers to the detoxification or clearance of toxic endogenous and exogenous substances through coordinated regulation of efflux processes (Liddle and Stedman, 2008). It is noteworthy that the most important phase-I drug metabolizing enzymes (cytochrome P450) and mainly CYP34A, are not expressed evenly throughout the hepatic lobular architecture with a preferable expression in zone 3 (pericentral zone) when compared to other zones (Yokose et al. 1999). Accordingly, uneven drug metabolizing activity is demonstrated related to the degree and zonal location of P450s expression (Yokose et al. 1999). One of the reasons that have emerged to explain disparate zonal metabolism may be to protect the majority of the liver from the toxic effects of electrophilic metabolites (Wilkinson, 2005). With zone 3 exhibiting the most potent expression of P450s and hence the most active metabolic potential, necrosis will be limited to the pericentral zone in case reactive molecules were not successfully eliminated (Park et al. 2005).

1.4.3.1 Role of drug metabolizing enzymes

Phase I metabolism is predominantly catalysed by P450 enzymes in the liver and is defined as the basic structural modulation of a drug molecule; whereas phase II metabolism refers to the attachment or "conjugation" of a water-soluble moiety to a certain drug (Liddle and Stedman, 2008). P450 enzymes consist of a gene superfamily with 57 members in the human genome (Ortiz de Montellano, 1995). A subset of around 15 P450 enzymes, belonging to the CYP 1, 2 and 3 gene families, mediate approximately 70–80% of all phase I-dependent metabolism of therapeutic drugs and many other xenobiotics (Ortiz de Montellano, 1995). P450s belonging to other families are implicated in a wide range of functions, the most important being the synthesis of cholesterol and steroid and the metabolism of fatty acids (Nebert et al. 2002; Wilkinson, 2005). P450 enzymes insert a single oxygen atom into their substrates; hence they are referred to as mono-oxygenases (Nebert et al. 2002). Alternatively, they may be referred to as 'mixed function oxidases' with respect to their vast substrate specificity (Nebert et al. 2002). Drug metabolizing P450s are located in the smooth endoplasmic reticulum within hepatocytes where they accomplish various metabolic reactions comprising aliphatic and aromatic hydroxylation, oxidative and reductive dehalogenation, *O*-, *S*- and *N*-dealkylation, *N*-oxidation and *N*-hydroxylation, demethylation and deamination (Ortiz de Montellano, 1995). Phase I metabolism predominately generates hydrophilic metabolites exhibiting sufficient water solubility for direct elimination; however in some cases it can produce certain substrates that require further metabolism by phase II enzymes (Mohutsky et al. 2010). These enzymes play a vital role in the detoxification of reactive toxic molecules generated by phase I metabolism (Park et al. 2005).

Phase II enzymes are collectively known as 'transferases' as they transfer a hydrophilic chemical moiety from a donor molecule to a recipient drug molecule (Mohutsky et al. 2010). These enzymes are commonly involved in detoxifying electrophiles and polarizing metabolites thus favouring their elimination (Mohutsky et al. 2010). Particularly, UDP glucuronosyltransferases (UGT) catalyse the transfer of α-glucuronic acid from the donor molecule, uridine-α-glucuronide to a certain substrate molecule (Shaffer and Myers, 2005). The majority of transferases belong to multi-membered gene families that catalyse the conjugation of different but often overlapping range of substrates. In particular, glutathione-S-transferases are involved in catalysing the conjugation of reduced glutathione, through a sulfhydryl group, to electrophilic metabolites of various drugs such as adriamycin, busulfan, chlorambucil and cyclophosphamide; sulphotransferases (SULT) catalyse the transfer of a sulphate group to several substrates including paracetamol, bile acids and steroid hormones; N-acetyl transferases catalyse the N-acetylation and the O-acetylation of arylamines and heterocyclic amines; and finally UDP-glucuronosyltransferases (UGT) catalyse the glucuronidation of substrates such as bilirubin, NSAIDs, morphine and zidovudine (Shaffer and Myers, 2005).

1.4.3.2 Role of drug transporters

Hepatic transport proteins play a vital role in the hepatic uptake of drugs and their subsequent biliary excretion (Shitara et al. 2005). Accordingly, a greater understanding of how modulated hepatic transport influences systemic and hepatic exposure of drugs and their metabolites is highly important from both toxicological and therapeutic point of views (Shitara et al. 2005). The adequate hepatic transport of xenobiotics is largely dependent on the polarized nature of hepatocytes with the basolateral (sinusoidal and lateral) membrane domain interfacing sinusoidal blood and the canalicular (apical) membrane domain facing the bile duct lumen (Wang and Boyer, 2004). Basolateral transport proteins facilitate the movement of compounds to and from the blood circulation, while canalicular transport proteins favour the efflux of compounds from hepatocytes into the bile (Jigorel et al. 2006). Nomenclature for several human hepatic transport proteins, their respective substrates and regulating nuclear receptors are provided in (Table 1.1)

1.4.3.2.1 Hepatic Uptake Transport Proteins

Hepatic uptake proteins, which are predominantly located on the basolateral membrane of hepatocytes comprise: the Na+-taurocholate cotransporting polypeptide (NTCP), organic anion transporting polypeptides (OATPs), multidrug resistance-associated proteins 1, 3 and 4 (MRP1, 3 and 4), and organic anion and cation transporters (OATs and OCTs family) (Mottino and Catania, 2008). NTCP is considered as one of the most important hepatic uptake transporter due to its extensive involvement in the sodium-dependent basolateral uptake of both conjugated and unconjugated bile acids with a 2:1 sodium-to-taurocholate stoichiometry (Kemp et al. 2005). The inward direction of the Na+ gradient, that is maintained by the activity of Na+/K+ ATPase acts as the driving force for NTCP (Meier and Stieger, 2002). Furthermore, NTCP also mediate the transport of sulfated bile acids (chenodeoxycholate-3-sulfate and taurolithocholate-3-sulfate), steroid sulfates, and certain drugs, namely rosuvastatin and cholorambucil-taurocholate (Trauner

and Boyer, 2003; Ho et al. 2006). Mounting evidence correlated reduced hepatic uptake of bile acids to ethnicity-dependent polymorphisms in the human NTCP gene, further confirming the role of the latter in transporting bile acids from the blood into the liver (Ho et al. 2004). The family of organic anion transporting polypeptides (OATPs) proved to be in charge of the sodium independent uptake of a wide variety of amphipathic compounds comprising organic anions, bile acids, hormones, eicosanoids, steroids and various drugs containing bulky molecules with cationic groups (Hagenbuch and Meier, 2004; Mottino and Catania, 2008). Amongst the identified 11 human OATP isoforms, the human liver expresses exclusively OATP1B1 and OATP1B3; in addition to OATP1A, OATP1A2 and OATP2B1, which are co-expressed in several tissues (Konig et al. 2000). Commonly, OATPs function as bi-directional transporters in a way that the uptake of substrates from the blood to the liver is driven by the counter-transport of compounds such as reduced glutathione from the liver to the blood (Mottino and Catania, 2008). For example, Oatp1a1 mediated a bi-directional transport of substrates and anions, precisely GSH and HCO3-) (Li et al. 1998; Li et al. 2000). OATP1B3 favoured the co-transport of bile acids and reduced glutathione (Briz et al. 2006). In spite of their varying substrate affinity profiles, there still exist overlapping substrates for OATP1A2, 1B1 and 1B3, such as bile acids, estrone-3-sulfate, bromosulfophthalein (BSP), and dehydroepiandrosterone sulfate (DHEAS) (Konig et al. 2000). In addition to NTCP and OATPs, the human liver also expresses the organic anion transporter 2 (OAT2) and the organic cation transporter 1 and 3 (OCT 1 and 3) that function as ion exchangers (Sun et al. 2001; Choi and Song 2008). OAT2 is implicated in the hepatic uptake of negatively charged compounds whereas OCTs mediate the transport of small, formerly known as type I cations (Sun et al. 2001; Choi and Song, 2008). OCT1, which is solely expressed in the liver, facilitates the transport of numerous therapeutic drugs such as imatinib, metformin, and famotidine, while OCT3 selectively transports endogenous substrates (Sun et al. 2001; Choi and Song, 2008) (Table 1.2). It is important to note that hepatic uptake transporters play crucial roles in determining the pharmacokinetics and toxicokinetics of drugs; since the hepatic clearance of drugs depends predominantly on their hepatic uptake (Shitara et al. 2006). Hence, drug-induced modification in the expression and/or activity of important uptake transporters may alter its hepatic elimination, leading to toxic drug accumulation in the plasma (Shitara et al. 2006).

1.4.3.2.2 Hepatic efflux Transport Proteins

The vast majority of canalicular drug efflux transport proteins, with the exception of the recently-identified MATE 1 organic cation-H+ exchanger, are members of the ATP binding cassette (ABC) family of proteins which, is extensively involved in the ATP-dependent transport of solutes (Terada and Inui, 2008). Several important ABC-drug efflux proteins are situated on the canalicular membrane of hepatocytes, these include: the multidrug resistance protein 1 (MDR1 and MDR 3), the bile salt export pump (BSEP), the multidrug resistance associated protein 2 (MRP2) and the breast cancer resistance protein (BCRP) (Annaert et al. 2007). MDR1 (ABCB 1), commonly known as P-glycoprotein, was discovered over two decades ago in multidrug resistant (MDR) tumor cells and represents the most thoroughly studied ABC transport

protein (Juliano and Ling, 1976). It is known to be extensively involved in the biliary excretion of large hydrophobic and cationic substrates such as anti-cancer agents, cardiac glycosides, immunosuppressants, and antiretrovirals (Petrovic et al. 2007). Given the tight correlation between MDR1and the efflux of various chemotherapeutic agents, much interest was attributed to the identification of selective MDR1 modulators with the ability to increase drug sensitivity during chemotherapy despite the overexpression of MDR1 on the surface of tumor cells (Honma et al. 2002). MDR3 (ABCB4) is defined as a phospholipid flippase that mediates the biliary secretion of phospholipids and cholesterol; hence MDR3 contributes essentially to normal liver physiology in human and rats since phospholipids and cholesterol are responsible for the micellar solubilisation of bile acids in the lumen of the bile canaliculus (Carrella and Roda, 1999). MDR3 gene mutations result in progressive familial intrahepatic cholestasis type 3 (PFIC3), which can eventually progress to biliary cirrhosis (Carrella and Roda, 1999).When compared to MDR1, MDR3 exhibit similar substrate specificity but lower rate of transport (Evers et al. 2000). The bile salt export pump BSEP (ABCB11), the major bile acid efflux transport protein, is predominantly involved in the biliary excretion of both conjugated and unconjugated bile acids (Petrovic et al. 2007). However, earlier studies demonstrated that BSEP is not strictly limited to bile acids but may also mediate the transport of other substrates such as pravastatin (Kullak-Ublick et al. 2000; Hirano et al. 2005). Mutations in the ABCB11 gene and absence of BSEP protein expression commonly result in progressive familial intrahepatic cholestasis type 2 (PFIC2) (Petrovic et al. 2007). The latter causes a potent increase in the intracellular concentration of detergent-like bile acids leading to hepatocellular injury and necrosis (Kullak-Ublick et al. 2004). Drug-induced inhibition of BSEP by troglitazone, bosentan, cyclosporine A and rifampicin has been closely correlated with drug-induced liver injury (Fattinger et al. 2001; Mita et al. 2006). MRP2 (ABCC2) plays a pivotal role in the biliary excretion of organic anions and anionic conjugates such as glutathione conjugates, bilirubin-diglucuronide, bile acid conjugates as well as several important drugs (Cantz et al. 2000) (Table 1.2). The absence of MRP2 has been associated with Dubin-Jonson syndrome, an autosomal recessive disorder that causes defective biliary excretion of conjugated bilirubin (Petrovic et al. 2007). BCRP, (ABCG2) is prominently expressed, as a functional homodimer, in the canalicular membrane of the liver as well as in the breast, intestine, and mostly in placenta (Petrovic et al. 2007). This transporter belongs to the ABC half-transporter protein family and is involved in the transport of estrone-3-sulfate as well as various sulfated steroid compounds and several anti-cancers (Petrovic et al. 2007). Prominent evidence elucidated that BCRP is predominantly responsible for the decreased efficacy of several anti-cancer drugs namely methotrexate, mitoxantrone, topotecan and doxorubicin by extensively favouring their rapid extracellular efflux (Vethanayagam et al. 2005). Lately, numerous mammalian orthologues of the bacterial multidrug and toxin extrusion (MATE)-type transporters have been identified (Otsuka et al. 2005). In humans, the isoform MATE1 resides on the hepatic bile canalicular membrane (Otsuka et al.2005). The inwardly directed H+ gradient is considered as the driving force for MATE1-mediated excretion of organic cations (Otsuka et al. 2005). Although the majority of efflux

transport proteins reside on the canalicular membrane of hepatocytes, some of them are settled on the basolateral membrane; the ATP-dependent multidrug resistance-associated protein subfamily represents the main class of efflux transport proteins on the hepatic basolateral membrane (Annaert et al. 2001). MRP1, 3, 5, and 6 mediate the efflux of both hydrophobic uncharged molecules and hydrophilic anionic compounds (Annaert et al. 2007). MRP1 (ABCC1) is involved in the efflux of various organic anions comprising glucuronide-, glutathione-, and sulfate-conjugated drugs (Annaert et al. 2007). MRP1 displays a poor expression on the basolateral membrane of a healthy liver; however its expression is readily induced during sever liver injury probably to protect the liver from the injurious threat (Wilson et al. 2003). MRP3 (ABCC3) mediates the hepatic excretion of glucuronide conjugates and methotrexate and elucidates an increased expression under cholestatic conditions (Hirohashi et al. 1999). MRP4 (ABCC4) and MRP5 (ABCC5) are implicated in the transport of cyclic nucleotides such as cAMP and cGMP, as well as the purine analogs 6-mercaptopurine and 6-thioguanine (Zhou et al. 2001). Specifically, MRP4 demonstrated a strong implication in the transport of both, nucleoside-like drugs (zidovudine, lamivudine, and stavudine) and non-nucleotide substrates (methotrexate), as well as the transcriptase inhibitor azidothymidine and certain sulfate conjugates of bile acids and steroids (Zelcer et al. 2003). The basal expression level of MRP5 is considered to be relatively low in healthy liver; however treatment with lipopolysaccharide (LPS) elicited a potent increase in the expression of MRP5, which is commonly compensated by a down-regulation in MRP2 expression (Annaert et al. 2007). The latter suggests that MRP5 may be implicated in the hepatic response to cholestasis (Donner et al. 2004). MRP6 is co-localized at the apical as well as at the lateral side domains; where it mediates the transport of glutathione conjugates and BQ-123, an endothelin receptor antagonist (Annaert et al. 2007). MRP6 gene mutation results in pseudoxanthoma elasticum (PXE), a familial systemic connective tissue disorder targeting the eyes, skin, and blood vessels (Perdu and Germain, 2001).

1.4.3.3 Role of nuclear receptors

In the light of the drastic species evolution, living organisms succeeded to establish efficient protective strategies against the harmful effects of various xenobiotics. These strategies generally comprise two major groups of players: i) nuclear receptors or xenosensors and ii) xenobiotic metabolising and transporter systems (XMTS) (Eloranta et al. 2005). It is noteworthy that these two groups interact together in a highly coordinated manner to protect the liver as well as other organs from xenobiotic-induced toxicity; however nuclear receptors have demonstrated prominent roles in the regulation of a wide array of drug metabolizing enzymes and efflux transporters (Teng and miller, 2008). Several nuclear receptors such as the aryl hydrocarbon receptor (AhR), the nuclear factor-erythoroid 2 p45-related factor 2 (Nrf2) and the orphan nuclear receptors proved to play crucial roles in the modification of both the expression and activity of a wide range of drug metabolizing enzymes and transporters (Xu et al. 2005). The AhR is a highly polymorphic nuclear receptor which is known to recognize a wide array of chemical structures comprising non-aromatic and non-halogenated compounds (Xu et al. 2005). It binds to DNA as a heteromeric and transcriptionally regulates the expression of several genes

involved in drug metabolism and elimination (Xu et al. 2005). The family of orphan nuclear is an important subset of nuclear receptors that predominantly interacts with steroid-based ligands; namely this family includes: pregnane X receptor (PXR), the constitutive androstane receptor (CAR), the farnesoid X receptor (FXR), the liver X receptor (LXR), the peroxisome proliferator-activated receptor (PPAR) and retinoid X receptor (RXR) (Xu et al. 2005). The majority if not all of the nuclear receptors possess two crucial functional domains, namely the N-terminal DNA-binding domain (DBD), which consists of two DNA-binding zinc fingers, and a C-terminal ligand-binding domain (LBD), which is known to form the hydrophobic pocket into which the ligand binds (Xie et al. 2004). These receptors are normally located in the cytosol where they are activated by a wide range of endogenous compounds and therapeutic drugs (Xu et al. 2005) (Table 1.1). They are known to function as ligand-induced transcription factors; accordingly binding of certain ligands stimulates their translocation from the cytosol to the nucleus, as heterodimers or homodimers, where they modulate the transcription of several genes by binding respectively to their specific DNA response elements (Xu et al. 2005). Two members of the orphan nuclear receptors family, precisely the xenobiotic receptors PXR and CAR, proved to regulate gene expression by forming heterodimers with the retinoid X receptor (RXR) (Xie et al. 2004). This regulation takes place after the subsequent binding of the PXR–RXR or CAR–RXR heterodimers to their specific xenobiotic response elements (XREs) present in the promoter regions of drug-metabolizing enzymes and transporters (Xie et al. 2004). The ability of PXR and CAR to interact with a broad array of small lipophilic molecules including various therapeutic drugs and xenobiotic in addition to the prominent presence of their respective response elements in the promoter region of numerous drug-metabolizing and transporter genes explained their implication in the regulation of nearly all phases of hepatic drug metabolism and elimination (Xie et al. 2004). PXR and CAR demonstrated overlapping ligand specificity; hence their activation may result in the increased expression of the same enzymes due to stimulation of similar response elements (Goodwin et al. 2001; Maglich et al. 2002). Recent evidence elucidated that these receptors are efficiently implicated in the regulation of numerous physiological and pathophysiological processes, such as glucose homeostasis, lipid metabolism and inflammatory response (Chang, 2009). Nuclear receptors that seem to bind exclusively to endogenous ligands such as the farnesoid X receptor (FXR) and the vitamin D receptor (VDR) have also demonstrated a marked implication in the regulation of drug-metabolizing pathways (Xu et al. 2005). Although PXR has been classified as a xenobiotic receptor, mounting evidence revealed an equally efficient potential of PXR as an ‘endobiotic receptor’ that responds to a vast array of endogenous compounds (Chang, 2009). FXR plays a central role in maintaining bile acid homestasis in the enterohepatic circulating system by regulating the expression of several enzymes and transporters related to the synthesis and elimination of bile acids (Xu et al. 2005). Furthermore, it has been proven that FXR also regulates triglyceride and cholesterol metabolism, hence constituting a molecular link between lipid and bile acids metabolism (Lefebvre et al. 2009). Interestingly, all of PXR, FXR and VDR proved capable of binding and responding to bile acids, emphasizing that drug metabolism is interrelated with cholesterol and bile acid

homeostasis (Eloranta et al. 2005). CAR seems to be implicated in bilirubin and energy homeostasis, although its explicit roles as an endogenous regulator remain unclear (Goodwin and Moore, 2004). Depending on the nature of both the receptor and the ligand, nuclear receptors may positively or negatively regulate the expression of genes encoding several metabolizing enzymes such as cytochrome P450 (CYP), UDP glucuronosyltransferase (UGT), glutathione S-transferase (GST), sulfotransferase (SULT) as well as various transport proteins (Xu et al. 2005)

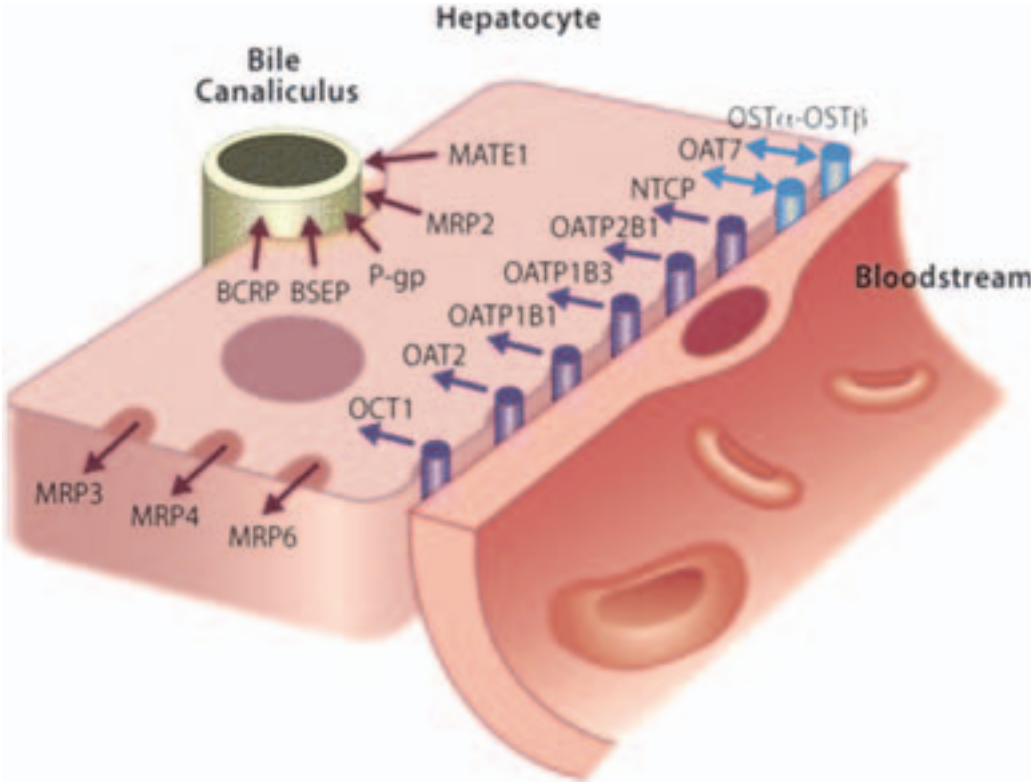


Figure 1.4. Illustration of the different transport protein found on the basolateral and apical membranes of hepatocytes. *(Adapted from Köck and Brouwer, 2012).*

Table 1.1. Major hepatic drug transporters: nomenclature, function and nuclear receptor regulation.
(Adapted from *Liddle and Stedman, 2008)*

Trivial Symbol (Gene Symbol)	Name	Nuclear Receptor	Function and Substrates
Basolateral membrane of hepatocyte			
OATP1B1 (SLCO1B1)a **OATP1B3 (SLCO1B3)b**	Organic anion-transporting proteins	FXR, HNF1α, PXR	Hepatic uptake of organic anions and cations like: enalapril, digoxin, HMG-CoA reductase inhibitors, BA, bilirubin, statins; Simvastatin, Irinotecan metabolite (SN-38), Methotrexate, Rifampin
OCT1 (SLC22A1)	Organic cation transporter		Hepatic uptake of hydrophilic organic cations e.g. cimetidine, choline, dopamine, acyclovir, zidovudine
OAT2 (SLC22A7)	Organic anion transporter		Hepatic uptake of organic anions and drugs e.g. methotrexate, NSAID, 5-Fluorouracil, Allopurinol, Erythromycin, Methotrexate, Ranitidine, Tetracycline,Zidovudine
NTCP (SLC10A1)	Na+-taurocholate cotransporting polypeptide	SHP/FXR	Na+-dependent uptake of conjugated BA from portal blood and other compounds like: Rosuvastatin, Bromosulfophthalein (BSP)
MRP1 (ABCC1)	Multidrug resistance associated proteins	PXR	Drug export from hepatocytes e.g. colchicine, etoposide, Daunorubicin, Doxorubicin, vindcristine, Methotrexate
MRP3 (ABCC3)		PXR, FTF	Organic solute transporter: extrudes BA conjugates, methotrexate, etoposide, Acetaminophen , Etoposide, and Fexofenadine
MRP4 (ABCC4)			Mediates glutathione efflux from hepatocytes into blood by cotransport with BA; also exports purine and nucleoside analogues, Azidothymidine and Methotrexate
Canalicular membrane of hepatocyte			
MDR1 (ABCB1)	Multidrug resistance-1	PXR	Excretion of organic cations, xenobiotics and cytotoxins to bile e.g. colchicine, doxirubicin, adriamycin, vinblastine, paclitaxel, vincristine, nelfinavir, Atorvastatin, Aldosterone; corticosterone; dexamethasone, Digoxin, Cyclosporin A, Mitoxantrone, Talinolol Erythromycin, Lovastatin, Doxorubicin, paclitaxel, Etoposide, Levofloxacin, Grapafloxacin, Losartan, Vinblastine, Verapamil
MDR3 (ABCB4)	Multidrug resistance-3	FXR, PPARα	Phospholipid export pump: translocates phosphatidylcholine from inner to outer leaflet of membrane bilayer
MRP2 (ABCC2)	Multidrug resistance-associated protein-2	PXR,CAR	Mediates multispecific organic anion transport into bile e.g. bilirubin diglucuronide, sulphates, glutathione conjugates,vinblastine, Camptothecin, Doxorubicin, Cerivastatin, Cisplatin, Vincristine, Etoposide, Glibenclamide, Indomethacin,
BSEP (ABCB11)	Canalicular bile salt export pump	FXR	ATP-dependent transport of monovalent bile salts, Fexofenadine, Pravastatin and paclitaxel into bile
FIC1 (ATP8B1)	Familial intrahepatic cholestasis-1		Potential aminophospholipid translocating ATPase
AE2 (SLC4A2)	Chloride–bicarbonate anion exchanger isoform-2		Facilitates bicarbonate secretion into bile, stimulates BA independent bile flow
ABCG5/ ABCG8	'Half ABC transporters'	FXR,PXR LXR	Transport sterols into bile and partially mediate biliary cholesterol secretion
BCRP (ABCG2)	'Half ABC transporters'		Mediates cellular extrusion of sulphated conjugates and various drugs like Daunorubicin, Doxorubicin, Metaxantrone, Prazosin, Ciprofloxacin, Ofloxacin, Norfloxacin, Cerivastatin, Dirithromycin; Erythromycin, Rifampicin, Irinotecan, Topotecan, Imatinib, Methotrexate, Zidovudine

Table 1.2. Overview of nuclear receptors including their ligand, target genes and function. *(Adapted from Zollner et al. 2010).*

Nuclear Receptor	Drug/Ligand	Transcriptional activation of genes	Function
Pregnane X receptor (PXR/RXR)	rifampicin, phenobarbital, dexamethasone, statins, clotrimazole, paclitaxel	CYP2B6-3A4-2C6-2C19-1A2-3A7-7A1-2C9, UGT1A2,SULT2A1,GSTs MRP2, BSEP, MDR1, MDR2,	BA synthesis, transport and detoxification Drug metabolism and detoxification
Constitutive androstane receptor (CAR/RXR)	bilirubin, phenobarbital, dimethoxycoumarin, dimethylesculetin, acetaminophen, phenytoin	CYP2A6-2B1-2B2-2B10-2B6-2C9-2C19-C29-3A4, SULT1A-2A1-2A9, UGT1A1, MRP2-3-4	BA synthesis, transport and detoxification Drug metabolism and detoxification
Farnesoid X receptor (FXR/RXR)	Deoxycholic, chenodeoxycholic and cholic acid	CYP3A4, SULT2A1, UGT2B4, UGT2B7, MRP2, ASBT, SHP, BSEP, MDR3, MDR2	Regulation, synthesis and transport of BA Drug detoxification
Liver X receptor (LXR/RXR)	Oxysterols, fatty acids, 6α-hydroxylated bile acids	CYP7A1, SULT2A9, MRP2-4	BA synthesis,transport and detoxification
Peroxisome proliferatoractivated receptor (PPARα)	Fatty acids, statins, eicasaonoids, leukotriens, NSAIDs	CYP7A1, SULT2A1, UGT2B4, UGT1A3, MDR2, ASBT	BA synthesis,transport, and detoxification Indirect induction of FXR
Aryl hydrocarbon receptor (AhR)	Halogenated & polycyclic aromatic hydrocarbons, tryptamine, bilirubin, biliverdin, lipoxin A4	CYP1A1-1A2-1B1, UGT1A1-1A6, GSTA1, MRP3-5-6, BCRP, NRF2	Drug detoxification
Nuclear factor E2-related factor 2 (NRF2)	Reactive oxygen species	MRP1-2-3-4, MRP3, BSEP, GSTS,UGTS CYP1A2-2A4-2A12-3A13-4A10-4A14-C39	BA transport Drug Metabolism

1.5 Role of drug metabolism in drug-induced hepatotoxicity

One of the liver's major physiological functions is the metabolism of lipophilic drugs into hydrophilic metabolites via hydrolytic phase I and conjugating phase II pathways in the aim of facilitating their elimination (Kalgutkar et al. 2005). The extensive involvement of the liver in drug metabolism and its continuous exposure to elevated concentrations of drugs/metabolites after oral administration renders it a primary target for drug-induced hepatotoxicity (Russman et al. 2009). Hepatotoxicity is defined as a hepatic injury correlated with impaired liver function following sufficient exposure to a drug or any other non-infectious agent (Navarro and Senior, 2006). Often, typical hepatic metabolism reactions are considered as detoxification processes that serve to attenuate the biological activity of the parent drug following exposure. However, occasionally and depending on the structural features of certain drugs, the same metabolic events may produce chemically reactive and toxic metabolites (Park et al. 2005). The metabolic transformation of relatively harmless compounds to reactive electrophilic metabolites is commonly referred to as metabolic activation or bioactivation (Park et al. 2005). Bioactivation proved to be a potent contributor to certain drug-induced toxicities, including hepatotoxicity, cutaneous ADRs, and blood dyscrasias (Kaplowitz, 2004). The precise pathogenic mechanism

underlying drug-induced hepatotoxicity has not been clearly elucidated to date; however sufficient evidence proved that DILI arises via two main pathways: direct hepatotoxicity and adverse immune reactions (Kaplowitz et al. 2004; Adams et al. 2010). Direct drug-induced hepatotoxicity proved to be tightly correlated with the process of bioactivation which yields reactive metabolites possessing the ability to covalently bind to proteins, nucleic acids, lipids and other cellular macromolecules resulting in protein dysfunction, DNA damage, lipid peroxidation, and oxidative stress (Holt and Ju, 2006). Furthermore, the bio-transformed reactive metabolites may cause loss of energy production and mitochondrial dysfunction, through depletion of intracellular calcium stores and disruption of ionic gradients, leading to hepatocellular death and liver failure (Holt and Ju, 2006). Subsequently, hepatic dysfunction and death of liver cells stimulate strong immune responses involving both innate and acquired immunological reactions (Adams et al. 2010). These reactions are mediated by several members of the innate immune system such as Kupffer cells (KC), natural killer (NK) cells, and natural killer T-cells after being activated by stimulatory signals released from stressed or damaged hepatocytes (Holt and Ju, 2006). These immune cells are known to produce pro-inflammatory factors and chemokines, such as TNF-α, IFN-gamma and IL-1β, which serve to recruit additional inflammatory cells to the liver promoting hepatic injury and tissue damage (Ramadori et al. 2008). Nevertheless, innate immune cells may also produce hepatoprotective factors such as IL-10 and IL-6 (Holt and Ju, 2006). Accordingly, an individual's increased susceptibility or sustained adaptation to DILI is largely dependent on the adequate balance between pro-inflammatory and hepatoprotective mediators. In addition to the evident implication of the innate immune system in the progression of drug induced liver injury adaptive immune reactions demonstrated a noticeable contribution in the pathogenesis of hepatotoxicity (Holt and Ju, 2006). This contribution is principally related to the hapten hypothesis, which suggests that the covalent binding of a drug or its reactive metabolites to essential hepatic proteins leads to the formation of "foreign" proteins (Holt and Ju, 2006). Subsequently, the latter will potently activate both B- and T-cells to release antibodies and cytotoxic mediators respectively; thus leading to a prolonged activation of the adaptive immune system which will consequently exacerbate hepatic injury (Matzinger, 2002).

1.6 Drug-induced liver injury (DILI)

Drug-induced liver injury and drug-induced hepatotoxicity are two different terms that are being used interchangeably to indicate hepatic damage caused by the toxic effects of a certain drug. Recent statistical studies attributed 50 % of acute liver failure cases to DILI considering it the leading cause for liver transplantation worldwide (Adams et al. 2010). Drug-induced hepatotoxicity is the most frequent reason for post-marketing drug withdrawal (Russman et al. 2010) with more than 1000 drugs being reported to cause liver injury (Weiler-Normann and Schramm, 2011). Recent examples of drugs withdrawn from the market or attributed "blackbox warnings" in USA and Europe are troglitazone, bromfenac, trovafloxacin, ebrotidine, nimesulide, nefazodone, telithromycin and ximelagatran (Shah, 1999; Mohapatra, 2005; Echols, 2010). With few exceptions, the majority of these drugs are implicated in idiosyncratic adverse reactions since the development of intrinsically hepatotoxic drugs is terminated during early

phases of drug development (Weiler-Normann and Schramm, 2012). Drugs may cause several types of hepatic injury depending on a convergence of drug-related and host-dependent features (Schjøtt, 2011). Accordingly, drug-induced hepatotoxicity is divided to two main subtypes: (1) intrinsic and (2) idiosyncratic hepatotoxicity (Russman et al. 2009). These subtypes display opposing characteristics and exhibit distinct modes of action (Roth and Ganey, 2010). Intrinsic hepatotoxicity is easily predictable and dose-dependent; it is often caused by the exposure of the liver to high doses of the parent drug or its respective metabolite, which overwhelms the hepatic defence system causing toxic adverse drug reactions (Schjøtt, 2011). Opposingly, idiosyncratic hepatotoxicity exhibits no clear relation to drug-dose and seems to arise in susceptible individuals based on genetic and environmental predisposing factors (Schjøtt, 2011). Table 1.3 summarizes the main differences between intrinsic and idiosyncratic hepatotoxicity. The susceptibility to DILI in addition to its pathogenic mechanisms and the clinical signs and symptoms accompanying it are poorly understood, complicating its adequate diagnosis by physicians and healthcare professionals (Ghabril et al. 2010). This is may be attributed to two main factors: Firstly, to the heterogenic mode of action of DILI and to its confounding clinical manifestation, which may be similar to any liver disease ranging from mild elevations in hepatic enzymes to fulminant liver failure on one hand; and secondly to the lack of accurate and specific 'gold standard' diagnostic tests for DILI (Andrade et al. 2007; Ghabril et al. 2010).

Table 1.3. Main differences between intrinsic an idiosyncratic hepatotoxicity.
(Adapted from Roth and Ganey, 2010).

Intrinsic Hepatotoxicity	Idiosyncratic Hepatotoxicity
Affects all individuals at certain dose → dose-dependent	Attacks only susceptible individuals → dose-independent
Related to pharmacological target of drug	Unrelated to pharmacological target of drug
Predictable latent period after exposure	Variable onset relative to exposure
Distinctive liver lesions	Variable liver pathology
Predictable using routine animal testing	Not predictable using routine animal tests

1.6.1 Pathways of drug-induced liver injury

Drug-induced liver injury remains to date an unsolved clinical problem leading to the majority of acute liver failure and transplantation cases worldwide (Grattagliano et al. 2009). This may be attributed partly to the ambiguity of hepatotoxic mechanisms and partly to the lack of efficient pre-marketing detection of potentially hepatotoxic drugs (Cosgrove et al. 2009). Accordingly, the identification of the mechanisms underlying drug-induced liver injury may provide helpful

insights for its prediction and optimal prevention (Grattagliano et al. 2009). The spectrum of injury following drug exposure extends from mild damage to severe hepatic failure; therefore it is highly improbable that DILI arises through a single mechanism of pathogenesis (Grattagliano et al. 2009). Most likely drug-induced hepatotoxicity is mediated via a complex interplay of several mechanisms belonging to more than one pathway (Holt and Ju, 2006). It has been acknowledged recently that intrinsic hepatotoxicity arises predominantly via direct drug-induced hepatocellular damage whereas idiosyncratic hepatotoxicity seems to be immune mediated (Grattagliano et al. 2009). Although these two pathways are distinct, significant evidence demonstrated that they are interactive since in most cases of DILI it is the initial drug induced hepatocyte damage that elicits the activation of an immune response; subsequently, activated immune cells will propagate an inflammatory reaction that will in its turn prevent hepatic regeneration and repair, promoting hepatocellular death (Holt and Ju, 2006).

1.6.1.1 Direct drug-induced hepatotoxicity

Direct hepatotoxicity is typically caused by the direct damaging action of a certain drug or more frequent its reactive metabolite on hepatocytes. The manner through which a drug or its metabolite directly induces damage to intracellular organelles defines the clinical manifestation of drug-induced liver injury (Lee, 2003). A drug or its reactive metabolite may induce initial hepatocellular damage through various mechanisms (Figure 1.5) including:

(1) Biotransformation and reactive metabolite formation

The biotransformation of hydrophobic drugs to more hydrophilic substances is primordial for their urinary or biliary excretion (Schjøtt, 2011). The presence of underlying genetic polymorphism or drug-induced modifications in key metabolic enzymes CYP450 may alter the process of biotransformation thus contributing to the toxic generation of reactive metabolites (Schjøtt, 2011). Likewise, drug-induced modulation of important nuclear receptors such as PXR and CAR proved to alter drug absorption, distribution, metabolism and elimination also leading to toxic accumulation of reactive metabolites (Zhou et al. 2005). The latter are normally detoxified by phase II enzymes, which catalyses the conjugation of electrophilic metabolites to hydrophilic molecules. However these enzymes may become saturated when the liver is exposed to an over dose of a damaging drug (Schjøtt, 2011). Consequently, the abundantly present reactive metabolites will covalently bind to important cellular components including proteins, lipids, and nucleic acids forming damaging adducts that will result in ATP depletion, reactive oxygen generation, ionic gradient reduction, and eventually cell swelling and rupture (Lee, 2003).

(2) Oxidative stress and lipid peroxidation

The biotransformation of drugs is frequently correlated with the generation of reactive oxygen species (ROS) (Schjøtt, 2011). Metabolic enzymes that contribute to ROS formation in the liver include CYP monooxygenases and NADPH oxidase. Normally, the generation of ROS is counter-balanced by anti-oxidant defences such as glutathione (Schjøtt, 2011). However during

depletion of anti-oxidants, the extensive intracellular presence of ROS will generate an oxidative stress; the latter will interfere with various molecular and biochemical processes involved in the regulation of cell differentiation, proliferation and death (Tarantino et al. 2009). This interference depends on key signalling proteins such as protein kinase (PKC), which demonstrated a crucial role in the progression of hepatic injury (Tarantino et al. 2009). The free radicals released during oxidative stress, trigger lipid peroxidation by attacking polyunsaturated fatty acids in the plasma membrane, setting off a free radical chain reaction sequence (Tarantino et al. 2009). Subsequently, lipid peroxidation results in loss of membrane integrity and leakage of microsomal enzymes. Furthermore, it will favour the production of reactive aldehydes that can form protein and DNA adducts, which in their turn may contribute to hepatotoxicity and carcinogenicity (Tarantino et al. 2009).

(3) Disruption of mitochondrial function

Drugs may impair normal mitochondrial function by disrupting the respiratory chain or by binding to mitochondrial DNA resulting in oxidative stress, lactic acidosis, anaerobic metabolism and fat accumulation. In addition, drugs may also lead to defective ATP production and fatty acid oxidation (Pessayre et al. 2001). The hepatic accumulation of fat accompanied with an inflammatory reaction may lead to drug-induced steatohepatitis (Lee, 2003). Direct mitochondrial dysfunction is mainly correlated with reactive metabolites generation, reduced glutathione depletion and protein alkylation (Grattagliano et al. 2009). The subsequent disruption in the mitochondrial outer membrane facilitates the release of proteins and cytochrome c leading to impairment of Ca2+ homeostasis and the accumulation of Na+ resulting eventually in hepatocyte death (Jaeschke and Bajt, 2006; Miller et al. 2008). The majority of the events that result in apoptosis or necrosis proved to be dependent on the impaired function of the mitochondria, on the permeabilization of its outer membrane and on ATP depletion (Pessayre et al. 1999). Drugs may exert their adverse effects on mitochondria either directly or indirectly. Direct mitochondrial damage results from drugs like isoniazid and rifampicin after their metabolic bioactivation; whereas indirect mitochondrial damage is mediated either through drug-induced functional impairment of the endoplasmic reticulum, as is the case with paracetamol or through lysosomal dysfunction (Chowdhury et al. 2006).

(4) Drug-induced disruption of bile flow

Drugs may impair normal bile flow either by disrupting subcellular actin filaments or by inhibiting key transport pumps; thus resulting in cholestasis and jaundice (Zollner et al. 2008). Consequently, the heptobiliary elimination of drugs and other endogenous compounds is impaired; causing hepatocellular damage which, either results from the accumulation of the damaging drug itself or from the toxic accumulation of bile acids (Zollner et al. 2008).

1.6.1.2 Immune mediated drug-induced hepatotoxicity

The liver is considered as a site of powerful immunological activity since it comprises numerous innate and adaptive immune cells including the biggest populations of kupffer cells and natural killer cells (Adams et al. 2010). As much as this fact is considered beneficial in enhancing the protective potential of the liver against a wide array of pathogens and external threats; it is detrimental in hepatotoxicity since it plays an important role in reducing the liver's regenerative potential thus progressing and aggravating liver injury. The mechanisms underlying immune mediated hepatotoxicity are largely dependent on one hand on the composition of hepatic innate and adaptive immune cells and on the other hand on the peculiar response of the liver to endobiotics and exobiotics (Holt and Ju, 2006). The initial drug-induced damage to hepatocytes seemed to trigger the activation of various innate immune cells in the liver resulting in strong inflammation and extensive cytokine release (Holt and Ju, 2006). The abundant release of cytokines, chemokines, reactive oxygen species and reactive nitrogen species from activated immune cells favoured the prolongation of liver injury either indirectly by sensibilizing the liver to the toxic effects of a certain drug or directly by inducing hepatocellular damage as is the case with interferon gamma and Fas ligand (Holt and Ju, 2006). In addition to the involvement of the innate system in immune mediated hepatotoxicity several clinical manifestations of DILI indicate that the adaptive immune system also plays an important role in drug-induced immune mediated liver injury (Holt and Ju, 2006). These manifestations comprise a delayed response with respect to the initial reaction (1-8 weeks) including the concurrent presence of fever, rash and increased eosinophil counts (Gunawan and Kaplowitz, 2004). In addition a speedy recurrence of toxicity is often observed upon drug re-exposure due to the presence of anti-drug antibodies or autoantibodies against altered hepatic proteins following drug-protein adduct formation (Gunawan and Kaplowitz, 2004). Several drugs like halothane, diclofenac, phenytoin tienilic acid, dihydralazine and carbamazepine demonstrated a prominent correlation with adaptive immune mediated hepatotoxicity (Zimmerman, 2000). This type of hepatotoxicity is predominantly based on two main concepts, the" *p-i* (*p*harmacological interaction of drugs with *i*mmune receptors) concept*"* and the "hapten/pro-hapten concept" (Schnyder and Pichler, 2009). The latter states that drugs or more frequently their reactive metabolites covalently bind to major intracellular proteins forming adducts that will express themselves as haptens on the surface of antigen presenting cells (Jose and Marta Castell, 2006). Subsequently this MHC-dependent immunogenic expression of haptens, on the surface of antigen presenting cells, will induce the production of antibodies against them on one hand; or auto-antibodies against modified drug-bound cellular components on the other hand (Russman et al. 2009). Dendritic cells play a central role in such immune responses since they serve to activate several adaptive immune reactions such as the activation of B and T-lypmphocytes (Schnyder and Pichler, 2009). The activation of B-lymphocytes stimulates the release of antibodies and kinines and activates the complement system; whereas the activation of both CD4-expressing and CD8- expressing T-lymphocytes induces the release of lymphokines or exhibit direct cytotoxicity via cell surface antigen expression and the secretion of granzymes and perforin, respectively (Swain, 2008). The

p-i concept implies that some drugs may illicit an immune mediated liver injury through the direct activation of T-cells by binding reversibly to various antigen-specific TCR in a direct way, indicating that not all drugs need to covalently bind to intracellular proteins and modulate them in order to illicit an immune response (Gerber and Pichler, 2006). The resultant drug-TCR interaction does not require prior metabolism or bioactivation, instead the parent drug seems to readily bind TCR just like it would do with non-immunological receptors (Gerber and Pichler, 2006). The *p-i* concept states that for T cells to be activated by drugs they must display three major properties: (1) prominent TCR expression favoring drug binding and the subsequent stimulation of a noticeable immune response; (2) low activity threshold allowing the response of T-cells to weak signals such as drug-TCR binding; (3) synergistic interaction between TCR and MHC on antigen presenting cells in addition to drug-TCR interaction which is required to amplify the drug-induced immune response (Schnyder and Pichler, 2009). Last but not least, it has been proven that the local supply of oxygen during drug metabolism exhibits a prominent influence on immune mediated hepatotoxicity (Grattagliano et al. 2009). Halothane serve as a good example of this fact since metabolizing it in the absence of oxygen leads to mild hepatitis; whereas its metabolism in the presence of abundant oxygen concentrations results in massive hepatic necrosis (Grattagliano et al. 2009).

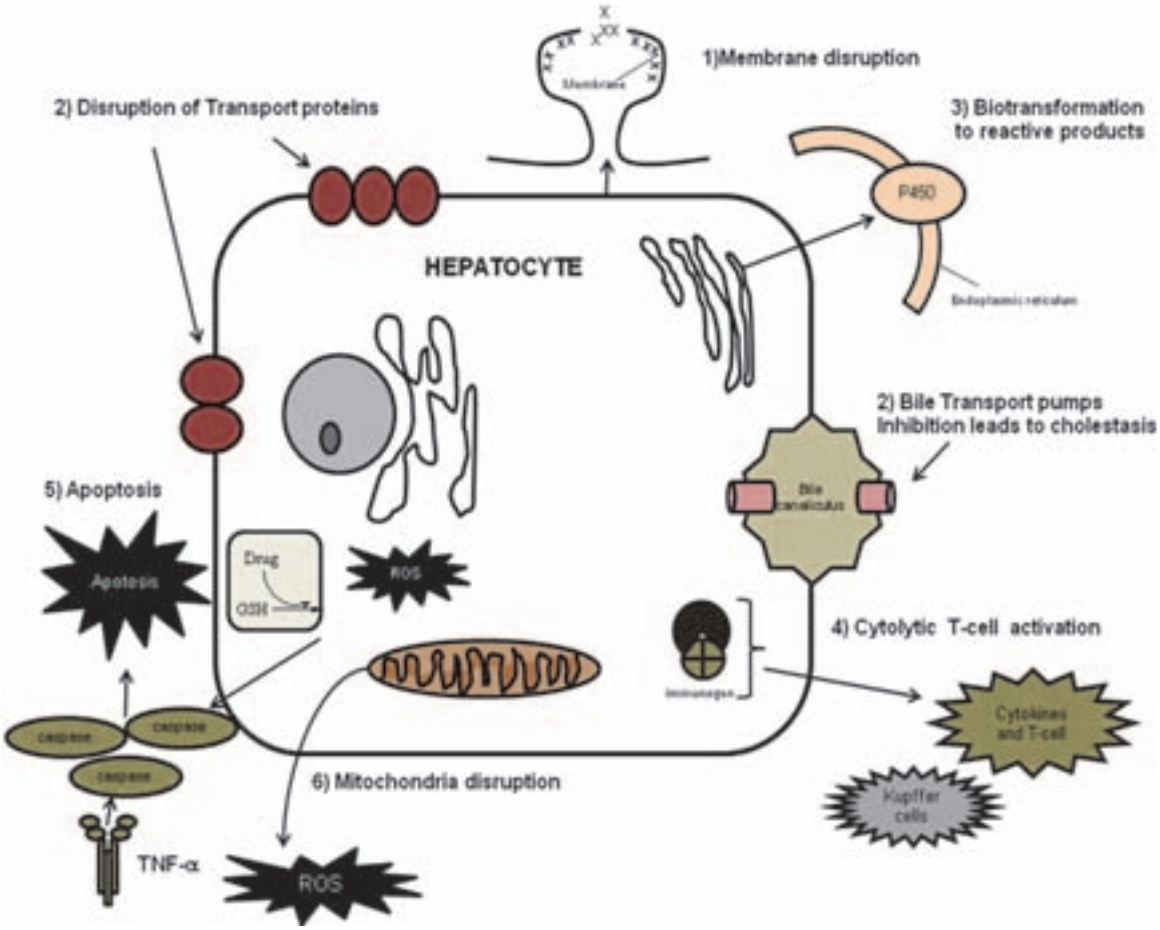


Figure 1.5. Mechanisms of hepatotoxic injury. Common cellular sites of hepatocyte injury are shown: 1) Membrane disruption (X) leads to disassembly of actin fibrils at the cell surface causing blebs and rupture of membrane; 2) Disruption of transport proteins or specifically bile transport proteins at the canalicular membrane interrupting bile flow; 3) Biotransformation by cytochrome P450 to reactive metabolites; 4) Cytolytic T-cell activation by resident macrophages, Kupffer cells in response to protein drug adduct acting as immunogen; 5) Apotosis by TNF-α or fas mediated activation of caspase pathway; and 6) Mitochondria disruption resulting in oxidative stress (ROS). *(Adapted from Lee, 2003).*

1.6.2 Major mechanisms of hepatocellular death

Regardless if drug-induced hepatotoxicity is mediated through direct drug-induced hepatocellular damage or through immune mediated liver injury; it is the resultant hepatocellular death that leads to severe liver injury resulting eventually in fatal hepatic failure (Kaplowitz, 2004; Chakraborty et al. 2012). Predominantly hepatocellular death occurs via two major mechanisms: Apoptosis and necrosis (Chakraborty et al. 2012).

1.6.2.1 Hepatocellular apoptosis

Apoptosis is defined as a physiologically normal process of programmed cell death (Chakraborty et al. 2012). It is distinguished from other modes of cell death by a series of synchronized

morphological events comprising mainly: cellular shrinkage, membrane blebbing, pyknosis (chromatin condensation) and karyorrhexis (nuclear fragmentation) (Malhi et al. 2010). The eventual scission of the cell into apoptotic bodies, which are defined as membrane-enclosed compartments containing intact cellular organelles and some nuclear fragments, is considered as the hallmark of apoptosis (Malhi et al. 2010). Subsequently these apoptotic bodies will display "eat me" signals, like phosphatidylserine, on the surface of their plasma membrane, promoting their phagocytosis by various immune cells, in particularly hepatic stellate and kupffer cells (Taylor et al. 2008; Jiang et al. 2009; Canbay et al. 2003). Drug-induced hepatocyte apoptosis is mediated via the activation of two main pathways: (1) The intrinsic pathway, which results via direct drug-induced severe hepatocellular stress; and (2) the extrinsic pathway, which is mainly correlated with mild cell stress and immune mediated reactions (Russman et al. 2009). The intrinsic pathway, which is related to direct drug-induced hepatotoxicity, is predominantly regulated by the mitochondria following severe intracellular stress signals (Grattagliano, 2009). These drug-induced stress signals activate the endoplasmic reticulum and the c-jun N-terminal kinase (JNK) pathways and promote lysosomal permeabilization (Russman et al. 2009). Subsequently, the extensive up-regulation of pro-apoptotic proteins like Bax, Bak, and Bad in addition to the down-regulation of apoptotic inhibitors belonging to the Bcl-2 family will lead to mitochondrial permeability transition (MPT) (Russman et al. 2009). The occurrence of MPT favors the entrance of protons into the inner mitochondrial membrane inducing mitochondrial ATP synthesis arrest (Malhi et al. 2010). The drug-induced ATP depletion favours the expansion of the extracellular matrix and causes the permeabilization and rupture of the mitochondrial membrane releasing cytochrome c and other pro-apoptotic mitochondrial proteins into the cytosol (Malhi et al. 2010; Haouzi et al. 2000; Feldmann et al. 2000). Cytosolic cytochrome c will then bind to other pro-apoptotic proteins constituting the famous "apoptotosome" which will eventually lead to the activation of several caspases resulting in cell death (Chakraborty et al. 2012). The extrinsic apoptotic pathway is correlated with drug-induced immune mediated hepatotoxicity and seems to be activated based on the "hapten hypothesis" (Lee, 2003). The latter states that drugs are too small to elicit an immune response; however upon covalently binding to intracellular proteins they form large immunogenic drug-protein adducts (Pichler, 2006). Subsequently these adducts will express themselves as neo-antigens on the surface of APC where they trigger the release of major death ligands such as TNF-α or Fas (Grattagliano, 2009). The interaction of these ligands with their respective receptors will form what is known as the death-inducing signalling complex (DISC), which leads to the trimerization of the receptor and to the clustering of death domains subsequently activating caspase-8 (Bantel and Schulze-Osthoff, 2012). Despite the capacity of caspase 8 to directly activate caspases 3, 6 and 7 in several cells, earlier studies suggest that this direct path is too weak to induce apoptosis in hepatocytes (Russman et al. 2009). Probably, hepatocellular apoptosis is mediated through an amplified mechanism involving the synergistic activation of both the mitochondrial and the death receptor pathways as follows: TNF-α will activate the cell death apoptotic pathway by binding to its receptor TNFR1 causing the activation of caspase 8; the latter in addition to activating

effector caspases leading to extrinsic apoptosis will also cleave Bid, a member of the Bcl-2 pro-apoptotic protein family, inducing its transformation to truncated Bid (tBid); subsequently tBid will bind to the mitochondrial outer membrane inducing cytochrome c release and promoting apoptosome formation; resulting eventually in the activation of the intrinsic apoptotic pathway (Chakraborty et al. 2012). Overall, the drug-induced activation of pro-apoptotic receptors will compete with activated cell survival signals leading to a complex interplay that will either progress liver injury or will promote hepatic regeneration depending on the strength of the opposing signals (Lee, 2003). It is important to note, that hepatocytes in particular are known to be resistant to TNF-α induced cytotoxicity under normal conditions (Wullaert et al. 2007). This resistance is attributed to the fact that the interaction of TNF-α with its specific receptor TNFR-1 activates synergistically two contradictory pathways, which counter-balance each other namely: the TNF-induced death receptor pathway which promotes extrinsic apoptosis vs. the NF-Kβ pathway, which mediates the transcription of survival genes in addition to anti-apoptotic proteins and enzymes (Bcl-2, NO synthase) (Wullaert et al. 2007). Hence, it is the severity and duration of the initial stimulus that eventually decides the fate of hepatocytes; for example the exposure of hepatocytes to TNF-α alone is most probably insufficient to trigger apoptosis, however the prolonged exposure of hepatocytes to TNF-α in addition to an extensive pool of cytokines during hepatic inflammation will most probably result in hepatocellular death (Grattagliano et al. 2009). Eventually both the intrinsic and the extrinsic pathways stimulate the activation of executioner caspases and nucleases leading to apoptotic hepatocellular death (Grattagliano et al. 2009). Although apoptosis is normally considered as "clean" cell death with rare inflammation and minimal injury, it is noteworthy that hepatic apoptosis may induce an inflammatory reaction in the liver depending on the abundant presence of hepatic immune cells and their enhanced activation (Malhi et al. 2010). Hepatocellular apoptosis activate resident kupffer and stellate cells to engulf resulting apoptotic bodies; consequently, kupffer cells will express several death ligands, namely TNF- α, TRAIL and FasL whereas stellate cells will produce pro-fibrogenic cytokines such as transforming growth factor-1 (TGF-1) and type I collagen (Canbay et al. 2003). The extensive activation of both types of hepatic cells acts as an inductor of prominent inflammation and a promoter of fibrosis (Malhi et al. 2010). Moreover, recent evidence suggests that apoptotic cells may secrete the nucleotides ATP and UTP, which can bind to P2Y2 receptors on the surface of hepatic macrophages and stellate cells; thus further promoting fibrogenesis (Elliott et al. 2009). All of these recent insights indicate that hepatocyte apoptosis plays a key role in inducing hepatic inflammation and fibrosis.

1.6.2.2 Hepatocellular necrosis

Unlike apoptosis, which takes place following mild hepatocellular stress provided that enough ATP is present to activate effector caspases; drug-induced hepatic necrosis results following severe hepatocellular injury provided that the cell is depleted of ATP (Bantel and Osthoff, 2012). Frequently, drug-induced necrosis is initiated by the opening of the mitochondrial membrane permeability transition (MPT) pores, causing the destruction of membrane potential and the inhibition of ATP synthesis (Labbe et al. 2008). Subsequently, massive cellular swelling results

in the rupturing of the outer plasma membrane leading to cytolysis (Zong and Thomson, 2006). Furthermore, necrosis is often correlated with massive homeostasis disturbance, cytoskeletal modifications, lysosomal breakdown and irreversible disruptions of electrical and ion gradients (Grattagliano, 2009). Frequently, the necrotic potential of drugs, like acetaminophen, is predominantly based on their CYP450-mediated metabolism to reactive metabolites (Grattagliano, 2009). During necrosis, the latter typically causes lipid and protein oxidation as well as GSH depletion (Grattagliano, 2009). Oxidized proteins and protein adduct possess immunogenic properties through which they may activate both Kupffer and PMN cells to further release reactive oxygen and nitrogen species (ROS and RNS) (Grattagliano, 2009). The increased accumulation of ROS and RNS in the liver in addition to the formation of protein disulfides often lead to increased oxidative stress, altered calcium homeostasis, loss of mitochondrial membrane potential, depleted glutathione and ATP stores and finally cell swelling (Hinson et al. 2010). The abnormal cellular swelling in necrosis is predominantly due to oxidative stress, speedy consumption of cellular energy, and mitochondrial dysfunction which stimulates anaerobic glycolysis, leading to a decrease in intracellular pH (Grattagliano, 2009). The resulting acidic environment is normally contrasted with sodium exchange; however due to the depletion of ATP in such cases sodium cannot be exchanged and hence will accumulate inside the cell (Grattagliano, 2009). The consequent osmotic pressure leads to cell swelling, blocking the apoptotic process, which necessitates a decrease in cell volume; thus promoting cell death by necrosis (Grattagliano, 2009). A synergistic increase in cytosolic Ca^{2+} may aggravate the resulting osmotic pressure in addition to other mechanisms like nucleotide alterations and protein synthesis disruption (Barros, 2001). Commonly, necrosis occurs in concert with an inflammatory reaction, which arises following necrotic cell lysis leading to the extensive release of pro-inflammatory mediators (Russman et al. 2009). The abundant presence of the latter will sensitize the liver to the toxic adverse effects of certain drugs thus amplifying hepatic injury (Russman et al. 2009). Finally it is noteworthy that distinguishing apoptosis from necrosis in drug-induced liver injury is not always simple despite the numerous mechanistic and morphological discrepancies existing between the two death mechanisms (Russman et al. 2009). It has been reported that the same drug may cause either apoptosis or necrosis or even exhibit a concurrent death mode involving both mechanisms; depending on one hand on the dose and severity of the initial death stimulus and on the other hand on the increased vulnerability of hepatocytes to such stimulus (Russman et al. 2009). For example the same drug may cause apoptosis at low doses and necrosis at high doses or even both in the presence of genetic or environmental predisposition to liver injury. Hepatic predisposition to a combined death mode, involving both apoptosis and necrosis, is often related to genetic alterations in important metabolic enzymes or to a concurrent episode of inflammation occurring during drug therapy (Bantel and Osthoff, 2012).

1.6.3 Susceptibility to drug-induced liver injury

The susceptibility to drug-induced liver injury is attributed predominantly to genetic polymorphisms in major drug-metabolizing enzymes that may either decelerate the metabolism

of toxic drugs or accelerate the generation of bioreactive drug metabolites (Tarantino et al. 2009). Genetic alterations in P450 enzymes led to the identification of four different phenotypes: poor metabolizers (PMs), who lack the active form of the enzyme; intermediary metabolizers (IMs), who are either heterozygous for one deficient allele or carry two alleles encoding reduced activity; extensive metabolizers (EMs), who carry two active alleles; and ultrarapid metabolizers (UMs), who carry multiple gene copies, a trait that is dominantly inherited (Ingelman-Sundberg, 2004). The difference in these phenotypes explains the inter-individual differences in drug metabolism and hence in susceptibility to drug-induced liver injury (Tarantino et al. 2009). In addition to genetic polymorphisms, environmental factors (concomitant drugs or alcohol abuse), age, gender, and underlying hepatic diseases (HIV or diabetes) proved to be important predisposing factors to drug-induced liver injury (Tarantino et al. 2009). For example adults exhibited an increased susceptibility to DILI when compared to children and women proved to be more sensible to drug induced hepatitis and acute liver failure than men; however advanced age predisposes men more than woman to drug induced cholestasis (David and Hamilton, 2010). Obesity, malnutrition and alcohol abuse have been correlated with an increased susceptibility to drug adverse effects especially in the case of acetaminophen (Rowden et al. 2005). Pre-existing hepatopathology is an important predisposing factor to the toxic effects of drugs that are metabolized by the liver (David and Hamilton, 2010). Hepatitis B and C serve as examples of diseases that may aggravate the severity of inflammatory reactions to anti-tuberculosis medication (Lee et al. 2005).

1.6.4 Clinical signs and symptoms of drug-induced hepatotoxicity

The precise diagnosis of hepatotoxicity constitutes a major challenge for health professionals since hepatotoxic drugs may exhibit clinically, biochemically and histoligically the same signs and symptoms as any primary liver disease (Bjornsson, 2010). Clinically, drug-induced liver injury is predominantly presented as acute hepatitis and/or cholestasis (Kaplowitz, 2004); however it is noteworthy that DILI may be manifested as any form of acute or chronic liver disease, including cirrhosis, chronic hepatitis, sinusoidal obstruction syndrome or neoplasm (Andrade et al. 2007). The symptoms of drug-induced liver injury are often similar to those of viral hepatitis and include: malaise, anorexia nausea, jaundice and abdominal pain (Zimmerman, 2000). Several clinical biomarkers have been used to distinguish liver injury; for example the increase in serum hepatic enzyme levels (AST, ALT, and ALP) was considered as a specific indicator of liver injury whereas elevations in total and conjugated bilirubin served to evaluate the liver's function as a whole (Kaplowitz, 2004). Subsequently the clinical pattern of drug-induced liver injury was characterized as either hepatocellular, exhibiting initially a prominent increase in the levels of ALT; or cholestatic demonstrating a predominant initial elevation in alkaline phosphatase (Navarro and Senior, 2006). It is important to note that the modulation of these biomarkers is not mutually exclusive in a way that liver injury may be presented as mixed clinical pattern (Kaplowitz, 2004). Acute hepatocellular (cytotoxic, cytolytic) liver injury is characterized by an increased in ALT levels (ALT > 2-fold that of ULN) or by an ALT/ALP ratio ≥ 5 (Benichou, 1990). Patients suffering from this particular type of liver damage exhibit

unspecific clinical features and occasional jaundice; sometimes they may show allergic symptoms such as rash, fever or peripheral eosinophilia (Goodman, 2002). Liver histology in acute hepatic injury demonstrates in varying intensity cell necrosis and inflammation, mainly in zone 3 of the hepatic accini, in addition to the abundant presence of eosinophils, which is typical in toxic etiology (Andrade et al. 2007). It has been proven that patients exhibiting drug-induced hepatocellular injury are more susceptible to acute liver failure than others with a mean mortality rate of 10%, according to "Hy's rule" (Andrade et al. 2007). Acute cholestatic injury is characterized by an increase in serum ALP levels (ALP > 2N) or by an ALT/ALP ≤ 2; this type of liver injury is further classified into two subtypes: canalicular cholestasis and hepatocanalicular hepatitis (acute cholestatic hepatitis) (Andrade et al. 2007). Canalicular cholestasis is often represented by an increase in conjugated bilirubin, AP and γ-glutamyl transpeptidase (γ-GT) with a slight impairment in serum transaminases (Andrade et al. 2007). It is often correlated with the intake of anabolic and contraceptive steroids. Liver biopsy of patients with canalicular cholestasis demonstrates in both hepatocyte cholestasis and dilated bile canals with marked bile plugs but without inflammatory or necrotic patterns (Goodman, 2002). Patients suffering from the hepatocanalicular hepatitis exhibit abdominal pain and fever similar to the acute biliary symptoms; in these cases, liver biopsy shows different degrees of necrosis and inflammation as well as marked cholestasis mainly in the centrilobular zone (Andrade et al. 2007). In mixed hepatic injury, features of either hepatocellular or cholestatic patterns may predominate with ALT/ALP ratio between 2 and 5 (Andrade et al. 2007). Frequently, patients exhibit allergic with a granulomatous liver biopsy specimen (Zimmerman, 2000). The majority of drugs that cause cholestatic injury can also cause a mixed hepatic injury; nevertheless hepatocellular injury proved to be much more correlated with acute liver failure than drug-induced cholestatic and mixed lesions (Andrade et al. 2007).

1.6.5 Clinical manifestations of drug-induced liver injury

Drug-induced liver injury may affect all types of hepatic cells (parenchymal and nonparenchymal cells) resulting in a vast variety of pathological conditions, such as acute and chronic hepatitis, steatohepatitis, cholestasis, fibrosis, and cirrhosis (Holt and Ju, 2006). Despite the fact that DILI may be manifested as any acute or chronic liver disease, hepatitis, cholestasis and a mixed presentation of both remain the predominant clinical manifestations of drug-induced liver injury (Kaplowitz, 2004).

1.6.5.1 Hepatitis

Acute hepatitis, with or without cholestasis is considered as the most frequent clinical manifestation of drug-induced liver injury (Ramachandran and Kakar, 2009). It often results from direct drug-induced damage and is characterized by several histological features like: (1) distinguished perivenular (acinar zone 3) necrosis, (2) mild hepatitis with canalicular cholestasis, (3) portal tract inflammation, (4) profuse neutrophils, (5) profuse eosinophils and (6) epitheloid-cells granulomas (Tajiri and Shimizu, 2008). The drug-induced hepatocellular injury may either induce spotty necrosis, affecting independent hepatocytes or confluent necrosis targeting at a time a group of hepatocytes (Ramachandran and Kakar, 2009). Centrizonal (zone 3) necrosis often characterizes drug-induced hepatitis, as is the case with acetaminophen and halothane (Ramachandran and Kakar, 2009). Commonly, fibrogenesis is absent in acute hepatitis; however hepatic regeneration occurs via increased hepatocyte proliferation (Ramachandran and Kakar, 2008). The persistence of these biochemical abnormalities for more than six months often results in chronic hepatitis (Batts and Ludwig, 1995). It has been defined that the onset of chronic hepatitis after three months is related to hepatocellular injury whereas a delayed 6 months onset is attributed to cholestatic or mixed injury (Ramachandran and Kakar, 2008). The latter presents a higher probability to progress into chronic hepatitis when compared with hepatocellular injury (Andrade et al. 2007). Chronic hepatitis is distinguished from acute hepatitis based on the presence of fibrosis and cirrhosis (Ramachandran and Kakar, 2008). Commonly, discontinuing the administration of hepatitis-causing drugs should attenuate the resulting symptoms provided that fibrosis is still in initial stages. In addition to acute and chronic hepatitis, several drugs may also cause auto-immune hepatitis (AIH) which is often distinguished from chronic hepatitis based on the presence of allergic features such as rash, fever, joint pain and increased eosinophil counts (Ramachandran and Kakar, 2008). AIH frequently results from drug-induced immune mediated liver injury based on the "hapten hypothesis" (Weiler-Normann and Schramm, 2013). The induced presence of autoantibodies and activated immune cells, particularly cytolytic T-cells, by drug-protein adduct formation represent the hallmark of AIH (Lohse and Mieli-Vergani, 2011).

1.6.5.2 Cholestasis

Cholestasis occurs when bile cannot flow from the liver to the duodenum; it can be caused by a functional defect in bile production at the hepatic level or by impairment in bile secretion at the duct level (Zollner et al. 2008). It is classified as "obstructive cholestasis" when the impairment in bile flow is due to a mechanical blockage at the bile duct level; or as "metabolic cholestasis" when the disruptions in bile formation are due to innate genetic defects or acquired adverse drug effects. While genetic mutations in important canalicular transporters often underlie hereditary cholestasis, various factors proved to be at the origin of acquired cholestasis (Zollner et al. 2008). For example pro-inflammatory cytokines proved to be causatively correlated with sepsis-induced cholestasis; hormones were associated with intrahepatic cholestasis of pregnancy (ICP) while various drugs induced drug-related cholestasis (Zollner et al. 2008). Frequently drugs result in cholestasis by modulating hepatic transporter proteins expression and function; however in rare

cases they may induce an immune reaction targeted against the bile duct epithelium leading eventually to a vanishing bile duct syndrome, which can evolve into biliary cirrhosis (Zollner et al. 2008). Approximately 30% of drug-induced liver injury cases are cholestatic; however it is not always the drugs themselves that cause cholestasis since the latter may be often caused by the inflammatory reaction accompanying drug adverse effects. For example idiosyncratic adverse drug reactions frequently involve: hypersensitivity, pro-inflammatory mediators and irregular metabolism; all of these factors proved to contribute to inflammatory cholestasis (cholestatic hepatitis) (Trauner et al. 2007; Pauli-Magnus et al. 2006). Several drugs have displayed an implication in cholestasis predominantly through the drug-or metabolite-induced functional inhibition of efflux proteins (Zollner et al. 2008). For example rifampin, cyclosporine, troglitazone, bosentan, troglitazone, and glibenclamide proved to *cis*-inhibit competitively the ATP-dependent efflux of taurocholate via Bsep (Milkiewicz et al. 2003; Fattinger et al. 2001; Funk et al. 2001). The hormonal metabolites of estrogen and progesterone demonstrated an indirect trans-inhibition of Bsep following their Mrp2-mediated secretion into bile (Stieger et al. 2000). In addition to direct drug-induced and inflammation-mediated transporter inhibition, polymorphisms in transporter genes also proved to be a predisposing factor to drug-induced cholestasis (Zollner et al. 2008). Mutations in MDR3 and BSEP served as recent examples correlating genetic variation to drug-induced cholestasis (Lang et al. 2007).

1.6.5.3 Steatosis and Steatohepatitis

Hepatic steatosis (fatty liver) is defined as the abnormal intracellular accumulation of lipids in the liver due to functional impairment in the normal processes of triglyceride synthesis and elimination (Anderson and Borlak, 2008). The excessive intracellular lipids are retained within vesicles that dislocate the cytoplasm; these vesicles may be large enough to deform the nucleus resulting in "macrovesicular steatosis" or relatively smaller in size keeping the nucleus intact in "microvesicular steatosis" (Anderson and Borlak, 2008). Steatosis is considered as a chronic situation that is often correlated with prolonged drug therapy (several weeks and even months), and probably hepatic drug accumulation (Lettéron et al. 2003). In most cases, prolonged exposure to certain drugs may lead to a reversible form of macrovesicular steatosis, which can progress in some patients into steatohepatitis, and eventually into cirrhosis (Donato et al. 2009). Rarely, some drugs may cause microvesicular steatosis, which may progress to fatal liver failure (Labbe et al. 2008). Commonly hepatic steatosis may evolve to "steatohepatitis" in case steatosis was accompanied by an inflammatory reaction involving hepatocellular necrosis (Nascimento et al. 2012). The predominant key mechanism underlying steatohepatitis proved to be related to mitochondrial dysfunction and, more specifically, respiratory chain deficiency regardless of the initial cause (Donato et al. 2009). Dysfunction in the mitochondrial membrane potential may disrupt the respiratory chain leading to ATP depletion and ROS generation (Donato et al. 2009). Subsequently, in a lipid-rich environment, increased ROS generation may readily stimulate lipid peroxidation leading to the release of aldehyde derivatives; the latter is responsible for the severe hepatocellular injury observed histologically in hepatocytes during drug-induced steatohepatitis (Begriche et al. 2006). Both steatosis and steatohepatitis are subtypes of nonalcoholic fatty liver

disease (NAFLD) which includes all form of liver damage, except those attributed to alcohol, from simple steatosis to hepatic cirrhosis and eventually severe liver failure (Nascimento et al. 2012). Several factors may be causatively related to NAFLD predominantly: obesity, insulin resistance, surgical intervention and last but not least drugs and xenobiotics (Anderson and Borlak, 2008). These factors cause hepatic steatosis through various molecular events leading to enhanced lipids uptake, up-regulated lipogenesis, defective lipid elimination, oxidative stress, reduction, fatty acid oxidation and inhibition of mitochondrial respiratory chain (Anderson and Borlak, 2008).

1.6.5.4 Fibrosis and Cirrhosis

Generally, liver fibrosis often results from chronic liver injury. Particularly, it is the exaggerated drug-induced hepatocyte apoptosis that will activate hepatic stellate cells, favouring the engulfment of apoptotic bodies (Chakraborty et al. 2012). In their activated state, hepatic stellate cells are referred to as myofibroblasts; the latter play a key role in promoting extracellular matrix deposition and scar formation in the liver (Chakraborty et al. 2012). The extracellular matrix, which comprises a wide array of heterogeneous macromolecules like collagens, glycoproteins and proteoglycans, is fundamentally involved in hepatic cell signaling and differentiation in addition to the maintenance of normal liver architecture (Schuppan et al. 2001). Hepatic fibrosis is correlated with prominent alterations in the composition, quantity and distribution of the extracellular matrix leading to modifications in cell phenotypes, disruption of normal liver architecture, impairment in hepatic blood flow and alterations in cell signalling (Schuppan et al. 2001). Recent evidence has elucidated that the hepatocyte-mediated disruption of Bcl-xL results in prominent hepatocyte apoptosis and fibrogenesis (Chakraborty et al. 2012). Furthermore, the co-occurrence of an inflammatory reaction during drug therapy and the consequent secretion of injurious pro-inflammatory cytokines, such as IL-6 and TNF-α, also demonstrated a key role in fibrogenesis (Chakraborty et al. 2012). This role is based on the activation of immune cells and their migration to the site of hepatic injury where they will secrete several cytokines, like the profibrogenic cytokine TGF-β, which will further progress inflammation and fibrosis (Canbay et al. 2003).

Cirrhosis is defined as the end-stage consequence of several hepatic diseases including fibrosis. It is often characterized by extensive fibrogenesis and nodule formation leading to impaired hepatic function and blood flow (Schuppan and Afdhal, 2008). Similar to fibrosis, cirrhosis results following exaggerated wound-healing response to chronic hepatic injury resulting from a variety of causes, predominantly drugs. Cirrhotic patients may exhibit a wide variety of clinical symptoms ranging from minor symptoms to liver failure depending on both the severity of the underlying cause and the magnitude of hepatic fibrosis (Schuppan and Afdhal, 2008). Most of the morbidity and mortality cases in patients with liver disease occur after the progression of the disease to cirrhosis (Poynard et al. 2000). The latter is correlated with several severe clinical symptoms, most importantly hepatic encephalopathy, ascites, variceal bleeding and renal failure; consequently, the only effective therapy is liver transplantation (Poynard et al. 2000). It has been

reported, that in addition to the detrimental consequences of cirrhosis on patients with chronic liver injury, it also predisposes them to hepatocellular carcinoma (Davis, 2002).

1.6.6 Prediction of drug-induced liver injury

The process of drug discovery is tremendously expensive and time consuming; in order for a new drug to successfully reach the market ten to twelve years are spent in research and development and over €800 million are invested (Allen et al. 2010). Most of this money is spent during clinical trials on rigorous toxicity studies that are necessary to ensure the safety of new compounds. Indeed, post marketing drug withdrawal implies a net loss of tremendous amount of money and time in addition to increased risks of drug-induced death cases (Li, 2004). Due to the extensive involvement of the liver in drug metabolism and elimination drug-induced hepatotoxicity proved to be the leading cause of drug development attrition (Allen et al. 2010). It has been reported that a slight improvement of 10% in predicting drug-induced liver failure pre-clinically, before initiating laborious, time-consuming and expensive clinical trials, could save around €100 million and reduce the exposure of humans to toxic chemicals (Allen et al. 2010). Approximately 50% of the drugs that were correlated with hepatic injury during clinical trials did not exhibit toxic effects during animal experiments, implying that ameliorated preclinical evaluation of drug safety is vitally needed (Olson et al. 2000). The inability of animal models to efficiently predict human related drug toxicity may be related to several factors the most important being: (1) Prominent interspecies differences between animals and humans related to drug absorption, distribution, metabolism and elimination mechanisms (Li, 2004); (2) Limited biological diversity since animals under well-controlled experimental settings are not representative of humans living in heterogeneous conditions (Xu et al. 2004); (3) Difficulty in distinguishing primary form secondary adverse drug effects due to complex interactions of the liver with other organs (Guillouzo 1998); (4) Inability of animal models to account for inter-human variations in drug response based on genetic, physio-pathological and/or environmental factors (Guillouzo and Guguen-Guillouzo, 2008). Due to all of these drawbacks, human-based *in vitro* experimental models seem to be much more pertinent in the prediction of drug induced hepatotoxicity as they are providing interesting insights on all levels of investigational toxicology (Groneberg et al. 2002). In particular, the recent application of advanced "omics" techniques like genomics and proteomics in toxicity studies provided valuable mechanistic insights on the key pathways underlying drug-induced liver injury, promoting the early detection of hepatotoxic candidates and enhancing the predictive ability of *in vitro* liver models.

1.6.6.1 *In vitro* liver models

There exist several models to assess hepatotoxicity *in vitro*, the most important being: isolated liver cell models, liver slices models and isolated perfused organs (Table 1.4) (Groneberg et al. 2002). These models present numerous advantages and disadvantages over hepatic cell lines and transgenic cells related to accurate toxicity assessment, availability and adaption to experimental studies (Guillouzo and Guguen-Guillouzo, 2008). Of all these models, isolated hepatic cells represent the most widely used *in vitro* model in the investigation of hepatotoxicity;

predominantly due to the fact that they express a complete metabolic system, which best reproduces the physiological process of drug metabolism. Therefore, the application of these models in investigative toxicology may provide valuable insights on the underlying mechanisms of hepatic injury (Guillouzo and Guguen-Guillouzo, 2008).

The isolated liver cell model is made up of isolated animal or human hepatocytes and has been validated long time as suitable for the study of toxicology and metabolism (Groneberg et al. 2002). Viable normal hepatocytes may be acquired *in vitro* either from isolated hepatic cells or from tissue slices through the enzymatic disruption of the liver (Guillouzo and Guguen-Guillouzo, 2008). This model was subjected to drastic evolution over the last decades; just after hepatocyte isolation by collagenase and hyaluronidase has been described an improved technique suggesting the perfusion of livers in situ by collagenase and hyaluronidase has been developed (Groneberg et al. 2002). Subsequently, the 2-step collagenase technique for short-term culture of animal and human hepatocytes was proposed; the latter was followed by several approaches for the improvement of long-term hepatocyte culture including the insertion of basement membrane, collagen sandwich culture and co-culture with other epithelial cells (Groneberg et al. 2002). Regardless of all these improvements, once isolated, hepatocytes will undergo rapid phenotypic modifications and short-term survival (Guillouzo and Guguen-Guillouzo, 2008). This is mainly attributed to the lack of several *in vivo* features such as (1) the three dimensional architecture and composition of the hepatic extracellular matrix, (2) homo-and heterotypic cell to cell interaction, (3) hepatic blood and bile flow, (4) physiological medium composition and endocrine factors, like oxygen supply and cytokine profiles and most recently (5) tissue organization and histotypic architecture (LeCluyse et al. 2012). There exist several sources of cellular material each exhibiting various advantages and disadvantages especially in terms of expression and activity of various metabolic enzymes, efflux transporters and nuclear receptors. The most used cellular sources for the study of drug-induced toxicity include (1) primary hepatocytes, (2) immortalized hepatic cell lines (Fa2N4, HepG2 and HepaRG) and (3) hepatic stem cells.

(1) Primary hepatocytes

Freshly isolated primary hepatocytes, is the preferred model for ADME/Tox investigations since it best reflects the *in vivo* hepatic functional responses to drug or chemical exposure (Guillouzo and Guguen-Guillouzo, 2008). At the moment of isolation from animal or human tissues, primary hepatocytes express all the metabolizing enzymes and transporter proteins in their indigenous hepatic levels and arrangement; however this expression is lost instantaneously after isolation (LeCluyse et al. 2012). This rapid loss in gene expression and concurrent lack of functionality has been tackled over the past years through the development of several techniques to improve the long term culture of functionally-stable primary hepatocytes; these techniques mainly included co-culturing primary hepatocytes with essential hepatic tissue-specific biomatrix components in presence of other hepatic cells in a three dimensional spheroid aggregate culture or in perfusion culture systems (LeCluyse et al. 2012). Despite these improvements, the unavailability of primary hepatocytes and the difficulty to obtain them especially from human

tissues represented a major obstacle for their routine experimental use (LeCluyse et al. 2012). The recent evolution of hepatocyte cryopreservation ameliorated the availability of primary hepatocytes and facilitated experimental reproducibility; however it did not resolve the initial difficulty of obtaining human tissues from which primary hepatocytes are isolated.

(2) Immortalized hepatic cell lines

Hepatic cell lines, which are commonly obtained from liver tumors, are defined as permanently established and genetically transformed clonal lineages in which daughter cells will proliferate indefinitely within adequate medium and growth substratum conditions (Guillouzo and Guguen-Guillouzo, 2008; LeCluyse et al. 2012). Contrarily to primary hepatocytes, hepatic cell lines are immortalized, and hence they are not limited to a restricted number of divisions mainly due to genetic polymorphisms in their growth control pathways (Mees et al. 2009). While these cell lines represent the model of choice in the investigation of toxicity and hepatic functionality *in vitro;* they are inappropriate for metabolic studies since they lack important metabolic enzymes key drug transporters and essential nuclear receptors *(*LeCluyse et al. 2012). The most important disadvantage of human hepatic cell lines is related to genomic instability and de-differentiation resulting in phenotypes that no longer corresponds to that of human hepatocytes *in vivo*; whereas their ultimate advantage lies in their spontaneous availability and their simple culturing procedures (LeCluyse et al. 2012). Various human hepatic cell lines including Fa2N-4, HepG2, C3A (a sub-clone of the hepatoma-derived HepG2 cell line) and HepaRG have been evaluated as alternatives to primary human hepatocytes in metabolism and toxicity studies (Youdim et al, 2007; Hariparsad et al. 2008; Kanebratt and Andersson, 2008b; Kanebratt and Andersson, 2008a; Jennen et al. 2010; Wilkening et al. 2003). Numerous studies demonstrated that these hepatic cell lines overcome the challenges faced by primary hepatocytes regarding availability and short-term viability however primary hepatocytes remain “the golden standard” in metabolic enzymes and receptors expression (LeCluyse et al. 2012). Table 1.5 summarizes the main differences in the mRNA expression levels of 44 important genes between HepG2, HepaRG and human hepatocytes.

Fa2N4

Fa2N-4 cells were obtained from the immortalization of primary human hepatocytes by SV40 large T-antigen transfection; however they proved to be non-tumorigenic (Mills et al. 2004; Vermeir et al. 2005; Ripp et al. 2006). Commonly, these cells preserve the morphological features of primary human hepatocytes and express several P450 enzymes (CYP3A4, CYP1A2, and CYP2C9) that proved to be inducible following exposure to typical stimuli (Mills et al. 2004). Furthermore, Fa2N4 exhibited similar expression levels of PXR and AhR when compared to primary human hepatocytes (Mills et al. 2004). Nevertheless, other fundamental nuclear receptors such as CAR in addition to important uptake transporters including OATPs are missing in Fa2N4 cells indicating that the use of these cells in the prediction of drug-induced hepatotoxicity is drastically limited (LeCluyse et al. 2012).

HepG2

HepG2 is the most widely used and best characterized human hepatic cell line (LeCluyse et al. 2012). HepG2 cells are isolated from a human liver tissue with a well-differentiated hepatocellular tumor (LeCluyse et al. 2012). They express 55 chromosomes and are known to be non-tumorigenic (LeCluyse et al. 2012). Morphologically, they appear similar to epithelial cells when cultured as monolayers or as small aggregates; whereas functionally, they are similar to normal hepatocytes in the production and secretion of indigenous hepatic plasma protein including transferrin, fibrinogen, albumin, plasminogen and α-2-macroglobulin (LeCluyse et al. 2012). HepG2 cells exhibit noticeable metabolic potential; they proved able to biotransform several but not all exogenous compounds including mutagens and carcinogens (LeCluyse et al. 2012). Remarkably, these cells do not display any p53 mutations; hence, unlike other cancerous cells, they possess the capacity to activate DNA damage response, provoke growth arrest and respond to apoptotic signals following the exposure to damaging stimuli (Wilkening et al. 2003). HepG2 are widely used in the fields of toxicogenomics and proteiomics, especially in studying the effect of cytokines on hepatic protein synthesis, predominantly due to their simple culturing and their functionality in these fields (Jennen et al. 2010). When compared to primary human hepatocytes HepG2 exhibit considerable discrepancies in basal gene expression (LeCluyse et al. 2012). For example, they over-express genes correlated in cell cycle regulation in addition to DNA, RNA and nucleotide metabolism, transcription, transport, and signal transduction (LeCluyse et al. 2012). Contrarily, they under-express several genes related to phase I drug-metabolism and to their regulating receptors (LeCluyse et al. 2012). This innate decreased expression and lack of activity of important P450-enzymes may result in metabolic-dependent toxicity for specific compounds regardless if the compound in itself is toxic or not (LeCluyse et al. 2012). This fact must be taken into consideration when using HepG2 in the prediction of drug-induced hepatotoxicity to avoid possible false positive results. Many subclones of HepG2 like HepG2/C3A were developed, by transfecting original HepG2 cells with constructs expressing high CYP levels, in the aim of surpassing the lack of their metabolic deficiency (LeCluyse et al. 2012). Overall, HepG2 and its subclones may provide valuable insights on mechanistic toxicology and hepatic functionality; however these cells remain incompetent facing primary hepatocytes in metabolic studies (LeCluyse et al. 2012).

HepaRG

HepaRG is a relatively recent cell line, isolated from the liver of a female suffering from hepatocellular carcinoma (Gripon et al. 2002). When compared to primary hepatocytes, these cells express rare karyotypic variations related mainly to a remodeled chromosome 7 and to a translocation t(12;22) with a loss of the 12p fragment (Gripon et al. 2002). When confluent, HepaRG exhibit peculiar morphological features and comprise two distinct cell types: Granular epithelial cells expressing hepatocytic markers and flattened clear-colored cells exhibiting cholangiocytic markers (LeCluyse et al. 2012). In order to accomplish hepatic functions HepaRG cells must undergo maximum differentiation to hepatocyte-like cells; the latter is attained

through treating the cells with 2% DMSO for two weeks (Guillouzo and Guguen-Guillouzo, 2008). When compared to primary hepatocytes, differentiated HepaRG cells express similar levels of principal P450 enzymes, Phase II enzymes, uptake and efflux transporters, and nuclear receptors (Aninat et al. 2006; LeVee et al. 2006). Overall HepaRG, represent the best alternative to primary human hepatocytes in the studies of metabolism and toxicity. Nevertheless, the fact that HepaRG display prominent resemblance with primary human hepatocytes regarding optimal metabolic enzyme expression depends greatly on the addition of high amount of DMSO (2%) (LeCluyse et al. 2012). It is noteworthy, that it is the high DMSO exposure that artificially leads to increased levels of CYP expression causing the activation of related receptor pathways such as CAR and PXR (LeCluyse et al. 2012). The addition of DMSO may not be innocent; therefore this factor must be taken into consideration in the assessment of toxicity and metabolism in order to avoid false negative or false positive results.

(3) Hepatocytes derived from stem cells

In view of the unavailability of primary human hepatocytes and the functional limitations of hepatic cell lines, the emergence of stem cell derived hepatocytes seems to be an efficient solution (LeCluyse et al. 2012). Hepatocytes may be obtained from the *in vitro* differentiation of stem cells; whereas stem cells are either obtained from embryos or from adult tissues (Guillouzo and Guguen-Guillouzo, 2008). Stem cells derived from embryos are known as totipotent embryonic stem cells whereas those derived from tissues are termed multipotent adult stem cells (Davila et al. 2004). Pluripotent stem cells (PSC) comprise embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) (LeCluyse et al. 2012). Several protocols defined the differentiation of PSC towards hepatocytes by stimulating the endodermal differentiation process through application of activin A, followed by the addition of fibroblast growth factors and Wnt3a to enhance differentiation towards hepatic lineages; subsequently, culture in medium containing typical hepatocyte culture supplements (e.g. insulin, dexamethasone, hepatocyte growth factor) in addition to oncostatin M promote functional maturation into hepatocyte-like cells over a 1–2 week period (LeCluyse et al. 2012). Adult stem cells possess limitless self-renewal ability and multipotent differentiation capacity; thus contribute efficiently to repair and generation of their resident tissue (LeCluyse et al. 2012). Similarly hepatic progenitor cells, a subtype of adult stem cells, reside in the liver and play significant roles in re-populating the epithelium during injury or hepatocellular death (LeCluyse et al. 2012). These cells are bi-potent and hence can either differentiate into hepatocytes or into cholangiocytes (Vessey and de la Hall, 2001; Forbes et al. 2002). It is important to note that there exist also extrahepatic stem cells that are able to differentiate into hepatocytes such as multipotent adult progenitor cells (MAPC) or bone marrow and adipose tissue derived mesenchymal stem cells (MSC) (Ong et al. 2006; Snykers et al. 2006). Adult stem cells, particularly iPSC and adipose derived MSC, exhibits an important advantage over other types of cells related to its ability to produce donor panels that elucidate important polymorphic alterations within a specific population (LeCluyse et al. 2012). Obviously, similar panels are hard to obtain with primary cells due to their scarce availability.

Despite the advantages of HSC in terms of availability and expansion, there exist considerable obstacles to their application as alternatives for primary hepatocytes. For example, some stem cell derived hepatocytes maintain the expression of fetal phenotype even though recent iPSC-based strategies seem to overcome this obstacle (Snykers et al. 2009; Guguen-Guillouzo et al. 2010). With respect to toxicity testing, it is very important that the cells refered to as "hepatocytes" express phase 1 and 2 metabolic enzymes in addition to principal uptake and efflux transporter (LeCluyse et al. 2012). To date, stem cells do not exhibit this feature (Guguen-Guillouzo et al. 2010); the lack in metabolic phase 1 activity in stem cell populations may be correlated with the heterogeneity of the "differentiated" population, as initially isolated stem cells express CYP3A4 activity similarly to primary human cells (Basma et al. 2009).
Finally in presence of all these different models, selecting the most appropriate one will depend most of the times on specific toxicological intrigues related particularly to a certain new drug candidate. However, a recent approach suggests that evaluating the safety of new drugs should be based on an investigative form of toxicology that integrates high-throughput genomics and proteomics techniques in the detection of hepatotoxic drugs and the investigation of their underlying toxic mechanisms (Ekins et al. 2006).

1.6.6.2 "Omics" technologies

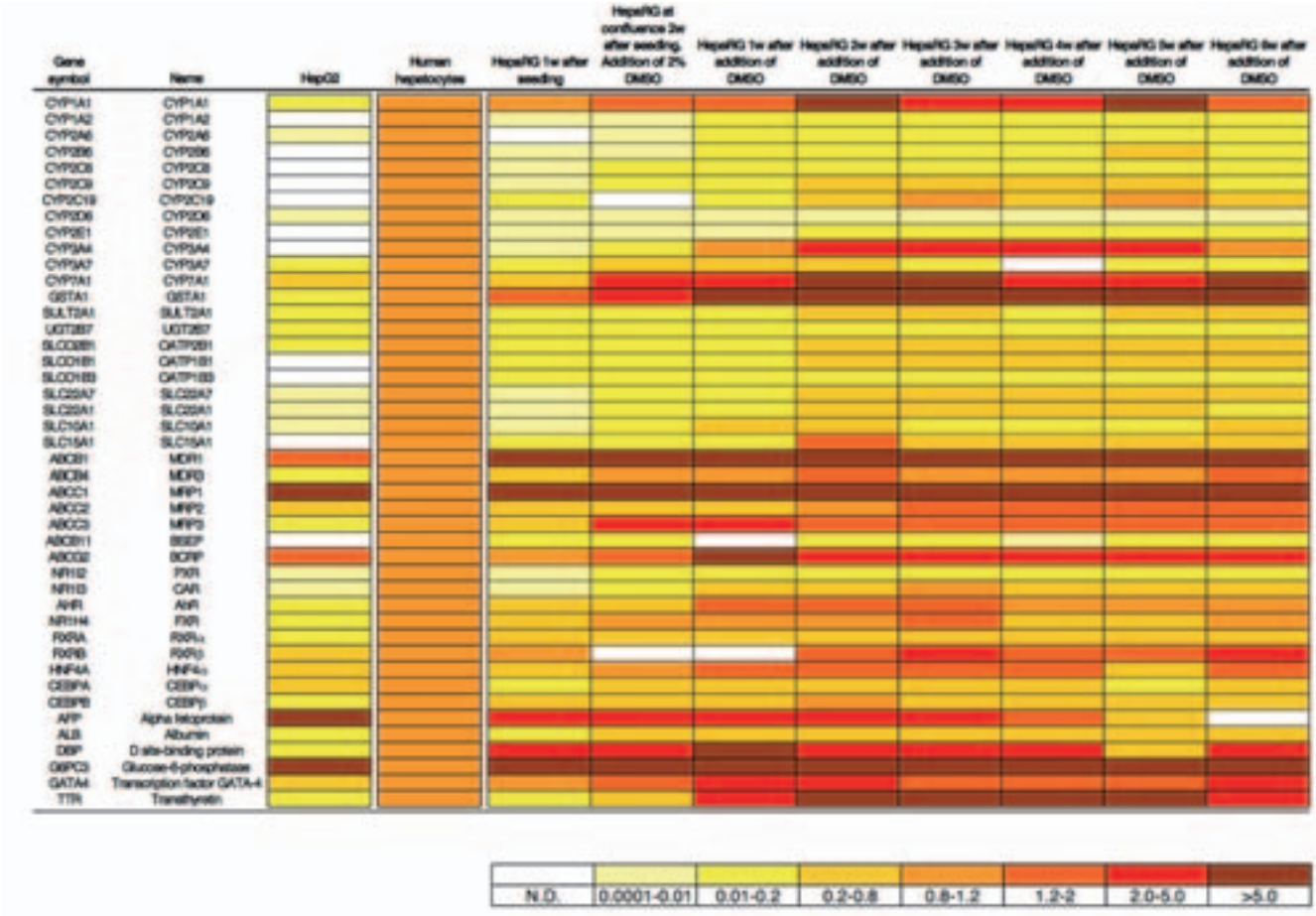
In light of the drastic evolution in the field of molecular biology during the last ten years, toxicogenomics has emerged based on the consideration of the entire genome's constitution and dynamics in investigative toxicology (Harrill and Rusyn, 2008). The remarkable development of high-throughput "omics" technologies such as genomics, proteomics and metabolomics and their implication in the evaluation of drug-induced hepatotoxicity provided valuable insights on the mechanisms underlying adverse drug reactions (Cui and Paules, 2010). Genomics represent the study of the whole genome gene expression using microarray profiling; whereas proteomics implies the implication of high-resolution 2D gel electrophoresis and mass spectrometry in the study of cell-and tissue-wide protein expression (Cui and Paules, 2010). Since proteins are the principal actors in drug metabolism and elimination, the implication of proteomics in toxicology should be more relevant than genomics; however proteome analysis is a very complex process (Guillouzo and Guguen-Guillouzo, 2008). Metabolomics is defined as the measurement of metabolic products using techniques such as mass spectrometry and nuclear magnetic resonance spectroscopy (Guillouzo and Guguen-Guillouzo, 2008). Recently microarray profiling has become the tool of interest in toxicology, since it has proved to be the most reliable technique for the large-scale detection of gene expression alterations in response to drug exposure (Cui and Paules, 2010). The ability to associate a chemical-induced phenotypic modification with gene expression variations is defined as "phenotypic anchoring" (Harrill and Rusyn, 2008). Integrating traditional toxicology with phenotypic anchoring as well as protein and metabolic profiling constituted a new approach in toxicology, referred to as "Systems toxicology" (Harrill and Rusyn, 2008). The latter seems to be incredibly promising in disclosing key mechanisms of adverse drug reactions, which will indeed ameliorated the prediction of human related drug-induced hepatotoxicity in early phases of drug development (Cui and Paules, 2010). Moreover, it

has been perceived that "omics" techniques contribute to the identification of innovative biomarkers for monitoring drug-induced liver injury (Searfoss et al. 2005). The discovery of specific and sensitive biomarkers is highly important in improving the detection of potentially toxic drugs thus preventing them from reaching the market (Waters and Fostel, 2004).

Table 1.4. Advantages and disadvantages of several hepatic cell line currently used for the investigation of drug-induced hepatotoxicity. (*Adapted from Groneberg et al. 2002; Guillouzo and Guguen-Guillouzo, 2008; Guillouzo, 1998).*

Model	Advantages	Disadvantages
Isolated Cells	➢ Available from animal or human ➢ May be cryopreserved ➢ Suitable for the study of toxicology and metabolism ➢ Easy to handle ➢ Allow the testing of several compounds at different concentrations	➢ Rapid phenotypic modifications post-isolation ➢ Short-term survival ➢ No bile measurement ➢ No cell-to-cell interaction ➢ No preserved anatomy
Liver Slices	➢ Preserve intra-and interlobular structure partly ➢ Maintain cell–cell and cell–matrix interactions ➢ Available from all species including humans ➢ Informative on cellular toxicity ➢ Allow the testing of several compounds at different concentrations	➢ Poor reproducibility and viability ➢ Unstable functionality of metabolic enzymes ➢ No bile measurement ➢ No cell-to-cell interaction ➢ No preserved anatomy
Isolated Organs	➢ Best mimics the *in vivo* situation ➢ Considers hemodynamics in the assessment of toxicology ➢ Preserves the 3D hepatic architecture ➢ Maintain cell–cell and cell–matrix interactions ➢ Allow bile collection and analysis	➢ Short-term functional integrity ➢ Unsuitable for multiple experimental conditions ➢ Cost tremendous amount of money ➢ Very complex to handle experimentally

Table 1.5. Expression of mRNA for 44 genes in cryopreserved human hepatocytes, HepG2 cells, and HepaRG cells at different weeks of culture. The expression is set to 1 in human hepatocytes for all genes measured. N.D: not detectable. *(Adapted from Kanebratt and Andersson, 2008).*



1.7 Idiosyncratic drug-induced livery injury (IDILI)

The term idiosyncrasy is defined in *Webster's Dictionary* as "a structure or behavioral characteristic unique to an individual or group;" this term is derived from the Greek words *idio*, meaning "own," *syn,* meaning "together," and *krasis*, meaning "mixture" (Ulrich, 2007). Accordingly, idiosyncratic hepatotoxicity may be defined, in the field of toxicology, as an adverse drug reaction resulting from a mixture of factors in a unique susceptible individual (Ulrich, 2007). This definition implies that drug exposure by itself cannot be considered as the cause of idiosyncratic hepatotoxicity; it is rather the convergence of host-specific predisposing factors in addition to drug-related toxic features that result in an idiosyncratic adverse reaction (Ulrich, 2007). IDILI is considered as an important subtype of drug-induced liver injury accounting for 13% of all drug-induced liver failure cases (Shaw et al. 2010). Although idiosyncratic hepatotoxicity occurs in a scarce subset of people, it is responsible for the majority of post-marketing drug withdrawal and use restrictions; thus representing a serious obstacle for pharmaceutical companies and an overwhelming challenge for health professionals (Shaw et al.

2010). This may be attributed to a great extent to the unpredictable mode of action of IDILI which, unlike intrinsic hepatotoxicity, is thought to be drug dose-independent, meaning that an idiosyncratic reaction may arise in certain individuals at a therapeutic dose that is completely safe to others (Shaw et al. 2010). In addition to being dose-independent IDILI exhibits several peculiar characteristics including: (1) poor correlation to the pharmacological target of the drug, (2) variable onset following drug exposure, (3) variable hepatic pathology, (4) irreproducibility and unpredictability (Deng et al. 2009). These characteristics imply that idiosyncratic hepatotoxicity is predominantly host-dependent rather than drug-dependent; probably, this dependency is based on various predisposing genetic or environmental factors that render a specific individual more susceptible to IDILI than another (Ulrich, 2007). Intrinsic and idiosyncratic hepatotoxicity exhibits different modes of action; while intrinsically toxic drugs or their reactive metabolites predominantly induce direct hepatocellular damage, idiosyncratic drugs rather cause immune-mediated hepatotoxicity (Russman et al. 2009). Clinically, intrinsic hepatotoxicity presents hepatocellular necrosis with negligible inflammation while idiosyncratic hepatotoxicity causes prominent inflammation-associated hepatic injury (Ramachandran and Kakar, 2008). Idiosyncratic hepatotoxicity is divided into two main subtypes: "metabolic idiosyncrasy" and "immunologic idiosyncrasy;" and the latter is further classified into allergic or non-allergic idiosyncratic reactions (Russman et al. 2009; Kaplowitz, 2005). Metabolic idiosyncrasy is based on inter-individual genetic variations in key metabolizing enzymes leading to the generation of reactive metabolites, which may either cause direct or immune-mediated hepatocellular damage; whereas immunologic idiosyncrasy is correlated with direct drug-induced activation of the immune system (Ramachandran and Kakar, 2008). Immune-mediated idiosyncratic hepatotoxicity is further classified into allergic or non-allergic reactions; while the former mainly involves an innate immune response the latter is correlated with a potent activation of the adaptive immune system in addition to the presence of typical allergic features such as rash, fever, high eosinophilia, autoantibodies and short latency period (Kaplowitz, 2005).

1.7.1 Mechanisms of idiosyncratic drug-induced liver injury

The poor predictability of IDILI and the lack of efficient *in vitro* and *in vivo* investigative models complicated the identification of its underlying key mechanism; however several hypotheses emerged through the years suggesting that idiosyncratic hepatotoxicity may arise through different modes of action (Roth and Ganey 2010). These mainly include: (1) Reactive intermediate hypothesis, (2) Genetic polymorphism hypothesis, (3) Hapten hypothesis, (4) Danger hypothesis, (5) Mitochondrial dysfunction hypothesis and (6) Inflammatory stress hypothesis (Shaw et al. 2010).

1.7.1.1 Reactive intermediate hypothesis

This hypothesis suggests that idiosyncratic liver injury arises following the biotransformation of a drug into its reactive metabolite which will covalently bind to vital intracellular proteins; this drug-protein interaction will subsequently disrupt membrane integrity and modify calcium

homeostasis in addition to other intracellular signalling pathways resulting cell death (Shaw et al. 2010). Although this hypothesis seems reasonable, it is important to note that it is not sufficient to induce idiosyncratic reactions, since several drugs that generate reactive metabolites were not correlated with idiosyncratic hepatotoxicity (Shaw et al. 2010). Accordingly, the presence of an underlying susceptibility factor, such as genetic polymorphisms in bioactivating enzymes would explain why the reactive metabolites of certain drug cause IDILI in some specific individuals and not in all the others (Shaw et al. 2010).

1.7.1.2 Genetic polymorphism hypothesis

This hypothesis states that a mutation in any cytoprotective gene decreases the toxicity threshold, predisposing individuals to IDILI at therapeutic drug doses (Shaw et al. 2010). A cytoprotective gene is any gene encoding important drug-metabolizing enzymes, uptake and efflux drug transporters, anti-inflammatory cytokines or anti-apoptotic proteins (Shaw et al. 2010). Polymorphisms in genes encoding P-450 enzymes lead to inter-individual variance in reactive metabolite formation thus explaining the difference in toxicity responses to drug exposure (Williams and Park, 2003). This hypothesis explains the inconsistent relation between the onset of idiosyncratic hepatotoxicity and drug exposure by the fact that people with a genetic mutation in one of the important metabolizing enzymes will metabolize the same drug at different rates; implying that the toxicity thresholds will be attained by some individuals sooner than the others (Shaw et al. 2010).

1.7.1.3 Hapten hypothesis

This hypothesis suggests that idiosyncratic hepatotoxicity is mediated by the immune system (Zhang et al. 2011). The hapten hypothesis indicates that a chemically active drug or its reactive metabolite covalently bind to intracellular proteins forming drug-protein adducts. Subsequently these adducts express themselves as foreign antigens, which will be either directly recognized by B-cells that will produce antibodies against them; or processed by antigen-presenting cells, which will present them to T-cells (Holt and Ju, 2006). The resultant expansion and activation of memory B-and cytolytic T-cells eventually induce an adaptive immune response, which will further propagate and aggravate hepatic injury (Shaw et al. 2010). When compared to others, the hapten hypothesis has been widely accepted in the investigation of idiosyncratic adverse reactions due to several consistent evidences. For example, several features characterizing IDILI like the variable onset of toxicity, the inconsistent relation to drug-dose and the high esosinophilic count accompanied by skin rash may be correlated with an adaptive immune response (Uetrecht, 2003). Furthermore, autoantibodies against altered self-protein have been detected in some patients with IDILI following exposure to chemically active drugs (Shaw et al. 2010). Nevertheless, to date a clear cause-and-effect interaction between autoantibodies and IDILI is still missing; additionally, experimental models correlating the presence of autoantibodies to drug-induced liver injury is lacking (Shaw et al. 2010).

1.7.1.4 Danger hypothesis

This hypothesis proposes a closely related theory to the hapten hypothesis indicating that the formation of drug-protein adduct is necessary to induce a immune response however it is insufficient to cause liver damage. This hypothesis suggests that the presence of a concurrent independent stress, "danger signal" in addition to drug-induced activation of an immune response is necessary to mediate hepatic injury. Commonly, the "danger signal" is either a simultaneous mild hepatocellular death or an infection resulting in cytokine release (Seguin and Uetrecht, 2003; Uetrecht, 1999).

1.7.1.5 Mitochondrial dysfunction hypothesis

This hypothesis proposes direct drug-induced mitochondrial damage or drug interaction with compromised mitochondria as an underlying cause of IDILI. Each individual exhibits a different capacity in accumulating dysfunctional mitochondria explaining the variable onset time of IDILI (Boelsterli and Lim, 2007). Impaired mitochondrial function may be caused by an imposed external disease or by a genetic mutation rendering the cells susceptible to idiosyncratic hepatotoxicity (Waring and Anderson, 2005). Significant evidence supported this hypothesis such as the correlation of IDILI-causing drugs like troglitazone, diclofenac and isoniazid with mitochondrial dysfunction (Shaw et al. 2010). Moreover, diclofenac and troglitazone demonstrated a mitochondrial- dependent cytotoxicity in HepG2 cells (Boelsterli, 2003; Tirmenstein et al. 2002).

1.7.1.6 Inflammatory stress hypothesis

The inflammatory stress hypothesis states that the occurrence of an acute episode of inflammation during drug therapy increases the liver's sensitivity to adverse drug effects (Shaw et al. 2010). This theory can be explained by the fact that a mild drug-induced hepatic injury might readily progress to severe tissue damage when accompanied with an extensive up-regulated production and release of pro-inflammatory mediators (Deng et al. 2009). In addition to promoting the hepatotoxic potential of drugs, inflammation may also cause a significant delay or even inhibition of hepatic tissue repair and regeneration; implying the prolongation of the injurious episode and hence the increased sensitivity of the liver (Deng et al. 2009). Nevertheless, it is noteworthy that not only inflammation may exacerbate the hepatotoxic potential of certain drugs but also drugs may aggravate the hepatic injury of an otherwise mild inflammatory stress (Deng et al. 2009). Overall, it is the synergistic interaction between the drug and pro-inflammatory mediators which results in idiosyncratic hepatotoxicity at a drug dose that is completely safe to all individuals in the absence of concurrent inflammatory reaction (Shaw et al. 2010). An episode of inflammation may be induced readily at any place and any time by various mechanisms mainly bacterial, viral and parasitic infections as well as hepatocellular death, which explains the unpredictability of IDILI (Ganey et al. 2004). Recently, several animal models have been established to validate this hypothesis through intentionally inducing inflammatory stress during drug therapy. These models were able to identify several

idiosyncratic drugs however could not provide a clear insight on the key mechanism underlying inflammation associated idiosyncratic hepatotoxicity due to the complex interactions of several inflammatory and toxicity pathways *in vivo* (Cosgrove et al. 2009).

1.8 Impact of inflammation on idiosyncratic drug-induced liver injury

Inflammation is a distinctive phenomenon which often results following tissue damage due to injury or infection; it displays characteristic features such as functional impairment and local irritation accompanied with swelling, pain, redness and heat (Deng et al. 2009). Hepatic inflammation is a frequent observation in a wide variety of liver diseases, including drug-induced liver injury, predominately due to the abundant presence of immune cells in the liver and their readiness to elicit a prominent inflammatory response following adequate stimulation (Adams et al. 2010). Significant evidence proved that hepatic inflammation alters the liver's homeostasis thereby enhancing tissue injury regardless of the underlying cause (Deng et al. 2009). Concerning idiosyncratic drug-induced hepatotoxicity, several reports documented in the literature demonstrated that the clinical use of non-steroidal anti-inflammatory drugs (NSAIDs) and antibiotics in patients with underlying inflammation is the most common cited cause of IDILI (Chalasani et al. 2008; Hussaini and Farrington, 2007). Furthermore, the concurrent presence of viral hepatitis during drug therapy increased the susceptibility of the liver so that hepatotoxicity was observed at therapeutic doses of an otherwise non-toxic drug (Hussaini and Farrington, 2007). These observations, in addition to others, exceeded simple hazard leading to the emergence of the inflammatory stress hypothesis, which suggests that an underlying episode of inflammation may predispose the liver to idiosyncratic hepatotoxicity from drugs which would otherwise remain safe in the absence of inflammation (Roth et al. 2003). This hypothesis links inflammation to hepatotoxicity by a series of successive events: Initially drug-induced injury results in lipid peroxidation, drug-protein adduct formation, bile flow impairment and calcium homeostasis disruption leading to focal zones of hepatocellular necrosis (Luster et al. 2000). Subsequently, hepatocellular necrosis will activate kupffer and other resident non-parenchymal cells, stimulating the secretion of an extensive pool of pro-inflammatory cytokines Roberts et al. 2007). The production and release of pro-inflammatory cytokines as well as other inflammatory mediators is further enhanced by the co-presence of an inflammagen like LPS, which will trigger the activation of kupffer cells and other immune cells leading to the exacerbation of the inflammatory reaction and promoting hepatic injury (Luster et al. 2000). Overall the increased sensitivity of the liver to the hepatotoxic potential of certain drugs, during hepatic inflammation, may be attributed predominantly to the extensive release of pro-inflammatory mediators, which will promote liver injury through various mechanisms. They may either cause direct hepatocelluar damage (Deng et al. 2009); or induce apoptotic and necrotic cell death signalling pathways (Deng et al. 2009); or even trigger transcriptional and functional alterations in important nuclear receptors and several drug metabolizing enzymes and transporters; thus modifying the toxicity profiles of certain drugs (LeVee et al. 2008).

1.8.1 Role of pro-inflammatory mediators in hepatocellular damage with emphasis on TNF-α

During inflammation associated drug-induced liver injury, pro-inflammatory mediators are initially released from resident hepatic cells following synergistic drug-inflammation activation (Deng et al. 2009; Ramadori et al. 2008). The released pro-inflammatory mediators predominantly include cytokines, chemokines and adhesion molecules, which will favour the infiltration of several extra-hepatic leukocytes to the liver (Table 1.6) (Adams et al. 2010). The inflammatory infiltrate often comprises monocytes, macrophages, lymphocytes (T and B cells), neutrophils, mast cells, eosinophils, natural killer cells and plasma cells (Stalnikowitz and Weissbrod, 2003). The abundant presence of active extra- and intra-hepatic cells in the liver will further amplify the production and release of a wide array of pro-inflammatory mediators (Ramadori et al. 2008). Consequently, hepatic homeostasis is disrupted and several cell death-signalling pathways are activated; thus exacerbating hepatic injury and predisposing the liver to adverse drug effects (Ramadori et al. 2008). Special interest has been attributed to kupffer cells, the resident macrophages of the liver, in inflammation associated idiosyncratic drug injury for several reasons. Firstly, kupffer cells proved to modulate acute hepatocyte injury and chronic hepatic responses to toxic compounds; secondly drug-induced modulation of kupffer cells proved to be causatively correlated with idiosyncratic hepatotoxicity; thirdly, kupffer cells prominently contribute to the exacerbation of hepatic inflammation through the extensive release of reactive oxygen and nitrogen species, proteases, thromboxane and prostaglandins in addition to a wide array of potent pro-inflammatory mediators (Roberts et al. 2007). These mediators either cause hepatocellular damage directly or indirectly by activating other cells like hepatic stellate cells causing sinusoidal contraction and neutrophils entrapment (Kharbanda et al. 2004); or sinusoidal endothelial cells favouring lymphoctes and neutrophils activation and transmigration to the parenchyma where they result in direct hepatocyte damage through the release of cytotoxic factors (Roberts et al. 2007; Adams et al. 2010). Furthermore, several drug-inflammation animal models elucidated that kuppfer cells play crucial roles in mediating idiosyncratic hepatotoxicity through the extensive release of potent damaging mediators (Roberts et al. 2007). Amongst the kupffer cells-released mediators, the major contributor to drug-cytokine synergistic hepatocellular damage is TNF-α; predominantly due to its significant contribution to hepatic homeostasis. Under normal physiological conditions, TNF-α promotes hepatocellular proliferation and hepatic generation (Nowatari et al. 2012); whereas during hepato-pathologies it will rather induce hepatocellular death (Cosgrove et al. 2008). Moreover, in various animal models of idiosyncratic hepatotoxicity, TNF-α proved to play crucial roles in predisposing the liver, to the toxic effects of several drugs (Shaw et al. 2009; Fredriksson et al. 2011). Since we are interested in investigating the impact of inflammation on idiosyncratic liver injury we will be focusing on the role of TNF-α in amplifying hepatic inflammation and mediating hepatocellular apoptosis.

1.8.1.1 Role of TNF-α in the amplification of hepatic inflammation

TNF-α is produced by various cell types such as macrophages, lymphocytes, and hepatic Kupffer cells either as membrane-bound cytokine termed, transmembrane TNF-α (tmTNF-α) or as a membrane-cleaved, soluble cytokine known as (sTNF-α) (Chastre et al. 2012). Functionally, this pleiotropic pro-inflammatory cytokine may perform a wide array of biological functions such as promoting cellular proliferation and survival, regulating the intensity and duration of immune responses, and even inducing hepatocellular apoptosis and necrosis (Chastre et al. 2012). Under normal physiological conditions, the production and release of TNF-α in the liver is negligible and therefore insufficient for the induction of hepatic damage (Wullaert et al. 2007). However during LPS-induced hepatic inflammation for example, resident kupffer cells will extensively produce and release TNF-α leading to a potent increase in its hepatic mRNA level followed by a rapid elevation in its proteinic concentration in the blood (Deng et al. 2009). Subsequently, TNF-α will perform a primordial role in orchestrating a prolonged and potent inflammatory reaction that will eventually lead to hepatocellular death and hepatic injury (Wullaert et al. 2007). In the liver, the potent pro-inflammatory role of TNF-α is initiated first of all by its binding to TNFR-1 (Newton and Dixit, 2012). Consequently, the latter will undergo a conformational change allowing it to recruit, TNF-R-associated death domain (TRADD), TNF-R associated factor-2 (TRAF2), and receptor interacting protein-1 (RIP-1) forming what is known as "Complex I" (Wullaert et al. 2007). The latter will then activate NF-κB, JNK and p38 pathways which will predominately mediate the pro-inflammatory role of TNF-α in the exacerbation of hepatic injury through several mechanisms. Initially the abundant production and release of TNF-α during an inflammatory reaction will lead to a prominent activation of the NF-κB pathway (Wullaert et al. 2007). The latter will mediate the TNF-induced transcriptional activation of various pro-inflammatory cytokines, chemokines and adhesion molecules, which will favor the recruitment of several immune cells to the liver and their subsequent adhesion to hepatocytes causing prominent cellular damage (Wullaert et al. 2007). Particularly, TNF- α will induce the prolonged up-regulation of IL-1β, IL-6, IL-8, IL-18 and IFN-γ, predominantly through the activation of the NF-κB pathway (Kearney et al. 2013; Tukov et al. 2007). The up-regulated production of these pro-inflammatory mediators either exerts direct hepatocellular damage or contributes to the amplification of the inflammatory reaction by inducing the production of monocytes /macrophages from the bone marrow and acute phase proteins from the liver (Kearney et al. 2013). Furthermore, the NF-κB-mediated TNF-induced up-regulation of several chemokines (IL-8, MIP-2, MCP-1, KC, and RANTES) and adhesion molecules (ICAM-1 and VCAM-1) will favour the abundant hepatic infiltration of several leukocytes, predominantly monocytes, nature killer cells, T and B-lymphocytes and neutrophils on one hand; and their adherence to hepatocytes causing hepatocellular damage on the other hand (Ramadori et al. 2008). In addition to recruiting them, TNF-α activates these cells in addition to resident immune hepatic cells further inducing the release of pro-inflammatory cytokines in addition to leukotriene, phosphatase A2, thromboxane A2, nitric oxide and reactive oxygen species (Yang et al. 2004); all of which result in cytotoxic hepatocellular death. In particular, neutrophils are thought to prominently exacerbate hepatic injury by a series of chronological steps: First of all neutrophils

are activated by the TNF-upregulated expression of Mac-1, which belongs to the β2-integrin family of adhesion molecules, on their cell surface (Jaeschke, 2006). Subsequently, the enhanced expression of β2-integrin will favor the extravasation of neutrophils into the parenchyma, which is a necessary step in neutrophils-mediated tissue damage (Ramaiah and Jaeschke, 2007). Finally, neutrophils will adhere to hepatocyte via the TNF-induced expression of ICAM-1 on the surface of hepatocytes causing respiratory burst and neutrophilic degranulation (Ramaiah and Jaeschke, 2007). The resulting release of proteases, nitric oxide, neutral proteinase, reactive oxygen species and other cytotoxic mediators diffuses into hepatocytes causing oncotic necrosis (Ramaiah and Jaeschke, 2007). Last but not least the increased release of TNF-α in addition to the up-regulated production of IL-1β and IL-6 may contribute to hepatic injury by favoring hemostasis (Tukov et al. 2007). These cytokines activate the coagulation system mainly by triggering tissue factor expression on monocytes, macrophages and endothelial cells leading to hepatic fibrin deposition (Shaw et al. 2009). In particular TNF-α and IL-1β demonstrated an increasing potential on the endothelial cell-mediated expression and release of PA-1, which is a potent inhibitor of fibrinolysis, further enhancing fibrin clot formation and deposition in the liver (Tukov et al. 2007). Furthermore, the TNF-induced sinusoidal endothelial cell damage seems to be also involved in inducing coagulation (Tukov et al. 2007).

Overall, TNF-α promotes hepatocellular damage and hepatic injury by several distinct mechanisms involving the contribution of a wide variety of immune cells. This observation further supports the hypothesis stating that idiosyncratic drug-induced hepatotoxicity is immune-mediated (Adams et al. 2010). Since cytokines including TNF-α cannot be stored intra-cellularly, their contribution in the regulation of inflammatory reactions depend primordially on the transcriptional pathways producing them. Concerning, hepatic TNF-α special interest has been given to NF-κB, since many of the immunological activities of kupffer cells, the main producers of TNF-α, is mediated through this precise pathway (Muriel et al. 2008). However it is important to note that other pathways, precisely JNK and p38 may also mediate the pro-inflammatory role of this cytokine (Wullaert et al. 2007). The exacerbating role of NF-κB in hepatic inflammation is predominantly based on its involvement in the transcription of a wide variety of pro-inflammatory cytokines, chemokines and adhesion molecules which favor the potent activation of immune cells, their abundant recruitment to the liver and their adhesion to hepatocytes causing cell death and promoting hepatic injury (Tak and Firestein, 2001). This role extremely contradicts the hepto-protective role of NF-κB, which favours the transcriptional activation of survival, proliferative and anti-apoptotic genes; thus protecting hepatocytes from TNF-induced cell death (Wullaert et al. 2007). Most probably this contradiction is attributed to the severity and duration of the stress signal. Precisely, when hepatocytes are exposed to TNF-α alone, the TNF-induced activation of NF-κB will most likely promote hepatocyte survival and resistance to apoptosis. However, when hepatocytes are exposed to an extensive pool of pro-inflammatory cytokines and cytotoxic mediators, like is the case during synergistic drug-cytokine exposure, the pro-inflammatory damaging role of NF-κB will be privileged on its hepato-protective role; thus

exacerbating the inflammatory reaction, inducing hepatocellular damage, and predisposing the liver to drug-cytokine hepatic injury.

1.8.1.2 Role of TNF-α in hepatocellular apoptosis

The role of TNF-α in mediating hepatocellular apoptosis is initiated by the binding of TNF-α to its receptor (TNFR-1) which will subsequently lead to the formation of complex 1 signalosome resulting in the activation of nuclear factor kappa-B (NF-κB), JNK and p38 MAPK signalling pathways in addition to effector caspases cascade (Papa et al. 2009). Under certain favouring cellular context, like the concurrent presence of predisposing pharmacological agents, an apoptosis-inducing complex II may originate from complex I following the release of TNFR1 from the membrane and the recruitment of procaspase-8 and FADD (Papa et al. 2009). Consequently, complex II or the death-inducing signalling complex (DISC) will lead to the activation of caspase-8 and the subsequent induction of the extrinsic apoptotic pathway (Cosgrove et al. 2008; Beyaert et al. 2002; Micheau and Tschopp, 2003). However, in hepatocytes the mere activation of caspase-8 is insufficient to exhibit TNF-α-induced hepatocellular death implying the need of an amplified apoptotic signal (Wullaert et al. 2007). This amplified signal is commonly provided through the concurrent activation of the mitochondrial apoptotic pathway (intrinsic pathway) along with the TNF-activated extrinsic pathway (Wullaert et al. 2007). The activation of the mitochondrial apoptotic pathways commonly occurs via caspase8-mediated truncation of Bid which results in the formation of activated tBid (Wullaert et al. 2007). The latter will then translocate to the mitochondria causing functional impairment and disrupting membrane integrity; eventually this results in the cytosolic release of cytochrome c by distinct mechanisms that involve: (1) promoting the oligomerization and insertion of Bak and Bax into the mitochondrial outer membrane forming pores; (2) favouring the opening of the mitochondrial permeability transition pores (MPT) by TNF-α which seems to play a pivotal role in TNF-induced hepatocellular apoptosis since specific inhibitors of MPT prevented the apoptotic effects of TNF *in vitro* and *in vivo* (Bradham et al. 1998; Zhao et al. 2003; Soriano et al. 2004); (3) enhancing the accumulation of reactive oxygen species by favouring the TNF-induced disruption of the mitochondrial transport chain and membrane potential; (4) mediating indirect TNF-α-induced mitochondrial impairment and cytochrome c release through the lysosomal secretion of cathepsin B (Wullaert et al. 2007). Following the cytosolic release of cytochrome c, the latter will bind to Apaf-1, deoxy-ATP, and procaspase-9 forming the apoptosome, which leads to the activation of caspase-9; subsequently, activated caspase 9 will induce the proteolytic activation of effector caspases which in turn will magnify the apoptotic signal by re-activating procaspase-8 and -9 (Wullaert et al. 2007). The overall extensive caspase activity generated from this amplification loop will eventually induce hepatocellular death. In addition to tBid-mediated activation of the mitochondrial apoptotic pathway, which sensitizes hepatocytes to TNF-α-induced apoptosis, it is noteworthy that in case of IDILI the activation of the intrinsic apoptotic pathway may be attributed to the toxic drug or its reactive metabolite. Several evidence suggesting drug-induced mitochondrial damage as an underlying mechanism of IDILI may support this hypothesis (Labbe et al. 2008; Jones et al.

2010). Hence hepatocellular death will result from the synergistic activation of the extrinsic apoptotic pathway by TNF-α and the intrinsic apoptotic pathway either by caspase-activated tBid or by direct drug-induced mitochondrial damage. It is important to note that TNFα may induce hepatocyte apoptosis by mechanisms that do not involve tBid such as the stimulation of acidic and neutral sphingomyelinase (A-SMase and N-SMase); or the generation of ROS independent of the mitochondria through 5-lipoxygenase-mediated metabolization of arachidonic acid, which is formed following the TNF-induced activation of cytosolic phospholipase A2 (Wullaert et al. 2007). Furthermore, other important pathways may also mediate liver injury during an inflammatory reaction, such as the JNK pathway which was first identified as a modulator of liver injury in animal models of APAP-induced hepatotoxicity; this pathway plays key roles in mediating cellular response to growth factors, cytokines and environmental stress (Singh and Czaja, 2007). The activation of JNK pathway may either favour proliferation and survival or promote hepatocellular death; depending on the severity and duration of the activating stimulus (Singh and Czaja, 2007). While temporal activation of JNK favours survival, prolonged activation rather promotes apoptosis (Singh and Czaja, 2007). TNF-α is known to extensively activate the JNK pathway through a ROS-dependent mechanism resulting in hepatocellular death (Schwabe, 2006). Particularly, TNF-induced ROS leads to the oxidization and inactivation of MAPK phosphatases (MKP), causing the de-phosphorylation of JNK and its prolonged activation (Schwabe and Brenner, 2006). However, the ROS-dependent prolonged activation of the JNK pathway requires the inhibition of the NF-κB pathway; since the latter is involved in the transcriptional activation of several anti-oxidant genes that will fight ROS preventing the activation of JNK (Schwabe and Brenner, 2006). In the case of inflammation associated idiosyncratic drug, the inhibition of NF-κB may be attributed to the toxic drug or its reactive metabolite, like is the case with diclofenac, thus favouring JNK-induced hepatocellular death (Fredriksson et al. 2011). The prolonged activation of JNK seems to sensitize hepatocyte to TNF-induced apoptosis through the activation of apoptotic genes upstream of the mitochondria (Schwabe et al. 2004). For example, JNK may induce the proteosomal degradation of cFLIP, which is an inhibitor of caspase-8 dependent TNF-induced apoptosis, through phosphorylating and activating Itch (Schwabe and Brenner, 2006). Earlier studies demonstrated that APAP-induced hepatotoxicity was attenuated in JNK deficient mice implying a direct involvement of the JNK pathway in drug-induced liver injury (Gunawan et al. 2006; Hanawa et al. 2008).

1.8.2 Role of pro-inflammatory mediators in the modulation of drug ADME

It is widely acknowledged that an inflammatory reaction may potently impair hepatic detoxification pathways predominantly by the action of potent pro-inflammatory cytokines such as IL-1β, IL-6, IFN-γ, and TNF-α (Fardel and LeVee, 2009). These cytokines, in addition to others, commonly induce a significant down-regulation in the expression and activity of important metabolizing enzymes, key drug transporters and related nuclear receptors; thus modifying the pharmacokinetics of certain drugs and altering their hepatotoxicity profiles (LeVee et al. 2008). Pro-inflammatory cytokines may modulate the expression and activity of their target genes either through direct interaction with their respective transcriptional pathway or

via the intermediary of regulatory nuclear receptors (Teng et al. 2007). In either case, NF-κB seems to be the most frequently involved pathway in inflammation-induced transcriptional repression of drug metabolizing enzymes and their regulatory nuclear receptors both *in vitro* and *in vivo* (Morgan et al. 2008). For example, IL-1β, IL-6, IFN-γ, and TNF-α may induce the down-regulation of CYP34A in a direct manner; or through the down-regulation of the nuclear receptor CAR, which is known to induce its expression (Fardel and LeVee, 2009). Particularly, IL-6 seems to inhibit the rifampicin- and phenobarbital-induced up-regulation of CYP2B6, CYP34A, CYP2C8 and CYP2C9 by inducing the down-regulation of both nuclear receptors CAR and PXR (Fardel and LeVee, 2009). Often during inflammation, the reduced expression and activity of drug-metabolizing enzymes and transporters are mediated by a down-regulation in their regulatory nuclear receptors (Ghose et al. 2009). Earlier studies demonstrated that the repressive potential of LPS, TNF and IL-1 on the expression and activity of CYP3A4 is mediated by the activation of the transcriptional pathway NF-κB (Aggarwal, 2004; Xiao and Ghosh, 2005); which subsequently down-regulates the PXR/RXR complex in HepG2 cell line and primary human hepatocytes (Gu et al. 2006). Nevertheless, it is noteworthy that post-translation down regulation of P450 enzymes, that involves enzyme modification and degradation, may also be an underlying mechanism of inflammation-induced variations in drug metabolism (Aitken et al. 2006). Lately, it has been suggested that a certain subset of patients, in advanced cancer stages, may suffer from anti-cancer drug induced severe toxicity most probably due to the reduced metabolic activity of P450 enzymes especially, CYP3A4 (Morgan et al. 2008). This may attributed predominantly to cancer-induced inflammation which is present in at least 60% of patients with advanced cancer; consistently, previous studies demonstrated cancer patients exhibiting significantly reduced CYP3A4 activity also presented increased plasma concentrations of inflammatory mediators, in particular IL-6 and C-reactive protein (Morgan et al. 2008). In addition to NF-κB, several transcription factors like NF-IL6, AP-1, and STAT, in addition to the MAPK pathways may be also involved in cytokine-induced alteration of drug detoxification hepatic proteins (Fardel and LeVee, 2009). Particularly, the MAPK pathway is thought to be one of the main pathways through which of IL-1β, IL-6, and TNF-α mediate their modulatory effects on hepatic drug transporters in human hepatocytes (Fardel and LeVee, 2009). In the latter, TNF-α demonstrated a decreasing potential on the mRNA expression levels of important sinusoidal influx transporters in addition to BSEP, while unaffecting all the other efflux transporters (LeVee et al. 2008). In previously established animal models, the administration of pro-inflammatory cytokines proved to down-regulate individual P450 isozymes and P-glycoprotein transporters at the gene transcriptional level causing a subsequent decrease in related mRNA, protein and enzyme/transporter activity (Morgan et al. 2002; Renton, 2004). Particularly, the administration of TNF-α and IL-1β resulted in several findings: firstly the downregulation of basolateral Ntcp and Oatp1/Oatp1a by both cytokines; secondly, TNF-α mainly repressed apical Bsep and Mrp2 while IL-1β modulated Mrp3; thirdly both cytokines demonstrated a potent suppression in the regulator of Ntcp, HNF-1α; finally both cytokines reduced nuclear protein availability and post-translational regulation through decreasing the

binding affinity of nuclear hormone receptors by reducing its heterodimerization associate, RXR (Geier et al. 2005). To date, the LPS model of inflammation in animals best demonstrate the repressive potential of pro-inflammatory cytokines on drug metabolizing enzymes and transporters; hence best elucidating the impact of inflammation on the resulting drug-induced hepatotoxicity. Cheng et al. demonstrated that rats treated with LPS exhibited a pronounced down regulation in the hepatic transcription of the CYP2C11, CYP3A2, and CYP2E1 genes (Cheng et al. 2003); probably mediated by the extensive presence of several LPS-induced cytokines which are known to be implicated in transcriptional suppression (Aitken et al. 2006). However, several investigations have shown that the speed with which P450 mRNAs are down-regulated in rat liver *in vivo* cannot be justified solely by this mechanism; suggesting the possibility of the co-occurrence of altered mRNA stability (Geier et al. 2005). Furthermore, the P450 mRNA suppression is not sufficiently rapid to explain the rapidly observed modulation in P450 protein expression and activity. Consistently, nitric oxide-mediated enzyme inhibition (Vuppugalla and Mehvar, 2005) and protein degradation (Ferrari et al. 2001) proved to be implicated in the functional suppression of some P450 isoforms in addition to mRNA suppression (Geier et al. 2005).

Table 1.6. Release of pro-inflammatory mediators from different hepatic cells during hepatic inflammation *(Adapted from Ramadori et al. 2008)*

Hepatic Cells	Mediator
Hepatocytes	IL-8, IP-10, MIG, MIP-1-2-3, KC
Sinusoidal Cells	RANTES, MCP-1, IL-8, MIP-1α, MIP-1β, MIG, ITAC
Kupffer Cells	IL-1, IL-6, IL-10, IL-18, TNF-α, TGF-β, MIPs, IL-8, IP-109, KC/GRO, RANTES
Hepatic Stellate Cells	IL-8, RANTES, MCP-1

1.9 Prediction of inflammation associated idiosyncratic drug-induced hepatotoxicity

The accurate prediction of idiosyncratic hepatotoxicity remains to date a main obstacle for pharmaceutical companies and unattained challenge for health care professionals (Kaplowitz, 2005). Idiosyncratic adverse drug reactions (IADRs) are by definition dose-independent, host-dependent, unpredictable and difficult to diagnose; thus complicating the development of effective models for the pre-marketing detection of idiosyncratic hepatotoxins on one hand and the thorough investigation of their underlying toxic mechanisms on the other hand (Deng et al. 2009). Despite the fact that the exact mechanisms underlying idiosyncratic adverse reactions in susceptible individuals have not been yet clearly elucidated, several hypotheses have emerged through the years proposing different mode of actions (Shaw et al. 2010). Recent interest has

been attributed recently to the inflammatory stress hypothesis, which led to the establishment of several cellular and animal models for the prediction of idiosyncratic hepatotoxicity.

1.9.1 *In vivo* models

Idiosyncratic drug-induced liver injury (IDILI) exhibits an extremely low incidence rate occurring barely in 1 in 10,000 patients; hence it was estimated that in order to detect IDILI clinically, 30,000 patients are required (Shaw et al. 2010). Assuming similar incidence rate in animals, 30,000 animals are required for the accurate detection of IDILI-causing drugs. Accordingly the application of animal models in the prediction of IDILI is very laborious and extremely expensive (Shaw et al. 2010). Nevertheless, some *in vivo* models were developed for the prediction of IDILI, predominantly based on the danger and the inflammatory stress hypothesis (Cosgrove et al. 2009). These animal models succeeded in reproducing idiosyncratic liabilities for some drugs in rodents based on the inflammatory stress hypothesis (Lee et al. 2000; Shaw et al. 2007; Buchweitz et al. 2002; Zou et al. 2009). Generally, in these animal models inflammation-associated idiosyncratic hepatotoxicity was assessed by the co-administration of non-toxic doses of bacterial LPS along with potentially hepatotoxic drug candidates to rodents (Deng et al. 2009, Shaw et al. 2010). The synergistic administration of both LPS and chlorpromazine induced amplified hepatotoxicity in rats as assessed by the increase in liver transferases and serum creatine kinase; when compared to the negligible hepatotoxic effect of either one of them alone (Buchweitz et al. 2002). The hepatotoxic potential of monocrotaline (MCT) was potently exacerbated in LPS-administered rats and the mortality rate was much more elevated when LPS was simultaneously administered with MCT than when it was given to rats one hour before MCT (Yee et al. 2000). The co-treatment of LPS with ranitidine and trovafloxacin revealed the hepatotoxic potential of these drugs which otherwise remained hidden in the absence of inflammation; the hepatotoxic potential of these drugs involved the activation and hepatic invasion of neutrophils since the depletion of neutrophils protected rats from drug-inflammation liver injury (Deng et al. 2006; Waring et al. 2005). The activation of neutrophils proved to be independently mediated by the pro-inflammatory cytokine TNF and plasminogen activator inhibitor-1, which contributed significantly to the synergistic hepatotoxic potential of LPS and sulindac in rats (Zou et al. 2009; Zou et al. 2011). Furthermore, ranitidine (RAN) treatment proved to sustain high plasmatic concentration of LPS-induced TNF much more potently than when LPS is administered alone to rats (Tukov et al. 2007). In addition to rat models there exist mice models for the prediction of IDILI. The administration of halothane to LPS-treated female mice revealed prominently its hepatotoxic potential; these findings were consistent with the fact that women are more susceptible to halothane-induced hepatitis (Walton et al. 1976). Similarly, the co-treatment of mice with non-toxic doses of LPS and trovafloxacin revealed the otherwise hidden hepatotoxic potential of the latter, which proved to be based on the extensive release of TNF and interferon gamma (IFN-γ) (Shaw Et al. 2010). This model proved to be selective to idiosyncratic drugs since the co-treatment of levofloxacin, the non-idiosyncratic analogue of trovafloxacin, did not cause hepatotoxicity (Shaw et al. 2007; Shaw et al. 2009).

Seemingly, the co-administration of LPS and candidate drugs in rodents improved the predictability of idiosyncratic hepatotoxicity in animals; however the extrapolation of these findings to human displayed limited success (Xu et al. 2004). Only 50% of the drugs which were identified as hepatotoxic in humans were previously detected in animals predominantly due to the following limitations: (1) Prominent interspecies differences between animals and humans related to drug absorption, distribution, metabolism and elimination mechanisms (Li, 2004). (2) Limited biological diversity since animals under well-controlled experimental settings are not representative of humans living in heterogeneous conditions (Xu et al. 2004). (3) Extremely low incidence rate of these reactions in animals since they predominantly result from human-based genetic and/or environmental susceptibility (Peters, 2005). Furthermore, the availability of animals for routine toxicity screening is being subjected to several obstacles related on one hand to the low throughput nature of animal models; and on the other hand to animal welfare and to the widespread desire to limit their scarification for experimental purposes. The drawbacks of predictive animal models may be circumvented by the development of efficient human-related *in vitro* models, that are applicable to high throughout toxicity screening and that offer a much more accurate prediction of idiosyncratic hepatotoxicity in humans.

1.9.2 *In vitro* models

Very recently *Thompson et al.* developed an extensive *in vitro* approach for the assessment of idiosyncratic drug-induced liver injury in THLE and HepG2 (Thompson et al. 2012). This approach included a panel of five *in vitro* assays, which evaluated the toxicity of 36 known drug candidates through the investigation of mitochondrial injury, drug covalent binding and drug-induced inhibition of human BSEP and rat Mrp2. Using this approach the authors identified 27 idiosyncratically hepatotoxic drugs (Thompson et al. 2012). In the last decade, the inflammatory stress hypothesis led to the development of few successful *in vitro* models for the prediction of idiosyncratic hepatotoxicity. In 2007, *Tukov et al.* co-cultured rat hepatocytes in addition to kupffer cells in the aim of establishing a cellular drug-inflammation co-culture model; that mimics the cell-to-cell interaction, which takes place *in vivo* in response to drug exposure during an inflammatory reaction (Tukov et al. 2007). Predominantly, this model tackled the impact of inflammation on the hepatotoxicity of monocrotaline (MCT). It elucidated that when the latter was administered alone to the co-culture, it did not affect the kupffer cell-mediated release of TNF and thus did not cause prominent hepatotoxicity; however, when LPS was co-administered with MCT to the co-culture, the drug caused an elevation in the levels of LPS-induced TNF (Tukov et al. 2007). Hence, the co-administration of MCT and LPS predisposed the liver to TNF-mediated hepatic injury revealing the potential hepatotoxicity of MCT (Tukov et al. 2007). These findings were consistent with the *in vivo* toxicity studies of MCT whereby the latter did not increase the plasmatic concentration of TNF when administered alone to rats; however it did potently increase it in presence of LPS (Tukov et al. 2007). Similar to LPS-administered animal models of idiosyncratic hepatotoxicity this model indicates that administering LPS to a co-culture of hepatocytes and Kupffer cells is an effective tool for the prediction of inflammation associated idiosyncratic liver injury (Tukov et al. 2007). However it should be emphasized that

the presence of hepatocytes in the same culture with kupffer cells is not innocent; the latter significantly alter the function and structure of hepatocytes. Particularly, several kupffer cell-released pro-inflammatory cytokines (TNF-α, IL-1 and IL-6) and acute phase proteins proved to down-regulate metabolizing enzymes and increase the number of ribosomes and lysosomes in hepatocytes (Panin et al. 2002; Sunman et al. 2004). Accordingly, this model displays a significant limitation in the investigation of metabolic idiosyncrasy; however it constitutes an accurate predictive tool for the detection of inflammation associated idiosyncratic drugs on one hand; and for the elucidation of the mechanisms underlying the synergistic drug-inflammation hepatocellular damage. In 2009, *Zou et al.* established a drug-inflammation *in vitro* model by administering TNF for the induction of an inflammatory reaction instead of LPS; since the latter is unable to activate inflammatory signalling pathways in hepatocytes (Ponzetti et al. 2010; Scott et al. 2009). The administration of TNF during drug therapy succeeded in revealing the hepatotoxic potential of sulindac and diclofenac in primary rat hepatocytes and HepG2 cells respectively (Zou et al. 2009; Fredriksson et al. 2011). The drawback of this model lies in the artificial induction of inflammation. The production of TNF is not readily auto-induced *in vivo* and requires always a stimulating hepatocellular stress like LPS for example. In addition to inducing the production and release of TNF, the latter activates a wide variety of intracellular signalling pathways, which contribute in different ways and to different extents in the promotion of inflammation associated hepatotoxicity (Liguori et al. 2010). Hence administering TNF alone to cells does not take into consideration the effects of these pathways on idiosyncratic drug induced hepatotoxicity; thus under-estimating the toxic mechanisms underlying drug-inflammation induced liver injury. Also in 2009, *Cosgrove et al.* established a drug-cytokine predictive cellular approach that succeeded in selectively predicting the hepatotoxic potential of several idiosyncratic drugs by co-administering them to HepG2 cells as well as primary human and rat hepatocytes along with LPS and a cytokine mix containing TNF, IFN-γ, IL-1α, and IL-6 (Cosgrove et al. 2009). This model represents an excellent pre-clinical tool for the detection of inflammation associated idiosyncratic drugs; however the direct administration of all these pro-inflammatory cytokines at a time to cells may induce a potent hepatotoxicity by itself resulting in an exacerbated hepatic injury which may not be necessarily correlated with the synergistic presence of an idiosyncratic drug.

1.10 Aims of this thesis

Firstly, this thesis aims at developing a high throughput cellular model for the prediction of inflammation associated idiosyncratic hepatotoxicity based on the inflammatory stress hypothesis. The latter states that: "the co-occurrence of an inflammatory reaction during drug therapy sensitizes the liver to adverse drug reactions, so that idiosyncratic hepatotoxicity arises at therapeutic doses from otherwise safe drugs" (Shaw et al. 2010). Experimentally, this theory implies that inducing a synergistic inflammatory reaction during the administration of new candidate drug may reveal its otherwise hidden hepatotoxic potential. Accordingly, we have co-administered HepG2 cells with an inflammatory mix, containing TNF and LPS, along with the

drugs in question in the aim of detecting their underlying hepatotoxic potential. LPS and TNF were particularly used to induce inflammation in HepG2 cells in the aim of reproducing the success of LPS-administered animal models in detecting the hepatotoxic potential of several idiosyncratic drugs in humans (Deng et al. 2009). Initially we co-administrated TNF and LPS along with four reference idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone), known to induce exacerbated hepatocellular damage in the presence of inflammation; and their non-idiosyncratic analogues (levofloxacin, aspirin, clarithromycin and buspirone) to validate the sensitivity and selectivity of our model. Subsequently, we applied this model to high throughput toxicity screening of several famous anti-cancer drugs in the aim of demonstrating its efficiency as a pre-clinical predictive tool of inflammation-associated idiosyncratic hepatotoxicity. Particularly, we have used this drug-inflammation model to screen the hepatotoxic potential of anti-cancer drugs due to the tight correlation between cancer and inflammation and hence the elevated risk for cancer patients to exhibit idiosyncratic adverse drug reactions. The development of this model aims at presenting an analogue *in vitro* approach of the previously discussed LPS-administered animal models of idiosyncratic hepatotoxicity, while providing prominent advantages on the latter. Mainly these advantages may be summarized as follows: (1) the presented model is high throughput in nature, hence it allows the simultaneous hepatotoxicity screening of a wide variety of new drug candidates in less time and significantly lower costs than animal models; (2) the presented model is based on human isolated cells and hence is much more representative of the liver's physiological response to idiosyncratic drug exposure during an inflammatory reaction, than are animals who present prominent inter-species differences in pharmacokinetics and toxicogenomics; (3) the established model elucidates exclusively the toxic mechanisms through which idiosyncratic drugs targets primary the liver; and is not confounded by extra-hepatic causes of hepatotoxicity like is the case *in vivo* whereby the liver is in a complex interaction with other organs which may result in liver-independent hepatotoxicity.

Secondly, we aimed at elucidating the cellular and molecular mechanisms underlying the inflammation-associated hepatotoxicity of four idiosyncratic drugs namely, trovafloxacin, nimesulide, telithromycin and nefazodone through the application of modern techniques such as capillary flow cytometry and toxicoproteiomics. We have focused on disclosing the major intracellular signalling pathways through which idiosyncratic drug synergizes with pro-inflammatory mediators to cause hepatocellular damage. Overall this thesis presents a new predictive drug-inflammation high throughput cellular approach, which may serve as an efficient pre-clinical tool for the prediction of inflammation-associated idiosyncratic hepatotoxicity. Furthermore, the presented work provides valuable mechanistic insights on the toxic mode of action of trovafloxacin, nimesulide, telithromycin and nefazodone in an inflammatory context with emphasis on the mechanisms and intracellular signalling pathways mediating drug-induced cholestasis, steatosis and hepatocellular apoptosis in susceptible individuals.

In the light of this view, the results attained in this thesis will be divided into 3 chapters based on the following 3 objectives:

(1) Developing a cellular model for the prediction of inflammation associated idiosyncratic hepatotoxicity and investigating its cellular underlying mechanisms

(2) Studying the implication of hepatic efflux transporters, namely MDR1 and MRP2, in the pathogenesis of inflammation associated idiosyncratic hepatotoxicity

(3) Investigating the molecular mechanisms underlying drug-induced hepatocellular death in inflammation associated idiosyncratic hepatotoxicity

Chapter 2
Material & Methods

2.1 Materials

2.1.1 HepG2 Cells

HepG2 is a human hepatocellular carcinoma cell line isolated from the hepatic tissue of a 15-year old Caucasian American Male suffering from a well-differentiated liver cancer. Morphologically, HepG2 are epithelial like adherent cells that grow as monolayers in small aggregates. HepG2 cells were obtained from the American Type Culture Collection (Maryland, U.S.A.) and was maintained in Minimum Essential Medium (MEM) supplemented with 10% Fetal bovine Serum (FBS), Penicillin/Streptomycin (100 unit/ml and 100 µg/ml) and Glutamine (2mM).

2.1.2 Drugs, pro-inflammatory mediators, reagents and compounds

All the drugs, pro-inflammatory mediators, reagents, and compounds used throughout this thesis are listed in Table 2.1 in addition to their provider and the final concentration administered to cells of each and every one of them. Unless otherwise mentioned, all the drugs and reagents are dissolved in water or DMSO depending on their solubility to attain a stock solution of 10 mM. Subsequently, the final concentration of every one of them is calculated as desired and administered to cells in 96- or 6-wells plates. The final concentration of DMSO never exceeded 0.5% in every well. The list of anti-cancer drugs used is detailed separately in Chapter 3 with their indication and mode of action.

Table 2.1. List of all the drugs, pro-inflammatory mediators, reagents and compounds used in this thesis in addition to their providers and final concentration administered to cells.

Materials	*Final Concentration*	*Provider*
DRUGS		
Trovafloxacin	450 µM	Sigma Aldrich
Nimesulide	450 µM	
Nefazodone	70 µM	
Levofloxacin	450 µM	
Aspirin	450 µM	
Buspirone	70 µM	
Clarithromycin	175 µM	
Benzbromarone	250 µM	
Verapamil	100 µM	
Telithromycin	175 µM	Tebu-Bio
PRO-INFLAMMATORY MEDIATORS		
Tumor necrosis factor alpha (TNF-α)	---	BD Pharmingen
Lipopolysaccharide (LPS from *Escherichia coli* 055:B5)	---	Sigma Aldrich
REAGENTS		
Dihydroethidium (DHE)	5 µM	Invitrogen Life Technologies
Bodipy 493/503	3.75 ng/ml	
Rhodamine 123	0.5 µM	
CDFDA	1 µM	
Propidium Iodide solution	1µg/mL	Miltenyi Biotec
COMPOUNDS		
Celastrol	100 µM	Sigma Aldrich
Thymquinone	100 µM	Sigma Aldrich
DMSO	0.5%	Sigma Aldrich

2.1.3 Kits

Table 2.2. List of all the kits used in this thesis in addition to their providers.

Kits	Provider
BD OptEIA Human Il-8 ELISA kit	Clinisciences
Annexin V-FITC apoptosis kit	Miltenyi Biotec
Guava Caspase 8 kit	Guava Merck Millipore

2.1.4 Buffers and solutions for protein preparation and immunodetection

Table 2.3. List of all the buffers and solutions used in protein extraction and western blot.

Buffers and Solutions	Composition
10X TBS (Tris buffered saline)	78.80 g/l Tris-HCl (500 mM), 87.66 g/l NaCl (150 mM), add H2O to 1 L
1 % BSA/PBS (Bovine Serum albumin)	1 % BSA in phosphate buffer saline (PBS)
6X Laemmli buffer	1.2 g SDS, 6 mg bromphenol blue, 4.7 ml glycerol, 1.2 ml Tris 0.5M (pH 6.8), 0.93g DTT, add to 10 ml
10X PBS (phosphate buffered saline)	2 g KCl, 82 g NaCl, 5.72g Na2HPO4 x 2 H2O, 2 g KH2PO4, add H2O to 1 L
1X PBS	1:10 dilution of 10X PBS in deionized water, calibrate to pH 7.4
PBST (phosphate buffered saline with Tween 20)	1 ml Tween 20 (0.1 %) add to 1 l 1x PBS (pH 7.4)
RIPA lysis buffer	50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 % NP-40, 0.1 % SDS, 1 mM NaF, 1 mM Na3VO4 and 5 µl/ml protease inhibitor cocktail
1X SDS-PAGE buffer (sodium dodecyl sulfate-polyacrylamide gel electrophoresis)	1:10 dilution of 10x SDS-PAGE buffer in deionized water
10X SDS-PAGE buffer	250 mM Tris, 1.92 M glycine, 1 % SDS in H2O1
Blocking solutions	1X TBST with 5 % nonfat dry milk
1X TBS	1:10 dilution of 10X TBS in deionized water, calibrate to pH 7.4
1X TBST (Tris buffered saline with Tween 20)	1 ml Tween 20 (0.1 %) add to 1 l 1x TBS (pH 7.4)
10X Transfer buffer	3 g/l Tris base (25 mM), 14.4 g/l glycin (192 mM), 20 % methanol, add H2O to 1 L
1X Transfer buffer	1:10 dilution of 10x transfer buffer in deionized water

2.1.5 Antibodies

Table 2.4. List of monoclonal (m) or polyclonal (p) primary and secondary antibodies used in immunolocalization, immunofluorescence and western blot.

Antibody	Host	M/P	Dilution	Provider
Anti-Pglycoprotein UIC2 (FITC)	Mouse	monoclonal	**1/100**	Abcam
Anti-MRP2	Mouse	monoclonal	**1/500**	Genetex
Anti-P53	Mouse	monoclonal	**1/500**	Santa Cruz Biotechnology
Anti-P21	Rabbit	polyconal	**1/500**	Santa Cruz Biotechnology
Anti-Bax T22-A (FITC)	Rabbit	monoclonal	**1/50**	Abcam
Anti-pERK1/2	Mouse	monoclonal	**1/500**	Santa Cruz Biotechnology
Anti-tBid	rabbit	polyclonal	**1/300**	Enogene
Anti-mouse IgG HRP-linked	rabbit	monoclonal	**1/2000**	Cell Signaling
Anti-rabbit IgG HRP-linked	mouse	monoclonal	**1/2000**	Cell Signaling

2.1.6 Capillary flow cytometer

All the cytometric experiments were performed using Guava EasyCyte Plus capillary flow cytometer (Merck Millipore, Life Science division, Merck KgaA, Darmstadt, Germany) equipped with two light scatter detectors that measure the forward scatter (an estimation of cell size) and the side scatter (an estimation of intracellular granularity); in addition to a 488nm excitation laser and four emission band pass filters at 530/40, 585/42, 675/30 and 780/30 (Figure 2.1). This instrument has automated 96-well plate handling built in, and a choice of optical layout. All cytometric results were computed using the Guava ExpressPro software (Merck Millipore Guava Tech) in terms of x-geometric mean arbitrary units (AU) or percent of fluorescent cells (%).

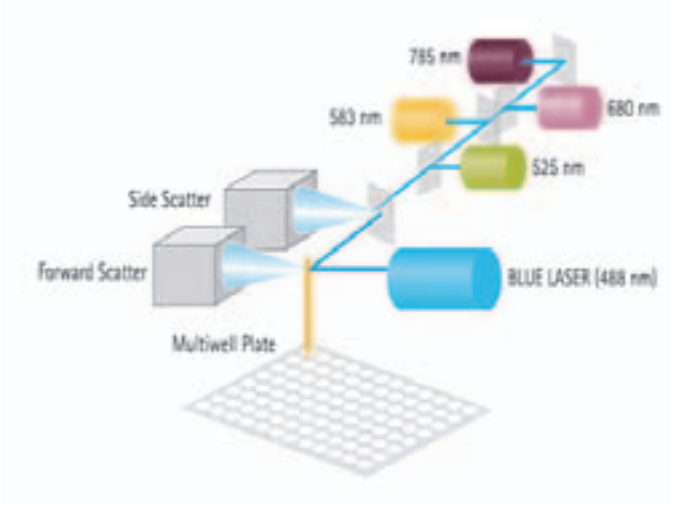


Figure 2.1. **Optical layout of Guava EasyCyte Plus capillary flow cytometer**.
(Adapted from http://www.millipore.com).

2.2 Methods

2.2.1 HepG2 culturing protocol

HepG2 cells were cultured in a special way in order to adapt them for capillary flow cytometric use. The cells were grown in 75cm^2 flasks at 37°C in 5% CO_2 until confluent. Subsequently, they were trypsinized and resuspended in fresh new medium. For experimental purposes HepG2 cells were seeded onto 96- and 6-well plates for cytometric and western blot analysis respectively.

2.2.2 Fluorescence microscopic detection of functional biliary poles using CDF assay

Before performing functional studies with HepG2 cells it is mandatory to confirm their functionality. It is widely recognized that for a hepatocyte to be functional it should be polarized. A polarized hepatocyte is one that that expresses the right membrane transporters on the right hepatic pole. In order to validate the polarity of the used HepG2 cells, the transport of CDFDA (5 and 6-carboxy-2',7'-dichlorofluorescein diacetate), the specific substrate of MRP2, was tracked using fluorescence microscopy. CDFDA is a non-fluorescent compound that enters the cell by passive diffusion where it is esterified to fluorescent CDF (5 and 6-carboxy-2',7'-dichlorofluorescein). In polarized hepatocytes, CDF should be transported to the apical (canalicular) pole where it will accumulate to be effluxed outside of the cell by MRP2. This is demonstrated under fluorescence microscopy by the accumulation of the green fluorescent CDF at the apical pole between two hepatocytes.

2.2.3 Experimental workflow

Overall this thesis aims at developing a cellular drug-inflammation model of idiosyncratic hepatotoxicity for the detection of inflammation associated hepatotoxic drugs on one hand; and at elucidating the underlying hepatotoxic mechanisms of four known idiosyncratic drugs namely trovafloxacin, nimesulide, telithromycin and nefazodone on the other hand. Accordingly, all the experiments performed follow three subsequent main steps. Firstly cells were cultured until confluent and then seeded either on 96-well pates or 6-well plates depending on the type of experiment. Secondly, cells were incubated for 24 hours with the drug under toxicity screening in presence and absence of an inflammatory context, which was stimulated using an inflammatory mix comprised of both LPS and TNF-α. Thirdly, drug-inflammation induced hepatotoxicity was assessed using several techniques including ELISA, capillary flow cytometry, immunofluorescence and western blot. ELISA was predominantly used to validate the inflammatory effect of the administered inflammatory mix. Capillary flow cytometry was used to evaluate inflammation-drug synergistic induction of hepatocellular death, steatosis, oxidative stress and cholestasis (Figure 2.2). Furthermore capillary flow cytometry was also used to investigate the expression of MDR1 and Bax proteins. Immunofluorescence was used to detect the presence of functional biliary poles in addition to the expression and localization of MRP2 proteins. Western blot was mainly used to investigate

the implication of several pro-apoptotic proteins in drug-inflammation induced hepatocellular death.

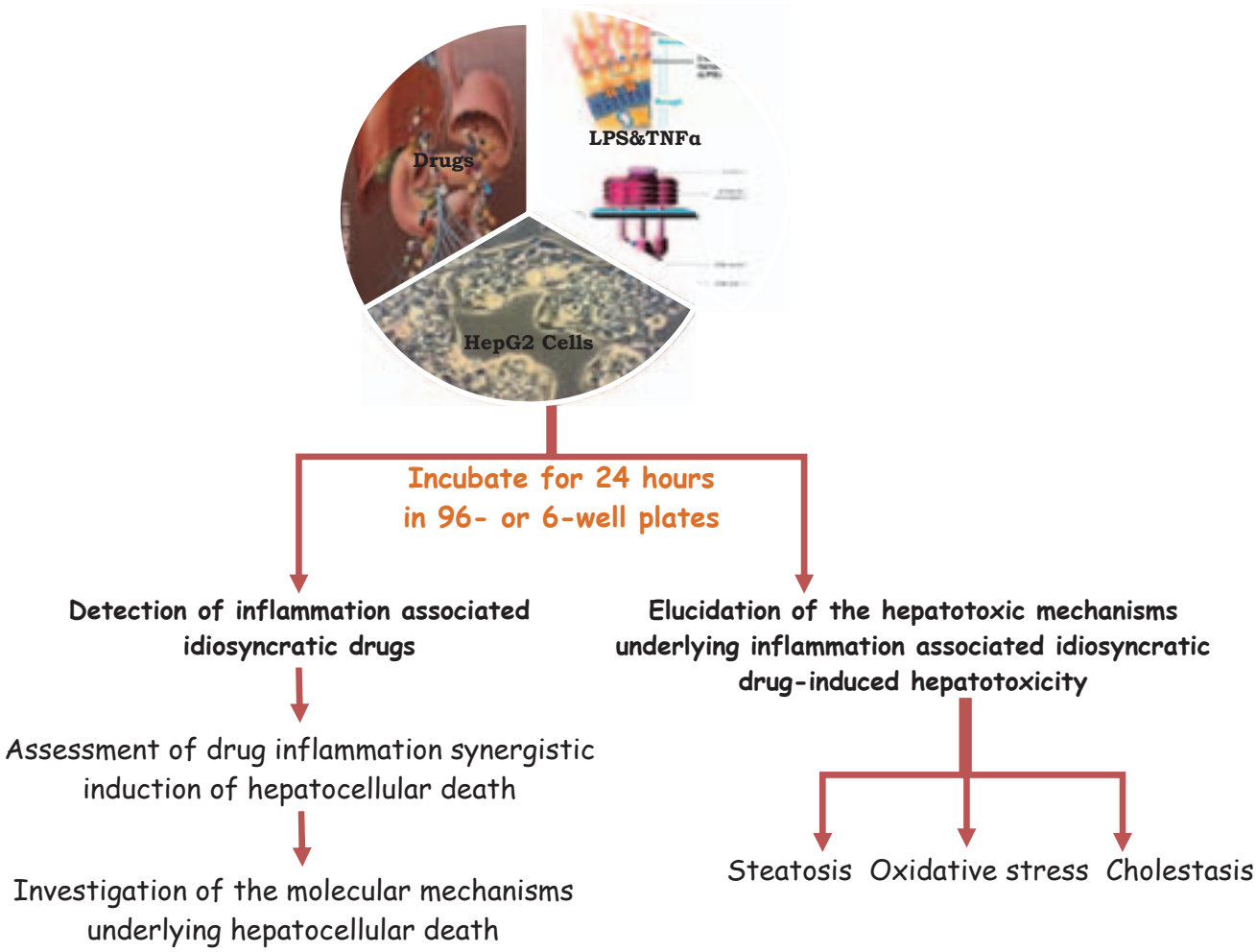


Figure 2.2. General overview of the experimental workflow.

2.2.4 IL-8 Enzyme linked immunosorbent assay (ELISA)

The principle of a double antibody sandwich ELISA is based on the reaction of a capture antibody bound to the surface of a polystyrene microtiter plate with the target protein antigen present in the sample followed by binding of a secondary biotinylated antibody to the bound antigen. The combination of two monoclonal antibodies that recognize two different non-overlapping epitopes of the antigen is used to enhance the sensitivity and specificity of the assay. The indirect detection method can be performed using the streptavidin-HRP system by covalent binding of the streptavidin to the biotin conjugates of the secondary antibody. The

addition of TMB (3,3',5,5'-tetramethylbenzidine), the most frequently used colorimetric substrate for HRP detection in ELISA, generates a blue color when TMB is oxidized during the enzymatic degradation of H2O2 by HRP. The enzyme-substrate reaction is stopped by the addition of sulfuric acid, resulting in a color change to yellow which can be measured spectrophotometrically at a wavelength of 450 nm. In order to validate the inflammatory effect of the administered inflammatory mix comprising LPS and TNF-α, IL-8 protein levels were quantified using BD OptEIA human IL-8 ELISA kit according to manufacturer's protocol. Briefly, HepG2 cells were incubated with both LPS and TNF-α for 24 hours. Subsequently, the supernatants were collected and the concentration of HepG2-released interleukin-8 (IL-8) was determined as follows: First of all 100 µL of anti-human IL-8 capture antibody (diluted 1/250 in coating buffer) was added to every well and incubated overnight at 4°C. Then the solution was aspirated and the plate was washed 3X with the wash buffer (PBS with 0.05% Tween 20) before 200 mL of assay diluent (PBS with 10% FBS pH 7.0.) were added to each well in order to block the unspecific binding sites. After aspirating the solution and washing 3X, 100 µL of standard recombinant human IL-8 or sample were added to each well and incubated at room temperature for 2 hours. The solution was then aspirated and the wells were washed 5X with the wash buffer. Subsequently, 100 µL of biotinylated anti-human IL-8 detection antibody (diluted 1/500 in PBS) was added to each well and incubated for 2 hours at room temperature while agitating. The wells were then washed 4X and incubated with 100 µL streptavidin-HRP (diluted 1/2000 in PBS-BSA) for one hour while agitating at room temperature. The solution was then aspirated and the wells were washed extensively (7X) before incubating each well with 100 µL of substrate solution (tetramethylbenzidine, TMB and hydrogen peroxide) for 30 minutes at room temperature in the dark. Finally 50 µL of stop solution (1M H3PO4 or 2 N H2SO4) were added to each well and the plate was then read at 450 nm within 30 minutes with λ correction at 570 nm.

The mean absorbance of each set of duplicate standards, controls and samples were calculated and a standard curve was plotted in order to determine the concentration of IL-8 released with respect to the optical density of the sample.

2.2.5 Capillary flow cytometric analysis of hepatocellular death using AnnexinV-FITC/PI apoptosis kit

Inflammation associated idiosyncratic drug-induced hepatotoxicity was assessed through analyzing drug-inflammation synergistic induction of hepatocellular death by capillary flow cytometry using AnnexinV-FITC/PI (Miltenyi Biotec) according to manufacturer's protocol. Briefly, HepG2 cells were incubated for 24 hours with the desired drug in presence and absence of pro-inflammatory stimuli. Subsequently, cells were washed with 1X Binding buffer and centrifuged at 300 *g* for 10 minutes. Then the supernatants were aspirated completely and the cell pellets were resuspended in 100 µL of 1X Binding buffer to which 10 µL of AnnexinV-FITC were added for 15 minutes in the dark at room temperature. Cells were then washed with 1X Binding buffer and centrifuged at 300 *g* for 10 minutes. The supernatants were aspirated completely and the cell pellets were resuspended in 500 µL of 1X Binding buffer to

which 2 µL of PI were added prior to analysis by capillary flow cytometry. No additional wash step is required. The double staining of cells with AnnexinV-FITC and PI allows the distinction between viable cells, apoptotic cells, dead cells and necrotic cells. In viable cells, the negatively charged phosphotidylserine (PS) is located on the cytosolic leaflet of the plasma membrane lipid bilayer whereas in apoptotic cells phosphotidylserine is translocated from the inner to the outer leaflet where it becomes exposed to FITC-labeled Annexin-V (a phospholipid-binding protein which has a high affinity for PS in the presence of physiological concentrations of calcium). Upon binding of FITC-labeled Annexin-V to the externalized PS a green fluorescence is emitted identifying the apoptotic cell population. Unlike apoptotic cells who are impermeable to PI, the ruptured cell membrane in necrotic cells is permeable to PI, which will enter the cell and bind to the fragmented DNA inside the nucleus emitting a red fluorescence what will identify the necrotic cell population. Overall dead cells are distinguished on a cytogram as follows: viable cells will stain negative for both AnnexinV-FITC and PI. Early apoptotic cells will stain positive solely for AnnexinV-FITC emitting a bright green fluorescence. Late apoptotic cells will stain positive for both AnnexinV-FITC and PI emitting both green and red fluorescence. Necrotic cells will stain positive only for PI emitting a bright red fluorescence (Figure 2.3).

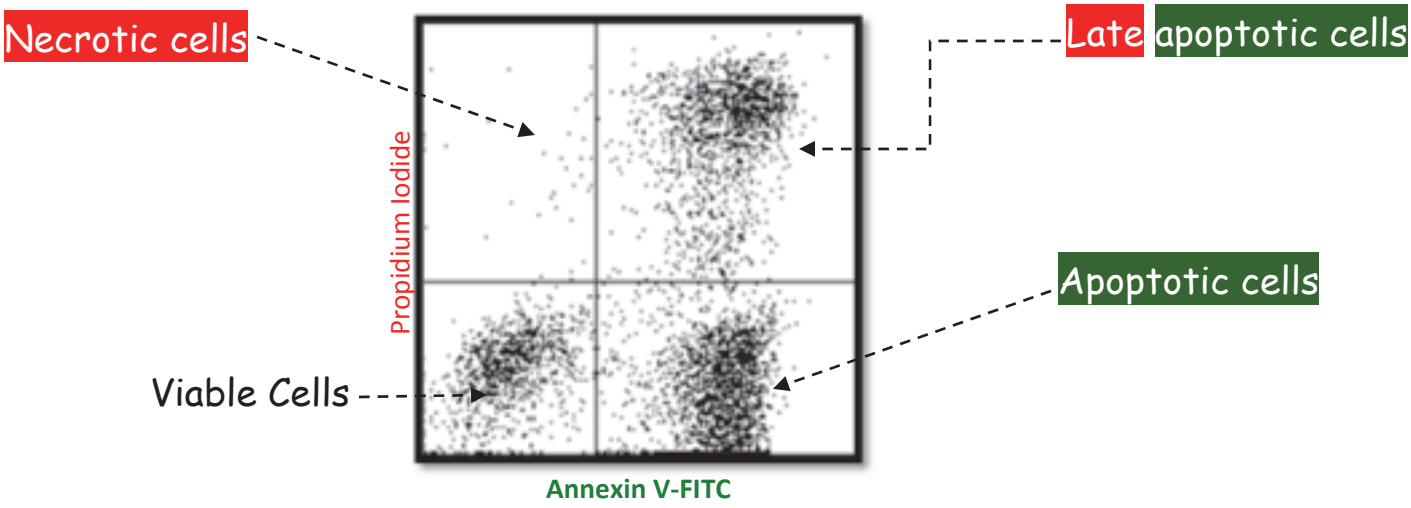


Figure 2.3. A representative cytogram demonstrating viable, early and late apoptotic and necrotic cells.
(Adapted from Miltenyi Biotec AnnexinV-FITC apoptosis assay manual).

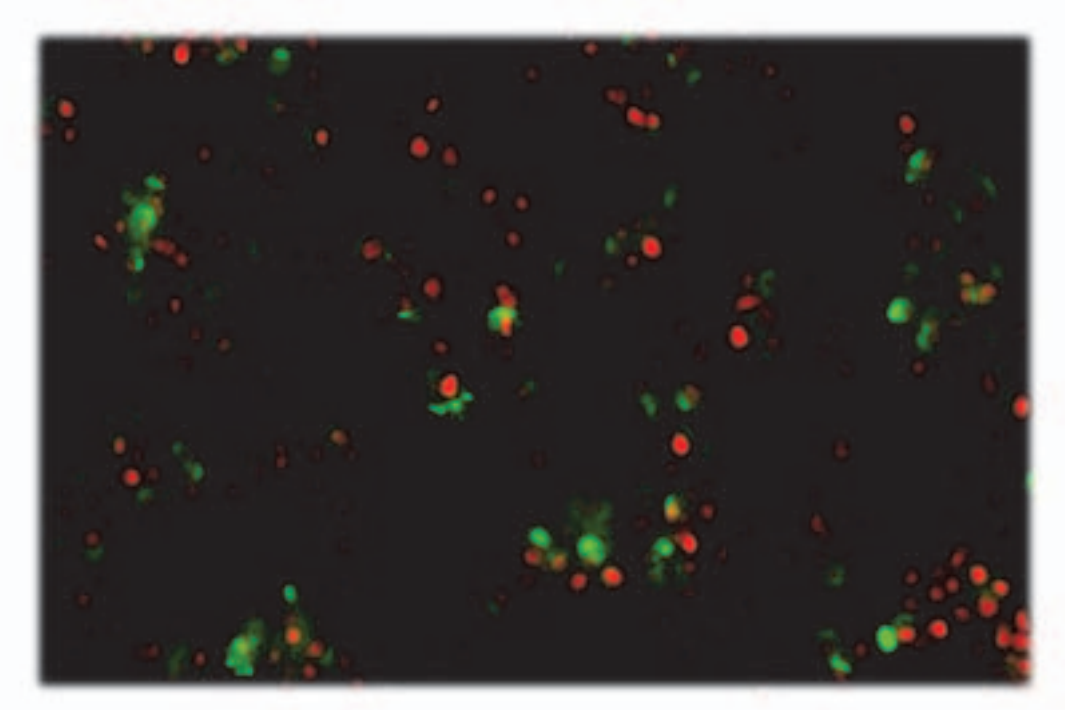


Figure 2.4. Double staining of HepG2 cells with AnnexinV-FITC (green) and PI (red). AnnexinV-FITC will bind to the translocated PS on the outer plasma membrane of apoptotic cells emitting a green fluorescence whereas PI will bind to the fragmented DNA in the nucleus of late apoptotic or necrotic cells emitting a red fluorescence. Image obtained by Celigo® (imaging cell cytometer) following the incubation of HepG2 cells with celastrol for 24 hours).

2.2.6 Capillary flow cytometric analysis of hepatocellular death using Guava Caspase 8 Kit

Caspase 8 plays a central role in apoptotic cell death and is engaged predominantly in the activation of the extrinsic apoptotic pathway; however it has been proven that in many cells such as hepatocytes, caspase 8 may also mediate the activation of the intrinsic apoptotic pathway through cleaving the pro-apoptotic protein Bid. The guava caspase 8 kit distinguishes four different populations of cells as follows: viable cells (Caspase reagent - /7-AAD -), mid-stage apoptotic cells (Caspase reagent + / 7-AAD -), late-stage apoptotic cells (Caspase reagent+ / PI+) and necrotic cells (Caspase reagent- / 7-AAD -) based on the simultaneous staining of cells with Caspase 8 FAM reagent and 7-AAD. The Caspase 8 FAM reagent is a cell permeable and non-toxic inhibitor of caspase 8 which consists of a peptide, specific for caspase 8 active site, conjugated to a carboxyfluorescein (FAM) fluorochrome as well as to a fluoromethyl ketone group (FMK), which covalently links the inhibitor to the activated caspase. As this reagent enters the cells it will bind to the activated caspase 8 and will be retained in the cell emitting a fluorescence, which is directly proportional to the number of intracellular active caspase 8 enzymes. Cells staining positive for Caspase 8 FAM reagent are committed to the extrinsic apoptotic pathway. 7-AAD is mainly an indicator of membrane

integrity and it is impermeable to viable cells as well as to early and mid-stage apoptotic cells; however it permeates late apoptotic and necrotic cells and stains the fragmented DNA in the nucleus emitting a red fluorescence. Cells are stained directly in 96-well plate with caspase 8 FAM reagent for one hour. After incubation cells are washed and stained with 7-AAD for 10 minutes before they were analyzed by Guava easycyte plus capillary cytometer.

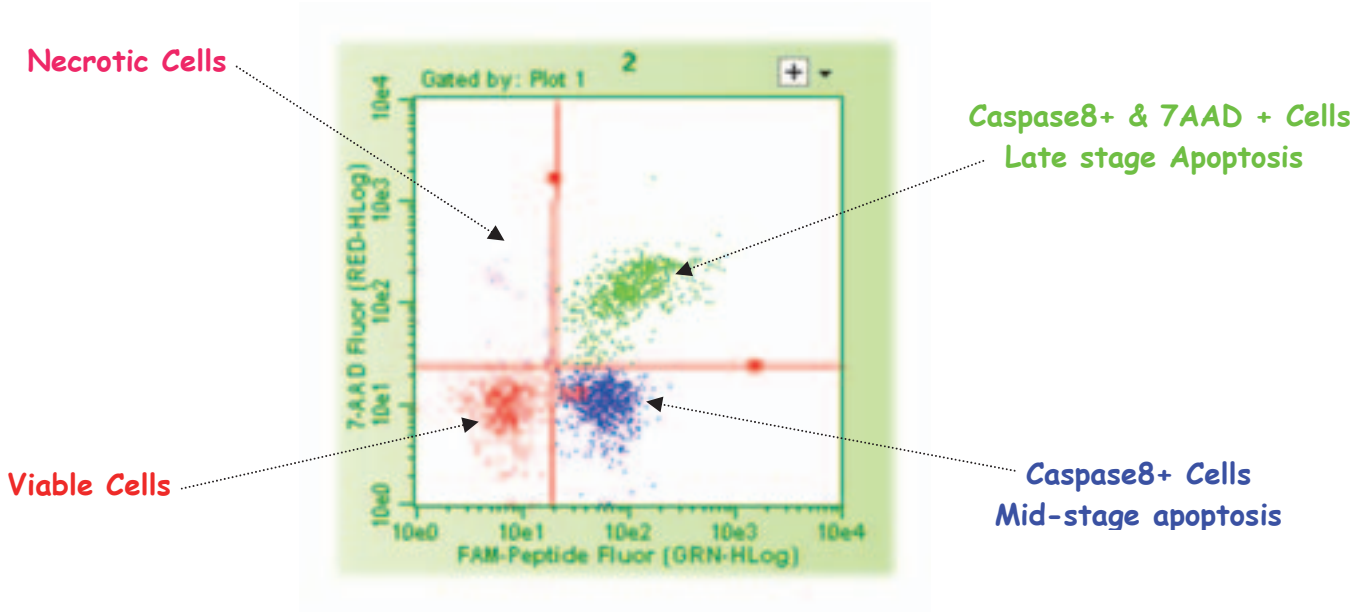


Figure 2.5. A representative cytogram demonstrating Caspase 8 FAM reagent and 7-AAD stained cells.
(Adapted from Guava caspase 8 apoptosis assay manual).

2.2.7 Capillary flow cytometric analysis of drug-induced steatosis and oxidative stress

Drug-induced liver steatosis and oxidative stress particularly superoxide anion generation were analyzed by Guava EasyCyte Plus capillary flow cytometer. Cell viability was determined by propidium iodide (PI) exclusion. PI is an intercalating agent that emits a strong red fluorescence at 617 nm upon binding to DNA. Viable cells with intact membranes are impermeable to PI whereas dead cells whose membrane is disrupted become permeable to PI, which will enter the cell and bind to the DNA in the nucleus emitting a strong red fluorescence. This allows the direct evaluation of cytotoxicity in addition to the exclusion of dead cells from the flow cytometric analysis, thus restricting further functional assessments to the live-cell population in each sample (Donato et al. 2009).

Drug-induced steatosis was assessed using BODIPY 493/503, which is a fluorescent probe used to detect intracellular lipid accumulation. This hydrophobic probe emits a green fluorescent signal with a narrow wave length range (excitation wavelength 480nm, maximum emission wavelength 515 nm) upon binding of its non-polar structure to neutral lipids in the

cell. Its hydrophobic nature facilitates its entry into non-polar environments whereas its narrow wavelength allows its application in combination with various fluorescent probes in multi-labeling experiments (Spangenburg et al. 2011). Significant evidence demonstrated that BODIPY 493/503 presents several advantages especially in terms of specificity when compared to the widely used Nile Red dye for the quantification of intracellular lipid droplets by flow cytometry (Donato et al. 2009, Spangenburg et al. 2011).

Drug-induced intracellular ROS generation, primarily superoxide anion ($O_2^{-\bullet}$), was evaluated using the cell permeable fluorogenic substrate dihydroethidium (DHE). This non-fluorescent probe diffuses passively into cells where it is oxidized rapidly by the superoxide anion $O_2^{-\bullet}$ to ethidium; which subsequently binds to the DNA in the nucleus emitting a strong red fluorescence (670 nm) (Cury-Boaventura and Curi, 2005). The emitted red fluorescence is directly proportional to the quantity of intracellular superoxide anion and hence permits the direct quantification of drug-induced $O_2^{-\bullet}$.

2.2.8 Capillary flow cytometric analysis of MDR1 and MRP2 efflux activity

Drug-induced cholestasis was evaluated by capillary flow cytometry using fluorescent transport assays. The effects of idiosyncratic drugs on the efflux activity of two important canalicular transporters, namely MDR1 and MRP2 were assessed using selective fluorescent substrates particularly Rhodamine 123 (Rho123) and 5 (and 6)-carboxy-2',7'-dichlorofluorescein diacetate (CDFDA). The fluorescent dye rhodamine 123 is a substrate for P-glycoprotein and its transport out of the cell has been demonstrated to reflect P-glycoprotein function. CDFDA is a non-fluorescent esterified form of 5 (and 6)-carboxy-2',7'-dichlorofluorescein (CDF) that freely diffuses into cells where it is cleaved by esterases to give CDF, a fluorescent dye effluxed by MRP2. Analysis of variation in rhodamine 123 and CDF intracellular fluorescence in presence and absence of the four tested idiosyncratic drugs determines if these drugs are inhibitors, enhancers or have no effect on the functionality of MDR1 and MRP2 and hence if the impairment of these transporters is implicated in inflammation associated idiosyncratic drug-induced hepatotoxicity. The inhibitory potential of the tested compounds on rhodamine-123 and CDF efflux was evaluated in comparison to the maximum inhibition obtained with 100μM verapamil and 250 μM benzbromarone, the standard inhibitors of MDR1 and MRP2, in the same experiment. Briefly, HepG2 cells were cultured in 24-well plates for 48 hrs until they were 80% confluent. Then cells were loaded with 0.5 μM rhodamine-123 (RH123) and 1 μM CDFDA for 30 min at 37°C in 5 % CO2 in the presence or absence of the 4 idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin, and nefazodone) in presence and absence of pro-inflammatory stimuli (accumulation phase). Cells were then immediately transferred on ice, washed once with ice-cold phosphate buffered saline (PBS) and resuspended in Rho123 and CDF-free medium for 120 min at 37°C to allow maximum efflux of fluorescent compounds (efflux phase). MDR1 and MRP2-mediated efflux of rhodamine 123 and CDF was monitored on a Guava EasyCyte Plus capillary flow cytometer (Merck Millipore, Life Science division, Merck KgaA, Darmstadt, Germany). The

accumulated intracellular fluorescence intensity of rhodamine 123 and CDF at 530/40 nm was computed on the Guava ExpressPro software (Merck/Millipore/Guava Tech) in terms of x-geometric mean arbitrary units (AU) and percent of fluorescent cells (%) respectively. Final concentration of DMSO applied to cells during incubation with tested drugs was 0.5%. In the tested setup, this concentration did not affect cell viability, cell morphology or rhodamine-123 and CDF efflux.

2.2.9 Capillary flow cytometric analysis of MDR1 protein (P-gp) expression

HepG2 cells were incubated with the four idiosyncratic drugs in presence and absence of an inflammatory context for 24 hours. Subsequently cells were harvested by gentle scraping using 2ml PBS/BSA buffer (Phosphate Buffered Saline pH 7.4 with 1% Bovine Serum Albumin). Cells were then transferred to a 15 ml conical tube containing 10 ml of PBS/BSA buffer and centrifuged at 400 *g* for 5 minutes. The supernatants were discarded and the cellular pellets were resuspended in PBS/BSA buffer. Then cells were incubated with FITC-labeled anti-Pgp reacting with an external surface epitope of P-gp for 30 minutes at room temperature (10µl of anti-MDR1 was added to 100µl of cells). Cells were then washed with PBS/BSA (200 µL) and centrifuged at 400 *g* for 5 minutes. The supernatants were discarded and cells were resuspended in PBS/BSA (200 µL). Data was acquired by Guava EasyCyte Plus capillary flow cytometer and results were analyzed using the Guava ExpressPro software (Merck/Millipore/Guava Tech) in terms of x-geometric mean arbitrary units (AU). The increase in FITC-emitted green fluorescence (530 nm) was directly proportional to the expression of MDR1 protein in HepG2 cells.

2.2.10 Western Blot analysis of MRP2 protein expression

After incubating HepG2 cells for 24 hours with the four idiosyncratic drugs in presence and absence of the inflammatory mix cells were scraped and transferred to 15 ml tubes and centrifuged at 500 *g* for 5 minutes. Cell pellets were washed three times with cold 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and solubilized in lysis buffer (RIPA). After spinning for 20 minutes at 14,000 *g* at 4°C, protein concentrations were determined in supernatants using a Bradford assay (BioRad). All samples, were denatured by heating for 5 minutes at 95° C, before being loaded onto a 8% gel for MRP2 protein detection. After SDS-PAGE, Western blotting followed. The transferred nitrocellulose blot was blocked with 5% skim milk powder in 50mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20 at room temperature for 2 hours. The membrane was then immunoblotted with mouse monoclonal MRP2 antibody (diluted 1/500 in BSA) overnight at 4°C. Subsequently the membrane was washed three times with 50 mM Tris-buffered saline and 0.05% Tween 20. Following incubation with horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (1:2000) for 2 hours at room temperature, the blots were developed using enhanced chemiluminescence (ECL).

2.2.11 Immunolocalization of MRP2 proteins

Localization of MRP2 in HepG2 cells was performed using indirect immunofluorescence, which is a two-step technique: Firstly, a primary unlabeled antibody binds to the target, in this case MRP2 protein; and secondly a fluorophore-labeled secondary antibody (directed against the Fc portion of the primary antibody) is used to detect the first antibody. This technique requires two incubation periods (one with the primary antibody and the other with the secondary) and hence is considered to be more complicated and time consuming than direct immunofluorescence; however, it is more sensitive because more than one secondary antibody can bind to each primary antibody, amplifying the fluorescence signal (Odell and Cook, 2012). Immunolocalization of MRP2 in HepG2 cells was performed in a 96-well plate using a tri-parametric immunostaining technique, which allowed the staining of cellular nuclei and actin filaments in addition to MRP2 proteins. MRP2 proteins were stained using a primary goat anti-MRP2 antibody followed by anti-goat dylight488-conjugated secondary antibody. The nuclei were stained using Hoechst whereas the actin filaments were stained using phalloidin. Phalloidin is a mushroom toxin known to bind with high affinity to small and large actin filaments and to be permeable to aldehyde-fixed cells (Capani et al. 2001).

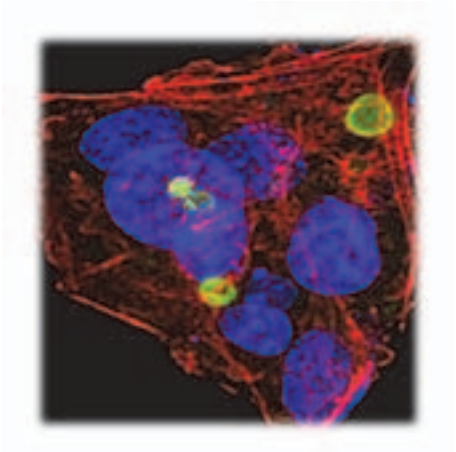


Figure 2.6. Immunostaining of HepG2 cells with anti-MRP2 (green), DY-554 phalloidin (red) and DRAQ5 (bleu pseudocolor). *(Adapted from Cell Signaling technology official website:* http://www.cellsignal.com/products/4446.html).

2.2.12 Western blot analysis of hepatocellular death signaling protein expression

After incubating HepG2 cells in 6-well plates for 24 hours with the four idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) in presence and absence of the inflammatory mix (LPS and TNF-α), cellular proteins were extracted from harvested cells for western blot analysis using RIPA buffer. Cell lysates were homogenized and quantified using BioRad DC Protein Assay. A standard dilution of Bovine Serum Albumin (BSA) (Bio-Rad) was prepared ranging from 0.125-4 mg/ml in RIPA buffer. Absorbance was read at 750nm

using a spectrophotometer and protein concentrations were calculated using the Softmax Pro5.2 software.

After quantification, and prior to electrophoresis, the sandwich gel was freshly prepared by mixing ingredients of stacking and separating gel. The polymerization of the gel is initiated by TEMED (Tetramethylethylenediamine) and APS (Ammonium persulfate). First the separating gel was poured to 3 cm from the top of the gel caster plate and overlaid with distilled water to remove air bubbles. After polymerization, the water is removed and the stacking gel solution was layered on top of the separating gel. The 10-well comb was carefully inserted into the stacking gel before polymerization. Before loading the extracted protein lysates onto the wells on the gel, they were mixed with 6x concentrate of laemmli sample buffer and denatured for 5 minutes at 95°C. The reducing agents DTT or ß-mercaptoethanol are used to remove tertiary and quaternary structure by breaking intra and inter-molecular disulfide bonds whereas bromophenol blue serves as a marker dye to enable visualization of proteins migration. The gel cassette was placed in the electrophoresis chamber and filled with 1X SDS-PAGE running buffer before loading 25 µl samples (20 µg) and 7 µl pre-stained molecular weight marker into gel wells.

Separation of protein samples was carried out under denaturing conditions using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). SDS denatures secondary and non-disulfide linked tertiary structures of proteins by adding negative charge to each protein in proportion to its mass. Thus negatively charged proteins are separated based on their molecular size, whereby smaller proteins move faster than larger proteins through the pores of polyacrylamide gel during the electrophoretic run. The separation of proteins was performed by applying an electric field of 100 V for approximately 75 minutes, causing the molecules to migrate across the gel towards the anode.

Immunoblotting (Western blotting) is a widely used technique for the detection of a specific protein on PVDF or nitrocellulose membranes using polyclonal or monoclonal antibodies. After separation of the proteins by SDS-PAGE, they can be transferred from the gel onto a membrane using a Tris-glycine-methanol transfer buffer. Methanol is used to strip complexed SDS from the protein molecules and ameliorate protein binding capacity to the membrane. After blocking of nonspecific binding sites of the membrane, the target protein is recognized by a specific primary antibody and a secondary horseradish peroxidase (HRP)-conjugated antibody, which binds directly to the primary antibody. This complex can be detected by chemiluminescence reaction in which HRP catalyzes the oxidation of the substrate luminol, resulting in emission of light. After separation the proteins were transferred onto nitrocellulose (NC) membranes at 200 V for 90 minutes on ice using 1X transfer buffer. The transfer of proteins from a gel to a membrane was accomplished by laying a gel upside down on top of filter paper, then a membrane on top of the gel with a stack of filter paper on top of the membrane. Furthermore, foam pads were placed between chambers to reduce vibration during the process of protein transfer. All blotting pads were placed in 1X transfer buffer for 10 min to equilibrate before blotting. Prior to immunodetection, the protein bands were visualized by

reversible staining of the membrane with Ponceau S solution for 1 minute to assess the efficiency of the transfer. After successful blotting, membranes were blocked in 5 % nonfat dry milk in TBST buffer (blocking buffer) for 2 hours at room temperature. Subsequently, the membranes were incubated with the desired primary antibody in blocking buffer overnight at 4 °C or for 1 hour at room temperature. After several washing steps with TBST for 15 min to remove unbound antibody, the membrane was incubated with HRP-conjugated secondary antibodies at adequate dilutions in blocking buffer for 1 hour at room temperature, followed by subsequent washing steps. Immunodetection of proteins was performed by chemiluminescence after incubating the membrane with 1.5 ml of a 1:1 mixture of Amersham ECL western blotting detection reagents (GE Healthcare) for 1 min. The protein bands were visualized by autoradiography after exposure to a light-sensitive hyperfilm.
The primary antibodies used were the following: anti-MRP2 (1/500), anti-p53 (1/500), anti-p21 (1/500), anti-pERK1/2 (1/500), anti-Bid (1/300).The secondary antibodies used rabbit anti-mouse and mouse anti-rabbit both coupled to HRP and used at a dilution of 1/2000.

2.2.13 Statistical analysis

Statistical analysis was performed using one-way and two-way analysis of variance (ANOVA) followed by Bonferroni post test in most of the experiments unless otherwise mentioned. One-way ANOVA is used to compare the means of drug-treated HepG2 cells with DMSO-treated (negative control) HepG2 cells for all parameters. Two-way ANOVA is used to compare the means of two data sets: HepG2 cells and inflamed HepG2 cells in all performed experiments.
* represents $P < 0.05$, ** represents $P < 0.01$ and *** represents $P < 0.001$. All graphs and statistics were produced using GraphPad Prism v5.00.

Chapter 3

Development of a cellular model for the prediction of inflammation associated idiosyncratic hepatotoxicity and investigation of its underlying mechanisms

3.1 Introduction

Idiosyncratic drug adverse reactions represent a leading cause of post-marketing drug withdrawal worldwide (Kaplowitz, 2005). While intrinsically toxic drugs exhibit a dose-dependent toxicity that is easily predicted during the early phases of drug development; idiosyncratic hepatotoxicity is dose-independent and unpredictable (Roth and Ganey, 2010). The low incidence rate of idiosyncratic drug adverse reactions and their correlation with a convergence of peculiar host-dependent susceptibility factors, commonly related to genetic and environmental predisposition, complicated their prediction during pre-clinical and even clinical trials (Deng et al. 2009; Ulrich et al. 2007). Hence, the hepatotoxic potential of idiosyncratic drugs was only revealed after the latter was administered to the wide population causing detrimental effects for both the developing pharmaceutical company and the adversely affected individual (Ulrich et al. 2007). Despite the lack of accurate knowledge concerning the mode of action of idiosyncratic drugs it was widely accepted that idiosyncratic adverse drug reactions are divided into two major subtypes: immunologic idiosyncrasy and metabolic idiosyncrasy; while the former is associated with typical allergic signs and symptoms (fever, rash, eosinophilia and antibody production) in addition to hepatotoxicity, the latter exhibits asymptomatic liver injury (Ramachandran and Kakar, 2009). However, definite evidence confirming either one of them remains lacking to date (Deng et al. 2009). The list of drugs causing idiosyncratic hepatotoxicity is extending continuously due to the prominent lack of effectively predictive models on one hand; and the ambiguity of the precise mechanisms causing idiosyncratic adverse reactions in certain individuals and not others, on the second hand (Roth and Ganey, 2010). Particularly, it has been reported that non-steroidal anti-inflammatory drugs and antibiotics, which are usually prescribed to treat inflammatory conditions, are the most implicated drugs in idiosyncratic hepatotoxicity (Deng et al. 2009). Furthermore, several anti-cancer drugs exhibited a sudden hepatotoxic potential in patients who presented potent inflammatory reaction at advanced cancer stages (Morgan et al. 2008). Although some anti-cancer drugs may elicit a dose-dependent intrinsic hepatotoxicity, the vast majority of chemotherapy-associated adverse drug reactions are idiosyncratic and dose-independent (Cheung et al. 2011). However, the mechanisms underlying anti-cancer drug-induced idiosyncratic hepatotoxicity in a certain patient and not the other remain to date unclear.

From the wide array of drugs that are correlated with hepatotoxicity, this chapter focuses predominantly on four specific idiosyncratic drugs namely, trovafloxacin, nimesulide, telithromycin and nefazodone; in addition to several famous anti-cancer drugs. Trovafloxacin, which is known commercially as Trovan® belongs to the fluoroquinolone family of antibiotics and exhibits a potent anti-bacterial potential, against a vast variety of gram + and gram - bacteria, by inhibiting the uncoiling of super-coiled bacterial DNA (Gootz et al. 1996). Trovafloxacin is mainly metabolized by the liver through glucuronidation and excreted by fecal elimination (Dalvie et al. 1997). This drug was withdrawn from the European market in 1999 due to frequent cases of severe hepatotoxicity (Lucena et al. 2000). Nimesulide, commercially known as

Nimalox®, is a non-steroidal anti-inflammatory drug which exhibits both anti-pyretic and analgesic properties; it exerts its pharmacological effect through the selective inhibition of COX-2 and is extensively metabolized to 16 different metabolites through different pathways involving cleavage of the parent drug molecule at its ether linkage site, reduction of the NO2 group to NH2, ring hydroxylation and conjugation with either glucuronic acid or sulphate (Macpherson et al. 2013). Nimesulide was withdrawn from the market in several countries in 2002 due to elevated hepatotoxicity risks (Traversa et al. 2003). Telithromycin, commercially known as Ketek®, is the first ketolide antibiotic approved by the FDA for the treatment of respiratory tract infections by interfering with bacterial protein synthesis (Clay et al. 2006). It is metabolized mainly by the liver through CYP3A4 and CYP1A isoenzymes and eliminated predominantly by the feces; however the majority of the drug's pharmacological effect is exerted by the parent molecule rather than by its metabolites (Zuckerman, 2004; Namour et al. 2001). In 2006, the use of telithromycin was restricted in several countries to the treatment of community acquired pneumonia (Echols, 2011). Nefazodone, commercially known as Nefadar®, is a potent anti-depressant that acts as an antagonist of the 5-HT-2A receptors; it is known to be both a substrate and an inhibitor of CYP34A (Khan et al. 2007; Bauman et al. 2008). It was withdrawn from the market in several countries in 2003 and from the US and Canada in 2004 due to several cases of liver injury.

3.1.1 Mode of action of drug-induced idiosyncratic hepatotoxicity

Several hypotheses have emerged to elucidate the hepatotoxic mode of action of idiosyncratic drugs, the most important being the danger hypothesis, the pharmacological interaction hypothesis and the inflammatory stress hypothesis (Uetrecht, 2007). All three of them suggest that the immune system is significantly involved in the precipitation of idiosyncratic hepatotoxicity (Uetrecht, 2007; Adams et al. 2010). The danger hypothesis supports the previously described hapten hypothesis, which states that following up-regulated biotransformation, the reactive metabolites of a certain drug covalently binds to cellular protein forming immunogenic foreign antigens, called hapten (Shaw et al. 2010). Subsequently, haptens will express themselves on the surface of antigen presenting cells (APC) where they activate B and T-lymphocytes eliciting an adaptive immune response (Uetrecht, 2007). However, the danger hypothesis considers the hapten-induced activation of the adaptive immune system as a primary signal that is insufficient to cause hepatic injury; hence this hypothesis proposes that a concurrent secondary signal is required to in order to further stimulate cytotoxic T-cells and antibody-producing B-cells; thus amplifying the adaptive immune response and promoting hepatic injury (Seguin and Uetrecht, 2003). This secondary signal may either be mild drug-induced cell death or even a synergistic episode of inflammation (Figure 3.1) (Kaplowitz, 2005). The pharmacological interaction hypothesis shares the same concept as the hapten hypothesis; however states that even in the absence of reactive metabolites a parent drug itself is capable of inducing an immune response by binding either to the major histocompatibility complex (MHC)

on antigen presenting cells or to T-cell receptors on T-helper cells (Figure 3.2) (Pichler et al. 2002). The inflammatory stress hypothesis, on which this thesis will be focused, states that the presence of an underlying episode of inflammation during drug therapy predisposes the liver to idiosyncratic drug adverse reactions so that hepatotoxicity will arise at therapeutic doses of otherwise non-toxic drugs; likewise the concurrent administration of a hepatotoxic drug during a mild episode of inflammation may exacerbate the inflammatory response rendering it injurious to the liver (Shaw et al. 2010). This hypothesis led to the establishment of several animal models that succeeded in identifying several idiosyncratically hepatotoxic drugs in humans (Table 3.1). Nevertheless, these models exhibited an overall limited success in the prediction of idiosyncratic hepatotoxicity since only 50% of hepatotoxic drugs in human were successfully detected in animals (Xu et al. 2004).

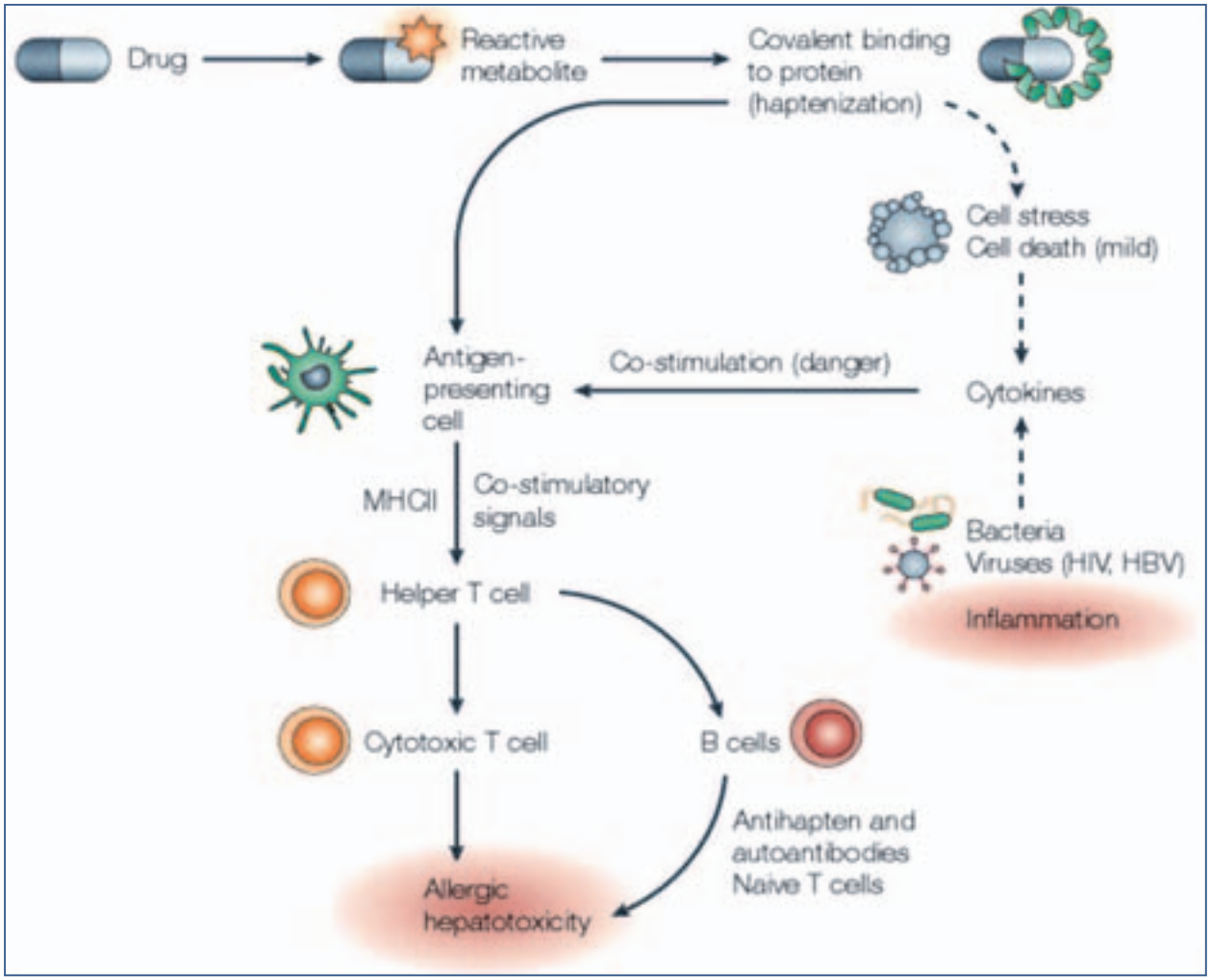


Figure 3.1. Illustration of the danger hypothesis. MHC II: Major histocompatibility complex II; HIV: human immunodeficiency virus; HBV: hepatitis B virus *(Adapted from Kaplowitz, 2005)*.

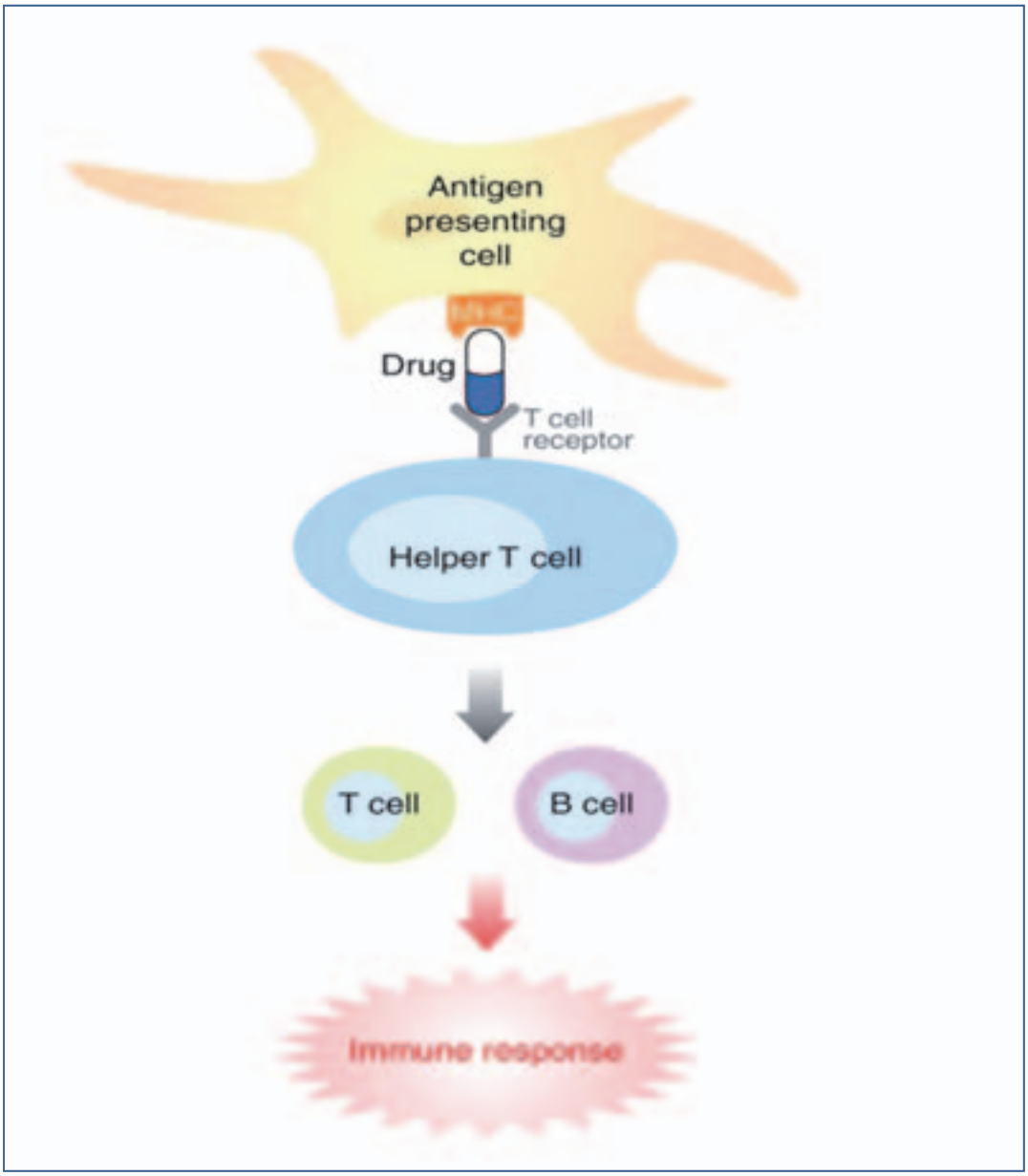


Figure 3.2. Illustration of the PI hypothesis. *(Adapted from Ueterecht, 2007).*

Table 3.1. List of idiosyncratic drugs known to cause adverse drug reactions in LPS-treated animals
(Adapted from Abdel-Bakky et al. 2010; Deng et al. 2009; Ikeda, 2011; Lu et al. 2012).

Drugs	Pharmacologic Class
Chlorpromazine	Antipschotic
Trovafloxacin	Antibiotic
Ranitidine	Histamine H2receptor antagonist
Halothane	Anaesthetic
Sulindac	Analgesic
Diclofenac	Analgesic
Troglitazone	Anti-diabetic
Amiodarone	Anti-arrhythmic

3.1.2 Mechanisms of drug-induced hepatocellular death

The low incident rate and the lack of efficient models for the study of idiosyncratic hepatotoxicity complicated the clear elucidation of its underlying mechanisms (Cosgrove et al. 2009). Similar to intrinsic hepatotoxicity, idiosyncratic adverse drug reactions may manifest as any acute or chronic liver disease which renders their clinical diagnosis a major challenge to physicians (Chalasani and Bjornsson, 2010). Drug-induced liver injury is most frequently manifested either as acute hepatitis or as cholestasis and sometimes as a mixed presentation of both pathologies (Kaplowitz, 2005). Regardless of the mode of action and even of the clinical manifestation of idiosyncratic hepatotoxicity, it is the resulting idiosyncratic drug-induced hepatocellular death which is causing hepatic failure in every case (Kaplowitz, 2005; Chakraborty et al. 2012). Hence, the investigation of the mechanisms underlying idiosyncratic drug-induced hepatocellular death may provide valuable insights for the prevention of fatal liver failure. Hepatocellular death is predominantly mediated through two major pathways: Apoptosis and Necrosis. *Russman et al.* proposed a mechanistic model elucidating the mechanisms through which a toxic drug causes hepatocellular death (Russman et al. 2009). This model is based on three subsequent steps:

Step 1: Initial mechanisms of toxicity including direct drug-induced hepatocellular stress, mitochondrial dysfunction and immune reactions.

Most frequently a drug or its reactive metabolites cause hepatocellular damage by inducing direct cellular stress, impairing mitochondrial function or stimulating the activation of the immune system as proposed by the hapten and PI hypothesis previously. Direct drug-induced hepatocellular stress may be mediated by several mechanisms including: glutathione depletion,

covalent binding to cellular components (enzymes, proteins, nucleic acid and lipids) and the inhibition of important drug transporters leading to the toxic accumulation of their substrates (Russman et al. 2009). In case of direct drug-induced mitochondrial dysfunction, toxic drugs or their reactive metabolites disrupt the mitochondrial respiratory chain leading to: (1) enhanced reactive oxygen species formation, (2) ATP depletion, (3) inhibition of β-oxidation leading to steatosis, (4) mitochondrial DNA damage and (5) mitochondrial permeability transition (MPT) (Russman et al. 2009). Immune reactions are most often evoked by the reactive metabolites of a toxic drug, which covalently binds to cellular proteins forming drug-protein adducts. These adducts express themselves as foreign antigens (Hapten formation) on the surface of antigen presenting cells bound to major histocompatibility complex (MHC) (Halt and Ju, 2006). Subsequently, this expression activates B-cells and cytotoxic T-cells; inducing the release of either antibodies against haptens or auto-antibodies against the modified cellular components in addition to cytotoxic mediators (Halt and Ju, 2006). It is important to note that a single hepatotoxic drug is not limited to one mode of action; on the contrary in most of the cases a drug causes several initial injurious mechanisms leading eventually to amplified hepatocellular death and hepatic failure. These initial injurious mechanisms may be considered as "upstream events" that will subsequently lead to the activation of "downstream events" such as the activation of the innate immune system, which will trigger the production and release of several pro- and anti-inflammatory responses; consequently favoring regeneration and recovery or promoting injury and hepatic failure (Russman et al. 2009).

Step 2: Direct or death receptor-mediated drug-induced mitochondrial permeability transition (MPT)

MPT refers to an increase in the mitochondrial permeability to small molecules (less than 1500 daltons) often resulting from the opening of the mitochondrial permeability transition pore (Martel et al. 2012). In case the drug was not severe enough to directly cause mitochondrial permeability transition leading to hepatocellular death; the latter may be mediated either through drug-induced hepatocellular stress or through the potent activation of the immune system, via two main pathways: (1) a direct pathway, referred to as the intrinsic pathway, which is often activated by a severe cell stress or (2) an indirect pathway, referred to either as the extrinsic pathway or the death receptor pathway, which is triggered by mild cell stress and/or immune reactions (Malhi et al. 2008). During the intrinsic pathway severe drug-induced hepatocellular stress promotes lysosomal permeabilization and activates the endoplasmic reticulum or the JNK pathway; subsequently the activation of pro-apoptotic (e.g. Bax, Bak, Bad) and the inhibition of anti-apoptotic (e.g. Bcl-2, Bcl-xL) proteins activates MPT (Russman et al. 2009).

In the extrinsic pathway an initial mild drug-induced injury may be exacerbated by a concurrent episode of inflammation, which will induce the production of pro-inflammatory mediators favoring the prevalence of signaling cytokines that promote injury on those that prevent it. Consequently, sensitized hepatic cells become more susceptible to the fatal effects of tumor necrosis factor alpha (TNF-α), Fas ligand (FasL) and interferon gamma (IFN-γ). Subsequently,

TNF-α and FasL will bind to their intracellular death receptors, triggering the activation of caspase 8 (Russman et al. 2009). Despite the capacity of initiator caspase 8 to directly activate effector caspases 3, 6 and 7, this direct path appears to be insufficient to cause hepatocellular apoptosis (Bantel and Schulze-Osthoff, 2012). Consequently, an amplification mechanism is required and is commonly mediated through the caspase 8-induced activation of the pro-apoptotic protein Bid in addition to signaling ceramides; leading to MPT (Russman et al. 2009).

Step 3: Drug-induced hepatocellular death

Finally MPT causes impaired mitochondrial function and energy production leading to apoptotic or necrotic hepatocellular death (Haouzi et al. 2000). MPT results following the opening of the mitochondrial pore leading to an increased permeability of the mitochondria; this allows the extensive influx of protons into the mitochondria which disrupts mitochondrial ATP synthesis (Haouzi et al. 2000). Consequently, matrix expansion and mitochondrial outer membrane permeabilization and rupture take place favoring the release of cytochrome c and other pro-apoptotic mitochondrial proteins into the cytosol (Bantel and Schulze-Osthoff, 2012). Subsequently, cytochrome c will bind to scaffold apaf-1 and pro-caspase 9 constituting a complex called apoptosome, which will subsequently activate effector caspases leading to cell death (Bantel and Schulze-Osthoff, 2012). However it is important to note that apoptosis unlike necrosis is ATP dependent; hence apoptosis can only take place if MPT did not rapidly and simultaneously occur in all mitochondria (Bantel and Schulze-Osthoff, 2012). Only if some mitochondria are left intact and continue to synthesize ATP, activated pro-caspase 9 and possibly also other pro-apoptotic mitochondrial proteins subsequently activate executioner caspase 3. Caspase 3 will then cleave specific cell proteins and further activate pro-caspases 6, 7 and 2 which have their own target proteins, eventually resulting in programmed apoptotic cell death (Bantel and Schulze-Osthoff, 2012).

Necrosis, in contrast, occurs if the initial drug-induced injury is so severe that MPT rapidly and simultaneously occurs in all mitochondria, resulting in total ATP depletion (Bantel and Schulze-Osthoff, 2012). It is important to note that necrotic cell death may also result from the activation of the extrinsic pathway in the absence of ATP (Bantel and Schulze-Osthoff, 2012). Necrosis is often accompanied with a severe inflammatory reaction which involves the extensive release of pro-inflammatory cytokines. The latter often exacerbates drug induced hepatotoxicity through sensitizing surrounding hepatocytes to injury causing further collateral damage (Russman et al. 2009).

Finally it is noteworthy that the differentiation between apoptosis and necrosis is not always easy. Mixed phenomena have been observed in some cases and the same hepatotoxin may cause apoptosis or necrosis, or even both, depending on the circumstances related to dose and preexisting vulnerability of hepatocytes (Formigli et al. 2000; Papucci et al. 2004). However, regardless of the mode of cell death it is evident that the mitochondria play a crucial role in determining cell fate predominantly because: i) the mitochondria are often a primary target for initial drug-induced toxicity; ii) MPT exhibits a fundamental role in mediating extrinsic and

intrinsic apoptosis; iii) mitochondria provide the majority of the cell's ATP supply and are also the main intracellular source of oxygen and nitrogen free radicals hence the extent of mitochondrial dysfunction eventually determines whether hepatocytes die by apoptosis or necrosis (Russman et al. 2009).

In this chapter we will be focusing on steatosis and reactive oxygen species formation, both of which are correlated with impaired mitochondrial function, as possible mechanisms of inflammation associated idiosyncratic hepatotoxicity. Reactive oxygen species (ROS) are toxic byproducts of several cellular processes such as mitochondrial respiration and CYP450-mediated drug biotransformation; however under normal physiological conditions ROS are eliminated by cellular antioxidants such as superoxide dismutase, catalase and glutathione peroxidase (Thannickal and Flanburg, 2000). In case the latter are overwhelmed by the extensive generation of ROS; oxidative injury is observed either from superoxide anion itself or from its reduced products H2O2 and hydroxyl radical (OH) (Luster et al. 2000). Extensive generation of ROS has been frequently correlated with drug-induced impairment of the mitochondrial function; nevertheless there exist other sources of ROS such as the endoplasmic reticulum and peroxisomes for example (Thannickal and Flanburg, 2000). Furthermore, it is widely recognized that pro-inflammatory cytokines such as TNF-α, IL-1 and IFN-γ are significantly involved in the generation of ROS in non-phagocytic cells (Thannickal and Flanburg, 2000). TNF-α-induced generation of ROS proved to be correlated with apoptotic cell death in several cell types including hepatocytes (Thannickal and Flanburg, 2000). Particularly, superoxide anion proved to be significantly implicated in TNF-mediated apoptosis since the over expression of superoxide dismutase 2 inhibited the apoptotic potential of TNF-α (Thannickal and Flanburg, 2000). TNF-α induces apoptosis through several mechanisms including: (1) ROS-dependent prolonged activation of cell death pathways such as JNK, (2) glutathione depletion, (3) redox-dependent formation of ceramide from sphingomyelin, and (4) activation of apoptosis signal-regulating kinase-1 (ASK-1) (Thannickal and Flanburg, 2000). Elevated ROS proved to be also correlated with the pro-inflammatory role of TNF-α in inducing the expression of cell adhesion molecules and chemokines such as IL-8 (Rahman et al. 1998; Shimada et al. 1999). The direct correlation of ROS with cell death and the wide implication of drugs and inflammation in ROS generation renders the latter a highly probable underlying mechanism of inflammation associated idiosyncratic liver injury.

Hepatic steatosis is a pathological condition referring to abnormal intracellular accumulation of lipids in the liver often leading to hepatomegaly (Anderson and Borlak, 2008). Hepatic fat accumulation may result from four different processes namely, (1) promoted de novo synthesis of hepatic free fatty acids, (2) increased delivery of free fatty acids to the liver, (3) decreased oxidation of free fatty acids, and (4) impaired synthesis or secretion of very low-density lipoprotein (VLDL) (Anderson and Borlak, 2008). Particularly, drug-induced steatosis has been predominantly correlated with inhibition of mitochondrial fatty acid oxidation (Reddy and Rao, 2006). In most cases the prolonged exposure to steatotic drugs induces macrovesicular steatosis,

whereby excessive intracellular lipids are retained in large vesicles able to dislocate the cytoplasm and deform the nucleus (Donato et al. 2009). One third of the patients with steatosis are expected to progress to steatohepatitis, which refers to inflammation-associated steatosis (Nascimento et al. 2012; Harrison et al. 2003). *Day and James* provided a pathophysiologic rationale for the progression of steatosis to steatohepatitis by proposing the "two-hit" model in 1998 (Day and James, 1998). This model suggests that the reversible intracellular deposition of triglycerides (TAG) constitutes the "first hit", which leads to several metabolic and molecular modifications sensitizing the liver to the "second hit," usually referred to as oxidative stress and cytokine-induced liver injury (Day and James, 1998). The "second hit" often results from increased mitochondrial β-oxidation of the free fatty acids, enhanced formation of reactive oxygen species and depletion of major antioxidants such as glutathione and vitamin E (Anderson and Borlak, 2008). Increased accumulation of ROS leads to oxidative stress which promotes cytokine production, especially TNF-α, through the upregulation of NF-κB. This combination of lipid peroxidation and cytokine production results in hepatocyte death (Siebler and Galle, 2006).

Drug-induced steatosis represent a major obstacle for pharmaceutical companies since it is considered as a critical manifestation of drug-induced hepatotoxicity leading to liver failure and thus to several post-marketing withdrawals and black box warnings (Donato et al. 2010). Accordingly, the development of efficient high throughput pre-clinical models able to detect steatotic drugs before they reach the market may be of tremendous benefit for pharmaceutical companies as much as for human health.

3.1.3 Drug-inflammation animal models of idiosyncratic hepatotoxicity

Generally, in these animal models inflammation-associated idiosyncratic hepatotoxicity was assessed by the co-administration of non-toxic doses of bacterial LPS along with the drug candidates under toxicity testing to rodents (Deng et al. 2009, Shaw et al. 2010). Idiosyncratic drugs, such as chlorpromazine, trovafloxacin, sulindac, amiodarone and halothane manifested their hepatotoxic potential solely in presence of an LPS-induced inflammatory reaction in rodents, while remaining non-toxic even at high doses in the absence of inflammation (Deng et al. 2009). This might explain why the idiosyncratically hepatotoxic potential of these drugs was not detected in pre-clinical and clinical phases of drug development. Similarly, a non-toxic dose of LPS is rendered significantly hepatotoxic, when co-administered with several idiosyncratically toxic drugs such as diclonfenac, sulindac, trovafloxacin, ranitidine and chlorpromazine; but not with their respective non-idiosyncratic analogues (Buchweitz et al. 2002; Luyendyk et al. 2003; Deng et. 2006; Shaw et al. 2007). Overall, these observations suggest that idiosyncratic hepatotoxicity arises following the synergistic damaging effects of both idiosyncratic drugs and LPS-induced pro-inflammatory cytokines (Kaplowitz, 2005; Ganey et al. 2004). The presence of either one without the other proved to be insufficient to induce hepatic injury; while the combined presence of both factors resulted in an amplified hepatocellular stress, which served to predispose the liver to injury from either one of them.

Significant evidence supported this suggestion in drug-inflammation animal models. For example, the administration of LPS in rats, induced a potent up-regulation in the plasma levels of various important pro-inflammatory cytokines namely tumor necrosis factor-α (TNF-α), interferon-gamma (IFNγ), interleukin-1α and -1β (IL-1α/β) and interleukin-6 (IL-6) (Bergheim et al. 2006). These cytokines are involved in the activation of several hepatocellular death signaling pathways in liver diseases and injury (Wullaert et al. 2007). Particularly, TNF-α, IL-1α/β, and LPS potently activated IKK–NF-κB, p38, and JNK pathways while IFNγ and IL-6 were correlated with the activation of STAT1 and STAT3 pathways (Schwabe and Brenner, 2006; Tacke et al. 2009; Malhi and Gores, 2008; Luedde and Trautwein, 2006). The concurrent induction of hepatocellular death in animals co-administered with LPS and idiosyncratic drugs, such as trovafloxacin, sulindac, amiodarone, cadmium and ranitidine, proved to be dependent predominantly on TNF-induced intracellular signaling pathways (Shaw et al. 2007, 2009; Tukov et al. 2007; Barton et al. 2001; Gardner et al. 2002; Kayama et al. 1995; Lu et al. 2012; Zou et al. 2009). *Shaw et al.* demonstrated that the increased trovafloxacin-induced hepatotoxicity in LPS-treated mice was predominantly due to the prolonged presence of TNF-α in the plasma (Shaw et al. 2007). Trovafloxacin is thought to increase the biosynthesis of TNF-α and decrease its elimination thus leading to high plasmatic TNF-α level which stimulates the secretion of several pro-inflammatory cytokines resulting in aggravated liver injury (Shaw et al. 2010). Seemingly the co-administration of LPS and candidate drugs in rodents improved the predictability of idiosyncratic hepatotoxicity in animals; however the extrapolation of these findings to human displayed limited success (Xu et al. 2004). Only 50% of the drugs which were identified as hepatotoxic in humans were previously detected in animals predominantly due to the following limitations: (1) Prominent interspecies differences between animals and humans related to drug absorption, distribution, metabolism and elimination mechanisms (Xu et al. 2004). (2) Limited biological diversity since animals under well-controlled experimental settings are not representative of humans living in heterogeneous conditions (Xu et al. 2004). (3) Extremely low incidence rate of these reactions in animals since they predominantly result from human-based genetic and/or environmental susceptibility (Peters, 2005). Furthermore, the availability of animals for routine toxicity screening is being subjected to several obstacles related on one hand to the low throughput nature of animal models (Cosgrove et al. 2009); and on the other hand to animal welfare and to the widespread desire to limit their scarification for experimental purposes. The drawbacks of predictive animal models may be circumvented by the development of efficient human-related *in vitro* models, that are applicable to high throughout toxicity screening and that offer a much more accurate prediction of idiosyncratic hepatotoxicity in humans.

3.1.4 Aims of chapter 3

In this part of our results, we aimed first of all at developing a high throughout cellular model for the prediction of inflammation associated idiosyncratic hepatotoxicity. This model aims at reproducing the success of the previously established LPS-administered animal models in detecting the hepatotoxic potential of idiosyncratic drugs on one hand; while offering several advantages on animal models and circumventing the numerous obstacles facing them on the other hand. Mainly these advantages may be summarized as follows: (1) the presented model is high throughput in nature, hence it allows the simultaneous hepatotoxicity screening of a wide variety of new drug candidates in less time and significantly lower costs than animal models; (2) the presented model is based on human isolated cells and hence is much more representative of the liver's physiological response to idiosyncratic drug exposure during an inflammatory reaction, than are animals who present prominent inter-species differences in pharmacokinetics and toxicogenomics; (3) the established model elucidates exclusively the toxic mechanisms through which idiosyncratic drugs targets primary the liver; and is not confounded by extra-hepatic causes of hepatotoxicity like is the case *in vivo* whereby the liver is in a complex interaction with other organs which may result in liver-independent hepatotoxicity (Deng et al. 2009); and (4) finally the application of cell lines in the prediction of idiosyncratic hepatotoxicity presents no ethical limitation and does not sacrifice a big number of animals for the sake of science, unlike animal models (Xu et al. 2004). Particularly, this advantage is very important in the prediction of idiosyncratic adverse reactions which exhibit an extremely low incidence rate (1 in 10,000); it has been estimated that in order to accurately detect the hepatotoxic effect of a single idiosyncratic drug 30,000 animals are needed (Shaw et al. 2010).

Second of all, this chapter aims at validating the efficiency of the developed drug-inflammation in high throughput toxicity screening. Accordingly this model was used to screen several anticancer drugs for inflammation-associated hepatotoxicity. The link between cancer and inflammation has been demonstrated more than hundred years ago; it has been suggested that a prolonged inflammatory reaction predisposes patients to cancer and hence inflammation was observed frequently in all types of cancer (Lu et al. 2006). Based on the inflammatory stress hypothesis, an underlying episode of inflammation predisposes the liver to adverse drug reactions from an otherwise safe drug in the absence of inflammation; accordingly, cancer patients exhibits a high risk of developing anti-cancer drug-induced hepatotoxicity (Shaw et al. 2010). Therefore the development of an efficient model able to accurately detect inflammation associated hepatotoxic drugs would save cancer patients tremendous suffering and would be of great help to health professionals and pharmaceutical companies.

Third of all, this chapter aims at elucidating the hepatotoxic mechanisms of trovafloxacin, nimesulide, telithromycin and nefazodone, in presence and absence of pro-inflammatory stimuli, with emphasis on steatosis and oxidative stress.

3.2 Experimental work

3.2.1 Development of a cellular model for the prediction of inflammation associated idiosyncratic drug-induced hepatotoxicity

This model is based on the application of our immortalized hepatic cell line HepG2 in the prediction of inflammation associated idiosyncratic drug-induced hepatotoxicity. It comprises three main constituents (Figure 3.3):

(1) HepG2 cells, which are commonly not recommended for metabolic studies due to the lack in important CYP450 enzymes and nuclear receptors; however are widely used in toxicity studies involving toxicogenomics and proteiomics (LeCluyse et al. 2012). It should be noted that the used HepG2 cells in this model are very different than the standard HepG2 cells. They are cultured in a particular way, which enhanced the expression and activity of several drug transporters and broadened the applications of this cell line. Most importantly our way of culturing these cells permitted their analysis by capillary flow cytometry similarly to cells in suspension without any problem despite their adherent nature

(2) Four known idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) and their non-idiosyncratic analogues (levofloxacin, aspirin, buspirone and clarithromycin) (Cosgrove et al. 2009). These drugs were initially used to validate the sensitivity and selectivity of the developed model since the four of them have previously proved to reveal an amplified hepatotoxic potential in the presence of an inflammatory context; whereas their non-idiosyncratic analogues did not synergize with pro-inflammatory mediators to induce amplified hepatotoxicity (Cosgrove et al. 2009). In all performed experiments, trovafloxacin and levofloxacin in addition to nimesulide and aspirin were administered at a final concentration of 450 μM; telithromycin and clarithromycin were administered at 175 μM, whereas nefazodone and buspirone were administered at 70 μM. These concentrations represent 100 fold the average plasma maximum concentration (*Cmax)* of the drug following single therapeutic dose administration in humans (Xu et al. 2008). Similar to the work published by Xu et al. and Cosgrove et al., the *Cmax* of these drugs is multiplied by 100 in our experimental conditions to encompass a scaling factor that will account for the numerous pharmacokinetic and toxicodynamic variances in the human population (Cosgrove et al. 2009). These drugs and their relevant concentrations were selected from a DILI drug database according to initial dosing studies, based on the following criteria: (i) when administered alone the drug elicits minimal drug-only hepatotoxicity, (ii) when co-administered with a representative cytokine mix, it induces robust supra-additive hepatotoxicity (iii) the administered drug is within a physiologically relevant dosing limit of 100-fold its *Cmax* value (Cosgrove et al. 2009; Xu et al. 2008).

(3) An inflammatory mix containing both LPS and TNF; both cytokines were used to induce an inflammatory reaction in the presented cellular model unlike animal models in which LPS is

administered alone. This is due to the fact that LPS commonly induces hepatic inflammation *in vivo* by stimulating macrophages, particularly Kupffer cells, to produce and release potent inflammatory mediators especially TNF-α (Luster et al. 2000). Kupffer cells are lacking in the developed model, which is constituted solely of HepG2 cells; hence both LPS and TNF-α were simultaneously administered to cells in order to investigate the implication of both cytokines in inflammation-associated idiosyncratic hepatotoxicity while circumventing the lack of Kuppfer cell-producing TNF-α. Eventually, these three constituents are set up together to make up our cellular model of inflammation-associated idiosyncratic hepatotoxicity. HepG2 cells were incubated for 24 hours with the four reference idiosyncratic drugs and their non-idiosyncratic analogues in presence and absence of an inflammatory reaction, which was stimulated by the administration of the inflammatory mix. Subsequently, the inflammation associated idiosyncratic hepatotoxicity of the administered drugs was assessed through the evaluation of synergistic drug-inflammation induced hepatocellular death.

Furthermore, this model was used to screen several anticancer drugs for inflammation associated hepatotoxicity, by assessing the synergistic anticancer drug-inflammation induced hepatocellular death, in the aim of demonstrating its efficiency as a predictive preclinical tool.

Eventually, this model was used to elucidate the possible mechanisms through which idiosyncratic drugs synergize with pro-inflammatory mediators to cause amplified hepatic injury with emphasis on oxidative stress and steatosis.

Hepatocellular death, steatosis and oxidative stress were assessed by capillary flow cytometry using fluorescent assays. Whereas the inflammatory potential of the administered inflammatory mix was assessed using enzyme-linked immunosorbent assay (ELISA).

3.2.2 Assessment of pro-inflammatory cytokine release

In order to validate if the inflammatory mix, comprising both TNF and LPS do indeed induce an inflammatory reaction in HepG2 cells, the release of pro-inflammatory cytokines was assessed using the IL-8 ELISA assay from BD Pharmingen according to manufacturer's instructions. Briefly, HepG2 cells were incubated for 24 hours with both LPS and TNF-α, after which the supernatants were used to assess the concentration of released IL-8.

3.2.3 Assessment of idiosyncratic drug-induced hepatocellular death

Hepatocellular death was assessed using Annexin V-FITC Kit (Miltenyi Biotec) according to manufacturer's protocol. Briefly, HepG2 cells were incubated with the four idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) and their non-idiosyncratic analogues (levofloxacin, aspirin, buspirone and clarithromycin); in presence and absence of the inflammatory mix (TNF-α & LPS) for 24 hours. Cells were then washed with phosphate buffered saline (PBS) and stained with 10µl AnnexinV-FITC and 5µl PI. Annexin V is a

phospholipid-binding protein which has a high affinity for phosphotidylserine (PS) in the presence of physiological concentrations of calcium. In viable cells, the negatively charged phosphotidylserine (PS) is located in the cytosolic leaflet of the plasma membrane lipid bilayer whereas in apoptotic cells phosphotidylserine is translocated from the inner to the outer leaflet where it becomes exposed to FITC-labeled Annexin V. The binding of FITC-labeled Annexin V to the externalized phosphotidylserine will emit a green fluorescence identifying the apoptotic cell population. PI is an intercalating agent, which binds to the DNA of dead cells (necrotic cells or late apoptotic cells) with disrupted plasma membranes emitting a red fluorescence. The fluorescence intensity of Annexin-V FITC stained cells at 530/40 nm and PI stained cells at 675/30 were analyzed by Guava EasyCyte Plus capillary flow cytometer and computed using the Guava ExpressPro software (Merck/Millipore/Guava Tech) in terms of x-geometric mean arbitrary units (AU). Final concentration of DMSO applied to cells during incubation with tested drugs was 0.5%. In the tested setup these concentrations had no adverse effects on cell viability or cell morphology. The apoptotic potential of the four tested drug was compared to the apoptotic potential of celastrol (100µM) in the same experiment. Using this assay we were able first of all to determine the mechanism through which the tested idiosyncratic drugs cause hepatocellular death (apoptosis or necrosis) and second of all to investigate the impact of inflammatory mediators (TNF and LPS) on these mechanisms of death.

3.2.4 Assessment of inflammation associated anticancer drug-induced hepatotoxicity

The ancestral link between inflammation and cancer in addition to the recent recognition of inflammation as a susceptibility factor for idiosyncratic hepatotoxicity, predispose cancer patient to a high risk of inflammation associated idiosyncratic hepatotoxicity. Accordingly, we were highly interested in using the developed drug-inflammation cellular model for the detection of inflammation associated hepatotoxic anti-cancer drugs. The hepatotoxic potential of these drugs was assessed through the investigation of hepatocellular death following the simultaneous staining of HepG2 cells with AnnexinV-FITC and PI (Miltenyi Biotec apoptosis kit) according to manufacturer's protocol. Briefly, after incubating the HepG2 cells with several anti cancer drugs (listed in Table 3.2) in presence and absence of an inflammatory context, cells were then washed with phosphate buffered saline (PBS) and stained with 10µl AnnexinV-FITC and 5µl PI. Subsequently the fluorescence intensity of AnnexinV FITC stained cells at 530/40 nm and PI stained cells at 675/30 were analyzed by Guava EasyCyte Plus capillary flow cytometer and computed using the Guava ExpressPro software (Merck/Millipore/Guava Tech) in terms of x-geometric mean arbitrary units (AU). All anticancer drugs were dissolved in DMSO in order to attain a final stock solution at 10 mM. The concentration of the administered anticancer drugs was relevant to 100 fold their *Cmax* in humans following single therapeutic dose. Final concentration of DMSO applied to cells during incubation with tested drugs was 0.5%. In the tested setup these concentrations had no adverse effects on cell viability or cell morphology. The

apoptotic potential of the four tested drug was compared to the apoptotic potential of celastrol (100µM) in the same experiment.

3.2.5 Assessment of idiosyncratic drug-induced oxidative stress

HepG2 cells were incubated with the four idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) in presence and absence of the inflammatory mix (TNF-α & LPS) for 24 hours. Subsequently, the cells were stained for 30 minutes at 37°C in the dark with dihydroethidium (DHE) at a final concentration of 5µM. DHE passively diffuses in the cells where it is oxidized by superoxide anion $O_2^{-\bullet}$ to ethidium (a red fluorescent compound). The emitted red fluorescence at 630nm was proportional to the quantity of superoxide anion found inside the cells. The results were analyzed using Guava ExpressPro software (Merck/Millipore/GuavaTech) in terms of x-geometric mean arbitrary units (AU). Final concentration of DMSO applied to cells during incubation with tested drugs was 0.5%. In the tested setup these concentrations had no adverse effects on cell viability or cell morphology. The oxidative potential of the four tested drug was compared to the oxidative potential of thymoquinone (100µM) in the same experiment.

3.2.6 Assessment of idiosyncratic drug-induced steatosis

HepG2 cells were incubated with the four idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) in presence and absence of the inflammatory mix (TNF-α & LPS) for 24 hours. Then cells were stained for 30 minutes at 37°C in the dark with BODIPY 493/503 in combination with PI at a final concentration of 3.75 ng/mL and 2.5µg/mL respectively. The cell suspensions were then analyzed by flow cytometry directly without any additional washing step. The emitted green fluorescence of BODIPY 493/503-stained cells at 515 nm was directly proportional to the quantity of accumulated intracellular lipids. All the measurements were restricted to live cells by gating the cells that excluded PI. The results were analyzed using Guava ExpressPro software (Merck/Millipore/GuavaTech) in terms of x-geometric mean arbitrary units (AU). Final concentration of DMSO applied to cells during incubation with tested drugs was 0.5%. In the tested setup these concentrations had no adverse effects on cell viability or cell morphology.

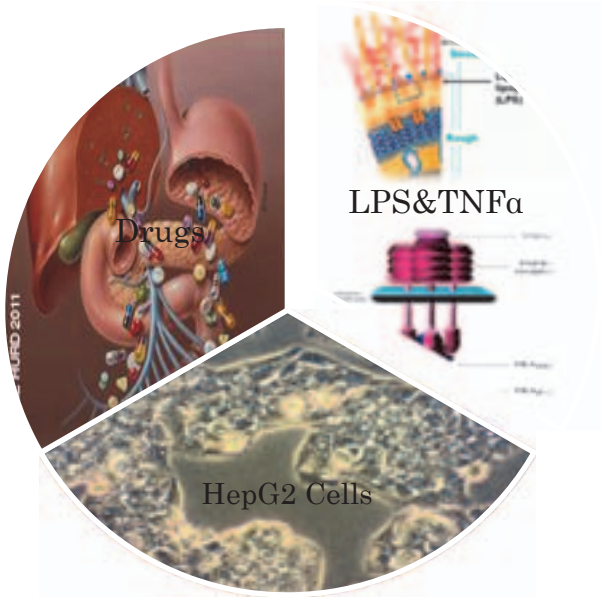


Figure 3.3 Illustration of the developed drug-inflammation cellular model.

Table 3.2. The anti-cancer drugs used in the developed drug-inflammation model and their respective indications.

Anti-cancer drugs	*Mode of action and Indication*
Azaguanine-8	Antimetabolite (purine analog) used in the treatment of acute leukemia
Nocodazole	Anti-neoplastic drug that interferes with the polymerization of microtubules
Thioguanine	Antimetabolite used in the treatment of acute myelogenous and lymphoblastic leukemia
Methotrexate	Antimetabolite (Folic acid antagonist) used in the treatment of ALL, NHL, breast, head and neck, lung, stomach, and esophagus cancers
Mitoxantrone	Anthracenedione used in the treatment of metastatic breast cancer, ALL, AML, NHL
Etoposide	Topoisomerase II inhibitor used in the treatment of Kaposi's and Ewing's sarcoma, glioblastoma, lung and testicular cancer
Daunorubicin HCl	Anthracycline used in the treatment of ALL and AML
Ifosfamide	Alkylating agent used in the treatment of testicular, breast, ovarian, cervical and lung cancer in addition to NHL and HL
Azacitidine-5	DNA methyltransferases inhibitor used in the treatment of MDS and CML
Chlorambucil	Alkylating agent used in the treatment of chronic lymphocytic leukemia, NHL and ovarian carcinoma
Cytarabine	Antimetabolite (Pyrimidine antagonist) used in the treatment of AML and NHL
Busulfan	Alkylating agent (Alkylsulfonate) used in the treatment of CML
Docetaxel	Antimitotic plant alkaloids (Taxanes) used in the treatment of breast, ovarian, prostate and non small-cell lung cancer
Temozolomide	Alkylating agent used in the treatment of glioblastoma multiforme and melanoma
Topotecan	Topoisomerase inhibitor plant alkaloids (Camptothecan analogs) used in the treatment of ovarian, cervical and small cell lung cancer
5-Fluorouracil	Antimetabolite (Thymidylate synthase inhibitor) used in the treatment of colorectal and pancreatic cancer
Erlotinib	Tyrosine kinase inhibitor (EGFR inhibitor) used in the treatment of lung and pancreatic cancer
Imatinib	Competitive tyrosine kinase inhibitor used in the treatment of CML and GISTS
Floxuridine	Antimetabolite used in the treatment of colorectal cancer
Vorinostat	Histone deacetylase inhibitor used in the treatment of cutaneous T-cell lymphoma
Cyclophosphamide	Alkylating agent used in the treatment of lymphoma, leukemia and brain cancer
Fludarabine	Antimetabolite (Adenosine deaminase inhibitor) used in the treatment of chronic lymphocytic leukemia and lymphomas

3.3 Results

3.3.1 LPS and TNF-induced IL-8 release

The main target of co-administering LPS and TNF-α along with the four idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) to HepG2 cells is to induce an inflammatory reaction which will serve to reveal the hepatotoxic potential of these drugs; based on the inflammatory stress hypothesis and the previously established drug-inflammation models (Deng et al. 2009; Cosgrove et al. 2009; Shaw et al. 2010). Therefore it is very important to ensure that the administered inflammatory mix satisfies this primary target.

Inflammatory reactions are predominantly evaluated based on the potent activation of intracellular inflammatory signaling pathways and on the extensive release of pro-inflammatory mediators. Accordingly, the levels of IL-8 were assessed using ELISA to confirm that the administered inflammatory mix do indeed induce a significant inflammatory reaction. IL-8-induced recruitment of leukocytes, especially neutrophils, to the site of inflammation is considered as the hallmark of acute inflammation (Harada et al. 1994). The attained results demonstrate that incubating HepG2 cells with both LPS and TNF-α for 24 hours, induces a potent increase in the concentration of secreted IL-8 corresponding to 1000 pg/ml (Figure 3.4).

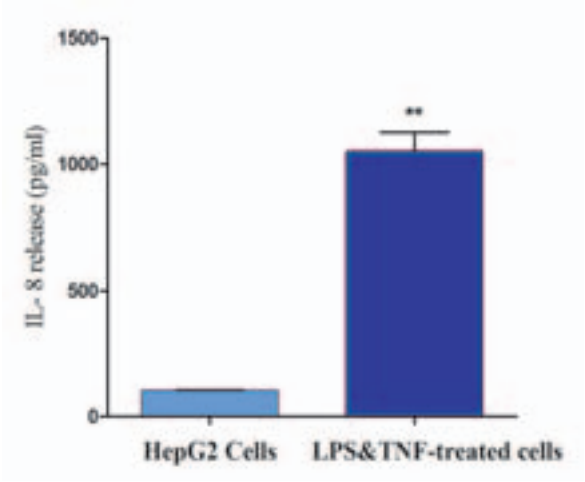


Figure 3.4. Effect of LPS and TNF-α on IL-8 release in HepG2 cells. After incubating HepG2 cells with LPS and TNF-α for 24 hours, a potent increase in IL-8 secretion is demonstrated corresponding to a concentration of 1000 pg/ml. Bars represent Mean ± S.E.M. Statistical analysis was performed using unpaired t-test. ** represents $P < 0.01$ and refers to the variation in IL-8 secretion between non-inflamed and inflamed HepG2 cells (LPS and TNF-α-treated cells).

3.3.2 Idiosyncratic drug-induced hepatocellular death

Hepatocellular death is predominantly the end result of every hepatotoxic drug, which leads to hepatic injury and eventually liver failure in severe cases; hence the investigation of drug-induced hepatocellular death provides direct valuable insights on the severity of the hepatotoxic potential of the administered drug. In this chapter, idiosyncratic drug induced hepatocellular death was assessed using a fluorescent apoptosis kit (Miltenyi Biotec) which distinguishes live cells, early apoptotic cells, late apoptotic cells and necrotic cells based on the simultaneous staining of cells with AnnexinV-FITC and PI. After incubating HepG2 cells for 24 hours with four idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) and their four non-idiosyncratic analogues (levofloxacin, aspirin, buspirone and clarithromycin) in presence

and absence of the inflammatory mix (TNF-α and LPS), the results attained demonstrated that in the absence of an inflammatory context only nefazodone and telithromycin exhibit a significant apoptotic potential when compared to the negative control (cells incubated with DMSO only) (Figure 3.5A). However, when these drugs were administered to cells along with TNF-α and LPS, trovafloxacin, nimesulide, nefazodone, clarithromycin, and telithromycin exhibited a more potent apoptotic effect than when they were administered to cells alone (Figure 3.5B). The attained results demonstrated that, only the four idiosyncratic drugs but not their non-idiosyncratic analogues, with the exception of clarithromycin, synergize with TNF-α and LPS to promote hepatocellular apoptosis (Figure 3.5C). The apoptotic potential of the investigated drugs is elucidated by an increase in green and red fluorescence of AnnexinV-FITC/PI stained cells as demonstrated in Figure 3.6.

3.3.3 Anticancer drug-induced hepatocellular death

Inflammation is correlated with approximately 60 % of advanced cancers (Morgan et al. 2008); therefore it represents a major susceptibility factor predisposing cancer patients to anti-cancer drug-induced hepatotoxicity which may significantly exacerbate their health state. Accordingly, the early prediction of inflammation associated anticancer drug-induced hepatotoxicity may save these patients tremendous amounts of suffering and in some cases may save their lives. Therefore, we utilized the developed cellular drug-inflammation model to detect the inflammation-associated hepatotoxic potential of several anti-cancer drugs through the investigation of synergistic inflammation-anticancer drug-induced hepatocellular death. After incubating the cells with several anti cancer drugs (listed in Table 3.2) in presence and absence of the inflammatory mix, the attained results demonstrated that azaguanine-8, nocodazole, methotrexate, etoposide, azacytidine-5, chlorambucil, cytarabine, busulfan, docetaxel, 5-fluorouracil, erlotinib, imatinib, and fludarabine synergize with LPS and TNF-α to induce an amplified apoptotic potential (Figure 3.7).

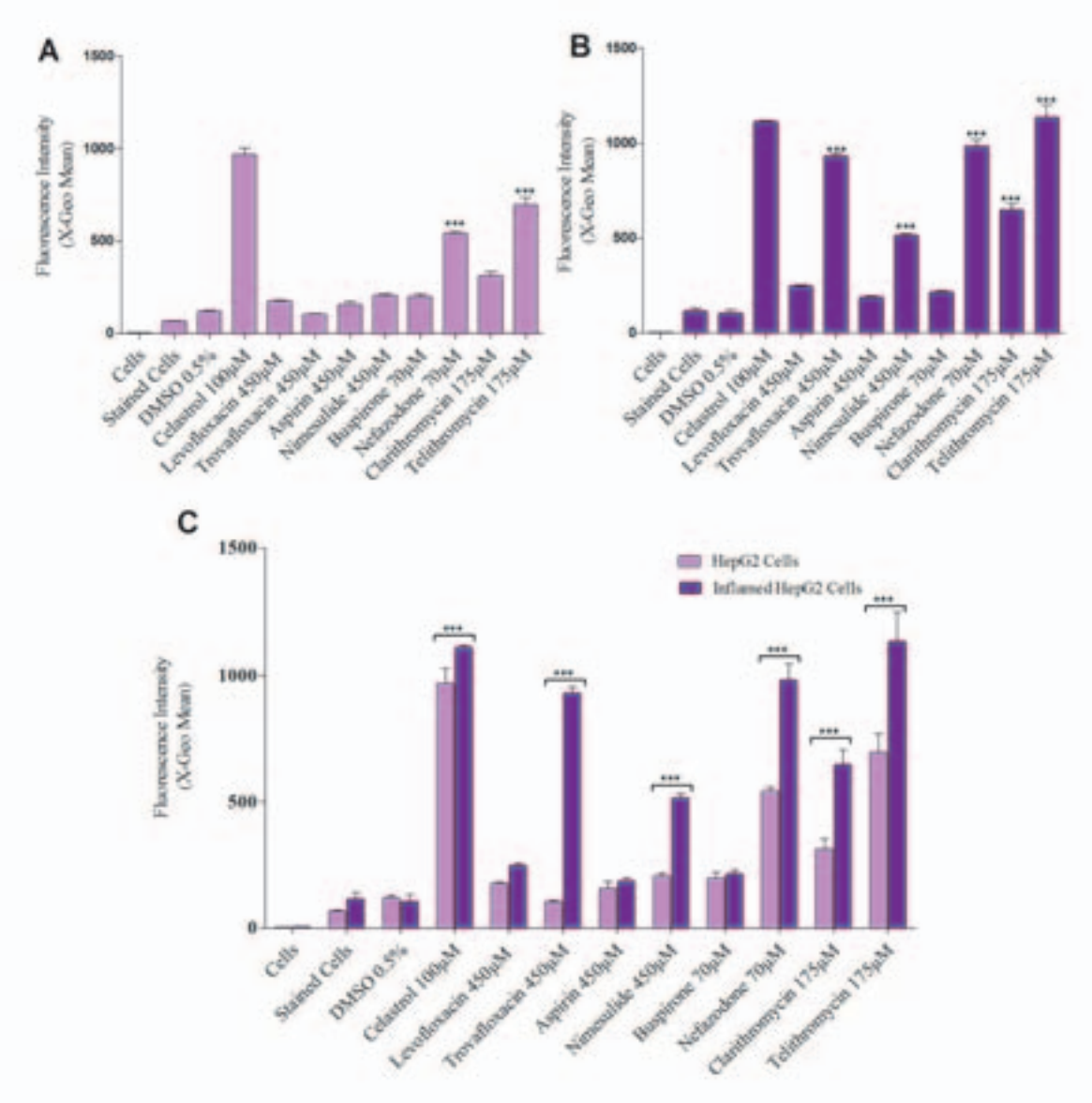


Figure 3.5. Apoptotic effect of four idiosyncratic drugs and their non-idiosyncratic analogues in non-inflamed and inflamed HepG2 cells. After incubating HepG2 cells with the four idiosyncratic drugs in absence (A) and presence of LPS and TNF-α (B) for 24 hours, hepatocellular death was assessed following the simultaneous staining of cells with AnnexinV-FITC and PI by capillary flow cytometry. Graph C represents the apoptotic effects of the four tested idiosyncratic drugs in absence and presence of LPS and TNF-α. Bars represent the emitted intracellular fluorescence of AnnexinV-FITC and PI stained cells in terms of X-geometric mean (AU). Data is represented as Mean ± S.E.M (n=3). Statistical analysis was performed using one way (for graphs A and B) and two way ANOVA (for graph C) followed by Bonferroni post test. *** represents $P < 0.001$ and refers to the variation in apoptotic potential between DMSO-treated and drug-treated HepG2 cells in graphs A and B. In graph C the p-value corresponds to the variation in apoptotic potential between non-inflamed and inflamed HepG2 cells.

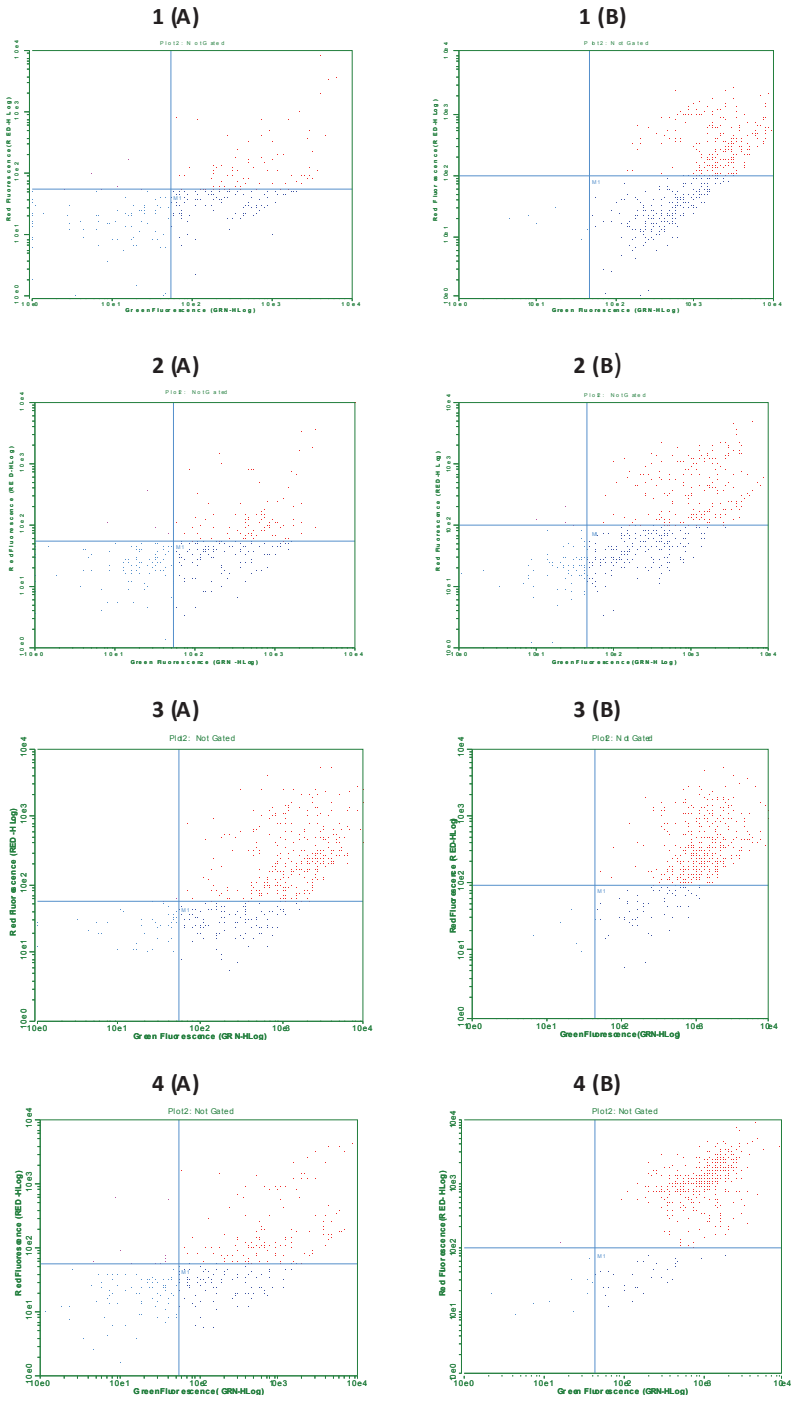


Figure 3.6. Cytograms of the four idiosyncratic drugs (1) trovafloxacin, (2) nimesulide, (3) nefazodone and (4) telithromycin in absence (A) and presence (B) of pro-inflammatory stimuli. After incubating HepG2 cells with the four idiosyncratic drugs in absence (A) and presence of LPS and TNF-α (B) for 24 hours, hepatocellular death was assessed following the simultaneous staining of cells with AnnexinV-FITC and PI by capillary flow cytometry. The amplified hepatocellular death observed when drugs were co-administered to cells along with LPS and TNF is illustrated in the presented cytograms by an increase in both green and red fluorescence.

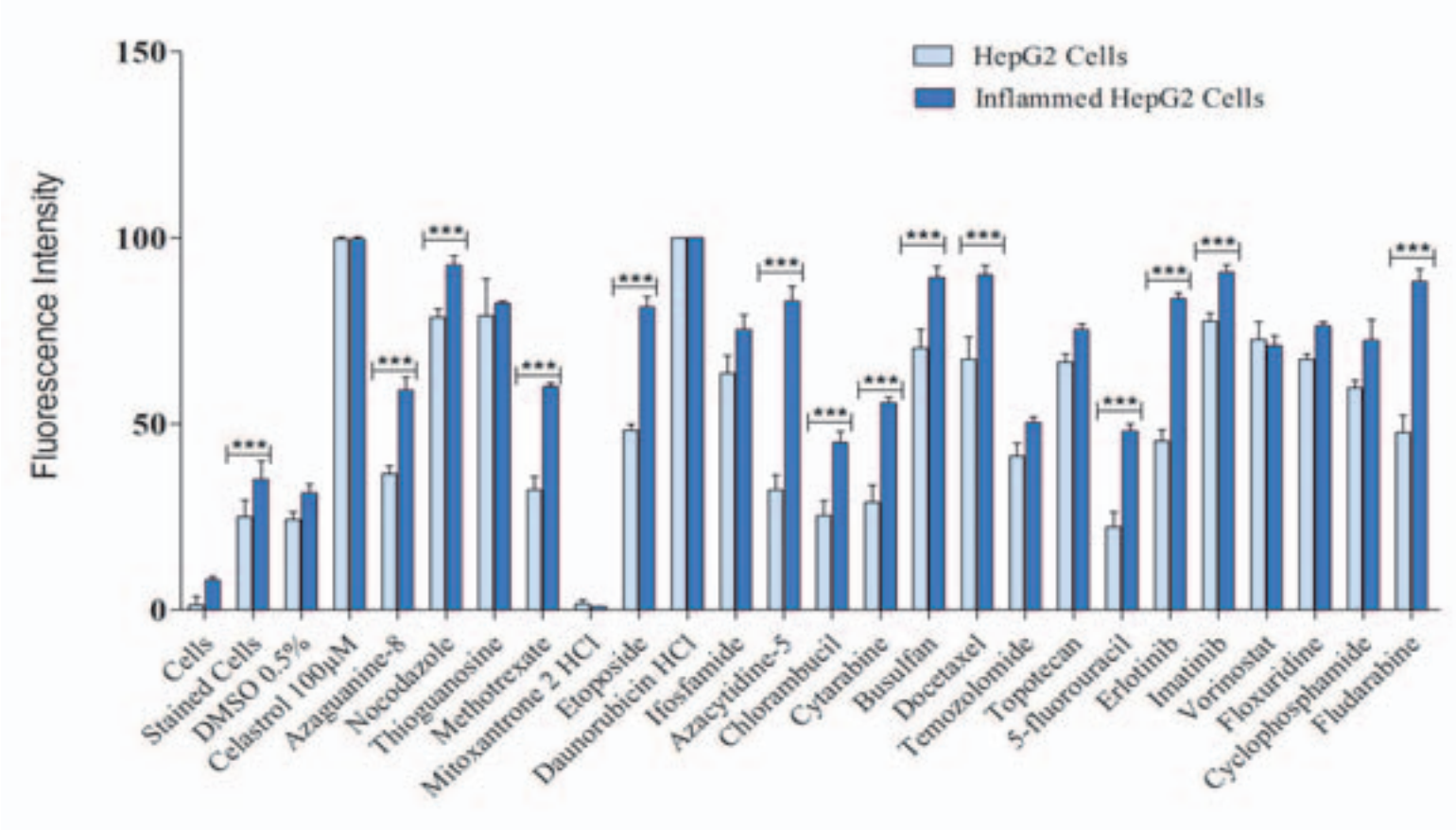


Figure 3.7. The apoptotic effect of anticancer drugs in non-inflamed and inflamed HepG2 cells. The developed cellular drug-inflammation model was used to detect the inflammation associated hepatotoxic potential of several anticancer drugs. After incubating HepG2 cells with several anticancer drugs in presence and absence of LPS and TNF-α for 24 hours, hepatocellular death was assessed following the simultaneous staining of cells with AnnexinV-FITC and PI by capillary flow cytometry. Bars represent the emitted intracellular fluorescence of AnnexinV-FITC and PI stained cells. Data is represented as Mean ± S.E.M (n=3). Statistical analysis was performed using two way ANOVA followed by Bonferroni post test.
*** represents $P < 0.001$ and refers to the variation in apoptotic potential between non-inflamed and inflamed HepG2 cells.

3.3.4 Idiosyncratic drug-induced superoxide anion generation

The extensive generation of ROS is a widely encountered observation during drug biotransformation. Superoxide anion is among the reactive oxygen species that promote liver injury in response to ischemia-reperfusion, oxidative stress, and many toxic chemicals (Jones et al. 2010). Accordingly, idiosyncratic-drug induced superoxide anion generation was assessed in this chapter in order to elucidate if oxidative stress is an underlying mechanism of inflammation associated drug-induced hepatotoxicity. After incubating HepG2 cells with trovafloxacin,

nimesulide, telithromycin and nefazodone for 24 hours the attained results demonstrated that, with the exception of nefazodone, none of the four idiosyncratic drugs induced the intracellular accumulation of superoxide anion (Figure 3.8A). However, following the co-incubation of HepG2 cells with these drugs along with LPS and TNF-α, the four idiosyncratic drugs favored the intracellular accumulation of superoxide anions when compared to the negative control (DMSO-treated cells) (Figure 3.8B). Upon comparing the oxidative potential of the four idiosyncratic drugs in presence and absence of an inflammatory context, particularly nimesulide and nefazodone induced the generation and accumulation of superoxide anion in a significant manner (Figure 3.8C).

3.3.5 Idiosyncratic drug-induced steatosis

Macrovasciluar steatosis is frequently linked with the prolonged exposure of the liver to steatotic drug leading to cirrhosis in some patients (Donato et al. 2009). Furthermore, several pro-inflammatory cytokines demonstrated a significant role in the progression of steatosis to steatohepatitis (Anderson and Borlak, 2008). The correlation of both drugs and inflammation in the progression of hepatic steatosis, urged us to investigate whether drug-induced steatosis may be one of the underlying mechanisms of inflammation associated idiosyncratic drug-induced hepatotoxicity. After incubating HepG2 cells with the four idiosyncratic drugs for 24 hours, the results attained demonstrate that trovafloxacin, telithromycin and nefazodone significantly induce the intracellular accumulation of lipids as demonstrated by the increase in bodipy493/503-emitted fluorescence with respect to untreated cells (Figure 3.8A). Nimesulide apparently favors the elimination lipids, since the accumulation of intracellular lipids in presence of nimesulide is less than the basal amount of accumulated lipids in untreated HepG2 cells (Figure 3.9A). However, when HepG2 cells were co-incubated with these four drugs in addition to LPS and TNF-α, only nefazodone maintained a significant steatotic potential whereas those of trovafloxacin and telithromycin were noticeably attenuated (Figure 3.9B). In contrary, the administration of LPS and TNF-α alone to HepG2 cells, significantly enhanced the intracellular accumulation of lipids when compared to untreated cells. These results indicate that the despite the fact that pro-inflammatory mediators, in particular LPS and TNF-α, promote the accumulation of intracellular lipids when administered to cells alone; they exhibit a significant attenuating effect on the stetatotic potentials of trovafloxacin and telithromycin (Figure 3.9C). Nevertheless, the extremely potent steatotic potential of nefazodone remained unchanged despite the presence of LPS and TNF-α.

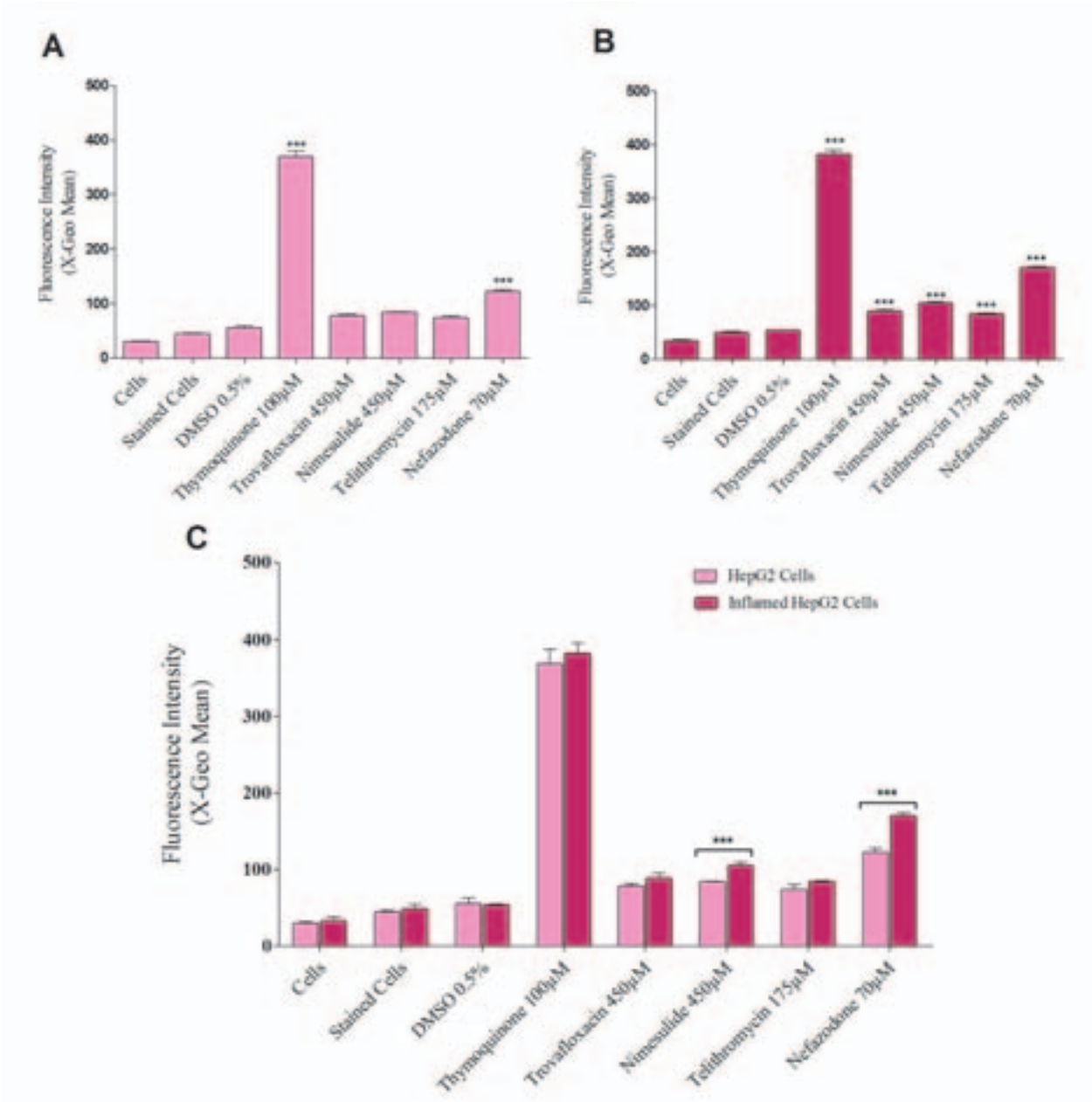


Figure 3.8. Effect of idiosyncratic drugs on the intracellular accumulation of superoxide anions in non-inflamed and inflamed HepG2 cells. After co-incubating HepG2 cells with the four idiosyncratic drugs in absence (A) and presence of LPS and TNF-α (B) for 24 hours, superoxide anion generation was assessed using DHE by flow cytometry. Graph C represents the oxidative potential of the four tested idiosyncratic drugs in absence and presence of LPS and TNF-α. Bars represent the accumulated intracellular ethidium-emitted red fluorescence in terms of X-geometric mean (AU). Data is represented as Mean ± S.E.M (n=3). Statistical analysis was performed using one way (for graphs A and B) and two way ANOVA (for graph C) followed by Bonferroni post test. *** represents $P < 0.001$ and refers to the variation in oxidative potential between DMSO-treated and drug-treated HepG2 cells in graphs A and B. In graph C the p-value corresponds to the variation in the oxidative potential between non-inflamed and inflamed HepG2 cells.

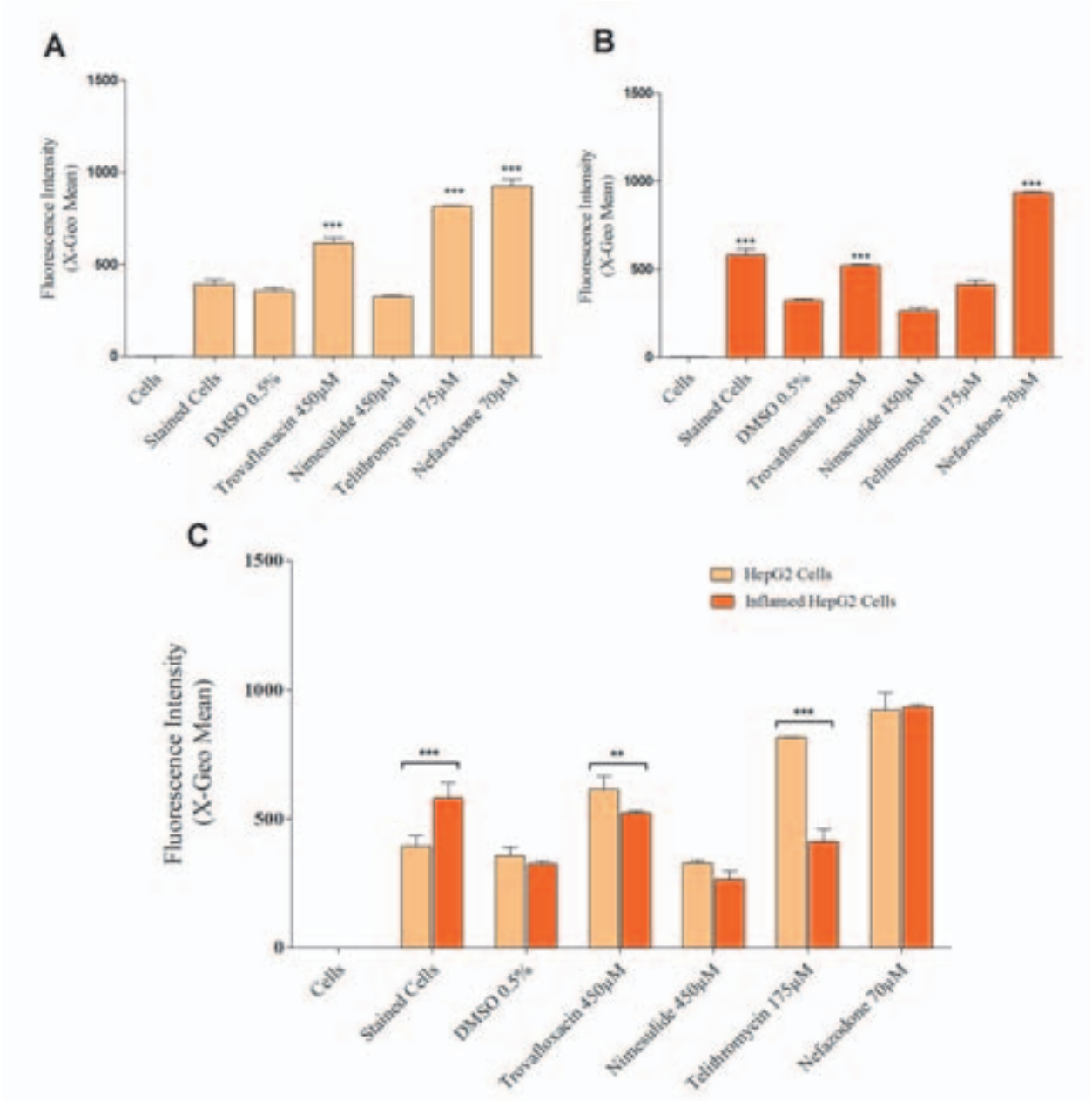


Figure 3.9. The steatotic effect of idiosyncratic drugs in non-inflamed and inflamed HepG2 cells. After co-incubating HepG2 cells with the four idiosyncratic drugs in absence (A) and presence of LPS and TNF-α (B) for 24 hours, the intracellular accumulation of lipids, otherwise known as steatosis was assessed using BODIPY 493/503 by flow cytometry. Graph C represents the steatotic potential of the four tested idiosyncratic drugs in absence and presence of LPS and TNF-α. Bars represent the accumulated intracellular fluorescence of BODIPY 493/503 in terms of X-geometric mean (AU). Data is represented as Mean ± S.E.M (n=3). Statistical analysis was performed using one way (for graphs A and B) and two way ANOVA (for graph C) followed by Bonferroni post test. ** and *** represents $P < 0.01$ and $P < 0.001$, respectively, and refers to the variation in steatotic potential between DMSO-treated and drug-treated HepG2 cells in graphs A and B. In graph C the p-value corresponds to the variation in steatotic potential between non-inflamed and inflamed HepG2 cells.

3.4 Discussion

Idiosyncratic drug-induced liver injury (IDILI) is the leading cause of post-marketing drug withdrawal worldwide. Several features of IDILI such as extremely low incidence rate, drug dose-independency and correlation with rare host-specific susceptibility factors complicated the development of efficient models for pre-marketing detection of idiosyncratic drugs and the understanding of their hepatotoxic mechanisms of action (Deng et al. 2009; Cosgrove et al. 2009). Therefore, the development of innovative preclinical tools that successfully identify potentially hepatotoxic idiosyncratic drugs is a paramount need for public health in general and the pharmaceutical industry in particular (Cosgrove et al. 2009). Overall, this chapter presents a high throughput cellular drug-inflammation model for the prediction of inflammation associated idiosyncratic drug-induced hepatotoxicity. This model aims at reproducing the success of LPS-administered animal models in the detection of idiosyncratically hepatotoxic drugs in humans while overcoming the limitations of animal models. In these animals the co-administration of LPS along with several idiosyncratic drugs (Table 3.1) succeeded in revealing their otherwise hidden hepatotoxic potential; which was predominantly dependent on the LPS-induced upregulation of pro-inflammatory mediators, especially TNF-α (Bergheim et al. 2006; Shaw et al. 2007). In the presented cellular drug-inflammation model of idiosyncratic hepatotoxicity, inflammation was induced by administering an inflammatory mix comprising both LPS and TNF-α to HepG2 cells; subsequently hepatotoxicity was assessed by evaluating synergistic drug-inflammation-induced hepatocellular death. The inflammatory potential of this mix was evaluated by assessing the concentration of secreted IL-8 following its incubation with HepG2 cells for 24 hours. IL-8 is a potent chemoattractant that appears early during an inflammatory reaction and plays significant role in leukocyte recruitment to the site of inflammation (Harada et al. 1994). The significant elevation in the concentration of HepG2-released IL-8 confirms the inflammatory potential of the administered mix and indicates that important hepatocellular inflammatory pathways such as NF-κβ, which normally mediates the transcriptional up-regulation of IL-8 are potently activated (Figure 3.4) (Osawa et al. 2002).These results are consistent with previous studies that demonstrated a potent up-regulation in the production of IL-8 following exposure of hepatocytes to TNF-α (Osawa et al. 2002).

Initially, the sensitivity and selectivity of the developed drug-inflammation model were validated using four idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) known to exhibit amplified hepatotoxic potentials in presence of pro-inflammatory mediators (Cosgrove et al. 2009). The attained results demonstrate that the four idiosyncratic drugs but not their non-idiosyncratic analogs (levofloxacin, aspirin and buspirone), exhibit an amplified apoptotic potential when co-administered to HepG2 cells with LPS and TNF-α (Figure 3.5). These results strongly suggest that the developed drug-inflammation model is sensitive since it successfully detected the enhanced apoptotic potential of the four idiosyncratic drugs known to induce supra-additive hepatotoxicity when co-administered with inflammatory cytokines (Cosgrove et al. 2009). Furthermore, this model proved to be selective since it detected the toxic potentials of

previously identified inflammation associated hepatotoxic drugs but not that of their non-toxic analogues. Exceptionally, clarithromycin (the analog of telithromycin), which is not identified as an inflammation associated hepatotoxic drug elucidated a significant elevation in its apoptotic potential when administered within an inflammatory context. This observation was consistent with the work published by *Cosgrove et al.* in which clarithromycin also exhibited an enhanced apoptotic potential within an inflammatory context, assessed using caspase 3/7 and LDH assays (Cosgrove et al. 2009). As is the case when idiosyncratic drugs are administered to cells in the absence of an inflammatory context, the administration of the inflammatory mix in the absence of idiosyncratic drugs barely induces a negligible elevation in hepatocellular death. This observation is consistent with several previous studies that confirm the resistance of hepatocytes to TNF-α and LPS-induced apoptosis (Schwabe and Brenner, 2006). It has been proven that LPS is unable to cause hepatotoxicity when administered alone to hepatocytes; despite the fact that the latter express TLR4 they exhibit a weak response following LPS exposure and are mainly involved in its biliary elimination (Cosgrove et al. 2009; Guo and Friedman, 2010). TNF-α is a pleiotropic pro-inflammatory cytokine that is extensively involved in hepatic pathophysiology due to its capacity to induce hepatocellular death through the activation of caspase-8 and Bid; in addition to hepatocyte survival through activating the transcription of NF-κβ-mediated survival genes (Wullaert et al. 2007). In the absence of a secondary stimulus, the simple administration of TNF-α to hepatocytes is insufficient to cause hepatocellular death since the latter is predominantly involved in the activation of NF-κβ, which favors the transcription of anti-apoptotic and survival genes (Wullaert et al. 2007). Accordingly, it has been suggested that for TNF-α to induce hepatocellular death it requires a concurrent secondary stimulus with the capacity to either block the activation of NF-κβ or to extensively induce the over-production and release of TNF-α for a prolonged period of time (Wullaert et al. 2007). Overall, the attained results in this chapter suggest that idiosyncratic hepatotoxicity, which is manifested by an increase in hepatocellular death, arises mainly following the synergistic exposure of HepG2 cells to both idiosyncratic drugs and the inflammatory mix. The presence of either one without the other proved to be insufficient to induce significant hepatic injury; while the combined presence of both factors resulted in an amplified hepatocellular stress, which served to predispose the liver to injury from either one of them. The exact mechanisms through which the tested idiosyncratic drugs synergize with pro-inflammatory mediators to cause exacerbated hepatic death remain ambiguous to date with the exception of trovafloxacin, whose hepatotoxic potential was previously studied in LPS-administered animal models (Shaw et al. 2007; Shaw et al. 2010). Probably the amplified apoptotic potential observed following the co-administration of the four idiosyncratic drugs along with the inflammatory mix to HepG2 cells is due to a drug-induced blockage of the NF-κβ pathway which sensitizes hepatocytes to drug-inflammation induced hepatocellular death. Subsequently, hepatocellular death may be mediated through the synergistic drug-inflammation activation of various death signaling pathways such as extrinsic and intrinsic apoptosis, JNK, p38 and JAK/STAT; all of which proved to be responsive to both

drugs and pro-inflammatory mediators (Singh and Czaja, 2007, Shaw et al. 2010; Gao, 2005; Fredriksson et al. 2011; Schwabe and Brenner, 2006). This theory was further elucidated and validated by *Fredriksson et al.* who demonstrated that the co-administration of dicolfenac with TNF-α to HepG2 cells induced amplified hepatocellular death mainly through the activation of caspase-8/Bid/APAF1 following diclofenac-mediated inhibition of the NF-κβ pathway (Fredriksson et al. 2011). Another probable mechanism for drug-inflammation synergistic induction of hepatocellular death may be related to drug-induced inhibition of the pathways implicated in the hepatic elimination of TNF an LPS; thus favoring prolonged TNF and LPS-induced activation of inflammatory pathways such as NF-κβ and TLR (Toll-like receptors). Consequently, the potent activation of these pathways will lead to the extensive production of hepatotoxic pro-inflammatory mediators such as chemokines and cytokines that will increase the liver's susceptibility to drug-induced liver injury (Shaw et al. 2010). These mediators often comprise hepatocyte-released pro-inflammatory factors such as IL-8, monocyte-induced gamma interferon (MIG), gamma-interferon-inducible protein (IP-10), cytokine-induced neutrophil chemoattractant (KC) and macrophage inflammatory proteins (MIPs) (Ramadori et al. 2008); which are predominantly involved in recruiting leukocytes to the liver that will in their turn release a wide variety of cytotoxic mediators, like ROS, nitric oxide, leukotrienes, phosphatase and proteases further predisposing the liver to injury. Logically, a stressed liver exposed to a certain drug is much more susceptible to its adverse reactions than a healthy intact one. Furthermore, inflammation may predispose the liver to drug-induced hepatotoxicity by altering the expression and activity of important drug metabolizing enzymes and drug transporters leading to toxic accumulation of parent drug molecule or its reactive metabolites leading to hepatocellular death (Morgan et al. 2008).

After validating the selectivity and sensitivity of the developed drug-inflammation cellular model in the detection of inflammation associated idiosyncratic drugs (Figure 3.5), this model was used to screen several anticancer drugs (Table 3.2) for inflammation associated hepatotoxicity. The main objective behind using the developed drug-inflammation model in the toxicity screening of a different category of drugs is to demonstrate that the presented model is not simply limited to the detection of hepatotoxic drugs with idiosyncratic liabilities but can rather detect any drug with the potential of exhibiting amplified toxicity in an inflammatory context. The screened drugs exhibit different modes of action and are not principally prescribed for the treatment of liver cancer; nevertheless the majority of them induced a significant apoptotic potential on HepG2 cells most probably due to the wild type expression of P53 in this hepatic cell line. After co-incubating HepG2 cells with anticancer drugs and the inflammatory mix for 24 hours the results attained demonstrate that azaguanine-8, nocodazole, metothrexate, etoposide, azacytidine-5, chlorambucil, cytarabine, busulfan, docetaxel, 5-fluorouracil, erlotinib, and fludarabine exhibit a significantly enhanced apoptotic potential in the presence of TNF-α and LPS (Figure 3.7). These results suggest that the developed drug-inflammation model is not limited to the detection of hepatotoxic drugs with previously identified idiosyncratic liabilities but may be used as an

efficient pre-clinical tool for the detection of inflammation associated hepatotoxic potential of a wide variety of drugs belonging to different pharmacological classes. It is important to note that the word "idiosyncratic" does not merely refer to a limited category of drugs; any drug may be considered idiosyncratic if it displays the features of idiosyncrasy (dose-independent, host-dependent, variable liver pathology, variable onset time, irreproducible and unpredictable) (Roth and Ganey, 2010). Hence, the drugs whose apoptotic potential was significantly amplified in the presence of pro-inflammatory mediators may now be considered as inflammation-associated idiosyncratic drugs. However, further tests are needed to confirm their idiosyncratic adverse reactions before restricting their administration to patients. The majority of the screened anticancer drugs cause cell death by inducing DNA damage; hence it is unlikely for an inflammation-induced alteration in their mode of action to be a possible mechanism for the amplified hepatocellular death observed when these drugs are co-administered with the inflammatory mix to cells. We rather propose an inflammation-induced modification in the expression and activity of important metabolizing enzymes and essential drug transporters as a possible mechanism predisposing HepG2 cells to enhanced anticancer drug-induced hepatotoxicity. Several evidence support the probability of this suggestion like for example the involvement of TNF-α in addition to other pro-inflammatory cytokines in the down regulation of important metabolizing enzymes and drug transporters (Fradel and Le Vee, 2009; Teng et al. 2008). Furthermore, it has been demonstrated that both LPS and TNF-α inhibit CYP34A, which is responsible for the metabolism of the majority of anticancer drugs, through activating the NF-κβ-mediated down-regulation of PXR/RXR complex in HepG2 cells (Aggarwal, 2004; Gu et al. 2006). Although the inhibition of CYP34A is irrelevant in the proposed model due to its weak expression in HepG2 cells, this remains a relevant mechanism of toxicity for inflammation associated hepatotoxic anticancer drugs in general. Moreover, the fact that docetaxel and etoposide are substrates of MDR1 whereas methotrexate is a substrate of MRP2 and erlotinib is a substrate of BCRP in addition to the fact that inflammation commonly modulates these transporters; suggest that drug-induced competitive inhibition of the stated transporters in combination with inflammation-induced down regulation of their mRNA or protein levels may be a probable mechanism for synergistic anticancer drug-inflammation enhanced apoptosis (Russel, 2010; Fardel and Le Vee, 2009; Xia et al. 2010). Optimally such toxicity studies must be performed on normal human hepatocytes and not on immortalized cell lines like HepG2 that lack several metabolic enzymes, transporters and nuclear receptors. Nevertheless, I tend to emphasize first of all that the HepG2 cells used express normal p53 and hence they are responsive to apoptosis; second of all in this model we are not testing the hepatotxic potential of drugs in general we are focusing on their increased toxicity in the presence of an inflammatory context, which is relatively unrelated to the metabolic potential of the cells. Indeed the increased toxicity of these drugs might be correlated with the poor basal expression of important metabolic enzymes such as CYP34A in HepG2 cells; however this model elucidates the difference between the hepatotoxic potential of a certain drug when administered alone to cells and when it is co-

administered along with an inflammatory mix, regardless of the primary causes underlying this toxic potential. The main focus of this model is to predict the hidden hepatotoxic potential of drugs which may be manifested if the latter are administered to a patient with an underlying inflammatory reaction and not to assess the general toxicity profile of these drugs which might be linked to the poor metabolic activity of HepG2 cells.

In addition to developing a high throughput *in vitro* model for the detection of inflammation associated hepatotoxic drugs our work aimed at elucidating the toxic mechanisms underlying inflammation associated drug-induced liver injury. A toxic drug or its reactive metabolite may induce hepatotoxicity through various pathways and mechanisms; however this chapter will focus on the implication of excessive reactive oxygen species, particularly superoxide anions, and steatosis in inflammation associated idiosyncratic hepatotoxicity. Accordingly, the oxidative and steatotic potential of trovafloxacin, nimesulide, telithromycin and nefazodone was investigated in presence and absence of pro-inflammatory stimuli (LPS and TNF-α).

In the aim of elucidating if oxidative stress is implicated in inflammation associated idiosyncratic hepatotoxicity the effect of the four idiosyncratic drugs on the intracellular accumulation of superoxide anions was assessed by capillary flow cytometry using dihydroethidium. The results attained demonstrated that the presence of LPS and TNF-α enhanced the oxidative potential of nimesulide and nefazodone without affecting that of trovafloxacin and telithromycin (Figure 3.8C). This indicates that both nimesulide and nefazodone synergize with LPS and TNF-α to promote idiosyncratic hepatotoxicity via favouring the generation and accumulation of superoxide anions. The co-presence of idiosyncratic drugs and pro-inflammatory mediators may promote the intracellular accumulation of superoxide anions either through the synergistic induction of mitochondrial damage, which in its turn promotes the generation of excessive amounts of superoxide anions; or through the synergistic inhibition of important anti-oxidant systems involved in the elimination of superoxide anions such as such as glutathione-S-transferase and superoxide dismutase. Both nimesulide and nefazodone have been generally correlated with mitochondrial dysfunction, raising the probability of their implication in extensive superoxide generation since mitochondrial perturbation is often associated with increased ROS formation (Berson et al. 2006; Mingatto et al. 2002; Dykens et al. 2008). These suggested theories are further validated by a previously published *in vivo* study, which demonstrated that heterozygous Sod2 knockout mice are much more susceptible to nimesulide-induced mitochondrial damage and hepatocellular apoptosis (Tan et al. 2007). Sod2 is a member of the superoxide dismutase family that catalyzes the transformation of superoxide anion into hydrogen peroxide and oxygen; hence the inhibition or the knock-out of Sod2 leads to the toxic intracellular accumulation of superoxide anion (Grimes et al. 2005). The excessive accumulation of superoxide anions has been frequently correlated with apoptosis, which may explain the amplified apoptotic potential of nimesulide and nefazodone when co-administered to cells along with LPS and TNF-α (Lim et al. 2008). Superoxide anion-induced apoptosis seems to be mediated by oxidation of Trx2 (mitochondrial thioredoxin) and subsequent induction of Ask1

(apoptosis signal-regulating kinase 1); consequently the Trx2/Ask1 pathway triggers the prolonged activation of JNK leading to hepatocellular death (Lim et al. 2008). Since the accumulation of reactive oxygen species proved to be necessary for the TNF-induced prolonged activation of JNK leading to hepatocellular death and since the inhibition of NF-κβ is necessary to sustain a high intracellular concentration of ROS due to the involvement of this pathway in the transcriptional activation of antioxidant genes (Schwabe and Brenner, 2006); it is highly probable that synergistic drug-inflammation-induced accumulation of superoxide anion mediates hepatocellular death as follows: In presence of an inflammatory context the elevation in the intracellular accumulation of superoxide anion results from the combination of drug-induced mitochondrial damage and enhanced ROS formation with drug-induced inhibition of the NF-κβ pathway; thus favoring the accumulation of ROS while blocking the transcription of anti-oxidant and survival genes (Schwabe and Brenner, 2006). Consequently, HepG2 cells are sensitized to TNF-induced apoptosis most likely through the ROS-mediated prolonged activation of the JNK pathway or possibly through the TNF-mediated activation of caspase-8 (Schwabe and Brenner, 2006). The results published by *Grimes et al.* further support this theory by demonstrating that nimesulide-induced inhibition of Sod2 and NF-κβ pathway sensitize human lung carcinoma cell line to apoptosis (Grimes et al. 2006).

In the aim of elucidating if steatosis is implicated in inflammation associated idiosyncratic hepatotoxicity the effect of the four idiosyncratic drugs on the intracellular accumulation of lipids was assessed by capillary flow cytometry using Bodipy 493/503. The results attained demonstrated that the presence of LPS and TNF-α attenuated the steatotic potential of trovafloxacin and telithromycin; however did not affect that of nefazodone (Figure 3.9C). Contrarily, the administration of LPS and TNF-α alone to cells induced a significant elevation in the intracellular accumulation of lipids. This observation is consistent with the fact that an inflammatory stress is commonly known to exacerbate the hepatic accumulation of lipids both *in vitro* and *in vivo* by several mechanisms such as favouring the expression of low-density lipoprotein receptor (LDLr) and promoting the hepatic influx of cholesterol while blocking its ABCA1-mediated efflux (Ma et al. 2008). Due to the implication of inflammation in the exacerbation of steatosis and in its progression to steatohepatitis we highly doubt that the administration of LPS and TNF-α protects hepatocytes from the steatotic potential of idiosyncratic drugs; especially with nefazodone maintaining its steatotic potential even within an inflammatory context. If inflammation exhibited a hepatoprotective role against idiosyncratic drug-induced steatosis in general then the steatotic potential of nefazadone should have been attenuated following its co-administration with LPS and TNF-α. We rather suppose that the significant decrease in the steatotic potential of trovafloxacin and telithromycin is possibly due to a "competition" between these drugs and the co-administered pro-inflammatory mediators for the same steatotic target thus leading to an overall attenuated steatotic potential. In all cases the attenuated steatotic potential of trovafloxacin and telithromycin within an inflammatory context, implies that steatosis is not implicated in their inflammation associated hepatotoxic potential.

However, steatosis is most probably significantly involved in inflammation associated nefazodone-induced hepatotoxicity. The potent steatotic potential of nefazodone may be correlated with the disruption of hepatic fatty acid oxidation either through drug-induced inhibition of mitochondrial β-oxidation enzymes or through sequestration of the cofactors involved in this metabolic pathway (Labbe et al. 2008). Alternatively, nefazodone may favor the accumulation of intracellular lipids by disrupting β-oxidation through severely impairing the mitochondrial function either by inhibiting the transfer of electrons along the components of the respiratory chain or by damaging mitochondrial DNA (Donato et al. 2009). Furthermore, it may enhance the accumulation of lipids by inhibiting microsomal triglyceride transfer protein, an enzyme involved in the formation of triglyceride-rich very-low density lipoproteins (VLDL); thus decreasing fatty acid oxidation and preventing hepatic fat removal via lipoprotein secretion (Donato et al. 2009).

Finally, this chapter presents an efficient high throughput cellular drug-inflammation model able to detect the inflammation associated hepatotoxic potential of a wide range of drugs on one hand; and to elucidate their underlying mechanisms of toxicity on the other hand. In this chapter the developed drug-inflammation model was particularly used to elucidate the unknown toxic mechanisms underlying the idiosyncratic hepatotoxicity of trovafloxacin, nimesulide, telithromycin and nefazodone with emphasis on steatosis and oxidative stress. The results attained demonstrated that both nimesulide and nefazodone most probably induce inflammation associated hepatotoxicity via favouring the accumulation of superoxide anions. Furthermore, the hepatotoxic potential of nefazodone proved to be also correlated with its potent steatotic effect, especially in presence of pro-inflammatory mediators. However, oxidative stress and steatosis do not seem to be implicated in the inflammation associated idiosyncratic hepatotoxicity of trovafloxacin and telithromycin. The presented work in this chapter confirmed the inflammatory stress hypothesis stating that: " a mild concurrent episode of inflammation predispose the liver to the adverse reactions of a certain drug resulting in a toxic response at otherwise safe drug doses in the absence of inflammation" (Shaw et al. 2010); and further validated the efficiency of drug-inflammation synergistic induction of hepatocellular death as a pre-clinical tool for the detection of inflammation associated hepatotoxic drugs. The fact that the developed drug-inflammation model was not limited to a precise drug category and was able to detect the inflammation associated hepatotoxic potential of antibiotics, anti-inflammatory, anti-depressant and anticancer drugs suggests that this model may be considered as successful surrogate for LPS-administered animal models, especially for high throughput toxicity screening.

Chapter 4

Implication of MDR1 and MRP2 in inflammation associated idiosyncratic drug-induced hepatotoxicity

The majority of this chapter has been published in Cytometry part A, 83A: 403-408, 2013 in the form of a brief report. Additional experiments were performed concerning the effect of trovafloxacin, nimesulide telithromycin and nefazodone on the expression of MDR1 and MRP1 in presence and absence of inflammatory context. Accordingly Chapter 4 will be divided to two parts:

Part I will include the published article: Saab L., Peluso J., Muller C. and Ubeaud-Sequier, G. 2013. Implication of hepatic transporters (MDR1 and MRP2) in inflammation associated idiosyncratic drug-induced hepatotoxicity investigated by microvolume cytometry. *Cytometry PartA,* 83A: 403-408.

Part II will include the experiments concerning the effect of trovafloxacin, nimesulide telithromycin and nefazodone on the expression of MDR1 and MRP2 in presence and absence of inflammatory context.

Chapter 4

Part I

Saab L., Peluso J., Muller C. and Ubeaud-Sequier, G. 2013. Implication of hepatic transporters (MDR1 and MRP2) in inflammation associated idiosyncratic drug-induced hepatotoxicity investigated by microvolume cytometry. *Cytometry PartA,* 83A: 403-408.

Cytometry PART A
Journal of the International Society for Advancement of Cytometry

Implication of Hepatic Transporters (MDR1 and MRP2) in Inflammation-Associated Idiosyncratic Drug-induced Hepatotoxicity Investigated by Microvolume Cytometry

Lea Saab, Jean Peluso, Christian D. Muller, Genevieve Ubeaud-Sequier*

UMR CNRS 7200, Laboratoire d'Innovation Thérapeutique, Université de Strasbourg, FMTS, Faculté de Pharmacie, Illkirch, France

Received 3 July 2012; Revision Received 11 October 2012; Accepted 15 January 2013

Grant sponsor: MAREX; Grant number: [FP 7; KBBE–3-245137 (EU)]

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Pr. G. Ubeaud-Sequier UMR CNRS 7200, Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, BP6024, F-67401 Illkirch Cedex, France

Email: ubeaud@unistra.fr

Published online 11 February 2013 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.22263

• Abstract

Idiosyncratic drug-induced hepatotoxicity accounts for about 13% of all cases of acute liver failure, therefore cited as the most frequent reason for post-marketing drug withdrawal. Despite this, the underlying mechanisms remain poorly understood due to lack in adequate screening assays and predictive in vitro models. Hepatic transporters play a crucial role in the absorption, distribution, and elimination of both endogenous substrates and xenobiotics. Defects in transporter function can lead to altered drug disposition, including toxicity and loss of efficacy. Inflammation is one condition for demonstrated variable drug response, attributed in part, to changes in function of drug transporters. The present study investigates the implication of two important hepatic transporters (MDR1 and MRP2) in idiosyncratic drug-induced hepatotoxicity in the presence and absence of an inflammatory context. The synergistic effect of idiosyncratic drugs (Trovafloxacin, nimesulide, telithromycin, and nefazodone) and inflammatory stimuli (TNF-α + LPS) on the efflux activity of hepatic transporters was studied using microvolume cytometry. Our results demonstrated on the one hand that both MDR1 and MRP2 are variably implicated in idiosyncratic drug-induced liver injury and on the other hand that the occurrence of an inflammatory reaction during idiosyncratic drug therapy can noticeably modulate this implication. In the absence of an inflammatory stress, none of the four tested drugs modulated the efflux activity of MRP2; nevertheless telithromycin and nefazodone inhibited the efflux activity of MDR1. Upon occurrence of an inflammatory stress, the inhibitory potential of trovafloxacin, nimesulide, and nefazodone on the efflux activity of MRP2 was noticeably revealed, while the telithromycin and nefazodone-induced inhibition of MDR1 was clearly attenuated. Knowledge of underlying mechanisms may significantly contribute to elimination of potential hepatotoxic drugs long before marketing and to prevention of drug-induced hepatotoxicity.

• Key terms

Idiosyncratic drug-induced hepatotoxicity; hepatic transporters; MDR1; MRP2; microvolume cytometry

Introduction

The liver is known to be the primary target for adverse drug reactions (ADRs) presumably due to its extensive involvement in drug metabolism and elimination (1). Idiosyncratic adverse drug reactions (IADRs) are considered as an important subset of ADRs, accounting for ~13% of all acute liver failure cases (2). IADRs represent one of the leading causes for post-marketing drug withdrawal (3), predominantly due to being host-dependent, poorly predicted by standard preclinical or early clinical trials and unrelated to the pharmacologic target of the drug (2). However, a recently developed drug-cytokine co-treatment approach proved to be highly efficient in the early prediction of idiosyncratic drug-induced hepatotoxicity, especially in animal models (4). This approach is consistent with the inflammatory stress

ISAC International Society for Advancement of Cytometry

hypothesis, which states that "the co-occurrence of an acute episode of inflammation during drug therapy results in sensitization of the liver causing liver injury from an agent that typically would not cause hepatotoxicity" (5). Nevertheless, the underlying mechanisms through which idiosyncratic drugs synergize with pro-inflammatory cytokines to precipitate serious liver injury need further investigation and understanding for a better prediction or even prevention of IADRs. In the present work, we investigated the effect of idiosyncratic drugs on the activity of important hepatic transporters in the absence and presence of an inflammatory context, in the aim of demonstrating that impaired drug efflux and elimination may be an important underlying mechanism of idiosyncratic hepatotoxicity. Hepatic membrane transporters play an essential role in the absorption, distribution, and elimination of both endogenous substrates and xenobiotics (6), therefore possessing the ability to significantly modulate the efficacy and toxicity of pharmacological agents (7). From the several transport proteins found on the canalicular membrane of hepatocytes, we will be focusing on the implication of MDR1 and MRP2 in idiosyncratic drug-induced liver injury. While MDR1 (ABCB1, P-glycoprotein, P-gp) is involved in the transport of a wide spectrum of structurally varying substances (many drugs, but also natural compounds) preferentially large hydrophobic and positively charged molecules, MRP2 is more involved in the efflux of both hydrophobic uncharged molecules and water-soluble anionic compounds (8). It is noteworthy, that drug interactions, multi-drug resistance, and inter-individual variations in drug response have been attributed to altered transporter expression or activity mainly during diseases associated with an inflammatory response, such as hypoxia and cancer (6). The close correlation between altered transporters functionality and inflammatory diseases is probably due to the fact that pro-inflammatory cytokines possess a modulatory effect on the expression and activity of drug transporters (9), thus rendering the liver more susceptible to drug adverse reactions.

In order to elucidate the importance of hepatic efflux transporters in protecting or sensitizing the liver to drug-induced hepatotoxicity, we have investigated in this work the implication of MDR1 and MRP2 in inflammation-associated idiosyncratic drug-induced liver injury by incubating HepG2 cells with four referent idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin, and nefazodone) in the presence and absence of an inflammatory environment. These drugs were specifically chosen because they are known to synergistically induce death when co-administered with a mix containing LPS and TNF-α (4). The effects of these idiosyncratic drugs on the efflux activity of MDR1 and MRP2 were analyzed by capillary flow cytometry using standard fluorescent transport assays.

Material and Methods

Chemicals, Drugs, and Cytokines

Rhodamine 123 (RH 123) and 5 (and 6)-carboxy-2',7'-dichlorofluorescein (CDF) and its diacetate promoiety (CDFDA) was purchased from Invitrogen. Verapamil, Trovafloxcacin, Nefazodone, Nimesulide, and Benzbromarone were purchased from Sigma-Aldrich. Telithromycin was obtained from Tebu-Bio. Unless otherwise noted, the following drug concentrations were used: 450 μM trovafloxacin, 70 μM nefazodone, 450 μM nimesulide, and 175 μM telithromycin. These drug concentrations were selected from initial dosing studies based on the criteria that the drug concentration (i) elicit minimal drug-only hepatotoxicity, (ii) induce robust supra-additive hepatotoxicity synergy with a representative cytokine mix, and (iii) be within a physiologically relevant dosing limit of 100-fold its Cmax value. Tumor necrosis factor-α was obtained from BD Pharmingen and was used at a final concentration of 100 ng/ml. Lipopolysaccharide (LPS) from *E. coli* serotype 055:B5 was purchased from Sigma-Aldrich and was used at 20 μg/ml.

Cell Staining and Flow Cytometry

The human hepatocellular carcinoma cell line HepG2 was obtained from the American Type Culture Collection (Maryland) and was maintained in DMEM supplemented with 10% FBS, P/S (100 unit/ml and 100 μg/ml) and glutamine (2 mM). Cells were grown at 37°C in a humidified incubator equilibrated with 5% CO_2. Cells were trypsinized and subcultured twice a week.

To discriminate between negative and positive events in the analysis, a non-stained control sample from each culture condition always accompanied acquisition of the stained cells to define their cut off. Gates were drawn around the appropriate cell populations using a forward scatter (FSC) versus side scatter (SSC) acquisition dot plot after excluding dead cells based on propidium iodide staining so that only viable cells were taken into consideration. Cytometers performances are checked weekly using the Guava easyCheck Kit 4500-0025 (Merck/Millipore/Guava Hayward, CA). Flow cytometric measurement of MDR1 functional activity using Rhodamine 123 efflux assay. The fluorescent dye rhodamine 123 is a substrate for P-glycoprotein and its transport out of the cell has been demonstrated to reflect P-glycoprotein function. Analysis of variation in rhodamine 123 intracellular fluorescence together with the effect of P-glycoprotein modulating agents (i.e., verapamil) investigates the role played by multidrug resistance protein in idiosyncratic drug-induced hepatotoxicity.

Briefly, HepG2 cells were cultured in 24-well plates at a density of 10^5 cells for 48 h until they were 80% confluent. Then cells were loaded with 0.5 μM rhodamine-123 (RH123) for 30 min at 37°C in 5% CO_2 in the presence or absence of 100 μM Verapamil, a standard P-gp inhibitor and four selected drugs (Trovafloxacin, Nimesulide, Telithromycin, and Nefazodone) (accumulation phase). Cells were then immediately transferred on ice, washed once with ice-cold phosphate buffered saline (PBS), and re-suspended in RH123-free medium for 120 min at 37°C to allow maximum efflux of fluorescent compounds (efflux phase). To be analyzed by capillary cytometry, cells were trypsinized and re-suspended in culture medium. MDR1-mediated efflux of rhodamine 123 was monitored on a Guava EasyCyte Plus capillary flow cytometer

(Merck Millipore, Life Science division, Merck KgaA, Darmstadt, Germany) equipped with a 488 nm excitation laser and four emission band pass filters at 530/40, 585/42, 675/30, and 780/30 nm. The accumulated intracellular fluorescence intensity of rhodamine 123 at 530/40 nm was computed on the Guava ExpressPro software (Merck/Millipore/Guava Tech) in terms of x-geometric mean arbitrary units (AU). Dead cells were excluded based on propidium iodide staining. Final concentration of DMSO applied to cells during incubation with tested drugs was 0.5%. In the tested setup, these concentrations had no adverse effects on cell viability, cell morphology, nor on rhodamine-123 efflux. The inhibitory potential of tested compounds on rhodamine-123 efflux was expressed relative to maximum inhibition obtained with 100 μM verapamil in the same experiment.

Flow cytometric measurement of MRP2 functional activity using the CDF efflux assay. MRP2 transport activity was investigated using the 5 (and 6)-carboxy-2',7'-dichlorofluorescein (CDF) and its diacetate promoiety (CDFDA) efflux assay. Briefly, HepG2 cells were cultured in 24-well plates at a density of 10^5 cells for 48 h until they were 80% confluent; then they were incubated with a medium containing 1 μM CDFDA for 20 min at 37°C in 5% CO_2 in the presence or absence of 250 μM benzbromarone, a known inhibitor of MRP2, and four selected drugs (Trovafloxacin, Nimesulide, Telithromycin, and Nefazodone). CDFDA is a nonfluorescent esterified form of CDF that freely diffuses into cells where it is cleaved by esterases to give CDF, a fluorescent dye effluxed by MRP2. The loaded cells were then washed three times with ice-cold medium and incubated in PBS for 120 min at 37°C to allow maximum efflux of CDF. To be analyzed by capillary cytometry, cells were trypsinized gently and re-suspended in culture media. MRP2-mediated efflux of CDF was monitored as described before on a Guava EasyCyte Plus System capillary cytometer. The accumulated intracellular fluorescence of CDF (530/40 nm) was computed on the Guava ExpressPro software in terms of percent of fluorescent cells. Dead cells were excluded based on propidium iodide uptake. Final concentration of DMSO applied to cells during incubation with tested drugs was 0.5%. In the tested setup, these concentrations had no adverse effects on cell viability, cell morphology, nor on CDF efflux results. The inhibitory potential of tested compounds on CDF efflux was expressed relative to maximum inhibition obtained with 250 μM benzbromarone in the same experiment.

Statistical Analysis

The data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using the unpaired *t*-test. Statistical significance was considered when $P < 0.05$.

Results

Idiosyncratic Drugs Effects on MDR1 Activity

To evaluate the implication of MDR1 in drug-induced idiosyncratic hepatotoxicity, we have studied the effect of four idiosyncratic drugs on the efflux activity of MDR1 in the presence and absence of LPS and TNF-α. In the absence of an inflammatory context our results demonstrated, when compared to the specific MDR1 inhibitor verapamil, that telithromycin and nefazodone elucidated an inhibitory potential on the efflux activity of MDR1; such inhibition is represented by an increase in the intracellular fluorescence of rhodamine 123-loaded cells (Fig. 1 and Fig. 3). However, co-treatment of hepG2 cells with a pro-inflammatory mix containing TNF-α and LPS along with idiosyncratic drugs for 24 h has noticeably reduced their inhibitory potential on the efflux activity of MDR1 as demonstrated by the decrease in fluorescence of rhodamine 123-loaded cells (Fig. 1B). It should be noted that even verapamil, which is considered as the standard MDR1 inhibitor, revealed a decreased inhibitory potential within an inflammatory context.

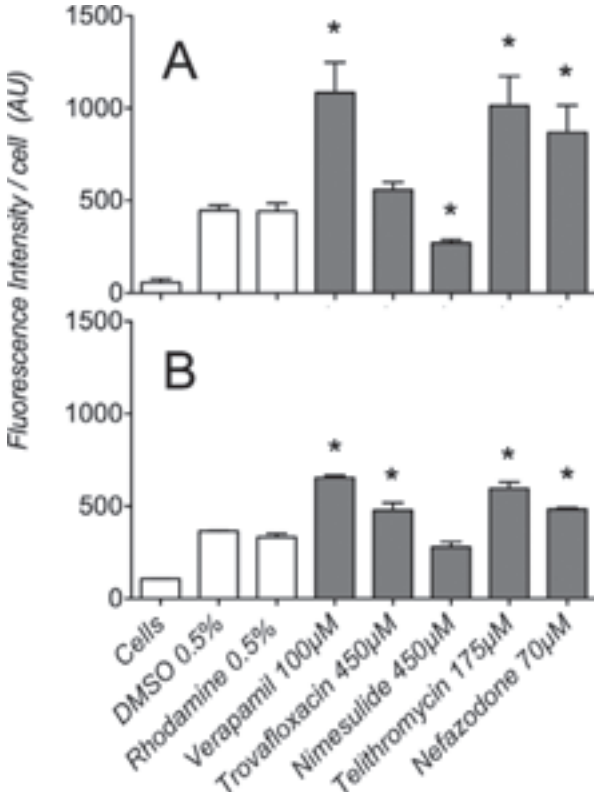


Figure 1. Effect of idiosyncratic drugs on the MDR1-mediated efflux of rhodamine 123. HepG2 cells were incubated for 24 h, in the absence (A) or presence (B) of TNF-α + LPS, with four drugs known to induce idiosyncratic hepatotoxicity. Bars represent the geometric mean values of fluorescence intensity ± SD ($n = 4$). *Represents $P < 0.05$.

Idiosyncratic Drugs Effects on MRP2 Activity

Based on the fact that MRP2 is extensively involved in protecting the liver of potentially toxic xenobiotics, we have investigated its role in idiosyncratic drug-induced hepatotoxicity in the presence and absence of our pro-inflammatory mix. Figure 2 demonstrates that besides trovafloxacin that exhibited a mild inhibitory potential on the efflux of MRP2,

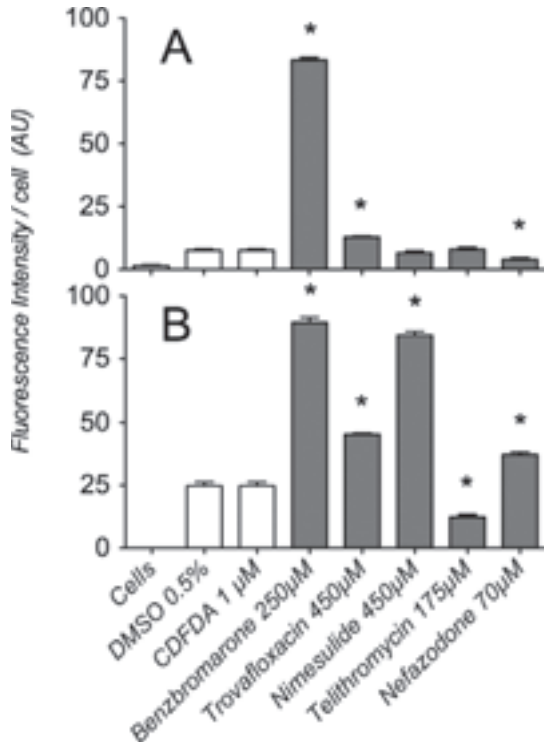


Figure 2. Effect of idiosyncratic drugs on the MRP2-mediated efflux of CDF. HepG2 cells were incubated for 24 h, in the absence (A) or presence (B) of TNF-α + LPS, with four drugs known to induce idiosyncratic hepatotoxicity. Bars represent geometric mean values of fluorescence intensity expressed ± SD (n = 4). *Represents $P < 0.05$.

none of the studied idiosyncratic drugs inhibited the efflux of CDF when compared with benzbromarone in the absence of an inflammatory context. Contrary to MDR1, MRP2 was strongly implicated in the drug-cytokine-induced hepatotoxicity. Our results demonstrate that trovafloxacin, nimesulide, and to a lesser extent nefazodone noticeably inhibited the efflux activity of MRP2 in an inflammatory context as represented by an increase in the fluorescence of CDF-loaded cells (Fig. 2B). The co-treatment of HepG2 cells with both TNF-α and LPS along with idiosyncratic drugs for 24 h revealed that these drugs possess a potent inhibitory potential that was suppressed in the absence of an inflammatory context, suggesting that inflammation associated idiosyncratic drug synergy may be an effective tool in revealing the roles played by this hepatic transporter in drug-induced liver injury.

Discussion

Idiosyncratic adverse drug reactions (IADRs) account for the majority of post-marketing drug withdrawal and "black box warnings" (4); nevertheless, the lack of adequately predictive pre-clinical and clinical assays (3) complicated the revelation and understanding of their underlying mechanisms. One of the hypotheses that have emerged to explain IADRs is that inflammatory stress induced by exogenous or endogenous inflammatory agents is a susceptibility factor for the precipitation of idiosyncratic drug-induced liver injury (2). Recently established animal models co-administering bacterial LPS to induce an inflammatory background during drug therapy have succeeded to predict the potential hepatotoxicity of certain drugs (4). However, the low throughput nature of these models necessitates the development of high throughput in vitro predictive models of idiosyncratic drug-induced hepatotoxicity to better understand its underlying mechanisms. Accordingly, we have developed in the present work a drug-cytokine cellular model in which we co-treated HepG2 cells with a mix of pro-inflammatory mediators (LPS and TNF-α) along with several idiosyncratic drugs, aiming at investigating the implication of two important efflux transporters, MDR1 and MRP2, in inflammation-associated idiosyncratic drug hepatotoxicity. We have chosen specifically LPS and TNF-α to induce inflammation for two main reasons: first, to mimic the in vivo situation of previously validated animal models in which the co-administration of minimal doses of LPS with idiosyncratic drugs better revealed their potential hepatotoxicity (3); second, to elucidate any implication of MDR1 and MRP2 in inflammation-associated drug-induced liver toxicity since the LPS-stimulated release of TNF-α is known to modulate hepatic drug transporters expression and activity (9).

Our results revealed that the co-occurrence of an episodic inflammatory reaction during drug therapy modulated in an opposing manner the efflux activity of MDR1 and MRPP2. Telithromycin and nefazodone proved to be potent inhibitors of the MDR1-mediated efflux of rhodamine 123 when compared to verapamil. Nefazodone is known to induce hepatotoxicity by inhibiting BSEP, thus leading to the accumulation of drug and bile acid in the liver (10). To our knowledge, no previous studies demonstrated a link between nefazodone and MDR1; however, our results showed that this drug possesses a potent inhibitory potential on MDR1 probably through a structure-specific interaction with this transporter as is the case with BSEP (10). Telithromycin is known to be a substrate of both MDR1 and MRP2; however, in our results it elucidated an inhibitory potential solely on MDR1. Being both a substrate and an inhibitor of MDR1 suggests that telithromycin blocks the efflux activity of this transporter by competitively inhibiting the extracellular translocation of rhodamine 123 (8). It is noteworthy that the inhibitory potential of nefazodone, telithromycin, and even verapamil proved to be reversible as it was noticeably lost within an inflammatory context, probably due to a TNF-α-stimulated induction of MDR1 protein expression and functionality (11). Concerning MRP2, besides trovafloxacin which exhibited a mild inhibitory effect on the efflux of CDF, none of the tested idiosyncratic drugs modulated the efflux activity of this transporter indicating a poor implication of MRP2 in idiosyncratic drug-induced liver injury. Conversely, the co-presence of pro-inflammatory mediators along with idiosyncratic drugs has markedly potentiated their inhibitory potential on the efflux activity of MRP2 (mainly trovafloxacin and nimesulide) probably due to a TNF-α-induced downregulation of MRP2 protein expression and activity (12). The fact that the hepatotoxicity of nimesu-

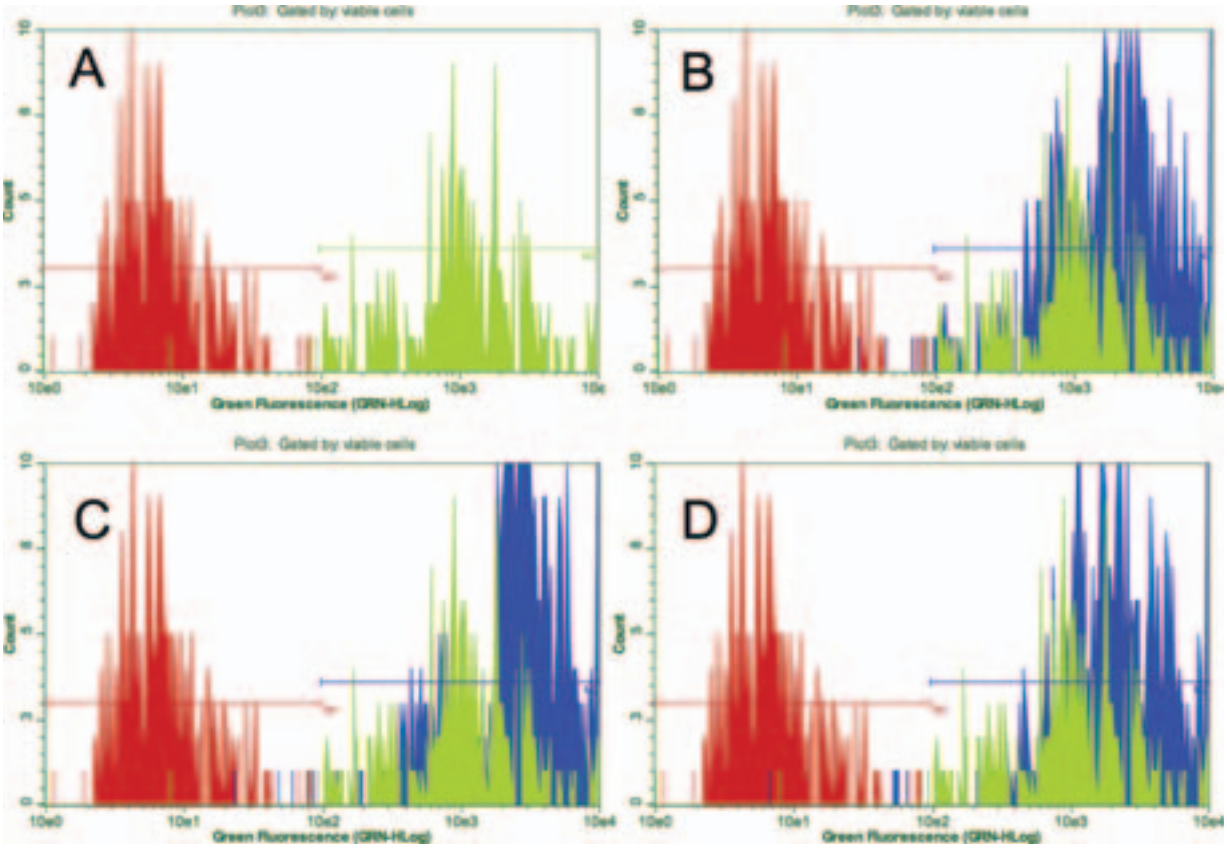


Figure 3. Intracellular retention of rhodamine 123 in HepG2 cells in the presence of 0.5 μM Rhodamine 123 (A), 100 μM Verapamil (B), 70 μM Nefazodone (C), and 175 μM Telithromycin (D). Prior to cytometry analysis cells were treated as described under *Materials and Method*. Histograms represent the cell counter numbers versus geo-mean fluorescent intensity (AU). In each figure, three overlaid histograms represent, from left to right, the peak from auto-fluorescence of control cells without rhodamine 123, the peak from rhodamine loaded-cells and the peak from rhodamine loaded-cells in the presence of verapamil (B), nefazodone (C), and telithromycin (D). [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

lide is often correlated with a remarkable increase in conjugated bilirubin and cholestatic injury (13) explains the observed nimesulide-induced inhibition of MRP2, since the latter is responsible for the canalicular excretion of conjugates, including bilirubin, glutathione, and bile salts (10).

Trovafloxacin proved to cause severe liver injury in animals only after synergizing its administration with an LPS-induced inflammatory stress (2). Consistently, our results demonstrated that trovafloxacin exhibited an inhibitory effect on the efflux activity of MRP2 only within an inflammatory context. The fact that this inhibitory potential was completely hidden in the absence of TNF-α and LPS confirms that the presence of pro-inflammatory mediators is necessary to reveal the toxicity of this idiosyncratic drug. This hepatotoxicity might be closely correlated to the presence of TNF, especially that trovafloxacin pre-treatment in vivo proved to prolong the LPS-induced increase in plasma concentration of TNF (2).

The regulatory pathways by which pro-inflammatory cytokines synergize with idiosyncratic drugs to alter human hepatic drug transporter expression and activity remain to be technically proven. However, important consideration must be given to MAPKs, since these kinases are known to be implicated in the phenotypic effect of pro-inflammatory cytokines (12). Moreover, recent studies proved that type II nuclear receptors, such as pregnane X receptor (PXR), constitutive androstane receptor (CAR), farnesoid X receptor (FXR), PPARα (peroxisome proliferator-activated receptor), and retinoic acid receptor (RAR) play important roles in the regulation of human drug transporters expression and activity in response to both xeno- and endobiotics during inflammation, cholestasis, and cancer (14). Much evidence proved that the regulation of both MDR1 and MRP2 is PXR-mediated especially that several of their inducers such as rifampicin, ritonavir, and saquinavir happen to be also PXR-activating ligands (Teng and Miller 2008). Accordingly, the observed drug-induced inhibition of MDR1 and/or MRP2 efflux activity may be attributed to drug-mediated suppression of PXR. Furthermore, PXR activators are known to minimize inflammation-mediated downregulation of transporters and attenuate cholestatic liver injury; thus, it is perfectly logical that PXR inhibitors may be strongly correlated to altered drug disposition and cholestasis during an inflammatory reaction (6).

In the present work, we showed that micro-volume cytometry is an efficient technique to demonstrate the altered

activity of both MDR1 and MRP2 during idiosyncratic drug-induced liver injury. Furthermore, it is noteworthy that the presence of pro-inflammatory mediators during idiosyncratic drug therapy can noticeably modulate such correlation. While MDR1 proved to be more implicated in idiosyncratic drug-induced hepatotoxicity than MRP2 in the absence of an inflammatory context, MRP2 exhibited a noticeable involvement in idiosyncratic drug-induced hepatotoxicity and this solely in the presence of an inflammatory context. Further research is needed to better elucidate the mechanisms through which the idiosyncratic drugs-inflammatory mediators synergy modulate hepatic transporters activity inducing liver toxicity.

Literature Cited

1. Russmann S, Kullak-Ublick GA, Grattagliano I. Current concepts of mechanisms in drug-induced hepatotoxicity. Curr Med Chem 2009;16:3041–3053.
2. Shaw PJ, Ganey PE, Roth RA. Idiosyncratic drug-induced liver injury and the role of inflammatory stress with an emphasis on an animal model of trovafloxacin hepatotoxicity. Toxicol Sci 2010;118:7–18.
3. Deng X, Luyendyk JP, Ganey PE, Roth RA. Inflammatory stress and idiosyncratic hepatotoxicity: hints from animal models. Pharmacol Rev 2009;61:262–282.
4. Cosgrove BD, King BM, Hasan MA, Alexopoulos LG, Farazi PA, Hendriks BS, Griffith LG, Sorger PK, Tidor B, Xu JJ, et al. Synergistic drug-cytokine induction of hepatocellular death as an in vitro approach for the study of inflammation-associated idiosyncratic drug hepatotoxicity. Toxicol Appl Pharmacol 2009;237:317–330.
5. Waring JF, Liguori MJ, Luyendyk JP, Maddox JF, Ganey PE, Stachlewitz RF, North C, Blomme EA, Roth RA. Microarray analysis of lipopolysaccharide potentiation of trovafloxacin-induced liver injury in rats suggests a role for proinflammatory chemokines and neutrophils. J Pharmacol Exp Ther 2006;316:1080–1087.
6. Teng S, Piquette-Miller M. Regulation of transporters by nuclear hormone receptors: implications during inflammation. Mol Pharm 2008;5:67–76.
7. Glavinas H, Krajcsi P, Cserepes J, Sarkadi B. The role of ABC transporters in drug resistance, metabolism and toxicity. Curr Drug Deliv 2004;1:27–42.
8. Yamaguchi S, Zhao YL, Nadai M, Yoshizumi H, Cen X, Torita S, Takagi K, Hasegawa T. Involvement of the drug transporters p glycoprotein and multidrug resistance-associated protein Mrp2 in telithromycin transport. Antimicrob Agents Chemother 2006;50:80–87.
9. Morgan ET, Goralski KB, Piquette-Miller M, Renton KW, Robertson GR, Chaluvadi MR, Charles KA, Clarke SJ, Kacevska M, Liddle C, et al. Regulation of drug-metabolizing enzymes and transporters in infection, inflammation, and cancer. Drug Metab Dispos 2008;36:205–216.
10. Kostrubsky SE, Strom SC, Kalgutkar AS, Kulkarni S, Atherton J, Mireles R, Feng B, Kubik R, Hanson J, Urda E, et al. Inhibition of hepatobiliary transport as a predictive method for clinical hepatotoxicity of nefazodone. Toxicol Sci 2006;90:451–459.
11. Fernandez C, Buyse M, German-Fattal M, Gimenez F. Influence of the pro-inflammatory cytokines on P-glycoprotein expression and functionality. J Pharm Pharm Sci 2004;7:359–371.
12. Fardel O, Le Vee M. Regulation of human hepatic drug transporter expression by pro-inflammatory cytokines. Expert Opin Drug Metab Toxicol 2009;5:1469–1481.
13. Bessone F. Non-steroidal anti-inflammatory drugs: What is the actual risk of liver damage? World J Gastroenterol 2010;16:5651–5661.
14. Jigorel E, Le Vee M, Boursier-Neyret C, Parmentier Y, Fardel O. Differential regulation of sinusoidal and canalicular hepatic drug transporter expression by xenobiotics activating drug-sensing receptors in primary human hepatocytes. Drug Metab Dispos 2006;34:1756–1763.

Chapter 4

Part II

Effect of idiosyncratic drugs on the expression of MDR1 and MRP2 in presence and absence of pro-inflammatory stimuli

4.1 Introduction

Drug-induced cholestasis accounts for approximately half of the reported cases of drug-induced hepatotoxicity (Björnsson et al. 2005); hence it is considered as one of the main mechanisms through which a hepatotoxic drug may induce liver injury (DILI) (Bohan et al. 2002). Cholestasis results predominantly either from a functional defect in bile synthesis at the hepatocyte level or from impairment in bile flow and secretion at the bile duct level (Zollner et al. 2008). Drugs mainly cause cholestasis by inhibiting hepatocellular transporter expression and activity and, in few cases, by inducing vanishing bile duct syndrome, which can readily progress to biliary cirrhosis (Zollner et al. 2008). Vanishing bile duct syndrome (VBDS) is a rare but critical complication of drug induced liver injury marked clinically by chronic cholestasis and histologically by loss of intrahepatic bile ducts (Okan et al. 2008).VBDS is typically correlated with drug-induced production of auto-antibodies against cytokeritin, which is a major constituent of bile ducts leading to their destruction (Okan et al. 2008). Predominantly, the drugs that have been reported to cause cholestatic injury are correlated with idiosyncratic hepatotoxicity, which is often associated with inflammation, hypersensitivity and impaired metabolism (Zollner et al. 2008). Inflammation itself has been frequently correlated with altered bile secretion resulting in inflammatory cholestasis referred to as ‘‘cholestatic hepatitis’’ (Trauner et al. 1999; Pauli-Magnus and Meier, 2006). The majority of idiosyncratic drug-induced cholestasis cases result from altered transporter expression or functional inhibition mediated by the drug itself or its reactive metabolites (Padda et al. 2011). Particularly, this chapter will investigate the effects of four known idiosyncratic drugs namely, trovafloxacin, nimesulide, telithromycin and nefazodone on the expression of MDR1 and MRP2, which are two important efflux transporters significantly involved in drug elimination.

4.1.1 Regulation of hepatic MDR1 and MRP2 protein expression

Hepatic drug transporters play primordial roles in hepatobiliary elimination of xenobiotics, particularly drugs (Jigorel et al. 2006). Specifically, P-glycoprotein encoded by the multidrug resistance 1 gene (MDR1) and multidrug resistance protein 2 (MRP2) belong to the ATP-binding cassette (ABC) superfamilies of transporters and are located on the hepatic canalicular pole where they mediate the secretion of drugs and their metabolites into bile (Figure 4.1) (Schinkel and Jonker, 2003; Fardel et al. 2005). While MDR1 is involved in the transport of neutral or positively charged hydrophobic large organic compounds with a polyaromatic skeleton including a wide variety of anticancer drugs; MRP2 predominantly transport phase II metabolism products such as glucuronide, glutathione, and sulfate conjugates of drugs in addition to unconjugated amphipathic drugs, organic anions, neutral or cationic anti-cancer drugs and bilirubin conjugates (Fardel and Le Vee, 2009; Kast et al. 2002). Similar to the majority of drug-metabolizing enzymes hepatic drug transporters are predominantly regulated by xenobiotic-activated nuclear receptors and transcription factors such as the pregnane X receptor (PXR), the constitutive androstane receptor (CAR), the farnesoid X receptor (FXR), the liver X receptor (LXR), the

peroxisome proliferator-activated receptor (PPAR), the aryl hydrocarbon receptor (AhR) and nuclear factor E2-related factor 2 (NrF2) (Teng and Miller, 2008; Jigorel et al. 2006). In particular, phenobarbital-activating CAR and rifampicin-activating PXR proved to induce MDR1 and MRP2 mRNA expression in human hepatocytes; whereas oltipraz-activating Nrf2 mediated solely the up-regulation of MRP2 mRNA expression (Jigorel et al. 2006). These drug-sensing receptors in additional to the transcriptional factor Nrf2 are normally located in the cytosol where they are activated by a wide range of endogenous compounds and therapeutic drugs. They are known to function as ligand-induced transcription factors; accordingly binding of certain ligands stimulates their translocation from the cytosol to the nucleus, as heterodimers or homodimers, where they modulate the transcription of several genes by binding respectively to their specific DNA response elements (Xie et al. 2004). A subset of nuclear receptors, precisely the xenobiotic receptors PXR and CAR, proved to regulate gene expression by forming heterodimers with the retinoid X receptor (RXR) (Xie et al. 2004). This regulation takes place after the subsequent binding of the PXR–RXR or CAR–RXR heterodimers to their specific xenobiotic response elements (XREs) present in the promoter regions of several drug transporters (Xie et al. 2004). The ability of PXR and CAR to interact with a broad array of small lipophilic molecules including various therapeutic drugs and xenobiotic in addition to the prominent presence of their respective response elements in the promoter region of numerous drug-metabolizing and transporter genes explained their implication in the regulation of nearly all phases of hepatic drug metabolism and elimination (Xie et al. 2004). PXR and CAR demonstrated overlapping ligand specificity; hence their activation may result in the increased expression of the same enzymes due to stimulation of similar response elements (Goodwin et al. 2001; Maglich et al. 2002). Recent evidence elucidated that these receptors are efficiently implicated in the regulation of numerous physiological and pathophysiological processes, such as glucose homeostasis, lipid metabolism, enterohepatic biliary transport and inflammatory response (Chang, 2009). Although PXR has been classified as a xenobiotic receptor, mounting evidence revealed an equally efficient potential of PXR as an 'endobiotic receptor' that responds to a vast array of endogenous compounds (Chang, 2009). Similarly, FXR plays a central role in maintaining bile acid homestasis in the enterohepatic circulating system by regulating the expression of several enzymes and transporters related to the synthesis and elimination of bile acids (Lefebvre et al. 2009). Furthermore, it has been proven that FXR also regulates triglyceride and cholesterol metabolism, hence constituting a molecular link between lipid and bile acids metabolism (Lefebvre et al. 2009). Interestingly, PXR and FXR proved capable of binding and responding to bile acids, emphasizing that drug metabolism is interrelated with cholesterol and bile acid homeostasis (Eloranta et al. 2005). CAR seems to be implicated in bilirubin and energy homeostasis, although its explicit roles as an endogenous regulator remain unclear (Goodwin et al, 2004). Other than orphan nuclear receptors, some signaling events proved to be also implicated in the regulation of hepatic transporters expression. For example, an estrogen response element has been identified in the BCRP promoter region implying that BCRP

expression may be influenced by estrogen (E1) and/or estradiol (E2) (Ee et al. 2004; Imai et al. 2005). Furthermore, BCRP expression proved to be also regulated by the PI3K-Akt signaling pathway via post-transcriptional/translational mechanisms, particularly in BCRP-overexpressing K562 cells (Nakanishi et al. 2006). Moreover, intracellular protein trafficking mechanisms proved to be also involved in the normal expression of transport proteins on their corresponding hepatic pole (Wakabayashi et al. 2006). Earlier studies demonstrated that the trafficking of MRP2 to the canalicular membrane is mediated by a protein kinase C-dependent mechanism (Beuers et al. 2001); hence, a drug-induced modulation in the expression or activity of protein kinase C may alter the expression and localization of MRP2 on the canalicular pole.

4.1.2 Hepatic protein trafficking and localization in polarized hepatocytes

Hepatocytes are considered as highly polarized epithelial cells; their plasma membrane is separated by tight junctions into sinusoidal, basolateral and canalicular domains, each expressing different sets of proteins and lipids (Wang and Boyer, 2004). A normal membrane polarity is primordially required for correct localization of hepatic proteins and normal hepatocytic functioning especially concerning canalicular bile and sinusoidal protein secretion (LeCluyse et al. 2012). The correct expression of transport proteins on their respective hepatic poles is mediated by three major intracellular trafficking pathways: Initially a membrane protein is synthesized in the endoplasmic reticulum, to be later on modified in Golgi complex, and finally sorted at the trans-Golgi network (TGN) (Wang and Boyer, 2004). From the TGN, proteins traffic along the post-Golgi biosynthetic pathway to the canalicular or sinusoidal membrane domain (Wang and Boyer, 2004). When the protein reaches the plasma membrane it is either retained or internalized, a process known as endocytosis; following endocytosis some proteins are transported to the opposite membrane domain of hepatocytes, by a process known as transcytosis (Wang and Boyer, 2004). Earlier studies elucidated that sinusoidal membrane proteins traffic directly from the Golgi complex to their final destination; whereas canalicular proteins predominantly traffic by an indirect route, from the Golgi complex to the sinusoidal membrane, followed by transcytosis through basolateral early endosomes and subapical compartments, before final localization on the canalicular plasma membrane domain (Wang and Boyer, 2004). The indirect transcytotic pathway was considered, for a long period of time, as the only route by which canalicular proteins reach their final destination in hepatocytes until recent evidence proved the contrary (Wang and Boyer, 2004). Recently MDR1 and BSEP proved to traffic directly from the Golgi complex to the canalicular domain whereas MRP2 rather follow an indirect trafficking route comprising transcytosis (Wang and Boyer, 2004). In a cholestatic liver hepatocyte polarity is disrupted; hence canalicular proteins are trapped in subapical compartments, the motility of transcytotic vesicles is decreased and tight junctions are impaired (Torok et al. 2001; Wang and Boyer, 2004). Overall these observations indicate that cholestasis significantly impairs transcytosis of canalicular proteins leading to a loss or even reversal of membrane polarity (Wang and Boyer, 2004). The mechanisms responsible for altered cell

polarity in cholestasis are incompletely understood. However earlier evidence suggested that increased amounts of taurolithocholic acid (TLCA), may be significantly involved in impaired transcytosis and altered MRP2 protein localization (Beuers et al. 2003; Beuers et al. 2001). It is equally plausible for the increased amounts of bile acids observed during cholestasis to affect directly or indirectly the trafficking of other plasma membrane proteins in hepatocytes (Wang and Boyer, 2004).

4.1.3 Impact of inflammation on the expression of hepatic MDR1 and MRP2

Inflammation is known to prominently impair drug transporters expression and functionality; thus altering drug pharmacokinetics and toxicity profiles (LeVee et al. 2008). In animals the effect of inflammation on hepatic transporters was predominantly studied following LPS and turpentine oil administration to rodents (Frenandez et al. 2004). Both pro-inflammatory agents induced a potent upregulation in the expression of a wide variety of pro-inflammatory cytokines mainly TNF-α, IL-1, IFN-γ and IL-6 (Frenandez et al. 2004; Teng and Miller, 2008). These cytokines proved to be correlated in different ways and to different extents with altered hepatic transporters mRNA and protein expression; consequently predisposing or protecting the liver from drug-induced cholestasis. Particularly, the effect of several pro-inflammatory cytokines on the expression of MDR1 and MRP2 may be summarized as follows: in mice, TNF-α increased the expression of Mdr1b but decreased the expression of Mrp2; IL-1 reduced the expression of Mdr1a and Mrp2; IL-6 decreased the expression of Mdr1b and Mrp2 whereas IFN-γ induced the expression of Mdr1 (Teng and Miller, 2008). The prominent interspecies differences between animals and humans in the ADME/TOX profile of drugs resulted in varying results concerning the impact of inflammation on human hepatic transporter expression. In human hepatocytes TNF-α neither affect MDR1 nor MRP2 mRNA expression levels; whereas IL-1β and IL-6 induce a decrease in the mRNA expression levels of both transporters (Le Vee et al. 2009). Similar to human hepatocytes IL-1β and IL-6 reduce mRNA expression of both MDR1 and MRP2 in the human hepatic cell line HepaRG (Fardel and LeVee, 2009). In contrast to human hepatocytes, TNF-α-treated HepaRG cells demonstrated reduced MDR1 and MRP2 mRNA expression levels (Fardel and LeVee, 2009). The mechanisms through which these cytokines impair human hepatic transport protein expression resulting in drug-induced cholestasis predominantly include: (i) pro-inflammatory cytokine-induced activation of nuclear factor-κβ (NF-κβ) and nuclear factor-IL6 (NF-IL6) which regulates the expression of hepatic transporter proteins by interacting with their respective putative binding sites on the promoter sequences of BCRP, MDR1 and MRP2 genes (Morgan et al. 2008); (ii) pro-inflammatory cytokine-induced downregulation of several orphan nuclear receptors (PXR, CAR, FXR and RXR) expression levels (Petrovic et al. 2007); pro-inflammatory cytokine-induced activation of several intracellular signaling pathways involved in transporter expression such as signal transducers and activators of transcription (STAT), CCAAT enhancer binding protein, activating protein 1 (AP-1), nuclear factor-B (NF-B) and MAPKs (Teng and Miller, 2008; Fardel and LeVee, 2009).

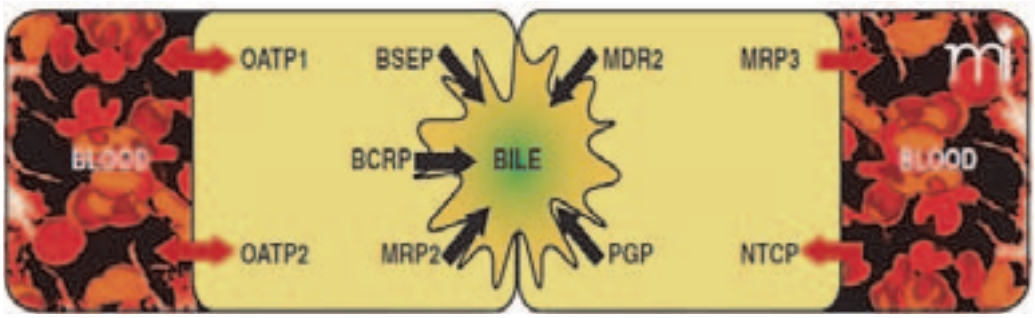


Figure 4.1. Hepatic drug transporters whose expression is influenced by inflammation.
(Adapted from Petrovic et al. 2007).

4.1.4 Aim of chapter 4

This chapter investigates the effect of four idiosyncratic drugs namely trovafloxacin, nimesulide, telithromycin and nefazodone on hepatic MDR1 and MRP2 protein expression in the aim of elucidating whether impaired hepatic transporter expression is causatively correlated with the previously demonstrated idiosyncratic drug induced functional inhibition of MDR1 and MRP2 (Chapter 4, Part I). Revealing the mechanisms underlying idiosyncratic drug-induced cholestasis may provide valuable insights on how to prevent it; this may help reduce the incidence rate of one of the most frequent and critical clinical manifestation of drug-induced liver injury.

4.2 Experimental workflow

4.2.1 Detection of functional biliary poles in HepG2 cells by CDF assay

HepG2 cells were cultured in a small culture flask until confluent. Then the culture media was removed and cells were washed with HBSS (Hank's Balanced Salt Solution). Subsequently, cells were incubated with CDFDA (5 μM) at 37°C for 20 minutes. CDFDA is the diacetate moiety of CDF, the fluorescent substrate of MRP2. It enters the cells by passive diffusion where it is esterified to fluorescent CDF by esterases. In polarized hepatocytes, CDF should be transported to the apical (canalicular) pole where it will accumulate to be effluxed outside of the cell by MRP2. The accumulated CDF will emit a green fluorescence that may be detected by a fluorescence microscope.

4.2.2 Immuolocalization of MRP2 proteins by indirect immunofluorescence

Localization of MRP2 in HepG2 cells was performed using indirect immunofluorescence as follows: HepG2 cells were incubated in a 96-well plate for 24 hours. Then cells were permeabilized by adding 80 µL of PBS/0.3%Triton-X100 solution per well for 20 minutes at room temperature. Cells were then blocked with PBS containing 5% donkey serum and 1% BSA (50µL/well) for 1 hour at room temperature. Subsequently cells were incubated overnight at 4°C with primary monoclonal goat anti-MRP2 diluted in PBS containing 5% donkey serum and 1% BSA (40µL/well). Cells were then washed with PBS (three times) for 10 minutes at room temperature with shaking before being incubated with anti-goat dylight488-conjugated secondary antibody (diluted 1/500 in PBS containing 5% donkey serum and 1% BSA) for 1 hour at room temperature (40µL/well). After incubation, cells were washed three times with PBS for 10 minutes at room temperature with shaking. Cells were imaged using the high throughput cellular imaging platform ImPACcell.

4.2.3 Western blot analysis of MRP2 protein expression

After incubating HepG2 cells for 24 hours in a 6-well plate with the four idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) in presence and absence of the inflammatory mix (LPS and TNF-α), cellular proteins were extracted for western blot analysis as described previously in Chapter 2. Proteins were then separated on polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. After blocking in Tris-buffered saline containing 5 % nonfat dry milk (blocking buffer), membranes were incubated overnight at 4°C with primary mouse monoclonal MRP2 antibody (M2III-6) diluted 1/500 in blocking buffer. Subsequently membranes were incubated with peroxidase-conjugated anti-mouse secondary antibodies diluted 1/2500 in blocking buffer. After extensive washing immunolabeled proteins were visualized by chemiluminescence.

4.2.4 Capillary flow cytometric analysis of MDR1 protein expression

HepG2 cells were incubated for 24 hours with the four tested idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) in presence and absence of the inflammatory mix (LPS and TNF-α). Subsequently, culture media was aspirated and cells were harvested by gentle scraping using 2ml of PBS/BSA buffer (Phosphate Buffered Saline pH 7.4 with 1% Bovine Serum Albumin). Cells were then transferred to a 15 ml conical tube containing 10 ml of PBS/BSA buffer and centrifuged at 400 *g* for 5 minutes. The supernatants were discarded and the cellular pellet was resuspended in PBS/BSA buffer. Then cells were incubated with FITC-labeled anti-Pgp reacting with an external surface epitope of P-gp for 30 minutes at room temperature (10µl of anti-MDR1 was added to 100 µl of cells). Cells were then washed with 0.2 ml of PBS/BSA and centrifuged at 400 *g* for 5 minutes. The supernatants were discarded and cells were resuspended in 0.2 ml of PBS/BSA. Data was acquired by Guava EasyCyte Plus capillary flow cytometer and results were analyzed using the Guava ExpressPro software

(Merck/Millipore/Guava Tech) in terms of x-geometric mean arbitrary units (AU). The increase in FITC-emitted green fluorescence (530nm) was directly proportional to the expression of MDR1 proteins in HepG2 cells.

4.3 Results

4.3.1 Accumulation of CDF at the apical pole between two polarized HepG2 cells

After incubating HepG2 cells with CDFDA for 20 minutes at 37°C, the latter freely entered the cell by passive diffusion and was hydrolyzed by esterases to CDF, the fluorescent substrate of MRP2. CDF accumulated at the apical (canalicular) pole of HepG2 cells emitting a bright green fluorescence which was detected by fluorescence microscopy (Figure 4.2). These observations indicate that the used HepG2 cells are correctly polarized, with the canalicular pole located at its normal location between two adjacent HepG2 cells. Furthermore, the fact that CDF accumulated at the canalicular pole strongly implies that the fluorescent substrate will be most probably effluxed outside the cell by its respective transporter, MRP2, indicating the presence of functional biliary poles.

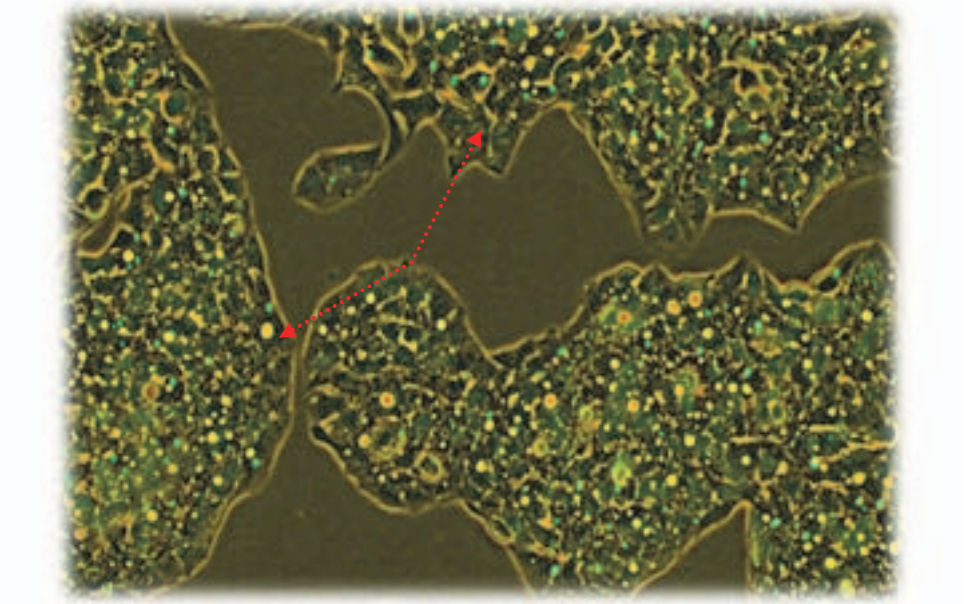


Figure 4.2. Accumulation of CDF at the bile canalicular pole between two adjacent HepG2 cells. The trafficking of CDF to the canalicular pole indicates that the used HepG2 cells exhibit correct membrane polarity, protein trafficking and cellular functionality. The accumulation of CDF at the canalicular pole is represented in the figure by the green fluorescent circles (some of which are indicated by the red arrows).

4.3.2 Localization of MRP2 proteins at the apical pole between two HepG2 cells

The triparametric staining of HepG2 cells with anti-MRP2 (green), Hoechst (bleu) and Phalloidin (red) indicated that HepG2 cells significantly express MRP2 proteins and confirmed that these proteins are correctly located at the apical poles between two adjacent HepG2 cells (Figure 4.3). These results further confirm the polarity of HepG2 cells and imply correct transport protein trafficking.

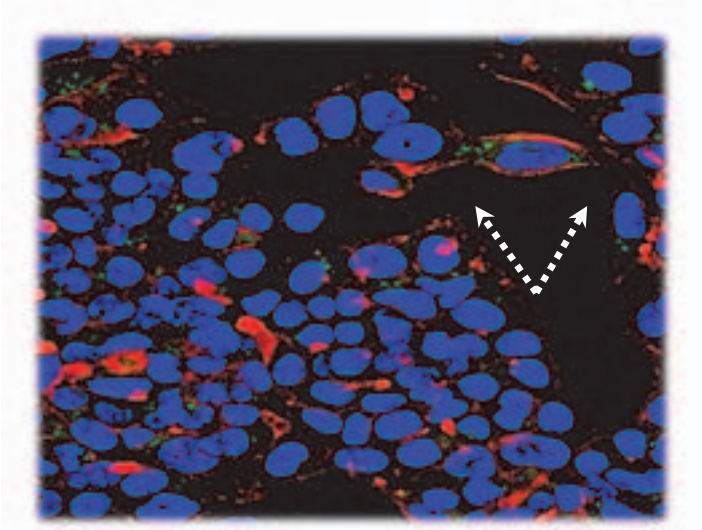


Figure 4.3. Immunolocalization of MRP2 proteins in HepG2 cells. The tri-parametric staining of HepG2 cells with the nucleus-staining dye Hoechst (blue color) and the actin-staining toxin phalloidin (red color) in addition to anti-MRP2 (green color) reveals the correct localization of MRP2 at the canalicular pole of HepG2 cells (indicated by the two white arrows).

4.3.3 Effect of idiosyncratic drugs on the expression of MRP2

Immunoblot analysis of MRP2 expression revealed first of all that HepG2 cells basally express a significant amount of MRP2 proteins. Following the 24-hours incubation of HepG2 cells with the four tested idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) the results attained demonstrated that in the absence of inflammation trovafloxacin and nefazodone induce the expression of MRP2 proteins whereas nimesulide and telithromycin reduce it. However, the co-administration of these drugs along with LPS and TNF-α to HepG2 cells completely suppressed the expression of MRP2 proteins in all samples except telithromycin (Figure 4.4 A and B). These results suggest that the administered pro- inflammatory mediators, namely LPS and TNF-α, prominently suppress the expression of MRP2 proteins in an extremely significant manner, which was strong enough to mask the inducing effects of trovafloxacin and nefazodone on its expression.

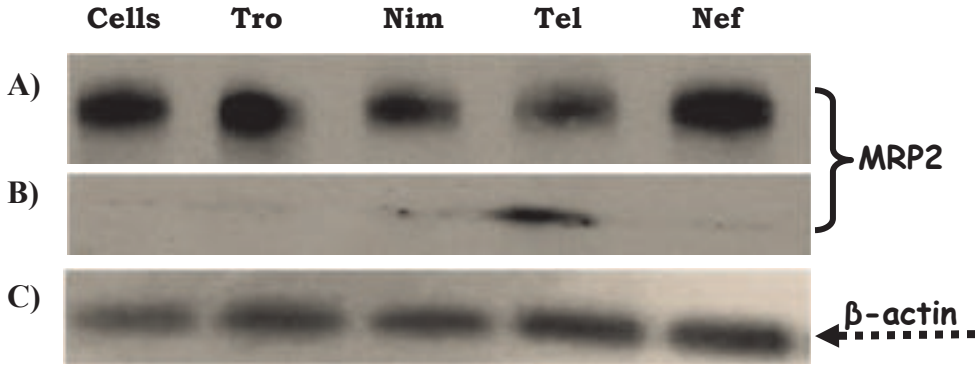


Figure 4.4 (A). Imunnoblot analysis of MRP2 protein expression in HepG2 cells in non-inflamed and inflamed HepG2 cells. Lane A corresponds to the expression of MRP2 proteins in HepG2 cells following their incubation with trovafloxacin, nimesulide, telithromycin and nefazodone for 24 hours in the absence of an inflammatory context. Lane B corresponds to the expression of MRP2 proteins in HepG2 cells co-treated with the four idiosyncratic drugs in addition to LPS and TNF-α. Lane C corresponds to the expression of β-actin which serves as an internal control for every loaded sample.

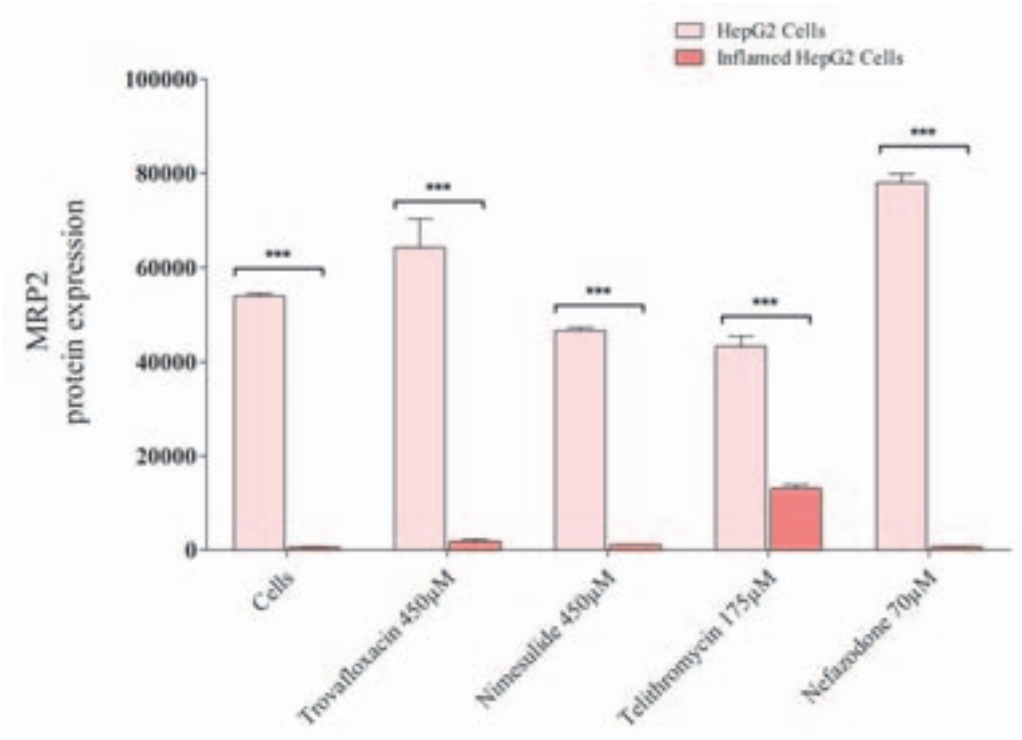


Figure 4.4 (B). Quantification of MRP2 protein expression by ImageJ in non-inflamed and inflamed HepG2 cells. After incubating HepG2 cells with the four idiosyncratic drugs in absence and presence of LPS and TNF-α for 24 hours, the expression of MRP2 protein was assessed by western blot and quantified by image J. Bars represent the densitometric analysis of three different experiments (n=3). Data is represented as Mean ± S.E.M. Statistical analysis was performed using two way ANOVA followed by Bonferroni post test. *** represents $P < 0.001$ and refers to the variation in MRP2 protein expression between non-inflamed and inflamed HepG2 cells. These results indicate that the co-presence of LPS and TNF-α along with idiosyncratic drugs favor the reduction of MRP2 protein expression in an extremely significant manner.

4.3.4 Effect of idiosyncratic drugs on the expression of MDR1

After incubating HepG2 cells for 24 hours with the four tested idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) in presence and absence of the inflammatory mix (LPS and TNF-α) the results attained demonstrated first of all that untreated HepG2 cells significantly express MDR1 proteins. Nevertheless this expression was modulated by the four idiosyncratic drugs as follows: In the absence of LPS and TNF-α, trovafloxacin, telithromycin and nefazodone induced the expression of MDR1 proteins whereas nimesulide reduced it in a statistically significant manner when compared to untreated cells (Figure 4.5A).However, when the four idiosyncratic drugs were co-administered to cells along with LPS and TNF-α, the inducing effects of trovafloxacin, telithromycin and nefazodone were drastically attenuated whereas the reducing effect of nimesulide was potentiated (Figure 4.5B). The effects of the four idiosyncratic drugs on the expression of MDR1 varies significantly upon their co-administration with LPS and TNF-α indicating that inflammation strongly suppresses the expression of MDR1 (Figure 4.5C). The inducing effect of trovafloxacin on the protein expression of MDR1 is demonstrated by a representative flow cytometric histogram in Figure 4.6.

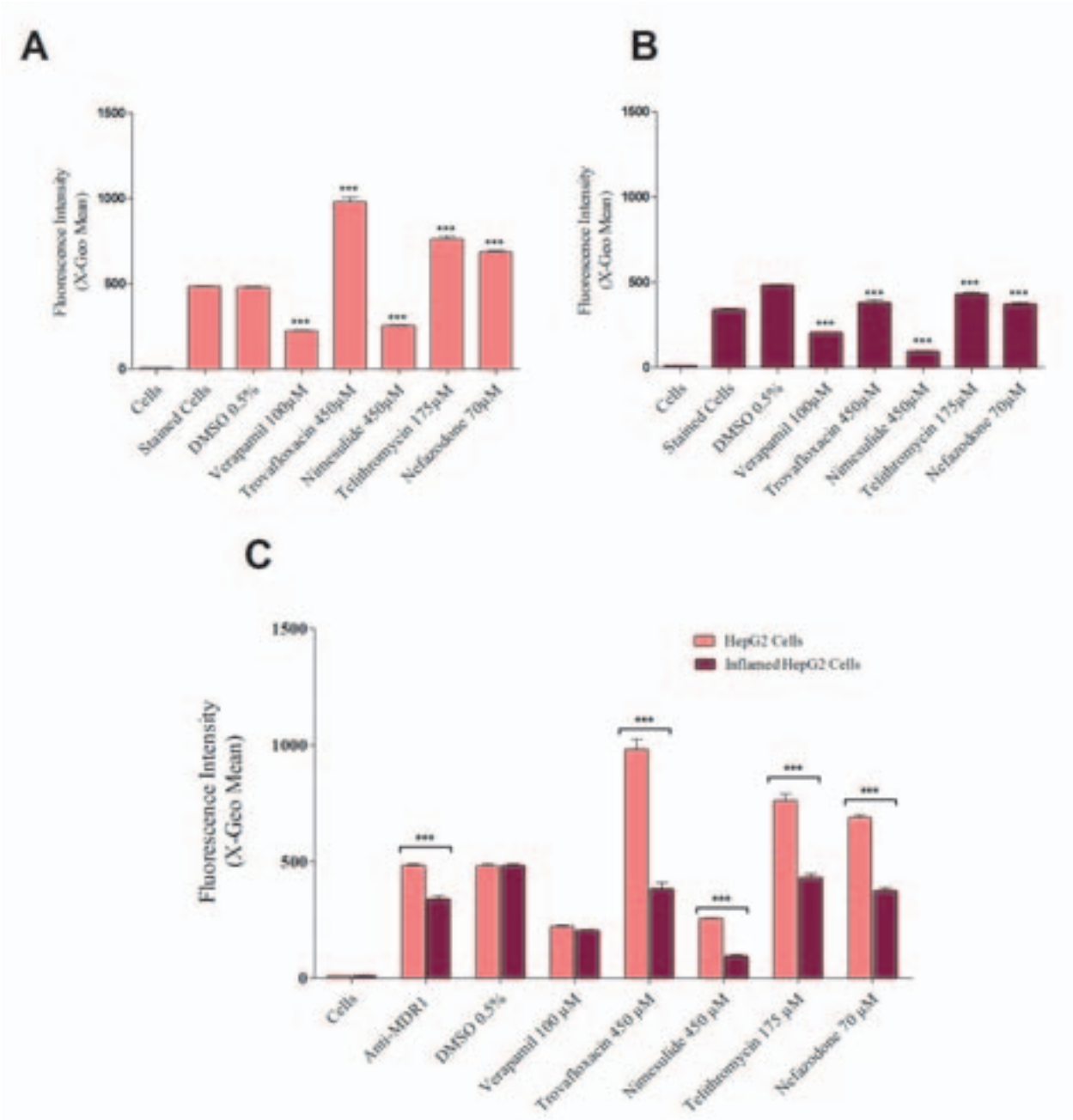


Figure 4.5. Effect of idiosyncratic drugs on MDR1 protein expression in non-inflamed and inflamed HepG2 cells. After co-incubating HepG2 cells with the four idiosyncratic drugs in absence (A) and presence of LPS and TNF-α (B) for 24 hours, the expression of MDR1 protein was assessed using FITC-conjugated anti-MDR1 by capillary flow cytometry. Graph C represents the effects of the four tested idiosyncratic drugs on the expression of MDR1 in non-inflamed and inflamed HepG2 cells. Bars represent the emitted intracellular fluorescence of FITC-conjugated MDR1 upon binding to its target in terms of X-geometric mean (AU). Data is represented as Mean ± S.E.M (n=3). Statistical analysis was performed using one way (for graphs A and B) and two way ANOVA (for graph C) followed by Bonferroni post test. *** represents $P < 0.001$ and refers to the variation in MDR1 protein expression between DMSO-treated and drug-treated HepG2 cells in graphs A and B. In graph C the p-value corresponds to the variation in MDR1 protein expression between non-inflamed and inflamed HepG2 cells.

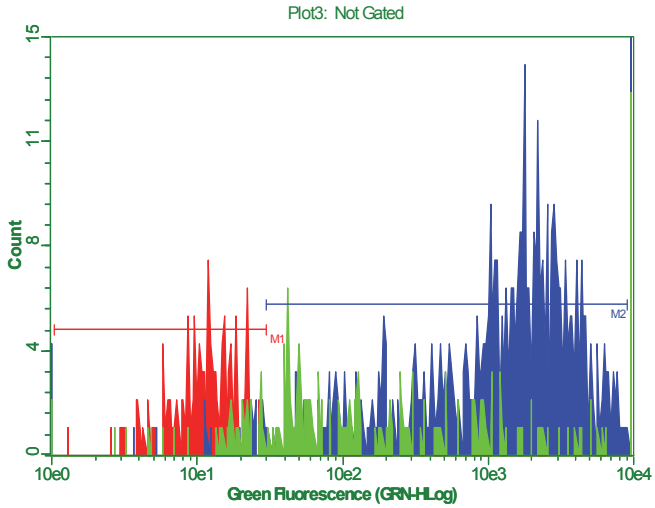


Figure 4.6. A representative capillary flow cytometric histogram revealing the effect of Trovafloxacin on MDR1 protein expression. The red fluorescent peak corresponds to the fluorescence emitted from untreated HepG2 cells. The green fluorescence peak refers to FITC-conjugated anti-MDR1 treated cells and corresponds to the basal expression of MDR1 proteins in HepG2 cells. Upon binding of FITC-conjugated anti-MDR1 to MDR1 proteins a green fluorescence, that is directly proportional to the quantity of MDR1 proteins present in HepG2 cells, is detected by capillary flow cytometry. The blue fluorescent peak corresponds to the fluorescence emitted from FITC-conjugated anti-MDR1 treated cells after being incubated with trovafloxacin for 24 hours. The increase in fluorescence intensity between the green and the blue fluorescence peaks implies that trovafloxacin significantly induces MDR1 protein expression.

4.4 Discussion

This part of chapter 4 investigated the effect of four idiosyncratic drugs namely trovafloxacin, nimesulide, telithromycin and nefazodone on the expression of MDR1 and MRP2, two important efflux transporters involved in the elimination of a vast variety of drugs, in the aim of elucidating whether idiosyncratic drug-induced alteration of MDR1 and MRP2 protein expression is causatively correlated with the impaired efflux function observed in the presence of these drugs in Chapter 4 part I. Before studying the effect of these drugs on the expression of MDR1 and MRP2, HepG2 membrane polarity and hence hepatic transport protein trafficking and localization were verified. The results attained demonstrated that CDF, the fluorescent substrate of MRP2, trafficed directly, after entering the cells by passive diffusion, to the canalicular pole of HepG2 cells where it accumulated emitting a green fluorescence These results indicate that the used HepG2 cells exhibit correct membrane polarity and protein trafficking with the canalicular membrane localized as expected between two adjacent cells and MRP2 localized at its normal place on the plasma membrane of the canalicular pole. MRP2 proteins are known to traffic to the canalicular pole of hepatocytes via the indirect transcytotic route; moreover unlike MDR1 and BSEP which undergo dynamic endocytosis and recycling, MRP2 proteins are

predominantly retained at the canalicular membrane (Wang and Boyer, 2004). The main mechanisms implicated in the apical retention of hepatic transport proteins involve principally the actin based cytoskeleton and proteins like ezrin-radixin-moesin (ERM). The latter directly bind to both actin and single transmembrane proteins and interact with proteins like MRP2 via their PDZ domains (Bretscher et al. 2002; Fanning et al. 1999). Accordingly, the demonstrated correct polarity and localization of MRP2 in HepG2 cells strongly suggest the correct functionality of these mechanisms. Subsequently, the effect of trovafloxacin, nimeuslide, telithromycin and nefazodone on the expression of MRP2 and MDR1 in presence and absence of an inflammatory context was studied by western blot and capillary flow cytometry respectively.

Concerning MRP2, the results attained demonstrated first of all a prominent basal expression of MRP2 proteins in HepG2 cells; nevertheless this expression was completely repressed in presence of LPS and TNF-α. Consistently several previous studies confirmed that inflammation potently downregulates the expression of MRP2 in several models of cholestasis including endotoxemia (Petrovic et al. 2007). Nevertheless, LPS-incubated human and rat liver slices demonstrated prominent inter-species differences with regard to transporters regulation; while LPS-treated rat liver slices demonstrated reduced MRP2 mRNA levels human hepatic slices MRP2 mRNA levels remained unchanged in presence of LPS (Petrovic et al. 2007). However, MRP2 protein expression in humans proved to be virtually non-existent 24 hours following LPS administration; implying that MRP2 is predominantly regulated at the post-transcriptional level (Petrovic et al. 2007; Zhang et al. 2010). The latter was further supported by the fact that intracellular redistribution of Mrp2 occurs during the early stages of cholestasis, while alterations in Mrp2 mRNA amounts take place at later times (Petrovic et al. 2007). The reported LPS-induced disappearance of MRP2 protein expression is extremely consistent with the results attained in our experimental conditions following the administration of the inflammatory mix comprising both LPS and TNF-α to HepG2 cells; implying that MRP2 is mainly altered by the presence of LPS. Nonetheless, LPS-induced pro-inflammatory cytokines especially TNF-α revealed an essential role in inflammation-induced alteration of hepatic transporters (Cherrington et al. 2004). This role was supported by three main evidences: Firstly, the administration of anti-TNF-α prevented LPS from impairing bile flow and bile acid secretion; secondly, the administration of recombinant TNF-α and IL-6 proved to downregulate NTCP and MRP2 and thirdly the administration of immunosuppressors such as dexamethasone, which prevents the LPS-induced release of cytokines partially blocks the down-regulation of MRP2 (Cherrington et al. 2004). These evidences suggest that TNF-α, just like LPS, may also be significantly involved in the suppression of MRP2 protein expression. Regarding the effect of the four tested idiosyncratic drugs on the expression of MRP2 the results attained demonstrated that in the absence of an inflammatory context trovafloxacin and nefazodone induce a remarkable upregulation in MRP2 protein expression whereas nimesulide and telithromycin downregulate the expression of this transporter. In the presence of an inflammatory context the modulatory effects of these drugs on MRP2 are masked due to the drastic inflammation-induced suppression

of MRP2 proteins. Nevertheless a mild expression of MRP2 proteins was maintained in the co-presence of telithromycin along with LPS and TNF-α; indicating that the presence of telithromycin attenuated the drastic suppressing effect of inflammation (Figure 4.4B). Since telithromycin reduced MRP2 protein expression when administered alone to cells, most probably it competes with inflammation for the same target responsible for downregulating the expression of MRP2 such as for example, PXR or CAR, thus attenuating the synergistic reducing effect of both the drug and the administered inflammatory mix, leading to a mild expression of MRP2.

The mechanisms through which inflammation prevents the protein expression of MRP2 remain unclear to date especially that this protein seems to be regulated on all levels: transcriptional, translational and post-translational (Zhang et al. 2010). On the transcriptional level, inflammation most probably suppresses MRP2 expression by downregulating its regulatory orphan nuclear receptors, namely PXR, FXR and CAR (Teng and Piquette-Miller, 2008). In particular IL-1β, an upregulated cytokine during LPS-induced endotoxemia, proved to suppress MRP2 expression either by downregulating the retinoic acid receptor α (RARα)/RXRα heterodimer or by inactivating the binding of the interferon regulatory factor (IRF3) to the interferon stimulatory response element (ISRE) on the MRP2 promoter in HepG2 cells (Denson et al. 2002; Denson et al. 2000; Hisaeda et al. 2004). TNF-α most likely alter MRP2 expression by activating NF-κβ, which is known to downregulate the activity of PXR by preventing the PXR-RXR heterodimer from binding to its responsive elements in the promoters of target genes (Gu et al. 2006; Teng and Piquette-Miller, 2008). Particularly, PXR, FXR and CAR proved to be involved in the regulation of MRP2 due to the presence of their binding sites in the promoter region of the *Mrp2* gene (Teng and Piquette-Miller, 2008). PXR elucidated a crucial role in LPS-induced suppression of MRP2 expression in mice; since this suppression was prominently obvious in $PXR^{+/+}$ mice but significantly attenuated in their PXR $^{-/-}$ compatriots (Teng and Piquette-Miller, 2008). Furthermore, hepatocyte enriched transcription factors (1, 3 or 4) whose expression is often altered during inflammation proved to be also involved in the transcriptional regulation of MRP2 (Fardel and Le Vee, 2009). On the post-translational level, LPS is thought to alter the canalicular localization of MRP2 by favoring its endocytic retrieval (membrane internalization) in rats; thus impairing its efflux function (Rost et al.1999). To date the mechanisms underlying the translational regulation of MRP2 remain extremely ambiguous; however most probably they involve the common factors controlling the translation of mRNA in eukaryotic cells, namely microRNA, mRNA binding proteins, translation initiation factors, and intrinsic characteristics of mRNA (5' and 3' untranslated regions (UTRs) (Zhang et al. 2010).

Concerning MDR1, the results attained demonstrate first of all a significant basal expression of MDR1 protein in HepG2 cells; however this expression was significantly reduced after incubating cells with LPS and TNF-α for 24 hours. Similarly verapamil, the standard inhibitor of MDR1 induced a remarkable suppression of MDR1 protein expression, which is consistent with the work published by *Komoto et al.* confirming that not only verapamil but several other Ca^{2+} antagonists probably possess an inhibitory effect on MDR1 expression (Komoto et al. 2007). The

obtained results are consistent with several previous studies that demonstrated the reducing effect of inflammation on the expression of MDR1 mRNA and protein levels. For example *LeVee and Piquette-Miller* demonstrated that TNF-α decreased MDR1 protein expression in HepG2 cells but not in human hepatocytes, in which TNF-α failed to alter MDR1 mRNA and protein expression (Lee and Piquette-Miller, 2003). *Morgan et al.* elucidated a decreased MDR1 protein expression in LPS-treated rodents further confirming the suppressing effect of inflammation on the expression of MDR1 (Morgan et al. 2008). *Fardel and LeVee* demonstrated that in contrast to human hepatocytes and consistent with HepG2 cells, the administration of TNF-α to HepaRG cells induced a significant decrease in MDR1 mRNA levels (Fardel and LeVee, 2009). Regarding the effects of idiosyncratic drugs on the expression of MDR1 proteins in HepG2 cells, the results attained demonstrated that, in the absence of pro-inflammatory mediators, trovafloxacin, telithromycin and nefazodone significantly induce the expression of MDR1 proteins in comparison with their basal expression in untreated cells (Figure 4.5A). Contrarily, nimesulide potently favors the suppression of MDR1 protein expression. However when these drugs were co-administered to cells in addition to LPS and TNF-α, the inducing potential of trovafloxacin, telithromycin and nefazodone was significantly attenuated whereas the reducing potential of nimesulide on MDR1 protein expression was potentiated (Figure 4.5B). These results most probably indicate that inflammation interferes in a preventive manner with the mechanisms through which trovafloxacin, telithromycin and nefazodone modulate the expression of MDR1; thus attenuating their inducing effects. In contrary, inflammation favored the suppressing effect of nimesulide on MDR1 protein expression probably through a synergistic drug-inflammation suppressive mechanism. Predominantly, pro-inflammatory cytokines may regulate the expression of MDR1 either by activating transcriptional pathways such as NF- κβ (nuclear factor-κβ) and NF-IL6 (nuclear factor-IL6), which may interact with their respective binding sites on the promoter sequence of the transporter's gene modulating its expression (Morgan et al. 2008); or by down-regulating the expression of specific orphan nuclear receptors known to regulate the expression of MDR1, such as PXR and CAR (Teng and Piquette-Miller, 2008); or by triggering the activation of intracellular signaling pathways involved in transporter expression such as STAT (signal transducers and activators of transcription), CCAAT enhancer binding protein, AP-1 (activating protein 1), NF-B (nuclear factor-B) and MAPKs (Teng and Miller, 2008; Fardel and LeVee, 2009). Given the fact that the inflammatory context in our experimental conditions is stimulated by the administration of LPS and TNF-α, both of which activate the NF-κβ pathway either indirectly through the prior activation of the TLR pathway or directly through interacting with TNFR-1 (Kaisho et al. 2001); it is highly probable that this pathway is involved in the inflammation-induced down regulation of MDR1. This probability is supported by the fact that LPS and TNF-α-induced activation of NF-κβ inhibits the activity of PXR by preventing the PXR-RXR heterodimer from binding to its responsive elements in the promoters of target genes (Gu et al. 2006; Teng and Piquette-Miller, 2008). Since MDR1 is a known target of PXR, it is highly probable for LPS and TNF-induced NF-κβ-mediated

downregulation of PXR to be the mechanism underlying the reduced expression of MDR1 in the presence of an inflammatory context. It is equally plausible for the four tested idiosyncratic drugs to alter the expression of MDR1 via a PXR-dependent mechanism since increased expression of PXR was frequently correlated with increased MDR1 expression in addition to the fact that several MDR1inducers such as paclitaxel, tamoxifen and ritonavir are also PXR activating ligands (Teng and Piquette-Miller, 2008). Furthermore, the fact that co-administrating LPS and TNF-α in addition to the three idiosyncratic drugs (trovafloxacin, telithromycin and nefazodone) reduced their inducing effect on MDR1 protein expression suggest that the administered pro-inflammatory mediators and these three idiosyncratic drugs interact with the same PXR-mediated regulatory pathway; with TNF and LPS potently downregulating this receptor thus attenuating its responsiveness to trovafloxacin, telithromycin and nefazodone leading to a decrease in their PXR-mediated inducing effect on MDR1 expression. In contrary to the inducing potential of these three idiosyncratic drugs, nimesulide exhibited a reducing effect on MDR1 protein expression which was further potentiated in presence of an inflammatory context suggesting most likely a drug-inflammation synergistic down-regulation of PXR expression.

Overall the results attained in this chapter reveal that inflammation, potently downregulates the expression of MDR1 and completely suppress the expression of MRP2 proteins in HepG2 cells; indicating that an inflammatory reaction is an important predisposing factor for cholestasis. Furthermore, these results suggest that inflammation may potentiate idiosyncratic drug-induced hepatotoxicity by impairing MDR1 and MRP2-mediated drug elimination through altering their protein expression.

This chapter aimed predominantly at elucidating if cholestasis is significantly implicated in the pathogenesis of inflammation associated idiosyncratic drugs and at determining whether MDR1 and MRP2 are involved in the cholestatic potential of these drugs. Particularly, this part of chapter 4 aimed at elucidating if idiosyncratic drug-induced alteration in MDR1 and MRP2 protein expression is correlated with idiosyncratic drug-induced inhibition of their efflux activity.

In chapter 4 Part I (the published article in cytometry part A), which tackled the effect of idiosyncratic drugs on the efflux activity of MDR1 and MRP2, telithromycin and nefazodone significantly inhibited MDR1 whereas nimesulide promoted its efflux activity in the absence of pro-inflammatory stimuli (LPS and TNF-α). Nevertheless, when these drugs were co-administered to cells along with LPS and TNF-α, their inhibitory effects on the MDR1-mediated efflux of rhodamine were attenuated. However these two drugs may still be considered as inhibitors of MDR1 when compared to the negative control (rhodamine-treated cells). Furthermore, the co-administration of the four idiosyncratic drugs along with LPS and TNF-α revealed the inhibitory potential of trovafloxacin on the efflux activity of MDR1.

Concerning the effects of idiosyncratic drugs on the efflux activity of MRP2 the results attained in Chapter 4 Part I demonstrated that none of the idiosyncratic drugs altered the activity of MRP2 in the absence of pro-inflammatory mediators. However, when these drugs were

administered to HepG2 cells along with LPS and TNF-α, each of trovafloxacin, nimesulide, and nefazodone revealed an inhibitory potential on the efflux activity of MRP2. By comparing the results of part I (presented in the published Cytometry part A article) and part II of Chapter 4 concerning MDR1, it may be concluded that in the absence of pro-inflammatory stimuli: trovafloxacin potently induces MDR1 expression while slightly inhibiting its efflux activity; nimesulide exhibits a reducing effect on MDR1 protein expression while favoring its efflux activity whereas telithromycin and nefazodone are at a time inducers of MDR1 protein expression and inhibitors of its efflux activity. However, when these drugs are co-administered to cells in addition to LPS and TNF-α, the expression of MDR1 is reduced with all of them when compared to the negative control (DMSO-treated cells). However, an overall increased efflux activity is observed upon co-incubating HepG2 cells with the four idiosyncratic drugs along with LPS and TNF-α as demonstrated by the decrease in the accumulated intracellular fluorescence of rhodamine 123 (please refer to figure 1B in the Cytometry part A article). Moreover, the inhibiting effects of telithromycin and nefazodone and even of verapamil, the standard MDR1 inhibitor, on the MDR1-mediated efflux of rhodamine were attenuated; nevertheless these drugs are still considered as inhibitors when compared to the negative control (rhodamine-treated cells). Regarding MRP2, it may be concluded that trovafloxacin and nefazodone are inducers of its expression with the former being an inhibitor and the latter being an inducer of MRP2 efflux activity in the absence of pro-inflammatory mediators. Concerning the expression and activity of MRP2 in HepG2 cells co-exposed to idiosyncratic drugs and pro-inflammatory stimuli, the results attained imply that the synergistic presence of LPS and TNF in addition to trovafloxacin, nimesulide, and nefazodone reduces significantly the expression of MRP2 while potently inhibiting its efflux activity.

Finally correlating the effects of idiosyncratic drugs on the expression of MDR1 and MRP2 with their effects on the functionality of these transporters indicated that a drug inducing the expression of a certain transporter is not obligatory an inducer of its activity and may be even an inhibitor. Similarly it is not astonishing for a certain drug to suppress the expression of a transporter while promoting its efflux activity. The regulation of expression and activity may occur via different unrelated pathways whereby the alteration of one does not necessarily affect the other. With regard to the implication of MDR1 and MRP2 in inflammation associated idiosyncratic hepatotoxicity, it may be deduced that MRP2 is more implicated than MDR1 in this type of toxicity; predominantly because the expression and activity of MRP2 was drastically suppressed in the co-presence of idiosyncratic drugs and pro-inflammatory mediators while MDR1 remained more or less functional. These results suggest that cholestasis is indeed one of the main mechanisms underlying the inflammation associated hepatotoxic potential of the four tested idiosyncratic drugs. Particularly, trovafloxacin and nefazodone may promote idiosyncratic hepatotoxicity via a cholestatic mechanism that involves the downregulation of both MDR1 and MRP2; whereas telithromycin and nimesulide specifically involve the downregulation of MDR1 and MRP2, respectively, in their cholestatic potential.

Chapter 5

Investigation of the molecular mechanisms underlying drug-induced hepatocellular death in inflammation associated idiosyncratic hepatotoxicity

5.1 Introduction

Drug-induced liver injury (DILI) is considered as the main cause of acute hepatic failure and liver transplantation in western countries (Grattagliano et al. 2009). The majority of the cases of post-marketing liver injury have been correlated with idiosyncratic drugs since the development of intrinsically toxic drugs is normally stopped before the latter reach the market (Grattagliano et al. 2009). This is mainly correlated with the unpredictable dose-independent nature of idiosyncratic hepatotoxicity which impeded the development of efficient predictive models and complicated the understanding of the toxic mechanisms underlying idiosyncratic drug-induced liver injury (IDILI) (Cosgrove at al. 2009). The spectrum of injury of hepatotoxic drugs is very wide, extending from mild damage to massive hepatic failure and including in between drug-induced cholestasis, hepatitis, steatosis, fibrosis and cirrhosis. Regardless of the clinical manifestation of drug-induced liver injury, acute liver failure predominantly occurs when the extent of hepatocellular death exceeds hepatic regenerative capacity (Bantel and Schulze-Osthoff, 2012). Hence the identification of the mechanisms leading to hepatocellular death may provide valuable insights on the prevention of drug-induced liver failure. It is widely acknowledged that hepatocellular death may proceed via several pathways including: apoptosis, necroptosis, necrosis and autophagic cell death (Malhi et al. 2010). The relative contribution of apoptosis or necrosis to hepatic failure remains controversial to date (Schulze-Osthoff and Bantel, 2011). Apoptosis represents an ATP-dependent cell death process whereas necrosis is typically considered as the consequence of acute metabolic perturbation occurring during ATP depletion (Bantel and Schulze-Osthoff, 2012). Nevertheless, the distinction between apoptosis and necrosis is not always obvious despite the morphological and biochemical alterations differentiating the two processes. In several cases, the same stimulus may induce both apoptosis and necrosis depending on the dose and duration of exposure; therefore, in many situations a dying cell will not follow a clear-cut form of cell death, but rather a mixed form exhibiting features of both apoptosis and necrosis (Bantel and Schulze-Osthoff, 2012). This chapter will focus predominantly on drug-induced apoptosis.

5.1.1 Pathologic apoptosis

Under normal physiological conditions apoptosis is a programmed form of cell death that plays a crucial role in the preservation of tissue homeostasis by counter-balancing cell proliferation and eliminating damaged, infected, or transformed cells (Bantel and Schulze-Osthoff, 2012). However, during hepatic disease or injury excessive apoptosis may eventually lead to tissue destruction and organ failure (Bantel and Schulze-Osthoff, 2012). Hepatocellular apoptosis is known to be the first cellular response to a wide variety of toxic stimuli, including drugs, and a frequent companion to several hepatic diseases such as viral hepatitis, alcohol-induced liver disease, nonalcoholic fatty liver disease, cholestatic liver diseases, and ischemia/reperfusion injury (Canbay et al. 2004). Particularly, patients suffering from alcoholic hepatitis and nonalcoholic steatohepatitis exhibit increasing apoptotic features, which are often causatively correlated with disease severity and hepatic failure (Natori et al. 2001; Feldstein et al. 2003).

Inspite of the pervasive concurrence of apoptotic features in hepatic damage, the correlation between apoptosis and hepatic inflammation has not been thoroughly explored probably because of the prevailing dogma stating that apoptosis is an "innocuous" form of cell death (Canbay et al. 2004). This presumption is held true when investigating physiologic apoptosis under healthy conditions in which it is tightly restricted to discrete subsets of cells both spatially and temporally; however during hepatic diseases or injury "pathologic apoptosis" is non-selective, affects a wide variety of cells and may be sustained for a long period of time causing serious hepatic damage (Canbay et al. 2004). Pathologic hepatocellular apoptosis may result following the exposure of the liver to various stress stimuli such as hepatotoxic drugs or inflammation acting in several times as an enhancer of the initial hepatic damage caused by these stimuli (Guicciardi and Gores, 2005). Predominantly, a toxic drug may induce hepatocellular apoptosis by itself or by the intermediary of its reactive metabolites either through causing mitochondrial dysfunction and hence activating the intrinsic apoptotic pathway or through directly triggering the activation of the death receptor pathway (Extrinsic pathway) (Holt and Ju, 2006). It is important to note that a toxic drug, depending on severity and exposure, may either cause hepatocellular apoptosis directly or rather induce distinct forms of hepatic damage such as oxidative stress, steatosis or cholestasis leading eventually to hepatocellular death (Russman et al. 2009). In all cases hepatocellular apoptosis will proceed either via the intrinsic pathway or via the extrinsic pathway. However these pathways are not mutually exclusive, as in some cells such as hepatocytes and cholangiocytes, the execution of apoptosis requires the concurrent activation of both pathways (Bantel and Schulze-Osthoff, 2012).

5.1.2 Intrinsic pathway of apoptosis (Mitochondrial pathway)

A hepatotoxic drug or its reactive metabolites commonly activate the intrinsic apoptotic pathway directly by causing mitochondrial dysfunction through several mechanisms including: mitochondrial respiratory chain inhibition leading to ATP depletion and elevated concentrations of reactive oxygen species (ROS), β-oxidation inhibition leading to steatosis, mitochondrial DNA damage and mitochondrial permeability transition (MPT), i.e. opening of the "MPT pore" located in their inner membrane (Russman et al. 2009). Nevertheless a hepatotoxic drug may also cause intrinsic apoptosis indirectly by inducing MPT through either activating lysosomal permeabilization, or the endoplasmic reticulum pathway, or c-jun N-terminal kinase (JNK) (Gunawan et al. 2006; Schwabe et al. 2006); which in their turn will favor the activation of pro-apoptotic (e.g. Bax, Bak, Bad) and the inhibition of anti-apoptotic (e.g. Bcl-2, Bcl-xL) proteins (Russman et al. 2009).The mitochondrial pathway of apoptosis is predominantly regulated by pro-apoptotic members of the Bcl-2 protein family which cause MPT resulting in the release of several apoptotic activators into the cytosol namely cytochrome *c*, second activator of mitochondrial apoptosis (SMAC), endonuclease G, high temperature requirement A2 (HrtA2), and apoptosis-inducing factor (AIF) (Danial and Korsmeyer, 2004; Rasola and Bernardi, 2007). The released cytochrome *c* from the mitochondrial intramembrane space into the cytosol forms in addition to pro-caspase 9 and apoptosis activating factor-1 (APAF-1) what is known as "the

apoptosome" (Fulda and Debatin, 2006). The latter subsequently activates caspase 9, which will trigger the activation of effector caspases 3, 7, and 6 leading to hepatocellular apoptosis (Russman et al. 2009). The Bcl-2 family includes both pro- and anti-apoptotic proteins, which are divided into three subsets: 1) the anti-apoptotic members which contain BH domains like Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1; *2*) the proapoptotic multi-domain effector proteins Bax, Bak, and Bok; and *3*) the BH3-only pro-apoptotic members Bid, Bim, Bad, Bik, Bmf, Hrk, Noxa, and Puma, which function as initial sensors of distinct apoptotic signals (Bouillet and Strasser, 2002). A highly important subset of the Bcl-2 family genes, which encompasses *Bax*, *Noxa*, *PUMA* and *Bid* proved to be p53 targets; implying that p53 contributes greatly to the regulation of intrinsic apoptosis (Haupt et al. 2003). Generally, the tumor suppressor gene p53 exhibits a primordial role in the regulation of cellular stress response, through the activation of genes involved in cell cycle control, DNA repair, and apoptosis (Amundson et al. 1998). Particularly in apoptosis, p53 is involved in apoptosome formation by favoring the release of cytochrome c through triggering the induction of target genes encoding BH3-only proteins and by enhancing the transcriptional expression of APAF-1(Haupt et al. 2003). Moreover, p53 can trigger the activation of caspase 8 and caspase 6 in a transcription-independent and transcription dependent manner respectively (Haupt et al. 2003); or it may directly cause mitochondrial damage by translocating to the mitochondria and causing mitochondrial membrane permeabilization (Figure 5.1) (Mihara et al. 2003).

5.1.3 Extrinsic pathway of apoptosis (death receptors pathway)

A toxic drug predominantly induces the extrinsic apoptotic pathway by prior activation of the immune system based on the hapten hypothesis (Holt and Ju, 2006). Briefly, the hapten hypothesis states that a drug or its reactive metabolites will covalently bind to cellular proteins forming "foreign" antigens, which will be expressed on the surface of antigen presenting cells where they will initiate an acquired immunological response (Shaw et al. 2010). However, it has been argued that the hapten hypothesis is insufficient to cause hepatic injury and that for the latter to occur a secondary stress signal is required for the activation of antigen presenting cells and the concurrent induction of the innate immune system (Uetrecht, 2008). *Kaplowitz* argued that a concomitant episode of inflammation occurring during drug therapy may act as the required secondary stress signal; which will activate the innate immune reaction further amplifying drug-induced activation of the acquired immune system leading to liver injury (Kaplowitz, 2005). Liver injury is often caused by increased hepatocellular death, which may be mediated through the extrinsic apoptotic pathway as follows: The simultaneous presence of a hepatotoxic drug and an inflammatory stress will lead to the concurrent activation of both the acquired and the innate immune systems leading to an extensive production and release of pro-inflammatory mediators, which will subsequently bind to their respective cell death receptors on the surface of hepatocytes (Russman et al. 2009). Hepatocytes are known to over express several death receptors such as TNF-R1, Fas and TRAIL-R1/2, which predisposes them to the lethal effects of TNF-α, Fas-L (Fas ligand) and TRAIL (Canbay et al. 2005). Following ligand

engagement, the corresponding receptors will undergo oligomerization, triggering the activation of intracellular death signaling pathways (Malhi et al. 2006). TNFR1 is distinct from Fas and TRAIL receptors in that activated TNFR1 initially activates NF-κβ and c-jun N-terminal kinase (JNK) pathways and then upon internalization activates the apoptotic cascade (Barnhart and Peter, 2003). This dual activity of TNFR1 explains the functional duplicity of TNF-α, which may either favor cell survival and proliferation or promote apoptosis depending on the cell type and context (Schwabe and Brenner, 2006). Particularly, TNF-α favors cell survival and proliferation by activating NF-κβ, p38 and c-Jun N-terminal kinase (JNK) pathways through binding to TNF-R1 on the surface of hepatocytes. Subsequently, TNFR1 will undergo a conformational change that allows it to recruit TNF-R-associated death domain (TRADD), TNF-R associated factor 2 (TRAF-2), and receptor interacting protein 1 (RIP-1) forming complex I. Complex I will then mediate the activation of these pathways (Figure 5.2) (Schwabe and Brenner, 2006). In contrary, in specific contexts, TNF-α may promote apoptosis rather than survival, by interacting with TNFR1 and inducing the recruitment of the adapter protein FADD, which will then recruit pro-caspase 8 by means of its dead effector domain in order to constitute the death-inducing signaling complex (DISC) (Wullaert et al. 2007). The clustering of pro-caspase 8 initiates the autoactivation of DISC leading to the proteolitic activation of several effector caspases (caspase 3, 6 and 7), which are responsible for the main destructive cellular events that result in apoptosis (Wullaert et al. 2007). However, in hepatocytes and other type II cells, the activation of caspase 8 is insufficient to induce apoptosis; these types of cells often require the activation of the mitochondrial pathway along with the death receptor pathway to respond to an apoptotic signal (Wullaert et al. 2007). TNF-α may induce the activation of the mitochondrial pathway by activating caspase 8, which will cleave the proapoptotic protein Bid yielding truncated Bid (tBid) (Bantel and Schulze-Osthoff, 2012). Truncated Bid will then translocate to the mitochondria where it will induce in concert with Bax and Bak the permeabilization of the outer mitochondrial membrane leading to the release of cytochrome c into the cytosol and the formation of the apoptosome, which will eventually trigger the activation of the intrinsic apoptotic pathway (Malhi et al. 2010). Similar to the intrinsic pathway, the extrinsic pathway may be also regulated by the tumor suppressor gene p53 (Haupt et al. 2003). However, p53-mediated regulation of extrinsic apoptosis is highly dependent on the cell type and comprises the induction of three transmembrane proteins namely Fas, DR5 and PERP (Haupt et al. 2003). For example p53-mediated induction of *Fas* takes place in the spleen, kidney and lung but not in the heart and the liver (Figure 5.1) (Haupt et al. 2003).

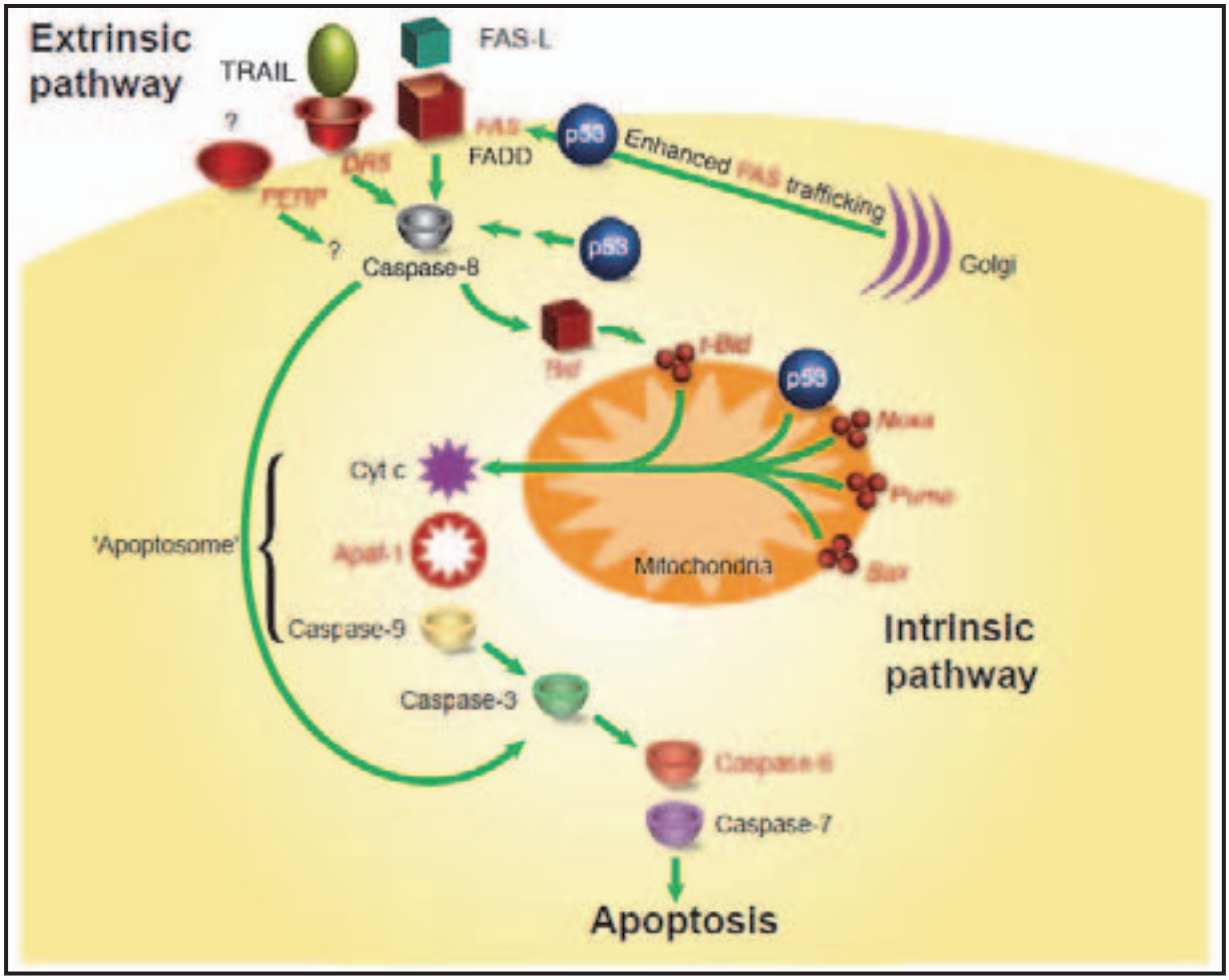


Figure 5.1. Involvement of p53 in the extrinsic and intrinsic apoptotic pathways.
P53 promotes intrinsic apoptosis by upregulating the expression of Bid, Noxa, Puma, Bax, Apaf-1 and caspase 6. P53 may also promote extrinsic apoptosis by favoring the transcription of PERP, DR5 and FAS. P53 target genes are shown in red. (*Adapted from Haupt et al. 2003).*

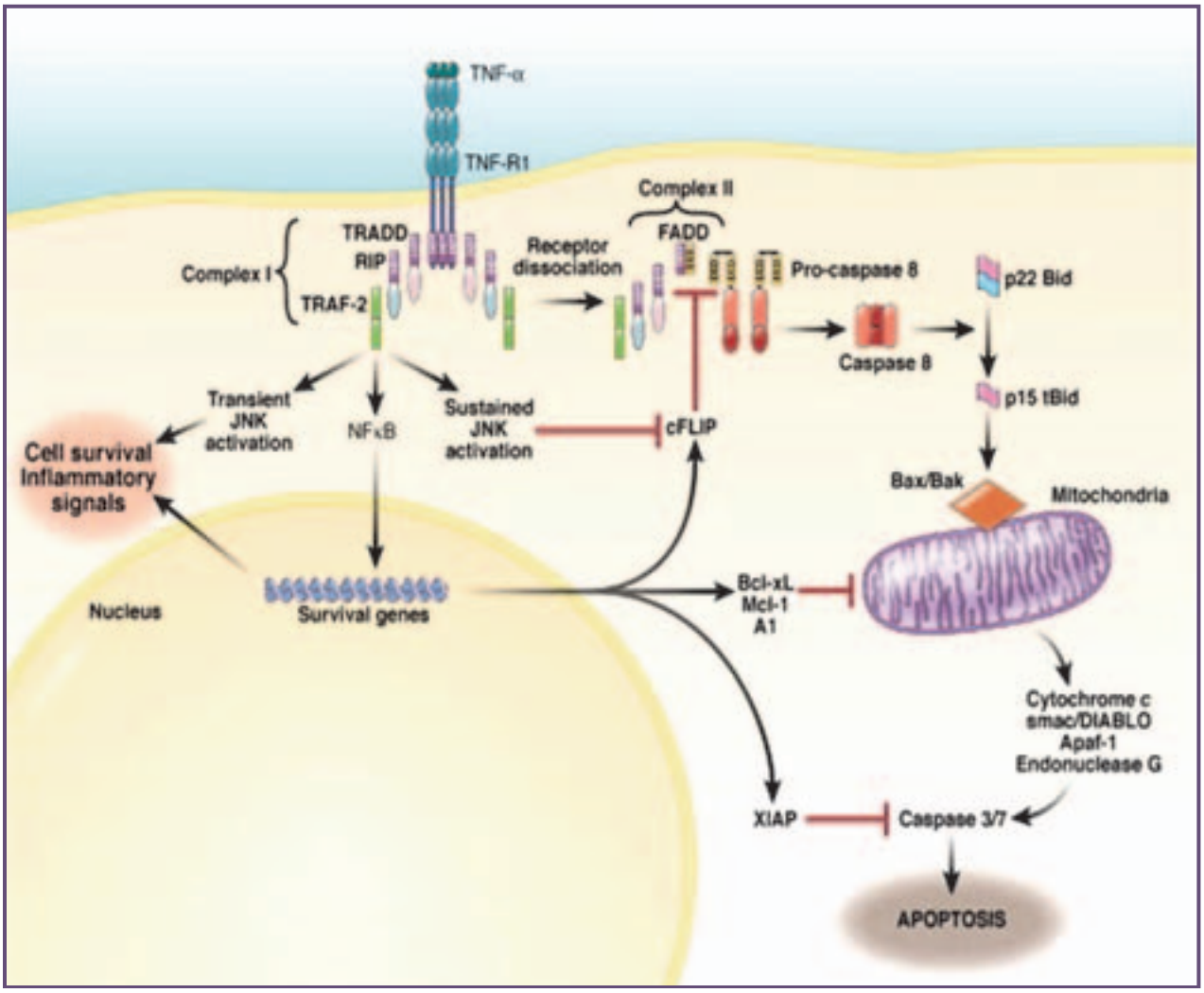


Figure 5.2. TNF-α signaling. TNF-α binds to its receptor, TNFR1 inducing a conformational change and favoring the recruitment of TNFR1-associated death domain protein (TRADD), receptor-interacting kinase (RIP) and TNF receptor–associated factor (TRAF2). This signal transduction complex is referred to as complex 1. The latter activates Nuclear factor κB (NF-κB) in addition to transient and prolonged JNK activation. Active NF-κB translocates to the nucleus, leading to the transcription of antiapoptotic genes like cFLIP (FLICE-like inhibitory protein), Bcl-xL, Mcl-1, A1, and XIAP, which regulate apoptosis at multiple levels. TRADD, receptor interacting protein, and TRAF2 then undergo receptor dissociation and recruit FADD. FADD contains a death effector domain (DED) that leads to activation of caspase 8, that will subsequently cleave Bid to tBid. tBid will then translocate to the mitochondria and activate Bax and Bak to trigger the release of proapoptotic factors resulting in apoptosis. *(Adapted from Malhi and Gores, 2008)*.

5.1.4 Correlation between hepatocellular apoptosis and inflammation

In opposition with the widely acknowledged fact linking inflammation to necrosis rather than to apoptosis several mechanisms demonstrated that inflammation is tightly correlated with pathological apoptosis (Canbay et al. 2004). Under pathological conditions, when the intensity of apoptosis overcomes the capacity of the liver to eliminate cellular debris, apoptotic bodies undergo spontaneous rupture and release their contents causing massive necrosis and

inflammation; frequently leading to the disruption of hepatocyte integrity (Patel et al. 1998). Furthermore death receptor-mediated apoptosis may contribute to hepatic inflammation, possibly by initiating inflammatory signaling cascades (Maher et al. 1997; Canbay et al. 2003). Particularly, Fas agonists induce the expression of various chemokines (e.g., macrophage inflammatory protein-2, CXC ligand-1), which in their turn will promote hepatic neutrophil infiltration, further exacerbating the inflammatory reaction (Faouzi et al. 2001). In consistency with this concept, *Jaeschke et al.* demonstrated that hepatocellular apoptosis potently stimulate neutrophil extravasation and promote endotoxin-induced liver injury (Lawson et al. 1998; Jaeschke et al. 2002). Moreover, TNF-α which is predominantly engaged in extrinsic apoptosis through the activation of TNFR1 also activates several intracellular signaling pathways such as NF-κβ and MAPK, particularly the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38-MAP kinase (Lu and Xu, 2006). The potent activation of these pathways may thus lead to the propagation of an injurious inflammatory signal, in addition to the apoptotic signal, which may amplify hepatocellular death in case prolonged (Schwabe and Brenner, 2006). Although the ERK pathway was attributed to survival in many cell types, significant evidence emerged in the last decade to prove its involvement in the mediation of apoptosis at the intrinsic as well as at the extrinsic level (Zhuang and Schnellmann, 2006) (Figure 5.3). ERK ½ is thought to regulate intrinsic apoptosis by up regulating the expression and activity of p53 and Bax; thus favoring cytochrome *c* release and caspase-3 activation (Zhuang and Schnellmann, 2006). Furthermore, ERK ½ proved to be also implicated in the induction of extrinsic apoptosis by upregulating the production of TNF-α and caspase 8 while favoring the suppression of important survival pathways such as the phosphatidylinositol 3-kinase/Akt pathway (Zhuang and Schnellmann, 2006). Finally, the disposition of apoptotic bodies may also correlate apoptosis to hepatic inflammation; for example, engulfment of hepatocyte-released apoptotic bodies by macrophages and/or Kupffer cells will trigger the latter to induce the expression of death ligands, especially Fas and TNF-α thereby amplifying apoptosis (Canbay et al. 2004).

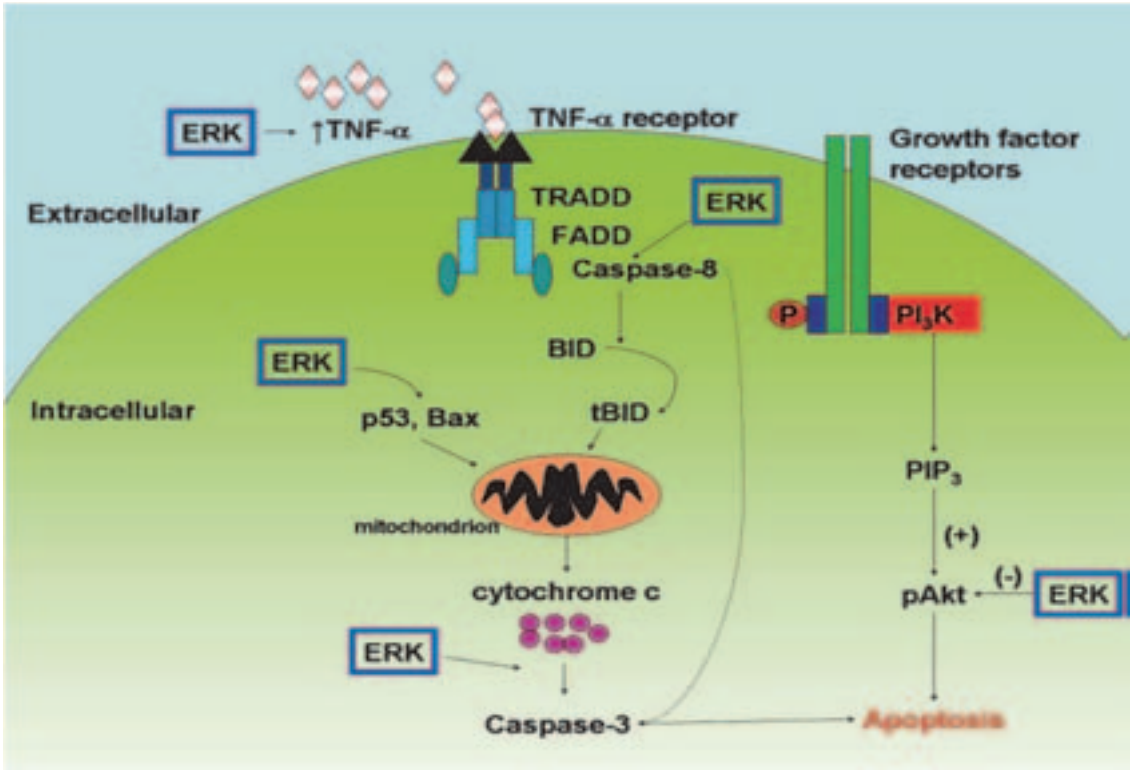


Figure 5.3. Mechanisms of ERK-mediated apoptosis. ERK may regulate apoptosis at multiple stages by upregulating p53 and BAX action, increasing caspase-3 and caspase-8 activities, decreasing Akt activity and increasing TNF-α production. *(Adapted from Zhuang and Schnellmann, 2006).*

5.1.5 Aim of chapter 5

This chapter aims at elucidating the molecular mechanisms through which pro-inflammatory mediators synergize with an idiosyncratic drug to cause hepatocellular death. Particularly, the effects of four known idiosyncratic drugs namely trovafloxacin, nimesulide, telithromycin and nefazodone on the expression of apoptotic mediators, belonging to both the intrinsic and the extrinsic pathways, were evaluated in presence and absence of pro-inflammatory mediators; in order to determine whether inflammation associated idiosyncratic drug-induced hepatocellular death is mediated by the mitochondrial pathway or rather by the death receptors pathway, or possibly by both pathways. Accordingly, we have tested the effect of the four idiosyncratic drugs on the expression and activity of specific proteins and enzymes, that are known to be significantly involved in the regulation of apoptosis and cellular growth namely: (1) p53 which is the central upstream regulator of intrinsic and extrinsic apoptosis during stress signals, (2) Bax, which is an important pro-apoptotic protein essential for the execution of intrinsic apoptosis, (3) Caspase 8 which is the initiator enzyme of the extrinsic apoptotic pathway, (4) (tBid) which is an important apoptotic protein that mediate the convergence of both apoptotic pathways, (5) p21 which is predominantly involved in cell growth arrest in response to DNA damage or cellular stress, (6) and finally pERK½, which is a member of the MAPK family, involved in the

regulation of intrinsic and extrinsic apoptosis at different levels and known to be activated by pro-inflammatory cytokines such as TNF-α.

5.2 Experimental workflow

5.2.1 Cell culture and treatment

HepG2 cells were seeded in 6-well plates (2mL/well) (western blot analysis) or 96-well plates (200µL/well) (capillary flow cytometric analysis) until confluent. Then cells were incubated with four known idiosyncratic drugs namely trovafloxacin (450 µM), nimesulide (450 µM), telithromycin (175 µM) and nefazodone (70 µM),) in the absence and presence of LPS and TNF-α for 24 hours. Prior to western blot analysis, cells were harvested and cellular proteins were extracted according to the protocol detailed in chapter 2.

5.2.2 Western blot analysis of p53, p21, tBid and pERK ½ protein expression

HepG2 protein extracts (20µg) were mixed with laemmli loading buffer, denatured at 95°C for 5 minutes, separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were then blocked in 5% milk for 2 hours at room temperature. Subsequently, the membranes were incubated overnight at 4°C with the following primary antibodies: mouse monoclonal anti-p53, rabbit polyclonal anti-p21, mouse monoclonal anti-pERK1/2, and rabbit polyclonal anti-tBid. All the primary antibodies were used at a dilution of 1/500 and purchased from Santa Cruz biotechnology with the exception of anti-tBid which was used at a dilution of 1/300 and bought from Enogene. Following extensive washing steps the membranes were incubated for 1 hour at room temperature with HRP-conjugated anti-rabbit (Cell Signaling) and anti-mouse secondary antibodies (Cell Signaling) at a dilution of 1/2000. Immunodetection was performed by chemiluminescence using Amersham ECL Western Blotting Detection Reagents (GE Healthcare).

5.2.3 Capillary flow cytometric analysis of Bax protein expression

Bax protein expression was analyzed by capillary flow cytometry following the fixation and permeabilization of HepG2 cells by BD Cytofix/Cytoperm kit according to manufacturer's protocol. Briefly HepG2 cells were seeded on a 96-well plate and incubated with the four idiosyncratic drugs in presence and absence of LPS and TNF-α for 24 hours. Subsequently cells were incubated with BD Cytofix/Cytoperm solution (100µL/well) for 20 minutes at 4°C. Then the wells were washed with BD Perm/Wash buffer (250µL/well) and incubated with 50 µL BD Perm/Wash buffer containing FITC conjugated anti-bax (Abcam) at a dilution of 1/50 for 30 minutes at 4°C. Subsequently cells were washed two times with BD Perm/ Wash buffer (250µL/well) and re-suspended in staining buffer before analysis by Guava EasyCyte Plus capillary flow cytometer (Merck Millipore, Life Science division, Merck KgaA, Darmstadt, Germany) equipped with two light scatter detectors that measure the forward scatter (an estimation of cell size) and the side scatter (an estimation of intracellular granularity); in addition

to a 488nm excitation laser and four emission band pass filters at 530/40, 585/42, 675/30 and 780/30. The green emitted fluorescence at 530 nm is directly proportional to the quantity of intracellular Bax proteins. Results were computed using the Guava ExpressPro software (Merck/Millipore/Guava Tech) in terms of fluorescent cells percentage (%).

5.2.4 Capillary flow cytometric analysis of caspase 8 activation

The detection of apoptotic cells dying via the extrinsic or death receptors pathway was performed using Guava Technologies Caspase 8 kit according to manufacturer's protocol. Briefly, HepG2 cells were seeded at a concentration of $1x10^5$ cells/mL (100µL/well) in a 96-well plate and incubated for 24 hours with the four idiosyncratic drugs in presence and absence of the inflammatory mix (LPS and TNF-α). Subsequently, cells were incubated with Caspase 8 reagent (10 µL/well) for 1 hour at 37°C. Then cells were washed two times with apoptosis wash buffer before being incubated with 7-AAD working solution (200 µL/well) for 10 minutes at room temperature. Cells were then analyzed by Guava EasyCyte Plus capillary flow cytometer (Merck Millipore, Life Science division, Merck KgaA, Darmstadt, Germany) equipped with two light scatter detectors that measure the forward scatter (an estimation of cell size) and the side scatter (an estimation of intracellular granularity); in addition to a 488nm excitation laser and four emission band pass filters at 530/40, 585/42, 675/30 and 780/30. The green emitted fluorescence at 530 nm corresponds to cells in the early stage of caspase-8 dependent apoptosis. The red emitted fluorescence at 670 nm corresponds to dead cells that have lost their membrane integrity. The simultaneous emission of green and red fluorescence corresponds to dead cells that have died via a caspase-8 dependent mechanism. Results were computed using the Guava ExpressPro software (Merck/Millipore/Guava Tech) in terms of x-geometric mean arbitrary units (AU).

5.3 Results

5.3.1 Effect of idiosyncratic drugs on p53 protein expression

Immunodetection of p53 protein expression demonstrated first of all that untreated HepG2 cells basally express significant amounts of p53 proteins. Upon incubation of cells, for 24 hours, with the four tested idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone), the results attained demonstrated that trovafloxacin induce significantly the expression of p53 proteins whereas nimesulide, telithromycin and nefazodone reduce it when compared to the negative control (DMSO-treated cells) (Figure 5.4A). However, when these drugs were co-administered to HepG2 cells along with LPS and TNF-α, the inducing effect of trovafloxacin on the expression of p53 proteins was attenuated whereas the reducing effects of telithromycin and nefazodone were greatly enhanced. The reducing effect of nimesulide on the expression of p53 was rather unaffected by the presence of LPS and TNF-α (Figure 5.4B).

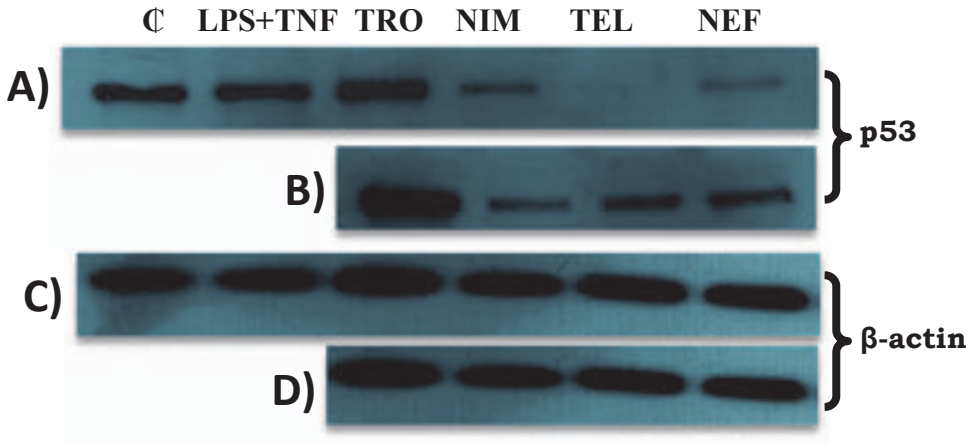


Figure 5.4(A). Effect of idiosyncratic drugs on the expression of p53 proteins in HepG2 cells in absence and presence of pro-inflammatory stimuli. Immunoblot analysis of p53 protein expression was performed following 24 hours incubation of HepG2 cells with the four idiosyncratic drugs in presence (Lane A) and absence (Lane B) of LPS and TNF-α. Lanes C and D demonstrate the expression of β-actin corresponding to every loaded sample (internal control). (₵: negative control; TRO: trovafloxacin, NIM: nimesulide; TEL: telithromycin; NEF: nefazodone).

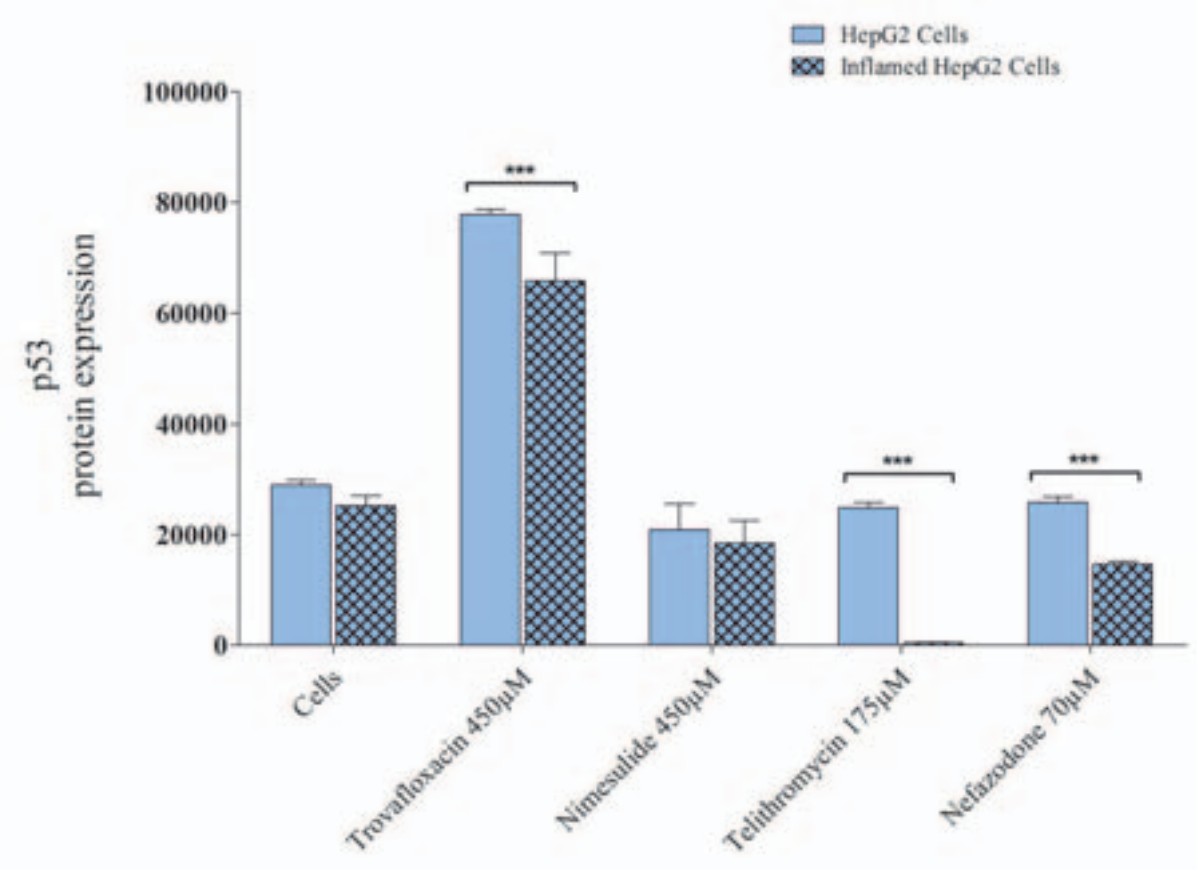


Figure 5.4(B). Quantification of p53 protein expression by Image J in non-inflamed and inflamed HepG2 cells. After incubating HepG2 cells with the four idiosyncratic drugs in absence and presence of LPS and TNF-α, the quantification of p53 protein expression was accomplished using Image J. Bars represent the densitometric analysis of p53 protein expression. Data is represented as Mean ± S.E.M (n=3). Statistical analysis was performed using two way ANOVA followed by Bonferroni post test. *** represents $P < 0.001$ and refers to the variation in p53 protein expression between non-inflamed and inflamed HepG2 cells. The results attained indicate that the inducing effect of trovafloxacin on p53 protein expression is attenuated within an inflammatory context and that telithromycin and nefazodone reduce the expression of p53 proteins when co-administered along with LPS and TNF-α in a much more significant manner than when administered alone to cells.

5.3.2 Effect of idiosyncratic drugs on Bax protein expression

After incubating HepG2 cells for 24 hours with trovafloxacin, nimesulide, telithromycin and nefazodone in the absence and presence of pro-inflammatory mediators, the intracellular expression of Bax proteins was assessed by capillary flow cytometry using an FITC-conjugated anti-Bax. The results attained demonstrated that the four idiosyncratic drugs induce the expression of Bax in the absence of an inflammatory context when compared to its basal expression in untreated cells. Nevertheless, the inducing effect of the four drugs was significantly potentiated following their co-administration to HepG2 cells along with LPS and TNF-α (Figure 5.5). These results elucidated that the presence of pro-inflammatory mediators promote the drug-induced up-regulation of Bax protein expression.

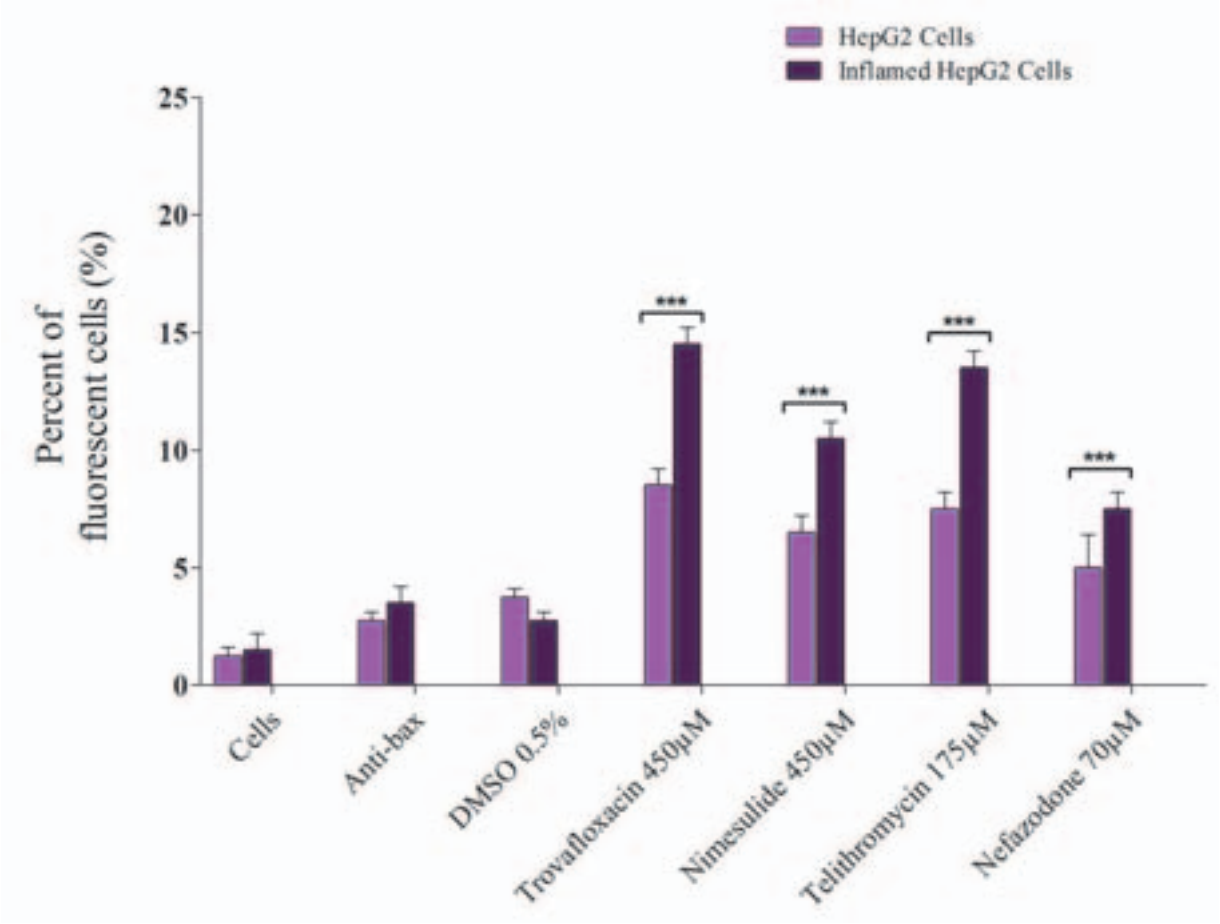


Figure 5.5. Effect of idiosyncratic drugs on the expression of Bax proteins in HepG2 cells in absence and presence of pro-inflammatory stimuli. After incubating HepG2 cells for 24 hours with the four idiosyncratic drugs in absence and presence of LPS and TNF-α, the expression of the pro-apoptotic protein Bax was assessed by capillary flow cytometry using an FITC-conjugated anti-Bax, which emits a green fluorescence upon binding to Bax proteins. Bars represent the emitted fluorescence which is proportional to the quantity of Bax proteins present in the cells. Data is represented as Mean ± S.E.M (n=3). Statistical analysis was performed using two way ANOVA followed by Bonferroni post test. *** represent $P < 0.001$ and refer to the variation in the expression of Bax proteins between non-inflamed and inflamed HepG2 cells. The attained results demonstrate that the presence of pro-inflammatory mediators favors idiosyncratic drug-induced expression of Bax proteins.

5.3.3 Effect of idiosyncratic drugs on p21 protein expression

After incubating HepG2 cells with the four tested idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) in presence and absence of pro-inflammatory stimuli, the results attained demonstrated first of all that p21 is not basally expressed in HepG2 cells. Furthermore, none of the idiosyncratic drugs induced its expression in a significant manner when administered alone to cells. However, when these drugs were co-administered along with LPS and TNF-α, telithromycin potently induced the expression of p21 proteins (Figure 5.6).

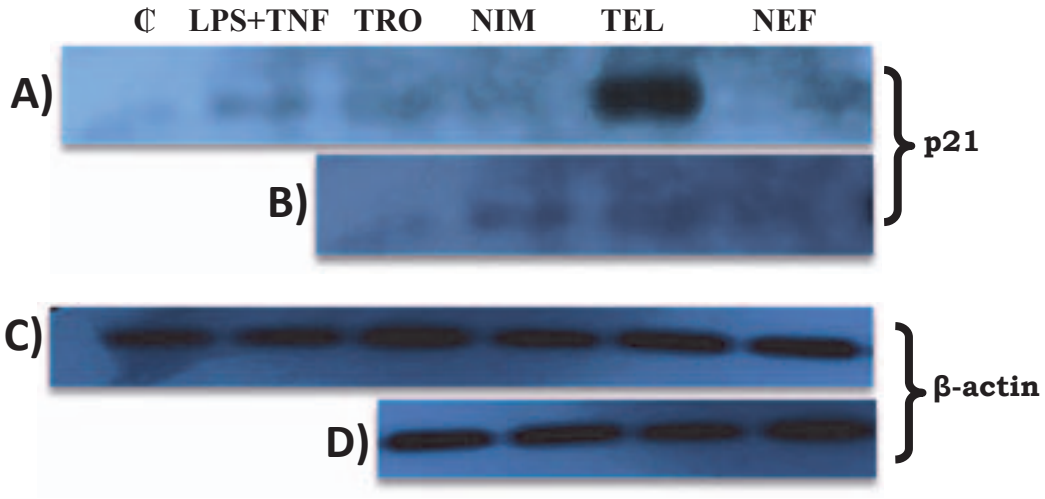


Figure 5.6. Effect of idiosyncratic drugs on the expression of p21 proteins in HepG2 cells in absence and presence of pro-inflammatory stimuli. Immunoblot analysis of p21 protein expression was performed following 24 hours incubation of HepG2 cells with the four idiosyncratic drugs in presence (Lane A) and absence (Lane B) of LPS and TNF-α. Lanes C and D demonstrate the expression of β-actin corresponding to every loaded sample (internal control).The attained results demonstrate that amongst the four tested idiosyncratic drugs only telithromycin was able to significantly induce the expression of p21 proteins when co-administered to cells along with LPS and TNF-α (₵: negative control; TRO: trovafloxacin, NIM: nimesulide; TEL: telithromycin; NEF: nefazodone).

5.3.4 Effect of idiosyncratic drugs on caspase 8 activation

After incubating HepG2 cells with the four tested idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) in presence and absence of LPS and TNF-α, the intracellular presence of active caspase 8 was assessed by capillary flow cytometry in the aim of identifying the cells dying by extrinsic apoptosis. The attained results demonstrated that nimesulide, telithromycin and nefazodone induce the activation of caspase 8 enzymes when administered alone to cells. The inducing effect of these drugs was significantly enhanced when they were co-administered to cells along with LPS and TNF-α (Figure 5.7). Contrarily, trovafloxacin did not modify the activity of caspase 8 neither in the absence nor in the presence of pro-inflammatory mediators. These results indicate that nimesulide, telithromycin and nefazodone induce apoptosis via the extrinsic pathway.

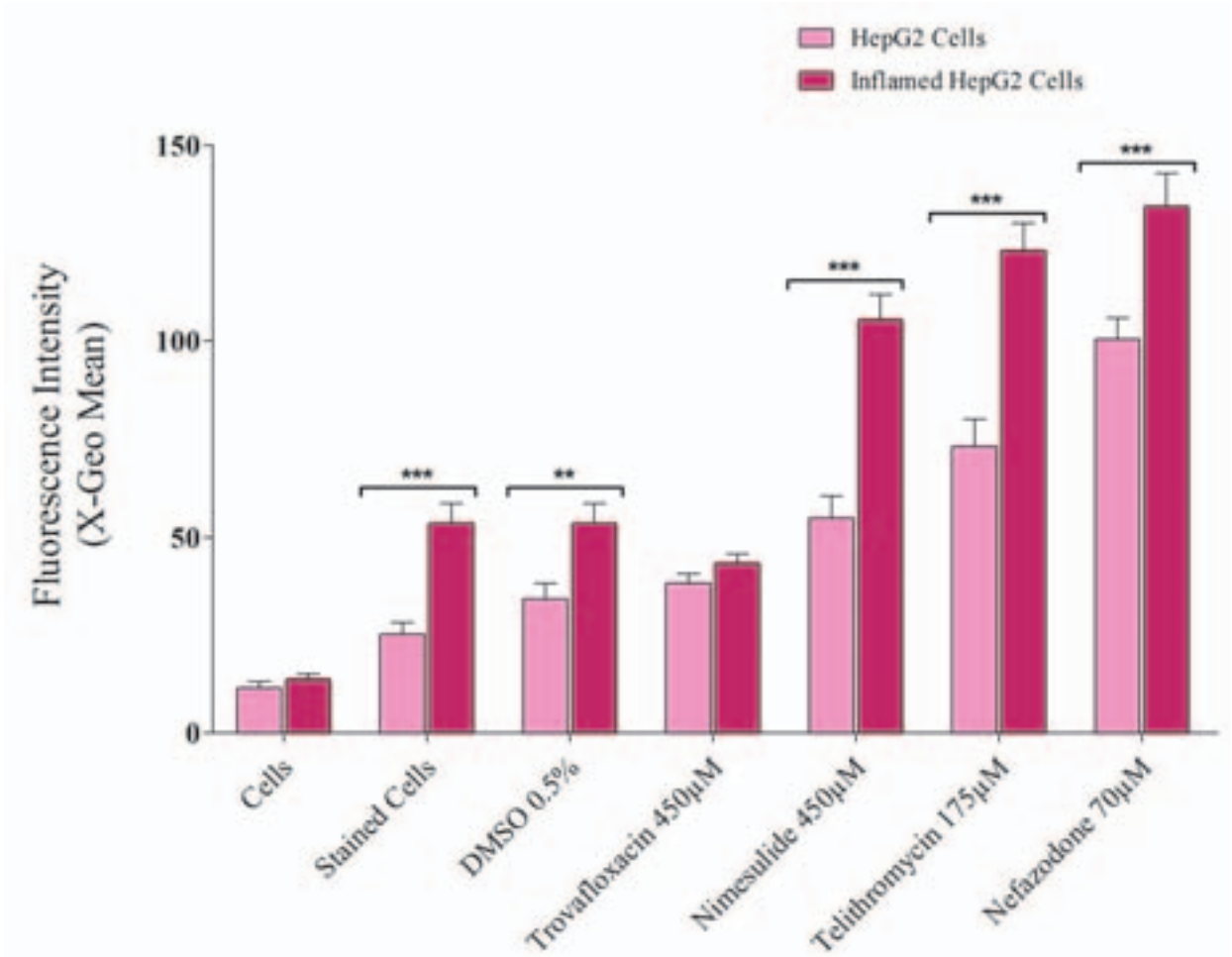


Figure 5.7. Idiosyncratic drug-induced activation of caspase 8 in non-inflamed and inflamed HepG2 cells. After co-incubating HepG2 cells with the four idiosyncratic drugs in absence and presence of LPS and TNF-α for 24 hours, apoptotic cells dying via caspase 8 activation (extrinsic pathway) were detected by capillary flow cytometry using Caspase 8 FAM reagent. Bars represent the emitted fluorescence which is proportional to the number of active caspase 8 enzymes found inside the cell. Data is represented as Mean ± S.E.M (n=3). Statistical analysis was performed using two way ANOVA followed by Bonferroni post test. ** and *** represent $P < 0.01$ and $P < 0.001$ respectively and refer to the variation in caspase 8 activation between non-inflamed and inflamed HepG2 cells. These results indicate that nimesulide, telithromycin and nefazodone induce the activation of caspase 8, when co-administered to cells along with LPS and TNF-α, in a much more significant manner than when administered alone.

5.3.5 Effect of idiosyncratic drugs on truncated Bid (tBid) protein expression

After incubating HepG2 cells with the four tested idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) in presence and absence of LPS and TNF-α, the results attained demonstrated that none of the administered idiosyncratic drugs, neither in the absence nor in the presence of pro-inflammatory mediators, induce the cleavage of Bid despite their inducing effect on caspase 8 activity (Data not shown).

5.3.6 Effect of idiosyncratic drugs on pERK ½ protein expression

After incubating HepG2 cells with the four idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) in presence and absence of LPS and TNF-α, the results attained demonstrate that in the absence of inflammation: nimesulide and telithromycin slightly induced the expression of phosphorylated ERK½; nefazodone mildly reduced it; whereas trovafloxacin did not affect it. However, when these drugs were co-administered to cells along with LPS and TNF-α, trovafloxacin, nimesulide and telithromycin induced the expression of phosphorylated ERK½ in a significant manner whereas nefazodone did not modulate it (Figure 5.8 A and B). These results indicate that LPS and TNF-α favor idiosyncratic drug-induce induction of pERK½ protein expression with the exception of nefazodone.

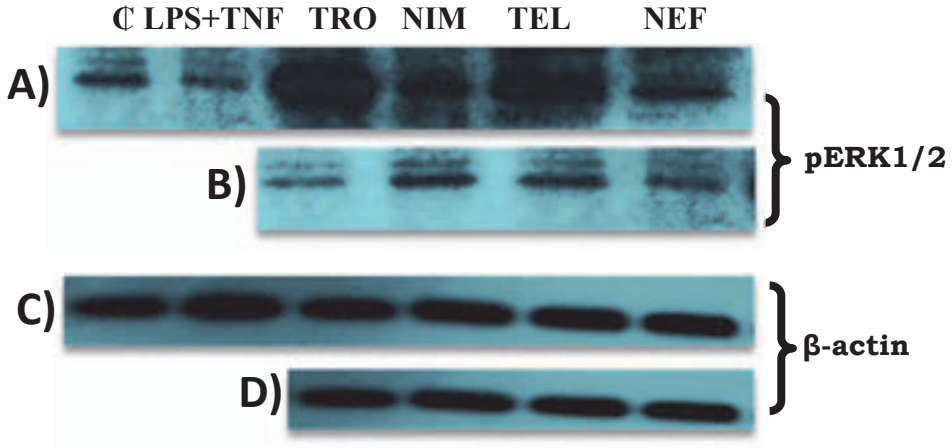


Figure 5.8 (A). Effect of idiosyncratic drugs on the expression of pERK ½ proteins in HepG2 cells in absence and presence of pro-inflammatory stimuli. Immunoblot analysis of pERK ½ protein expression was performed following 24 hours incubation of HepG2 cells with the four idiosyncratic drugs in presence (Lane A) and absence (Lane B) of LPS and TNF-α. Lanes C and D demonstrate the expression of β-actin corresponding to every loaded sample (internal control).The attained results demonstrate that trovafloxacin, nimesulide and telithromycin induce the expression of pERK ½ when co-administered to HepG2 cells along with LPS and TNF-α (Ć: negative control; TRO: trovafloxacin, NIM: nimesulide; TEL: telithromycin; NEF: nefazodone).

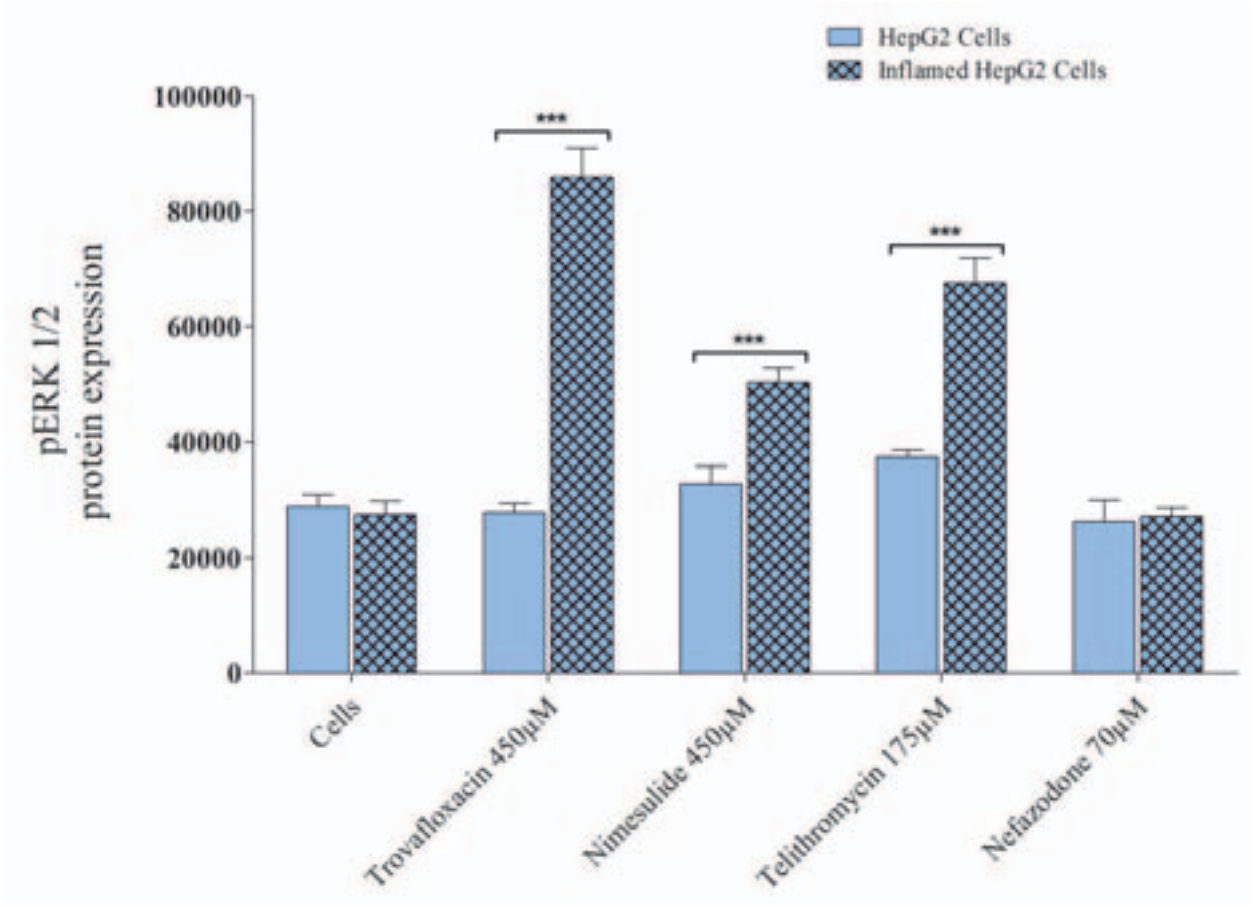


Figure 5.8 (B). Quantification of pERK ½ protein expression by Image J in non-inflamed and inflamed HepG2 cells After incubating HepG2 cells with the four idiosyncratic drugs in absence and presence of LPS and TNF-α , the quantification of pERK ½ protein expression was accomplished using Image J. Bars represent the densitometric analysis of pERK ½ protein expression. Data is represented as Mean ± S.E.M (n=3). Statistical analysis was performed using two way ANOVA followed by Bonferroni post test. *** represents $P < 0.001$ and refers to the variation in pERK ½ protein expression between non-inflamed and inflamed HepG2 cells. The results attained indicate that trovafloxacin, nimesulide and telithromycin induce the expression of pERK ½ proteins when co-administered to cells along with LPS and TNF-α, in a much more significant manner than when administered alone.

5.4 Discussion

This chapter aimed at elucidating the various molecular mechanisms underlying inflammation associated idiosyncratic drug-induced hepatotoxicity. Accordingly, the effects of four known idiosyncratic drugs namely trovafloxacin, nimesulide, telithromycin and nefazodone were tested, in absence and presence of LPS and TNF-α, on the expression and activity of various pro-apoptotic proteins and enzymes, belonging to different cell death signaling pathways; in order to identify the exact apoptotic mechanisms through which pro-inflammatory mediators synergize with idiosyncratic drugs to cause amplified hepatocellular death. Particularly, the effects of the

four idiosyncratic drugs were tested on the expression of p53, Bax, p21, tBid and pERK½, as well as on the activity of caspase 8 in absence and presence of pro-inflammatory mediators.

P53 is a widely known tumor suppressor gene referred to as the "guardian of genome" based on its significant role in protecting cells from severe stress, especially DNA damage, either by triggering apoptosis or by favoring cell growth arrest at the G1 phase (Amundson et al. 1998; He et al. 2005). The fact that p53 acts as the central upstream player in the regulation of intrinsic and extrinsic apoptosis in addition to growth arrest, aroused our interest to test its expression in inflammation associated idiosyncratic hepatotoxicity. The attained results demonstrated first of all that HepG2 cells basally express a significant amount of p53 proteins; these results were consistent with the widely acknowledged fact stating that HepG2 cells, despite being cancerous cells, express wild type p53 that enables them to respond to DNA damage, apoptosis and growth arrest signals (LeCluyse et al. 2012). However, after incubating these cells with the four idiosyncratic drugs the expression of p53 was prominently modulated. In the absence of an pro-inflammatory stimuli, trovafloxacin potently induced the expression of p53 whereas nimesulide telithromycin and nefazodone decreased it when compared to it its basal expression in untreated cells (Figure 5.4). The induction of p53 protein expression is predominantly regulated at the post-translational level and closely correlated with protein stability (Xu, 2003); hence the inducing effect of trovafloxacin on p53 protein expression implies that this drug most likely induce nuclear or mitochondrial DNA damage, which will upregulate the expression of p53 at the post-translational level. This upregulation is most probably mediated by trovafloxacin-induced downregulation of MDM2, which is a RING finger type E3 ligase that promotes the poly-ubiquitylation and proteasomal degradation of p53 (Meek and Anderson, 2009). It is equally plausible for the induction of p53 protein expression to be promoted by trovafloxacin-induced activation of specific kinases such as DNA-activated protein kinase, which will phosphorylate p53 promoting its stability (Gijssel et al. 1997). Similarly, the reducing effect of nimesulide, telithromycin and nefazodone on the expression of p53 may be also correlated with MDM2; however in an opposite manner. For example, these drugs may induce the expression and activity of MDM2 favoring the ubiquitylation and degradation of p53. Alternatively, trovafloxacin may induce the upregulation of p53 by favoring the generation of reactive oxygen species (ROS), since previous studies demonstrated that oxidative stress is significantly implicated in the post-translational modifications of p53. Particularly, ROS proved be involved in the phosphorylation and activation of p53 via oxidative-stress-induced activity of platelet-derived growth-factor-β (PDGF-β) and ataxia-telangiectasia mutated protein kinase (ATM kinase) (Chen et al. 2003). In our experimental conditions, the co-administration of LPS and TNF-α along with the four idiosyncratic drugs, attenuated the inducing effect of trovafloxacin on p53 protein expression while enhancing the reducing effects of telithromycin and nefazodone. In contrary, the administration of LPS and TNF-α to cells alone did not modulate the expression of p53 despite the fact that pro-inflammatory mediators commonly exhibit an NF-κβ-mediated down-regulatory potential on the expression and activity of p53 (Gudkov et al. 2011). It has been previously reported that inflamed cells with up-regulated NF-κβ activity shut down the p53

pathway and reciprocally cells with prominently active p53 downregulate their NF-κβ signaling pathway (Gudkov et al. 2011). The results attained in our experimental conditions suggest that the co-administration of LPS and TNF-α was insufficient to potently activate NF-κβ and hence could not reduce the expression of p53 proteins as expected. However, the fact that the presence of pro-inflammatory mediators reduced the inducing effect of trovafloxacin while extremely promoting the reducing effects of telithromycin and nefazodone on the expression of p53 proteins is consistent with the previously demonstrated negative correlation between upregulated inflammatory pathways and the expression of p53 in times of cellular stress (Gudkov et al. 2011). These results imply that for inflammation to downregulate the expression of p53 in HepG2 cells, an amplifying secondary stress signal able to enhance LPS and TNF-induced activation of NF-κβ is required. Apparently, in our experimental conditions the concurrent presence of idiosyncratic drugs, particularly telithromycin and nefazodone, acted as the required secondary signal, as these two drugs promoted the reducing effects of inflammation on p53 protein expression. Possibly, telithromycin and nefazodone may act as agonists of TNF-α or inducers of TNFR1 expression; thus favoring the TNFR1-mediated activation of NF-κβ pathway. This pathway proved to down-regulate the expression and activity of p53 through various mechanisms. For example, NF-κβ may favor the down-regulation of p53 through competing with the latter for limited pools of the transcriptional co-activator, CBP (CREB binding protein) which is required for the optimal activity of both NF-κB and p53 (Gudkov et al. 2011). Alternatively, NF-κB may promote the reduction of p53 protein expression through favoring the transcription of its major inhibitor MDM2 (Egan et al. 2004; Tergaonkar et al. 2002). Moreover IKKβ, which is an essential component of the NF-κB pathway, proved to phosphorylate p53 leading to its ubiquitination and degradation independent of MDM2; thus pro-inflammatory cytokines that activate IKK may lead to long-term inhibition of p53 activity (Xia et al. 2009). Furthermore, typical NF-κB-regulated anti-apoptotic genes can also suppress p53 function by acting against its positively regulated pro-apoptotic downstream targets (Perkins, 2007).

Bax belongs to the Bcl-2 family of proteins and act as a pro-apoptotic molecule by translocating to the mitochondria and triggering the release of pro-apoptotic proteins from the intermembrane space into the cytosol; thus promoting the formation of the "apoptosome" (Degli Esposti and Dive, 2003). Bax is predominantly regulated by p53 and is mainly involved in the intrinsic apoptotic pathway (Haupt et al. 2003). However, the activation of Bax is also possible through the extrinsic pathway via caspase8-mediated truncation of Bid, which in its turn translocates to the mitochondria and activates Bax and Bak. Subsequently, these two pro-apoptotic proteins will promote mitochondrial permeability transition, pro-apoptotic protein release and apoptosome formation; leading eventually to apoptosis (Haupt et al. 2003). The results attained demonstrated that the four idiosyncratic drugs induce the expression of Bax proteins when co-administered to cells along with pro-inflammatory mediators, in a much more significant manner than when administered alone (Figure5.5). Accordingly, it may be deduced that the co-occurrence of an inflammatory reaction during idiosyncratic drug-therapy may promote hepatocellular death via the up-regulation of Bax proteins, which in their turn will favor the induction of intrinsic

apoptosis. Commonly, Bax is known to be regulated by p53 implying that an increase in the expression of Bax proteins is preceded by an increase in the expression of p53 (Haupt et al. 2003). However, in our experimental conditions the expression of p53, in contrary to that of Bax, is reduced in the synergistic presence of pro-inflammatory mediators and idiosyncratic drugs; indicating that the upregulation of Bax is mediated via a p53-independent mechanism. The ERK ½ pathway proved to be one of the p53-independent mechanisms, which regulates apoptosis by favoring the expression of Bax proteins; suggesting that the observed increase in the expression of Bax proteins may be mediated via an ERK½-dependent mechanism (Zhuang and Schnellmann, 2006). This suggestion was further supported by the fact that the three idiosyncratic drugs (trovafloxacin, nimesulide and telithromycin), which potently induced the expression of Bax proteins in the presence of LPS and TNF-α, also promoted the expression of phosphorylated ERK½ (Figure5.8). Nefazodone also induced the expression of Bax proteins in the presence of pro-inflammatory mediators however to a lesser extent than the other drugs; this observation was consistent with the fact that nefazodone induced a weaker phosphorylation of ERK ½ when co-administered to cells with LPS and TNF-α.

After studying the effect of idiosyncratic drugs on the expression of Bax proteins to determine if Bax-mediated apoptosis is implicated in idiosyncratic drug-induced hepatotoxicity, we studied the effects of these drugs on the expression of p21 in the aim of demonstrating if drug-induced induction of cell cycle arrest is also involved in idiosyncratic hepatotoxicity. Immunodetection of p21 proteins was performed after incubating HepG2 cells with the four idiosyncratic drugs in absence and presence of the inflammatory mix. The results attained demonstrated that solely telithromycin is able to significantly induce the expression of p21 proteins when co-administered to cells along with LPS and TNF-α (Figure 5.6). Commonly, p21 is tightly regulated by p53 and is reported to be the main contributor of p53-induced cell growth arrest by inhibiting G1 cyclin-dependant kinases (cyclinA/cdk2, cyclinE/cdk2, cyclinD/cdk4 complexes) and triggering cell cycle arrest at the G1 phase (Harper et al. 1993). However, the fact that telithromycin completely repressed p53 protein expression in the presence of pro-inflammatory mediators, suggests that its inducing effect on the expression of p21 is most probably mediated by a p53-independent mechanism. This suggestion is not very astonishing since several p53-independent mechanisms have been proposed to explain the modulation of p21 in the absence of p53, although the former is a principle target of the latter and a central component in a variety of p53-mediated stress responses (Aliouat-Denis et al. 2005). These mechanisms predominantly implicate a variety of transcription factors, each of which is induced by a different signaling pathway, namely Sp1 and Sp3 (specificity protein 1 and 3), Ap2 (activating protein 2), STATs (signal transducer and activator of transcription), C/EBP α and β (CCAAT-enhancer binding proteins α and β), and the basic loop-helix-loop proteins BETA2 and MyoD (Gartel and Tyner, 2002). For example, *Moustakas and Kardassis* demonstrated that Smad proteins, which play crucial roles in signal transduction, mediate p21 induction in response to TGF-β via the transcription factor Sp1 (Moustakas and Kardassis, 1998). In addition to TGF-β, TNF-α, IFN-γ and histone deacetylase inhibitors proved to induce p21 trans-activation via p53-independent mechanisms (Gartel and

Tyner, 2002). Particularly TNF-α induced the expression of p21 via the activation of NF-κβ in highly malignant Ewing tumor cells as well as in MCF-7 breast carcinoma cells; whereas IFN-γ induced p21 by a STAT1-dependent, p53-independent mechanism (Gartel and Tyner, 2002). Histone deacetylase inhibitors proved to induce p21 in a p53-independent manner through interacting with Sp1 binding sites in the p21 promoter (Gartel and Tyner, 2002). The fact that telithromycin induced p21 protein expression only within an inflammatory context highly proposes the contribution of pro-inflammatory cytokines in this induction. However, the simple addition of LPS and TNF-α to HepG2 cells without the concurrent presence of telithromycin was insufficient to promote the expression of p21 proteins in our experimental conditions; implying that enhanced p21 expression require the synergistic action of telithromycin in addition to LPS and TNF-α. Most probably the synergistic drug-inflammation induction of p21 protein expression is mediated by an amplified activation of the NF-κβ pathway. Alternatively p21 protein expression could be mediated by telithromycin induced upregulation of TGF-β, which is often extensively released during drug-induced lipid peroxidation and steatohepatitis (Jaeschke et al. 2002).

After investigating the effects of trovafloxacin, nimesulide, telithromycin and nefazodone on the expression of pro-apoptotic proteins belonging to the intrinsic pathway, their effects on the activity of caspase 8, which is known to be the principal mediator of the extrinsic apoptotic pathway, were studied (Bantel and Schulze-Osthoff, 2012); in the aim of elucidating if these drugs promote hepatocellular death via the extrinsic apoptotic pathway. The results attained demonstrated that nimesulide, telithromycin and especially nefazodone activate caspase 8, in a much more significant manner when co-administered along with pro-inflammatory mediators than when administered alone to cells. These results suggest that these three drugs promote hepatocellular death in inflammation associated idiosyncratic hepatotoxicity via the extrinsic apoptotic pathway. Nimesulide, telithromycin and nefazodone may activate caspase 8 by acting as death ligands (TNF-α, Fas-L and TRAIL) agonists and thus interacting with death receptors (TNFR1, Fas and TRAIL-R1/2) leading to receptor oligomerization and recruitment of adaptor protein FADD, which will form with pro-caspase 8 the death inducing signaling complex (DISC) (Bantel and Schulze-Osthoff, 2012). Subsequently, this complex will induce the dimerization and the proteolytic cleavage of pro-caspase 8 generating an active caspase 8 enzyme that will initiate the death receptor (extrinsic) apoptotic pathway (Bantel and Schulze-Osthoff, 2012). Alternatively, these three drugs may induce the activity of caspase 8 independently of death receptors through a mechanism involving the mitochondrial apoptotic pathway (Sohn et al. 2005). It has been reported that several anticancer drugs such as taxol and etoposide induce the activity of caspase 8 via this same mechanism, which is predominantly based on drug-induced mitochondrial permeability transition, cytochrome c release, apoptosome formation and activation of effector caspases (Sohn et al. 2005). Amongst these caspases, particularly caspase 3 and 6 proved to be essential for the interchain proteolytic death receptor-independent activation of caspase 8 (Sohn et al. 2005; Wieder et al. 2001). However, the fact that nimesulide, telithromycin and nefazodone trigger the activation of caspase 8 in a more significant manner in

the presence of LPS and TNF-α suggests most probably that these drugs induce the expression of TNFR1, consequently leading to an enhanced interaction between TNF-α and TNFR1. Subsequently, this interaction will further promote the activation of caspase 8 eventually leading to amplified extrinsic apoptosis. Nevertheless, it is noteworthy that these two mechanisms are not mutually exclusive; or at least no valid reported proof prevents nimesulide, telithromycin and nefazodone from inducing the activity of caspase 8 via two simultaneous mechanisms: the first being the induction of TNFR1 expression leading to amplified TNF-TNFR1 interaction; and the second being the induction of mitochondrial damage leading to activation of caspase 3 and 6, which in their turn will lead to the proteolytic activation of caspase 8 (Sohn et al. 2005).

It is commonly known that hepatocytes are resistant to TNF-induced apoptosis because the interaction of TNF-α with TNFR1 generate low amounts of initiator caspase 8 that are usually insufficient to cause hepatocellular death (Bantel and Schulze-Osthoff, 2012). Usually, an extrinsic apoptotic stimulus requires the activation of the mitochondrial pathway in addition to the extrinsic pathway in order to effectuate hepatocellular death. The activation of the mitochondrial pathway is commonly induced through the caspase-8-mediated cleavage of Bid, a pro-apoptotic Bcl-2 family protein, which subsequently initiates together with Bak and Bax the release of mitochondrial pro-apoptotic mediators (Schwerk and Schulze-Osthoff, 2005). Accordingly, we have tested the effect of the four idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) on the expression of truncated Bid in presence and absence of pro-inflammatory mediators. The results attained demonstrated that neither in the absence nor in the presence of an inflammatory context did any of the four tested idiosyncratic drugs induce the expression of truncated Bid. The fact that nimesulide, telithromycin and nefazodone potently activated caspase 8 without triggering the truncation of Bid either suggests that these drugs are powerful enough to cause hepatocellular death via the extrinsic apoptotic pathway and do not require the involvement of the mitochondrial pathway; or that these drugs do involve the mitochondrial pathway however through a tBid-independent mechanism.

It is widely acknowledged that during the activation of apoptotic pathways a concomitant induction of survival signaling pathways, mainly the phosphatidylinositol 3-kinase/Akt and the ERK pathway, often occurs to counter-balance apoptosis and prevent organ injury (Zhuang and Schnellmann, 2006). Particularly, the ERK pathway proved to be implicated in cellular death in addition to survival and proliferation and hence was investigated in this chapter to assess its involvement in inflammation associated idiosyncratic drug-induced hepatotoxicity. The results attained demonstrated that trovafloxacin, nimesulide and telithromycin potently induce the expression of phosphorylated ERK ½ when co-administered to cells along with LPS and TNF-α (Figure 5.8); indicating that the concurrent presence of these drugs along with pro-inflammatory mediators promotes a potent activation of the ERK½ pathway. In contrary, nefazodone did not alter the expression of pERK ½ neither in absence nor in presence of pro-inflammatory mediators; indicating that the ERK pathway is not involved in the hepatotoxic potential of this drug (Figure 5.8). The fact that the administration of the inflammatory mix alone did not promote the expression of phosphorylated ERK indicates that the activation of the ERK pathway

is predominantly based on the synergistic action of both inflammation and idiosyncratic drugs and that the administration of either one of them alone is insufficient to induce this activation. The ERK pathway is involved in the regulation of the intrinsic and extrinsic apoptotic pathways by upregulating p53 and Bax and favoring the synthesis of TNF-α and the activation of caspase 8 respectively (Zhuang and Schnellmann, 2006). Accordingly, it is highly probable for trovafloxacin to induce the expression of p53 through promoting the phosphorylation of ERK ½, and for nimesulide and telithromycin to induce the activation of caspase 8 via an ERK-dependent pathway. However, nefazodone most likely induces the activity of caspase 8 via an ERK-independent mechanism, which may be related to the activation of the mitochondrial pathway for example.

In conclusion, this chapter elucidated the molecular mechanisms underlying inflammation associated idiosyncratic drug-induced hepatocellular death in the aim of providing valuable insights for the development of efficient preventive and therapeutic techniques, which may serve to reduce the severity of drug-induced hepatic injury and liver failure. Particularly, trovafloxacin proved to induce hepatocellular death via the intrinsic apoptotic pathway through the up-regulation of p53 and Bax protein. This induction is apparently mediated via an ERK-dependent mechanism. Nimesulide most probably favors hepatocellular death via the induction of both apoptotic pathways. Predominantly, this drug activates the intrinsic apoptotic pathway by promoting the expression of Bax via an ERK-dependent mechanism while activating the extrinsic apoptotic pathway through enhancing the activation of caspase 8. Similarly, telithromycin promotes hepatocellular death via the activation of both, the intrinsic (via ERK-dependent upregulation of Bax) and the extrinsic (through favoring the activation of caspase 8) apoptotic pathways. In addition to apoptosis, telithromycin also induce p21-dependent cell cycle arrest most probably at the G1 phase. Nefazodone predominantly favor hepatocellular death via the extrinsic apoptotic pathway through up-regulating the activity of caspase 8 enzymes; nevertheless the intrinsic pathway is involved in the apoptotic potential of this drug. Despite the fact that the majority of the tested idiosyncratic drugs implicate both apoptotic pathways in their hepatotoxic potential, the convergence of these pathways proved to be tBid-independent. Most probably, the amplified hepatotoxic potential observed in the synergistic presence of idiosyncratic drugs and pro-inflammatory mediators is mediated via the concomitant independent activation of both the intrinsic and the extrinsic apoptotic pathways, as follows: idiosyncratic drugs may act as agonists of TNF-α, either by upregulating its biosynthesis or by promoting the expression of TNFR1, leading to amplified activation of the extrinsic pathway; while synergistically causing mitochondrial damage and subsequently triggering the up-regulation of Bax, mitochondrial permeability transition and release of pro-apoptotic factors leading to the execution of intrinsic apoptosis. Although this presumption appears as a logical mechanism that may mediate amplified hepatocellular death in inflammation associated drug-induced hepatotoxicity, it remains to be proven by tangible observations and valid evidences.

Chapter 6
Conclusion and Perspectives

6.1 Conclusion

Idiosyncratic adverse drug reactions represent the leading cause of drug-induced liver injury and the most frequent reason for post-marketing drug withdrawal; predominantly due to the lack of efficient predictive models and the ambiguity of the toxic mechanisms underlying idiosyncratic hepatotoxicity (Shaw et al. 2010; Deng et al. 2009). Several hypotheses have emerged through the years to try and explain the mode of action of idiosyncratic adverse drug reactions; however this thesis focused on the inflammatory stress hypothesis, which states that: "a mild episode of inflammation occurring synergistically during drug therapy, may predispose the liver to drug-induced damage, resulting in a toxic response at an otherwise safe drug dose" (Shaw et al. 2009). This hypothesis led to the development of animal models that succeeded in revealing the idiosyncratic hepatotoxic potential of several drugs; however these models presented an overall limited predictive success due to the prominent interspecies differences existing between animals and humans with respect to drug absorption, distribution, metabolism and elimination (Xu et al. 2004). Furthermore, these models are low throughput in nature, which renders them incompatible with the high throughput demands of preclinical pharmaceutical screening (Cosgrove et al. 2009).

In view of the current lack of effective predictive models able to detect the inflammation associated hepatotoxic potential of an idiosyncratic drug candidate before reaching the market on one hand; and the ambiguity of the toxic mechanisms underlying synergistic drug-inflammation induced amplified hepatotoxicity on the other hand, this thesis had three main objectives: Firstly, we focused on developing a high throughput human-based *in vitro* model for the prediction of inflammation associated idiosyncratic hepatotoxicity and the elucidation of its underlying mechanisms. Particularly, the developed drug-inflammation model was used to elucidate the hepatotoxic mechanisms of four known idiosyncratic drugs namely trovafloxacin, nimesulide, telithromycin and nefazodone with emphasis on oxidative stress, steatosis and cholestasis.

Secondly, we aimed at investigating the implication of two important efflux transporters (MDR1 and MRP2) in the pathogenesis of inflammation associated idiosyncratic hepatotoxicity; predominantly because drug-induced cholestasis has been reported to be one of the main clinical manifestations of drug-induced hepatotoxicity (Kaplowitz, 2005).

Thirdly, we focused on elucidating the molecular mechanisms through which the four selected idiosyncratic drugs synergize with pro-inflammatory mediators to cause amplified hepatocellular death using the developed drug-inflammation model.

Based on these fundamental objectives the results attained in this thesis were divided into three main chapters: Chapter 3, 4 and 5.

The work presented in chapter 3 focused first of all on developing a high throughput *in vitro* cell culture model of inflammation associated idiosyncratic drug-induced hepatotoxicity, which is based on the synergistic exposure of HepG2 cells to pro-inflammatory mediators (LPS and TNF-α) and potentially idiosyncratic drugs. In order to validate the sensitivity and specificity of the developed drug-inflammation model, it was initially used to the assess the hepatotoxic potential of four idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone), known to

cause amplified hepatotoxicity in presence of pro-inflammatory stimuli, and their non-idiosyncratic analogues (levofloxacin, aspirin, clarithromycin and buspirone) in the absence and presence of LPS and TNF-α. The results attained in this chapter demonstrated that developed model may be considered sensitive and specific since it detected the inflammation associated hepatotoxic potentials of the four idiosyncratic drugs but not of their non-idiosyncratic analogues. Subsequently, this model was used to screen several anticancer drugs for inflammation associated hepatotoxicity in order to test its efficacy in high throughput toxicity screening of a different category of drugs. In particular, anticancer drugs were chosen based on the reported correlation between underlying episodes of inflammation and sudden drug-induced hepatotoxic reactions in cancer patients (Morgan et al. 2008). A considerable number of the screened anticancer drugs, namely azaguanine-8, nocodazole, methotrexate, etoposide, azacytidine-5, chlorambucil, cytarabine, busulfan, docetaxel, 5-fluorouracil, erlotinib, imatinib, and fludarabine exhibited an enhanced apoptotic effect in presence of LPS and TNF-α; indicating that the stated drugs may exhibit amplified hepatotoxicity if administered to patients suffering from a concurrent episode of inflammation. These results indicate that the developed drug-inflammation model is applicable to high throughput toxicity screening and is able to detect the inflammatory associated hepatotoxic potential of any drug regardless of its category or mode of action. Accordingly, this model represents an efficient pre-clinical tool for the detection of inflammation associated idiosyncratic drugs, which if combined with a complementary clinical predictive approach may constitute an effective strategy for the prevention of post-marketing idiosyncratic drug-induced hepatotoxicity cases.

After validating the sensitivity and selectivity of the developed drug-inflammation model, the latter was used in the investigation of the toxic mechanisms through which the four idiosyncratic drugs synergized with LPS and TNF-α, to cause amplified hepatotoxicity with emphasis on oxidative stress and steatosis. The attained results demonstrated that nimesulide and nefazodone predominantly induce an amplified hepatotoxic potential in presence of LPS and TNF-α via favoring the intracellular accumulation of superoxide anions. Furthermore, nefazodone also potently induced the intracellular accumulation of lipids; indicating that steatosis may be considered as an underlying mechanism of nefazodone-induced idiosyncratic hepatotoxicity. In our experimental conditions, oxidative stress and steatosis were not implicated in the inflammation associated idiosyncratic hepatotoxicity of trovafloxacin and telithromycin.

In order to further elucidate the mechanisms underlying the amplified hepatotoxic potential of trovafloxacin, nimesulide, telithromycin and nefazodone within an inflammatory context, their effects on the expression and activity of MDR1 and MRP2 were tested in Chapter 4. This chapter aimed predominantly at elucidating if cholestasis is significantly implicated in the pathogenesis of inflammation associated idiosyncratic drugs and at determining whether MDR1 and MRP2 are involved in the cholestatic potential of these drugs. The attained results demonstrated that trovafloxacin, telithromycin and nefazodone reduce the expression and inhibit the efflux activity of MDR1 in the presence of an inflammatory context; indicating that these three drugs induce inflammation associated idiosyncratic hepatotoxicity via a cholestatic mechanism that involves

the down-regulation of the efflux transporter MDR1. Concerning the effect of the four idiosyncratic drugs on the expression and activity of MRP2, within an inflammatory context, our results indicated that the co-presence of LPS and TNF along with trovafloxacin, nimesulide and nefazodone completely repressed the expression of MRP2 proteins while significantly reducing it with telithromycin. Furthermore, it was demonstrated that trovafloxacin, nimesulide and nefazodone potently inhibit the efflux activity of MRP2. The obtained results imply that these three drugs induce inflammation associated idiosyncratic hepatotoxicity via a cholestatic mechanism that involves the down-regulation of MRP2. Finally it may be inferred that the cholestatic potential of trovafloxacin and nefazodone is mediated via the downregulation of both MDR1 and MRP2; whereas that of telithromycin and nimesulide is predominantly based on the separate downregulation of MDR1 and MRP2 respectively.

Overall each and every idiosyncratic drug from the four drugs tested exhibited a different mechanism of action; however they all resulted in amplified apoptosis in presence of an inflammatory context. Therefore, we were interested to investigate the molecular mechanisms through which idiosyncratic drugs synergize with pro-inflammatory mediators to promote hepatocellular apoptosis. Accordingly, the effects of the four idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) on the expression of several proteins involved in apoptosis and cell growth arrest (p53, Bax, caspase 8, tBid, p21 and pERK ½), were tested in the absence and presence of an inflammatory context. Amongst the four idiosyncratic drugs tested only trovafloxacin induced the expression of p53 proteins suggesting that this drug may induce hepatocellular death via a p53-mediated mechanisms; however the remaining three idiosyncratic drugs rather promoted apoptotic cell death via p53-independent mechanisms.

Overall, the enhanced apoptotic potentials of the four idiosyncratic drugs within an inflammatory context were mediated as follows:

Trovafloxacin induced the expression of Bax proteins, most probably via an ERK½-dependent mechanism; indicating that this drug induced apoptosis predominantly via the intrinsic pathway. Nimesulide induced the expression of Bax proteins and the activation of caspase 8 enzymes also via an ERK½-dependent mechanism; suggesting that this drug induces apoptosis via the intrinsic as well as the extrinsic pathway. Similarly, telithromycin demonstrated an inducing effect on the expression of Bax proteins and on the activation of caspase 8 enzymes via a p53-independent ERK½-dependent mechanism; implying that both the intrinsic and the extrinsic apoptotic pathways are implicated in the apoptotic potential of this drug. Furthermore, telithromycin exhibited a potent inducing effect on the expression of p21 within an inflammatory context, indicating that this drug favors cell growth arrest, in addition to apoptosis, also via a p53-independent mechanism. Nefazodone demonstrated the most potent inducing effect on the activation of caspase 8, indicating that this drug predominantly promote hepatocellular death via the extrinsic apoptotic pathway. Nevertheless, this drug also induced the expression of Bax proteins but to a lesser extent than the other three tested drugs. These results imply that the apoptotic effect of nefazodone is predominantly mediated via the extrinsic apoptotic pathway; however it involves the activation of the mitochondrial pathway via the moderate up-regulation

of Bax. The effect of the four idiosyncratic drugs on the expression of truncated Bid (tBid) was tested to explain if the involvement of the intrinsic pathway in the apoptotic potential of these drugs is mediated via caspase 8-dependent truncation of Bid. The fact that none of the tested drugs induced the expression of tBid in the presence of pro-inflammatory mediators suggests that the involvement of the mitochondrial pathway is effectuated by direct drug-induced damage to the mitochondria rather than through the intermediary of truncated Bid. Finally, it seems that the amplified apoptotic potential observed in the synergistic presence of idiosyncratic drugs and pro-inflammatory mediators is mediated via the activation of both apoptotic pathways, the intrinsic and the extrinsic; however through a tBid-independent mechanism. Most probably idiosyncratic drugs act as agonists of TNF-α, either by upregulating its synthesis or by promoting the expression of its corresponding receptor (TNFR1) thus promoting the activation of caspase 8 and amplifying the induction of the extrinsic apoptotic pathway; while concurrently causing mitochondrial damage and subsequently triggering the upregulation of Bax, the release of pro-apoptotic factors, the formation of the apoptosome and the activation of effector caspases leading to the execution of intrinsic apoptosis. The fact that the mitochondrial pathway proved to be also involved in the caspase 3-mediated proteolytic activation of caspase 8, further supports the hypothesis stating that both apoptotic pathways are significantly implicated in inflammation associated idiosyncratic drug-induced hepatotoxicity (Sohn et al. 2005). It is noteworthy, that the amplified apoptotic potential of the four idiosyncratic drugs in presence of LPS and TNF-α, whether mediated by the intrinsic or the extrinsic pathway, is predominantly based on idiosyncratic drug-induced upregulation of phosphorylated ERK ½. However, the mechanisms through which these drugs promote the expression of pERK½ remain ambiguous; accordingly their elucidation constitutes an interesting perspective for future experiments.

Overall this thesis presented a cellular model for the prediction of inflammation associated idiosyncratic drug-induced hepatotoxicity, that is applicable to high throughput toxicity screening on hand and that allows the investigation of the cellular and molecular mechanisms underlying inflammation associated idiosyncratic hepatotoxicity and hepatocellular death on the other hand; thus providing valuable insights on how to prevent idiosyncratic adverse drug reactions and how to identify patients who are at an elevated risk of developing them (Holt and Ju, 2006). These insights may pave the way for the development of a complete therapeutic and preventive strategy that can avert human suffering and save several lives especially that 75% of idiosyncratic adverse drug reactions result in severe hepatic failure and death (Pandit et al. 2012).

6.2 Perspectives

This thesis presented an efficient high throughput cellular model for the prediction of inflammation associated drug-induced idiosyncratic hepatotoxicity on one hand; and for the elucidation of the toxic mechanisms through which idiosyncratic drugs synergize with pro-inflammatory mediators to cause amplified hepatocellular death on the other hand. Although the attained results provided valuable insights on the prediction and prevention of inflammation associated idiosyncratic hepatotoxicity; several future perspectives may be envisaged to further develop and ameliorate this model in order to improve its accuracy and efficacy in the prediction of inflammation associated idiosyncratic drug-induced liver injury. The proposed perspectives will be divided into two main sections: The first section will tackle the improvements that may be done in order to ameliorate the efficacy and accuracy of the proposed predictive model. These improvements will be predominately correlated with the replacement of HepG2 cells with more advanced hepatocyte culture systems that reproduce more appropriately the physiological hepatic genotype and phenotype. The second section will predominantly propose the adoption of "systems biology" approach and the integration of advanced "Omics" techniques in the presented drug-inflammation predictive model; in order to provide a more complete investigation and assessment of the toxic mechanisms underlying inflammation associated idiosyncratic drug-induced liver injury.

6.2.1 Use of advanced hepatocyte cell culture systems to improve the prediction of inflammation associated idiosyncratic drug-induced hepatotoxicity

The first proposed perspective is to re-establish the presented predictive model of inflammation associated idiosyncratic hepatotoxicity using a culture system that better reproduce the physiological response of the liver upon simultaneous exposure to an inflammatory stress and an idiosyncratically toxic drug. For example, replacing HepG2 cells with HepaRG cells or with human hepatocytes, which better express important metabolic enzymes (such as CYP450), drug transporters (such as BSEP) and nuclear receptors (such as PXR) will enhance the predictive potential of the presented drug-inflammation model while providing broader insights on the probable mechanisms underlying inflammation associated idiosyncratic hepatotoxicity. Using cells which better express PXR and CAR will certainly ameliorate the understanding of the regulatory mechanisms underlying the modulatory effects of idiosyncratic drugs on the expression of MDR1 and MRP2. Particularly, using human hepatocytes instead of HepG2 cells will definitely circumvent the inability of the latter to be used in the assessment of metabolic idiosyncrasy, which is an important subtype of idiosyncratic hepatotoxicity related to acquired or innate impaired drug metabolism (Uetrecht, 2008). Furthermore, replacing the 2D standard culture techniques with more advanced tissue-engineered hepatocyte cell culture systems that better mimic the *in vivo* physiological situation will significantly ameliorate the efficacy and accuracy of the proposed drug-inflammation model in the prediction idiosyncratic hepatotoxicity. These culture systems may include: the culture of hepatocytes on hepatic

biomatrix scaffolds, three-dimensional spheroid aggregate culture, co-culture systems and perfusion culture systems.

Hepatic biomatrix is constituted of a partially purified extract of extracellular matrix, derived from whole rat liver and comprises types I–IV collagen, fibronectin and extracellular matrix glycoproteins, including a number of important proteoglycans and growth factors (Wang et al. 2011). Liver biomatrix enhances hepatocyte attachment and long-term survival (3 weeks or more in culture) while maintaining normal cell shape and cytoarchitecture, which in turn promotes the native physiological expression of a wide variety of genes and transcription factors that regulate cell growth, differentiation and apoptosis (LeCluyse et al. 2012). Furthermore, the chemical composition and biophysical characteristics of extracellular matrices used in hepatocyte cultures prominently promote an *in vivo*-like liver-specific gene expression and cellular response to extracellular soluble signals; this will better mimic the physiological response of the liver to toxic xenobiotics within an inflammatory context while enhancing the accuracy of the proposed drug-inflammation model.

The three-dimensional spheroid aggregate culture, is made up of hepatocytes that are cultured on non-adhesive surfaces under suitable conditions whereby they small sphere-shaped aggregates or "spheroids" that will deposit extracellular matrix components such as laminin, fibronectin, and collagen on their outer surface (Landry et al. 1985; Li et al.1992; Dilworth et al. 2000). This type of culture proved to maintain several hepatic functions for prolonged periods of time (Brophy et al. 2009; Sakai et al. 2010). Many of the beneficial effects of spheroidal aggregate culture are attributed to the retention of a three-dimensional cytoarchitecture, the presence of important extracellular matrix components, and the establishment of crucial cell to cell contacts (Landry et al.1985; Takabatake et al. 1991; Yuasa et al. 1993; Luebke-Wheeler et al. 2009; Sakai et al. 2010). The main advantages presented by the replacement of standard 2D culturing techniques with advanced 3D hepatocyte culture systems in the proposed drug-inflammation model are predominantly correlated with the maintenance of stable CYP enzymes expression especially following induction; which will allow the application of this model in the study of metabolic idiosyncrasy. Studying the impact of inflammation on metabolic idiosyncrasy was impossible using HepG2 cells since they exhibit basal metabolic-dependent toxicity which will lead to high false positivity. Furthermore, the 3D culture of hepatocytes proved to ameliorate the expression of hepatic transporters and the circulation of bile flow thus allowing a better assessment of inflammation associated idiosyncratic drug-induced cholestasis (Du et al. 2008).

The co-culture system, whereby hepatocytes are co-cultured with other non-parenchymal cells, such as kupffer cells for example, in the presence of adhesion molecule like cadherins and integrins will reproduce the complexity of the liver *in vivo*; thus providing a native expression of liver-specific transcription factors and maintaining integral phenotypic structure and function (LeCluyse et al. 2012). Co-culture systems proved highly efficient in studying direct and indirect signaling pathways involved in certain drug- and cytokine-induced effects on hepatocyte functions (Wandzioch et al. 2004; Sunman et al. 2004; Tukov et al. 2007). Accordingly, co-culturing HepG2 cells with kupffer cells, which play significant roles in the regulation of hepatic

inflammation, will most definitely ameliorate the accuracy of the presented drug-inflammation predictive model by reproducing an inflammatory reaction *in vitro* that better resembles hepatic inflammation *in vivo*. Kupffer cells are known to play a significant role in the regulation of hepatic inflammation by producing and releasing a wide array of pro-inflammatory factors including TNF-α (Roberts et al. 2007). Accordingly, co-culturing kupffer cells with hepatocytes in the presented drug-inflammation model permits the investigation of the roles played by various pro-inflammatory cytokines in idiosyncratic hepatotoxicity. The use of such co-culture systems will not only improve the accuracy of the presented drug-inflammation model but will also provide broader insights on the mechanistic basis of inflammation associated idiosyncratic drug-induced hepatotoxicity especially concerning the various cytokine-induced signaling pathways that may be possibly involved in the promotion of hepatocellular death or survival. Nevertheless, it is important to note that co-culturing kupffer cells with hepatocytes do not only provide advantages. One of the main disadvantages of this model is the kupffer cell-induced alteration in the functions and structure of hepatocytes. Particularly, several kupffer cell-released pro-inflammatory cytokines (TNF-α, IL-1 and IL-6) and acute phase proteins proved to down-regulate metabolizing enzymes and increase the number of ribosomes and lysosomes in hepatocytes (Panin et al. 2002; Sunman et al. 2004).

Perfusion culture systems, whereby primary liver cells are maintained under constant dynamic flow generally improve cell viability and metabolic performance (LeCluyse et al. 2012). They reproduce perfectly the *in vivo* situation by providing three dimensional cytoarchitecture, *in vivo*-like expression of drug metabolizing enzymes and transporters and functional bile canaliculi (LeCluyse et al. 2012). Using perfusion culture systems that reproduce the physiological functionality of the bile canaliculi will most definitely allow a more accurate assessment of inflammation associated idiosyncratic drug-induced cholestasis; however these systems present several obstacles related to the their unavailability, complexity and low throughput nature.

These proposed advanced hepatocyte culture systems that better sustain hepatic drug metabolism and transport over a chronic time scale (more than seven days), could be employed in the development of more physiologically relevant models of inflammation associated idiosyncratic drug-induced hepatotoxicity on one hand; and be utilized in the study of combined acute and chronic hepatocyte responses following concurrent exposure to idiosyncratic drugs and inflammatory stress on the other hand.

6.2.2 Use of advanced techniques to better elucidate the mechanisms underlying inflammation associated idiosyncratic drug-induced hepatotoxicity

In this thesis several techniques have been used to elucidate the mechanisms through which four idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) synergized with pro-inflammatory mediators (LPS and TNF-α) to cause amplified hepatocellular death. These techniques mainly included ELISA, capillary flow cytometry, immunofluorescence, and western blot. Although these techniques constituted the basis of scientific research for several years, recent advances in the fields of molecular biology and biotechnology led to the emergence of

several innovative techniques that shifted toxicology from an empirical practice to an investigative discipline; whereby the prediction of toxicity is based on a "systems biology" approach rather than on a reductionist approach that investigates the alteration in individual components of biological systems in response to a toxic exposure (Vliet, 2011). Systems biology, a recently emerging field in life sciences, aims at integrating data from different approaches (e.g., genomics, proteomics, metabolomics, and imaging) in order to identify the various molecular and biochemical pathways implicated in homeostasis on one hand, while discovering the dynamic interactions between their components and the ways through which they maintain and control homeostasis on the other hand (Heijne et al. 2005; Vliet, 2011). In order to adopt "systems biology" approach in the assessment of drug-induced toxicity several different properties need to be investigated (Kitano, 2002). According to *Kitano,* initially the identification of the individual structural components constituting the system, namely its networks of genes, proteins, and biochemical pathways is a must. Particularly, omics technologies combined with molecular techniques such as gene knock-out and silencing are appropriate for identifying the interactions and relationships between individual components (Kitano, 2002). Next, the system dynamics should be thoroughly investigated and understood through the quantitative measurement of single components at normal homeostasis conditions and after inducing specific perturbations (Kitano, 2002). In order to better understand the dynamics in a biological system it is crucial to understand the temporal and spatial dynamics of the molecular and biochemical processes constituting it (Vliet, 2011). This is mainly achieved through the use of innovative real-time imaging techniques and models using a variety of mathematical and computational approaches (Kherlopian et al. 2008; Sadot et al. 2013). Another requirement is the development of data standardization and quality assurance guidelines to allow international studies (Vliet, 2011). Recent advances in the field of bioinformatics led to the development of visualization programs that formulate detailed graphical and mathematical models able to generate hypotheses of the system's behavior in response to a specific perturbation, such as toxic drug exposure, in the aim of providing systems level understanding of toxicity pathways (Vliet, 2011).

Accordingly in order to follow "systems biology" approach in the investigation of inflammation associated idiosyncratic drug-induced hepatotoxicity, several "Omics" technologies should be integrated in the proposed drug-inflammation model. The Omics technology, is constituted of different methods, namely: **Genomics**, "The study of genes and their function;" **Proteomics**, "The study of proteins;" **Metabolomics**, "The study of molecules involved in cellular metabolism;" **Transcriptomics**, "The study of the mRNA; "**Glycomics**, "The study of cellular carbohydrates" and **Lipomics**, "The study of cellular lipids" (Bhanushali et al. 2010). Particularly, genomics, transcriptomics, proteomics and metabolomics are extensively used to assess modifications at the transcript and protein level following the exposure to a toxic compound in order to provide a better prediction and understanding of drug adverse reactions (Ge and He, 2009). The combination of toxicology and genomics led to the establishment of a new sub-discipline, called toxicogenomics (Nuwaysir et al. 1999). The concept of toxicogenomics was developed by considering the structure and dynamics of the entire genome

in the study of drug-induced toxicity (Cui and Paules, 2010). Toxicogenomics employ advanced tools such as microarray and molecular imaging in order to reveal drug-induced alterations in gene expression; thus providing insights on the molecular or cellular hepatic processes that might be correlated with these alterations and generating mechanistic hypotheses of toxic drug responses (Cui and Paules, 2010; Suter et al. 2010). The thorough understanding of the mechanisms underlying drug-induced toxicity is primordial for accurate safety evaluation in the early phase of drug development (Cui and Paules, 2010). Furthermore, it has been recognized that omics methods provide new possibilities for the discovery of innovative biomarkers for target organ toxicities (Searfoss et al. 2005). The identification of sensitive and specific biomarkers for monitoring drug-induced liver injury is important to improve the detection of potentially toxic drugs in early preclinical stages (Searfoss et al. 2005; Waters and Fostel, 2004). Additionally, toxicogenomics profiles enable the classification of compounds exhibiting similar pathologies; based on the assumption that similar pathological phenotypes affect the same or related molecular mechanisms of toxicity that might be correlated with similar changes in gene expression (Waring et al. 2001). The identification of such toxicity-related gene expression signatures (fingerprints) may be used as a diagnostic or predictive tool in toxicity screening (Suter et al. 2004; Fielden et al. 2007; Rohrbeck et al. 2010; Zidek et al. 2007). Experimentally, the integration of toxicogenomics in the proposed drug-inflammation model of idiosyncratic hepatotoxicity could be processed as follows: HepG2 cells, HepaRG or human hepatocytes may be cultured as recommended and incubated for 24 hours with the four tested idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) in absence and presence of LPS and TNF-α. Subsequently the synergistic effect of idiosyncratic drugs and pro-inflammatory mediators is investigated by microarray technology following total extraction of RNA and preparation of cRNA (Liguori et al. 2008). Fragmented and labeled cRNA may then be hybridized to an Affymetrix human genome arrays, which contain sequences corresponding to roughly 22,200 transcripts, for microarray analysis according to the standard protocol provided by Affymetrix (Santa Clara, CA) (Liguori et al. 2008). The microarray scanned image and intensity files may be imported in Rosetta Resolver gene expression analysis software version 6.0 (Rosetta Inpharmatics, Kirkland, WA) (Liguori et al. 2008). Ratios for each treatment array versus its respective control may be built using Resolver's Affymetrix error model (Liguori et al. 2008). Agglomerative or divisive cluster analysis is subsequently performed using the average link heuristic criteria (agglomerative only) and the Euclidean distance metric for similarity measure (Liguori et al. 2008). Some microarray data may be also evaluated using the Panther Classification system (Liguori et al. 2008). Nowadays, microarray profiling has become one of the most used omics tool in the characterization of drug toxicities, predominantly because it allows large-scale determination of the gene expression changes that are correlated with a defined pathology (Harrill and Rusyn, 2008). The most important step in toxicogenomics is the ability to correlate a chemical-elicited phenotypic modification with alteration in gene expression changes; this phenomenon is known as "phenotypic anchoring" (Harrill and Rusyn, 2008). Experimentally determined gene expression signatures constitute a fundamental framework for

the identification of sensitive biomarkers that are indicative of toxicological responses before the appearance of severe clinical symptoms (Harrill and Rusyn, 2008). *Heinloth et al*. further confirmed the utility of this approach by demonstrating that patterns of gene expression perturbations observed at subtoxic doses of acetaminophen in rats indicated mild cellular injury that was not detectable by histopathology or clinical chemistry methods within the liver (Heinloth et al. 2004). At toxic doses, expression changes in the same subset of genes were correlated with mitochondrial dysfunction and oxidative stress (Heinloth et al. 2004). These observations imply that gene expression profiling has the potential to identify subtle markers of cellular injury that precede sudden overt organ toxicity (Harrill and Rusyn, 2008). Employing microarray profiling in the identification of innovative biomarkers that may detect inflammation associated idiosyncratic hepatotoxicity may avert severe suffering since this type of toxicity appears impulsively and without prior notice accompanied by critical clinical symptoms. Not only does toxicogenomics improve toxicity assessment but also it aids in identifying predisposed individuals to idiosyncratic hepatotoxicity; based on alterations the expression of relevant genes (Harrill and Rusyn, 2008). This further supports the urge of integrating toxicogenomics in the predictive models of idiosyncratic hepatotoxicity; especially that the latter is predominantly based on host-dependent innate or acquired genetic predisposition. With no doubt toxicogenomics present valuable insights on gene expressions and pathways that are modified by idiosyncratic drugs; however these insights are frequently descriptive and may not reflect changes at the protein level (Harrill and Rusyn, 2008). Genomic studies are indeed more comprehensive than high-throughput metabolite or protein analysis; however they fail to characterize the full complement of cellular proteins, which are often subject to drug-induced post-translational modifications and hence implicated in several drug adverse reactions (Harrill and Rusyn, 2008). To address this concern, a complete "toxicology systems" approach, including transcriptomics, metabolomics and proteomics, must be integrated in the predictive models of idiosyncratic hepatotoxicity; this will with no doubt optimize the efficacy and accuracy of these models.

Despite the fact that several interesting perspectives may ameliorate the accuracy and efficacy of the proposed drug-inflammation model, it is important to note that the work presented in this thesis constitute the cornerstone of these perspectives by validating the efficiency of the drug-inflammation synergistic approach as an important *in vitro* tool for the prediction of inflammation associated idiosyncratic hepatotoxicity. Furthermore, the results attained provide a framework for further development of more sophisticated similar *in vitro* models that are able to better elucidate the complex interplay of the various mechanisms underlying this type of toxicity.

Résumé de la thèse

I. Contexte scientifique

Le foie, organe privilégié du métabolisme et de l'élimination des xénobiotiques, est la cible principale des effets indésirables lors de l'administration des médicaments. Les hépatopathies médicamenteuses survenant sur un mode idiosyncrasique sont à l'origine de 13% des cas d'insuffisance hépatique aiguë. Elles représentent un obstacle majeur au développement de médicaments et sont à l'origine du retrait du marché de nombreux d'entre eux. Cette hépatotoxicité idiosyncrasique résulte généralement d'une hypersensibilité induite par les médicaments qui se produit de manière imprévisible, indépendamment de la dose et de la cible pharmacologique, chez des individus sensibles. Cette hépatotoxicité survient chez un petit nombre de patients à des doses thérapeutiques et non toxiques. Ces différences individuelles dans l'apparition de ces effets indésirables hépatotoxiques peuvent être principalement expliquées par des susceptibilités génétiques et environnementales. Ce type de toxicité représente un obstacle majeur au développement de médicaments en raison i) d'une part, du manque de tests de dépistage préclinique et de modèles prédictifs in vivo et in vitro et ii) d'autre part, de la complexité des mécanismes cellulaires et moléculaires sous jacents. Durant ces dernières années, plusieurs hypothèses ont été proposées pour expliquer ces hépatopathies idiosyncrasiques, en particulier, l'hypothèse du « stress inflammatoire ». Ainsi, la survenue d'un épisode inflammatoire aigu lors d'un traitement médicamenteux, sensibiliserait le foie pour les effets indésirables des médicaments, mettant en évidence leur toxicité idiosyncrasique. Ceci a été observé sur des modèles de rongeurs dans lesquels une inflammation aigue induite par du LPS lors de l'administration de médicaments a révélé leur toxicité idiosyncrasique. Cependant compte tenu de la très faible incidence des réactions idiosyncrasiques et de la nature non automatisable des modèles animaux ainsi que de la réglementation des « 3R », le développement de modèles cellulaires humains à haut débit devient nécessaire afin de mieux comprendre et d'élucider les mécanismes d'hépatotoxicité idiosyncrasique.

II- Objectif

Ainsi, notre travail s'articule sur 3 axes :

i) Développer un modèle prédictif *in vitro* à haut débit d'hépatotoxicité idiosyncrasique liée à un stress inflammatoire.

ii) Evaluer l'implication des transporteurs d'efflux ABC (MDR1 et MRP2) dans ce type de toxicité et comprendre l'impact de l'inflammation sur la modulation de leur activité.

iii) Etudier les mécanismes moléculaires sous jacents de la mort cellulaire induite par une hépatotoxicité idiosyncrasique couplée à un stress inflammatoire.

Dans une première partie, nous avons mis en place un modèle cellulaire prédictif d'hépatotoxicité idiosyncrasique dans un contexte inflammatoire. Ce modèle est basé sur l'hypothèse du stress inflammatoire qui explique qu'un épisode inflammatoire modeste peut réduire le seuil d'hépatotoxicité d'un médicament en réduisant la fenêtre thérapeutique aboutissant ainsi à une réaction toxique à des doses thérapeutiques (Shaw et al. 2010). En effet, la présence de nombreux médiateurs de l'inflammation au cours d'un traitement médicamenteux sensibilise le foie aux effets indésirables des médicaments (Roth et Ganey, 2010). Cependant, il est également possible que le médicament lui même puisse prolonger la réaction inflammatoire en aggravant l'hépatopathie médicamenteuse. Ainsi, induire une réaction inflammatoire au cours de la thérapie médicamenteuse peut révéler le potentiel caché de certains médicaments hépatotoxiques dès les premiers stades de développement d'un médicament (Shaw et al. 2010). Le stress inflammatoire peut être induit par divers mécanismes comme une infection, un traumatisme, des brûlures, des blessures, etc… (Ganey et al 2004). Les lipopolysaccharides (LPS), composants essentiels de la membrane externe des bactéries, se sont avérés être de puissants inducteurs de stress inflammatoire *in vivo*. Dans les modèles animaux d'hépatotoxicité idiosyncrasique développés antérieurement, des doses non toxiques de médicaments à potentiel idiosyncrasique deviennent hépatotoxiques lors de la co-exposition à une dose non toxique mais modestement inflammatoire de LPS (Shaw et al. 2010). Plusieurs médicaments connus pour leur potentiel d'hépatotoxicité idiosyncrasique chez l'homme, ont été évalués comme la trovafloxacine, la ranitidine, le sulindac, la chlorpromazine, l'halothane, l'amiodarone, et le diclofénac. Tous ces médicaments se sont révélés hépatotoxiques chez des rongeurs en cas de co-administration avec une quantité non toxique de médiateurs inflammatoires (Buchweitz et. al 2002; Cheng et al, 2009; Deng et al, 2006; Luyendyk et al, 2003; Waring et al, 2006; Zou et al 2009). Cependant, les analogues structuraux de ces médicaments de la même classe pharmacologique qui ne possèdent pas ce potentiel idiosyncrasique chez l'homme, n'ont pas entraîné d'hépatotoxicité en synergie avec le stress inflammatoire sur les modèles de rongeurs (Waring et al. 2006). Bien que ces modèles animaux se soient avérés être de bons modèles pour révéler le potentiel hépatotoxique idiosyncrasique de certains médicaments, ils présentent plusieurs limites dans la prédiction de l'hépatotoxicité idiosyncrasique des médicaments induite chez l'homme (Xu et al, 2004). En effet, (1) il existe d'importantes différences inter-espèces entre animaux et humains, liées à la pharmacocinétique des médicaments rendant les modèles peu prédictibles (Li, 2004). (2) La diversité biologique n'est pas représentée puisque les animaux sous des conditions expérimentales bien contrôlées, ne sont pas représentatifs des êtres humains vivant dans des conditions hétérogènes (Xu et al. 2004). (3) Il est difficile de distinguer les effets primaires des effets secondaires dus à des interactions complexes entre le foie et d'autres organes (Guillouzo, 1998). (4) Le taux d'incidence de ces réactions chez les animaux est extrêmement faible car ce mode de toxicité idiosyncrasique résulte principalement des susceptibilités génétiques et/ou environnementales chez l'homme (Peters, 2005). En raison de tous ces inconvénients, des modèles cellulaires *in vitro* humains sont de plus en plus recherchés pour être plus adaptés à la

prédiction de la toxicité hépatique médicamenteuse idiosyncrasique chez l'homme et fournir des indications intéressantes sur les mécanismes de toxicité (Gronenberg et al. 2002).

A l'heure actuelle, il existe peu de modèles cellulaires prédictifs capables de détecter les médicaments qui seront potentiellement hépatotoxiques dans un contexte inflammatoire avant leur mise sur le marché. De plus, les mécanismes toxiques sous jacents de l'hépatotoxicité idiosyncrasique liée à un stress inflammatoire ne sont pas clairement élucidés. Aussi, notre travail vise principalement à développer un nouveau modèle cellulaire prédictif d'hépatotoxicité idiosyncrasique dans un contexte inflammatoire et à élucider les mécanismes cellulaires et moléculaires qui sous-tendent l'hépatotoxicité de quatre médicaments idiosyncrasiques (la trovafloxacine, le nimésulide, la télithromycine et la néfazodone) en mettant l'accent sur l'apoptose, la stéatose, le stress oxydatif et la cholestase. Les résultats obtenus dans cette thèse indiquent que ce modèle peut être considéré comme un outil préclinique efficace pour la prédiction de l'hépatotoxicité idiosyncrasique associé à un stress inflammatoire et l'élucidation des mécanismes toxiques qui sous-tendent l'amplification des lésions hépatiques médicamenteuse dans un contexte inflammatoire, fournissant ainsi des indications précieuses sur les moyens de prévenir les effets indésirables des médicaments idiosyncrasiques et sur la manière d'identifier les patients qui ont un risque élevé de développer ces réactions (Holt et Ju, 2006).

Dans une première partie, cette thèse présente un nouveau modèle cellulaire, adaptable au criblage haut débit, pour la prédiction de l'hépatotoxicité idiosyncrasique associé à un stress inflammatoire. Ce modèle est basé sur l'exposition synergique des cellules HepG2, cultivées dans des conditions particulières, à des médiateurs pro-inflammatoires et des médicaments potentiellement idiosyncrasiques durant 24 heures. Initialement, l'hépatotoxicité de quatre médicaments idiosyncrasiques connus (la trovafloxacine, le nimésulide, la télithromycine et la néfazodone) et leurs analogues non idiosyncrasiques (la lévofloxacine, l'aspirine, la clarithromycine et la buspirone) a été évaluée en absence et présence d'un contexte inflammatoire pour valider la sensibilité et la spécificité du modèle. La réaction inflammatoire a été provoquée par incubation des cellules avec un mélange contenant du LPS et du TNF-α. Ce mélange a induit une sécrétion élevée d'IL-8 au bout de 24 heures ce qui confirme son effet inflammatoire sur les cellules HepG2. L'hépatotoxicité des quatre médicaments idiosyncrasiques et de leurs analogues non idiosyncrasiques a été évaluée en mesurant leur effet apoptotique. Cet effet a été étudié par un test d'apoptose fluorescent (AnnexinV-FITC/PI, Miltenyi Biotec) et analysé par la cytométrie en flux (Figure 7.1). En effet, le modèle développé s'est avéré être sensible et spécifique puisque les quatre médicaments idiosyncrasiques, mais pas leurs analogues non idiosyncrasiques, induisent un effet apoptotique amplifié en présence d'un contexte inflammatoire. Par la suite, ce modèle a été utilisé pour détecter les effets hépatotoxiques potentiels lors d'un stress inflammatoire, de plusieurs médicaments anticancéreux, principalement en raison de la corrélation rapportée entre une réaction inflammatoire et la survenue d'effets indésirables médicamenteux chez les patients cancéreux (Morgan et al 2008). Après criblage d'un grand nombre de médicaments anticancéreux, plusieurs ont présenté un effet

apoptotique accru lorsqu'ils sont incubés avec les cellules en présence de LPS et de TNF-α, spécifiquement l'azaguanine-8, le nocodazole, le méthotrexate, l'étoposide, l'azacytidine-5, le chlorambucile, la cytarabine, le busulfan, la docétaxel, le 5-fluorouracile, l'erlotinib, l'imatinib, et la fludarabine. Ces résultats indiquent que ces médicaments peuvent entraîner une hépatotoxicité sévère s'ils sont administrés à des patients souffrant d'une inflammation aigue (Figure 7.2). En conséquence, ce modèle représente un outil préclinique de choix pour la détection des médicaments qui seront potentiellement hépatotoxiques dans un contexte inflammatoire; cet outil combiné avec une approche prédictive clinique complémentaire peut constituer une stratégie efficace pour la prévention des effets idiosyncratiques indésirables des médicaments. Les mécanismes toxiques exacts par lesquels un médicament idiosyncrasique agit en synergie avec des médiateurs pro-inflammatoires tels que LPS et TNF-α, pour entraîner des hépatopathies médicamenteuses sévères, restent encore à ce jour mal connu. Cependant, ce travail de thèse a démontré que le stress oxydatif, la stéatose et la cholestase sont impliqués à des degrés divers dans l'hépatotoxicité idiosyncrasique liée à un stress inflammatoire. Dans cette première partie, les potentiels oxydatifs et stéatosiques des quatre médicaments idiosyncrasiques (la trovafloxacine, le nimésulide, la télithromycine et la néfazodone) ont été étudiés en absence et en présence d'un contexte inflammatoire (Figures 7.3 et 7.4).

Concernant les potentiels oxydatifs des quatre médicaments idiosyncrasiques, nos résultats montrent que seule la néfazodone induit une accumulation significative de radicaux superoxydes lorsqu'elle est incubée seule avec les cellules (Figure 7.3A). Par contre, les quatre médicaments favorisent l'accumulation intracellulaire de radicaux superoxydes d'une manière significative lorsqu'ils sont incubés en présence de LPS et TNF-α (Figure 7.3B). Cependant seuls les potentiels oxydatifs du nimésulide et de la néfazodone sont significativement augmentés lors d'un stress inflammatoire par comparaison à leur effets oxydatifs lorsqu'ils sont incubés seuls avec les cellules (Figure 7.3C).

En ce qui concerne les effets stéatosiques des quatre médicaments idiosyncrasiques, nos résultats montrent que la trovafloxacine, la télithromycine et la néfazodone induisent significativement l'accumulation intracellulaire des triglycérides lorsqu'ils sont incubés seuls avec les cellules (Figure 7.4A). Par contre, lorsque les quatre médicaments sont incubés en présence de LPS et TNF-α, seules la trovafloxacine et la néfazodone s'avèrent significativement stéatosique par rapport au contrôle négatif (cellules incubées avec 0.5% DMSO) (Figure 7.4B). En comparant les potentiels stéatosiques des quatre médicaments en absence et en présence d'un stress inflammatoire, nos résultats indiquent que les effets stéatosiques de la trovafloxacine et de la télithromycine diminuent significativement alors que l'effet de la néfazodone reste inchangé lors d'un stress inflammatoire par rapport à leur effets stéatosiques lorsqu'ils sont incubés seuls avec les cellules (Figure 7.3C).

Globalement, nos résultats montrent que le stress oxydatif lié à l'accumulation de radicaux superoxydes est impliqué dans l'hépatotoxicité idiosyncrasique associée à un stress inflammatoire du nimésulide et de la néfazodone alors que la stéatose semble être fortement impliquée dans la toxicité idiosyncrasique de la néfazodone elle-même.

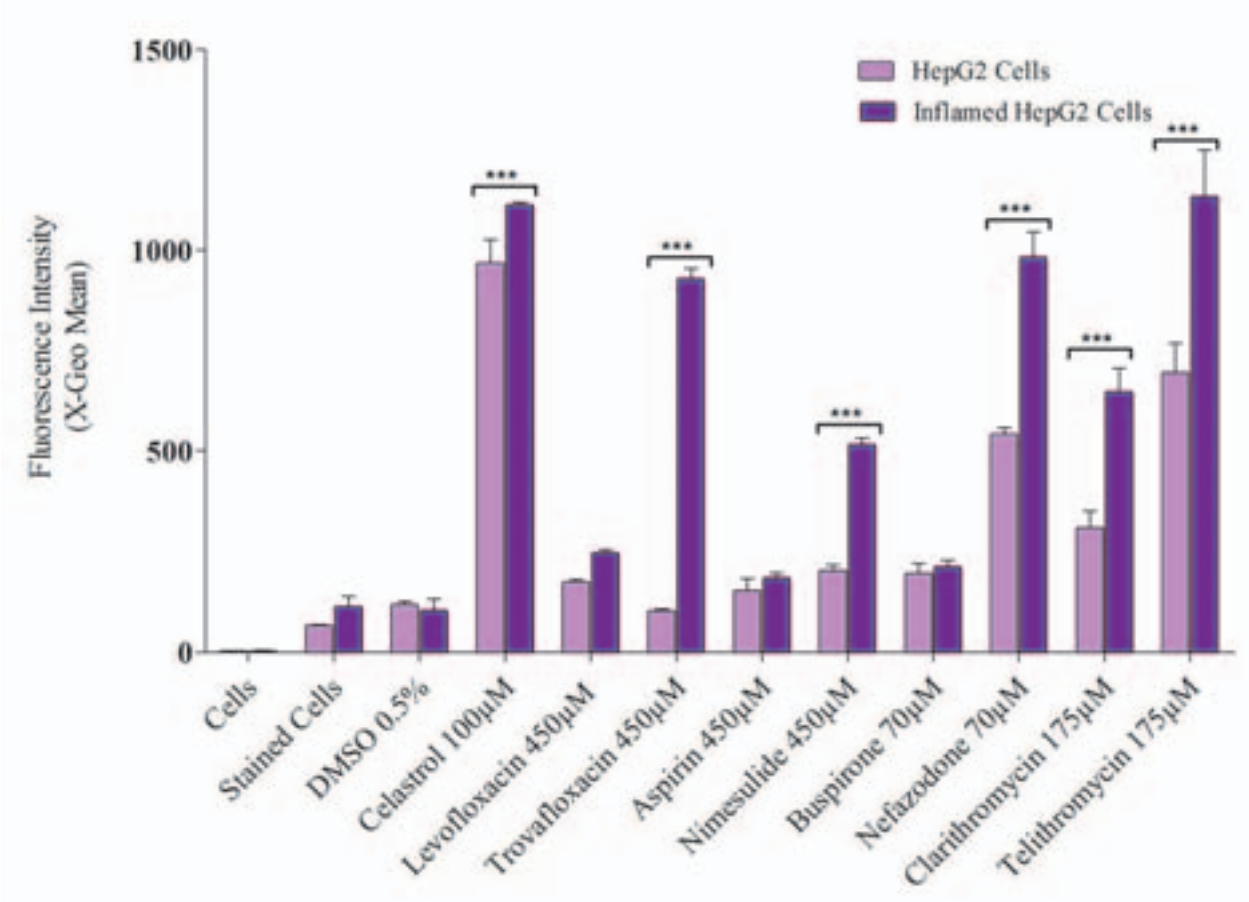


Figure 7.1. Effet apoptotique des quatre médicaments idiosyncrasiques et de leurs analogues structuraux sur cellules HepG2 en absence et en présence de stimuli inflammatoires. Après incubation des cellules HepG2 avec les quatre médicaments idiosyncrasiques en présence et en absence de LPS et de TNF-α pendant 24 heures, la mort hépatocellulaire est évaluée suivant le double marquage de cellules avec AnnexinV-FITC et PI par cytométrie en flux. Les données sont représentées sous forme de moyenne ± S.E.M (n = 3). L'analyse statistique est réalisée par test ANOVA suivi de Bonferroni post test.
*** = P <0.001 et représente la variation de l'effet apoptotique entre les cellules HepG2 non-enflammées et enflammées.

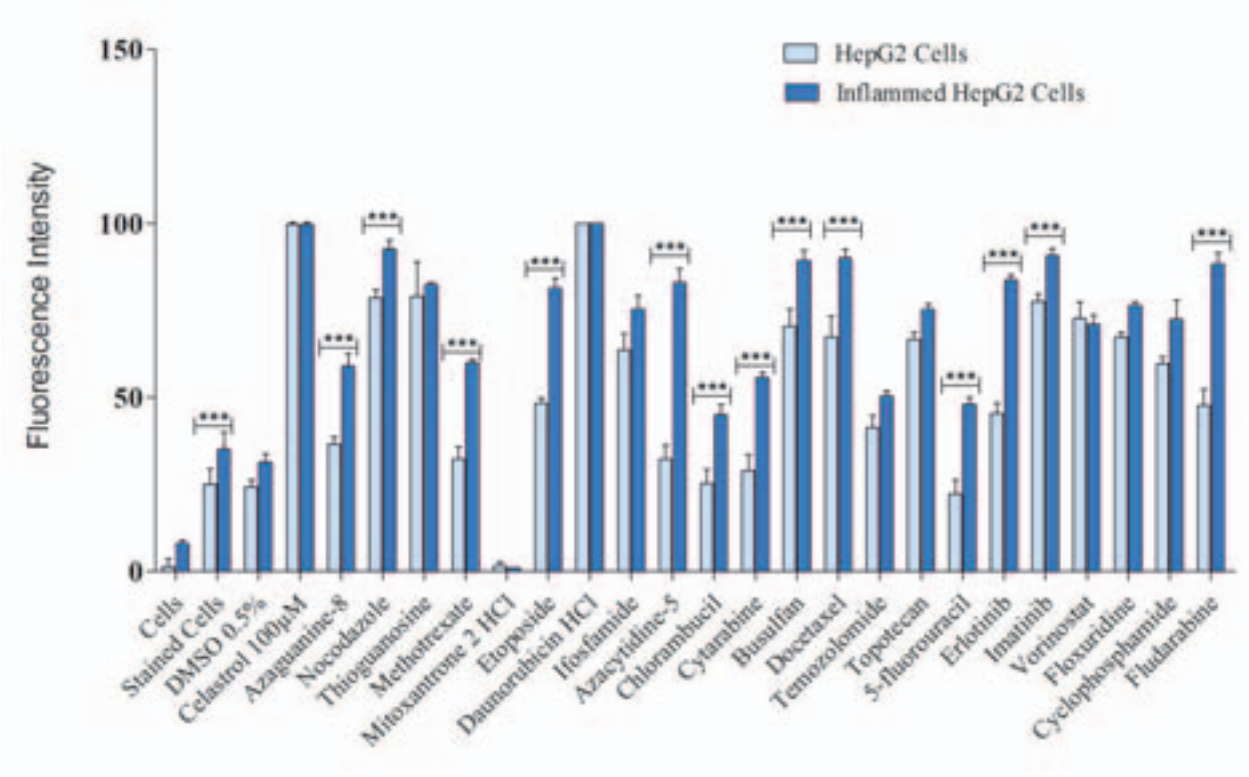


Figure 7.2. Effet apoptotique des médicaments anticancéreux sur cellules HepG2 en absence et en présence de stimuli inflammatoires. Après incubation des cellules HepG2 avec 22 médicaments anticancéreux en présence et en absence de LPS et de TNF-α pendant 24 heures, la mort hépatocellulaire est évaluée suivant le double marquage de cellules avec AnnexinV-FITC et PI par cytométrie en flux. Les données sont représentées sous forme de moyenne ± S.E.M (n = 3). L'analyse statistique a été réalisée par test ANOVA suivi de Bonferroni post test.
*** = P <0.001 et représente la variation de l'effet apoptotique entre les cellules HepG2 non-enflammées et enflammées.

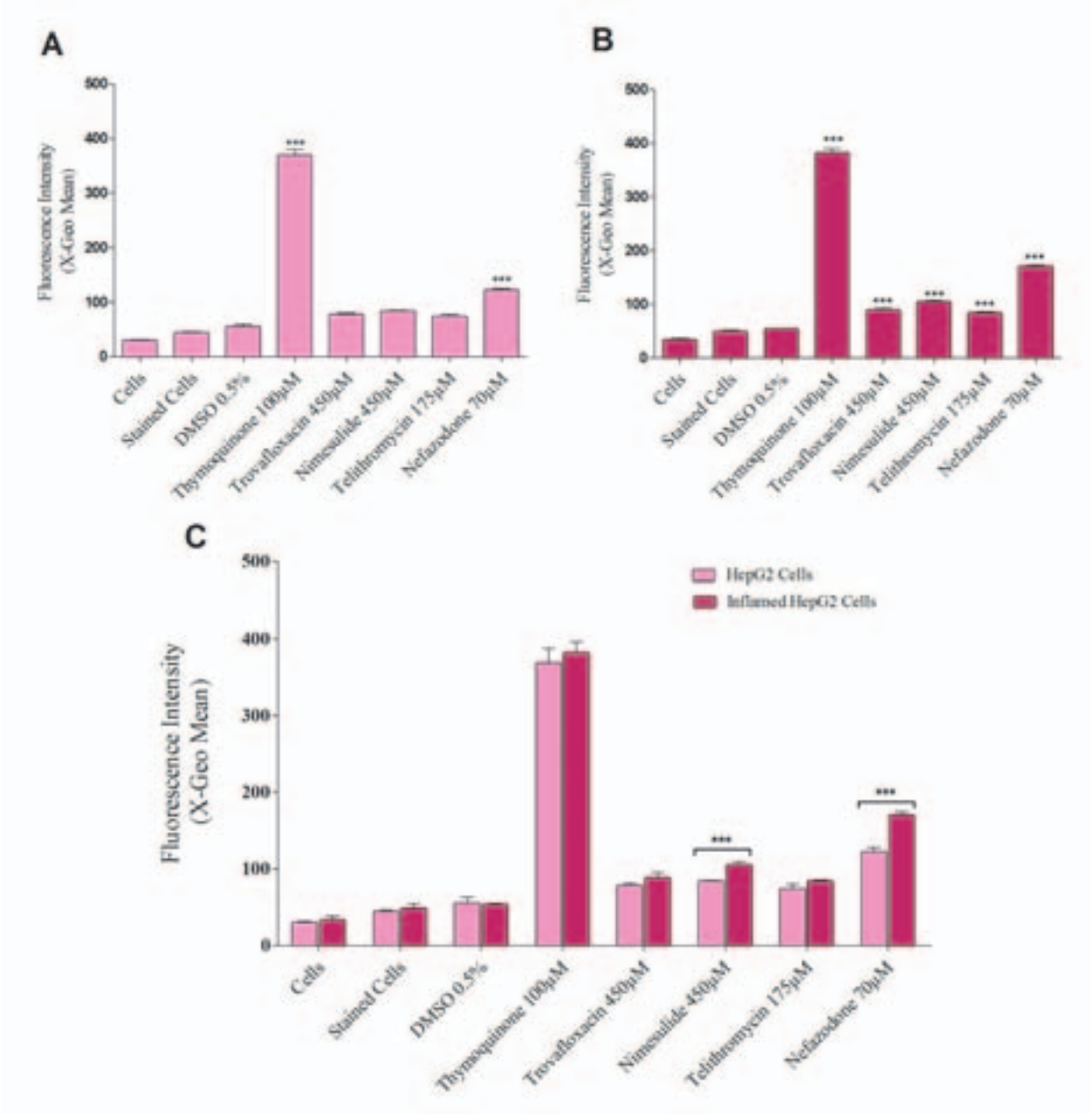


Figure 7.3. Effet oxydatif des quatre médicaments idiosyncrasiques sur cellules HepG2 en absence et en présence de stimuli inflammatoires. Les graphes représentent les effets oxydatifs des molécules testées après incubation avec des cellules HepG2 (A), en présence d'un mélange de LPS et TNF-α (B) et en absence et en présence du mélange LPS et TNF-α pendant 24 heures (C). L'accumulation intracellulaire de radicaux superoxydes est évaluée en utilisant DHE (dihydroéthidium) par cytométrie en flux. Les barres représentent la fluorescence intracellulaire rouge d'éthidium. Les données sont représentées sous forme de moyenne ± S.E.M (n = 3). L'analyse statistique est réalisée par test ANOVA suivi de Bonferroni post test. *** = $P < 0.001$ et représente sur les graphes A et B la variation de l'effet oxydatif entre les cellules traitées et non-traitées (contrôle négatif). Sur le graphe C, la valeur p représente la variation de l'effet oxydatif entre les cellules HepG2 non-enflammées et enflammées.

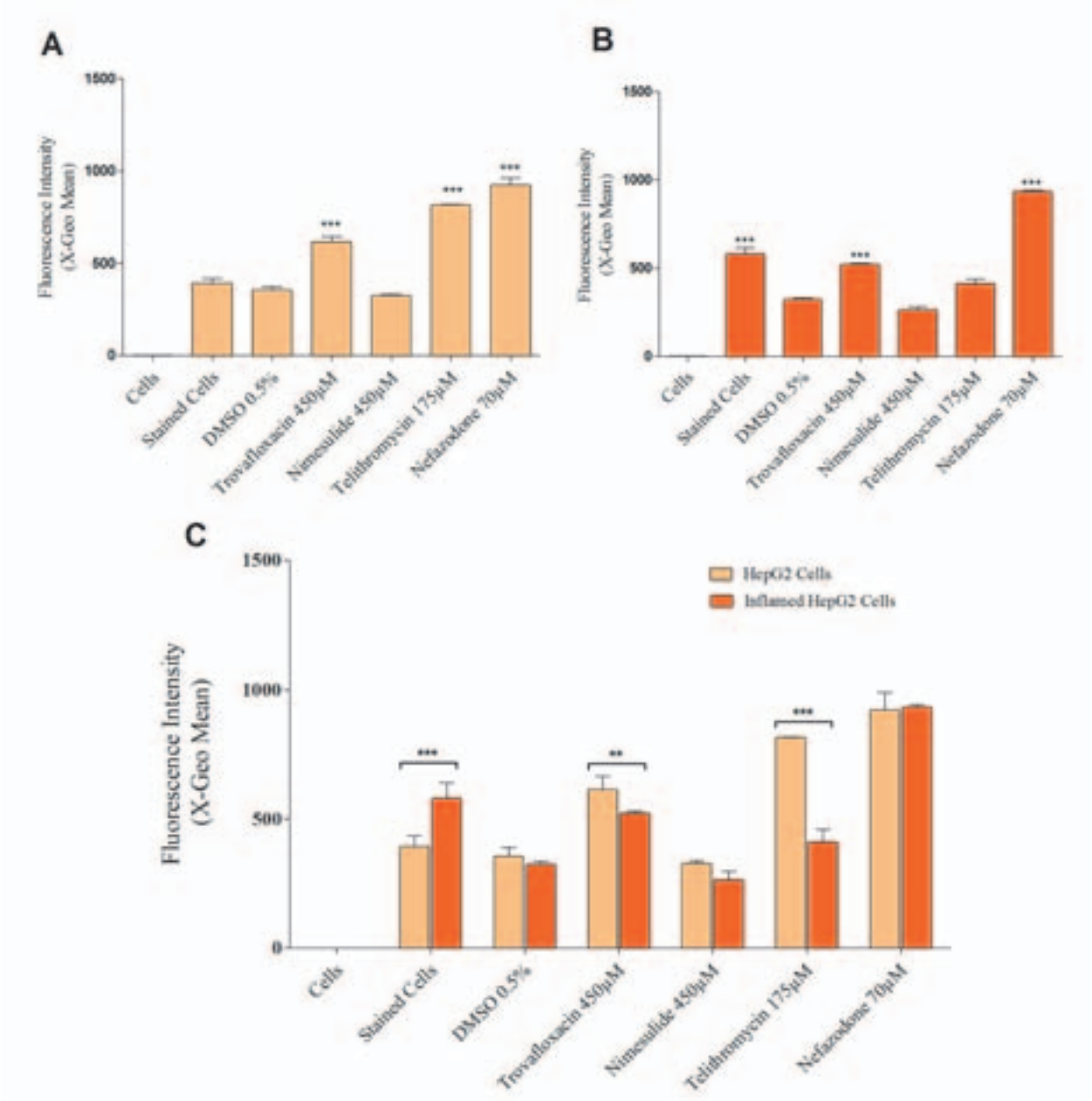


Figure 7.4. Effet stéatosique des quatre médicaments idiosyncrasiques sur cellules HepG2 non enflammées et enflammées. Les graphes représentent les effets stéatosiques des quatre médicaments testées après leur incubation dans les cellules HepG2 (A), en présence de LPS et de TNF-α (B) et en absence et en présence de LPS et de TNF-α pendant 24 heures (C). L'effet stéatosique est évalué en utilisant BODIPY 493/503 par cytométrie en flux. Les barres représentent la fluorescence intracellulaire accumulée de BODIPY 493/503. Les données sont représentées sous forme de moyenne ± S.E.M (n = 3). L'analyse statistique est réalisée par test ANOVA suivi de Bonferroni post test. ** Et *** = P <0,01 et P <0,001 respectivement. Sur les graphes A et B, la valeur p représente la variation de l'effet stéatosique entre les cellules traitées et non-traitées (contrôle négatif). Sur le graphe C, la valeur p représente la variation de l'effet stéatosique entre les cellules HepG2 non-enflammées et enflammées.

Dans une deuxième partie, nous avons recherché si la cholestase pouvait être un des mécanismes sous-jacents de l'hépatotoxicité idiosyncrasique. Pour cela, nous avons étudié l'effet de ces quatre médicaments (la trovafloxacine, le nimésulide, la télithromycine et la néfazodone) sur l'expression et l'activité de deux transporteurs ABC d'efflux (MDR1 et MRP2) dans notre modèle d'hépatotoxicité idiosyncrasique. En effet, les transporteurs membranaires jouent un rôle essentiel dans la pharmacocinétique des xénobiotiques, et en particulier dans leur élimination biliaire pouvant ainsi moduler l'efficacité et la toxicité des médicaments. Néanmoins, ce rôle dépend principalement de la polarité des cellules hépatiques et de la localisation des protéines membranaires spécifiques à leurs pôles respectifs (transporteurs SLC sur la membrane basolatérale et transporteurs ABC sur les membranes apical et sinusoïdal). Parmi ces transporteurs, nous nous sommes intéressés à l'implication des protéines MDR1 et MRP2 dans les mécanismes d'hépatotoxicité idiosyncrasique.

Avant d'étudier l'implication de ces deux transporteurs dans l'hépatotoxicité idiosyncrasique, nous avons vérifié s'ils étaient correctement situés sur le pôle apical des cellules HepG2, confirmant ainsi la polarité correcte des cellules hépatiques et leur fonctionnalité. Pour cela, nous avons incubé les cellules en présence d'un substrat fluorescent (5-(6)-carboxy-2',7' dichloro-fluorescéine (CDF)), qui s'accumule sur le pôle apical entre deux hépatocytes polarisés. Nos résultats montrent que nos cellules sont bien polarisées (Figure 7.5).

Afin de voir l'effet modulateur de ces médicaments sur l'expression et l'activité de ces transporteurs et d'étudier l'impact de l'inflammation sur cette modulation, nous avons incubé les 4 médicaments tests dans nos cellules hépatiques soumises ou non à un stress inflammatoire. L'effet sur l'expression de ces transporteurs est étudié par cytométrie en flux en utilisant un anticorps monoclonal couplé à un fluorochrome fluorescent (pour l'étude de MDR1) (Figure 7.6), et par western blot (pour l'étude de MRP2) (Figure7.8). L'effet sur l'activité d'efflux de MDR1 et de MRP2 est analysé par cytométrie en flux, en utilisant des tests de transport avec des substrats fluorescents spécifiques pour respectivement MDR1 et MRP2 (Rhodamine 123 et CDF) (Figure7.7 et 7.9).

Concernant les effets des quatre médicaments sur l'expression du MDR1, nos résultats montrent que la trovafloxacine, la télithromycine et la néfazodone induisent significativement l'expression des protéines MDR1 lorsqu'ils sont incubés seuls avec les cellules (Figure 7.6A). Par contre, les quatre médicaments induisent l'effet inverse lorsqu'ils sont incubés avec les cellules en présence de LPS et TNF-α. Par rapport au contrôle négatif (cellules traitées avec 0.5% DMSO), les quatre médicaments réduisent significativement l'expression des protéines MDR1 (Figure 7.6B). Nos résultats indiquent que la trovafloxacine, le nimésulide, la télithromycine et la néfazodone diminuent significativement l'expression des protéines MDR1 lors d'un stress inflammatoire par rapport à leurs effets lorsqu'ils sont incubés seuls avec les cellules (Figure 7.6C).

Par rapport à l'effet des quatre médicaments sur l'activité d'efflux du transporteur MDR1, les résultats obtenus montrent que lorsqu'ils sont incubés seuls avec les cellules, la télithromycine et la néfazodone inhibent significativement l'activité d'efflux de ce transporteur alors que le nimésulide la favorise (Figure 7.7A). Cependant, lorsque ces quatre médicaments sont incubés

avec les cellules en présence de LPS et de TNF-α, les effets inhibiteurs de la télithromycine et de la néfazodone sont fortement atténués (Figure 7.7B). Malgré cette atténuation, ces deux médicaments restent des inhibiteurs de l'activité d'efflux du transporteur MDR1. De plus, la trovafloxacine s'avère être un inhibiteur de l'efflux du transporteur MDR1 uniquement en présence des stimuli inflammatoires.

Concernant les effets des quatre médicaments sur l'expression du MRP2, nos résultats montrent que lorsqu'ils sont incubés seuls avec les cellules, la trovafloxacine et la néfazodone induisent significativement l'expression des protéines MRP2 alors que le nimésulide et la télithromycine la réduisent. Par contre, lors de l'incubation de ces médicaments avec les cellules en présence de LPS et TNF-α, l'expression des protéines MRP2 est complètement supprimée. Cependant une très faible expression est observée lorsque la télithromycine est co-incubée avec les cellules en présence de LPS et TNF-α (Figure 7.8).

Par rapport à l'effet des quatre médicaments idiosyncrasiques sur l'activité du transporteur MRP2, nos résultats montrent qu'en absence de stimuli inflammatoires, la trovafloxacine inhibe l'activité d'efflux du MRP2 alors que la néfazodone l'active en comparant au contrôle négatif (cellules traitées avec 0.5% DMSO) (Figure 7.9A). Par contre, lorsque les quatre médicaments idiosyncrasiques sont incubés avec les cellules en présence de LPS et TNF-α, la trovafloxacine, le nimésulide et la néfazodone s'avèrent des inhibiteurs de l'efflux du transporteur MRP2 (Figure 7.9B).

Globalement les résultats obtenus démontrent que lors d'un stress inflammatoire, la trovafloxacine diminue drastiquement l'expression des protéines MDR1 et MRP2 et inhibe leurs activités d'efflux. Le nimésulide réduit d'une manière significative l'expression de MDR1 et MRP2 et inhibe fortement l'activité d'efflux de MRP2. La télithromycine diminue fortement l'expression des protéines MDR1 et MRP2 et s'avère être un inhibiteur de MDR1 même si son effet en présence de LPS et de TNF-α est moins puissant que lorsqu' elle est incubée seule avec les cellules. La néfazodone supprime complètement l'expression des protéines MRP2 tout en réduisant celle des protéines MDR1 et inhibe l'activité d'efflux des deux transporteurs. Ces résultats indiquent que la cholestase peut être considérée comme un mécanisme sous-jacent de l'hépatotoxicité idiosyncrasique de ces médicaments. Le nimésulide s'avère cholestatique via un mécanisme qui implique également la réduction de l'expression et l'inhibition de l'activité des protéines MRP2. Par contre, la télithromycine semble provoquer la mort des cellules hépatiques, dans un contexte inflammatoire, par un mécanisme cholestatique qui implique la diminution et l'inhibition des protéines MDR1 et non des protéines MRP2. La néfazodone et la trovafloxacine semblent impliquer l'inhibition de l'expression et de l'activité des deux transporteurs MDR1 and MRP2 dans leurs effets cholestatiques.

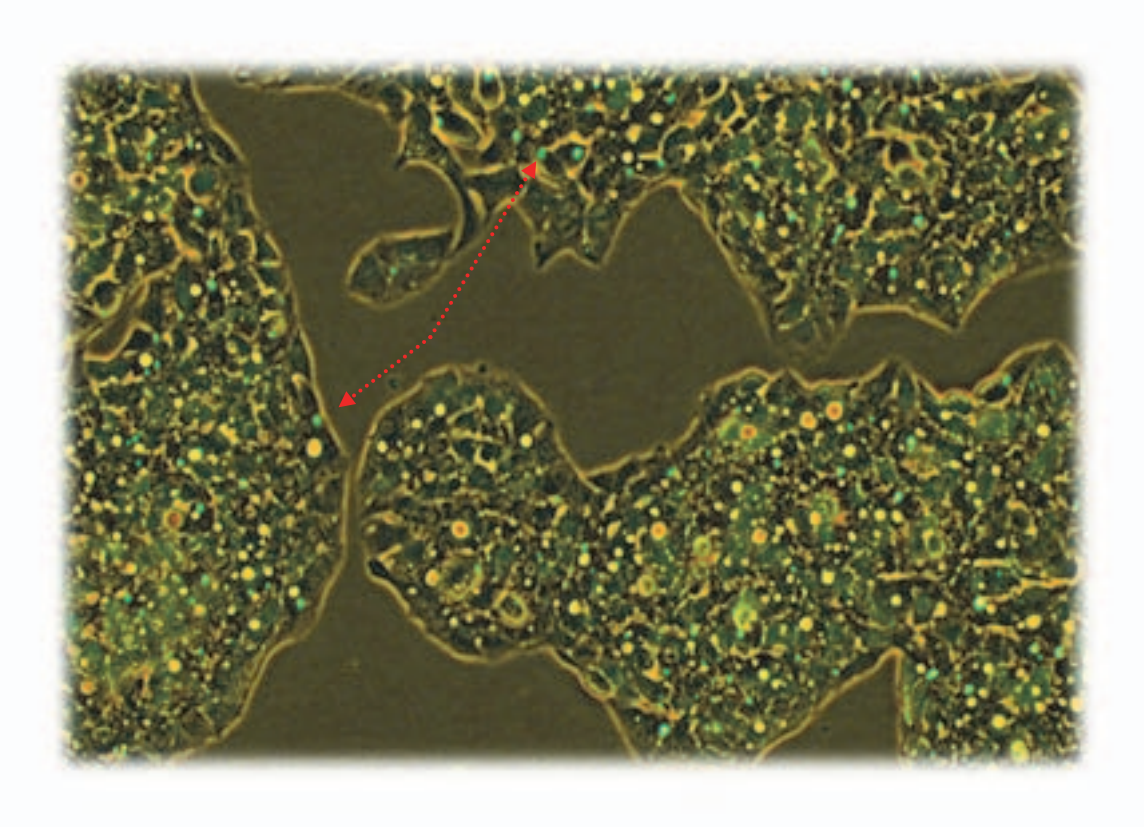


Figure 7.5. Localisation des pôles biliaires. Après incubation des cellules HepG2 avec du CDFDA, le diacetate de fluorescéine rendu fluorescent par estérification dans la cellule (CDF) est pris en charge par les transporteurs, et s'accumule aux pôles biliaires au niveau des canalicules si les pôles biliaires sont présents et fonctionnels. Les flèches rouges désignent ces pole biliaires (point vert brillant).

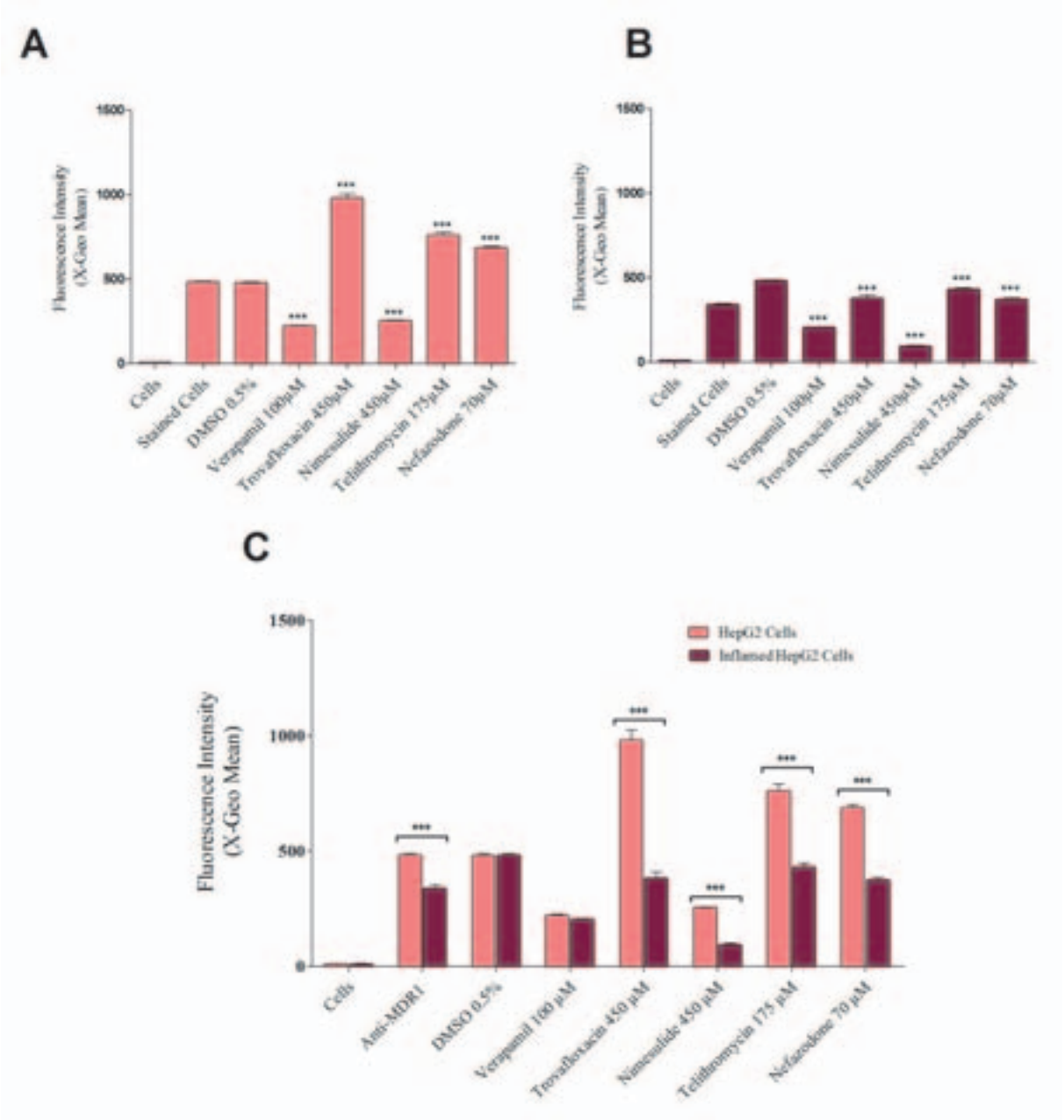


Figure 7.6. Effet des quatre médicaments idiosyncrasiques sur l'expression des protéines MDR1 dans les cellules HepG2 non enflammées et enflammées. Dans la figure A, les quatre médicaments idiosyncrasiques sont incubés dans les cellules HepG2. Dans la figure B les mêmes médicaments sont incubés dans les cellules HepG2 en présence de LPS et de TNF-α pendant 24 heures. La figure C représente l'effet des quatre médicaments sur l'expression MDR1 en absence et en présence de LPS et de TNF-α pendant 24 heures. L'expression des protéines MDR1 est évaluée à l'aide d'un anticorps monoclonal couplé à un fluorochrome (FITC) par cytométrie en flux. Les barres représentent l'intensité de la fluorescence intracellulaire émise suite à la liaison de l'anticorps anti-MDR1 à sa cible. La fluorescence détectée est directement proportionnelle à la quantité des protéines MDR1 présentes dans les cellules HepG2. Les données sont représentées sous forme de moyenne ± S.E.M (n = 3). L'analyse statistique est réalisée par test ANOVA suivi de Bonferroni post test. *** = P <0,001 et représente sur les graphes A et B la variation de l'effet des médicaments sur l'expression MDR1 entre les cellules traitées et non-traitées (contrôle négatif). Sur le graphe C, la valeur p représente la variation de l'effet des médicaments sur l'expression MDR1 entre les cellules HepG2 non-enflammées et enflammées.

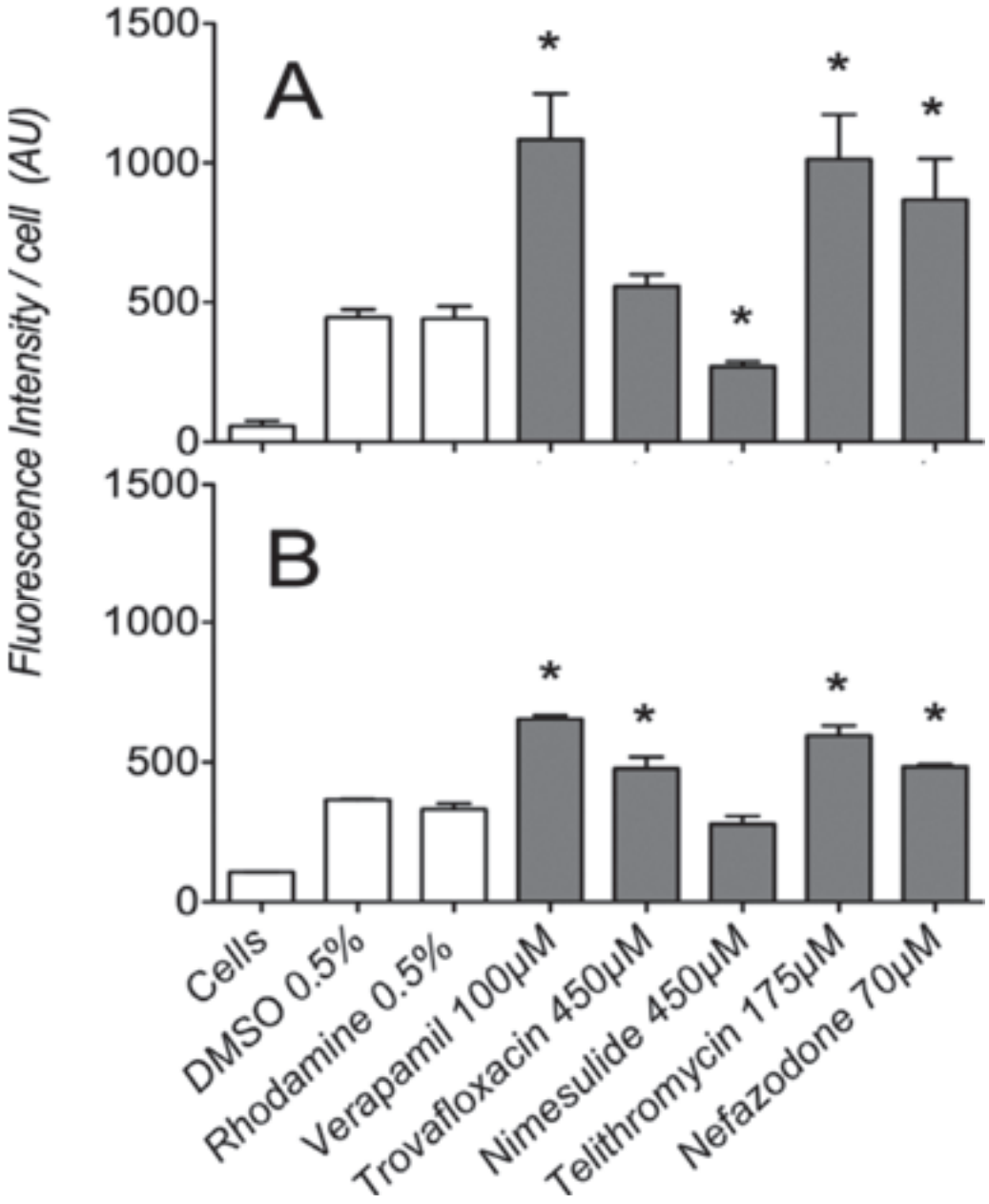


Figure 7.7. L'effet des quatre médicaments idiosyncrasiques sur l'activité des transporteurs MDR1 en absence et présence de stimuli inflammatoires. Après incubation de cellules HepG2 avec les quatre médicaments idiosyncrasiques en absence (A) ou en présence (B) de LPS et de TNF-α pendant 24 heures, l'activité des transporteurs MDR1 est évaluée à l'aide d'un test de transport fluorescent (Rhodamine 123) par cytométrie en flux. Les barres représentent l'intensité de la fluorescence intracellulaire accumulée de la rhodamine 123. Les données sont représentées sous forme de moyenne ± S.E.M (n = 4). L'analyse statistique est réalisée à l'aide d'un test t non apparié

* = P <0.05 et représente la variation de l'activité des transporteurs MDR1 entre les cellules HepG2 traitées et non traitées (contrôle négatif).

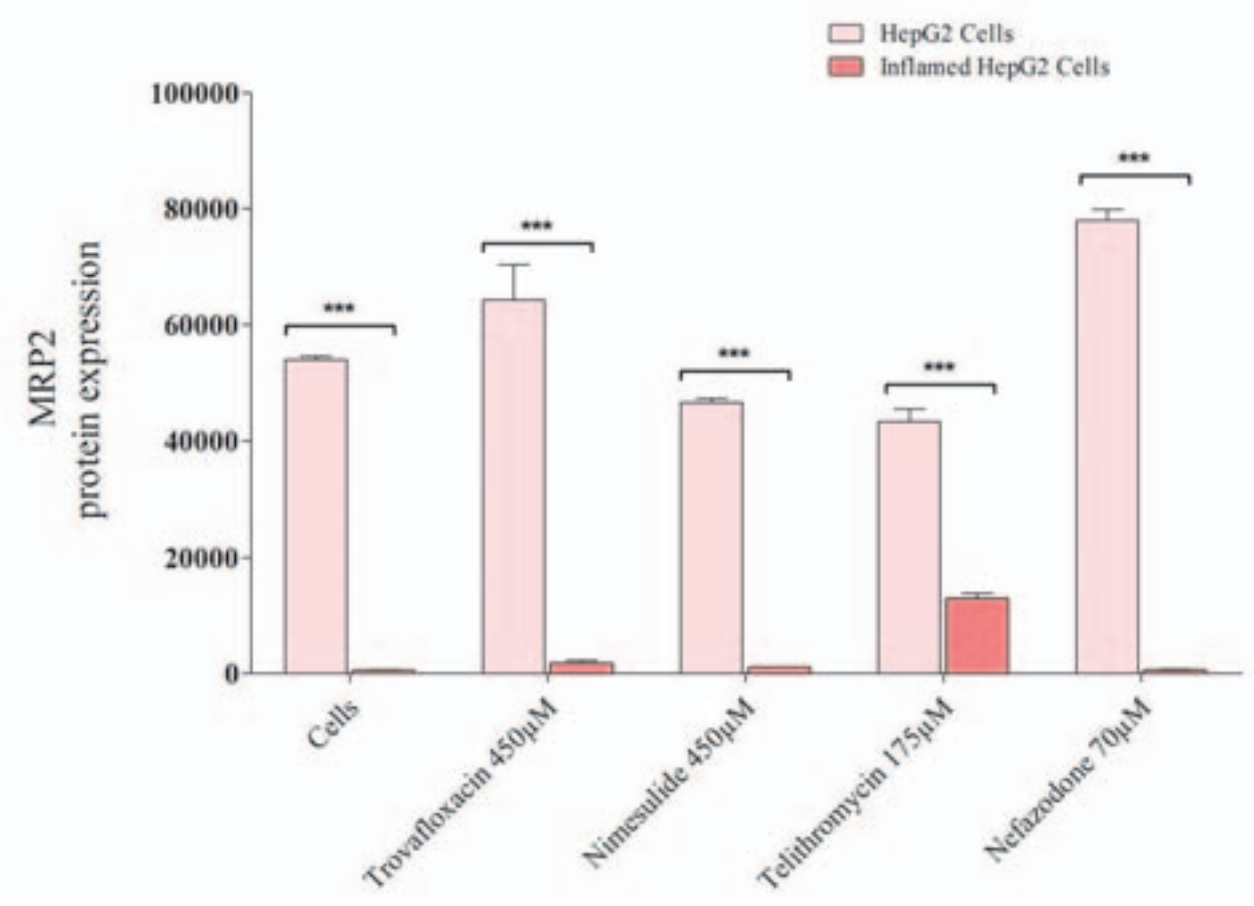


Figure 7.8. Effet des quatre médicaments idiosyncrasiques sur l'expression des protéines MRP2 en absence et présence de stimuli inflammatoires. Après incubation de cellules HepG2 avec les quatre médicaments idiosyncrasiques en présence et en absence de LPS et de TNF-α pendant 24 heures, l'expression des protéines MRP2 est évaluée par western blot. Cette expression est également quantifiée par le logiciel Image J. Les barres représentent l'analyse densitométrique de trois expériences différentes (n = 3). Les données sont représentées sous forme de moyenne ± S.E.M. L'analyse statistique est réalisée à l'aide d'ANOVA suivie par Bonferroni post test *** = P < 0.001 et représente la variation de l'expression des protéines MRP2 entre les cellules HepG2 non enflammées et enflammées.

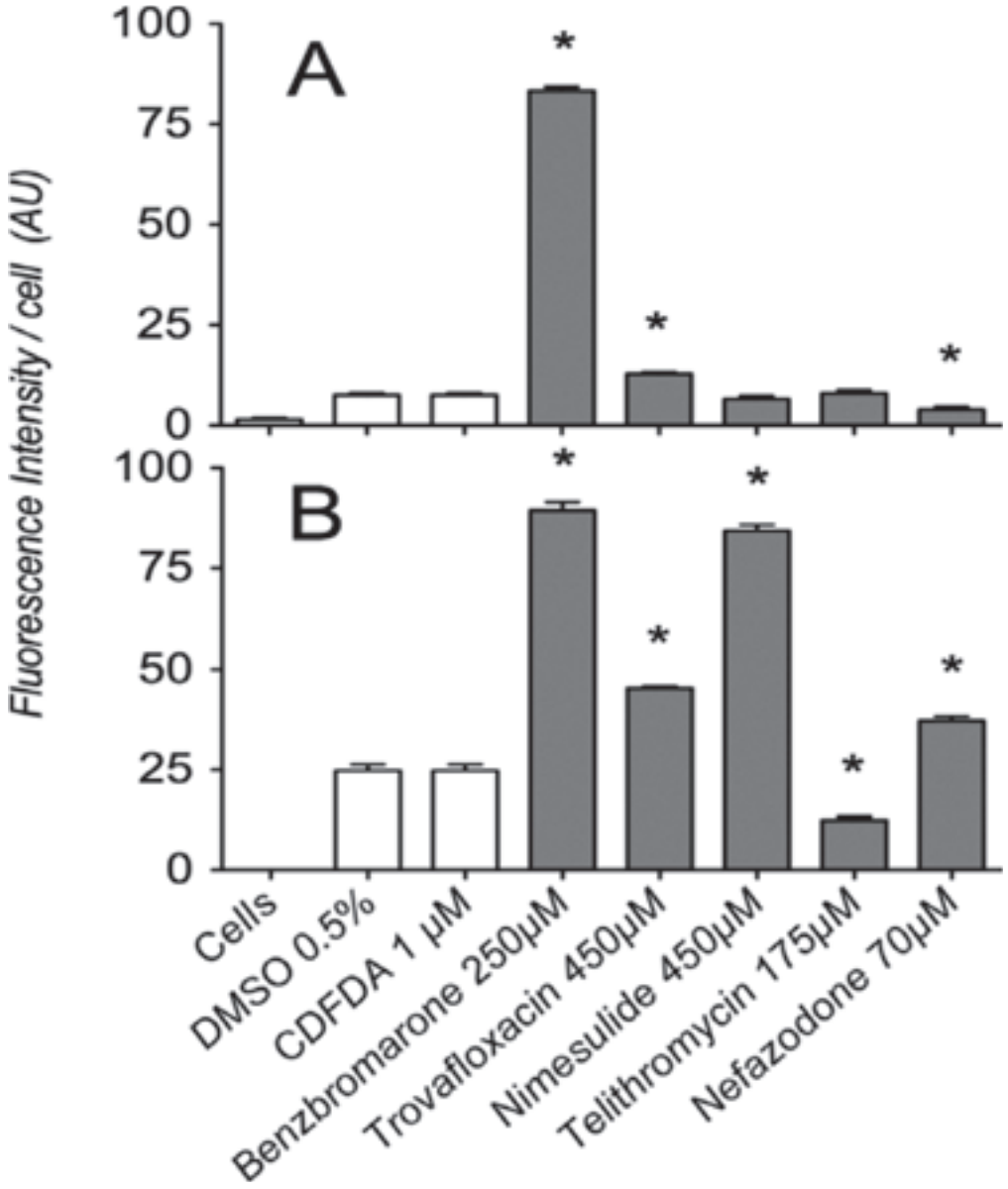


Figure 7.9. Effet des quatre médicaments idiosyncrasiques sur l'activité des transporteurs MRP2 dans les cellules HepG2 non enflammées et enflammées. Après incubation de cellules HepG2 avec les quatre médicaments idiosyncrasiques en absence (A) ou en présence (B) de LPS et de TNF-α pendant 24 heures, l'activité des transporteurs MRP2 est évaluée à l'aide d'un test de transport fluorescent (CDF) par cytométrie en flux. Les barres représentent l'intensité de la fluorescence intracellulaire accumulée du CDF. Les données sont représentées sous forme de moyenne ± S.E.M (n = 4). L'analyse statistique est réalisée à l'aide d'un test t non apparié.
* = P <0.05 et représente la variation de l'activité des transporteurs MDR1 entre les cellules HepG2 traitées et non traitées (contrôle négatif).

Dans une troisième partie nous avons étudié les mécanismes moléculaires sous-jacents la mort cellulaire observée lors d'une hépatotoxicité idiosyncrasique liée à un stress inflammatoire. Bien que chaque médicament idiosyncrasique des quatre médicaments testés ait montré un mécanisme d'action différent, ils ont tous induit un effet apoptotique amplifié en présence d'un contexte inflammatoire. Principalement, un médicament toxique peut induire l'apoptose des cellules hépatiques (par lui-même ou par l'intermédiaire de ses métabolites réactifs) soit indirectement en induisant une dysfonction mitochondriale qui va mener à l'activation de la voie apoptotique intrinsèque, soit directement en interagissant avec les récepteurs de mort exprimés sur la surface des hépatocytes activant la voie apoptotique extrinsèque (Holt et Ju, 2006). Plusieurs protéines sont impliquées dans la régulation de l'apoptose hépatocellulaire. Particulièrement nous avons étudié dans cette partie la modulation de l'expression de p53, Bax, caspase 8, tBid, p21 et pERK½ lors d'une hépatotoxicité idiosyncrasique liée à un stress inflammatoire.

P53 est un gène suppresseur de tumeur dénommé le «gardien du génome» par son rôle important dans la protection des cellules contre un stress sévère, notamment des dommages de l'ADN, soit en déclenchant l'apoptose, soit en favorisant l'arrêt du cycle cellulaire à la phase G1 (Amundson et al, 1998; He et al 2005). Le fait que p53 soit considéré comme le pivot central dans la régulation de l'apoptose intrinsèque et extrinsèque, a suscité notre intérêt pour évaluer son implication dans l'hépatotoxicité idiosyncrasique associé à un stress inflammatoire.

Bax est une protéine apoptotique importante qui favorise l'ouverture des pores de transition de perméabilité des mitochondries et la libération de molécules proapoptotiques comme le cytochrome c, déclenchant ainsi la phase effectrice de l'apoptose intrinsèque. Ainsi, une surexpression de Bax conduit à une augmentation de la sensibilité de la cellule à l'apoptose intrinsèque (Russman et al. 2009 ; Haupt et al. 2003). La caspase 8 est considérée comme la caspase initiatrice ou activatrice de la voie apoptotique extrinsèque. Cette caspase est normalement activée suite à la liaison des ligands spécifiques (FasL, TNF, etc…) sur certains récepteurs de mort cellulaire (CD95/Apo-1/Fas, TNFR1, etc..) exprimés à la surface des hepatocytes (Bantel and Schulze-Osthoff, 2012). Dans les cellules de type I (monocytes), la quantité de caspase 8 activée au niveau des récepteurs est suffisante pour promouvoir directement l'activation des caspases effectrices en aval (caspase-3, -7 et -6) et générer les changements morphologiques caractéristiques de l'apoptose (Bantel and Schulze-Osthoff, 2012). Par contre, dans les cellules de type II comme les hépatocytes, cette quantité de caspase 8 activée est insuffisante et doit engager la voie apoptotique mitochondriale, par l'intermédiaire de la protéine proapoptotique Bid, pour entraîner la mort cellulaire (Bantel and Schulze-Osthoff, 2012). Suite au clivage de Bid par caspase 8, le fragment C-terminal de la protéine (tBid) est translocalisé vers la mitochondrie où il induit la libération de cytochrome c (Li et al.1998; Luo et al.1998).

P21 est impliquée dans le contrôle du cycle cellulaire et est étroitement liée à la différenciation et à la mort cellulaire programmée (Gartel and Tyner, 2002). Le déroulement du cycle cellulaire est permis par l'activation séquentielle de complexes protéiques particuliers, les dimères cycline/Cdk (Harper et al. 1993). La protéine p21Waf1/Cip1 est capable, en se liant à ces complexes d'en

inhiber l'activité, permettant l'arrêt du cycle (Harper et al. 1993). La surexpression de cette protéine est observée dans les cellules ayant subi des stimulis, entraînant, la réparation de l'ADN, la différenciation, la sénescence, ou l'apoptose des cellules (Harper et al. 1993).

Bien que la voie MAPK, particulièrement la famille ERK, soit attribuée à la survie dans de nombreux types cellulaires, de nombreuses études plus récentes ont montré que ERK ½ est également impliquée dans la médiation de l'apoptose intrinsèque et extrinsèque (Zhuang et Schnellmann, 2006). ERK ½ régulerait ainsi l'apoptose intrinsèque en induisant la sur-expression des protéines p53 et Bax, favorisant ainsi la libération de cytochrome c et l'activation de la caspase-3 (Zhuang et Schnellmann, 2006). De plus, ERK ½ s'est avéré être également impliquée dans l'induction de l'apoptose extrinsèque en favorisant la production de TNF-α et l'activation de la caspase 8 (Zhuang et Schnellmann, 2006).

En conséquence, l'effet des quatre médicaments idiosyncrasiques (la trovafloxacine, le nimésulide, la télithromycine et la néfazodone) a été testé sur l'expression de plusieurs protéines impliquées dans l'apoptose intrinsèque et extrinsèque (p53, Bax, caspase 8, tBid, p21 et pERK½), en présence ou non d'un contexte inflammatoire. Les effets des quatre médicaments idiosyncrasiques sur l'expression de p53, Bax, caspase 8, et pERK½ sont illustrés dans les Figures 7.10, 7.11, 7.12 et 7.13.

Nos résultats montrent que la trovafloxacine induit l'expression de la protéine p53 alors que le nimésulide, la télithromycine et la néfazodone favorisent sa réduction. Ceci indique que seul l'effet apoptotique de la trovafloxacine pourrait être régulé par la p53 alors que les trois autres médicaments entraînent la mort cellulaire par des mécanismes indépendamment de p53.

Particulièrement, la trovafloxacine induit significativement l'expression des protéines Bax et pERK ½. Par contre son effet sur l'activité de la caspase 8 est relativement faible. Ces résultats indiquent que la trovafloxacine probablement entraine la mort cellulaire par la voie intrinsèque en favorisant l'expression des protéines Bax par un mécanisme dépendant de l'activation de la voie MAPK, plus spécifiquement de la famille ERK ½.

Le nimésulide induit l'expression des protéines Bax également par l'intermédiaire d'un mécanisme dépendant d'ERK ½, mais elle induit également l'activation de la caspase 8 d'une manière significative. Ces résultats indiquent que ce médicament induit l'apoptose par l'intermédiaire des deux voies, l'intrinsèque et l'extrinsèque.

La télithromycine induit fortement l'expression des protéines Bax et pERK ½ , indiquant que cette molécule induit l'apoptose via la voie intrinsèque par un mécanisme qui dépend largement de l'activation de ERK ½. En outre, la télithromycine présente un effet inducteur puissant sur l'expression de p21 dans un contexte inflammatoire, ce qui indique que ce médicament favorise l'arrêt du cycle cellulaire, en plus de l'apoptose.

La néfazodone induit l'expression des protéines Bax à un degré moindre que les autres trois autres médicaments testés mais il est cependant l'inducteur le plus puissant de l'activité caspase 8. Ces résultats indiquent que l'effet apoptotique de la néfazodone est principalement médié par la voie apoptotique extrinsèque, mais il implique l'activation de la voie mitochondriale via l'induction modérée de Bax.

Nous avons testé l'effet des quatre médicaments idiosyncrasiques sur l'expression de la forme tronquée de Bid (tBid) pour expliquer si l'implication de la voie intrinsèque dans le potentiel apoptotique de ces médicaments passe par le clivage de Bid dépendant de la caspase 8. Le fait qu'aucun des médicaments étudié n'ait induit l'expression de tBid en présence d'un contexte inflammatoire suggère que l'implication de la voie mitochondriale est effectuée directement par des dommages mitochondriaux d'origine médicamenteuse plutôt que par l'intermédiaire de tBID. Enfin, il semble que le potentiel apoptotique amplifié observé en présence des médicaments idiosyncrasiques en synergie avec des médiateurs pro-inflammatoires soit médié par l'activation simultanée des deux voies de l'apoptose, intrinsèque et extrinsèque, mais par un mécanisme indépendant de tBid. Le mécanisme d'activation concomitante des deux voies apoptotiques lors d'une hépatotoxicité idiosyncrasique liée à un stress inflammatoire pourrait être expliqué de la manière suivante. Les médicaments idiosyncrasiques pourraient agir comme des agonistes du TNF-α, soit en induisant sa synthèse soit en favorisant l'expression de son récepteur (TNFR1) favorisant ainsi l'activation de la caspase 8 et l'induction de la voie apoptotique extrinsèque. Simultanément, ces mêmes médicaments provoqueraient des dommages mitochondriaux qui déclencheraient par la suite, la surexpression de Bax, la libération de facteurs pro-apoptotiques, la formation de l'apoptosome et l'activation des caspases effectrices conduisant à l'exécution de l'apoptose intrinsèque. Il est à noter que le potentiel apoptotique amplifié des quatre médicaments idiosyncrasiques en présence d'un contexte inflammatoire, que ce soit par la voie intrinsèque ou extrinsèque, est principalement basé sur la surexpression des protéines ERK ½ phosphorylées.

En conclusion, cette thèse présente un modèle cellulaire fiable pour la prédiction de l'hépatotoxicité idiosyncrasique associée à un stress inflammatoire et de ses mécanismes cellulaires et moléculaires sous-jacents. Les résultats présentés dans cette thèse ont élucidé les mécanismes de toxicité inconnus de quatre médicaments idiosyncrasiques (la trovafloxacine, le nimésulide, la télithromycine et la néfazodone) indiquant que ce modèle peut être utilisé non seulement comme un outil préclinique efficace pour l'identification de nouveaux médicaments qui pourraient être potentiellement hépatotoxiques lors d'un stress inflammatoire, mais aussi pour l'élucidation de leurs mécanismes. L'identification des voies par lesquelles un médicament idiosyncrasique exerce ses effets indésirables fournit sans doute des indications précieuses sur la prévention de l'hépatotoxicité idiosyncrasique de nouvelles molécules et pourrait contribuer au développement de marqueurs biologiques pour identifier des patients à risque.

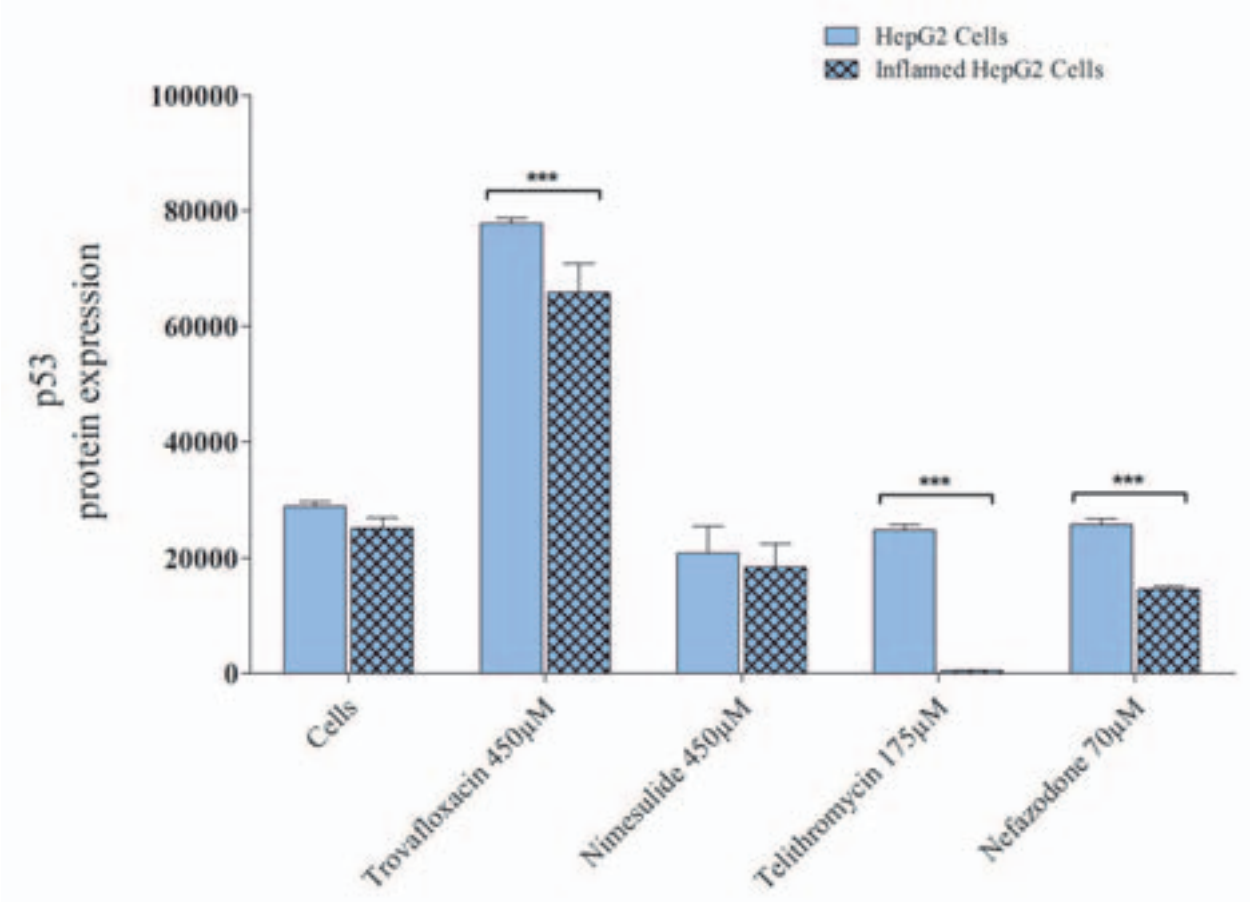


Figure 7.10. Effet des quatre médicaments idiosyncrasiques sur l'expression des protéines p53 dans les cellules HepG2 non enflammées et enflammées. Après incubation des cellules HepG2 avec les quatre médicaments idiosyncrasiques en présence et en absence de LPS et TNF-α pendant 24 heures, l'expression des protéines p53 est évaluée par western blot. Cette expression est également quantifiée par le logiciel Image J. Les barres représentent l'analyse densitométrique de trois expériences différentes (n = 3). Les données sont représentées sous forme de moyenne ± S.E.M. L'analyse statistique est réalisée à l'aide d'ANOVA suivie par Bonferroni post test.
*** = $P < 0,001$ et représente la variation de l'expression des protéines p53 entre les cellules HepG2 non enflammées et enflammées.

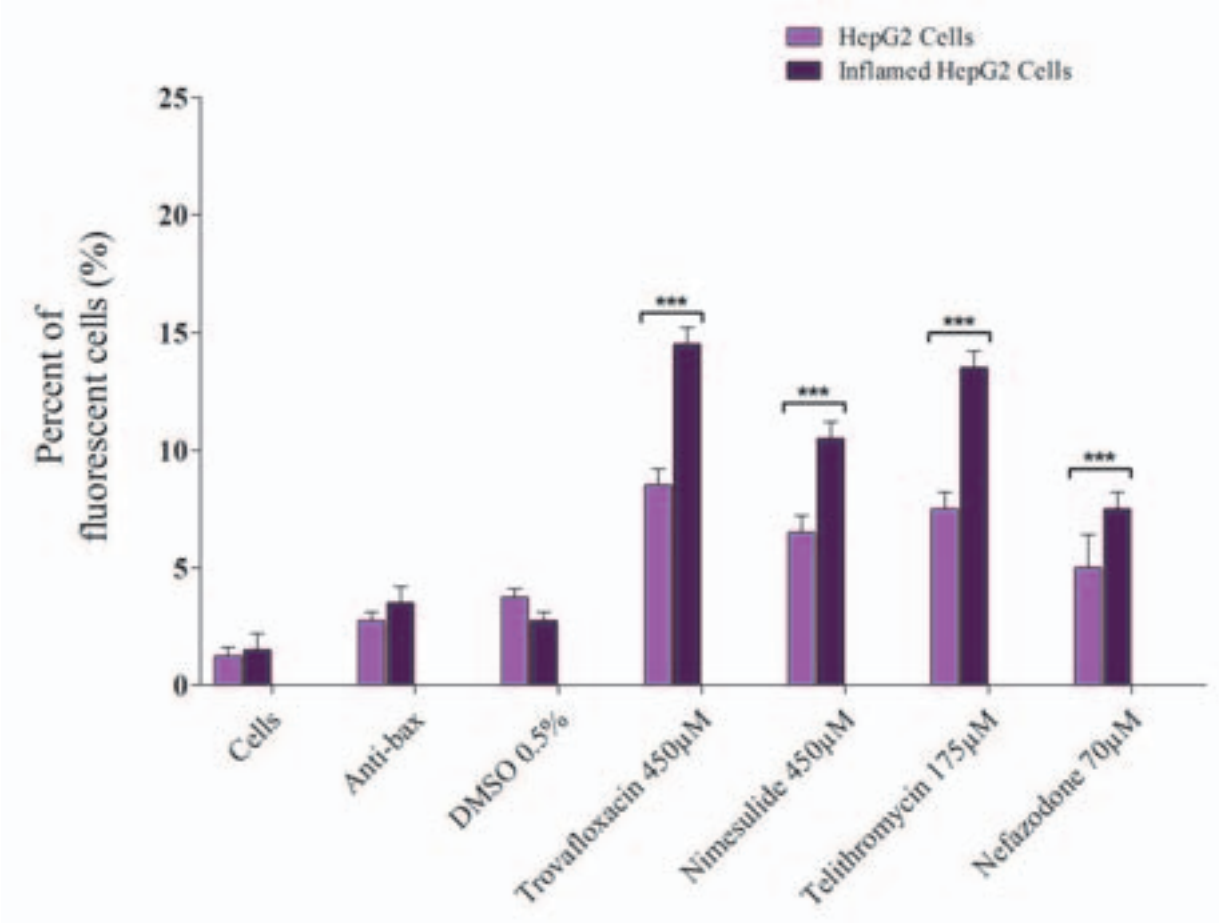


Figure 7.11. Effet des quatre médicaments idiosyncrasiques sur l'expression des protéines Bax dans les cellules HepG2 non enflammées et enflammées. Après incubation des cellules HepG2 avec les quatre médicaments idiosyncrasiques en présence et en absence de LPS et TNF-α pendant 24 heures, l'expression des protéines p53 est évaluée par western blot. Cette expression est également quantifiée par le logiciel Image J. Les barres représentent l'analyse densitométrique de trois expériences différentes (n = 3). Les données sont représentées sous forme de moyenne ± S.E.M. L'analyse statistique est réalisée à l'aide d'ANOVA suivie par Bonferroni post test.
*** = P < 0,001 et représente la variation de l'expression des protéines Bax entre les cellules HepG2 non enflammées et enflammées.

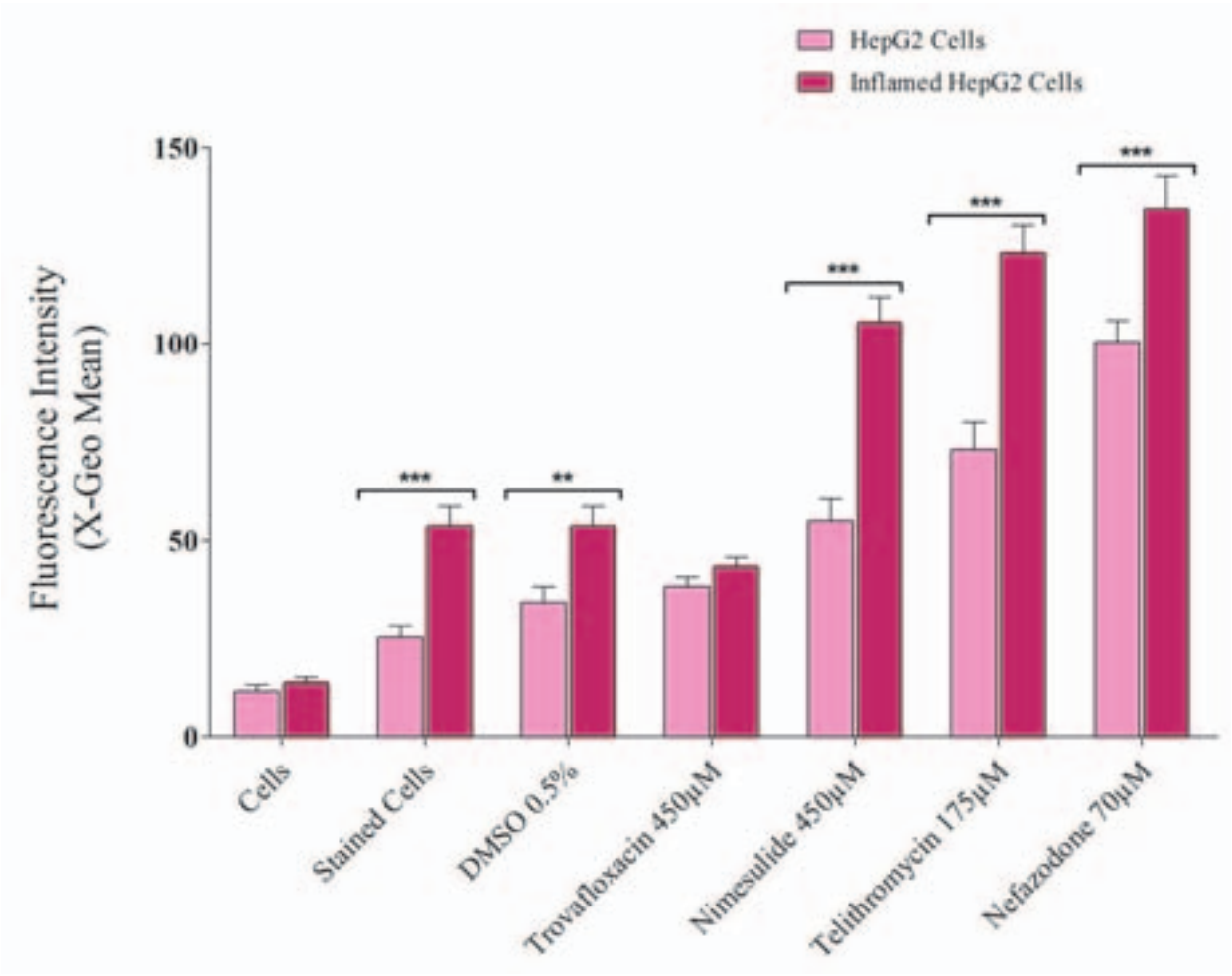


Figure 7.12. Effet des quatre médicaments idiosyncrasiques sur l'activité des caspases 8 dans les cellules HepG2 non enflammées et enflammées. Après incubation des cellules HepG2 avec les quatre médicaments idiosyncrasiques en absence et en présence de LPS et de TNF-α pendant 24 heures, l'activité des caspases 8 est mesurée par cytométrie en flux. Les barres représentent la fluorescence émise qui est proportionnelle au nombre des caspases 8 actives présentes dans les cellules. Les données sont représentées sous forme de moyenne ± S.E.M (n = 3). L'analyse statistique est réalisée à l'aide d'ANOVA suivie par Bonferroni post test.
** et*** : $P < 0,01$ et $P < 0,001$ respectivement; la valeur p représente la variation de l'activité des enzymes caspases 8 entre les cellules HepG2 non enflammées et enflammées.

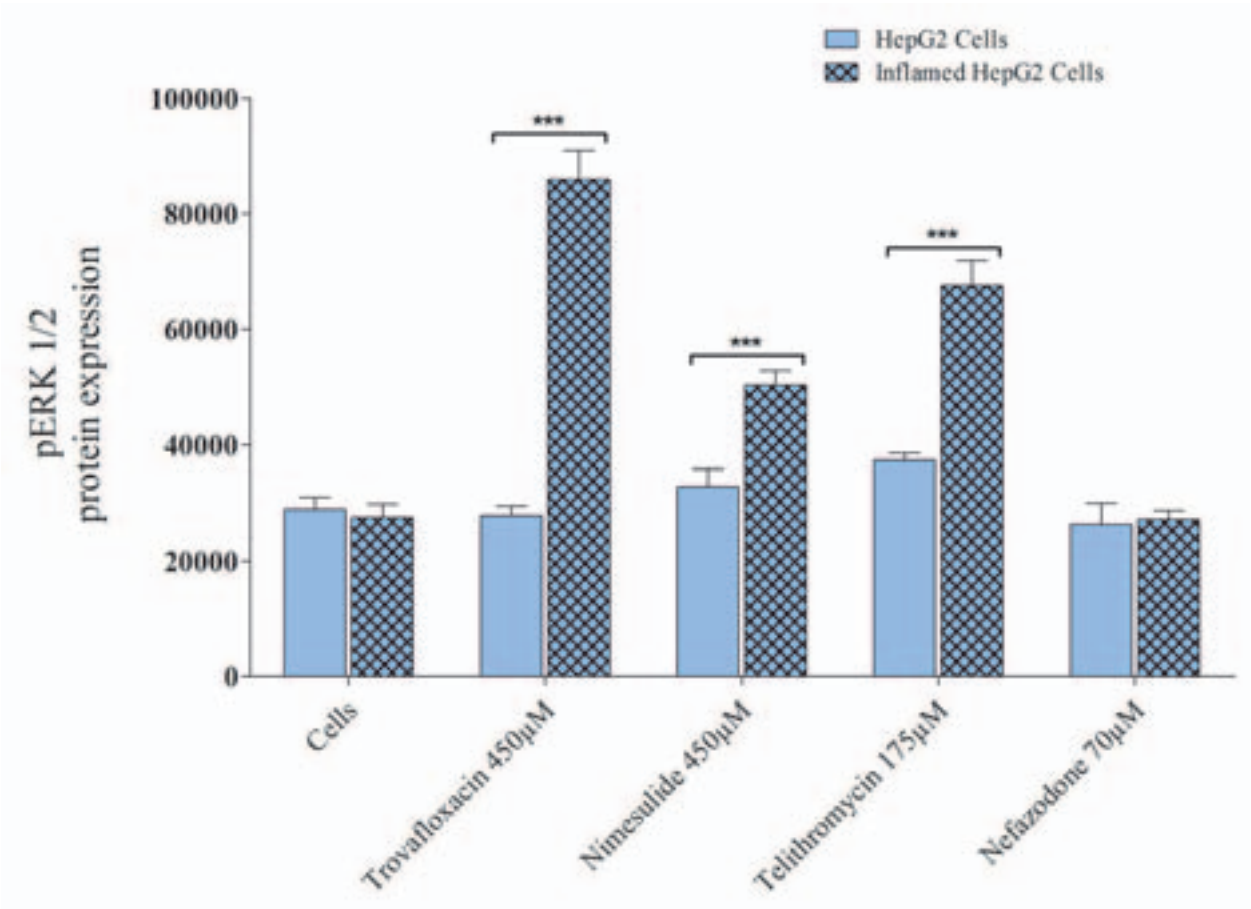


Figure 7.13. Effet des quatre médicaments idiosyncrasiques sur l'expression des protéines pERK ½ dans les cellules HepG2 non enflammées et enflammées. Après incubation de cellules HepG2 avec les quatre médicaments idiosyncrasiques en absence et en présence de LPS et de TNF-α pendant 24 heures, l'expression des protéines pERK ½ est évaluée par western blot. Cette expression est également quantifiée par le logiciel Image J. Les barres représentent l'analyse densitométrique de trois expériences différentes (n = 3). L'analyse statistique est réalisée à l'aide d'ANOVA suivie par Bonferroni post test.
*** = P < 0,001 et représente la variation de l'expression des protéines pERK ½ entre les cellules HepG2 non enflammées et enflammées.

References

Abdel-Bakky, M. S., Hammad, M. A., Walkerit, L. A., & Ashfaqi, M. K. (2010). Developing and characterizing a mouse model of hepatotoxicity using oral pyrrolizidine alkaloid (monocrotaline) administration, with potentiation of the liver injury by co-administration of LPS. *Nat Prod Commun, 5*(9), 1457-1462.

Abdel-Misih, S. R., & Bloomston, M. (2010). Liver anatomy. *Surg Clin North Am, 90*(4), 643-653. doi: 10.1016/j.suc.2010.04.017

Adams, D. H., Ju, C., Ramaiah, S. K., Uetrecht, J., & Jaeschke, H. (2010). Mechanisms of immune-mediated liver injury. *Toxicol Sci, 115*(2), 307-321. doi: 10.1093/toxsci/kfq009

Aggarwal, B. B. (2004). Nuclear factor-kappaB: the enemy within. *Cancer Cell, 6*(3), 203-208. doi: 10.1016/j.ccr.2004.09.003

Aitken, A. E., Richardson, T. A., & Morgan, E. T. (2006). Regulation of drug-metabolizing enzymes and transporters in inflammation. *Annu Rev Pharmacol Toxicol, 46*, 123-149. doi: 10.1146/annurev.pharmtox.46.120604.141059

Aliouat-Denis, C. M., Dendouga, N., Van den Wyngaert, I., Goehlmann, H., Steller, U., van de Weyer, I., Vialard, J. E. (2005). p53-independent regulation of p21Waf1/Cip1 expression and senescence by Chk2. *Mol Cancer Res, 3*(11), 627-634. doi: 10.1158/1541-7786.MCR-05-0121

Allen, J., Jeffrey, P., Williams, R., & Ratcliffe, A.J. (2010). Approaches to assessing drug safety in the discovery phase. *Drugs Fut, 35*(1).

Amundson, S. A., Myers, T. G., & Fornace, A. J., Jr. (1998). Roles for p53 in growth arrest and apoptosis: putting on the brakes after genotoxic stress. *Oncogene, 17*(25), 3287-3299. doi: 10.1038/sj.onc.1202576

Anderson, N., & Borlak, J. (2008). Molecular mechanisms and therapeutic targets in steatosis and steatohepatitis. *Pharmacol Rev, 60*(3), 311-357. doi: 10.1124/pr.108.00001

Andrade, R. J., Robles, M., Fernandez-Castaner, A., Lopez-Ortega, S., Lopez-Vega, M. C., & Lucena, M. I. (2007). Assessment of drug-induced hepatotoxicity in clinical practice: a challenge for gastroenterologists. *World J Gastroenterol, 13*(3), 329-340.

Aninat, C., Piton, A., Glaise, D., Le Charpentier, T., Langouet, S., Morel, F., . . . Guillouzo, A. (2006). Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. *Drug Metab Dispos, 34*(1), 75-83. doi: 10.1124/dmd.105.006759

Annaert, P. (2007). Drug transport in the liver. In Y. Guofeng & M. Morris (Eds.), Drug Transporters: Molecular Characterization and Role in Drug Disposition: John Wiley & Sons, Inc.

Annaert, P. P., Turncliff, R. Z., Booth, C. L., Thakker, D. R., & Brouwer, K. L. (2001). P-glycoprotein-mediated in vitro biliary excretion in sandwich-cultured rat hepatocytes. *Drug Metab Dispos, 29*(10), 1277-1283.

Asahina, K., Tsai, S. Y., Li, P., Ishii, M., Maxson, R. E., Jr., Sucov, H. M., & Tsukamoto, H. (2009). Mesenchymal origin of hepatic stellate cells, submesothelial cells, and perivascular mesenchymal cells during mouse liver development. *Hepatology, 49*(3), 998-1011. doi: 10.1002/hep.22721

Asirvatham, A. J., Schmidt, M., Gao, B., & Chaudhary, J. (2006). Androgens regulate the immune/inflammatory response and cell survival pathways in rat ventral prostate epithelial cells. *Endocrinology, 147*(1), 257-271. doi: 10.1210/en.2005-0942

Bantel, H., & Schulze-Osthoff, K. (2012). Mechanisms of cell death in acute liver failure. *Front Physiol, 3*, 79. doi: 10.3389/fphys.2012.00079

Barnhart, B. C., & Peter, M. E. (2003). The TNF receptor 1: a split personality complex. *Cell, 114*(2), 148-150.

Barros, L. F., Hermosilla, T., & Castro, J. (2001). Necrotic volume increase and the early physiology of necrosis. *Comp Biochem Physiol A Mol Integr Physiol, 130*(3), 401-409.

Barton, C. C., Barton, E. X., Ganey, P. E., Kunkel, S. L., & Roth, R. A. (2001). Bacterial lipopolysaccharide enhances aflatoxin B1 hepatotoxicity in rats by a mechanism that depends on tumor necrosis factor alpha. *Hepatology, 33*(1), 66-73. doi: 10.1053/jhep.2001.20643

Basma, H., Soto-Gutierrez, A., Yannam, G. R., Liu, L., Ito, R., Yamamoto, T., . . . Fox, I. J. (2009). Differentiation and transplantation of human embryonic stem cell-derived hepatocytes. *Gastroenterology, 136*(3), 990-999. doi: 10.1053/j.gastro.2008.10.047

Batts, K. P., & Ludwig, J. (1995). Chronic hepatitis. An update on terminology and reporting. *Am J Surg Pathol, 19*(12), 1409-1417.

Bauman, J. N., Frederick, K. S., Sawant, A., Walsky, R. L., Cox, L. M., Obach, R. S., & Kalgutkar, A. S. (2008). Comparison of the bioactivation potential of the antidepressant and hepatotoxin nefazodone with aripiprazole, a structural analog and marketed drug. *Drug Metab Dispos, 36*(6), 1016-1029. doi: 10.1124/dmd.108.020545

Begriche, K., Igoudjil, A., Pessayre, D., & Fromenty, B. (2006). Mitochondrial dysfunction in NASH: causes, consequences and possible means to prevent it. *Mitochondrion, 6*(1), 1-28. doi: 10.1016/j.mito.2005.10.004

Benichou, C. (1990). Criteria of drug-induced liver disorders. Report of an international consensus meeting. *J Hepatol, 11*(2), 272-276.

Bergheim, I., Luyendyk, J. P., Steele, C., Russell, G. K., Guo, L., Roth, R. A., & Arteel, G. E. (2006). Metformin prevents endotoxin-induced liver injury after partial hepatectomy. *J Pharmacol Exp Ther, 316*(3), 1053-1061. doi: 10.1124/jpet.105.092122

Bergom, C., Gao, C., & Newman, P. J. (2005). Mechanisms of PECAM-1-mediated cytoprotection and implications for cancer cell survival. *Leuk Lymphoma, 46*(10), 1409-1421. doi: 10.1080/10428190500126091

Berson, A., Cazanave, S., Descatoire, V., Tinel, M., Grodet, A., Wolf, C., . . . Pessayre, D. (2006). The anti-inflammatory drug, nimesulide (4-nitro-2-phenoxymethane-sulfoanilide), uncouples mitochondria and induces mitochondrial permeability transition in human hepatoma cells: protection by albumin. *J Pharmacol Exp Ther, 318*(1), 444-454. doi: 10.1124/jpet.106.104125

Beuers, U., Bilzer, M., Chittattu, A., Kullak-Ublick, G. A., Keppler, D., Paumgartner, G., & Dombrowski, F. (2001). Tauroursodeoxycholic acid inserts the apical conjugate export pump, Mrp2, into canalicular membranes and stimulates organic anion secretion by protein kinase C-dependent mechanisms in cholestatic rat liver. *Hepatology, 33*(5), 1206-1216. doi: 10.1053/jhep.2001.24034

Beuers, U., Denk, G. U., Soroka, C. J., Wimmer, R., Rust, C., Paumgartner, G., & Boyer, J. L. (2003). Taurolithocholic acid exerts cholestatic effects via phosphatidylinositol 3-kinase-dependent mechanisms in perfused rat livers and rat hepatocyte couplets. *J Biol Chem, 278*(20), 17810-17818. doi: 10.1074/jbc.M209898200

Beyaert, R., Van Loo, G., Heyninck, K., & Vandenabeele, P. (2002). Signaling to gene activation and cell death by tumor necrosis factor receptors and Fas. *Int Rev Cytol, 214*, 225-272.

Bioulac-Sage, P., Lebail, B. , & Balabaud, C. (2007). Liver and biliary tract histology. In R. J., B. J.P., B. A.T., R. J. & R. M. (Eds.), Textbook of hepatology: from basic science to clinical practice. (3rd edition ed.): Malden: Wiley-Blackwell Publishing.

Bird, T. G., Lorenzini, S., & Forbes, S. J. (2008). Activation of stem cells in hepatic diseases. *Cell Tissue Res, 331*(1), 283-300. doi: 10.1007/s00441-007-0542-z

Bjornsson, E. (2010). Review article: drug-induced liver injury in clinical practice. *Aliment Pharmacol Ther, 32*(1), 3-13. doi: 10.1111/j.1365-2036.2010.04320.x

Bjornsson, E., & Olsson, R. (2005). Outcome and prognostic markers in severe drug-induced liver disease. *Hepatology, 42*(2), 481-489. doi: 10.1002/hep.20800

Boelsterli, U. A. (2003). Idiosyncratic drug hepatotoxicity revisited: new insights from mechanistic toxicology. *Toxicol Mech Methods, 13*(1), 3-20. doi: 10.1080/15376510309824

Boelsterli, U. A., & Lim, P. L. (2007). Mitochondrial abnormalities--a link to idiosyncratic drug hepatotoxicity? *Toxicol Appl Pharmacol, 220*(1), 92-107. doi: 10.1016/j.taap.2006.12.013

Bogert, P. T., & LaRusso, N. F. (2007). Cholangiocyte biology. *Curr Opin Gastroenterol, 23*(3), 299-305. doi: 10.1097/MOG.0b013e3280b079fb

Bohan, A., & Boyer, J. L. (2002). Mechanisms of hepatic transport of drugs: implications for cholestatic drug reactions. *Semin Liver Dis, 22*(2), 123-136. doi: 10.1055/s-2002-30099

Bonilha, V. L., Marmorstein, A. D., Cohen-Gould, L., & Rodriguez-Boulan, E. (1997). Apical sorting of influenza hemagglutinin by transcytosis in retinal pigment epithelium. *J Cell Sci, 110 (Pt 15)*, 1717-1727.

Bouillet, P., & Strasser, A. (2002). BH3-only proteins - evolutionarily conserved proapoptotic Bcl-2 family members essential for initiating programmed cell death. *J Cell Sci, 115*(Pt 8), 1567-1574.

Bradham, C. A., Plumpe, J., Manns, M. P., Brenner, D. A., & Trautwein, C. (1998). Mechanisms of hepatic toxicity. I. TNF-induced liver injury. *Am J Physiol, 275*(3 Pt 1), G387-392.

Braet, F., & Wisse, E. (2002). Structural and functional aspects of liver sinusoidal endothelial cell fenestrae: a review. *Comp Hepatol, 1*(1), 1.

Bretscher, A., Edwards, K., & Fehon, R. G. (2002). ERM proteins and merlin: integrators at the cell cortex. *Nat Rev Mol Cell Biol, 3*(8), 586-599. doi: 10.1038/nrm882

Briz, O., Romero, M. R., Martinez-Becerra, P., Macias, R. I., Perez, M. J., Jimenez, F., . . . Marin, J. J. (2006). OATP8/1B3-mediated cotransport of bile acids and glutathione: an export pathway for organic anions from hepatocytes? *J Biol Chem, 281*(41), 30326-30335. doi: 10.1074/jbc.M602048200

Brophy, C. M., Luebke-Wheeler, J. L., Amiot, B. P., Khan, H., Remmel, R. P., Rinaldo, P., & Nyberg, S. L. (2009). Rat hepatocyte spheroids formed by rocked technique maintain differentiated hepatocyte gene expression and function. *Hepatology, 49*(2), 578-586. doi: 10.1002/hep.22674

Buchweitz, J. P., Ganey, P. E., Bursian, S. J., & Roth, R. A. (2002). Underlying endotoxemia augments toxic responses to chlorpromazine: is there a relationship to drug idiosyncrasy? *J Pharmacol Exp Ther, 300*(2), 460-467.

Burd, I., Breen, K., Friedman, A., Chai, J., & Elovitz, M. A. (2010). Magnesium sulfate reduces inflammation-associated brain injury in fetal mice. *Am J Obstet Gynecol, 202*(3), 292 e291-299. doi: 10.1016/j.ajog.2010.01.022

Canbay, A., Feldstein, A. E., Higuchi, H., Werneburg, N., Grambihler, A., Bronk, S. F., & Gores, G. J. (2003). Kupffer cell engulfment of apoptotic bodies stimulates death ligand and cytokine expression. *Hepatology, 38*(5), 1188-1198. doi: 10.1053/jhep.2003.50472

Canbay, A., Friedman, S., & Gores, G. J. (2004). Apoptosis: the nexus of liver injury and fibrosis. *Hepatology, 39*(2), 273-278. doi: 10.1002/hep.20051

Canbay, A., Kip, S. N., Kahraman, A., Gieseler, R. K., Nayci, A., & Gerken, G. (2005). Apoptosis and fibrosis in non-alcoholic fatty liver disease. *Turk J Gastroenterol, 16*(1), 1-6.

Cantz, T., Nies, A. T., Brom, M., Hofmann, A. F., & Keppler, D. (2000). MRP2, a human conjugate export pump, is present and transports fluo 3 into apical vacuoles of Hep G2 cells. *Am J Physiol Gastrointest Liver Physiol, 278*(4), G522-531.

Carrella, M., & Roda, E. (1999). Evolving concepts in the pathophysiology of biliary lipid secretion. *Ital J Gastroenterol Hepatol, 31*(7), 643-648.

Castell, J. V., & Castell, M. (2006). Allergic hepatitis induced by drugs. *Curr Opin Allergy Clin Immunol, 6*(4), 258-265. doi: 10.1097/01.all.0000235898.80052.80

Chakraborty, J. B., Oakley, F., & Walsh, M. J. (2012). Mechanisms and biomarkers of apoptosis in liver disease and fibrosis. *Int J Hepatol, 2012*, 648915. doi: 10.1155/2012/648915

Chalasani, N., & Bjornsson, E. (2010). Risk factors for idiosyncratic drug-induced liver injury. *Gastroenterology, 138*(7), 2246-2259. doi: 10.1053/j.gastro.2010.04.001

Chalasani, N., Fontana, R. J., Bonkovsky, H. L., Watkins, P. B., Davern, T., Serrano, J., . . . Drug Induced Liver Injury, Network. (2008). Causes, clinical features, and outcomes from a prospective study of drug-induced liver injury in the United States. *Gastroenterology, 135*(6), 1924-1934, 1934 e1921-1924. doi: 10.1053/j.gastro.2008.09.011

Chang, T. K. (2009). Activation of pregnane X receptor (PXR) and constitutive androstane receptor (CAR) by herbal medicines. *AAPS J, 11*(3), 590-601. doi: 10.1208/s12248-009-9135-y

Chapman, L. M., & Eddy, E. M. (1989). A protein associated with the mouse and rat hepatocyte junctional complex. *Cell Tissue Res, 257*(2), 333-341.

Chastre, A., Belanger, M., Beauchesne, E., Nguyen, B. N., Desjardins, P., & Butterworth, R. F. (2012). Inflammatory cascades driven by tumor necrosis factor-alpha play a major role in the progression of acute liver failure and its neurological complications. *PLoS One, 7*(11), e49670. doi: 10.1371/journal.pone.0049670

Chen, Q., Crosby, M., & Almasan, A. (2003). Redox Regulation of Apoptosis before and after Cytochrome C Release. *Korean J Biol Sci, 7*(1), 1-9. doi: 10.1080/12265071.2003.9647675

Cheng, P. Y., Wang, M., & Morgan, E. T. (2003). Rapid transcriptional suppression of rat cytochrome P450 genes by endotoxin treatment and its inhibition by curcumin. *J Pharmacol Exp Ther, 307*(3), 1205-1212. doi: 10.1124/jpet.103.057174

Cherrington, N. J., Slitt, A. L., Li, N., & Klaassen, C. D. (2004). Lipopolysaccharide-mediated regulation of hepatic transporter mRNA levels in rats. *Drug Metab Dispos, 32*(7), 734-741.

Cheung, M. C., Jones, R. L., & Judson, I. (2011). Acute liver toxicity with ifosfamide in the treatment of sarcoma: a case report. *J Med Case Rep, 5*, 180. doi: 10.1186/1752-1947-5-180

Choi, M. K., & Song, I. S. (2008). Organic cation transporters and their pharmacokinetic and pharmacodynamic consequences. *Drug Metab Pharmacokinet, 23*(4), 243-253.

Chowdhury, A., Santra, A., Bhattacharjee, K., Ghatak, S., Saha, D. R., & Dhali, G. K. (2006). Mitochondrial oxidative stress and permeability transition in isoniazid and rifampicin induced liver injury in mice. *J Hepatol, 45*(1), 117-126. doi: 10.1016/j.jhep.2006.01.027

Clay, K. D., Hanson, J. S., Pope, S. D., Rissmiller, R. W., Purdum, P. P., 3rd, & Banks, P. M. (2006). Brief communication: severe hepatotoxicity of telithromycin: three case reports and literature review. *Ann Intern Med, 144*(6), 415-420.

Cogger, V. C., McNerney, G. P., Nyunt, T., DeLeve, L. D., McCourt, P., Smedsrod, B., . . . Huser, T. R. (2010). Three-dimensional structured illumination microscopy of liver sinusoidal endothelial cell fenestrations. *J Struct Biol, 171*(3), 382-388. doi: 10.1016/j.jsb.2010.06.001

Cosgrove, B. D., Cheng, C., Pritchard, J. R., Stolz, D. B., Lauffenburger, D. A., & Griffith, L. G. (2008). An inducible autocrine cascade regulates rat hepatocyte proliferation and apoptosis responses to tumor necrosis factor-alpha. *Hepatology, 48*(1), 276-288. doi: 10.1002/hep.22335

Cosgrove, B. D., King, B. M., Hasan, M. A., Alexopoulos, L. G., Farazi, P. A., Hendriks, B. S., . . . Lauffenburger, D. A. (2009). Synergistic drug-cytokine induction of hepatocellular death as an in vitro approach for the study of inflammation-associated idiosyncratic drug hepatotoxicity. *Toxicol Appl Pharmacol, 237*(3), 317-330. doi: 10.1016/j.taap.2009.04.002

Cui, Y., & Paules, R. S. (2010). Use of transcriptomics in understanding mechanisms of drug-induced toxicity. *Pharmacogenomics, 11*(4), 573-585. doi: 10.2217/pgs.10.37

Cury-Boaventura, M. F., & Curi, R. (2005). Regulation of reactive oxygen species (ROS) production by C18 fatty acids in Jurkat and Raji cells. *Clin Sci (Lond), 108*(3), 245-253. doi: 10.1042/CS20040281

Dalvie, D. K., Khosla, N., & Vincent, J. (1997). Excretion and metabolism of trovafloxacin in humans. *Drug Metab Dispos, 25*(4), 423-427.

Dambach, D. M., Andrews, B. A., & Moulin, F. (2005). New technologies and screening strategies for hepatotoxicity: use of in vitro models. *Toxicol Pathol, 33*(1), 17-26. doi: 10.1080/01926230590522284

Danial, N. N., & Korsmeyer, S. J. (2004). Cell death: critical control points. *Cell, 116*(2), 205-219.

David, S., & Hamilton, J. P. (2010). Drug-induced Liver Injury. *US Gastroenterol Hepatol Rev, 6*, 73-80.

Davila, J. C., Cezar, G. G., Thiede, M., Strom, S., Miki, T., & Trosko, J. (2004). Use and application of stem cells in toxicology. *Toxicol Sci, 79*(2), 214-223. doi: 10.1093/toxsci/kfh100

Davis, G. L. (2002). Managing complications of cirrhosis. *Rev Gastroenterol Disord, 2*(3), 143-145.

Day, C. P., & James, O. F. (1998). Hepatic steatosis: innocent bystander or guilty party? *Hepatology, 27*(6), 1463-1466. doi: 10.1002/hep.510270601

Deaciuc, Iv Iv, D'Souza, N. B., Fortunato, F., Hill, D. B., Sarphie, T. G., & McClain, C. J. (2001). Alcohol-induced sinusoidal endothelial cell dysfunction in the mouse is associated with exacerbated liver apoptosis and can be reversed by caspase inhibition. *Hepatol Res, 19*(1), 85-97.

Degli Esposti, M., & Dive, C. (2003). Mitochondrial membrane permeabilisation by Bax/Bak. *Biochem Biophys Res Commun, 304*(3), 455-461.

DeLeve, L. D. (2007). Hepatic microvasculature in liver injury. *Semin Liver Dis, 27*(4), 390-400. doi: 10.1055/s-2007-991515

Deleve, L. D., Wang, X., & Guo, Y. (2008). Sinusoidal endothelial cells prevent rat stellate cell activation and promote reversion to quiescence. *Hepatology, 48*(3), 920-930. doi: 10.1002/hep.22351

DeLeve, L. D., Wang, X., Hu, L., McCuskey, M. K., & McCuskey, R. S. (2004). Rat liver sinusoidal endothelial cell phenotype is maintained by paracrine and autocrine regulation. *Am J Physiol Gastrointest Liver Physiol, 287*(4), G757-763. doi: 10.1152/ajpgi.00017.2004

Deng, X., Luyendyk, J. P., Ganey, P. E., & Roth, R. A. (2009). Inflammatory stress and idiosyncratic hepatotoxicity: hints from animal models. *Pharmacol Rev, 61*(3), 262-282. doi: 10.1124/pr.109.001727

Deng, X., Stachlewitz, R. F., Liguori, M. J., Blomme, E. A., Waring, J. F., Luyendyk, J. P., . . . Roth, R. A. (2006). Modest inflammation enhances diclofenac hepatotoxicity in rats: role of neutrophils and bacterial translocation. *J Pharmacol Exp Ther, 319*(3), 1191-1199. doi: 10.1124/jpet.106.110247

Denson, L. A., Auld, K. L., Schiek, D. S., McClure, M. H., Mangelsdorf, D. J., & Karpen, S. J. (2000). Interleukin-1beta suppresses retinoid transactivation of two hepatic transporter genes involved in bile formation. *J Biol Chem, 275*(12), 8835-8843.

Denson, L. A., Bohan, A., Held, M. A., & Boyer, J. L. (2002). Organ-specific alterations in RAR alpha:RXR alpha abundance regulate rat Mrp2 (Abcc2) expression in obstructive cholestasis. *Gastroenterology, 123*(2), 599-607.

Deopurkar, R., Ghanim, H., Friedman, J., Abuaysheh, S., Sia, C. L., Mohanty, P., . . . Dandona, P. (2010). Differential effects of cream, glucose, and orange juice on inflammation, endotoxin, and the expression of Toll-like receptor-4 and suppressor of cytokine signaling-3. *Diabetes Care, 33*(5), 991-997. doi: 10.2337/dc09-1630

Deslauriers, N., Oudghiri, M., Seguin, J., & Trudel, L. (1986). The oral immune system: dynamics of salivary immunoglobulin production in the inbred mouse. *Immunol Invest, 15*(4), 339-349.

Dilworth, C., Hamilton, G. A., George, E., & Timbrell, J. A. (2000). The use of liver spheroids as an in vitro model for studying induction of the stress response as a marker of chemical toxicity. *Toxicol In Vitro, 14*(2), 169-176.

Donato, M. T., Martinez-Romero, A., Jimenez, N., Negro, A., Herrera, G., Castell, J. V., . . . Gomez-Lechon, M. J. (2009). Cytometric analysis for drug-induced steatosis in HepG2 cells. *Chem Biol Interact, 181*(3), 417-423. doi: 10.1016/j.cbi.2009.07.019

Donner, M. G., Warskulat, U., Saha, N., & Haussinger, D. (2004). Enhanced expression of basolateral multidrug resistance protein isoforms Mrp3 and Mrp5 in rat liver by LPS. *Biol Chem, 385*(3-4), 331-339. doi: 10.1515/BC.2004.029

Dykens, E. M., & Roof, E. (2008). Behavior in Prader-Willi syndrome: relationship to genetic subtypes and age. *J Child Psychol Psychiatry, 49*(9), 1001-1008. doi: 10.1111/j.1469-7610.2008.01913.x

Dykens, J. A., Jamieson, J., Marroquin, L., Nadanaciva, S., Billis, P. A., & Will, Y. (2008). Biguanide-induced mitochondrial dysfunction yields increased lactate production and cytotoxicity of aerobically-poised HepG2 cells and human hepatocytes in vitro. *Toxicol Appl Pharmacol, 233*(2), 203-210. doi: 10.1016/j.taap.2008.08.013

Dykens, J. A., Jamieson, J. D., Marroquin, L. D., Nadanaciva, S., Xu, J. J., Dunn, M. C., . . . Will, Y. (2008). In vitro assessment of mitochondrial dysfunction and cytotoxicity of nefazodone, trazodone, and buspirone. *Toxicol Sci, 103*(2), 335-345. doi: 10.1093/toxsci/kfn056

Echols, R. M. (2011). Understanding the regulatory hurdles for antibacterial drug development in the post-Ketek world. *Ann N Y Acad Sci, 1241*, 153-161. doi: 10.1111/j.1749-6632.2011.06300.x

Echols, R. M. (2012). A long and winding road; evolution of antimicrobial drug development - crisis management. *Expert Rev Anti Infect Ther, 10*(11), 1311-1319. doi: 10.1586/eri.12.131

Ee, P. L., Kamalakaran, S., Tonetti, D., He, X., Ross, D. D., & Beck, W. T. (2004). Identification of a novel estrogen response element in the breast cancer resistance protein (ABCG2) gene. *Cancer Res, 64*(4), 1247-1251.

Egan, L. J., Eckmann, L., Greten, F. R., Chae, S., Li, Z. W., Myhre, G. M., . . . Kagnoff, M. F. (2004). IkappaB-kinasebeta-dependent NF-kappaB activation provides radioprotection to the intestinal epithelium. *Proc Natl Acad Sci U S A, 101*(8), 2452-2457.

Ekins, S., Andreyev, S., Ryabov, A., Kirillov, E., Rakhmatulin, E. A., Sorokina, S., . . . Nikolskaya, T. (2006). A combined approach to drug metabolism and toxicity assessment. *Drug Metab Dispos, 34*(3), 495-503. doi: 10.1124/dmd.105.008458

Elliott, M. R., Chekeni, F. B., Trampont, P. C., Lazarowski, E. R., Kadl, A., Walk, S. F., . . . Ravichandran, K. S. (2009). Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature, 461*(7261), 282-286. doi: 10.1038/nature08296

Eloranta, J. J., Meier, P. J., & Kullak-Ublick, G. A. (2005). Coordinate transcriptional regulation of transport and metabolism. *Methods Enzymol, 400*, 511-530. doi: 10.1016/S0076-6879(05)000285

Evers, R., Kool, M., Smith, A. J., van Deemter, L., de Haas, M., & Borst, P. (2000). Inhibitory effect of the reversal agents V-104, GF120918 and Pluronic L61 on MDR1 Pgp-, MRP1- and MRP2-mediated transport. *Br J Cancer, 83*(3), 366-374. doi: 10.1054/bjoc.2000.1260

Fanning, A. S., Mitic, L. L., & Anderson, J. M. (1999). Transmembrane proteins in the tight junction barrier. *J Am Soc Nephrol, 10*(6), 1337-1345.

Faouzi, S., Burckhardt, B. E., Hanson, J. C., Campe, C. B., Schrum, L. W., Rippe, R. A., & Maher, J. J. (2001). Anti-Fas induces hepatic chemokines and promotes inflammation by an NF-kappa B-independent, caspase-3-dependent pathway. *J Biol Chem, 276*(52), 49077-49082. doi: 10.1074/jbc.M109791200

Fardel, O., Jigorel, E., Le Vee, M., & Payen, L. (2005). Physiological, pharmacological and clinical features of the multidrug resistance protein 2. *Biomed Pharmacother, 59*(3), 104-114. doi: 10.1016/j.biopha.2005.01.005

Fardel, O., & Le Vee, M. (2009). Regulation of human hepatic drug transporter expression by pro-inflammatory cytokines. *Expert Opin Drug Metab Toxicol, 5*(12), 1469-1481. doi: 10.1517/17425250903304056

Fattinger, K., Funk, C., Pantze, M., Weber, C., Reichen, J., Stieger, B., & Meier, P. J. (2001). The endothelin antagonist bosentan inhibits the canalicular bile salt export pump: a potential mechanism for hepatic adverse reactions. *Clin Pharmacol Ther, 69*(4), 223-231. doi: 10.1067/mcp.2001.114667

Fava, G., Glaser, S., Francis, H., & Alpini, G. (2005). The immunophysiology of biliary epithelium. *Semin Liver Dis, 25*(3), 251-264. doi: 10.1055/s-2005-916318

Feldmann, G., Haouzi, D., Moreau, A., Durand-Schneider, A. M., Bringuier, A., Berson, A., . . . Pessayre, D. (2000). Opening of the mitochondrial permeability transition pore causes matrix expansion and outer membrane rupture in Fas-mediated hepatic apoptosis in mice. *Hepatology, 31*(3), 674-683. doi: 10.1002/hep.510310318

Feldstein, A. E., Canbay, A., Angulo, P., Taniai, M., Burgart, L. J., Lindor, K. D., & Gores, G. J. (2003). Hepatocyte apoptosis and fas expression are prominent features of human nonalcoholic steatohepatitis. *Gastroenterology, 125*(2), 437-443.

Feng, D., Wang, Y., Xu, Y., Luo, Q., Lan, B., & Xu, L. (2009). Interleukin 10 deficiency exacerbates halothane induced liver injury by increasing interleukin 8 expression and neutrophil infiltration. *Biochem Pharmacol, 77*(2), 277-284. doi: 10.1016/j.bcp.2008.09.028

Fernandez, C., Buyse, M., German-Fattal, M., & Gimenez, F. (2004). Influence of the pro-inflammatory cytokines on P-glycoprotein expression and functionality. *J Pharm Pharm Sci, 7*(3), 359-371.

Ferrari, L., Peng, N., Halpert, J. R., & Morgan, E. T. (2001). Role of nitric oxide in down-regulation of CYP2B1 protein, but not RNA, in primary cultures of rat hepatocytes. *Mol Pharmacol, 60*(1), 209-216.

Feudtner, C., Dai, D., Hexem, K. R., Luan, X., & Metjian, T. A. (2012). Prevalence of polypharmacy exposure among hospitalized children in the United States. *Arch Pediatr Adolesc Med, 166*(1), 9-16. doi: 10.1001/archpediatrics.2011.161

Fielden, M. R., Brennan, R., & Gollub, J. (2007). A gene expression biomarker provides early prediction and mechanistic assessment of hepatic tumor induction by nongenotoxic chemicals. *Toxicol Sci, 99*(1), 90-100. doi: 10.1093/toxsci/kfm156

Forbes, S., Vig, P., Poulsom, R., Thomas, H., & Alison, M. (2002). Hepatic stem cells. *J Pathol, 197*(4), 510-518. doi: 10.1002/path.1163

Formigli, L., Papucci, L., Tani, A., Schiavone, N., Tempestini, A., Orlandini, G. E., . . . Orlandini, S. Z. (2000). Aponecrosis: morphological and biochemical exploration of a syncretic process of cell death sharing apoptosis and necrosis. *J Cell Physiol, 182*(1), 41-49. doi: 10.1002/(SICI)1097-4652(200001)182:1<41::AID-JCP5>3.0.CO;2-7

Fredriksson, L., Herpers, B., Benedetti, G., Matadin, Q., Puigvert, J. C., de Bont, H., . . . van de Water, B. (2011). Diclofenac inhibits tumor necrosis factor-alpha-induced nuclear factor-kappaB activation causing synergistic hepatocyte apoptosis. *Hepatology, 53*(6), 2027-2041. doi: 10.1002/hep.24314

Friedman, S. L. (2008). Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev, 88*(1), 125-172. doi: 10.1152/physrev.00013.2007

Friedman, S. L., & Bansal, M. B. (2006). Reversal of hepatic fibrosis -- fact or fantasy? *Hepatology, 43*(2 Suppl 1), S82-88. doi: 10.1002/hep.20974

Fulda, S., & Debatin, K. M. (2006). Resveratrol modulation of signal transduction in apoptosis and cell survival: a mini-review. *Cancer Detect Prev, 30*(3), 217-223. doi: 10.1016/j.cdp.2006.03.007

Funk, C., Pantze, M., Jehle, L., Ponelle, C., Scheuermann, G., Lazendic, M., & Gasser, R. (2001). Troglitazone-induced intrahepatic cholestasis by an interference with the hepatobiliary export of bile acids in male and female rats. Correlation with the gender difference in troglitazone sulfate formation and the inhibition of the canalicular bile salt export pump (Bsep) by troglitazone and troglitazone sulfate. *Toxicology, 167*(1), 83-98.

Funk, C., Ponelle, C., Scheuermann, G., & Pantze, M. (2001). Cholestatic potential of troglitazone as a possible factor contributing to troglitazone-induced hepatotoxicity: in vivo and in vitro interaction at the canalicular bile salt export pump (Bsep) in the rat. *Mol Pharmacol, 59*(3), 627-635.

G.R., Wilkinson. (2005). Drug metabolism and variability among patients in drug response. *N Engl J Med, 352* (21), 2211–2221.

Gandhi, A., Guo, T., & Ghose, R. (2010). Role of c-Jun N-terminal kinase (JNK) in regulating tumor necrosis factor-alpha (TNF-alpha) mediated increase of acetaminophen (APAP) and chlorpromazine (CPZ) toxicity in murine hepatocytes. *J Toxicol Sci, 35*(2), 163-173.

Ganey, P. E., Luyendyk, J. P., Maddox, J. F., & Roth, R. A. (2004). Adverse hepatic drug reactions: inflammatory episodes as consequence and contributor. *Chem Biol Interact, 150*(1), 35-51. doi: 10.1016/j.cbi.2004.09.002

Gao, B. (2005). Cytokines, STATs and liver disease. *Cell Mol Immunol, 2*(2), 92-100.

Gao, J., Liu, X., & Rigas, B. (2005). Nitric oxide-donating aspirin induces apoptosis in human colon cancer cells through induction of oxidative stress. *Proc Natl Acad Sci U S A, 102*(47), 17207-17212. doi: 10.1073/pnas.0506893102

Gao, N., Rahmani, M., Dent, P., & Grant, S. (2005). 2-Methoxyestradiol-induced apoptosis in human leukemia cells proceeds through a reactive oxygen species and Akt-dependent process. *Oncogene, 24*(23), 3797-3809. doi: 10.1038/sj.onc.1208530

Gao, Q., Li, Y., & Chopp, M. (2005). Bone marrow stromal cells increase astrocyte survival via upregulation of phosphoinositide 3-kinase/threonine protein kinase and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase pathways and stimulate astrocyte trophic

factor gene expression after anaerobic insult. *Neuroscience, 136*(1), 123-134. doi: 10.1016/j.neuroscience.2005.06.091

Gao, R., & Brigstock, D. R. (2005). Activation of nuclear factor kappa B (NF-kappaB) by connective tissue growth factor (CCN2) is involved in sustaining the survival of primary rat hepatic stellate cells. *Cell Commun Signal, 3*, 14. doi: 10.1186/1478-811X-3-14

Gao, X., Deeb, D., Jiang, H., Liu, Y. B., Dulchavsky, S. A., & Gautam, S. C. (2005). Curcumin differentially sensitizes malignant glioma cells to TRAIL/Apo2L-mediated apoptosis through activation of procaspases and release of cytochrome c from mitochondria. *J Exp Ther Oncol, 5*(1), 39-48.

Gao, Z., Sasaoka, T., Fujimori, T., Oya, T., Ishii, Y., Sabit, H., . . . Sasahara, M. (2005). Deletion of the PDGFR-beta gene affects key fibroblast functions important for wound healing. *J Biol Chem, 280*(10), 9375-9389. doi: 10.1074/jbc.M413081200

Gardner, C. R., Laskin, J. D., Dambach, D. M., Sacco, M., Durham, S. K., Bruno, M. K., . . . Laskin, D. L. (2002). Reduced hepatotoxicity of acetaminophen in mice lacking inducible nitric oxide synthase: potential role of tumor necrosis factor-alpha and interleukin-10. *Toxicol Appl Pharmacol, 184*(1), 27-36.

Gartel, A. L., & Tyner, A. L. (2002). The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. *Mol Cancer Ther, 1*(8), 639-649.

Gaudio, E., Carpino, G., Cardinale, V., Franchitto, A., Onori, P., & Alvaro, D. (2009). New insights into liver stem cells. *Dig Liver Dis, 41*(7), 455-462. doi: 10.1016/j.dld.2009.03.009

Gautherot, I., Burdin, N., Seguin, D., Aujame, L., & Sodoyer, R. (2002). Cloning of interleukin-4 delta2 splice variant (IL-4delta2) in chimpanzee and cynomolgus macaque: phylogenetic analysis of delta2 splice variant appearance, and implications for the study of IL-4-driven immune processes. *Immunogenetics, 54*(9), 635-644. doi: 10.1007/s00251-002-0510-4

Ge, F., & He, Q. Y. (2009). Genomic and proteomic approaches for predicting toxicity and adverse drug reactions. *Expert Opin Drug Metab Toxicol, 5*(1), 29-37. doi: 10.1517/17425250802661895

Geier, A., Dietrich, C. G., Voigt, S., Ananthanarayanan, M., Lammert, F., Schmitz, A., . . . Gartung, C. (2005). Cytokine-dependent regulation of hepatic organic anion transporter gene transactivators in mouse liver. *Am J Physiol Gastrointest Liver Physiol, 289*(5), G831-841. doi: 10.1152/ajpgi.00307.2004

Gerber, B. O., & Pichler, W. J. (2006). Noncovalent interactions of drugs with immune receptors may mediate drug-induced hypersensitivity reactions. *AAPS J, 8*(1), E160-165. doi: aapsj080119

Ghabril, M., Chalasani, N., & Bjornsson, E. (2010). Drug-induced liver injury: a clinical update. *Curr Opin Gastroenterol, 26*(3), 222-226. doi: 10.1097/MOG.0b013e3283383c7c

Ghose, R., White, D., Guo, T., Vallejo, J., & Karpen, S. J. (2008). Regulation of hepatic drug-metabolizing enzyme genes by Toll-like receptor 4 signaling is independent of Toll-interleukin 1 receptor domain-containing adaptor protein. *Drug Metab Dispos, 36*(1), 95-101. doi: 10.1124/dmd.107.018051

Gieseler, R. K., Marquitan, G., Schlattjan, M., Sowa, J. P., Bechmann, L. P., Timm, J., . . . Canbay, A. (2011). Hepatocyte apoptotic bodies encasing nonstructural HCV proteins amplify hepatic stellate cell activation: implications for chronic hepatitis C. *J Viral Hepat, 18*(11), 760-767. doi: 10.1111/j.1365-2893.2010.01362.x

Glaser, S., Francis, H., Demorrow, S., Lesage, G., Fava, G., Marzioni, M., . . . Alpini, G. (2006). Heterogeneity of the intrahepatic biliary epithelium. *World J Gastroenterol, 12*(22), 3523-3536.

Glaser, S. S., Gaudio, E., Miller, T., Alvaro, D., & Alpini, G. (2009). Cholangiocyte proliferation and liver fibrosis. *Expert Rev Mol Med, 11*, e7. doi: 10.1017/S1462399409000994

Goldman, S. E., Malow, B. A., Newman, K. D., Roof, E., & Dykens, E. M. (2009). Sleep patterns and daytime sleepiness in adolescents and young adults with Williams syndrome. *J Intellect Disabil Res, 53*(2), 182-188. doi: 10.1111/j.1365-2788.2008.01140.x

Goodman, Z. D. (2002). Drug hepatotoxicity. *Clin Liver Dis, 6*(2), 381-397.

Goodwin, B., & Moore, J. T. (2004). CAR: detailing new models. *Trends Pharmacol Sci, 25*(8), 437-441. doi: 10.1016/j.tips.2004.06.007

Goodwin, B., Moore, L. B., Stoltz, C. M., McKee, D. D., & Kliewer, S. A. (2001). Regulation of the human CYP2B6 gene by the nuclear pregnane X receptor. *Mol Pharmacol, 60*(3), 427-431.

Gootz, T. D., Zaniewski, R., Haskell, S., Schmieder, B., Tankovic, J., Girard, D., . . . Polzer, R. J. (1996). Activity of the new fluoroquinolone trovafloxacin (CP-99,219) against DNA gyrase and topoisomerase IV mutants of Streptococcus pneumoniae selected in vitro. *Antimicrob Agents Chemother, 40*(12), 2691-2697.

Graham, J. M., Jr., Visootsak, J., Dykens, E., Huddleston, L., Clark, R. D., Jones, K. L., . . . Stevenson, R. E. (2008). Behavior of 10 patients with FG syndrome (Opitz-Kaveggia syndrome) and the p.R961W mutation in the MED12 gene. *Am J Med Genet A, 146A*(23), 3011-3017. doi: 10.1002/ajmg.a.32553

Grattagliano, I., Bonfrate, L., Diogo, C. V., Wang, H. H., Wang, D. Q., & Portincasa, P. (2009). Biochemical mechanisms in drug-induced liver injury: certainties and doubts. *World J Gastroenterol, 15*(39), 4865-4876.

Grimes, K. R., Daosukho, C., Zhao, Y., Meigooni, A., & St Clair, W. (2005). Proteasome inhibition improves fractionated radiation treatment against non-small cell lung cancer: an antioxidant connection. *Int J Oncol, 27*(4), 1047-1052.

Gripon, P., Rumin, S., Urban, S., Le Seyec, J., Glaise, D., Cannie, I., . . . Guguen-Guillouzo, C. (2002). Infection of a human hepatoma cell line by hepatitis B virus. *Proc Natl Acad Sci U S A, 99*(24), 15655-15660. doi: 10.1073/pnas.232137699

Groneberg, D. A., Grosse-Siestrup, C., & Fischer, A. (2002). In vitro models to study hepatotoxicity. *Toxicol Pathol, 30*(3), 394-399.

Gu, X., Ke, S., Liu, D., Sheng, T., Thomas, P. E., Rabson, A. B., . . . Tian, Y. (2006). Role of NF-kappaB in regulation of PXR-mediated gene expression: a mechanism for the suppression of cytochrome P-450 3A4 by proinflammatory agents. *J Biol Chem, 281*(26), 17882-17889. doi: 10.1074/jbc.M601302200

Gudkov, A. V., Gurova, K. V., & Komarova, E. A. (2011). Inflammation and p53: A Tale of Two Stresses. *Genes Cancer, 2*(4), 503-516. doi: 10.1177/1947601911409747

Guguen-Guillouzo, C., Corlu, A., & Guillouzo, A. (2010). Stem cell-derived hepatocytes and their use in toxicology. *Toxicology, 270*(1), 3-9. doi: 10.1016/j.tox.2009.09.019

Guicciardi, M. E., & Gores, G. J. (2005). Apoptosis: a mechanism of acute and chronic liver injury. *Gut, 54*(7), 1024-1033. doi: 10.1136/gut.2004.053850

Guillouzo, A. (1998). Liver cell models in in vitro toxicology. *Environ Health Perspect, 106 Suppl 2*, 511-532.

Guillouzo, A., & Guguen-Guillouzo, C. (2008). Evolving concepts in liver tissue modeling and implications for in vitro toxicology. *Expert Opin Drug Metab Toxicol, 4*(10), 1279-1294. doi: 10.1517/17425255.4.10.1279

Gunawan, B., & Kaplowitz, N. (2004). Clinical perspectives on xenobiotic-induced hepatotoxicity. *Drug Metab Rev, 36*(2), 301-312. doi: 10.1081/DMR-120034148

Gunawan, B. K., Liu, Z. X., Han, D., Hanawa, N., Gaarde, W. A., & Kaplowitz, N. (2006). c-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity. *Gastroenterology, 131*(1), 165-178. doi: 10.1053/j.gastro.2006.03.045

Guo, J., & Friedman, S. L. (2010). Toll-like receptor 4 signaling in liver injury and hepatic fibrogenesis. *Fibrogenesis Tissue Repair, 3*, 21. doi: 10.1186/1755-1536-3-21

Hagenbuch, B., & Meier, P. J. (2004). Organic anion transporting polypeptides of the OATP/ SLC21 family: phylogenetic classification as OATP/ SLCO superfamily, new nomenclature and molecular/functional properties. *Pflugers Arch, 447*(5), 653-665.

Hanawa, N., Shinohara, M., Saberi, B., Gaarde, W. A., Han, D., & Kaplowitz, N. (2008). Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in

acetaminophen-induced liver injury. *J Biol Chem, 283*(20), 13565-13577. doi: 10.1074/jbc.M708916200

Haouzi, D., Lekehal, M., Moreau, A., Moulis, C., Feldmann, G., Robin, M. A., . . . Pessayre, D. (2000). Cytochrome P450-generated reactive metabolites cause mitochondrial permeability transition, caspase activation, and apoptosis in rat hepatocytes. *Hepatology, 32*(2), 303-311. doi: 10.1053/jhep.2000.9034

Harada, A., Sekido, N., Akahoshi, T., Wada, T., Mukaida, N., & Matsushima, K. (1994). Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J Leukoc Biol, 56*(5), 559-564.

Hariparsad, N., Carr, B. A., Evers, R., & Chu, X. (2008). Comparison of immortalized Fa2N-4 cells and human hepatocytes as in vitro models for cytochrome P450 induction. *Drug Metab Dispos, 36*(6), 1046-1055. doi: 10.1124/dmd.108.020677

Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., & Elledge, S. J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell, 75*(4), 805-816.

Harrill, A. H., & Rusyn, I. (2008). Systems biology and functional genomics approaches for the identification of cellular responses to drug toxicity. *Expert Opin Drug Metab Toxicol, 4*(11), 1379-1389. doi: 10.1517/17425255.4.11.1379

Harrison, S. A., Torgerson, S., & Hayashi, P. H. (2003). The natural history of nonalcoholic fatty liver disease: a clinical histopathological study. *Am J Gastroenterol, 98*(9), 2042-2047. doi: 10.1111/j.1572-0241.2003.07659.x

Haupt, S., Berger, M., Goldberg, Z., & Haupt, Y. (2003). Apoptosis - the p53 network. *J Cell Sci, 116*(Pt 20), 4077-4085. doi: 10.1242/jcs.00739

Haussler, S., Ziesing, S., Rademacher, G., Hoy, L., & Weissbrodt, H. (2003). Evaluation of the Merlin, Micronaut system for automated antimicrobial susceptibility testing of Pseudomonas aeruginosa and Burkholderia species isolated from cystic fibrosis patients. *Eur J Clin Microbiol Infect Dis, 22*(8), 496-500. doi: 10.1007/s10096-003-0974-7

He, C. W., Liu, F., Zhang, Y. F., Liang, T., & Zhou, K. Y. (2005). Inducing apoptosis of human nasopharyngeal carcinoma cell line CNE-2Z by bcl-xL short hairpin RNA. *Ai Zheng, 24*(6), 646-652.

Heijne, W. H., Kienhuis, A. S., van Ommen, B., Stierum, R. H., & Groten, J. P. (2005). Systems toxicology: applications of toxicogenomics, transcriptomics, proteomics and metabolomics in toxicology. *Expert Rev Proteomics, 2*(5), 767-780. doi: 10.1586/14789450.2.5.767

Heinloth, A. N., Irwin, R. D., Boorman, G. A., Nettesheim, P., Fannin, R. D., Sieber, S. O., . . . Paules, R. S. (2004). Gene expression profiling of rat livers reveals indicators of potential adverse effects. *Toxicol Sci, 80*(1), 193-202. doi: 10.1093/toxsci/kfh145

Higuchi, S., Wu, R., Zhou, M., Ravikumar, T. S., & Wang, P. (2007). Downregulation of hepatic cytochrome P-450 isoforms and PPAR-gamma: their role in hepatic injury and proinflammatory responses in a double-hit model of hemorrhage and sepsis. *J Surg Res, 137*(1), 46-52. doi: 10.1016/j.jss.2006.04.019

Hildebrandt, H., Becker, C., Gluer, S., Rosner, H., Gerardy-Schahn, R., & Rahmann, H. (1998). Polysialic acid on the neural cell adhesion molecule correlates with expression of polysialyltransferases and promotes neuroblastoma cell growth. *Cancer Res, 58*(4), 779-784.

Hildebrandt, H., Becker, C., Murau, M., Gerardy-Schahn, R., & Rahmann, H. (1998). Heterogeneous expression of the polysialyltransferases ST8Sia II and ST8Sia IV during postnatal rat brain development. *J Neurochem, 71*(6), 2339-2348.

Hinson, J. A., Roberts, D. W., & James, L. P. (2010). Mechanisms of acetaminophen-induced liver necrosis. *Handb Exp Pharmacol*(196), 369-405. doi: 10.1007/978-3-642-00663-0_12

Hirano, M., Maeda, K., Hayashi, H., Kusuhara, H., & Sugiyama, Y. (2005). Bile salt export pump (BSEP/ABCB11) can transport a nonbile acid substrate, pravastatin. *J Pharmacol Exp Ther, 314*(2), 876-882. doi: 10.1124/jpet.105.084830

Hirohashi, T., Suzuki, H., & Sugiyama, Y. (1999). Characterization of the transport properties of cloned rat multidrug resistance-associated protein 3 (MRP3). *J Biol Chem, 274*(21), 15181-15185.

Hisaeda, K., Inokuchi, A., Nakamura, T., Iwamoto, Y., Kohno, K., Kuwano, M., & Uchiumi, T. (2004). Interleukin-1beta represses MRP2 gene expression through inactivation of interferon regulatory factor 3 in HepG2 cells. *Hepatology, 39*(6), 1574-1582. doi: 10.1002/hep.20216

Ho, R. H., Leake, B. F., Roberts, R. L., Lee, W., & Kim, R. B. (2004). Ethnicity-dependent polymorphism in Na+-taurocholate cotransporting polypeptide (SLC10A1) reveals a domain critical for bile acid substrate recognition. *J Biol Chem, 279*(8), 7213-7222. doi: 10.1074/jbc.M305782200

Ho, R. H., Tirona, R. G., Leake, B. F., Glaeser, H., Lee, W., Lemke, C. J., . . . Kim, R. B. (2006). Drug and bile acid transporters in rosuvastatin hepatic uptake: function, expression, and pharmacogenetics. *Gastroenterology, 130*(6), 1793-1806. doi: 10.1053/j.gastro.2006.02.034

Hoebe, K. H., Witkamp, R. F., Fink-Gremmels, J., Van Miert, A. S., & Monshouwer, M. (2001). Direct cell-to-cell contact between Kupffer cells and hepatocytes augments endotoxin-induced hepatic injury. *Am J Physiol Gastrointest Liver Physiol, 280*(4), G720-728.

Holt, M., & Ju, C. (2010). Drug-induced liver injury. *Handb Exp Pharmacol*(196), 3-27. doi: 10.1007/978-3-642-00663-0_1

Holt, M. P., & Ju, C. (2006). Mechanisms of drug-induced liver injury. *AAPS J, 8*(1), E48-54. doi: 10.1208/aapsj080106

Honma, W., Shimada, M., Sasano, H., Ozawa, S., Miyata, M., Nagata, K., . . . Yamazoe, Y. (2002). Phenol sulfotransferase, ST1A3, as the main enzyme catalyzing sulfation of troglitazone in human liver. *Drug Metab Dispos, 30*(8), 944-949.

Horani, A., Muhanna, N., Pappo, O., Melhem, A., Alvarez, C. E., Doron, S., . . . Safadi, R. (2007). Beneficial effect of glatiramer acetate (Copaxone) on immune modulation of experimental hepatic fibrosis. *Am J Physiol Gastrointest Liver Physiol, 292*(2), G628-638. doi: 10.1152/ajpgi.00137.2006

Hussaini, S. H., & Farrington, E. A. (2007). Idiosyncratic drug-induced liver injury: an overview. *Expert Opin Drug Saf, 6*(6), 673-684. doi: 10.1517/14740338.6.6.673

Ikeda, T. (2011). Drug-induced idiosyncratic hepatotoxicity: prevention strategy developed after the troglitazone case. *Drug Metab Pharmacokinet, 26*(1), 60-70.

Imai, Y., Ishikawa, E., Asada, S., & Sugimoto, Y. (2005). Estrogen-mediated post transcriptional down-regulation of breast cancer resistance protein/ABCG2. *Cancer Res, 65*(2), 596-604.

Ingelman-Sundberg, M. (2004). Human drug metabolising cytochrome P450 enzymes: properties and polymorphisms. *Naunyn Schmiedebergs Arch Pharmacol, 369*(1), 89-104. doi: 10.1007/s00210-003-0819-z

Iqbal, J., & Hussain, M. M. (2009). Intestinal lipid absorption. *Am J Physiol Endocrinol Metab, 296*(6), E1183-1194. doi: 10.1152/ajpendo.90899.2008

Iqbal, S., & Stevens, P. D. (2009). Cholangiopancreatoscopy for targeted biopsies of the bile and pancreatic ducts. *Gastrointest Endosc Clin N Am, 19*(4), 567-577. doi: 10.1016/j.giec.2009.06.005

Ito, Y., Bethea, N. W., Abril, E. R., & McCuskey, R. S. (2003). Early hepatic microvascular injury in response to acetaminophen toxicity. *Microcirculation, 10*(5), 391-400. doi: 10.1038/sj.mn.7800204

Ito, Y., & McCuskey, R. (2007). Hepatic microcirculation. . In R. J., B. J., B. A., R. J. & R. M. (Eds.), Textbook of

Hepatology: From Basic Sciences to Clinical Practice. (3rd edition. ed.): Malden, MA: Wiley-Blackwell Publishing.

Izumi, N., Mizuguchi, S., Inagaki, Y., Saika, S., Kawada, N., Nakajima, Y., . . . Ikeda, K. (2006). BMP-7 opposes TGF-beta1-mediated collagen induction in mouse pulmonary myofibroblasts through Id2. *Am J Physiol Lung Cell Mol Physiol, 290*(1), L120-126. doi: 10.1152/ajplung.00171.2005

Jaeschke, A. (2007). Kupffer cells. In J. Rodes, J. Benhamou, A. Blei, J. Reichen & M. Rizzetto (Eds.), Textbook of hepatology: from basic science to clinical practice. (3rd edition ed.). Malden, MA: Wiley-Blackwell Publishing.

Jaeschke, H. (2006). Mechanisms of Liver Injury. II. Mechanisms of neutrophil-induced liver cell injury during hepatic ischemia-reperfusion and other acute inflammatory conditions. *Am J Physiol Gastrointest Liver Physiol, 290*(6), G1083-1088. doi: 10.1152/ajpgi.00568.2005

Jaeschke, H., & Bajt, M. L. (2006). Intracellular signaling mechanisms of acetaminophen-induced liver cell death. *Toxicol Sci, 89*(1), 31-41. doi: 10.1093/toxsci/kfi336

Jaeschke, H., Gores, G. J., Cederbaum, A. I., Hinson, J. A., Pessayre, D., & Lemasters, J. J. (2002). Mechanisms of hepatotoxicity. *Toxicol Sci, 65*(2), 166-176.

Jennen, D. G., Magkoufopoulou, C., Ketelslegers, H. B., van Herwijnen, M. H., Kleinjans, J. C., & van Delft, J. H. (2010). Comparison of HepG2 and HepaRG by whole-genome gene expression analysis for the purpose of chemical hazard identification. *Toxicol Sci, 115*(1), 66-79. doi: 10.1093/toxsci/kfq026

Jiang, J. X., Venugopal, S., Serizawa, N., Chen, X., Scott, F., Li, Y., . . . Torok, N. J. (2010). Reduced nicotinamide adenine dinucleotide phosphate oxidase 2 plays a key role in stellate cell activation and liver fibrogenesis in vivo. *Gastroenterology, 139*(4), 1375-1384. doi: 10.1053/j.gastro.2010.05.074

Jiang, W., Sun, R., Zhou, R., Wei, H., & Tian, Z. (2009). TLR-9 activation aggravates concanavalin A-induced hepatitis via promoting accumulation and activation of liver CD4+ NKT cells. *J Immunol, 182*(6), 3768-3774. doi: 10.4049/jimmunol.0800973

Jigorel, E., Le Vee, M., Boursier-Neyret, C., Parmentier, Y., & Fardel, O. (2006). Differential regulation of sinusoidal and canalicular hepatic drug transporter expression by xenobiotics activating drug-sensing receptors in primary human hepatocytes. *Drug Metab Dispos, 34*(10), 1756-1763. doi: 10.1124/dmd.106.010033

Jones, D. P., Lemasters, J. J., Han, D., Boelsterli, U. A., & Kaplowitz, N. (2010). Mechanisms of pathogenesis in drug hepatotoxicity putting the stress on mitochondria. *Mol Interv, 10*(2), 98-111. doi: 10.1124/mi.10.2.7

Juliano, R. L., & Ling, V. (1976). A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta, 455*(1), 152-162.

Kaisho, T., Takeuchi, O., Kawai, T., Hoshino, K., & Akira, S. (2001). Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J Immunol, 166*(9), 5688-5694.

Kalgutkar, A. S., Gardner, I., Obach, R. S., Shaffer, C. L., Callegari, E., Henne, K. R., . . . Harriman, S. P. (2005). A comprehensive listing of bioactivation pathways of organic functional groups. *Curr Drug Metab, 6*(3), 161-225.

Kanebratt, K. P., & Andersson, T. B. (2008). Evaluation of HepaRG cells as an in vitro model for human drug metabolism studies. *Drug Metab Dispos, 36*(7), 1444-1452. doi: 10.1124/dmd.107.020016

Kanebratt, K. P., & Andersson, T. B. (2008). HepaRG cells as an in vitro model for evaluation of cytochrome P450 induction in humans. *Drug Metab Dispos, 36*(1), 137-145. doi: 10.1124/dmd.107.017418

Kaplowitz, N. (2004). Drug-induced liver injury. *Clin Infect Dis, 38 Suppl 2*, S44-48. doi: 10.1086/381446

Kaplowitz, N. (2005). Idiosyncratic drug hepatotoxicity. *Nat Rev Drug Discov, 4*(6), 489-499. doi: 10.1038/nrd1750

Kashuba, E. V., Gradin, K., Isaguliants, M., Szekely, L., Poellinger, L., Klein, G., & Kazlauskas, A. (2006). Regulation of transactivation function of the aryl hydrocarbon receptor by the Epstein-Barr virus-encoded EBNA-3 protein. *J Biol Chem, 281*(2), 1215-1223. doi: 10.1074/jbc.M509036200

Kast, H. R., Goodwin, B., Tarr, P. T., Jones, S. A., Anisfeld, A. M., Stoltz, C. M., . . . Edwards, P. A. (2002). Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. *J Biol Chem, 277*(4), 2908-2915. doi: 10.1074/jbc.M109326200

Katoonizadeh, A., Nevens, F., Verslype, C., Pirenne, J., & Roskams, T. (2006). Liver regeneration in acute severe liver impairment: a clinicopathological correlation study. *Liver Int, 26*(10), 1225-1233. doi: 10.1111/j.1478-3231.2006.01377.x

Kawakami, M., Nakamura, T., Okamura, N., Komoto, C., Markova, S., Kobayashi, H., . . . Sakaeda, T. (2007). Knock-down of sorcin induces up-regulation of MDR1 in HeLa cells. *Biol Pharm Bull, 30*(6), 1065-1073.

Kayama, F., Yoshida, T., Elwell, M. R., & Luster, M. I. (1995). Role of tumor necrosis factor-alpha in cadmium-induced hepatotoxicity. *Toxicol Appl Pharmacol, 131*(2), 224-234. doi: 10.1006/taap.1995.1065

Kearney, C. J., Sheridan, C., Cullen, S. P., Tynan, G. A., Logue, S. E., Afonina, I. S., . . . Martin, S. J. (2013). Inhibitor of apoptosis proteins (IAPs) and their antagonists regulate spontaneous and tumor necrosis factor (TNF)-induced proinflammatory cytokine and chemokine production. *J Biol Chem, 288*(7), 4878-4890. doi: 10.1074/jbc.M112.422410

Kemp, D. C., Zamek-Gliszczynski, M. J., & Brouwer, K. L. (2005). Xenobiotics inhibit hepatic uptake and biliary excretion of taurocholate in rat hepatocytes. *Toxicol Sci, 83*(2), 207-214. doi: 10.1093/toxsci/kfi020

Kershenobich Stalnikowitz, D., & Weissbrod, A. B. (2003). Liver fibrosis and inflammation. A review. *Ann Hepatol, 2*(4), 159-163.

Key, A. P., & Dykens, E. M. (2008). 'Hungry Eyes': visual processing of food images in adults with Prader-Willi syndrome. *J Intellect Disabil Res, 52*(Pt 6), 536-546. doi: 10.1111/j.1365-2788.2008.01062.x

Khan, A. Y., Preskorn, S. H., & Horst, W. D. (2007). Coadministration of nefazodone and desipramine: a pharmacokinetic interaction study. *J Pak Med Assoc, 57*(5), 230-235.

Khan, Z., Crawford, J.M. , & Stolz, D. (2007). Ultrastructure of the hepatocyte. In R. J., B. J., B. A., R. J. & M. Rizzett (Eds.), Textbook of Hepatology: From Basic Science to CLinical Practice. (Third ed.): Malden, MA: Wiley-Blackwell Publishing. doi: 10.1002/9780470691861.ch1c

Kharbanda, K. K., Rogers, D. D., 2nd, Wyatt, T. A., Sorrell, M. F., & Tuma, D. J. (2004). Transforming growth factor-beta induces contraction of activated hepatic stellate cells. *J Hepatol, 41*(1), 60-66. doi: 10.1016/j.jhep.2004.03.019

Kherlopian, A. R., Song, T., Duan, Q., Neimark, M. A., Po, M. J., Gohagan, J. K., & Laine, A. F. (2008). A review of imaging techniques for systems biology. *BMC Syst Biol, 2*, 74. doi: 10.1186/1752-0509-2-74

Khetani, S. R., & Bhatia, S. N. (2008). Microscale culture of human liver cells for drug development. *Nat Biotechnol, 26*(1), 120-126. doi: 10.1038/nbt1361

Kinoshita, K., Iimuro, Y., Otogawa, K., Saika, S., Inagaki, Y., Nakajima, Y., . . . Ikeda, K. (2007). Adenovirus-mediated expression of BMP-7 suppresses the development of liver fibrosis in rats. *Gut, 56*(5), 706-714. doi: 10.1136/gut.2006.092460

Kipp, H., & Arias, I. M. (2000). Newly synthesized canalicular ABC transporters are directly targeted from the Golgi to the hepatocyte apical domain in rat liver. *J Biol Chem, 275*(21), 15917-15925. doi: 10.1074/jbc.M909875199

Kitano, H. (2002). Systems biology: a brief overview. *Science, 295*(5560), 1662-1664. doi: 10.1126/science.1069492

Kiyosawa, N., Ando, Y., Manabe, S., & Yamoto, T. (2009). Toxicogenomic biomarkers for liver toxicity. *J Toxicol Pathol, 22*(1), 35-52. doi: 10.1293/tox.22.35

Klaassen, C. D., & Aleksunes, L. M. (2010). Xenobiotic, bile acid, and cholesterol transporters: function and regulation. *Pharmacol Rev, 62*(1), 1-96. doi: 10.1124/pr.109.002014

Kmiec, Z. (2001). Cooperation of liver cells in health and disease. *Adv Anat Embryol Cell Biol, 161*, III-XIII, 1-151.

Kolios, G., Valatas, V., & Kouroumalis, E. (2006). Role of Kupffer cells in the pathogenesis of liver disease. *World J Gastroenterol, 12*(46), 7413-7420.

Konig, J., Cui, Y., Nies, A. T., & Keppler, D. (2000). A novel human organic anion transporting polypeptide localized to the basolateral hepatocyte membrane. *Am J Physiol Gastrointest Liver Physiol, 278*(1), G156-164.

Kullak-Ublick, G. A., Stieger, B., Hagenbuch, B., & Meier, P. J. (2000). Hepatic transport of bile salts. *Semin Liver Dis, 20*(3), 273-292. doi: 10.1055/s-2000-9426

Kullak-Ublick, G. A., Stieger, B., & Meier, P. J. (2004). Enterohepatic bile salt transporters in normal physiology and liver disease. *Gastroenterology, 126*(1), 322-342.

Kuschk, P., Wiessner, A., Kappelmeyer, U., Weissbrodt, E., Kastner, M., & Stottmeister, U. (2003). Annual cycle of nitrogen removal by a pilot-scale subsurface horizontal flow in a constructed wetland under moderate climate. *Water Res, 37*(17), 4236-4242. doi: 10.1016/S0043-1354(03)00163-5

Labbe, G., Pessayre, D., & Fromenty, B. (2008). Drug-induced liver injury through mitochondrial dysfunction: mechanisms and detection during preclinical safety studies. *Fundam Clin Pharmacol, 22*(4), 335-353. doi: 10.1111/j.1472-8206.2008.00608.x

Landry, J., Bernier, D., Ouellet, C., Goyette, R., & Marceau, N. (1985). Spheroidal aggregate culture of rat liver cells: histotypic reorganization, biomatrix deposition, and maintenance of functional activities. *J Cell Biol, 101*(3), 914-923.

Lang, C., Meier, Y., Stieger, B., Beuers, U., Lang, T., Kerb, R., . . . Pauli-Magnus, C. (2007). Mutations and polymorphisms in the bile salt export pump and the multidrug resistance protein 3 associated with drug-induced liver injury. *Pharmacogenet Genomics, 17*(1), 47-60. doi: 10.1097/01.fpc.0000230418.28091.76

Lawson, J. A., Fisher, M. A., Simmons, C. A., Farhood, A., & Jaeschke, H. (1998). Parenchymal cell apoptosis as a signal for sinusoidal sequestration and transendothelial migration of neutrophils in murine models of endotoxin and Fas-antibody-induced liver injury. *Hepatology, 28*(3), 761-767. doi: 10.1002/hep.510280324

Le Vee, M., Gripon, P., Stieger, B., & Fardel, O. (2008). Down-regulation of organic anion transporter expression in human hepatocytes exposed to the proinflammatory cytokine interleukin 1beta. *Drug Metab Dispos, 36*(2), 217-222. doi: 10.1124/dmd.107.016907

Le Vee, M., Jigorel, E., Glaise, D., Gripon, P., Guguen-Guillouzo, C., & Fardel, O. (2006). Functional expression of sinusoidal and canalicular hepatic drug transporters in the differentiated human hepatoma HepaRG cell line. *Eur J Pharm Sci, 28*(1-2), 109-117. doi: 10.1016/j.ejps.2006.01.004

Le Vee, M., Lecureur, V., Moreau, A., Stieger, B., & Fardel, O. (2009). Differential regulation of drug transporter expression by hepatocyte growth factor in primary human hepatocytes. *Drug Metab Dispos, 37*(11), 2228-2235. doi: 10.1124/dmd.109.028035

LeCluyse, E. L., Witek, R. P., Andersen, M. E., & Powers, M. J. (2012). Organotypic liver culture models: meeting current challenges in toxicity testing. *Crit Rev Toxicol, 42*(6), 501-548. doi: 10.3109/10408444.2012.682115

Lee, B. H., Koh, W. J., Choi, M. S., Suh, G. Y., Chung, M. P., Kim, H., & Kwon, O. J. (2005). Inactive hepatitis B surface antigen carrier state and hepatotoxicity during antituberculosis chemotherapy. *Chest, 127*(4), 1304-1311. doi: 10.1378/chest.127.4.1304

Lee, G., & Piquette-Miller, M. (2003). Cytokines alter the expression and activity of the multidrug resistance transporters in human hepatoma cell lines; analysis using RT-PCR and cDNA microarrays. *J Pharm Sci, 92*(11), 2152-2163. doi: 10.1002/jps.10493

Lee, P. J., Ghorashian, N., Gaige, T. A., & Hung, P. J. (2007). Microfluidic System for Automated Cell-based Assays. *JALA Charlottesv Va, 12*(6), 363-367. doi: 10.1016/j.jala.2007.07.001

Lee, W. M. (2003). Acute liver failure in the United States. *Semin Liver Dis, 23*(3), 217-226. doi: 10.1055/s-2003-42641

Lee, W. M. (2003). Drug-induced hepatotoxicity. *N Engl J Med, 349*(5), 474-485. doi: 10.1056/NEJMra021844

Lefebvre, P., Cariou, B., Lien, F., Kuipers, F., & Staels, B. (2009). Role of bile acids and bile acid receptors in metabolic regulation. *Physiol Rev, 89*(1), 147-191. doi: 10.1152/physrev.00010.2008

Letteron, P., Sutton, A., Mansouri, A., Fromenty, B., & Pessayre, D. (2003). Inhibition of microsomal triglyceride transfer protein: another mechanism for drug-induced steatosis in mice. *Hepatology, 38*(1), 133-140. doi: 10.1053/jhep.2003.50309

Li, A. P. (2004). Accurate prediction of human drug toxicity: a major challenge in drug development. *Chem Biol Interact, 150*(1), 3-7. doi: 10.1016/j.cbi.2004.09.008

Li, A. P., Colburn, S. M., & Beck, D. J. (1992). A simplified method for the culturing of primary adult rat and human hepatocytes as multicellular spheroids. *In Vitro Cell Dev Biol, 28A*(9-10), 673-677.

Li, L., Lee, T. K., Meier, P. J., & Ballatori, N. (1998). Identification of glutathione as a driving force and leukotriene C4 as a substrate for oatp1, the hepatic sinusoidal organic solute transporter. *J Biol Chem, 273*(26), 16184-16191.

Li, L., Meier, P. J., & Ballatori, N. (2000). Oatp2 mediates bidirectional organic solute transport: a role for intracellular glutathione. *Mol Pharmacol, 58*(2), 335-340.

Libbrecht, L., Desmet, V., Van Damme, B., & Roskams, T. (2000). Deep intralobular extension of human hepatic 'progenitor cells' correlates with parenchymal inflammation in chronic viral hepatitis: can 'progenitor cells' migrate? *J Pathol, 192*(3), 373-378. doi: 10.1002/1096-9896(2000)9999:9999<::AID-PATH700>3.0.CO;2-5

Liguori, M. J., Blomme, E. A., & Waring, J. F. (2008). Trovafloxacin-induced gene expression changes in liver-derived in vitro systems: comparison of primary human hepatocytes to HepG2 cells. *Drug Metab Dispos, 36*(2), 223-233. doi: 10.1124/dmd.107.017608

Liguori, M. J., Ditewig, A. C., Maddox, J. F., Luyendyk, J. P., Lehman-McKeeman, L. D., Nelson, D. M., . . . Blomme, E. A. (2010). Comparison of TNFalpha to Lipopolysaccharide as an Inflammagen to Characterize the Idiosyncratic Hepatotoxicity Potential of Drugs: Trovafloxacin as an Example. *Int J Mol Sci, 11*(11), 4697-4714. doi: 10.3390/ijms11114697

Lim, P. L., Liu, J., Go, M. L., & Boelsterli, U. A. (2008). The mitochondrial superoxide/thioredoxin-2/Ask1 signaling pathway is critically involved in troglitazone-induced cell injury to human hepatocytes. *Toxicol Sci, 101*(2), 341-349. doi: 10.1093/toxsci/kfm273

Lohse, A. W., & Mieli-Vergani, G. (2011). Autoimmune hepatitis. *J Hepatol, 55*(1), 171-182. doi: 10.1016/j.jhep.2010.12.012

Lu, H., Ouyang, W., & Huang, C. (2006). Inflammation, a key event in cancer development. *Mol Cancer Res, 4*(4), 221-233. doi: 10.1158/1541-7786.MCR-05-0261

Lu, J., Jones, A. D., Harkema, J. R., Roth, R. A., & Ganey, P. E. (2012). Amiodarone exposure during modest inflammation induces idiosyncrasy-like liver injury in rats: role of tumor necrosis factor-alpha. *Toxicol Sci, 125*(1), 126-133. doi: 10.1093/toxsci/kfr266

Lu, Z., & Xu, S. (2006). ERK1/2 MAP kinases in cell survival and apoptosis. *IUBMB Life, 58*(11), 621-631. doi: 10.1080/15216540600957438

Luebke-Wheeler, J. L., Nedredal, G., Yee, L., Amiot, B. P., & Nyberg, S. L. (2009). E-cadherin protects primary hepatocyte spheroids from cell death by a caspase-independent mechanism. *Cell Transplant, 18*(12), 1281-1287. doi: 10.3727/096368909X474258

Luedde, T., & Trautwein, C. (2006). Intracellular survival pathways in the liver. *Liver Int, 26*(10), 1163-1174. doi: 10.1111/j.1478-3231.2006.01366.x

Luster, M. I., Simeonova, P. P., Gallucci, R. M., Matheson, J. M., & Yucesoy, B. (2000). Immunotoxicology: role of inflammation in chemical-induced hepatotoxicity. *Int J Immunopharmacol, 22*(12), 1143-1147.

Luyendyk, J. P., Maddox, J. F., Cosma, G. N., Ganey, P. E., Cockerell, G. L., & Roth, R. A. (2003). Ranitidine treatment during a modest inflammatory response precipitates idiosyncrasy-like liver injury in rats. *J Pharmacol Exp Ther, 307*(1), 9-16. doi: 10.1124/jpet.103.054288

M., Bhanushali, V., Bagale, Shirode, A., Joshi, Y. , & Kadam, V. (2010). An in-vitro toxicity testing - a reliable alternative to toxicity testing by reduction, replacement and refinement of animals. *International journal of advances in pharmaceutical sciences, 1*(1), 15-31. doi: 10.5138/ijaps.2010.0976.1055.01002

Ma, K. L., Ruan, X. Z., Powis, S. H., Chen, Y., Moorhead, J. F., & Varghese, Z. (2008). Inflammatory stress exacerbates lipid accumulation in hepatic cells and fatty livers of apolipoprotein E knockout mice. *Hepatology, 48*(3), 770-781. doi: 10.1002/hep.22423

Mackie, J. T., Atshaves, B. P., Payne, H. R., McIntosh, A. L., Schroeder, F., & Kier, A. B. (2009). Phytol-induced hepatotoxicity in mice. *Toxicol Pathol, 37*(2), 201-208. doi: 10.1177/0192623308330789

Maglich, J. M., Stoltz, C. M., Goodwin, B., Hawkins-Brown, D., Moore, J. T., & Kliewer, S. A. (2002). Nuclear pregnane x receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. *Mol Pharmacol, 62*(3), 638-646.

Maher, J. J., Scott, M. K., Saito, J. M., & Burton, M. C. (1997). Adenovirus-mediated expression of cytokine-induced neutrophil chemoattractant in rat liver induces a neutrophilic hepatitis. *Hepatology, 25*(3), 624-630. doi: 10.1002/hep.510250322

Malarkey, D. E., Johnson, K., Ryan, L., Boorman, G., & Maronpot, R. R. (2005). New insights into functional aspects of liver morphology. *Toxicol Pathol, 33*(1), 27-34. doi: 10.1080/01926230590881826

Malhi, H., & Gores, G. J. (2008). Cellular and molecular mechanisms of liver injury. *Gastroenterology, 134*(6), 1641-1654. doi: 10.1053/j.gastro.2008.03.002

Malhi, H., Gores, G. J., & Lemasters, J. J. (2006). Apoptosis and necrosis in the liver: a tale of two deaths? *Hepatology, 43*(2 Suppl 1), S31-44. doi: 10.1002/hep.21062

Malhi, H., Guicciardi, M. E., & Gores, G. J. (2010). Hepatocyte death: a clear and present danger. *Physiol Rev, 90*(3), 1165-1194. doi: 10.1152/physrev.00061.2009

Mann, K. K., Davison, K., Colombo, M., Colosimo, A. L., Diaz, Z., Padovani, A. M., . . . Miller, W. H., Jr. (2006). Antimony trioxide-induced apoptosis is dependent on SEK1/JNK signaling. *Toxicol Lett, 160*(2), 158-170. doi: 10.1016/j.toxlet.2005.06.017

Martel, C., Huynh le, H., Garnier, A., Ventura-Clapier, R., & Brenner, C. (2012). Inhibition of the Mitochondrial Permeability Transition for Cytoprotection: Direct versus Indirect Mechanisms. *Biochem Res Int, 2012*, 213403. doi: 10.1155/2012/213403

Marzioni, M., Glaser, S. S., Francis, H., Phinizy, J. L., LeSage, G., & Alpini, G. (2002). Functional heterogeneity of cholangiocytes. *Semin Liver Dis, 22*(3), 227-240. doi: 10.1055/s-2002-34501

Matter, K., & Mellman, I. (1994). Mechanisms of cell polarity: sorting and transport in epithelial cells. *Curr Opin Cell Biol, 6*(4), 545-554.

Matzinger, P. (2002). The danger model: a renewed sense of self. *Science, 296*(5566), 301-305. doi: 10.1126/science.1071059

Matzinger, P. (2002). An innate sense of danger. *Ann N Y Acad Sci, 961*, 341-342.

Meek, D. W., & Anderson, C. W. (2009). Posttranslational modification of p53: cooperative integrators of function. *Cold Spring Harb Perspect Biol, 1*(6), a000950. doi: 10.1101/cshperspect.a000950

Mees, C., Nemunaitis, J., & Senzer, N. (2009). Transcription factors: their potential as targets for an individualized therapeutic approach to cancer. *Cancer Gene Ther, 16*(2), 103-112. doi: 10.1038/cgt.2008.73

Meier, P. J., & Stieger, B. (2000). Molecular Mechanisms in Bile Formation. *News Physiol Sci, 15*, 89-93.

Meier, P. J., & Stieger, B. (2002). Bile salt transporters. *Annu Rev Physiol, 64*, 635-661. doi: 10.1146/annurev.physiol.64.082201.100300

Meier, Y., Pauli-Magnus, C., Zanger, U. M., Klein, K., Schaeffeler, E., Nussler, A. K., . . . Stieger, B. (2006). Interindividual variability of canalicular ATP-binding-cassette (ABC)-transporter expression in human liver. *Hepatology, 44*(1), 62-74. doi: 10.1002/hep.21214

Melhem, A., Muhanna, N., Bishara, A., Alvarez, C. E., Ilan, Y., Bishara, T., . . . Safadi, R. (2006). Anti-fibrotic activity of NK cells in experimental liver injury through killing of activated HSC. *J Hepatol, 45*(1), 60-71. doi: 10.1016/j.jhep.2005.12.025

Micheau, O., & Tschopp, J. (2003). Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell, 114*(2), 181-190.

Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., & Moll, U. M. (2003). p53 has a direct apoptogenic role at the mitochondria. *Mol Cell, 11*(3), 577-590.

Milkiewicz, P., Gallagher, R., Chambers, J., Eggington, E., Weaver, J., & Elias, E. (2003). Obstetric cholestasis with elevated gamma glutamyl transpeptidase: incidence, presentation and treatment. *J Gastroenterol Hepatol, 18*(11), 1283-1286.

Miller, J. T., & Btaiche, I. F. (2008). Oxandrolone in pediatric patients with severe thermal burn injury. *Ann Pharmacother, 42*(9), 1310-1315. doi: 10.1345/aph.1L162

Miller, T. J., Knapton, A., Adeyemo, O., Noory, L., Weaver, J., & Hanig, J. P. (2008). Cytochrome c: a non-invasive biomarker of drug-induced liver injury. *J Appl Toxicol, 28*(7), 815-828. doi: 10.1002/jat.1347

Mills, J. B., Rose, K. A., Sadagopan, N., Sahi, J., & de Morais, S. M. (2004). Induction of drug metabolism enzymes and MDR1 using a novel human hepatocyte cell line. *J Pharmacol Exp Ther, 309*(1), 303-309. doi: 10.1124/jpet.103.061713

Mingatto, F. E., dos Santos, A. C., Rodrigues, T., Pigoso, A. A., Uyemura, S. A., & Curti, C. (2000). Effects of nimesulide and its reduced metabolite on mitochondria. *Br J Pharmacol, 131*(6), 1154-1160. doi: 10.1038/sj.bjp.0703667

Mita, S., Suzuki, H., Akita, H., Hayashi, H., Onuki, R., Hofmann, A. F., & Sugiyama, Y. (2006). Inhibition of bile acid transport across Na+/taurocholate cotransporting polypeptide (SLC10A1) and bile salt export pump (ABCB 11)-coexpressing LLC-PK1 cells by cholestasis-inducing drugs. *Drug Metab Dispos, 34*(9), 1575-1581. doi: 10.1124/dmd.105.008748

Mohapatra, R., Tran, M., Gore, J. M., & Spencer, F. A. (2005). A review of the oral direct thrombin inhibitor ximelagatran: not yet the end of the warfarin era. *Am Heart J, 150*(1), 19-26. doi: 10.1016/j.ahj.2005.02.012

Mohutsky, M. A., Romeike, A., Meador, V., Lee, W. M., Fowler, J., & Francke-Carroll, S. (2010). Hepatic drug-metabolizing enzyme induction and implications for preclinical and clinical risk assessment. *Toxicol Pathol, 38*(5), 799-809. doi: 10.1177/0192623310375099

Moon, K. H., Lee, Y. M., & Song, B. J. (2010). Inhibition of hepatic mitochondrial aldehyde dehydrogenase by carbon tetrachloride through JNK-mediated phosphorylation. *Free Radic Biol Med, 48*(3), 391-398. doi: 10.1016/j.freeradbiomed.2009.11.008

Moore, K. A., & Lemischka, I. R. (2006). Stem cells and their niches. *Science, 311*(5769), 1880-1885. doi: 10.1126/science.1110542

Morgan, E. T. (2009). Impact of infectious and inflammatory disease on cytochrome P450-mediated drug metabolism and pharmacokinetics. *Clin Pharmacol Ther, 85*(4), 434-438. doi: 10.1038/clpt.2008.302

Morgan, E. T., Goralski, K. B., Piquette-Miller, M., Renton, K. W., Robertson, G. R., Chaluvadi, M. R., . . . Sinal, C. J. (2008). Regulation of drug-metabolizing enzymes and transporters in infection, inflammation, and cancer. *Drug Metab Dispos, 36*(2), 205-216. doi: 10.1124/dmd.107.018747

Morgan, E. T., Li-Masters, T., & Cheng, P. Y. (2002). Mechanisms of cytochrome P450 regulation by inflammatory mediators. *Toxicology, 181-182*, 207-210.

Mottino, A. D., & Catania, V. A. (2008). Hepatic drug transporters and nuclear receptors: regulation by therapeutic agents. *World J Gastroenterol, 14*(46), 7068-7074.

Moustakas, A., & Kardassis, D. (1998). Regulation of the human p21/WAF1/Cip1 promoter in hepatic cells by functional interactions between Sp1 and Smad family members. *Proc Natl Acad Sci U S A, 95*(12), 6733-6738.

Muriel, P., & Rivera-Espinoza, Y. (2008). Beneficial drugs for liver diseases. *J Appl Toxicol, 28*(2), 93-103. doi: 10.1002/jat.1310

Murray, K. F., Hadzic, N., Wirth, S., Bassett, M., & Kelly, D. (2008). Drug-related hepatotoxicity and acute liver failure. *J Pediatr Gastroenterol Nutr, 47*(4), 395-405. doi: 10.1097/MPG.0b013e3181709464

Nakanishi, T., Shiozawa, K., Hassel, B. A., & Ross, D. D. (2006). Complex interaction of BCRP/ABCG2 and imatinib in BCR-ABL-expressing cells: BCRP-mediated resistance to imatinib is attenuated

by imatinib-induced reduction of BCRP expression. *Blood, 108*(2), 678-684. doi: 10.1182/blood-2005-10-4020

Namour, F., Wessels, D. H., Pascual, M. H., Reynolds, D., Sultan, E., & Lenfant, B. (2001). Pharmacokinetics of the new ketolide telithromycin (HMR 3647) administered in ascending single and multiple doses. *Antimicrob Agents Chemother, 45*(1), 170-175. doi: 10.1128/AAC.45.1.170-175.2001

Nascimento, A. C., Maia, D. R., Neto, S. M., Lima, E. M., Twycross, M., Baquette, R. F., & Lobato, C. M. (2012). Nonalcoholic Fatty liver disease in chronic hepatitis B and C patients from Western Amazon. *Int J Hepatol, 2012*, 695950. doi: 10.1155/2012/695950

Natori, S., Rust, C., Stadheim, L. M., Srinivasan, A., Burgart, L. J., & Gores, G. J. (2001). Hepatocyte apoptosis is a pathologic feature of human alcoholic hepatitis. *J Hepatol, 34*(2), 248-253.

Navarro, V. J., & Senior, J. R. (2006). Drug-related hepatotoxicity. *N Engl J Med, 354*(7), 731-739. doi: 10.1056/NEJMra052270

Nebert, D. W., & Russell, D. W. (2002). Clinical importance of the cytochromes P450. *Lancet, 360*(9340), 1155-1162. doi: 10.1016/S0140-6736(02)11203-7

Newton, K., & Dixit, V. M. (2012). Signaling in innate immunity and inflammation. *Cold Spring Harb Perspect Biol, 4*(3). doi: 10.1101/cshperspect.a006049

Nowatari, T., Fukunaga, K., & Ohkohchi, N. (2012). Regulation of signal transduction and role of platelets in liver regeneration. *Int J Hepatol, 2012*, 542479. doi: 10.1155/2012/542479

Nuwaysir, E. F., Bittner, M., Trent, J., Barrett, J. C., & Afshari, C. A. (1999). Microarrays and toxicology: the advent of toxicogenomics. *Mol Carcinog, 24*(3), 153-159.

Okan, G., Yaylaci, S., Peker, O., Kaymakoglu, S., & Saruc, M. (2008). Vanishing bile duct and Stevens-Johnson syndrome associated with ciprofloxacin treated with tacrolimus. *World J Gastroenterol, 14*(29), 4697-4700.

Olson, H., Betton, G., Robinson, D., Thomas, K., Monro, A., Kolaja, G., . . . Heller, A. (2000). Concordance of the toxicity of pharmaceuticals in humans and in animals. *Regul Toxicol Pharmacol, 32*(1), 56-67. doi: 10.1006/rtph.2000.1399

Ong, S. Y., Dai, H., & Leong, K. W. (2006). Hepatic differentiation potential of commercially available human mesenchymal stem cells. *Tissue Eng, 12*(12), 3477-3485. doi: 10.1089/ten.2006.12.3477

Ortiz de Montellano, P. R. (1995). The 1994 Bernard B. Brodie Award Lecture. Structure, mechanism, and inhibition of cytochrome P450. *Drug Metab Dispos, 23*(11), 1181-1187.

Osawa, Y., Nagaki, M., Banno, Y., Brenner, D. A., Asano, T., Nozawa, Y., . . . Nakashima, S. (2002). Tumor necrosis factor alpha-induced interleukin-8 production via NF-kappaB and phosphatidylinositol 3-kinase/Akt pathways inhibits cell apoptosis in human hepatocytes. *Infect Immun, 70*(11), 6294-6301.

Otsuka, M., Matsumoto, T., Morimoto, R., Arioka, S., Omote, H., & Moriyama, Y. (2005). A human transporter protein that mediates the final excretion step for toxic organic cations. *Proc Natl Acad Sci U S A, 102*(50), 17923-17928. doi: 10.1073/pnas.0506483102

Padda, M. S., Sanchez, M., Akhtar, A. J., & Boyer, J. L. (2011). Drug-induced cholestasis. *Hepatology, 53*(4), 1377-1387. doi: 10.1002/hep.24229

Pandit, A., Sachdeva, T., & Bafna, P. (2012). Drug-Induced Hepatotoxicity: A Review. *Journal of Applied Pharmaceutical Science, 02*(05), 233-243. doi: 10.7324/JAPS.2012.2541

Panin, L. E., Maksimov, V. F., Khoshchenko, O. M., & Korostyshevskaia, I. M. (2002). [Effect of combined glucocorticoids and low density lipoproteins on structural and functional changes in hepatocytes and Kupffer cells]. *Tsitologiia, 44*(12), 1149-1156.

Papa, S., Bubici, C., Zazzeroni, F., & Franzoso, G. (2009). Mechanisms of liver disease: cross-talk between the NF-kappaB and JNK pathways. *Biol Chem, 390*(10), 965-976. doi: 10.1515/BC.2009.111

Papucci, L., Formigli, L., Schiavone, N., Tani, A., Donnini, M., Lapucci, A., . . . Capaccioli, S. (2004). Apoptosis shifts to necrosis via intermediate types of cell death by a mechanism depending on c-myc and bcl-2 expression. *Cell Tissue Res, 316*(2), 197-209. doi: 10.1007/s00441-004-0872-z

Park, B. K., Kitteringham, N. R., Maggs, J. L., Pirmohamed, M., & Williams, D. P. (2005). The role of metabolic activation in drug-induced hepatotoxicity. *Annu Rev Pharmacol Toxicol, 45*, 177-202. doi: 10.1146/annurev.pharmtox.45.120403.100058

Parola, M., & Pinzani, M. (2009). Hepatic wound repair. *Fibrogenesis Tissue Repair, 2*(1), 4. doi: 10.1186/1755-1536-2-4

Patel, T., Roberts, L. R., Jones, B. A., & Gores, G. J. (1998). Dysregulation of apoptosis as a mechanism of liver disease: an overview. *Semin Liver Dis, 18*(2), 105-114. doi: 10.1055/s-2007-1007147

Pauli-Magnus, C., & Meier, P. J. (2006). Hepatobiliary transporters and drug-induced cholestasis. *Hepatology, 44*(4), 778-787. doi: 10.1002/hep.21359

Perdu, J., & Germain, D. P. (2001). Identification of novel polymorphisms in the pM5 and MRP1 (ABCC1) genes at locus 16p13.1 and exclusion of both genes as responsible for pseudoxanthoma elasticum. *Hum Mutat, 17*(1), 74-75. doi: 10.1002/1098-1004(2001)17:1<74::AID-HUMU14>3.0.CO;2-F

Perkins, N. D. (2007). Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat Rev Mol Cell Biol, 8*(1), 49-62. doi: 10.1038/nrm2083

Perri, R. , & Shah, V. (2005). Hepatic sinusoidal endothelial cells. In J. F. Dufour, P. A. Clavien, C. Trautwein & R. Graf (Eds.), Signaling pathways in liver disease. Berlin: Springer.

Pessayre, D., Berson, A., Fromenty, B., & Mansouri, A. (2001). Mitochondria in steatohepatitis. *Semin Liver Dis, 21*(1), 57-69.

Peters, T. S. (2005). Do preclinical testing strategies help predict human hepatotoxic potentials? *Toxicol Pathol, 33*(1), 146-154. doi: 10.1080/01926230590522121

Petrovic, V., Teng, S., & Piquette-Miller, M. (2007). Regulation of drug transporters during infection and inflammation. *Mol Interv, 7*(2), 99-111. doi: 10.1124/mi.7.2.10

Pichler, W. J. (2002). Modes of presentation of chemical neoantigens to the immune system. *Toxicology, 181-182*, 49-54.

Pichler, W. J. (2003). Delayed drug hypersensitivity reactions. *Ann Intern Med, 139*(8), 683-693.

Pineiro-Carrero, V. M., & Pineiro, E. O. (2004). Liver. *Pediatrics, 113*(4 Suppl), 1097-1106.

Ponzetti, K., King, M., Gates, A., Anwer, M. S., & Webster, C. R. (2010). Cyclic AMP-guanine exchange factor activation inhibits JNK-dependent lipopolysaccharide-induced apoptosis in rat hepatocytes. *Hepat Med, 2010*(2), 1-11. doi: 10.2147/HMER.S7673

Porth, C. M. (1995). Understanding the cholesterol transport system. *Nursing, 25*(4), 32T-32U.

Postic, C., Dentin, R., & Girard, J. (2004). Role of the liver in the control of carbohydrate and lipid homeostasis. *Diabetes Metab, 30*(5), 398-408.

Powers, M. J., Domansky, K., Kaazempur-Mofrad, M. R., Kalezi, A., Capitano, A., Upadhyaya, A., . . . Griffith, L. G. (2002). A microfabricated array bioreactor for perfused 3D liver culture. *Biotechnol Bioeng, 78*(3), 257-269.

Poynard, T., Ratziu, V., Benmanov, Y., Di Martino, V., Bedossa, P., & Opolon, P. (2000). Fibrosis in patients with chronic hepatitis C: detection and significance. *Semin Liver Dis, 20*(1), 47-55.

Rahman, A., Kefer, J., Bando, M., Niles, W. D., & Malik, A. B. (1998). E-selectin expression in human endothelial cells by TNF-alpha-induced oxidant generation and NF-kappaB activation. *Am J Physiol, 275*(3 Pt 1), L533-544.

Rahman, S., Aitken, A., Flynn, G., Formstone, C., & Savidge, G. F. (1998). Modulation of RGD sequence motifs regulates disintegrin recognition of alphaIIb beta3 and alpha5 beta1 integrin complexes. Replacement of elegantin alanine-50 with proline, N-terminal to the RGD sequence, diminishes recognition of the alpha5 beta1 complex with restoration induced by Mn2+ cation. *Biochem J, 335 (Pt 2)*, 247-257.

Ramachandran, R., & Kakar, S. (2009). Histological patterns in drug-induced liver disease. *J Clin Pathol, 62*(6), 481-492. doi: 10.1136/jcp.2008.058248

Ramadori, G., Moriconi, F., Malik, I., & Dudas, J. (2008). Physiology and pathophysiology of liver inflammation, damage and repair. *J Physiol Pharmacol, 59 Suppl 1*, 107-117.

Ramaiah, S. K., & Jaeschke, H. (2007). Hepatic neutrophil infiltration in the pathogenesis of alcohol-induced liver injury. *Toxicol Mech Methods, 17*(7), 431-440. doi: 10.1080/00952990701407702

Rappaport, A. M. (1977). Microcirculatory units in the mammalian liver. Their arterial and portal components. *Bibl Anat*(16 Pt 2), 116-120.

Rasola, A., & Bernardi, P. (2007). The mitochondrial permeability transition pore and its involvement in cell death and in disease pathogenesis. *Apoptosis, 12*(5), 815-833. doi: 10.1007/s10495-007-0723-y

Reddy, J. K., & Rao, M. S. (2006). Lipid metabolism and liver inflammation. II. Fatty liver disease and fatty acid oxidation. *Am J Physiol Gastrointest Liver Physiol, 290*(5), G852-858. doi: 10.1152/ajpgi.00521.2005

Reinero, C. R., Brownlee, L., Decile, K. C., Seguin, B., Berghaus, R. D., Nelson, R. W., & Gershwin, L. J. (2006). Inhaled flunisolide suppresses the hypothalamic-pituitary-adrenocortical axis, but has minimal systemic immune effects in healthy cats. *J Vet Intern Med, 20*(1), 57-64.

Renton, K. W. (2004). Cytochrome P450 regulation and drug biotransformation during inflammation and infection. *Curr Drug Metab, 5*(3), 235-243.

Ripp, S. L., Mills, J. B., Fahmi, O. A., Trevena, K. A., Liras, J. L., Maurer, T. S., & de Morais, S. M. (2006). Use of immortalized human hepatocytes to predict the magnitude of clinical drug-drug interactions caused by CYP3A4 induction. *Drug Metab Dispos, 34*(10), 1742-1748. doi: 10.1124/dmd.106.010132

Roberts, R. A., Ganey, P. E., Ju, C., Kamendulis, L. M., Rusyn, I., & Klaunig, J. E. (2007). Role of the Kupffer cell in mediating hepatic toxicity and carcinogenesis. *Toxicol Sci, 96*(1), 2-15. doi: 10.1093/toxsci/kfl173

Rodés, J., Benhamou, J.P., Blei, A. , Reichen, J., & Rizzetto, M. (2007). *Textbook of Hepatology: From Basic Science to Clinical Practice.*

Rohrbeck, A., Salinas, G., Maaser, K., Linge, J., Salovaara, S., Corvi, R., & Borlak, J. (2010). Toxicogenomics applied to in vitro carcinogenicity testing with Balb/c 3T3 cells revealed a gene signature predictive of chemical carcinogens. *Toxicol Sci, 118*(1), 31-41. doi: 10.1093/toxsci/kfq246

Rost, D., Kartenbeck, J., & Keppler, D. (1999). Changes in the localization of the rat canalicular conjugate export pump Mrp2 in phalloidin-induced cholestasis. *Hepatology, 29*(3), 814-821. doi: 10.1002/hep.510290319

Roth, R. A., & Ganey, P. E. (2010). Intrinsic versus idiosyncratic drug-induced hepatotoxicity--two villains or one? *J Pharmacol Exp Ther, 332*(3), 692-697. doi: 10.1124/jpet.109.162651

Roth, R. A., Luyendyk, J. P., Maddox, J. F., & Ganey, P. E. (2003). Inflammation and drug idiosyncrasy--is there a connection? *J Pharmacol Exp Ther, 307*(1), 1-8. doi: 10.1124/jpet.102.041624

Rowden, A. K., Norvell, J., Eldridge, D. L., & Kirk, M. A. (2005). Updates on acetaminophen toxicity. *Med Clin North Am, 89*(6), 1145-1159. doi: 10.1016/j.mcna.2005.06.009

Russel, W. B., & Ortega, S. (2010). Graduate education: the future is now. *Science, 329*(5991), 511. doi: 10.1126/science.329.5991.511-b

Russmann, S., Kullak-Ublick, G. A., & Grattagliano, I. (2009). Current concepts of mechanisms in drug-induced hepatotoxicity. *Curr Med Chem, 16*(23), 3041-3053.

Sacerdoti, D., Gatta, A., & McGiff, J. C. (2003). Role of cytochrome P450-dependent arachidonic acid metabolites in liver physiology and pathophysiology. *Prostaglandins Other Lipid Mediat, 72*(1-2), 51-71.

Sadot, A., Sarbu, S., Kesseli, J., Amir-Kroll, H., Zhang, W., Nykter, M., & Shmulevich, I. (2013). Information-theoretic analysis of the dynamics of an executable biological model. *PLoS One, 8*(3), e59303. doi: 10.1371/journal.pone.0059303

Sakai, Y., Yamagami, S., & Nakazawa, K. (2010). Comparative analysis of gene expression in rat liver tissue and monolayer- and spheroid-cultured hepatocytes. *Cells Tissues Organs, 191*(4), 281-288. doi: 10.1159/000272316

Santoni-Rugiu, E., Jelnes, P., Thorgeirsson, S. S., & Bisgaard, H. C. (2005). Progenitor cells in liver regeneration: molecular responses controlling their activation and expansion. *APMIS, 113*(11-12), 876-902. doi: 10.1111/j.1600-0463.2005.apm_386.x

Schjøtt, J. (2011). Adverse Effects of Drugs and Toxins on the Liver, Liver Biopsy in Modern Medicine, Dr., & Yoshiaki Mizuguchi (Ed.), ISBN: 978-953-307-883-0. (2011). Adverse Effects of Drugs and Toxins on the Liver. In Y. Mizuguchi (Ed.), Liver Biopsy in Modern Medicine: Intechopen.

Schnyder, B., & Pichler, W. J. (2009). Mechanisms of drug-induced allergy. *Mayo Clin Proc, 84*(3), 268-272. doi: 10.1016/S0025-6196(11)61145-2

Schrenk, D., Eisenmann-Tappe, I., Gebhardt, R., Mayer, D., el Mouelhi, M., Rohrdanz, E., . . . Bock, K. W. (1991). Drug metabolizing enzyme activities in rat liver epithelial cell lines, hepatocytes and bile duct cells. *Biochem Pharmacol, 41*(11), 1751-1757.

Schulze-Osthoff, K., & Bantel, H. (2011). Necrosis versus apoptosis in acetaminophen-induced hepatotoxicity. *Hepatology, 53*(3), 1070. doi: 10.1002/hep.24027

Schuppan, D., & Afdhal, N. H. (2008). Liver cirrhosis. *Lancet, 371*(9615), 838-851. doi: 10.1016/S0140-6736(08)60383-9

Schuppan, D., Ruehl, M., Somasundaram, R., & Hahn, E. G. (2001). Matrix as a modulator of hepatic fibrogenesis. *Semin Liver Dis, 21*(3), 351-372. doi: 10.1055/s-2001-17556

Schwabe, R. F. (2006). Cell death in the liver-all roads lead to JNK. *Gastroenterology, 131*(1), 314-316. doi: 10.1053/j.gastro.2006.05.029

Schwabe, R. F., & Brenner, D. A. (2006). Mechanisms of Liver Injury. I. TNF-alpha-induced liver injury: role of IKK, JNK, and ROS pathways. *Am J Physiol Gastrointest Liver Physiol, 290*(4), G583-589. doi: 10.1152/ajpgi.00422.2005

Schwabe, R. F., Uchinami, H., Qian, T., Bennett, B. L., Lemasters, J. J., & Brenner, D. A. (2004). Differential requirement for c-Jun NH2-terminal kinase in TNFalpha- and Fas-mediated apoptosis in hepatocytes. *FASEB J, 18*(6), 720-722. doi: 10.1096/fj.03-0771fje

Schwerk, C., & Schulze-Osthoff, K. (2005). Methyltransferase inhibition induces p53-dependent apoptosis and a novel form of cell death. *Oncogene, 24*(47), 7002-7011. doi: 10.1038/sj.onc.1208855

Scott, M. J., Liu, S., Shapiro, R. A., Vodovotz, Y., & Billiar, T. R. (2009). Endotoxin uptake in mouse liver is blocked by endotoxin pretreatment through a suppressor of cytokine signaling-1-dependent mechanism. *Hepatology, 49*(5), 1695-1708. doi: 10.1002/hep.22839

Searfoss, G. H., Ryan, T. P., & Jolly, R. A. (2005). The role of transcriptome analysis in pre-clinical toxicology. *Curr Mol Med, 5*(1), 53-64.

Seguin, B., & Uetrecht, J. (2003). The danger hypothesis applied to idiosyncratic drug reactions. *Curr Opin Allergy Clin Immunol, 3*(4), 235-242. doi: 10.1097/01.all.0000083953.99396.a2

Shaffer, E.A., & Myers, R. P. (2005). *First principles of gastroenterology : the basis of disease and an approach to management* E. A. Shaffer & A. B. R. Thomson (Eds.),

Shah, R. R. (1999). Drug-induced hepatotoxicity: pharmacokinetic perspectives and strategies for risk reduction. *Adverse Drug React Toxicol Rev, 18*(4), 181-233.

Shao, Z. Y., Chen, B. A., Ding, J. H., Xia, G. H., Zhu, H. G., & Gao, X. Z. (2005). [Inhibition effect of vitamin K2 on human MDS-JSN04 cell line and its possible mechanism]. *Zhongguo Shi Yan Xue Ye Xue Za Zhi, 13*(6), 1028-1032.

Shaw, P. J., Beggs, K. M., Sparkenbaugh, E. M., Dugan, C. M., Ganey, P. E., & Roth, R. A. (2009). Trovafloxacin enhances TNF-induced inflammatory stress and cell death signaling and reduces TNF clearance in a murine model of idiosyncratic hepatotoxicity. *Toxicol Sci, 111*(2), 288-301. doi: 10.1093/toxsci/kfp163

Shaw, P. J., Ditewig, A. C., Waring, J. F., Liguori, M. J., Blomme, E. A., Ganey, P. E., & Roth, R. A. (2009). Coexposure of mice to trovafloxacin and lipopolysaccharide, a model of idiosyncratic hepatotoxicity, results in a unique gene expression profile and interferon gamma-dependent liver injury. *Toxicol Sci, 107*(1), 270-280. doi: 10.1093/toxsci/kfn205

Shaw, P. J., Ganey, P. E., & Roth, R. A. (2009). Trovafloxacin enhances the inflammatory response to a Gram-negative or a Gram-positive bacterial stimulus, resulting in neutrophil-dependent liver injury in mice. *J Pharmacol Exp Ther, 330*(1), 72-78. doi: 10.1124/jpet.109.151068

Shaw, P. J., Ganey, P. E., & Roth, R. A. (2009). Tumor necrosis factor alpha is a proximal mediator of synergistic hepatotoxicity from trovafloxacin/lipopolysaccharide coexposure. *J Pharmacol Exp Ther, 328*(1), 62-68. doi: 10.1124/jpet.108.143792

Shaw, P. J., Ganey, P. E., & Roth, R. A. (2010). Idiosyncratic drug-induced liver injury and the role of inflammatory stress with an emphasis on an animal model of trovafloxacin hepatotoxicity. *Toxicol Sci, 118*(1), 7-18. doi: 10.1093/toxsci/kfq168

Shaw, P. J., Hopfensperger, M. J., Ganey, P. E., & Roth, R. A. (2007). Lipopolysaccharide and trovafloxacin coexposure in mice causes idiosyncrasy-like liver injury dependent on tumor necrosis factor-alpha. *Toxicol Sci, 100*(1), 259-266. doi: 10.1093/toxsci/kfm218

Shen, D. D., Kunze, K. L., & Thummel, K. E. (1997). Enzyme-catalyzed processes of first-pass hepatic and intestinal drug extraction. *Adv Drug Deliv Rev, 27*(2-3), 99-127.

Shen, Y. C., Wang, M. Y., Wang, C. Y., Tsai, T. C., Tsai, H. Y., Lee, Y. F., & Wei, L. C. (2007). Clearance of intravitreal voriconazole. *Invest Ophthalmol Vis Sci, 48*(5), 2238-2241. doi: 10.1167/iovs.06-1362

Shimada, T., Watanabe, N., Hiraishi, H., & Terano, A. (1999). Redox regulation of interleukin-8 expression in MKN28 cells. *Dig Dis Sci, 44*(2), 266-273.

Shitara, Y., Horie, T., & Sugiyama, Y. (2006). Transporters as a determinant of drug clearance and tissue distribution. *Eur J Pharm Sci, 27*(5), 425-446. doi: 10.1016/j.ejps.2005.12.003

Shitara, Y., Sato, H., & Sugiyama, Y. (2005). Evaluation of drug-drug interaction in the hepatobiliary and renal transport of drugs. *Annu Rev Pharmacol Toxicol, 45*, 689-723. doi: 10.1146/annurev.pharmtox.44.101802.121444

Siebler, J., & Galle, P. R. (2006). Treatment of nonalcoholic fatty liver disease. *World J Gastroenterol, 12*(14), 2161-2167.

Sigal, S. H., Rajvanshi, P., Gorla, G. R., Sokhi, R. P., Saxena, R., Gebhard, D. R., Jr., . . . Gupta, S. (1999). Partial hepatectomy-induced polyploidy attenuates hepatocyte replication and activates cell aging events. *Am J Physiol, 276*(5 Pt 1), G1260-1272.

Simpkins, J. W., & Dykens, J. A. (2008). Mitochondrial mechanisms of estrogen neuroprotection. *Brain Res Rev, 57*(2), 421-430. doi: 10.1016/j.brainresrev.2007.04.007

Singh, R., & Czaja, M. J. (2007). Regulation of hepatocyte apoptosis by oxidative stress. *J Gastroenterol Hepatol, 22 Suppl 1*, S45-48. doi: 10.1111/j.1440-1746.2006.04646.x

Snykers, S., De Kock, J., Rogiers, V., & Vanhaecke, T. (2009). In vitro differentiation of embryonic and adult stem cells into hepatocytes: state of the art. *Stem Cells, 27*(3), 577-605. doi: 10.1634/stemcells.2008-0963

Snykers, S., Vanhaecke, T., Papeleu, P., Luttun, A., Jiang, Y., Vander Heyden, Y., . . . Rogiers, V. (2006). Sequential exposure to cytokines reflecting embryogenesis: the key for in vitro differentiation of adult bone marrow stem cells into functional hepatocyte-like cells. *Toxicol Sci, 94*(2), 330-341; discussion 235-339. doi: 10.1093/toxsci/kfl058

Sohn, D., Schulze-Osthoff, K., & Janicke, R. U. (2005). Caspase-8 can be activated by interchain proteolysis without receptor-triggered dimerization during drug-induced apoptosis. *J Biol Chem, 280*(7), 5267-5273. doi: 10.1074/jbc.M408585200

Soriano, M. E., Nicolosi, L., & Bernardi, P. (2004). Desensitization of the permeability transition pore by cyclosporin a prevents activation of the mitochondrial apoptotic pathway and liver damage by tumor necrosis factor-alpha. *J Biol Chem, 279*(35), 36803-36808. doi: 10.1074/jbc.M405297200

Spangenburg, E. E., Pratt, S. J., Wohlers, L. M., & Lovering, R. M. (2011). Use of BODIPY (493/503) to visualize intramuscular lipid droplets in skeletal muscle. *J Biomed Biotechnol, 2011*, 598358. doi: 10.1155/2011/598358

Stedman, Chris Liddle and Catherine A.M. (2008). Hepatic metabolism of drugs. In J. Rodés, Benhamou, J.P., Blei, A., Reichen, J., and Rizzetto,M. (Ed.), Textbook of Hepatology: From Basic Science to Clinical Practice (3rd edition ed.): Blackwell Publishing Ltd.

Sun, W., Wu, R. R., van Poelje, P. D., & Erion, M. D. (2001). Isolation of a family of organic anion transporters from human liver and kidney. *Biochem Biophys Res Commun, 283*(2), 417-422. doi: 10.1006/bbrc.2001.4774

Sunman, J. A., Hawke, R. L., LeCluyse, E. L., & Kashuba, A. D. (2004). Kupffer cell-mediated IL-2 suppression of CYP3A activity in human hepatocytes. *Drug Metab Dispos, 32*(3), 359-363. doi: 10.1124/dmd.32.3.359

Suter, L., Babiss, L. E., & Wheeldon, E. B. (2004). Toxicogenomics in predictive toxicology in drug development. *Chem Biol, 11*(2), 161-171. doi: 10.1016/j.chembiol.2004.02.003

Suter, L., Schroeder, S., Meyer, K., Gautier, J. C., Amberg, A., Wendt, M., . . . Pfannkuch, F. (2011). EU framework 6 project: predictive toxicology (PredTox)--overview and outcome. *Toxicol Appl Pharmacol, 252*(2), 73-84. doi: 10.1016/j.taap.2010.10.008

Swain, M. G. (2008). Hepatic NKT cells: friend or foe? *Clin Sci (Lond), 114*(7), 457-466. doi: 10.1042/CS20070328

Tacke, F., Luedde, T., & Trautwein, C. (2009). Inflammatory pathways in liver homeostasis and liver injury. *Clin Rev Allergy Immunol, 36*(1), 4-12. doi: 10.1007/s12016-008-8091-0

Tajiri, K., & Shimizu, Y. (2008). Practical guidelines for diagnosis and early management of drug-induced liver injury. *World J Gastroenterol, 14*(44), 6774-6785.

Tak, P. P., & Firestein, G. S. (2001). NF-kappaB: a key role in inflammatory diseases. *J Clin Invest, 107*(1), 7-11. doi: 10.1172/JCI11830

Takabatake, H., Koide, N., & Tsuji, T. (1991). Encapsulated multicellular spheroids of rat hepatocytes produce albumin and urea in a spouted bed circulating culture system. *Artif Organs, 15*(6), 474-480.

Tan, H. H., Ong, W. M., Lai, S. H., & Chow, W. C. (2007). Nimesulide-induced hepatotoxicity and fatal hepatic failure. *Singapore Med J, 48*(6), 582-585.

Tang, W. (2007). Drug metabolite profiling and elucidation of drug-induced hepatotoxicity. *Expert Opin Drug Metab Toxicol, 3*(3), 407-420. doi: 10.1517/17425255.3.3.407

Tarantino, G., Di Minno, M. N., & Capone, D. (2009). Drug-induced liver injury: is it somehow foreseeable? *World J Gastroenterol, 15*(23), 2817-2833.

Taylor, R. C., Cullen, S. P., & Martin, S. J. (2008). Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol, 9*(3), 231-241. doi: 10.1038/nrm2312

Teng, S., & Piquette-Miller, M. (2008). Regulation of transporters by nuclear hormone receptors: implications during inflammation. *Mol Pharm, 5*(1), 67-76. doi: 10.1021/mp700102q

Terada, T., & Inui, K. (2008). Physiological and pharmacokinetic roles of H+/organic cation antiporters (MATE/SLC47A). *Biochem Pharmacol, 75*(9), 1689-1696. doi: 10.1016/j.bcp.2007.12.008

Tergaonkar, V., Pando, M., Vafa, O., Wahl, G., & Verma, I. (2002). p53 stabilization is decreased upon NFkappaB activation: a role for NFkappaB in acquisition of resistance to chemotherapy. *Cancer Cell, 1*(5), 493-503.

Thannickal, V. J., & Fanburg, B. L. (2000). Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol, 279*(6), L1005-1028.

Thompson, R. A., Isin, E. M., Li, Y., Weidolf, L., Page, K., Wilson, I., . . . Kenna, J. G. (2012). In vitro approach to assess the potential for risk of idiosyncratic adverse reactions caused by candidate drugs. *Chem Res Toxicol, 25*(8), 1616-1632. doi: 10.1021/tx300091x

Tietz, P. S., & Larusso, N. F. (2006). Cholangiocyte biology. *Curr Opin Gastroenterol, 22*(3), 279-287. doi: 10.1097/01.mog.0000218965.78558.bc

Tirmenstein, M. A., Hu, C. X., Gales, T. L., Maleeff, B. E., Narayanan, P. K., Kurali, E., . . . Schwartz, L. W. (2002). Effects of troglitazone on HepG2 viability and mitochondrial function. *Toxicol Sci, 69*(1), 131-138.

Torok, N. J., Larusso, E. M., & McNiven, M. A. (2001). Alterations in vesicle transport and cell polarity in rat hepatocytes subjected to mechanical or chemical cholestasis. *Gastroenterology, 121*(5), 1176-1184.

Trauner, J., & Buning, J. (2007). Germ-cell cluster formation in the telotrophic meroistic ovary of Tribolium castaneum (Coleoptera, Polyphaga, Tenebrionidae) and its implication on insect phylogeny. *Dev Genes Evol, 217*(1), 13-27. doi: 10.1007/s00427-006-0114-3

Trauner, M., & Boyer, J. L. (2003). Bile salt transporters: molecular characterization, function, and regulation. *Physiol Rev, 83*(2), 633-671. doi: 10.1152/physrev.00027.2002

Trauner, M., Fickert, P., & Stauber, R. E. (1999). Inflammation-induced cholestasis. *J Gastroenterol Hepatol, 14*(10), 946-959.

Trauner, M., Fickert, P., & Wagner, M. (2007). MDR3 (ABCB4) defects: a paradigm for the genetics of adult cholestatic syndromes. *Semin Liver Dis, 27*(1), 77-98. doi: 10.1055/s-2006-960172

Traversa, G., Bianchi, C., Da Cas, R., Abraha, I., Menniti-Ippolito, F., & Venegoni, M. (2003). Cohort study of hepatotoxicity associated with nimesulide and other non-steroidal anti-inflammatory drugs. *BMJ, 327*(7405), 18-22. doi: 10.1136/bmj.327.7405.18

Tukov, F. F., Luyendyk, J. P., Ganey, P. E., & Roth, R. A. (2007). The role of tumor necrosis factor alpha in lipopolysaccharide/ranitidine-induced inflammatory liver injury. *Toxicol Sci, 100*(1), 267-280. doi: 10.1093/toxsci/kfm209

Turner, R., Lozoya, O., Wang, Y., Cardinale, V., Gaudio, E., Alpini, G., . . . Reid, L. M. (2011). Human hepatic stem cell and maturational liver lineage biology. *Hepatology, 53*(3), 1035-1045. doi: 10.1002/hep.24157

Uetrecht, J. (2003). Screening for the potential of a drug candidate to cause idiosyncratic drug reactions. *Drug Discov Today, 8*(18), 832-837.

Uetrecht, J. (2007). Idiosyncratic drug reactions: current understanding. *Annu Rev Pharmacol Toxicol, 47*, 513-539. doi: 10.1146/annurev.pharmtox.47.120505.105150

Uetrecht, J. (2008). Idiosyncratic drug reactions: past, present, and future. *Chem Res Toxicol, 21*(1), 84-92. doi: 10.1021/tx700186p

Uetrecht, J. P. (1999). New concepts in immunology relevant to idiosyncratic drug reactions: the "danger hypothesis" and innate immune system. *Chem Res Toxicol, 12*(5), 387-395. doi: 10.1021/tx980249i

Ulrich, R. G. (2007). Idiosyncratic toxicity: a convergence of risk factors. *Annu Rev Med, 58*, 17-34. doi: 10.1146/annurev.med.58.072905.160823

Utesch, D., & Oesch, F. (1992). Dependency of the in vitro stabilization of differentiated functions in liver parenchymal cells on the type of cell line used for co-culture. *In Vitro Cell Dev Biol, 28A*(3 Pt 1), 193-198.

van Gijssel, H. E., Maassen, C. B., Mulder, G. J., & Meerman, J. H. (1997). p53 protein expression by hepatocarcinogens in the rat liver and its potential role in mitoinhibition of normal hepatocytes as a mechanism of hepatic tumour promotion. *Carcinogenesis, 18*(5), 1027-1033.

van Montfoort, J. E., Hagenbuch, B., Groothuis, G. M., Koepsell, H., Meier, P. J., & Meijer, D. K. (2003). Drug uptake systems in liver and kidney. *Curr Drug Metab, 4*(3), 185-211.

van Vliet, E. (2011). Current standing and future prospects for the technologies proposed to transform toxicity testing in the 21st century. *ALTEX, 28*(1), 17-44.

Vaschetto, M., Weissbrod, T., Bodle, D., & Guner, O. (2003). Enabling high-throughput discovery. *Curr Opin Drug Discov Devel, 6*(3), 377-383.

Vee, M. L., Lecureur, V., Stieger, B., & Fardel, O. (2009). Regulation of drug transporter expression in human hepatocytes exposed to the proinflammatory cytokines tumor necrosis factor-alpha or interleukin-6. *Drug Metab Dispos, 37*(3), 685-693. doi: 10.1124/dmd.108.023630

Verdine, B. N., Troseth, G. L., Hodapp, R. M., & Dykens, E. M. (2008). Strategies and correlates of jigsaw puzzle and visuospatial performance by persons with Prader-Willi syndrome. *Am J Ment Retard, 113*(5), 343-355. doi: 10.1352/2008.113:342-355

Vessey, C. J., & de la Hall, P. M. (2001). Hepatic stem cells: a review. *Pathology, 33*(2), 130-141.

Vethanayagam, R. R., Wang, H., Gupta, A., Zhang, Y., Lewis, F., Unadkat, J. D., & Mao, Q. (2005). Functional analysis of the human variants of breast cancer resistance protein: I206L, N590Y, and D620N. *Drug Metab Dispos, 33*(6), 697-705. doi: 10.1124/dmd.105.003657

Vig, P., Russo, F. P., Edwards, R. J., Tadrous, P. J., Wright, N. A., Thomas, H. C., . . . Forbes, S. J. (2006). The sources of parenchymal regeneration after chronic hepatocellular liver injury in mice. *Hepatology, 43*(2), 316-324. doi: 10.1002/hep.21018

Vollmar, B., & Menger, M. D. (2009). The hepatic microcirculation: mechanistic contributions and therapeutic targets in liver injury and repair. *Physiol Rev, 89*(4), 1269-1339. doi: 10.1152/physrev.00027.2008

Vuppugalla, R., & Mehvar, R. (2005). Enzyme-selective effects of nitric oxide on affinity and maximum velocity of various rat cytochromes P450. *Drug Metab Dispos, 33*(6), 829-836. doi: 10.1124/dmd.105.003848

Wakabayashi, Y., Kipp, H., & Arias, I. M. (2006). Transporters on demand: intracellular reservoirs and cycling of bile canalicular ABC transporters. *J Biol Chem, 281*(38), 27669-27673. doi: 10.1074/jbc.R600013200

Walton, B., Hamblin, A., Dumonde, D. C., & Simpson, R. (1976). Absence of cellular hypersensitivity in patients with unexplained hepatitis following halothane. *Anesthesiology, 44*(5), 391-397.

Wandzioch, E., Edling, C. E., Palmer, R. H., Carlsson, L., & Hallberg, B. (2004). Activation of the MAP kinase pathway by c-Kit is PI-3 kinase dependent in hematopoietic progenitor/stem cell lines. *Blood, 104*(1), 51-57. doi: 10.1182/blood-2003-07-2554

Wandzioch, E., Kolterud, A., Jacobsson, M., Friedman, S. L., & Carlsson, L. (2004). Lhx2-/- mice develop liver fibrosis. *Proc Natl Acad Sci U S A, 101*(47), 16549-16554. doi: 10.1073/pnas.0404678101

Wandzioch, E., & Zaret, K. S. (2009). Dynamic signaling network for the specification of embryonic pancreas and liver progenitors. *Science, 324*(5935), 1707-1710. doi: 10.1126/science.1174497

Wang, F., Dumstrei, K., Haag, T., & Hartenstein, V. (2004). The role of DE-cadherin during cellularization, germ layer formation and early neurogenesis in the Drosophila embryo. *Dev Biol, 270*(2), 350-363. doi: 10.1016/j.ydbio.2004.03.002

Wang, F., Kim, B. E., Petris, M. J., & Eide, D. J. (2004). The mammalian Zip5 protein is a zinc transporter that localizes to the basolateral surface of polarized cells. *J Biol Chem, 279*(49), 51433-51441. doi: 10.1074/jbc.M408361200

Wang, J. R., Lee, C. R., Lee, M. R., & Fuh, A. Y. (2004). Photorefractive effect induced by polarization gratings in dye-doped liquid crystals. *Opt Lett, 29*(1), 110-112.

Wang, L., & Boyer, J. L. (2004). The maintenance and generation of membrane polarity in hepatocytes. *Hepatology, 39*(4), 892-899. doi: 10.1002/hep.20039

Wang, L., Kolachala, V., Walia, B., Balasubramanian, S., Hall, R. A., Merlin, D., & Sitaraman, S. V. (2004). Agonist-induced polarized trafficking and surface expression of the adenosine 2b receptor in intestinal epithelial cells: role of SNARE proteins. *Am J Physiol Gastrointest Liver Physiol, 287*(5), G1100-1107. doi: 10.1152/ajpgi.00164.2004

Wang, R., Mair, R. W., Rosen, M. S., Cory, D. G., & Walsworth, R. L. (2004). Simultaneous measurement of rock permeability and effective porosity using laser-polarized noble gas NMR. *Phys Rev E Stat Nonlin Soft Matter Phys, 70*(2 Pt 2), 026312.

Wang, W. H., Gill, J., & Boutin, C. (2004). Spindle imaging in living mammalian oocytes using the liquid-crystal polarized light microscope and its practical use. *Methods Mol Biol, 253*, 215-225. doi: 10.1385/1-59259-744-0:215

Wang, W. W., Khetani, S. R., Krzyzewski, S., Duignan, D. B., & Obach, R. S. (2010). Assessment of a micropatterned hepatocyte coculture system to generate major human excretory and circulating drug metabolites. *Drug Metab Dispos, 38*(10), 1900-1905. doi: 10.1124/dmd.110.034876

Wang, X., Matsumoto, H., Zhao, X., Das, S. K., & Paria, B. C. (2004). Embryonic signals direct the formation of tight junctional permeability barrier in the decidualizing stroma during embryo implantation. *J Cell Sci, 117*(Pt 1), 53-62. doi: 10.1242/jcs.00826

Wang, X. M., Cui, F. Z., Ge, J., & Wang, Y. (2004). Hierarchical structural comparisons of bones from wild-type and liliput(dtc232) gene-mutated Zebrafish. *J Struct Biol, 145*(3), 236-245. doi: 10.1016/j.jsb.2003.10.028

Wang, Y., Cui, C. B., Yamauchi, M., Miguez, P., Roach, M., Malavarca, R., . . . Reid, L. M. (2011). Lineage restriction of human hepatic stem cells to mature fates is made efficient by tissue-specific biomatrix scaffolds. *Hepatology, 53*(1), 293-305. doi: 10.1002/hep.24012

Wang, Y., Nakagawa, Y., Liu, L., Wang, W., Ren, X., Anghel, A., . . . Liu, Y. (2011). Tissue-specific dysregulation of hexose-6-phosphate dehydrogenase and glucose-6-phosphate transporter production in db/db mice as a model of type 2 diabetes. *Diabetologia, 54*(2), 440-450. doi: 10.1007/s00125-010-1956-9

Wang, Y., Yao, H. L., Cui, C. B., Wauthier, E., Barbier, C., Costello, M. J., . . . Reid, L. M. (2010). Paracrine signals from mesenchymal cell populations govern the expansion and differentiation of human hepatic stem cells to adult liver fates. *Hepatology, 52*(4), 1443-1454. doi: 10.1002/hep.23829

Wang, Z. L. (2004). Functional oxide nanobelts: materials, properties and potential applications in nanosystems and biotechnology. *Annu Rev Phys Chem, 55*, 159-196. doi: 10.1146/annurev.physchem.55.091602.094416

Waring, J. F., & Anderson, M. G. (2005). Idiosyncratic toxicity: mechanistic insights gained from analysis of prior compounds. *Curr Opin Drug Discov Devel, 8*(1), 59-65.

Waring, J. F., Ciurlionis, R., Jolly, R. A., Heindel, M., & Ulrich, R. G. (2001). Microarray analysis of hepatotoxins in vitro reveals a correlation between gene expression profiles and mechanisms of toxicity. *Toxicol Lett, 120*(1-3), 359-368.

Waters, M. D., & Fostel, J. M. (2004). Toxicogenomics and systems toxicology: aims and prospects. *Nat Rev Genet, 5*(12), 936-948. doi: 10.1038/nrg1493

Weiler-Normann, C., & Schramm, C. (2011). Drug induced liver injury and its relationship to autoimmune hepatitis. *J Hepatol, 55*(4), 747-749. doi: 10.1016/j.jhep.2011.02.024

Weiler-Normann, C., Schramm, C., Quaas, A., Wiegard, C., Glaubke, C., Pannicke, N., . . . Lohse, A. W. (2013). Infliximab as a rescue treatment in difficult-to-treat autoimmune hepatitis. *J Hepatol, 58*(3), 529-534. doi: 10.1016/j.jhep.2012.11.010

Wieder, T., Essmann, F., Prokop, A., Schmelz, K., Schulze-Osthoff, K., Beyaert, R., . . . Daniel, P. T. (2001). Activation of caspase-8 in drug-induced apoptosis of B-lymphoid cells is independent of CD95/Fas receptor-ligand interaction and occurs downstream of caspase-3. *Blood, 97*(5), 1378-1387.

Wilkening, S., Stahl, F., & Bader, A. (2003). Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. *Drug Metab Dispos, 31*(8), 1035-1042. doi: 10.1124/dmd.31.8.1035

Will, Y., Dykens, J. A., Nadanaciva, S., Hirakawa, B., Jamieson, J., Marroquin, L. D., . . . Jessen, B. A. (2008). Effect of the multitargeted tyrosine kinase inhibitors imatinib, dasatinib, sunitinib, and sorafenib on mitochondrial function in isolated rat heart mitochondria and H9c2 cells. *Toxicol Sci, 106*(1), 153-161. doi: 10.1093/toxsci/kfn157

Williams, D. P., & Park, B. K. (2003). Idiosyncratic toxicity: the role of toxicophores and bioactivation. *Drug Discov Today, 8*(22), 1044-1050.

Wilson, Z. E., Rostami-Hodjegan, A., Burn, J. L., Tooley, A., Boyle, J., Ellis, S. W., & Tucker, G. T. (2003). Inter-individual variability in levels of human microsomal protein and hepatocellularity per gram of liver. *Br J Clin Pharmacol, 56*(4), 433-440.

Wu, R., Cui, X., Dong, W., Zhou, M., Simms, H. H., & Wang, P. (2006). Suppression of hepatocyte CYP1A2 expression by Kupffer cells via AhR pathway: the central role of proinflammatory cytokines. *Int J Mol Med, 18*(2), 339-346.

Wu, Y. M., Joseph, B., Berishvili, E., Kumaran, V., & Gupta, S. (2008). Hepatocyte transplantation and drug-induced perturbations in liver cell compartments. *Hepatology, 47*(1), 279-287. doi: 10.1002/hep.21937

Wullaert, A., van Loo, G., Heyninck, K., & Beyaert, R. (2007). Hepatic tumor necrosis factor signaling and nuclear factor-kappaB: effects on liver homeostasis and beyond. *Endocr Rev, 28*(4), 365-386. doi: 10.1210/er.2006-0031

Xia, X., Francis, H., Glaser, S., Alpini, G., & LeSage, G. (2006). Bile acid interactions with cholangiocytes. *World J Gastroenterol, 12*(22), 3553-3563.

Xia, X., Yang, J., Li, F., Li, Y., Zhou, X., Dai, Y., & Wong, S. T. (2010). Image-based chemical screening identifies drug efflux inhibitors in lung cancer cells. *Cancer Res, 70*(19), 7723-7733. doi: 10.1158/0008-5472.CAN-09-4360

Xia, Y., Padre, R. C., De Mendoza, T. H., Bottero, V., Tergaonkar, V. B., & Verma, I. M. (2009). Phosphorylation of p53 by IkappaB kinase 2 promotes its degradation by beta-TrCP. *Proc Natl Acad Sci U S A, 106*(8), 2629-2634. doi: 10.1073/pnas.0812256106

Xiao, C., & Ghosh, S. (2005). NF-kappaB, an evolutionarily conserved mediator of immune and inflammatory responses. *Adv Exp Med Biol, 560*, 41-45. doi: 10.1007/0-387-24180-9_5

Xie, G., Wang, L., Wang, X., Wang, L., & DeLeve, L. D. (2010). Isolation of periportal, midlobular, and centrilobular rat liver sinusoidal endothelial cells enables study of zonated drug toxicity. *Am J Physiol Gastrointest Liver Physiol, 299*(5), G1204-1210. doi: 10.1152/ajpgi.00302.2010

Xie, W., Uppal, H., Saini, S. P., Mu, Y., Little, J. M., Radominska-Pandya, A., & Zemaitis, M. A. (2004). Orphan nuclear receptor-mediated xenobiotic regulation in drug metabolism. *Drug Discov Today, 9*(10), 442-449. doi: 10.1016/S1359-6446(04)03061-2

Xiong, H., Hou, J., Gao, W. R., & Chen, Q. B. (2005). [Investigation of the effect of 2-methoxyestradiol and arsenic trioxide on the apoptosis-associated gene expression profile of myeloma cells]. *Zhonghua Xue Ye Xue Za Zhi, 26*(4), 200-204.

Xu, C., Li, C. Y., & Kong, A. N. (2005). Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch Pharm Res, 28*(3), 249-268.

Xu, J. J., Diaz, D., & O'Brien, P. J. (2004). Applications of cytotoxicity assays and pre-lethal mechanistic assays for assessment of human hepatotoxicity potential. *Chem Biol Interact, 150*(1), 115-128. doi: 10.1016/j.cbi.2004.09.011

Xu, J. J., Henstock, P. V., Dunn, M. C., Smith, A. R., Chabot, J. R., & de Graaf, D. (2008). Cellular imaging predictions of clinical drug-induced liver injury. *Toxicol Sci, 105*(1), 97-105. doi: 10.1093/toxsci/kfn109

Xu, Y. (2003). Regulation of p53 responses by post-translational modifications. *Cell Death Differ, 10*(4), 400-403. doi: 10.1038/sj.cdd.4401182

Yang, D., Chen, Q., Rosenberg, H. F., Rybak, S. M., Newton, D. L., Wang, Z. Y., . . . Howard, O. M. (2004). Human ribonuclease A superfamily members, eosinophil-derived neurotoxin and pancreatic ribonuclease, induce dendritic cell maturation and activation. *J Immunol, 173*(10), 6134-6142.

Yee, S. B., Kinser, S., Hill, D. A., Barton, C. C., Hotchkiss, J. A., Harkema, J. R., . . . Roth, R. A. (2000). Synergistic hepatotoxicity from coexposure to bacterial endotoxin and the pyrrolizidine alkaloid monocrotaline. *Toxicol Appl Pharmacol, 166*(3), 173-185. doi: 10.1006/taap.2000.8968

Yokose, T., Doy, M., T., Taniguchi, T., Shimada, M., Kakiki, T., Horie, . . . K., Mukai. (1999). Immunohistochemical study of cytochrome P450 2C and 3A in human non-neoplastic and neoplastic tissues. *Virchows Arch. , 434*(5), 401-411.

Youdim, K. A., Tyman, C. A., Jones, B. C., & Hyland, R. (2007). Induction of cytochrome P450: assessment in an immortalized human hepatocyte cell line (Fa2N4) using a novel higher throughput cocktail assay. *Drug Metab Dispos, 35*(2), 275-282. doi: 10.1124/dmd.106.012864

Yuasa, C., Tomita, Y., Shono, M., Ishimura, K., & Ichihara, A. (1993). Importance of cell aggregation for expression of liver functions and regeneration demonstrated with primary cultured hepatocytes. *J Cell Physiol, 156*(3), 522-530. doi: 10.1002/jcp.1041560311

Zelcer, N., Reid, G., Wielinga, P., Kuil, A., van der Heijden, I., Schuetz, J. D., & Borst, P. (2003). Steroid and bile acid conjugates are substrates of human multidrug-resistance protein (MRP) 4 (ATP-binding cassette C4). *Biochem J, 371*(Pt 2), 361-367. doi: 10.1042/BJ20021886

Zhan, S. S., Jiang, J. X., Wu, J., Halsted, C., Friedman, S. L., Zern, M. A., & Torok, N. J. (2006). Phagocytosis of apoptotic bodies by hepatic stellate cells induces NADPH oxidase and is associated with liver fibrosis in vivo. *Hepatology, 43*(3), 435-443. doi: 10.1002/hep.21093

Zhang, L., Theise, N., Chua, M., & Reid, L. M. (2008). The stem cell niche of human livers: symmetry between development and regeneration. *Hepatology, 48*(5), 1598-1607. doi: 10.1002/hep.22516

Zhang, X., Liu, F., Chen, X., Zhu, X., & Uetrecht, J. (2011). Involvement of the immune system in idiosyncratic drug reactions. *Drug Metab Pharmacokinet, 26*(1), 47-59.

Zhang, Y., Zhao, T., Li, W., & Vore, M. (2010). The 5'-untranslated region of multidrug resistance associated protein 2 (MRP2; ABCC2) regulates downstream open reading frame expression through translational regulation. *Mol Pharmacol, 77*(2), 237-246. doi: 10.1124/mol.109.058982

Zhao, X., Monson, C., Gao, C., Gouon-Evans, V., Matsumoto, N., Sadler, K. C., & Friedman, S. L. (2010). Klf6/copeb is required for hepatic outgrowth in zebrafish and for hepatocyte specification in mouse ES cells. *Dev Biol, 344*(1), 79-93. doi: 10.1016/j.ydbio.2010.04.018

Zhao, Y., Ding, W. X., Qian, T., Watkins, S., Lemasters, J. J., & Yin, X. M. (2003). Bid activates multiple mitochondrial apoptotic mechanisms in primary hepatocytes after death receptor engagement. *Gastroenterology, 125*(3), 854-867.

Zhou, J., Zhang, J., & Xie, W. (2005). Xenobiotic nuclear receptor-mediated regulation of UDP-glucuronosyl-transferases. *Curr Drug Metab, 6*(4), 289-298.

Zhou, J. F., Xiao, W. Q., Zheng, Y. C., Dong, J., & Zhang, S. M. (2006). Increased oxidative stress and oxidative damage associated with chronic bacterial prostatitis. *Asian J Androl, 8*(3), 317-323. doi: 10.1111/j.1745-7262.2006.00144.x

Zhou, S., Schuetz, J. D., Bunting, K. D., Colapietro, A. M., Sampath, J., Morris, J. J., . . . Sorrentino, B. P. (2001). The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med, 7*(9), 1028-1034. doi: 10.1038/nm0901-1028

Zhuang, S., & Schnellmann, R. G. (2006). A death-promoting role for extracellular signal-regulated kinase. *J Pharmacol Exp Ther, 319*(3), 991-997. doi: 10.1124/jpet.106.107367

Zidek, N., Hellmann, J., Kramer, P. J., & Hewitt, P. G. (2007). Acute hepatotoxicity: a predictive model based on focused illumina microarrays. *Toxicol Sci, 99*(1), 289-302. doi: 10.1093/toxsci/kfm131

Zimmerman, H. J. (2000). Drug-induced liver disease. *Clin Liver Dis, 4*(1), 73-96, vi.

Zollner, G., & Trauner, M. (2008). Mechanisms of cholestasis. *Clin Liver Dis, 12*(1), 1-26, vii. doi: 10.1016/j.cld.2007.11.010

Zollner, G., Wagner, M., & Trauner, M. (2010). Nuclear receptors as drug targets in cholestasis and drug-induced hepatotoxicity. *Pharmacol Ther, 126*(3), 228-243. doi: 10.1016/j.pharmthera.2010.03.005

Zong, W. X., & Thompson, C. B. (2006). Necrotic death as a cell fate. *Genes Dev, 20*(1), 1-15. doi: 10.1101/gad.1376506

Zou, W., Beggs, K. M., Sparkenbaugh, E. M., Jones, A. D., Younis, H. S., Roth, R. A., & Ganey, P. E. (2009). Sulindac metabolism and synergy with tumor necrosis factor-alpha in a drug-inflammation interaction model of idiosyncratic liver injury. *J Pharmacol Exp Ther, 331*(1), 114-121. doi: 10.1124/jpet.109.156331

Zou, W., Devi, S. S., Sparkenbaugh, E., Younis, H. S., Roth, R. A., & Ganey, P. E. (2009). Hepatotoxic interaction of sulindac with lipopolysaccharide: role of the hemostatic system. *Toxicol Sci, 108*(1), 184-193. doi: 10.1093/toxsci/kfn259

Zou, W., Roth, R. A., Younis, H. S., Malle, E., & Ganey, P. E. (2011). Neutrophil-cytokine interactions in a rat model of sulindac-induced idiosyncratic liver injury. *Toxicology, 290*(2-3), 278-285. doi: 10.1016/j.tox.2011.10.005

Zsembery, A., Thalhammer, T., & Graf, J. (2000). Bile Formation: a Concerted Action of Membrane Transporters in Hepatocytes and Cholangiocytes. *News Physiol Sci, 15*, 6-11.

Zuckerman, J. M. (2004). Macrolides and ketolides: azithromycin, clarithromycin, telithromycin. *Infect Dis Clin North Am, 18*(3), 621-649, xi-. doi: 10.1016/j.idc.2004.04.010

Development of a cellular predictive model of inflammation associated idiosyncratic drug-induced hepatotoxicity and investigation of its underlying cellular and molecular mechanisms

Abstract:

Idiosyncratic adverse drug reactions (IADRs) are considered as an important subset of ADRs, accounting for approximately 13% of all acute liver failure cases and representing one of the leading causes for post-marketing drug withdrawal (Shaw et al. 2010). The lack of effective *in vitro or in vivo* models able to predict the hepatotoxic potential of idiosyncratic drugs before being approved for marketing on one hand, and the ambiguity of the mechanisms underlying their hepatic pathogenesis on the other hand render IADRs a perplexing human health problem (Shaw et al. 2010). Accordingly, the work presented in this thesis was based on three main objectives:

1) Development of a high throughput human-based cellular model for the prediction of inflammation associated idiosyncratic drug-induced hepatotoxicity; based on the synergistic exposure of HepG2 cells to potentially hepatotoxic drugs and pro-inflammatory mediators (LPS and TNF-α).
2) Elucidation of the hepatotoxic mechanisms underlying four known idiosyncratic drugs (trovafloxacin, nimesulide, telithromycin and nefazodone) with emphasis on oxidative stress, steatosis and cholestasis.
3) Investigation of the molecular mechanisms underlying drug-inflammation synergistic induction of hepatocellular death

Firstly, the results attained in this thesis demonstrated that the developed model is sensitive, specific and applicable to high throughput toxicity screening of different categories of drugs. Secondly, our results demonstrated that the inflammation associated hepatotoxic potentials of the four tested idiosyncratic drugs are mediated as follows: trovafloxacin exerts a cholestatic potential that involves the down-regulation of both MDR1 and MRP2. Nimesulide promotes the intracellular accumulation of superoxide anions in addition to potently inhibiting MRP2. Telithromycin promotes hepatotoxicity predominately via a cholestatic mechanism that involves the down-regulation of MDR1. Nefazodone favors the accumulation of superoxide anions in addition to its prominent steatotic potential and inhibitory effect on both MDR1 and MRP2. Although each of the idiosyncratic drugs exhibited a different mechanism of toxicity they all induced amplified hepatocellular death in presence of LPS and TNF-α, which proved to be mediated via the intrinsic apoptotic pathway for trovafloxacin, the extrinsic for nefazodone and both apoptotic pathways for nimesulide and telithromycin. The amplified apoptotic potential of the four drugs proved to be based on the up-regulation of Bax and caspase 8 via an ERK½-dependent mechanism. These results indicate that the presented drug-inflammation model constitute an effective pre-clinical tool not only for the detection of inflammation-associated hepatotoxic drugs but also for the elucidation of their underlying mechanisms.

Hépatotoxicité idiosyncrasique liée à un stress inflammatoire: modèle de prédiction et mécanismes cellulaires et moléculaires

Résumé :

Les hépatopathies médicamenteuses survenant sur un mode idiosyncrasique représentent un obstacle majeur au développement de médicaments et sont à l'origine du retrait du marché de nombreux d'entre eux. Un des mécanismes mis en cause est lié à la survenue d'un épisode inflammatoire aigu qui, lors d'un traitement médicamenteux, sensibiliserait le foie pour les effets indésirables des médicaments, mettant en évidence leur toxicité idiosyncrasique. A l'heure actuelle, la détection précoce de médicaments potentiellement hépatotoxiques dans un contexte inflammatoire avant leur mise sur le marché, reste encore difficile et leurs mécanismes sous-jacents ne sont pas clairement élucidés.

Dans ce contexte, notre travail s'articule sur 3 axes :

1) Développer un nouveau modèle cellulaire *in vitro* humain, adaptable au criblage à haut débit prédictif d'hépatotoxicité idiosyncrasique liée à un stress inflammatoire, basé sur l'exposition synergique des cellules HepG2 cultivées dans des conditions particulières exposées à des médicaments potentiellement idiosyncrasiques et des médiateurs pro-inflammatoires (LPS et TNF-α).
2) Elucider les mécanismes sous jacents de la toxicité de 4 médicaments idiosyncrasiques connus (trovafloxacine, nimésulide, télithromycine et néfazodone), en mettant l'accent sur le stress oxydatif, la stéatose et la cholestase
3) Etudier les mécanismes moléculaires sous-jacents de la mort cellulaire observée lors d'une hépatotoxicité idiosyncrasique liée à un stress inflammatoire.

Ainsi, nous avons développé un modèle cellulaire humain prédictif d'hépatotoxicité idiosyncrasique liée à un stress inflammatoire sensible, spécifique et applicable au criblage en haut débit d'un grand nombre de médicaments. Pour cela, nous avons étudié dans ce modèle, les effets toxiques de quatre médicaments testés et élucidé leurs mécanismes. La trovafloxacine exerce un effet cholestatique par diminution de l'expression et de l'activité de MDR1 et MRP2. Le nimésulide favorise l'accumulation intracellulaire de radicaux superoxydes en plus de son potentiel cholestatique par inhibition de l'activité MRP2. La télithromycine favorise une hépatotoxicité principalement via un mécanisme cholestatique impliquant l'inhibition de MDR1. La néfazodone favorise l'accumulation des radicaux superoxydes en plus de son potentiel stéatosique important et de son effet inhibiteur sur les deux transporteurs MDR1 et MRP2. Bien que chaque médicament idiosyncrasique testé présente un mécanisme de toxicité différent, ils ont tous entraîné une mort hépatocellulaire amplifiée en présence de LPS et TNF-α, via la voie apoptotique intrinsèque pour la trovafloxacine, extrinsèque pour la néfazodone et les deux voies de l'apoptose pour le nimésulide et la télithromycine. Le potentiel apoptotique amplifié des quatre médicaments s'est avéré être médié par la surexpression de Bax et de caspase 8 via un mécanisme dépendant de ERK ½. Nos résultats indiquent que notre modèle peut être utilisé non seulement comme un outil préclinique pour l'identification de nouveaux médicaments qui pourraient être potentiellement hépatotoxiques lors d'un stress inflammatoire, mais aussi pour l'élucidation de leurs mécanismes.