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**UNIVERSITE DE STRASBOURG**

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**Maria Elena MALDONADO-CELIS**

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*Titre :*

# APOPTOTIC PATHWAYS TRIGGERED BY APPLE PROCYANIDINS IN HUMAN COLON ADENOCARCINOMA CELLS AND THEIR DERIVED METASTATIC CELLS

**Directeur de Thèse: Dr. F. RAUL**

**Membres du jury:**

| | |
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| **Prof. Claude KEDINGER** | **Examinateur** |
| **Prof. Jacques MAGDALOU** | **Rapporteur Externe** |
| **Prof. Marie-Paule VASSON** | **Rapporteur Externe** |
| **Dr. Pierre BISCHOFF** | **Rapporteur Interne** |
| **Dr. Francis RAUL** | **Directeur de Thèse** |

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*To my husband, for his infinite love,*
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# GENERAL INDEX

# LIST OF ABREVIATIONS

Ac- DEVD-pNA: Acetyl-Asp-Glu-Val-Asp-p-nitroaniline

Ac-IETD-pNA: Acetyl-Ile-Glu-Thr-Asp-p-nitroaniline

Ac-LEDH-pNA: N-Acetyl-Leu-Glu-His-Asp-p-nitroaniline

AdoMetDC: S-adenosylmethionine decarboxylase

AIF: Apoptosis Inducing Factor

ANOVA: Analysis of Variances

APC: adenomatous polyposis coli

Bax: Bcl-2 associated X protein

Bcl-2: B-cell lymphoma 2

BH: Bcl-2 Homology

BSA: Bovine Serum Albumin

CARD: Caspase Activated Recruitment Domain

CRC: colorectal cancer

CRD: Cysteine-rich domain

DD: death domain

DED: Death Effector Domain

$DiOC_2(3)$: 3,3'-DiethylOxacarboCyanine iodide

DISC: Death-Inducing Signaling Complex

DMSO: Dimethylsulfoxide

DR: Death Receptors

DR4: Death Receptor 4

DR5: Death Receptor 5

$\Delta\Psi_m$: Mitochondrial membrane potential

dUTP: deoxy uridiltriphosphate nucleotide

EGCG: Epigallocatechin gallate

EGF: epidermal growth factor

ELISA: Enzyme Linked Sorbed Assay

Endo G: Endonuclease G

ERK: Extracellular signal-Regulated Kinase

FACS: Fluorescence Activated Cell Sorter

FADD: Fas Associated Death Domain

FasL: Fas Ligand

FITC: Fluoroscein Isothiocyanate

5-FU: 5-floruracilo

FOLFIRI: folic acid, 5-FU, irinotecan

FOLFOX: 5-fluorouracilo / leucovorina / oxiplatina

HDAC: Histone deacetylase

$H_2O_2$: hydrogen peroxyde

IAP: Inhibitor of Apoptosis Proteins

IFL: irinotecan, 5-FU, leucovorin

JNK: c-Jun N-terminal Kinase

MAPK: Mitogen Activated Protein Kinase

MCL-1: Myeloid cell leukemia sequence 1

MDL 72527: N1,N4-bis(2,3-butanedienyl)-1,4-butaneamine dihydrochloride

MFI: mean fluorescence intensity

MPT: Mitochondrial Permeability Transition

NF-κB: Nuclear Factor – kappa B

ODC: Ornithine Decarboxylase

OMM: Outer Mitochondrial Membrane

OR : odds ratio

PAO: Polyamine Oxydase

PARP1: poly(ADP–ribose) polymerase-1

PBS: Phosphate Buffer Sodium

Pcy: Procyanidins

PI: propidium iodide

PI3K: Phosphoinositol-3-Kinase

PKC: Protein Kinase C

p-NA: para-Nitroaniline

ROS: Reactive Oxygen Species

RT-PCR: reverse transcription- polymerase chain reaction

SSAT: Spermidine/Spermine $N^1$-acetyl transferase

tBid: truncated Bid

TdT: Terminal deoxynucleotidil transferase

TNFR: Tumor Necrosis Factor Receptor

TRAIL: TNF- related apoptotic inducer ligand

TUNEL: Terminal deoxynucleotide transferase (TdT) d-UTP-biotin nick-end labeling

# INDEX OF FIGURES

# OBJECTIVES OF THE STUDY

Colorectal cancer (CRC) represents one of the major causes of death from cancer worldwide. Epidemiological studies support an inverse relationship between regular consumption of fruits and vegetables and the risk of CRC. Individual non-nutritive compounds present in fruits and vegetables have been identified as inhibitory agents of colon carcinogenesis. This health beneficial effect has been attributed in part to the polyphenolic compounds which can be divided into various clases on the basis of their molecular structure, with flavonoids being one of the main groups occurring in human diet. Apples are a rich source of flavonoids, and flavanols represent the major subclass of flavonoids, containing monomers (epicatechin and catechin) and polymeric forms (procyanidins).

During this work, I have been interested in studying the pro-apoptotic properties of apple procyanidins (Pcy). Our laboratory has previously reported that apple Pcy inhibited growth and induced the apoptosis of human colon carcinoma-derived metastatic SW620 cells. However, the cellular and molecular mechanisms by which it occurs are not well understood. The objective of this thesis was to identify the mechanisms of action by which apple Pcy triggered cellular signaling apoptotic pathway in two related human colon cancer cell lines with different proliferative characteristics. The SW480 cells correspond to a primary human colon adenocarcinoma and the SW620 cells are derived from the primary tumor and isolated from a mesenteric lymph node metastasis of the same patient.

# *INTRODUCTION*

# I. COLORECTAL CANCER

## 1. General epidemiological and etiological aspects

Colorectal cancer (CRC) represents the third cause of mortality for cancer in the world (Parkin et al., 2005; WHO, 2003). In terms of impact in developed countries CRC ranks third in frequency in men after prostate and lung cancer and, second in women after breast cancer (Figure 1). In general, rates of incidence of CRC are increasing rather rapidly in countries where overall risk was formerly low, while in high risk countries trends are either gradually increasing, stabilizing (North and West Europe), or declining with time (North America) (Figure 2) (Parkin et al, 2005). These cancers are rare in South America, Asia and Africa (Figure 2) (Parkin et al, 2005). These differences do not appear to be related to a genetic polymorphism of populations because immigrants lose all risk associated with their country or region of origin (Parkin and Hlat, 1996). From the study of migrants moved from low-risk to high-risk areas, the incidence of CRC increases rapidly within the first generation, implying that epigenetic (dietary and other environmental) factors constitute a major component of risk (Parkin et al, 2005).

It is accepted that about 70% of CRC cases are linked to diet (Bingham, 2000). Thus, food is one of the factors on which it is possible to act in order to increase primary prevention (INCa, 2007, 2009). Numerous epidemiological studies have established that a high intake of red or processed meat increases the risk of developing adenomas or colon cancer (Chao et al, 2005; Norat et al 2002; Willet, 2005). This risk increases in proportion to their consumption (Sandhu et al, 2001). Also, the physical inactivity and obesity are factors that promote CRC risk increase (Giovanucci, 2002), while regular physical activity is associated with a reduction in cancer risk (Giovanucci, 2002; Slattery and Potter, 2002). In addition, various studies suggest that consumption of alcohol is a risk factor for CRC (INCa 2007, 2009).

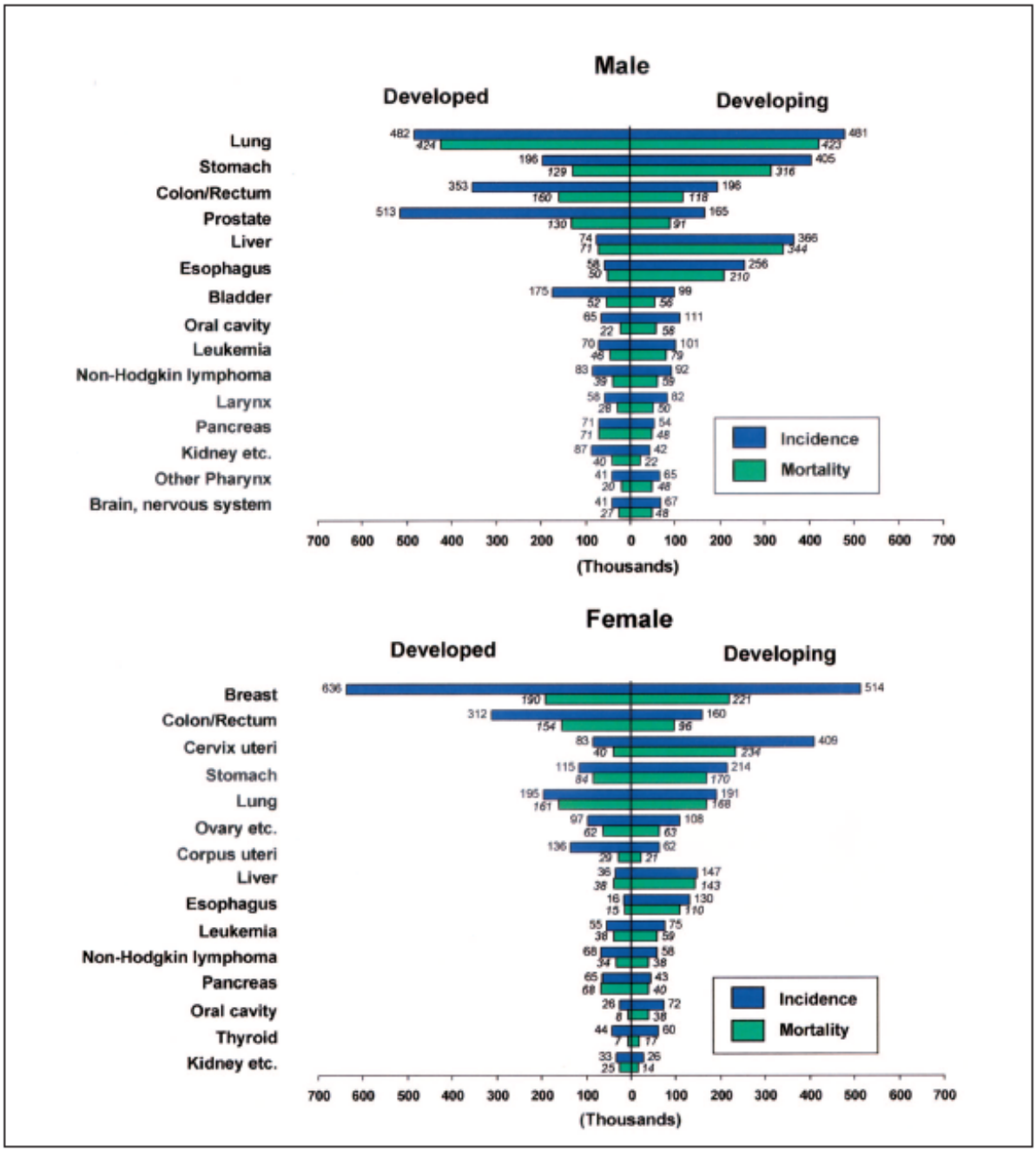


***Figure 1. Estimated incidence and mortality of cancer in the world.*** *Data shown in thousands for developing and developed countries by cancer site and sex (Parkin et al, 2005).*

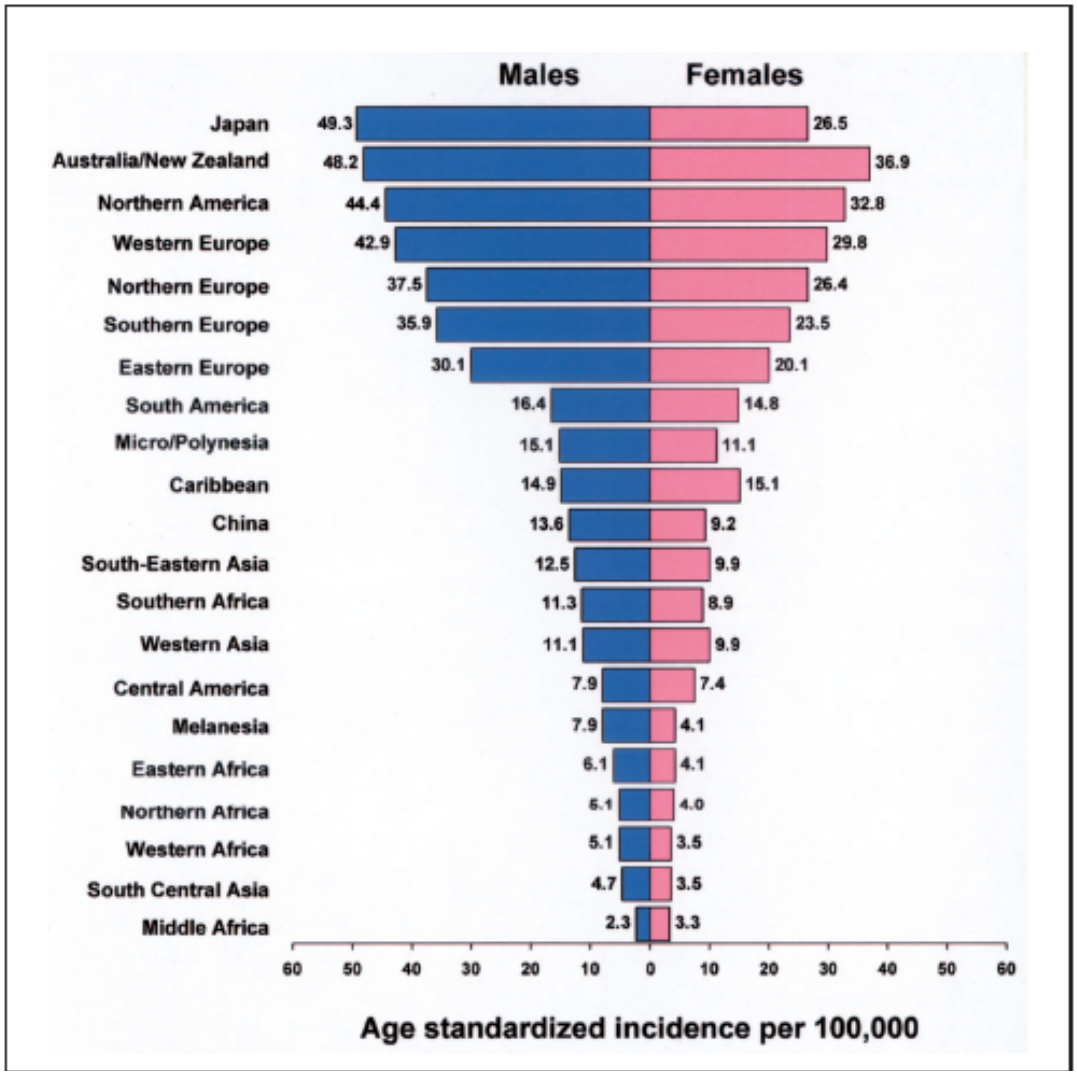


***Figure 2. Age-standardized incidence rates for colorectal cancer.*** *Data shown per 100,000 by sex (Parkin et al, 2005).*

### 1. 1. Colorectal cancer in France

It is one of the most frequent cancers in both sexes. The average age of diagnosis is approximately 70 years old, is rare before 50 years old but incidence is increasing rapidly (Bouvier et al, 2004) at least because of the increasing life expectancy (Bouvier et al, 2004; Colonna et al, 2001). However, the mortality rate has remained stable over the last 20 years, suggesting an improvement in prognosis (Menegoz et al, 1997). Possible explanations are that both early detection and treatment of this cancer have improved. Adjuvant therapy developed in the last decade may also have had a role to play in this improvement (Faivre-Finn et al, 2002). Related to the geographical distribution shows that the greater CRC incidence for the period 1980-2000 is higher in the Nord-East regions (Bouvier et al, 2004).

### 1.2. Colorectal cancer in Colombia

Colombia is an example of a country that belongs to the low-risk area for CRC; however it has been described an increase of incidence rates (INC, 2006, 2007). In Colombia, it was estimated for 1995 that 1500 people died of CRC, occupying the sixth leading cause of death by cancer. In 2005, CRC was the fifth cause of death by cancer. The rates of incidence are increasing significantly for CRC suggesting that it will equal gastric cancer (the first cause of death for cancer) in the year 2045, if current trends continue (Beltrán, 2004). The ages of diagnosis occurs between 30 to 74 years (INC, 2007). The incorporation of sedentary lifestyles, overweight, obesity associated with high consumption of foods rich in fat, refined sugars, low intake of fiber and micronutrients (ENSIN, 2005) may be considered important risk factors in CRC incidence.

## 2. Colon carcinogenesis

The central paradigm for the origin and development of the sporadic form of CRC is the adenoma–carcinoma sequence (Winaver, 1999). According to this model, human colorectal carcinomas begin as noninvasive adenomatous polyps, a small proportion of which become malignant over a period of 10–20 years (Hill et al, 1978). The earliest lesion to become visible is an aberrant crypt focus, which appears as a localized cluster of enlarged crypts (Renehan et al, 2002). The morphological changes of the adenoma–carcinoma sequence are associated with progressive gene alterations, including acquisition of somatic mutations affecting proto-oncogenes or tumor suppressor genes to undergo full malignant transformation (Vogelstein et al, 1988).

Fearon and Vogelstein proposed for the first time in 1990 a genetic model describing the various stages of development of a CRC from a normal epithelium until the development of metastases. This model identifies oncogenes and tumor suppressor genes (Ras oncogene, the adenomatous polyposis coli (APC), the deleted in colorectal cancer and the p53 genes), necessary for all stages of evolution of CRC (Figure 3). The sequence of events proposed during the development of the CRC is: (i) inactivation of tumor suppressor genes, (ii) activation of oncogenes, (iii) mutations of multiple genes and (iv) accumulation of changes (Cho and Volgestein, 1992).

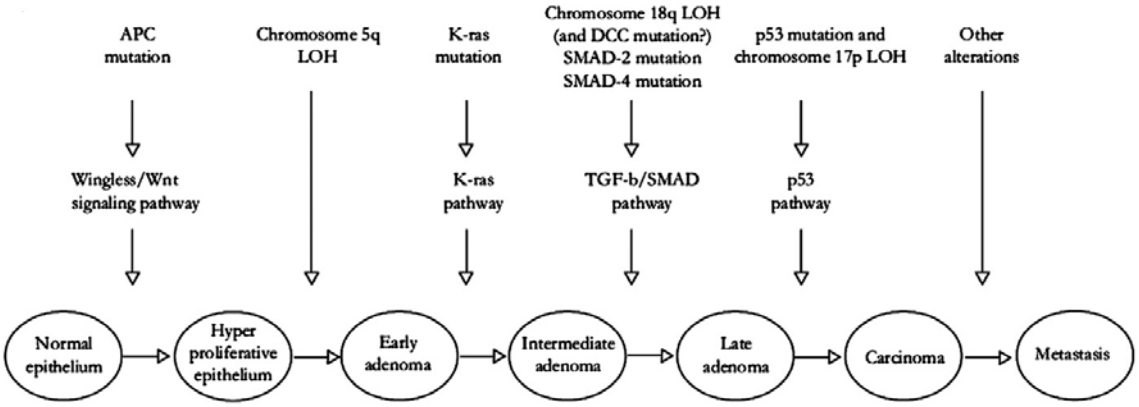


***Figure 3. The adenoma-carcinoma sequence****. Chronology of different mutations or lost of genes involved in the initiation and development of CRC (Fearon and Vogelstein, 1990).*

This adenoma-carcinoma sequence, which describes originally the development of sporadic cancers that accounts for nearly 85%, can also be applied to Familial Adenomatous Polyposis, which is caused also by mutations in the APC gene, an autosomal dominant syndrome (Robbins and Itzkowitz, 2002) with 80% to 100% penetrance, and an estimated prevalence of 1 in 5000 to 1 in 10 000, which results in the development of hundreds of polyps by the third decade of life (Bisgaard et al, 1994).

### 3. Colon cancer treatments

The adenomas and early cancers are often asymptomatic and potentially curable if found and removed, as compared to symptomatic CRC that is more likely to be advanced in stage which implies increased resistance of tumors to chemotherapeutic treatment, and an increase in long-term recurrence of the disease (Carethers, 2008).

For colon cancers, surgery with wide resection margins is the only therapy for stage I and II disease, although some stage II patients receive chemotherapy (Boland et al, 2000). For stage III disease, adjuvant 5-Fluorouracil (5-FU)-leucovorin or FOLFOX (5-FU, leucovorin, and oxaliplatin) has been shown to improve survival (de Gramont et al 2000; Goldberg et al 2004). For stage IV, surgery may be curative in highly selected patients with resectable bowel disease and resectable isolated hepatic or pulmonary metastases (Carethers, 2008). Chemotherapy is offered but may not improve overall survival. Palliative chemotherapy regimens for stage IV colon cancer include 5-FUleucovorin, FOLFOX, and FOLFIRI (folic acid, 5-FU, irinotecan), and IFL

(irinotecan, 5-FU, leucovorin), although IFL is no longer used due to high toxicity (de Gramont et al 2000; Goldberg et al 2004) (Figure 4).

Targeted therapies hold the promise of interrupting key cell pathways that are essential for the growth of the tumor, and are involved in the cancer cell survival and metastasis. Some of these targeted therapies are antibodies that can inhibit a receptor. Given their specific targeted nature, side effects might be less than general systemic chemotherapies. Specific growth factor inhibitors, such as bevacizumab (anti-VEGF, vascular endothelial growth factor) (Cilley et al, 2007) or cetuximab (anti-EGFR receptor, epidermal growth factor receptor) (Neyns et al, 2008) are available to treat advanced stages of the disease (Figure 4).

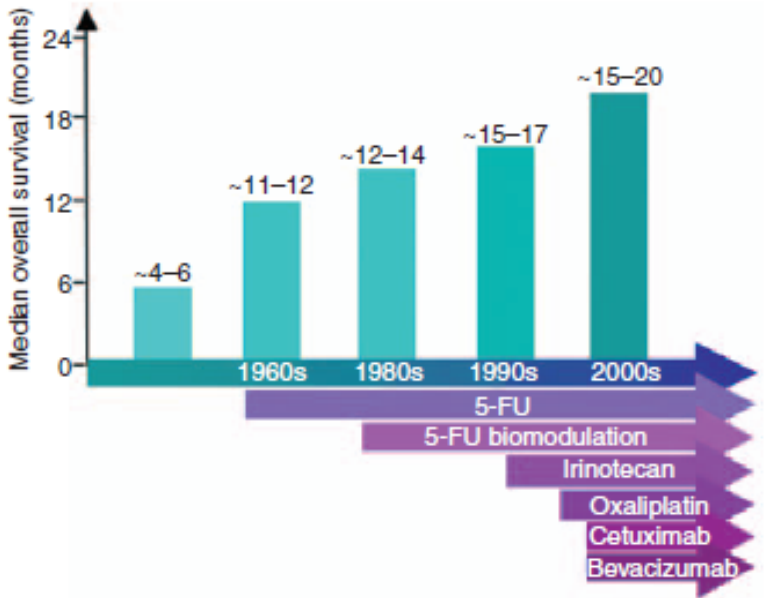


***Figure 4. Improved survival of patients with metastatic CRC by introduction of new therapies (Carethers, 2008).***

## 4. Nutritional factors involved in development and protection of CRC

Four principal classes of food-related mutagens that may be implicated in carcinogenesis are:

i) Heterocyclic and polycyclic aromatic amines formed during cooking of red and processed meat (Goldman et al, 2003);

ii) Nitrosamines formed during food processing and also by the colonic flora, acting on digestive residues of meat protein (Bingham, 1999);

iii) The production of reactive oxygen species (ROS) and pro-inflammatory cytokines associated to the excess of hemo iron from read meat (WCRF, 1997; INCa, 2009). Concerning processed meats their possible role could be related to the preservatives used, such as nitrite. Nitro compounds can be transformed into dialkylnitrosamines compounds that are carcinogenic (Riboli and Norat, 2003; Sugimura, 2000). It has been estimated that the risk of CRC has increased by 29% per 100 g of red meat consumed per day and 21% per serving of 50 g of processed meat consumed per day (INCa, 2009). Chao et al. (2005) investigated the link between prolonged meat consumption and the risk of colorectal cancer using data from the Cancer Prevention Study II (CPSII) in 148 610 adults between the ages of 50 and 74 years old. The main finding was that the risk of cancer to the distal portion of the large intestine might be increased by long-term with high intake of processed and red meat;

iv) A positive association was found between the consumption of sugar and starches (corn, wheat, rice) refined, and increased risk of CRC (WCRF, 1997). Two mechanisms have been proposed. First, a high consumption of sugar causes an increase in blood glucose may activate the process of carcinogenesis through hyperinsulinemia for insulin resistance indicated above. The second mechanism involves the cooking of sugar, which generates 5-hydroxymethyl-2-furaldehyde. This compound was found responsible for the initiation and promotion of tumors in rats (Giovannucci, 1995; WCRF, 1997).

The most consistent finding on diet as a determinant of cancer risk prevention is the consumption of vegetables and fruits. Convincing epidemiological evidence for this preventive action exists for CRC (Jonhson, 2004; Terry, 2001; van Breda et al, 2008; WCRF, 1997). The protective role of vegetables and fruits is attributed to micronutrients like carotenoids, vitamins E, C and A, folic acid and selenium; dietary fiber and, recently the focus and emphasis have shifted to the phytochemicals, non-nutritive bioactive compounds with no known nutritional value as flavonoids, phenolic acids, stilbilens, lignans (Terry et al, 2001; Manach et al 2004; van Breda et al, 2008) which influence multiple cellular mechanisms in a preventive approach.

The National Cancer Institute of the United States has determined in animal and *in vitro* studies that more than 1000 different phytochemicals possess cancer-preventive

activity, either comes from direct action (i.e. radical scavenging) or by interactions with metabolic and molecular processes (Surh, 2003). It has been estimated that there could be more than 100 different phytochemicals with anti-cancer properties in just a single serving of vegetables such as garlic, soybeans, ginger, onion, turmeric, tomatoes and cruciferous vegetables (for example, broccoli, cabbage, cauliflower and Brussels sprouts) (Figure 5) (Surh, 2003).

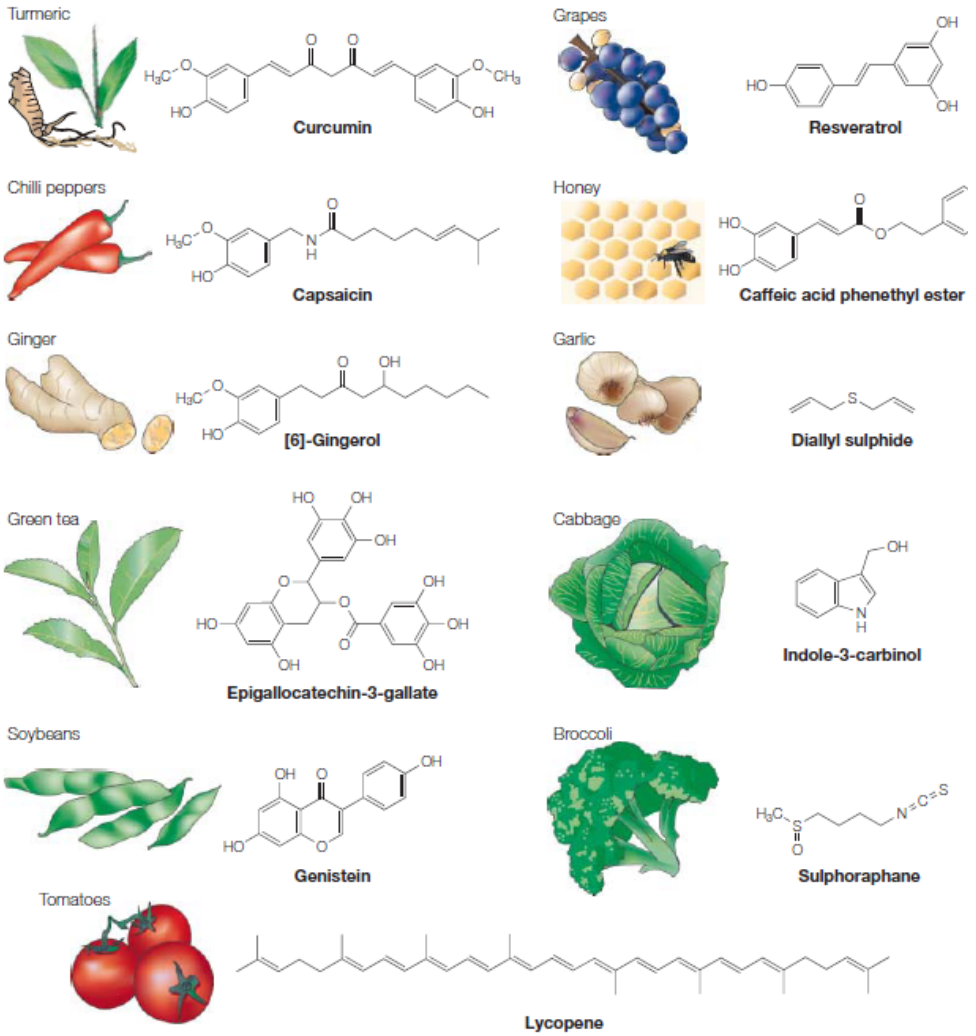


***Figure 5. Representative chemopreventive phytochemicals and their dietary sources (Surh, 2003).***

# II. CELL DEATH

## 1. Generalities

Cell death is a fundamental cellular response that has a crucial role in shaping our bodies during development and in regulating tissue homeostasis by eliminating altered unwanted cells. Cell death is a process that can occur either spontaneously or in the presence of cytotoxic agents. The first programmed cell death to be characterized was apoptosis, defined mechanistically as a programmed cell death that involves the sequential activation of caspases, controlled by B-cell lymphoma protein-2 (Bcl-2) family members, a process that will be described below. A second major type of cell death well described is necrosis, a degenerative death triggered accidentally or intentionally in the presence of cytotoxic agents. From a morphological level, cells dying by necrosis are characterized by organelle swelling, mitochondrial dysfunction, massive oxidative stress and rapid plasma-membrane permeabilization. The general view of the relationship between apoptosis and necrosis is that milder insults to the cell cause apoptosis, whereas more intense insults induce uncontrollable necrosis (McConkey, 1998).

In the past few years, evidence has emerged for a number of regulated non-apoptotic cell death pathways, including some with morphological features that were previously attributed to necrosis (Zong and Thompson, 2006) that are only relevant in rare circumstances when the apoptotic machinery is not activated. However, an understanding of the molecular mechanisms underlying non-apoptotic cell death *in vitro* has recently begun to emerge, as well as the importance of these processes. The three emerging regulated non-apoptotic cell death types are: type II cell death or authophagy, necroptosis or paraptosis, and poly(ADP–ribose) polymerase-1 (PARP1)-mediated-necrotic death.

The type II cell death or autophagy is characterized by the accumulation of double-membrane-enclosed vesicles. Autophagy is an intracellular catabolic mechanism that operates at low levels under normal conditions to mediate the degradation of cytoplasmic components, protein aggregates and expired intracellular organelles by forming double-membrane-enclosed vesicles called autophagosomes. The contents of autophagosomes are degraded by lysosomal enzymes after their fusion with lysosomes

(Kroemer and Jäätelä, 2005). Under conditions of nutrient deprivation, autophagy promotes cell survival by generating energy and intermediates for protein synthesis. Autophagy is regulated by a large group of *ATG* (autophagy-related) genes that are conserved from yeast to humans (Figure 6) (Degterev and Yuan, 2008).

The necroptosis is a type of programmed necrosis, which is activated through DRs by their respective death ligands when apoptosis is inhibited by caspase inhibitors, or mutations of caspase-8 or FADD (Figure 6) (Degterev and Yuan, 2008; Golstein and Kroemer, 2006). The morphological features of necroptosis are organelle swelling, rapid mitochondrial dysfunction, plasma membrane permeabilization, and lack of nuclear fragmentation (Degterev and Yuan, 2008). Emerging evidence suggests that the initiation of the necroptotic programme by TNFα occurs at the receptor level through the recruitment and activation of an intracellular signaling complex that involves the adaptor molecule RIP1, which is translocated into the mitochondria leading to a rapid mitochondrial dysfunction because of disruption of the association of ADP-ATP translocase (Zheng et al, 2006).

In the PARP1-mediated cell death, PARP1 is a nuclear enzyme that maintains genome stability, which is activated by DNA-strand breaks and recruits DNA-repair factors by attaching ADP–ribose units to chromatin-associated proteins (Degterev and Yuan, 2008) and leads to the release of inflammatory cytokines to alert immune cells of the presence of cells with DNA damage (Ditsworth et al, 2007). The PARP1-mediated cell death can be activated by two pathways: the energy collapse and the apoptosis-inducing factor (AIF) translocation (figure 6). In the first, the alkylating DNA damage promotes rapid PARP1-mediated depletion of cytosolic $NAD^+$, which leads to a necrotic death by 'energy collapse' in glycolytic cells (Zong et al, 2004). This mechanism can be viewed as an extension of the genome-surveillance function of PARP1, as it provides a way to differentially regulate DNA-damage responses in rapidly proliferating glycolytic cells and in cells in a vegetative state, relying on mitochondrial respiration for maintaining ATP levels (Zong et al, 2004). The second pathway by which PARP1 also mediates cell death is induced by secondary DNA damage associated with acute neuronal injury characterized by the translocation of the poly(ADP–ribose)-polymer into the cytosol and triggered translocation of AIF from the mitochondria to the nuclei (Andrabi et al, 2006; Yu et al, 2006).

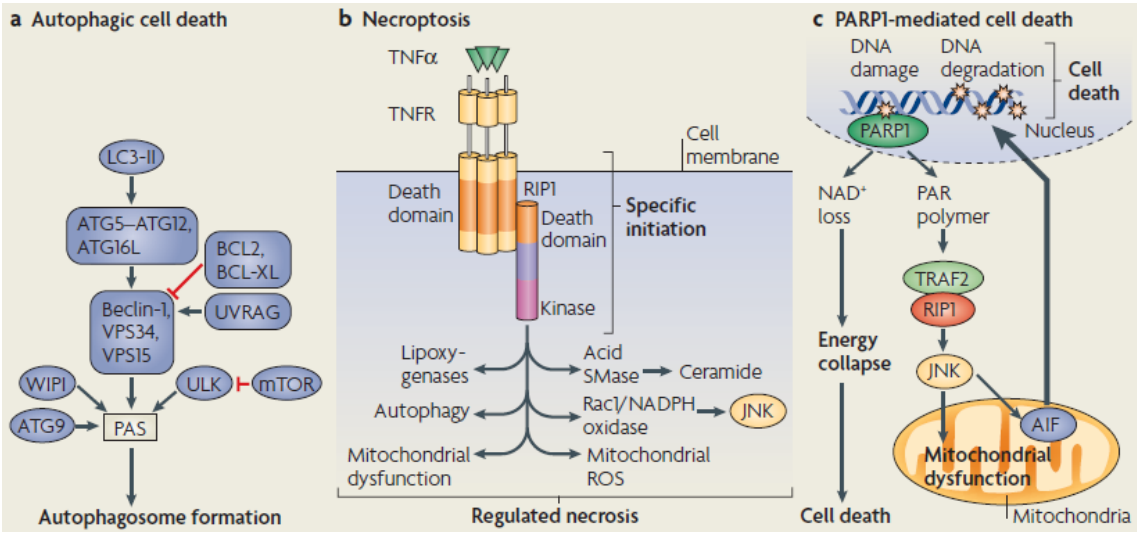


***Figure 6. The regulated non-apoptotic cell death pathways (Degterev and Yuan, 2008).***

## 2. Apoptosis

### 2.1 General characteristics

Apoptosis is a recognized physiological process in the removal of cells following exposure to toxic compounds as well as during development and in degenerative disorders. Apoptosis is a nontoxic model of cell death, which affects single cells in the midst of living tissues without eliciting an inflammatory response (Kim et al, 2006). Induction of apoptosis is considered to be one of the important targets in a cancer preventive approach. In this context, apoptosis provides a physiological mechanism for the elimination of abnormal cells, and could have beneficial effects on carcinogenesis. At the surface of the normal colonic mucosa, senescent colonocytes are constantly extruded into the gut lumen from the intercryptal zones (Johnson et al, 2004). This is consistent with a number of animal studies in which experimental enhancement of crypt-cell apoptosis has been shown to suppress the induction of neoplasia by chemical carcinogens (Gossé et al, 2005; Kozoni et al, 2000).

Apoptosis is characterized, at the morphological level, by cell shrinking, membrane blebbing, nuclear pyknosis, chromatin condensation, and cellular fragmentation into so-called apoptotic bodies rapidly phagocyted and digested by macrophages or neighboring cells, avoiding the inflammatory response that would occur after release of intracellular material (Lauber et al, 2004). These macroscopic changes are preceeded by biochemical events such as the redistribution of membrane lipids, the

loss of mitochondrial membrane potential, the activation of intracellular proteases named caspases, the proteolytic degradation of selected proteins in the cell that normally contribute to structural integrity of the nucleus (Earnshaw et al, 1999), and the fragmentation of DNA at internucleosomal sites (Kaufmann et al, 2000).

There are two main pathways involved in the induction of apoptosis; a death receptor-pathway (known as the extrinsic pathway) and a mitochondrial pathway (known as the intrinsic pathway) (Figure 7). The apoptotic signal involves the activation of many proteins that are part of two major families: the caspases and the Bcl-2 proteins. Caspases play an important role in the process of degradation of cellular organelles, whereas Bcl-2 proteins participate in maintenance and propagation of the signal (Kim et al., 2006).

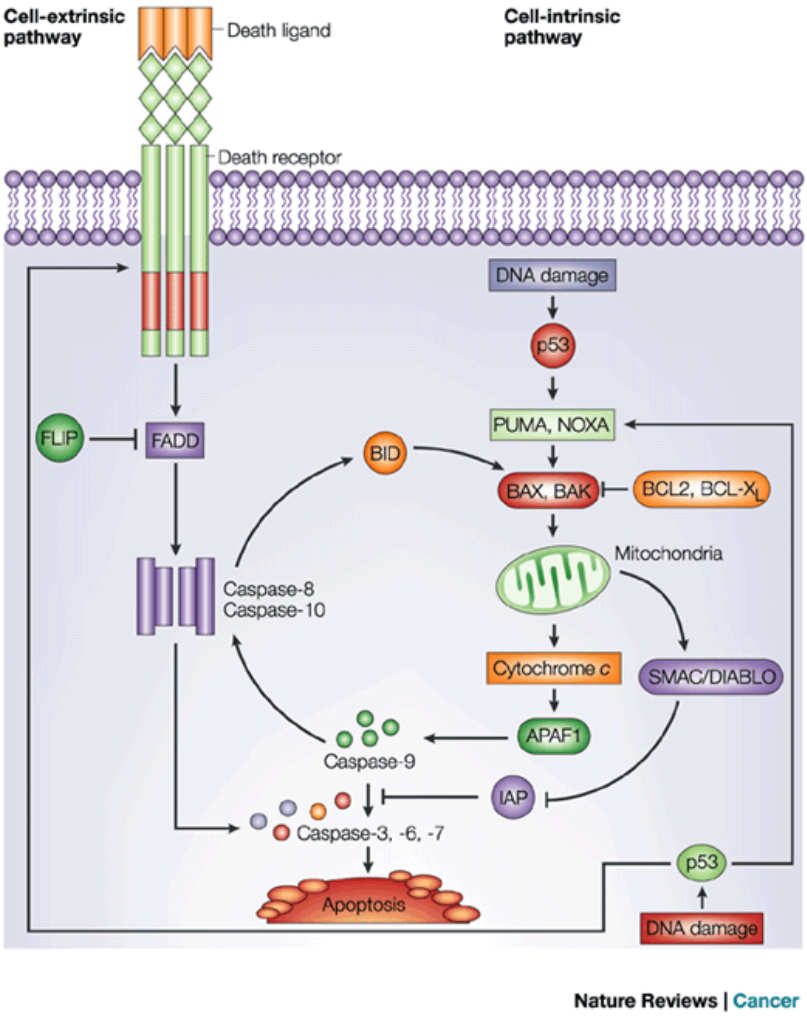


***Figure 7. The extrinsic and the intrinsic apoptotic pathways (Ashkenazi, 2002)***

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### 2.2 The caspases

Caspases (cysteine aspartate-specific proteases) are a family of intracellular proteins involved in the initiation and execution of apoptosis. Of the fourteen known mammalian caspases identified, seven (caspases-2, -3, -6, -7, -8, -9, and -10) are involved in apoptosis, others in inflammation. One current classification scheme divides these apoptotic caspases into two classes: 1) effector (or "downstream") caspases, which are responsible for most of the cleavages that disassemble the cell; and 2) initiator (or "upstream") caspases, which initiate the proteolytic cascade (Ashe et al, 2003; Kaufmann et al, 2000).

Caspases are synthesized as procaspases that are then proteolytically processed, at critical aspartate residues to their active forms (Kumar, 2007). The $NH_2$ terminal domain is of variable length depending on the functional category of the caspase. Initiator and inflammatory caspases possess long prodomains (>100 amino acids), whereas effector caspases have short prodomains (< 30 amino acids). Long prodomains contain specific motifs essential for caspase activity. These motifs may be either death effector domains (DEDs) as in caspases 8 and 10, or caspase recruitment domains (CARDs) as in caspases 1, 2, 4, 5, 9, 11, 12, 13, and 14 (Figure 8). These domains mediate interactions between caspases and a variety of adaptor molecules involved in cell signaling. Both DED-containing caspases are initiator caspases, whereas CARD-containing caspases may be either initiator caspases (caspases 2 and 9) (Kumar, 2007) or inflammatory caspases (1, 4, 5, 11, 12, 13, 14) (Scott and Saleh, 2007).

The induction of apoptosis through extrinsic or intrinsic death mechanisms results in the activation of initiator caspases. The death receptors (DRs) through adaptor molecules recruit initiator caspases 2, 8, or 10, while intrinsic death signals result in the activation of caspase 9. Activation of initiator caspases is the first step of a highly regulated, irreversible, self amplifying proteolytic pathway. Initiator caspases are able to cleave procaspases, and thus, are able to activate effector caspases (caspases 3, 6, and 7) or are able to amplify the caspase cascade by increased activation of initiator caspases.

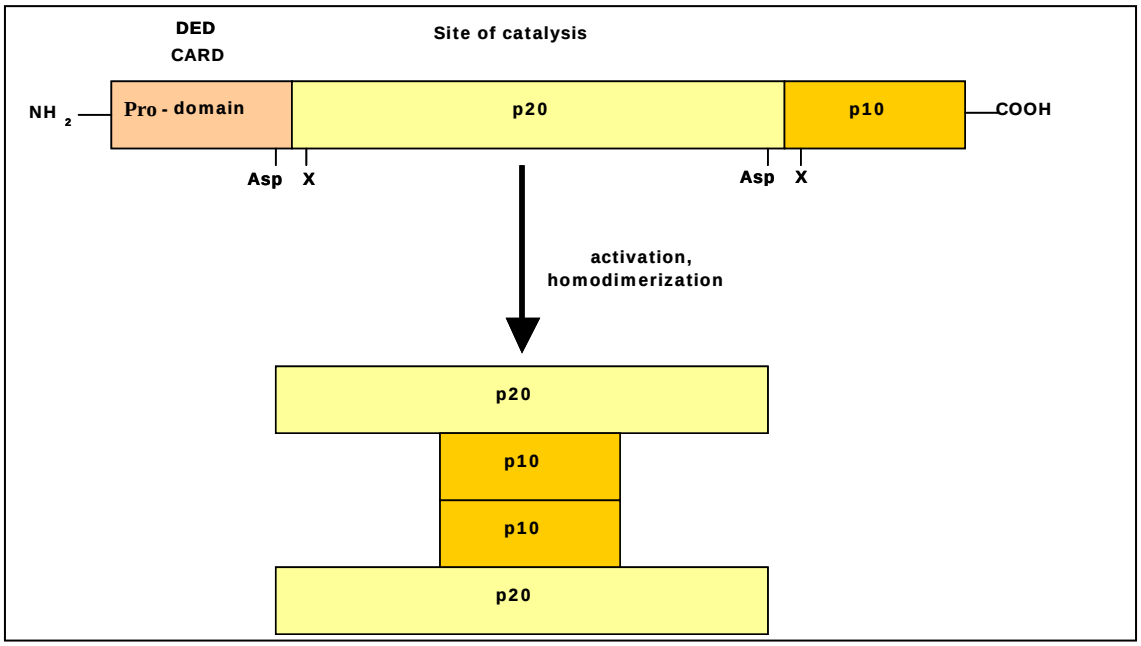



***Figure 8. Pattern of caspase activation***

## 2.3 Bcl-2 family proteins

The proteins of the Bcl-2 family are key regulators of apoptosis and their main function is to control mitochondrial permeability and particularly, the release of apoptogenic proteins from this organelle. The Bcl-2 family of proteins can be divided into three groups based on their structure and their role in apoptosis (Figure 9) (Er et al, 2006; Kirkin et al, 2004). These proteins can form homodimers and/or heterodimers, essentially through the interaction of their BH3 domain (Antignani et al, 2006; Er et al, 2006). The BH4 domain of anti-apoptotic proteins (Figure 9) is implicated in the control of their anti-death functions (Huang et al, 1998).

### **2.3.1** *Bid-protein*

Bid was first reported in 1996, it is widely expressed in various tissues (Wang et al, 1996). In a resting cell, Bid is predominantly cytoplasmic. Bid protein interacts with a hydrophobic groove on anti-apoptotic Bcl-2 family members and blocks their function. Following TNF-α, Fas or TRAIL treatment, the caspase-8 cleaved Bid (tBid) form homotrimers in the mitochondrial membrane which allows its interaction directly with Bax (Eskes et al, 2000) and induces its oligomerization in the outer mitochondrial membrane (OMM). Moreover, tBid-mediated lipid and cardiolipin redistribution could induce Bax to bind, intercalate and permeabilize the mitochondrial membrane resulting

in release of cytochrome c or Smac/DIABLO, activation of the apoptosome, and subsequent induction of apoptosis (Kirkin et al, 2004; Skommer et al, 2007). In addition, tBid has the ability to form channels in the mitochondrial outer membrane (Antignani et al, 2006).

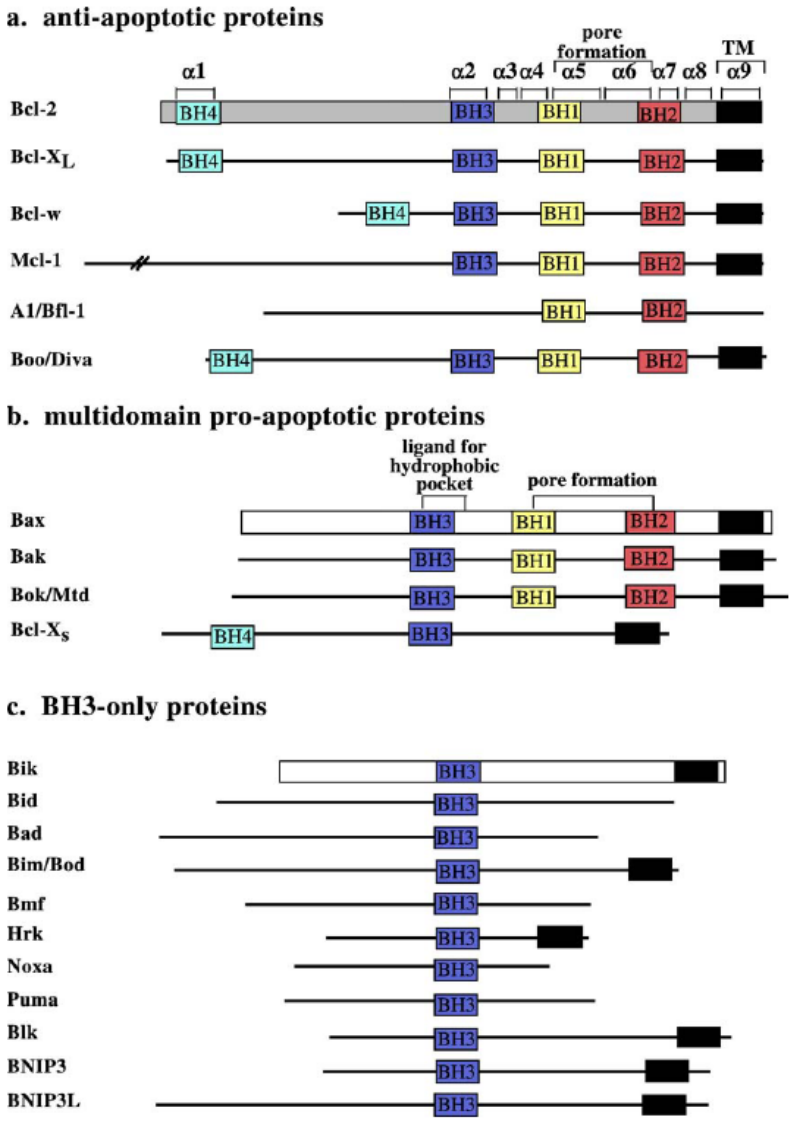



***Figure 9. Representation of mammalian Bcl-2 family members***. *Bcl-2 homology regions 1–4 (BH1–4) are indicated. TM indicates a putative transmembrane region that mediates localization to intracellular membranes (Er et al, 2006).*

### **2.3.2** *Bcl-2 protein*

Bcl-2 protein contains all four BH domains; possess established roles in the inhibition of apoptosis, although their exact mechanism remains elusive. Interestingly, recent work shows that Bcl-2 change conformation during apoptosis allowing it to bind

the N-terminal region of Bax, and consequently inhibit mitochondrial membrane permeabilization (Dlugosz et al, 2006).

#### **2.3.3** *Bax protein*

The mitochondrial form of Bax found in non-apoptotic cells is a 21 kDa monomer weakly associated with the OMM or soluble in the cytosol. Upon the induction of apoptosis, this monomer evolves into a high molecular complex (96 to 260 kDa) inserting into the OMM, suggesting that an oligomerization accompanies this insertion (Guihard et al, 2004). These complexes appear to be homo-oligomers of 6 to 8 molecules of Bax. Electron microscopy indicates that Bax translocation to the OMM is the first step in Bax activation and that the mitochondria-associated oligomer is the biologically active pro-apoptotic structure (Nechushtan et al, 2001).

As well as Bcl-2, Bax may be controlled by phosphorylation. Depending on the nature of the stimulus and/or site of phosphorylation (i.e., Ser163 or Ser184), Bax could be activated or inactivated (Nomura et al, 2003). Several studies have reported that JNK and p38 regulate positively Bax activation in diverse cellular systems (Weston et al, 2007). It was also showed that Akt kinase directly prevented Bax translocation to mitochondria via a phosphorylation of Ser184 (Gardai et al, 2004). Akt-dependent phosphorylation of Bax promoted its sequestration to the cytoplasm. This phosphorylated Bax was shown to hetero-dimerize with Mcl-1 and Bcl-$x_L$ in the cytoplasm of neutrophils (Gardai et al, 2004).

### **2.4 Extrinsic cell death pathway**

#### **2.4.1** *Death receptors (DRs)*

Cell surface DRs belong to the tumor necrosis factor receptor (TNFR) superfamily. They transmit their apoptotic signals following binding of death ligands. The best-characterized family members include Fas (also known as Apo1 or CD95) (Ashkenazi et al, 1998; Nagata et al, 1994, 1995). Additional members of the TNFR superfamily include DR4 also (known as TRAIL-R1) (Pan et al., 1997a) and DR5 (also known as TRAIL-R2) (Pan et al., 1997b). These receptors are transmembrane receptors characterized by extracellular cysteine-rich domains (CRD) and intracellular death domains (DDs). The extracellular CRD are responsible for receptor self-association

(CRD1) as well as receptor–ligand interactions (CRD2 and CRD3) (Siegel et al., 2000). Members of the TNFR superfamily require self-association prior to ligand binding; therefore, the CRD1 domain has also been termed the pre-ligand binding assembly domain (Chan et al., 2000). Following receptor–ligand binding, other DD containing proteins are recruited and function as adaptor proteins in the signal transduction cascade (see below). These adaptor proteins interact with a variety of other proteins to complete the DR signaling pathways.

### **2.4.2** *Signaling by CD95/Fas*

Fas (Apo-1/CD95) and Fas-ligand (FasL) system is recognized as a major pathway for the induction of apoptosis in cells and tissues (Nagata et al, 1994, 1995). This pathway induce the structural rearrangement of the receptor complex and recruitment of the adapter molecule Fas-associated death domain (FADD) and procaspase-8 to a death-inducing signal complex (DISC) which involve activation of caspase-8 that triggers the protease cascade leading to the activation of caspase-3, -6 and -7. Activation of Fas pathway may also induce a mitochondria-dependent apoptotic event (Figure 10). In other words, the extrinsic apoptotic pathway may recruit an intrinsic apoptotic pathway.

It has become clear that there is a cross-talk between the two canonical caspase activation pathways. Scaffidi *et al.* (1999) have distinguished two types of cellular responses after Fas ligation. In so-called “type I” cells, large amounts of caspase-8 are activated at the Fas/FADD complex; and activation of effector caspases proceeds as described above. In so-called “type II” cells, on the other hand, only small amounts of caspase-8 are initially recruited and activated. This active caspase-8 then cleaves the Bcl-2 family member Bid (Figure 10), generating an active fragment that interacts with Bax and facilitates cytochrome c release from mitochondria thereby activating caspase-9 and the downstream caspases (Desagher et al, 1999). What determines whether a cell requires this amplification pathway is currently unknown. Nonetheless, this cross-talk is important, since it determines whether factors that affect the mitochondrial pathway (i.e, Bcl-2 overexpression) will also render the cells resistant to apoptosis induced by death receptor ligation.

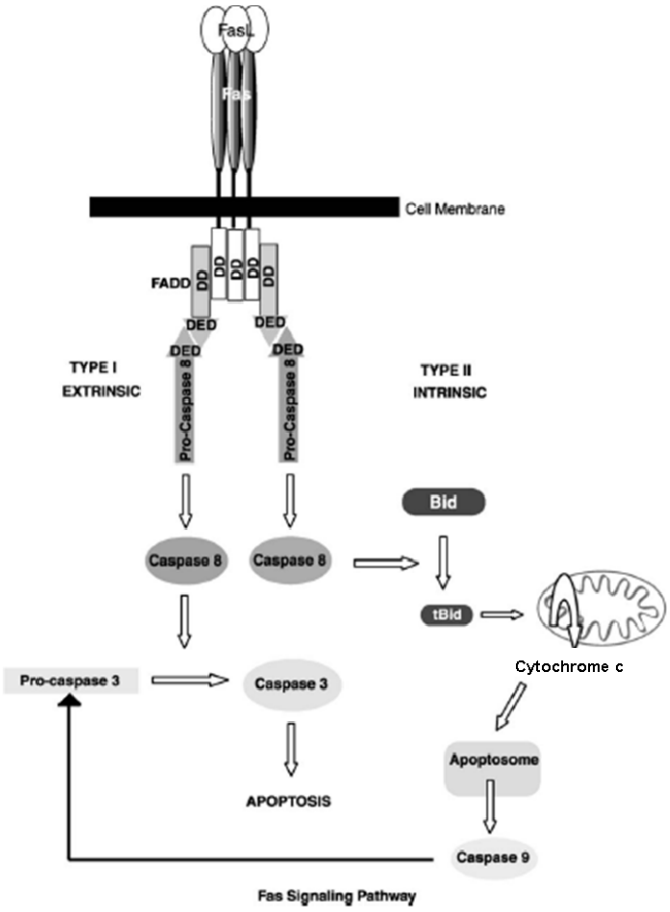



***Figure 10. Fas-mediated apoptotic signaling (Ashe et al, 2003).***

### **2.4.3** *Signaling by TRAIL*

Tumor necrosis factor α-related apoptosis-inducing ligand (TRAIL) is a death ligand expressed in the majority of human tissues (Pitti et al., 1996). Four DRs have been shown to bind specifically to TRAIL: two cell death-inducing receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5) and two non-cell-death-inducing receptors (TRAIL-R3/DcR1 and TRAIL-R4/DcR2), (Ashkenazi, 2002; Pan et al., 1997a,b). Like TRAIL, these receptors are expressed in a wide variety of tissues. However, it has been reported that TRAIL demonstrates selective toxicity to cancer cells, but not to normal cells (Ashkenazi et al, 1998; Kaufmann et al, 2005). DR4 and DR5, similar to Fas, signal apoptosis through an interaction with FADD and caspase 8 (Sulinam et al, 2001; Walczak et al, 1999), which may recruit the intrinsic mitochondrial cell death pathway similar to that following Fas ligation by FasL (Sulinam et al, 2001). In addition to apoptosis, TRAIL ligation of DR4 and DR5 can activate NF-kB and p53 (Kreuz et al, 2004; Lin et al, 2000; Rathore et al, 2004).

### 2.5 Intrinsic cell death pathway

Intrinsic cell death pathway activated by pro-apoptotic signals originate within the cell, resulting in the rupture of the OMM and the release of pro-apoptotic proteins (Chen et al, 2003; Kroemer, 1999). It can occur by the opening of the mitochondrial permeability transition pore resulting in a loss of mitochondrial membrane potential ($\Delta\psi m$), associated to an influx of fluid into the mitochondria; and/or through the pro-apoptotic Bax protein (Skommer et al, 2007). These processes can lead to cell death through three discernible and not mutually exclusive mechanisms (Kroemer, 1999; Green et al, 2004):

(1) Release of mitochondria-residing factors that promote caspase-dependent (cytochrome c, smac/DIABLO and omi/HtrA2) or caspase-independent (AIF, endonuclease G and omi/HtrA2) cell death. (Figure 11). Cytochrome c released into the cytosol interacts with apoptotic protease-activating factor-1, ATP/dATP, and caspase 9 to form the apoptosome (Chen et al, 2003). After proteolytic activation of caspase 9, the enzyme can then directly activate caspases-3 and -7. The smac/DIABLO, released from the mitochondria into the cytosol binds to inhibitors of apoptosis proteins (IAPs) to remove their inhibitory effect on caspase activity (Du et al., 2000; Madesh et al, 2002). A third mitochondrial factor implicated in apoptosis is the apoptosis inducing factor (AIF), which is translocated to the nucleus to induce partial DNA fragmentation and chromatin condensation (Susin et al., 1999);

(2) Loss of mitochondrial functions imperative for cell survival; Cytochrome *c* holds a fundamental role in respiration, transferring electrons from complex III to complex IV of the electron transport chain, and hence allowing mitochondrial transmembrane potential ($\Delta\psi m$) to be maintained. The $\Delta\psi m$ dissipation lead to: (i) cessation of the import of most proteins synthesized in the cytosol; (ii) $Ca^{2+}$ and glutathione release from the mitochondrial matrix; (iii) uncoupling of oxidative phosphorylation with cessation of ATP synthesis, oxidation of $NAD(P)H_2$ and glutathione; (iv) hyperproduction of superoxide anion by the uncoupled respiratory chain; (v) a decreased rate of electron transfer that lessens the consumption of mitochondrial pyruvate and its conversion into lactate, which in turn leads to cytoplasmic acidification;

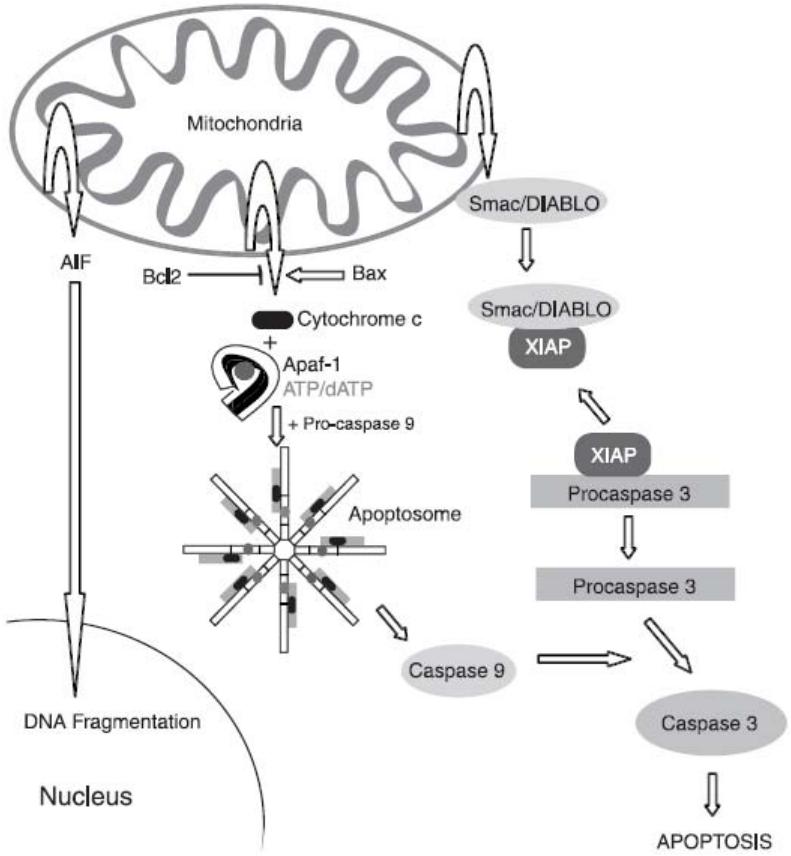



***Figure 11. Intrinsic pathway of apoptosis signaling (Ashe et al, 2003).***

(3) Induction of ROS. The oxidative stress leads to lipid peroxidation, calcium mobilization, mitochondrial permeability transition, ATP depletion, protein oxidation, loss of electron transport and/or DNA damage, and hence promotes cell death (Chen et al, 2003; Kroemer, 1999; Skommer et al, 2007).

## 2.6 Regulator mechanisms of apoptosis

### **2.6.1** *Pro-apoptotic signals*

The regulation of apoptosis at different levels is essential to maintain a balance between survival and cell death in the normal tissues. Alterations of this orchested machinery are responsible of proliferative signals and carcinogenesis. The tumor cells become resistant leading to gene-over expression and appearance of mutations in genes involved in genetic regulation.

The p53 tumor suppressor, functions as a transcription factor which becomes activated by a number of diverse stress stimuli including, DNA damage, hypoxia,

oncogene over-expression or metabolic limitations. Upon activation, p53 triggers growth arrest or apoptosis in order to eliminate damaged cells. In the latent form, p53 has low affinity to its specific DNA sequences but once activated, p53 regulates the expression of many target genes whose products are important players in cell-cycle regulation, apoptosis, senescence, metabolism and many others (Fuster et al, 2007; Millau et al, 2008). The tumor supressor activity of p53 is mediated by its ability to induce expression of pro-apoptotic proteins (figure 12) of the Bcl-2 family (i.e Bax, PUMA, Noxa, Bid) involved in the intrinsic but also Fas, TRAIL-DR4 and -DR5 extrinsic mediated apoptosis. The p53 protein is also able to interact directly with the mediators of apoptosis such as $Bcl_{XL}$, Bcl-2, Mcl-1 (Fuster et al, 2007). Many p53 mutations give rise to losses of p53 transcriptional function leading to a total loss of function when p53 mutants exert a dominant negative effect within tetramers (Millau et al, 2008). However, some p53 mutants may retain some transcriptional activities varying from one effector gene to another. Petitjean et al. (2007) showed that a dominant negative effect is an important mechanism for turning off p53 transcriptional activity since 80% of the most common mutants exhibit the capacity to exert dominant negative effect. Mutations in the p53 gene have been identified with a high incidence (about 50%) in cancers of lung, colon, stomach and esophagus (Soussi, 2000).


***Figure 12. Target genes regulated by p53 (Reed, 2003).***

The Rel/nuclear factor-κB (NF-κB) family of eukaryotic transcription factors is composed of a number of structurally related proteins that form homodimers and heterodimers (p50/p105, p52, p100, RelA (p65), c-Rel, and Rel B) (Chen et al, 1999). These dimers are sequestered in the cytoplasm bound to IκBa. Upon the appropriate stimulation, IκBa is phosphorylated by the IKKα or IKKβ kinase at specific serines, which then allows IκBα to undergo proteolysis through the proteosome pathway with

the subsequent NF-κB translocation to the nucleus. These dimers then bind to specific DNA consensus sequences in promoters and regulate the expression of a number of genes (Figure 13) (Chen et al, 1999; Lentsch et al, 1999). Many observations have also implicated NF-κB activation in the induction of apoptosis (Abbadie et al 1993; Ryan et al, 2000). NF-kB activation also cooperates with p53 to induce apoptosis (Chen et al, 2008; Muller et al, 1998; Ryan et al, 2000; Wu et al, 1997). However, the regulation of NF-kB transcriptional activity that leads to up-regulation of pro-apoptotic genes is unclear.

The JNK pathway (c-Jun N-terminal Kinase) and p38 mitogen-activated protein kinase (MAPK) signaling pathways are activated in response to cellular stress and can induce apoptosis (figure 14) (Lenassi and Plemenitas, 2006; Weston et al, 2007). The JNK and p38 are involved in the modulation of various pro-and anti-apoptotic pathways that depends of stimulus and cellular type. The activation of JNK pathway leads to the phosphorylation of transcription factors such as c-Jun or ATF-2 that regulate the expression of pro-apoptotic proteins such as FasL and TNFα, and inhibits by phosphorylation anti-apoptotic proteins that belongs to the Bcl-2 family proteins (Herr et al, 2001).

The mechanisms by which p38 contributes to an enhanced pro-apoptotic response include the phosphorylation and translocation of pro-apoptotic proteins from the Bcl-2 family (Park et al, 2003; Zhuang et al, 2000), the transforming growth factor-β-induced activation of caspase 8 (Schrantz et al, 2001) as well as the regulation of membrane blebbing and nuclear DNA condensation (Deschesnes et al, 2001). At the transcriptional level, expression of monoamine oxidase (DeZutter et al, 2001) or growth arrest and DNA damage (GADD)-inducible genes (Sarkar et al, 2002) have been shown to mediate pro-apoptotic effects of p38.

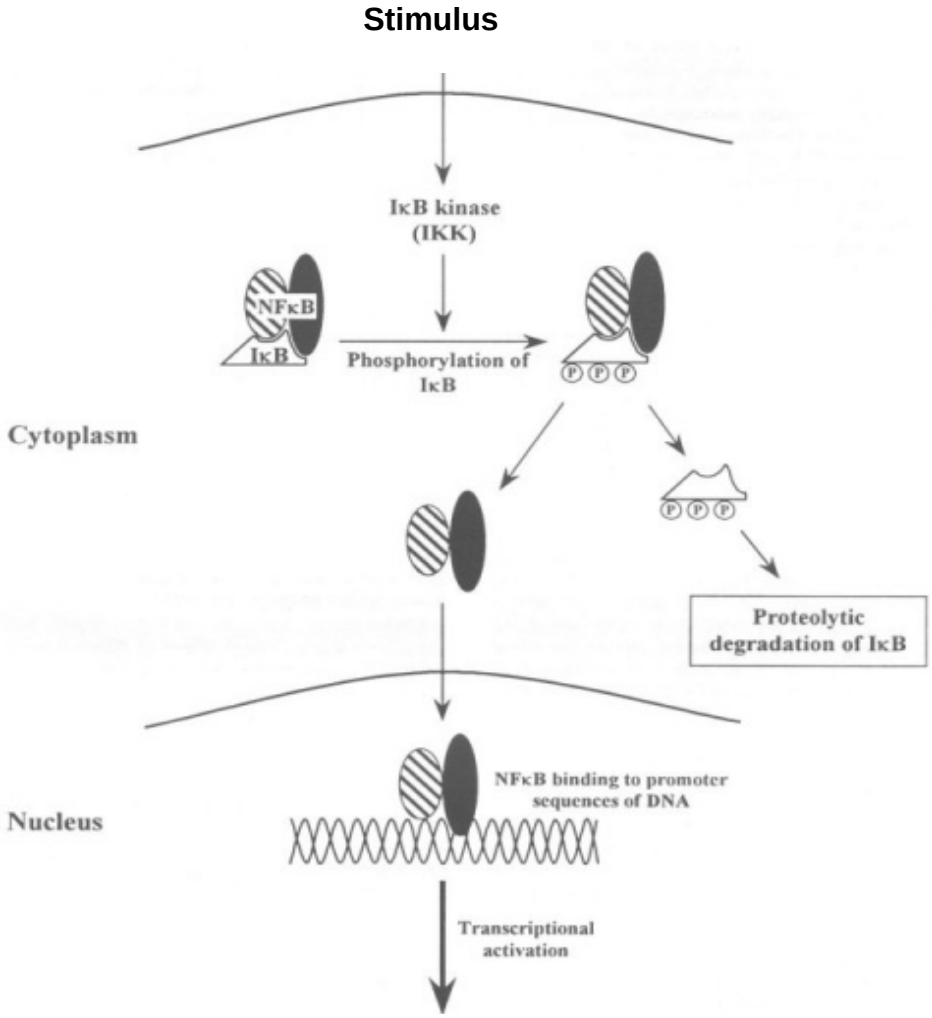



***Figure 13. Mechanism of NF-κB activation (Adapted from Lentsch et al, 1999).***

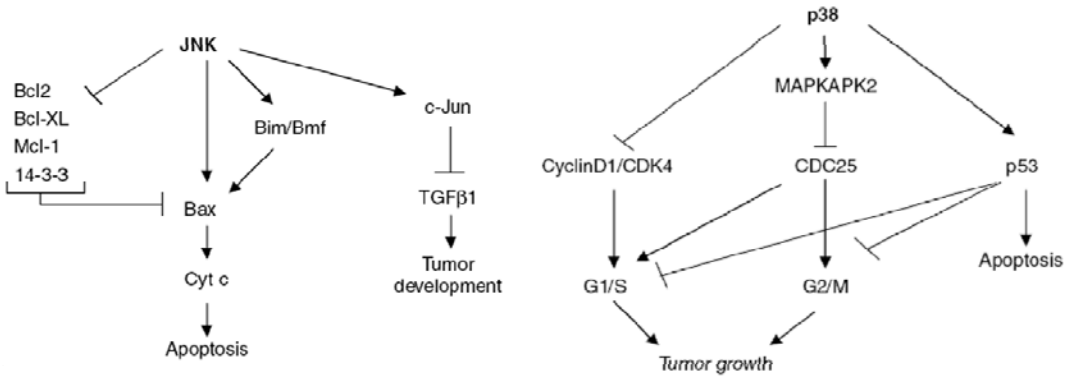



***Figure 14. The JNK and p38 MAPK signaling pathways activating tumor suppression mechanisms (Whitmarsh and Davis, 2007).***

### **2.6.2** *Inhibition of apoptosis by proliferative signals*

The activation of proliferation pathways such as the phosphadidylinositol 3 kinase (PI3K)-Akt (figure 15), the activation of NF-κB and Ras-Raf-MEK-ERK (Extracellular Regulating Kinase) pathway may interfere with the induction of apoptosis and, an exacerbation of their activity may be involved in carcinogenesis and chemoresistance (Kabore et al, 2004).

The PI3K-Akt pathway activated by receptors of growth factor with tyrosine kinase (TKRs) activity has been involved in modulating apoptotic pathways by phosphorylation reactions mediated by Akt (also known as protein kinase I PKB). Akt can inactivate by phosphorylation transcription factors such as family FoxO (forkhead box O) that control the expression of pro-apopototic and cell cycle inhibitors genes such as FasL, TRAIL and Bim (Calnan and Brunet, 2008). Akt can also phosphorylate pro-apoptotic proteins from the Bcl-2 family such as Bad. It has been shown that mutations in the PIK3CA gene that encodes the catalytic subunit of PI3K are common in breast cancer and ovarian cancer. These mutations lead to constitutive activation of Akt pathway. Akt can be overexpressed in ovary, pancreas and breast cancers. An inhibitor of the Akt pathway, PTEN (Phosphatase and Tensin homolog) may also be deleted or mutated for activating constitutively this pathway in certain cancers (Plati et al, 2008).

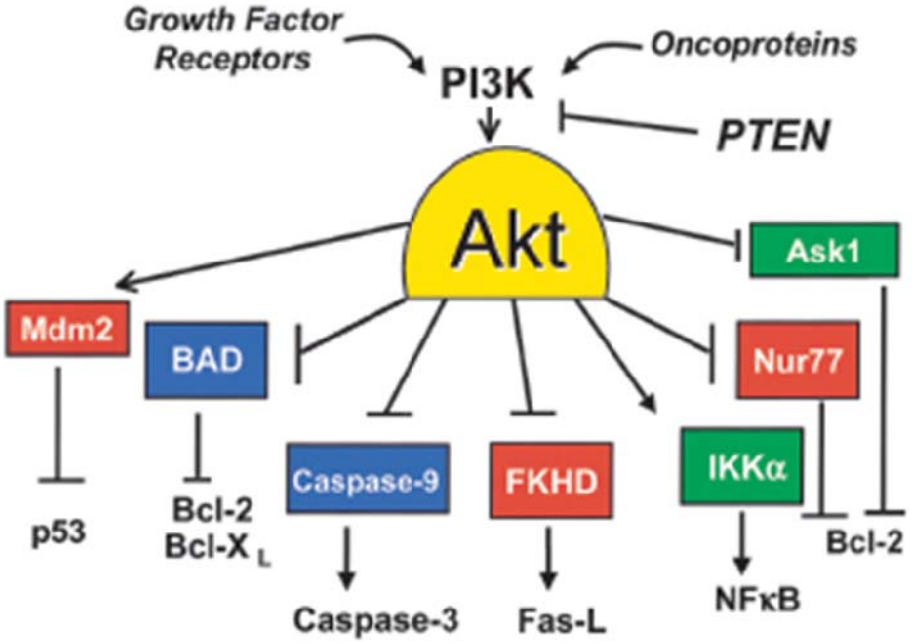



***Figure 15. Target protein and genes regulated byPI3K-AKT pathway (Reed, 2003).***

The NF-κB is also a pro-inflammatory transcription factor that has emerged as an important role in the development and progression of malignant cancers. NF-κB targets genes that promote tumor cell proliferation, survival, metastasis, inflammation, invasion (Sethi et al, 2008). In a number of systems, it has been demonstrated that NF-κB has an anti-apoptotic function. Knock-out of relA results in an embryonic lethal phenotype which was found to be caused by TNFα induced apoptosis within the fetal liver (Begg et al, 1996). In addition, NF-κB inhibits TNF-mediated apoptosis in Jurkat T cells, primary rat and human fibroblasts, and in MCF-7 breast carcinoma cell lines (Liv et al, 1996; van Antwerd et al, 1996). NF-κB has also been shown to protect against chemotherapy-mediated apoptosis in a number of malignant cell lines (Wang et al, 1996).

The G proteins of the Ras family also play an anti-apoptotic and anti-proliferative role through the activation of TKRs triggering the MAPK pathway Raf/MEK/ERK that leads to the activation of anti-apoptotic proteins such as p90RSK (90 kDa Ribosomal S6 Kinase Protein). This pathway is also able to interact with that of PI3K/Akt producing a synergistic anti-apoptotic effect. Activating mutations of Ras are found in more than 30% of human cancers. Mutations of the genes HRAS, KRAS and NRAs lead to a blocking of Ras protein in its activated state bound to GTP, therefore Ras pathway will be active in a constitutive form (Plati et al, 2008). Moreover, the TKRs may be mutated and permanently activate the anti-apoptotic pathways that are under its control. A well known example is the Epidermal Growth Factor (EGF) receptor, which overexpression in numerous cancers is correlated with carcinogenesis, poor prognosis and/or poor response to treatments. Binding of EGF to their receptor stimulates activation or repression of gene transcription and consequently up- or down-regulation of protein expression. Consequently, over-expression of hormone/growth factors and their receptors might present a growth advantage for preneoplastic cells (Plati et al, 2008).

**3. The oxidative stress**

The Oxidative stress is caused by an imbalance between the production of ROS and the antioxidant capacity of cells to prevent oxidative damage. The ROS include superoxide radical ($O_2$ •), hydrogen peroxide ($H_2O_2$) and hydroxyl radical (• OH). The

cell has developed a system composed of several antioxidant enzymes such as superoxide dismutase, which reduces O2 • by $H_2O_2$, catalase and glutathione peroxidase which reduces $H_2O_2$ to $H_2O$. There are also non-enzymatic antioxidant molecules such as flavonoids, vitamins A, C and E, carotenoids as well as the thiols glutathione, the ubiquinone, thioredoxin, etc (Valko et al., 2006).

In mammalian cells, the sources of ROS production are mainly from enzymatic origin. For example the NAD(P)H oxidase and the membrane enzyme complex III of respiratory chain in the mitochondria (Figure 16). Other sources in the cytosol or present within the cellular organelles also play a role in modulating cell signaling, for example intracellular increased of polyamine catabolism (production of $H_2O_2$ and amidopropanal). Overproduction of ROS and / or dysfunction of the antioxidant system are involved in pathophysiologic mechanisms of certain diseases (atherosclerosis, neurodegenerative diseases, and cancers). In other hand, the ROS are also important players in cell signaling and regulation of metabolism. This apparent paradox may be explained as the cell type, source producer as well as the level of ROS (Delattre et al., 2005). Cancer cells can produce large quantities of $H_2O_2$ (Szatrowski et al, 1991). Some tissues of colon cancer produce ROS in excess (Keshavarian et al., 1992), which could maintain the proliferation of tumor cells at high levels due to increased DNA mutations.

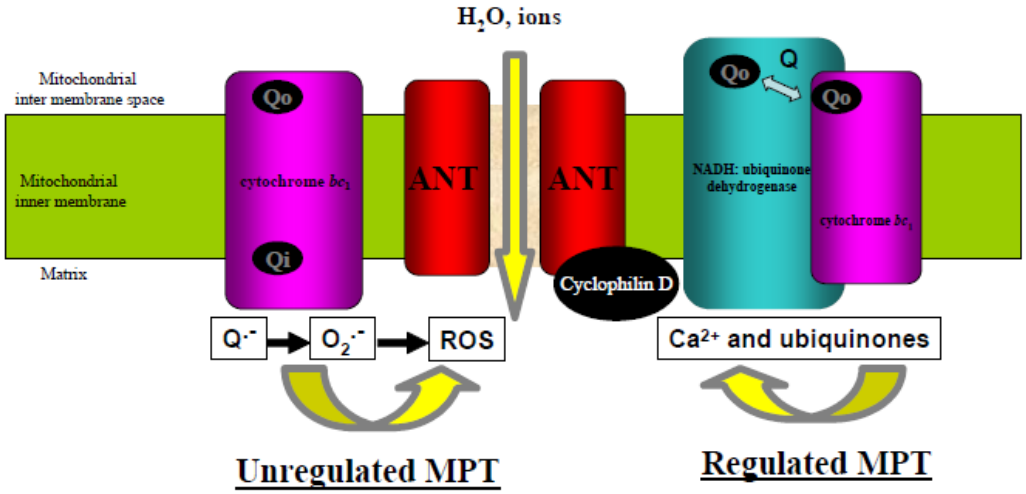



***Figure 16. Regulated and unregulated mitochondrial potential transmembrane (MPT) (Armstrong et al, 2004).***

Although ROS promote carcinogenesis, certain findings show that they can contribute to the amplification of death signals (Chen et al, 2003; Roussi et al, 2007).

Indeed, ROS may facilitate the disruption of the mitochondrial membrane and promote the opening of channels (Chen et al, 2003; Kim et al., 2006). The anti-apoptotic protein Bcl-2, enhances the resistance of cells to oxidative stress because its role is to maintain the closure of the mitochondrial pore (Armstrong et al, 2004). Thus, the use of antioxidants could sometimes be anti-apoptotic.

## 4. Polyamines: putrescine, spermidine and spermine

The putrescine (Put), spermidine (Spd) and spermine (spm), are aliphatic amines known like polyamines, which are formed and stored by nearly all eukaryotic cells. They are involved in multiple ways in cell proliferation and the maintenance of cell viability (Gerner et al, 2009).

The polyamines metabolism consist of biosynthesis and catabolic or retroconversion pathways (Figure 17). The two key enzymes involved in polyamine biosynthesis are ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (AdoMetDC). ODC catalyzes the formation of putrescine from L-ornithine, and AdoMetDC. decarboxylates *S*-adenosylmethionine (AdoMet). The product of this reaction, decarboxylated *S*-adenosylmethionine (dcAdoMet), is the aminopropyl group donor for spermidine and spermine synthesis. In the catabolic pathway (or retro-conversion pathway) acetylated polyamines are formed by spermine/spermidine acetyltransferase (SSAT), and are used as substrates by a flavin-dependent polyamine oxidase (PAO), which catalyzes their conversion back to spermidine and finally putrescine (Seiler, 1990).

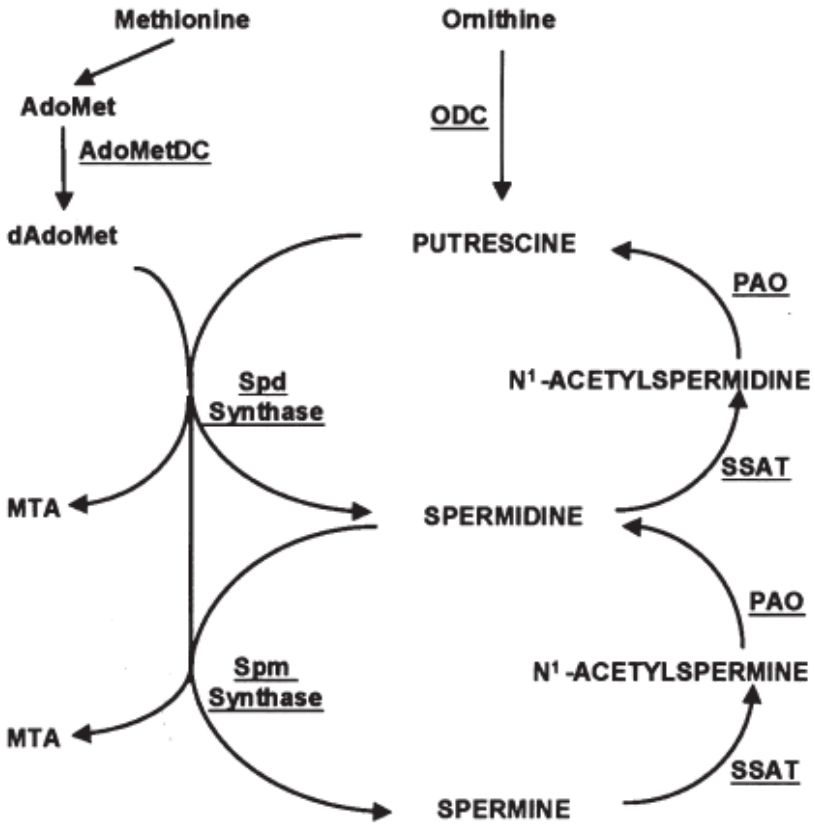



***Figure 17. Polyamine interconversion pathway***. *AdoMetDC : S-Adenosyl Methione Decarboxylase, ODC: Ornithine Decarboxylase, Spd Synthase : Spermidine Synthase, Spm : Spermine Synthase, SSAT : Spermidine/spermine $N^1$-acetyltransferase, PAO : Polyamine Oxidase (Gossé et al, 2006).*

The natural polyamines have four different ways of exerting physiological functions in cells, including apoptosis:

i) Binding to anionic sites by forming ion bonds: these polyamines form ion bonds with a great variety of negatively charged molecules. The binding energy increases with the number of positive charges from Put to Spd to Spm. Interactions with nucleic acids and proteins have been studied, being more selective interactions with anionic groups of proteins than nucleic acids. Electrostatic interactions of polyamines stabilize secondary and tertiary structures, or they induce conformational changes, and thus alter physical and biological properties of polyanions and proteins. An apoptosis related example of polyamines-protein interactions is the inhibition of apoptotic endonucleases by Spm. The most important example of conformational stabilization and packaging in this context, the observed destabilization of the chromatin structure due to depletion of polyamines. Exogenous polyamines stabilize

chromatin structure in cell nuclei, while polyamine depletion provokes an increased sensitivity of chromatin to degradation by different nucleases, impairs DNA repair mechanisms, and increases the sensitivity of DNA and chromatin to irradiation, heat and cytotoxic compounds (Basu et al, 1992; Williams et al, 1994).

ii) Formation of covalent bonds: polyamines alter physicochemical and biological properties of proteins. In view of their role in apoptosis, structural modifications of proteins by transglutaminase-catalysed linking of polymines to proteins are a possible reaction in apoptosis that should be important in situations of excessive polyamine accumulation (Facchiano et al, 2001).

iii) Scavenging radicals (and complexing cations): the polyamines have been suggested to protect cells against ROS; however the mechanisms by which this occurs are unknown. Khan et al. (1992a,b) proposed a mechanism by which spermidine and spermine could protect DNA from singlet oxygen attack, but did not demonstrate a direct involvement of the polyamines in the protection. Additionally, Ha et al (1998) suggested that Spm in high concentrations protects DNA from ROS attack in a manner similar to the classical antioxidants, because Spm is closely associated with chromatin, although available data have not showed a protective function of natural polyamines to ROS-induced damage under physiological or pathological conditions.

iv) Formation of cytotoxic aldehydes and ROS as products of oxidative deaminations: an extensive literature describes apoptogenic effects of the products of polyamine oxidation. The toxicity of the products of oxidative deamination of polyamines increase in the order Put<Spd<Spm, indicating that not only $H_2O_2$, but the aldehydes formed from these amines are also important cytotoxic agents (Seiler and Raul, 2005). There is an example for the rapid formation of cytotoxic products inside the cells which express PAO, with apoptosis as a consequence, inducers of SSAT. This effect can be explained by the massive production of N-acetyl derivatives of Spd and Spm, which follows induction of SAT, which react with PAO to form H2O2 and 3-acetamidopropanal. Their formation and apoptosis is prevented by inhibition of PAO by a selective inactivator of this enzyme (Ha et al, 1998; Gossé et al, 2006).

# III. CANCER CHEMOPREVENTION WITH DIETARY POLYPHENOLS

## 1. Definition of cancer chemoprevention

The concept of chemoprevention was introduced by Sporn in 1976 in a study concerning the preventive properties of natural forms of vitamin A in epithelial carcinogenesis (Sporn, 1976). Today chemoprevention refers to the use of natural, synthetic, or biological chemical agents to reverse, suppress, or prevent either the initial phase of carcinogenesis or the progression of neoplastic cells to cancer (Figure 17).

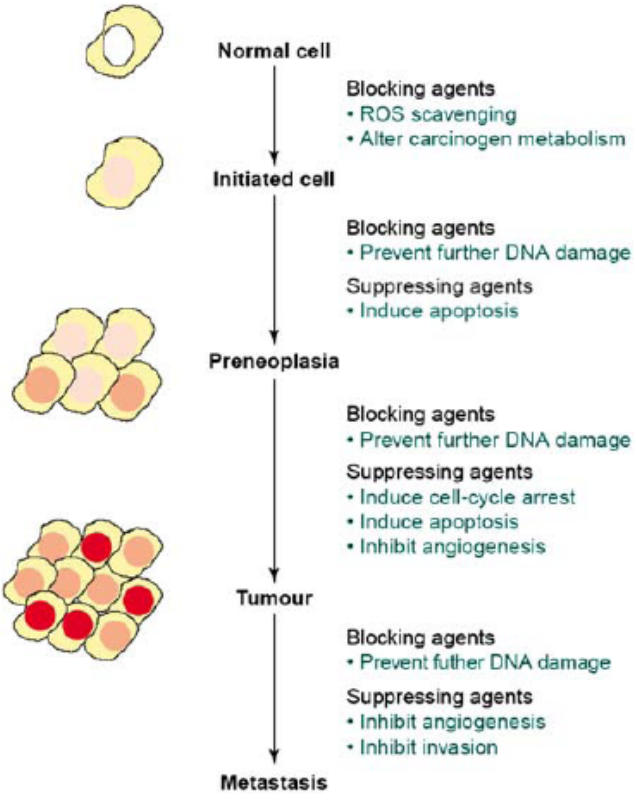



***Figure 18. Multistage model of carcinogenesis and chemopreventive targets (Manson, 2003).***

There are three strategies for cancer chemoprevention: (i) Primary chemoprevention involve interventions designed to help healthy individuals to prevent the development of a certain cancer. These individuals may have high-risk features (*e.g.* genetic mutations) and/or predisposing to cancer development; (ii) Secondary chemoprevention, is designed to provide treatment of premalignant lesions (*e.g.* colon adenomas) with the

aim to prevent progression to cancer; (iii) Tertiary chemoprevention aims to help patients with a history of treated cancer to prevent the development of a second primary cancer (Bonovas et al, 2008).

## 2. Identification of potential chemopreventive agents

At the present the National Cancer Institute of USA, based on numerous reports describing the anticancer activity of naturally occurring molecules (Aggarwal and Shisodia, 2006; Surh, 2003), have identified about 40 phytochemcials possessing potential chemopreventive. Although, the chemopreventive effects of these compounds are primarily based on cell culture and animal model studies, and only few of them are entering clinical trials. The phytochemicals are isolated from their natural sources, purified and assayed to test their ability in killing precancerous and cancerous cells with *in vitro* studies, animal models and phases I to III clinical trials (Figure 18) (Russo, 2007). By definition, the effectiveness of a chemopreventive agent is evaluated by its ability to interfere with the early stages of carcinogenesis, preventing the appearance of preneoplastic lesions in individuals at risk, and limiting the emergence of new neoplastic cells in patients previously treated for cancer (Russo, 2007).

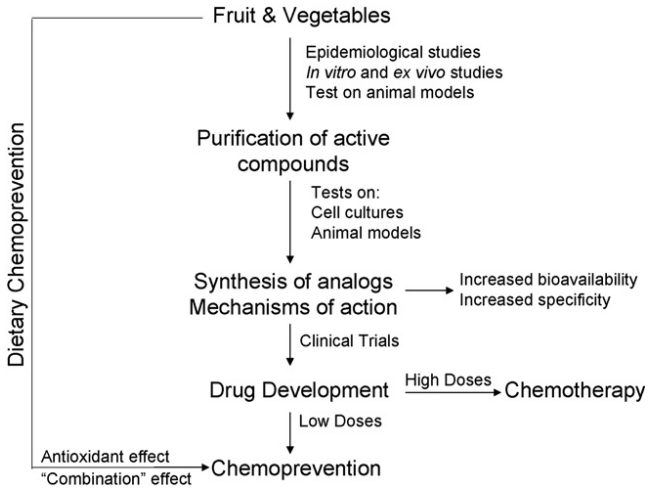



***Figure 19. Identification process of dietary chemopreventive agents (Russo, 2007)***

Carcinogenesis is a multistage process (Figure 17) (Manson, 2003). These stages are tumor initiation (several days), promotion (up to 10-20 years) and progression (1 to 5 years). Initiation is a rapid process that include the initial uptake of or exposure to a carcinogenic agent where the covalent interaction of ROS with target-cell DNA, leading

to genotoxic damage. In tumor promotion, epigenetic mechanisms are involved, and is a process relatively lengthy and reversible leading to accumulation of pre-malignant cells abnormally dividing. Progression is generally irreversible, and leads to the final stage of carcinogenesis with tumor growth and acquisition of invasiveness and metastatic potential (Manson, 2003; Surh, 2003). The passage from pre-malignant to malignant cell involves activation of proto-oncogenes and/or inactivation of tumor suppressor genes. Both categories of genes, when mutated, cause alterations in key cellular processes linked to cell growth and proliferation. Thus, a good chemopreventive agent should be able to interfere with one or more phases of the multistep carcinogenesis process.

The study of their mechanisms of action has allowed classifying many phytochemicals present in a diet rich in fruit and vegetables in terms of their ability to block the initiation stage of carcinogenesis (cancer-blocking agents) or to suppress (cancer-supressing agents) the proliferative capacity of preneoplastic lesions in the stages of tumor promotion and progression (Figure 19).

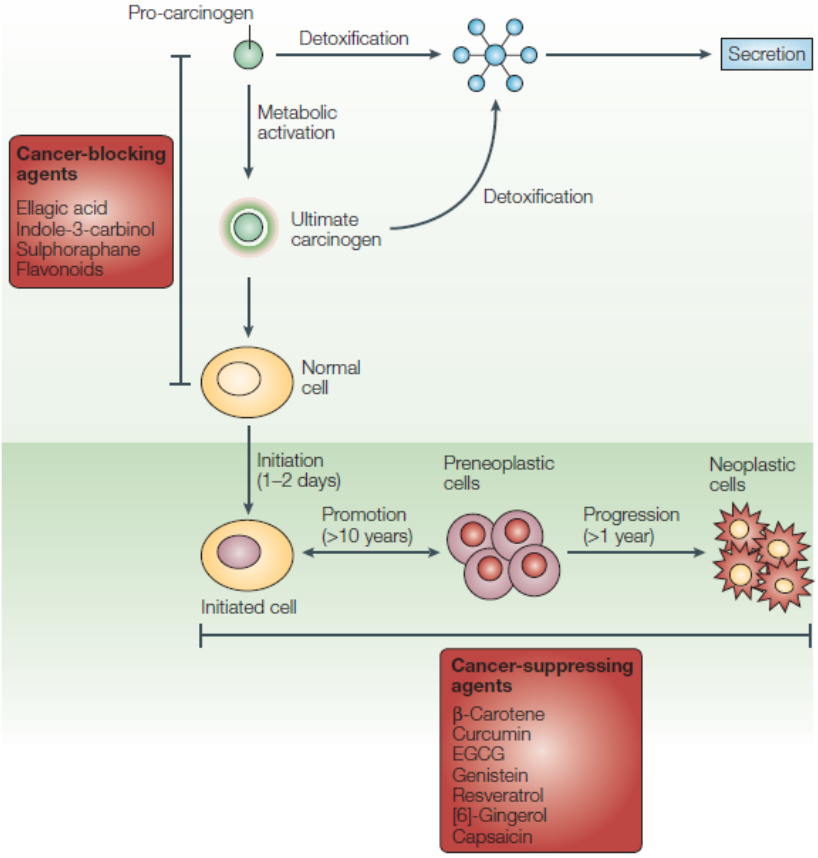



***Figure 20. Dietary phytochemicals that block or suppress multistage carcinogenesis (Surh, 2003).***

## 3. Polyphenols: general characteristics

Polyphenol compounds are a class of phytochemicals abundant throughout the plant kingdom. These molecules are secondary metabolites of plants and provide color to fruits and vegetables, and they are found in a wide variety of human foods and beverages. Much of the flavour and aroma of chocolates, tea, coffee and wine dependents upon the complex variety of phenolic compounds present in these products (Manach et al, 2004). These molecules are generally involved in protecting plants from the ultraviolet radiation, aggression by pathogens, and ROS. Polyphenols can be divided into 10 general classes based on their chemical structure (number of phenol rings, structural elements that bind these rings to one another, association with carbohydrates and organic acids), and more than 8000 different compounds have been described (Manach et al, 2005). The most abundantly occurring polyphenols in plants are phenolic acids, flavonoids, stilbenes and lignans, of which flavonoids and phenolic acids account for 60% and 30%, respectively, of dietary polyphenols (Ramos, 2007, van Breda et al, 2008).

Early interest in polyphenols was related to their "anti-nutritional" effects, i.e. decreasing absorption and digestibility of food because of their ability to bind proteins and minerals (Yang et al, 2001). Current interests are the antioxidative, antiinflammatory, and anticarcinogenic activities of polyphenolic compounds.

## 4. Flavonoids

### 4.1 Characteristics and dietary sources

Flavonoids are the largest class of phenolic compounds; over 6500 structurally different, naturally occurring flavonoids have been described (Ramos, 2007). The flavonoids share a common structure consisting of 2 aromatic rings (A and B) that are bound by 3 carbon atoms that form an oxygenated heterocycle (ring C). They may be divided into 6 subclasses (Figure 20) as a function of the type of heterocycle involved: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols (catechins, epicatechins and procyanidins) (Manach et al, 2004). Representative groups of flavonoids are presented in figure 21 together with their molecular structure, the best-known members of each group and food sources in which they are present.

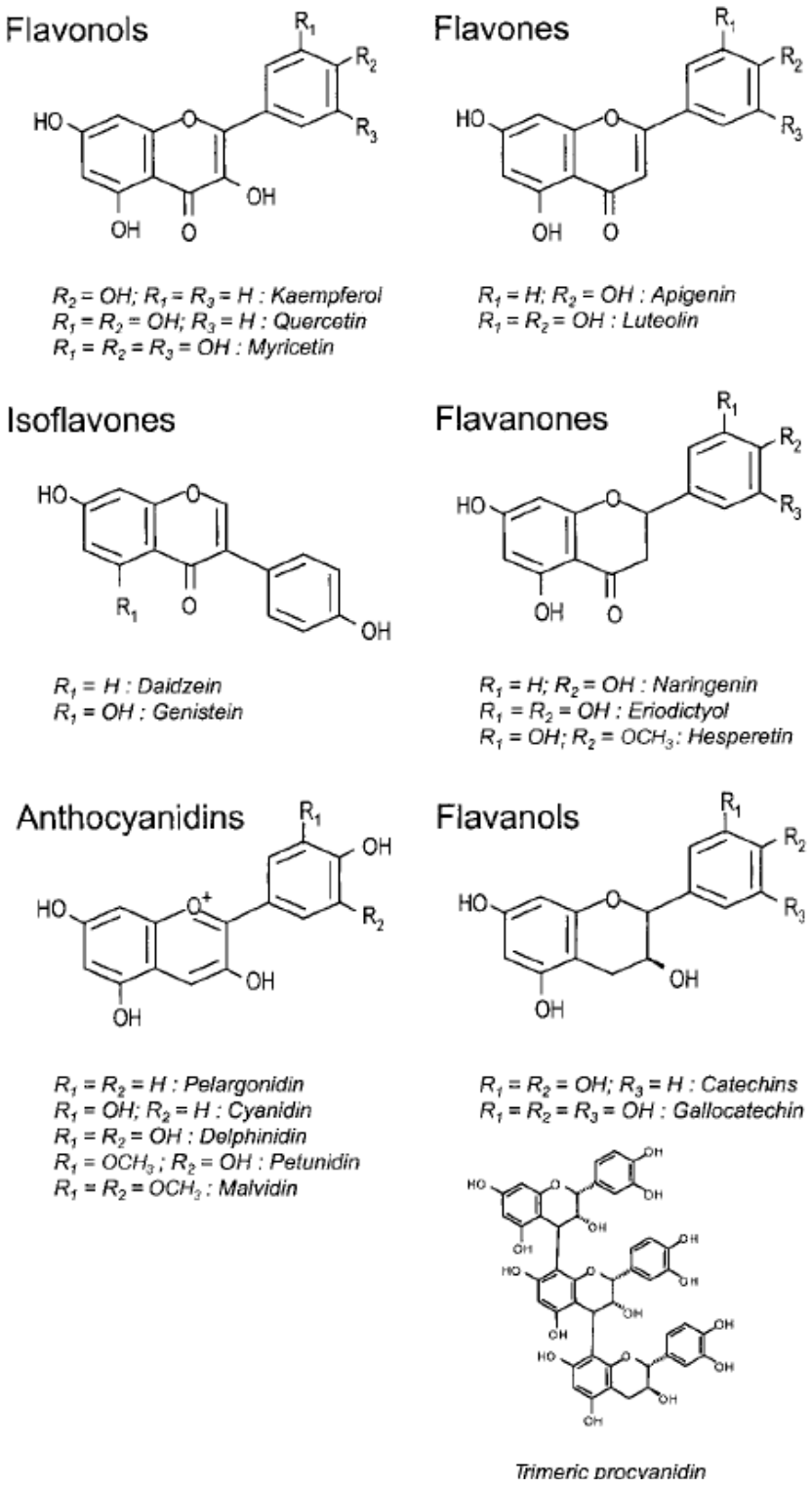



***Figure 21. Chemical structure of flavonoids (Manach et al, 2004).***

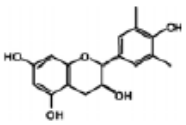
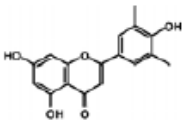
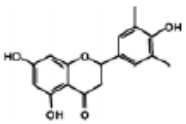
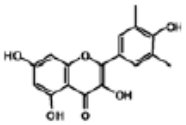
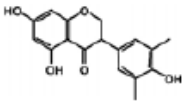
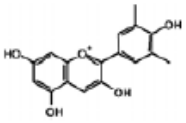
| Flavonoid subgroup | Representative flavonoids | Food source |
|---|---|---|
| Flavanols  | EGCG<br>Epigallocatechin<br>Catechin<br>Epicatechin | Chocolate, beans, tea, red wine, apple, cherry, apricot |
| Flavones  | Luteolin<br>Apigenin<br>Chrysin | Capsicum pepper, thyme<br>Celery<br>Parsley |
| Flavanones  | Naringenin<br>Hesperidin<br>Eriodictyol | Orange<br>Grapefruit |
| Flavonols  | Quercetin<br>Myricetin<br>Kaempherol<br>Rutin | Onion, apple, cherry, broccoli, tomato, berries, tea, red wine, leek |
| Isoflavonoids  | Genistein<br>Daidzein | Soya beans, legumes |
| Anthocyanindins  | Pelargonidin<br>Cyanidin<br>Malvidin | Rhubarb, cherry, strawberry, red wine |

***Figure 22. Basic chemical structure and major dietary sources of commonly occurring flavonoids (Ramos, 2007).***

### 4.2 Bioavailability of flavonoids

The metabolism of flavonoids has not been well characterized; studies have shown that there is great variability in preferential pathways among individuals probably due to differences in gut microflora populations (Manach et al, 2004, 2005). In food, most of the phenolic compounds are present as esters, glycosides or polymers, which can either be absorbed in these forms, or be hydrolyzed by intestinal enzymes or the colonic microflora; consequently aglycones or hydrolyzed products can be absorbed

in the small intestine. During absorption, flavonoids undergo extensive metabolism in the small intestine and later in the liver and other organs: phenolic compounds are conjugated by methylation, sulfation, glucuronidation or a combination in order to decrease their hydrophobicity and to facilitate their urinary and biliary excretion. Manach et al. (2004) have reported the existence of intermolecular bonds between albumin and quercetin conjugates, which supports its slow elimination from the body. Similarly, (-)-epigallocatechin-3-gallate (EGCG) possesses a high affinity for blood proteins (Sazuka et al, 1996) and, consequently, contributes to extend its half-life. Moreover, flavonoids may be secreted in bile to the duodenum and then reabsorbed, which results in enterohepatic cycling and evokes a longer half-life for conjugates.

### 4.3 Anticarcinogenic properties

A recent scientific focus dedicates research to understanding the mechanisms behind fruit and vegetable protective effects against various types of cancers (Nichenamettla et al, 2006). Animal studies and cell models suggest that flavonoids act as anticarcinogens through influencing molecular events in the initiation, promotion and progression stages of cancer.

#### **4.3.1** *Blocking mechanisms*

Flavonoids blocking agents prevent carcinogens by protecting DNA from nitrogen reactive intermediates and ROS attack because of its scavenging potential. Examples include quercetin (Bub et al, 2003 ; Chen et al, 2006), genistein (Win et al, 2002). Flavonoids also induce carcinogen detoxifying systems, (Canivenc-Lavier et al, 1996); alter metabolism of procarcinogens in favor of conjugation and excretion of reactive metabolites. Several flavonoids from citrus fruits and curcumin can interact with the aryl hydrocarbon receptor as agonists or antagonists, depending on structure and cell context (Johnson et al, 2007). Such interactions influence the expression of drug metabolizing enzymes such as cytochromes P450 as well as induction of phase II enzymes which may facilitate the elimination of certain carcinogens or their reactive intermediates (Zhang et al, 2003).

### **4.3.2.** *Supressing mechanisms*

These mechanisms result in suppression or elimination of tumor cells by interfering with cell cycle regulation, signal transduction pathways, transcriptional regulation, inhibition of cyclooxygenase activity, suppression of oncogenes and tumour formation, and induction of apoptosis of cancer cells (Ramos, 2007; Scalbert et al, 2005).

A significant number of flavonoids, alone or in combination, have been shown to induce G2/M arrest in CaCo2 and SW620 human colon carcinoma cells (Dorai et al, 2004; Gossé et al, 2005). Other suppressing mechanism considered is the inhibitory effect on arachidonic acid metabolism which leads to the production of many pro-inflammatory or mitogenic metabolites such as certain prostaglandins and ROS. Curcumin was one of the first chemopreventive phytochemicals shown to possess significant COX-2 inhibiting activity through the suppression of NF-kB (Dorai et al, 2004; Johnson et al, 2007).

During the carcinogenic process, both hypermethylation of the promoter regions of tumor suppressor genes and hypomethylation of oncogenes can occur, resulting in under- or over-expression. Both EGCG (Fang et al, 2005) and genistein (Fang et al, 2003) have been shown to reactivate a number of key genes, such as the cell cycle inhibitor p16 and the retinoic acid receptor, in several different cancer cell types. The mechanism proposed was through inhibition of DNA methyltransferase, which in the case of EGCG, involved a direct interaction with the enzyme.

In a majority of studies over the past few years, it has been focused and demonstrated that flavonoids like EGCG (Chen et al, 2003), quercetin (van Erk et al, 2005), luteolin (Horinaka et al, 2005), apigenin (Horinaka et al, 2006), and genistein (Dave et al, 2005) can trigger apoptosis through the modulation of a number of key elements in cellular signal transduction pathways linked to apoptosis. A decreased risk for different types of cancer (Depeint, 2003; Gee et al, 2002; Riboli et al, 2003; Su et al, 2002) or a diminished recurrence of lung (Le Marchand et al, 2000) or breast (Nakachi et al 1998) cancer has been reported after the consumption of flavonoids or certain foods or drinks (tea) rich in these phenolic compounds.

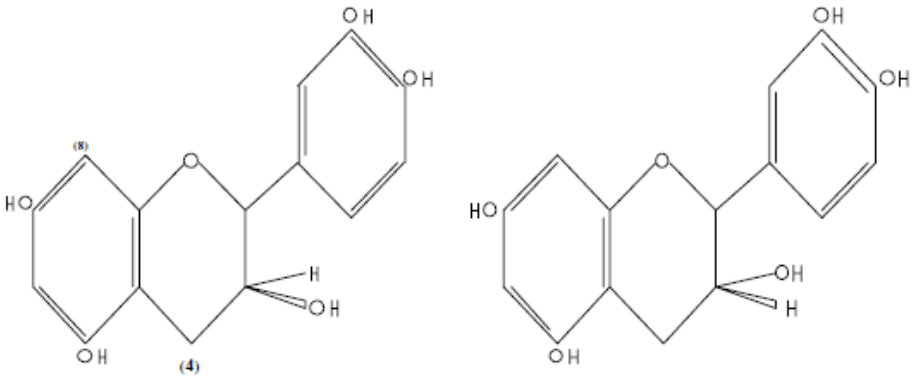
# IV. THE APPLE PROCYANIDINS

## 1. General characteristics and dietary sources

The procyanidins (Pcy), also known as condensed tannins, represent the second most abundant class of plant polyphenols (Gerhäusser, 2008a). Pcy are widely distributed in plants-derived food, especially in fruit, legume seeds, cereal grains, and a variety of beverages including wine, beer, tea, cocoa, and cider. They are concentred in the peel of fruits, and their content is decreased by industrial food processing methods as fruit peeling, decortications, juice filtration, maceration, drying or long-term storage. For example, natural cloudy apple juice contains about 2.5-fold more Pcy than processed clear apple juice (Manach et al, 2004; Mullen et al, 2007).

Pcy in foods affect parameters such as astringency, bitterness, sourness, sweetness, salivary viscosity, aroma, and color formation (Gerhauser, 2008b; Santos-Buelga et al, 2000). They are of interest in nutrition and medicine because of their potent antioxidant capacity and possible effects on human health in reducing the risk of chronic diseases such as cardiovascular diseases and cancers.

Pcy are di-, tri- and oligomeric condensation products of flavan-3-ol monomers of (+)-cathechin and (-)-epicathechins (Figure 22), and generally posses 12 – 16 phenolic OH-groups and 5-7 aromatic rings per 1000 units of relative molecular mass, and posses an average molecular weight of 1000-6000. Their mean degree of polymerization (DP) in foods has rarely been determined. However, in cider apples, the mean degree of polymerization ranges from 4 to 11 (Guyot et al, 1998). Dimeric Pcy are the named B-type Pcy which have one 4→ 6 or 4→8 interflavan linkages, whereas A-type Pcy are more rigid and the monomer subunits are further linked by an unusual second linkage. The C-type Pcy are trimers composed of three flavan-3-ol subunits with 4→6 or 4→8 bonds (Figure 23) (Aron and Kennedy, 2008; Auger et al, 2004; Gerhäuser, 2008b; Manach et al, 2004).



(+)-Catechin (-)-Epicatechin

| NAME | STRUCTURE |
|---|---|
| Procyanidin B1 | (-)-epicatechin-(4-8)-(+)-catechin |
| Procyanidin B2 | (-)-epicatechin-(4-8)-(-)-epicatechin |
| Procyanidin B3 | (+)-catechin-(4-8)-(+)-catechin |
| Procyanidin B4 | (+)-catechin-(4-8)-(-)-epicatechin |
| Procyanidin C1 | (-)-epicatechin-(4-8)-(-)-epicatechin-(4-8)-(-)-epicatechin |
| Procyanidin C2 | (-)-epicatechin-(4-8)-(-)-epicatechin-(4-8)-(+)-catechin |

***Figure 23. Structure of cathechin, epicathechin and procyanidins (Auger et al, 2004).***

In an analytical study the Pcy content of red wine, chocolate, cranberry juice and cloudy apple juice (Hammerstone et al, 2000; Huemmer et al, 2008) and varieties of apples (Guyot et al, 2001a,b; Hammerston et al, 2000; Sanoner et al, 1999; Vrhovsek et al, 2004) has been determined. On average, chocolate and apples contained the largest Pcy content per serving, 164.7 and 147.1 mg respectively, in apples this correspond to 63 to 77% of apple polyphenols. More over a serving size of cloudy apple juice contains 48-61% of Pcy, whereas red wine and cranberry juice contains 22.0 and 31.9 mg, respectively. The Pcy content varied greatly between apple samples (12.3–252.4 mg/serving) with the highest amounts on average observed for the Red Delicious (207.7 mg/ serving) and Granny Smith (183.3 mg/serving) varieties and the lowest amounts in the Golden Delicious (92.5 mg/serving) and McIntosh (105.0 mg/serving) varieties.

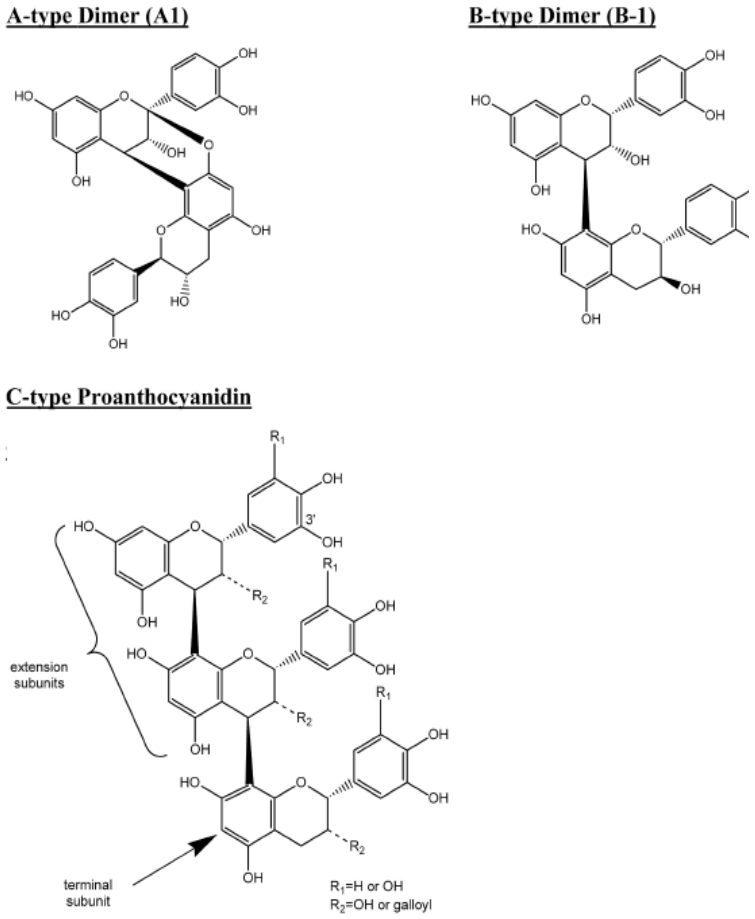



***Figure 24. Structure of procyanidins dimmers and trimers of the A-, B- and C-type (Aron and Kennedy, 2008).***

## 2. Physicochemical properties

### 2.1 Interaction with proteins

Pcy are generally capable to form unspecific complexes with proteins. Reaction with proteins is directly related to the DP (Khanbabaee et al, 2001). Pcy-protein interaction is a dynamic, generally reversible surface phenomenon driven by hydrophobic effects, which is reinforced by the establishment of hydrogen bonds. Phenolic hydroxyl groups of Pcy serve as proton donors and carbonyl groups of the peptide bonds as proton acceptors. Proline-rich proteins (such as collagen, gelatine, and salivary proteins) were found to have highest binding affinity through hydrophobic interaction. Also, carbohydrate residues in glycoproteins may enhance affinity and specificity of the interaction. Binding is also influenced by the molecular weight, and

the pattern of hydroxylation. Larger Pcy with a DP of more than 3 increase the affinity for protein (Gerhäuser, 2008b).

### 2.2 Antioxidant and radical scavenging capacity

Flavan3-ols have been shown to behave as antioxidants via several mechanisms including the scavenging of free radicals, chelation of transition metals, as well as the mediation and inhibition of enzymes (Cos et al, 2003). Pcy like all polyphenols are able to scavenge ROS, including singlet oxygen as well as superoxide anion-, hydroxyl-, peroxyl-, and nitric oxide radicals, through electron-donating properties and generation of relatively stable phenoxyl radicals, which are generally less harmful than the initial radical species (Santos-Buelga et al, 2000). Additionally, Pcy antioxidant activity increase from monomer to trimer and then decrease from trimer to tetramer. Pcy are able to complex with Fe(III) as well as Al(III) and Cu(II) reducing levels of free iron and copper which reduces the risk of hydroxyl radical formation through Fenton or Haber-Weiss reactions (Aron and Kennedy, 2008).

## 3. Bioavailability and metabolism

Flavonoids may influence mechanisms that contribute to cancer prevention. However, to do so *in vivo* they must be absorbed and achieve effective concentrations at the target site in the correct metabolic form (Milner, 2006). The least well absorbed apple polyphenols are the Pcy and anthocyanidins (if present). They are relatively stable in the gastric juice and reach the colon where they may exert a local effect before they are degraded by the microflora (Deprez et al, 2000; Hackman et al, 2008; Kahle et al, 2007; Manach et al, 2004;). Bioavailability of small Pcy up to trimers has been reported (Prior et al, 2005). Procyanidin dimers have been detected in urine, only catechin glucuronides and methylated glucuronide metabolites were measurable in plasma, kidney, and liver (Aron and Kennedy, 2008). Pcy with a DP > 3 are generally poorly bioavailable due to their molecular size, low permeability through paracellular absorption, and their likely complexation with luminal and mucosal proteins (Aron and Kennedy, 2008). *In vitro* incubations with human colonic microflora suggest that Pcy will be catabolized into low-molecular-weight phenolic acids such as mono- and dihydroxy-phenylacetic acid, hydroxyphenylpropionic acid, and phenylvaleric acid when they reach the colon (Deprez et al, 2000). These phenolic acids will then be

absorbed, further metabolized by conjugation, and excreted in urine (Manach et al, 2004).

A study confirmed these *in vitro* observations but in patients. Kahle et al. performed an apple juice intervention study with ileostomy patients (Kahle et al, 2005, 2007). Eleven volunteers drank 1 L of cloudy apple juice after an overnight fast. Ileostomy bags were collected immediately before 1 h and after 8h apple juice consumption. In this study was detected about 90% of the ingested Pcy in ileostomy bags (Kahle et al, 2007). The mean DP of Pcy was reduced from 5.7 (juice) to 3.4 within 2 h and further declined with time.

## 4. Cancer chemopreventive properties of apple polyphenols

### 4.1 *In vitro* studies

Apple constituents have been shown to exert potential cancer-protective effects via regulation of cell cycle progression and signal transduction pathways of cell growth and proliferation, induction of apoptosis, antioxidant and anti-inflammatory activities, DNA repair and tumor formation and antiangiogenic mechanisms (Figure 24) (Barth et al, 2005; Gossé et al, 2005, 2006; McCann et al, 2007).

#### 4.1.1 *Antioxidant activities*

Eberhardt et al. (2000) demonstrated that radical scavenging activity of fresh apples was mainly attributed to the phytochemical content rather than to that of vitamin C (Eberhardt et al, 2000). A comprehensive comparison of radical scavenging activity of apple extracts, fractions and subfractions with their phytochemical composition revealed that all major classes of apple phytochemicals contribute to antioxidant activity against peroxyl radicals measured in the ORAC assay, whereas DPPH (1,1-diphenyl-2-picrylhydrazyl) and superoxide anion radicals were potently scavenged by more lipophilic fractions containing quercetin-glycosides and Pcy (Zessner et al, 2008).

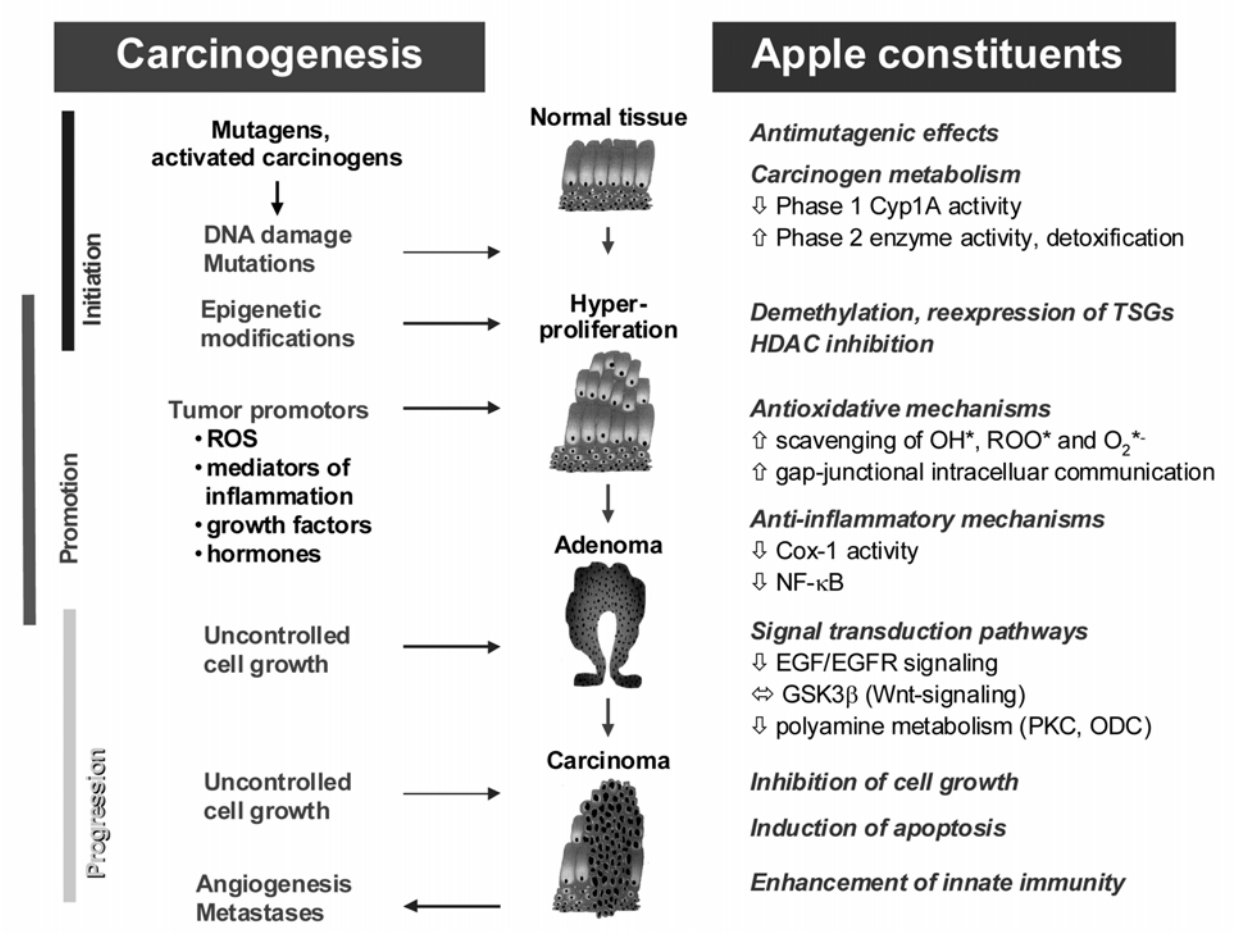



***Figure 25. Anticarcinogenic mechanisms of apple constituents represented in colon carcinogenesis.*** *OH● ,hydroxyl radicals; ROO●, peroxyl radicals; O2●–, superoxide anion radicals; TSG, tumor supressor gene; HDAC, histone deacetylase (Gerhäuser, 2008a).*

### **4.1.2** *Inhibition of signaling pathways*

Kern et al. (2005) and Fridrich et al. (2007) investigated the potential of apple juice extracts and apple juice polyphenols to influence EGF signaling. They found that Pcy dimers B1 and B2 as well as two quercetin glycosides possessed substantial EGFR-inhibitory properties. A Pcy-rich fraction also blocked the signaling cascade leading to the induction of ornithine decarboxylase (ODC), which is essential for cellular proliferation by formation of polyamines (Gossé et al, 2005). This fraction potently inhibited protein kinase C (PKC) activity by 70% in human colon a cancer-derived SW620 cell which was associated with down-regulation of polyamine biosynthesis and activation of polyamine catabolism, and activation of apoptosis (Gosé et al, 2005).

### **4.1.3.** *Inhibition of cell proliferation*

Apple Pcy has shown to contribute to a substantial part to the antiproliferative activity of apple extracts (Veeriah et al, 2006). Pcy obtained from a flavonoid mixture of apples inhibited proliferation of HT29 (Veeriah et al, 2006) and CaCo-2 (Kuntz et al, 1999) colon cancer cells at subcytotoxic concentrations and beneficially influenced the expression of several key genes associated with the transformation of xenobiotics. Growth inhibition of SW620 metastatic cells was observed with an enriched extract of apple Pcy. In contrast, an apple fraction containing the monomeric polyphenols: catechin, epicatechin and quercetin but no procyanidins showed no effect on cell growth. Pcy inhibited cell growth by perturbating cell cycle traverse leading to the accumulation of cells in the G2/M phase (Gossé et al, 2005).

### **4.1.4** *Induction of apoptosis*

Kern et al. analyzed the potential of apple juice polyphenol extract AE02 to induce apoptosis in HT29 cells (Kern et al, 2007). The AE02 extract potently induced caspase-3 activity and DNA fragmentation measured by an ELISA assay, although at relatively high concentrations. Since these experiments were performed under serum-free conditions without addition of catalase, formation of $H_2O_2$ may be responsible for part of these results.

Pro-apoptotic effects of Pcy have been described only in B16 mouse melanoma cells, human mammary (Miura et al, 2008), prostate LNCaP (Wu et al, 2007) and colon carcinoma-derived metastatic cells (Gossé et al, 2005), also in KATO III human stomach cancer cells, although at extremely high concentrations of up to 5 mg/mL (Hibasami et al, 2004). However, none of these studies mention the cellular and molecular mechanisms by which Pcy induce apoptosis in cancer cells.

## **4.2 Cancer chemopreventive properties in animal studies**

*In vitro* studies on molecular mechanisms will provide a hint to potential cancer preventive effects *in vivo.* Chemopreventive efficacy can only be demonstrated in animal models or human intervention studies with tumor incidence and multiplicity (e. g., number of tumors per animal) as endpoints. When considering application of a

compound or product for prevention of cancer in humans, toxicological and safety issues also have to be considered (Gerhäuser, 2008a).

One study has addressed toxicology and safety of a polyphenol rich extract from unripe apples (Applephenon®), which is sold in Japan as a food additive and nutritional supplement. The product contains high levels of Pcy (64%, dimers to 15-mers), 12% flavan-3-ol monomers, 7% flavonoids, and 18% non-flavonoids (Ohnishi-Kameyama, 1997). One gram of extract was reported to contain polyphenols equivalent to approximately four apples (Akazome, 2004). In the Ames mutagenicity test, only one out of five bacterial strains showed a slight increase in revertants indicative of mutagenic potential. No signs of mutagenicity were detected in the chromosomal aberration test in Chinese hamster lung cell culture and the micronucleus test in Sprague-Dawley rats. Also, no signs of toxicity at a dose of 2000 mg/kg body weight were observed in an acute and subchronic toxicity test. The extract was therefore regarded as safe (Shoji et al, 2004).

As a first indication of cancer chemopreventive efficacy *in vivo*, apple products have been tested in experimental animal models for chemically- or genetically-induced tumors of colon (Gossé et al, 2005), as well as in xenograft models for solid tumors and melanoma (Miura et al, 2008). Apple Pcy applied at 1% in drinking water, inhibited the growth of transplanted B16 mouse melanoma cells *in vivo*, and increased the survival rate of the host mice transplanted with B16 cells (Miura et al, 2008). The potentially important role of Pcy for colon cancer prevention was demonstrated by Gosse et al. (2005, 2006). In the azoxymethane-induced rat model, a procyanidin-enriched fraction from apples at a very low dose (0.01% in drinking water) significantly reduced the number of ACF/colon by 50% (Gossé et al, 2005).

### 4.3 Epidemiological evidence of apple consumption on cancer incidence

Although extrapolation from animal studies to the human situation is difficult, there is consistent evidence from epidemiological observations that regular consumption of one or more apples per day may contribute to the prevention of CRC. Recent publications indicate preventive effects of apple consumption on colorectal carcinogenesis. In the Nurses' Health Study, a large prospective cohort study conducted

in the USA reported that 20% of women who consumed the most apples had a significantly reduced risk of developing colorectal adenomas (odds ratio (OR) of 0.83 (95% CI = 0.70-0.98) in comparison to the 20% with the lowest intake (OR of 1.00, $P_{trend} = 0.05$) (Michels et al, 2006). In a case control study conducted in Uruguay, apple consumption was associated with a significant, dose-dependent reduction in CRC risk in men and women (Deneo-Pellegrini et al, 1996). In a South-Korean case-control study, fruit consumption (apples combined with banana, pear and watermelon) lowered the risk for colon cancer in men (adjusted OR: 0.36, 95% CI = 0.16-0.84), but not in women (adjusted OR: 1.14, 95% CI = 0.54-2.40) (Lee et al, 2005). A meta-analysis of multicenter case control studies conducted in Italy revealed that consumption of ≥1 apple/day in comparison with ≤ 1 apple/day significantly reduced the odds ratio for CRC (OR: 0.80, 95% CI = 0.71–0.90) as well as for cancers of the oral cavity (OR: 0.79, 95% CI = 0.62-1.00), larynx (OR: 0.58, 95% CI = 0.44-0.76), breast (OR: 0.82, 95% CI = 0.73-0.92) and ovary (OR: 0.85, 95% CI = 0.72-1.00) (Gallus et al, 2005).

# *RESULTS*

**CHAPTER 1**

# TRAIL-MEDIATED APOPTOSIS TRIGGERED BY APPLE PROCYANIDINS IN HUMAN ADENOCARCINOMA SW480 CELLS AND THEIR DERIVED METASTATIC SW620 CELLS

**I. Modulation by polyamines of apoptotic pathways triggered by procyanidins in human metastatic SW620 cells (publication)**

**II. Differential induction of apoptosis by Apple procyanidins in TRAIL-Sensitive Human Colon Tumor Cells and Derived TRAIL-Resistant Metastatic Cells (publication)**

**Modulation by polyamines of apoptotic pathways triggered by procyanidins in human metastatic SW620 cells**

M. E. MALDONADO-CELIS, S. ROUSSI C. FOLTZER-JOURDAINNE, F. GOSSE, A. LOBSTEIN, C. HABOLD, A. ROESSNER, R. SCHNEIDER-STOCK and F. RAU

**Cellular and Molecular Life Sciences, 2008, vol. 65, n° 9, pages 1425-1434**

# 3. Results: PUBLICATION

**Modulation by polyamines of apoptotic pathways triggered by procyanidins in human metastatic SW620 cells**

*Maldonado-Celis ME, Roussi S, Foltzer-Jourdainne C, Gossé F, Lobstein A, Habolde C, Roessner A, Schneider-Stockf R, Raul F.*

***Cell Molecular Life Science 2008; 65: 1425-1434.***

# 4. Complementary results

## 4.1 Effect of Pcy and MDL 72527 (MDL) on the activity of caspase-8, -3 and -9 in SW620 cells

Since potentiation of Pcy-induced apoptosis by MDL was inhibited with blocking antibodies for DR4 and DR5, this led us to hypothesize that MDL may potentiate the apoptotic effects of Pcy through the activation of the extrinsic apoptotic pathway mediated by TRAIL-death receptors DR4 and DR5. The activation of DR4 and DR5 receptors results in the formation of the DISC complex that leads to the activation of caspase-8 (Sulinam et al, 2001) which may activate two distinct death pathways. A mitochondrial-independent pathway leading directly to the activation of caspase-3, and subsequent DNA fragmentation. The second caspase-8 activated pathway involves a cross-talk with the intrinsic (mitochondrial) pathway through the cleavage of Bid protein producing tBid, which translocates into the mitochondria causing mitochondrial dysfunctions and release of cytochrome *c* into the cytosol (Luo et al, 1998). These events favor the activation of caspase-9 and the downstream activation of caspase-3 (Sulinam et al, 2001).

In order to characterize the apoptotic pathway triggered by Pcy in association with MDL in SW620 cells, we measured the activity of caspase-8, -3 and -9 as downstream markers of extrinsic and/or intrinsic apoptotic pathways by measuring the colorimetric reaction produced during the cleavage of their specific substrates for caspase-8 (Ac-IETD-pNA), caspase-3 (Ac- DEVD-pNA), caspase-9 (LEHD-pNA). As shown in Figure 26, Pcy used as a single drug or combined with MDL increased significantly ($P < 0.05$) caspase-8 and caspase–3 activities in SW620 cells. On the contrary, MDL reduced by 66% for 48 h of treatment the ability of Pcy to activate caspase-9. These data suggest that MDL enhanced Pcy-triggered apoptosis through the activation of the extrinsic apoptotic pathway involving at least TRAIL-death receptors DR4/DR5.

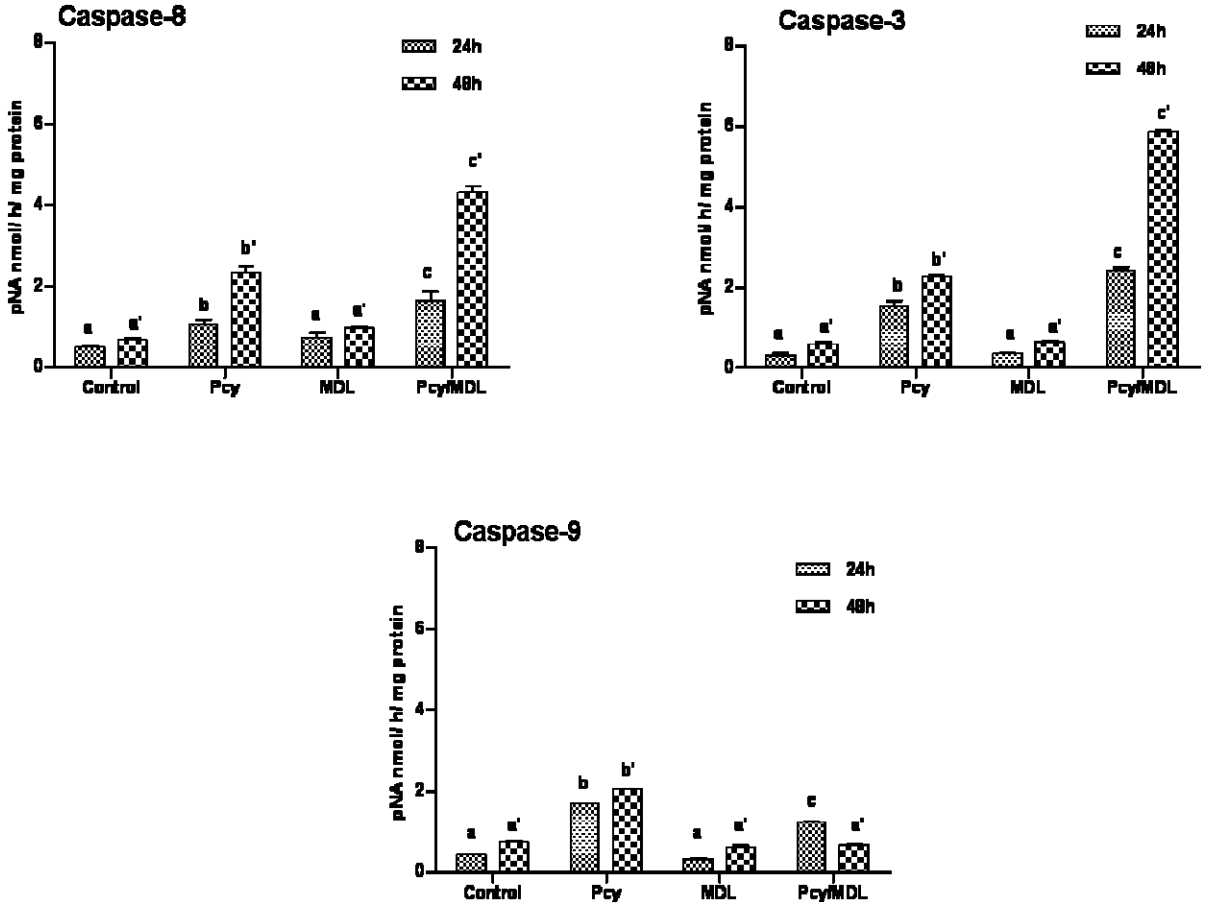


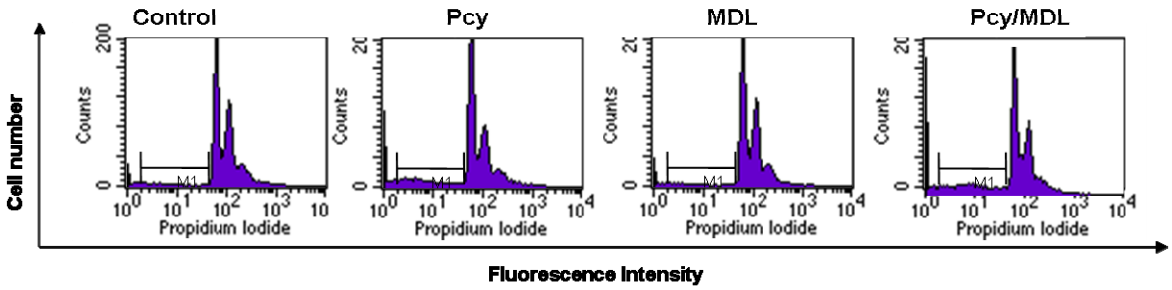

***Figure 26. Effects of Pcy and MDL on the caspase activities****. SW620 cells treated with 0.1% DMSO (control), 80 μg/ml Pcy, 50 μM MDL for 48 h. Data are indicated as nmol pNA released/ mg of total protein. Data are presented as the mean value ± SE of at the least three independent experiments and columns not sharing the same superscript differ significantly (P<0.05).*

### 4.2 Effects of Pcy and MDL 72527 on SW480 cell death

To obtain comparative information about the effects of MDL and Pcy treatments on human adenocarcinoma SW480 cells, we performed flow cytometry analysis of hypodiploid cell population with PI staining, mitochondrial membrane potential, expression and inactivation of DR4 and DR5 death receptors in SW480 cells treated with Pcy single or combined with MDL under the same conditions as for SW620 cells (Figures 27 -31).

First, we measured the amount of hypodiploid apoptotic cells (subG0/G1) 48 h after Pcy (80 μg/ml), MDL (50 μM) or Pcy/MDL treatments. As shown in figure 27,

MDL used as a single drug did not increase the subG0/G1 cell population when compared with control cells. The ability of Pcy to increase the number of hypodiploid cell population (13%) was not potentiated significantly by MDL.




| | % Hypodiploid cells |
|---|---|
| Control | 1.7 ± 0.1%[a] |
| Pcy | 13 ± 0.2%[b] |
| MDL | 1.5 ± 0.1%[a] |
| Pcy/MDL | 16 ± 1.5%[b] |

***Figure 27. Analysis of cell hypodiploid population after Pcy and MDL treatments****. SW480 cells treated with 0.1% DMSO (control), 80 µg/ml Pcy, 50 µM MDL and Pcy/MDL for 48 h. For the measurement of hypodiploid bodies, cells were collected and permeabilized, stained with PI and submitted to flow cytometric analysis as described in Maldonado-Celis et al. (2008). Flow cytometry data are represented. The percentage of cells in the subG0/G1 region (M1) is indicated in the table. Data are the mean ± SE of three separate experiments and statistical differences are expressed by superscript letters (a≠b, P<0.05).*

### 4.3 Effects of Pcy and MDL on mitochondrial membrane potential of SW480 cells

We examined by flow cytometry whether Pcy and Pcy/MDL combined treatments have an effect on mitochondrial membrane potential (ΔΨm) after staining the cells with DiOC2(3). As shown in Figure 28, the percentage of cells with mitochondrial membrane depolarized induced by Pcy (20%) was not modified significantly by MDL (18%) after 48 h of treatment. These results indicate that the mitochondrial membrane perturbation observed with Pcy in SW480 cells is an event independent of polyamine catabolism controlled by the PAO enzyme.

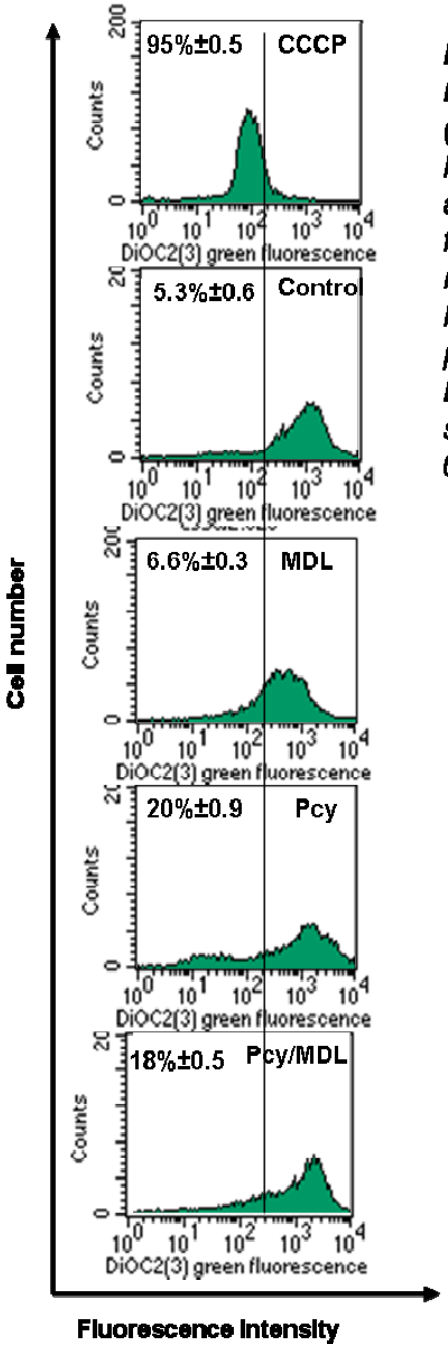
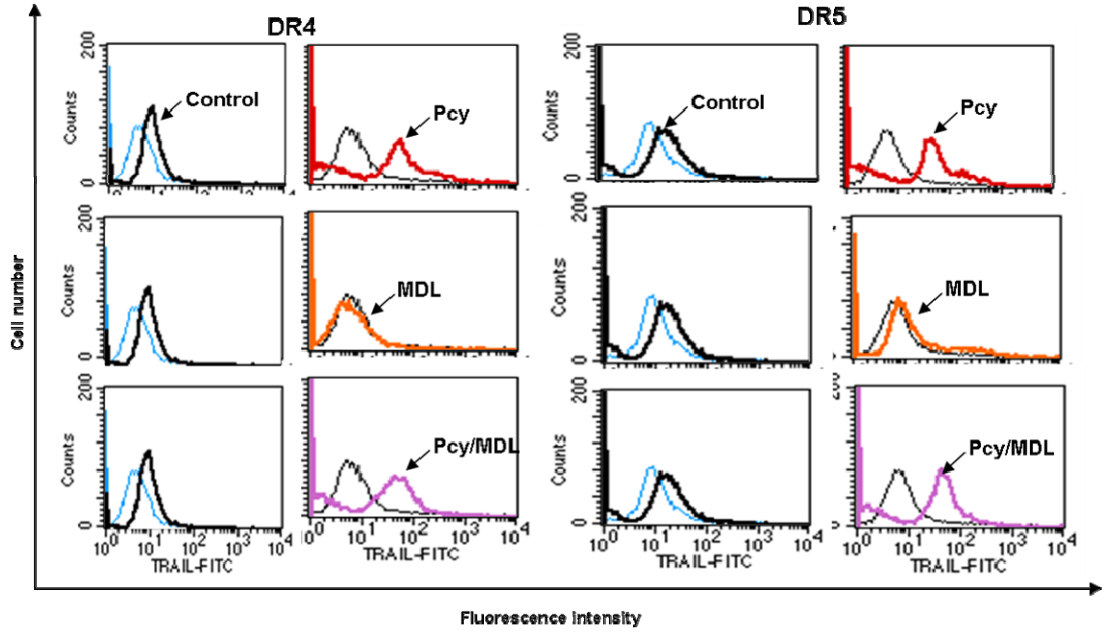



***Figure 28. Flow cytometry analyses of mitochondrial membrane potential**. Cells were treated with 0.1%DMSO (control), 80 µg/ml Pcy, 50 µM MDL and Pcy/MDL for 48 h. Cells were harvested, stained with DiOC2(3) reagent and analyzed by flow cytometry. Reduction of green fluorescence corresponds to the loss of mitochondrial membrane potential (ΔΨm). Cytometry data and histograms are shown. Data are presented as the percentages of cells with reduced green fluorescence. Data are obtained as the mean ± SE of at least three separate experiments. Control versus treated cells: *P < 0.05.*

### 4.4 Effects of Pcy and MDL on TRAIL death receptors expression and function in SW480 cells

We analyzed the cell surface expression of both TRAIL-death receptors DR4 and DR5 by flow cytometry using specific antibodies. As shown in figure 29, we observed that TRAIL-DR4 and –DR5 receptors were already present at the cell surface (control). Pcy increased significantly ($P<0.05$) the number of cells expressing DR4 (70%) and DR5 (74%), but no significant changes of DR4 (75%) and DR5 (78%) cell surface expression were observed with combined MDL/Pcy treatment (figure 29).




**% of Cells Expressing TRAIL Death receptors**

| | DR4 | DR5 |
|---|---|---|
| Control | 19.1±0.6[a] | 5.2±0.6[a] |
| Pcy | 70.6±2.4[b] | 74.0±2.6[b] |
| MDL | 16.4±0.3[a] | 24.1±2.2[a] |
| Pcy/MDL | 75.4±2.8[b] | 78.0±1.7[b] |

***Figure 29. Expression of TRAIL-death receptors DR4 and DR5 in SW480 cells****. Cell surface expression of TRAIL-DR4 and -DR5 death receptors analyzed by flow cytometry in cells treated with 0.1% DMSO (control), 80 µg/ml Pcy, 50 µM MDL and Pcy/MDL for 48 h. Cells were harvested and stained with FITC-conjugated monoclonal antibodies against the two types of TRAIL receptors (DR4 and DR5). Data are represented on cytometry histograms and the fluorescence shifts to the right are indicative of cell death receptor expression on cell membrane (overlays). The percentage of cells with enhanced fluorescence is given in the table. Data are the mean value ± SE of at least three separate experiments and statistical differences are expressed by superscript letters (a≠b, P<0.05).*

To investigate the correlation between cell surface DR4/DR5 receptors expression and apoptosis, SW480 cells were pre-treated with anti-DR4 and anti-DR5 blocking antibodies for 24 h before Pcy and/or MDL treatments and the rate of apoptosis was evaluated by flow cytometry. As shown in Figure 30, the amount of apoptotic cells dropped from 14% to 8% with Pcy and from 16% to 9% with Pcy/MDL-combined treatments after addition of blocking antibodies, indicating that TRAIL-death receptor pathway was involved in the apoptotic effects triggered by Pcy in these cells.

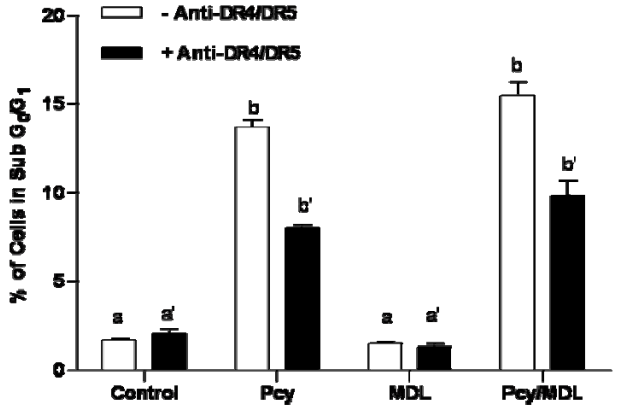



***Figure 30. Analysis of hypodiploid cells in the presence of blocking antibodies against DR4 and DR5 receptors.*** *SW480 cells treated with 0.1% DMSO (control), 80 µg/ml Pcy, 50 µM MDL for 48 h. Antibodies anti-human cell death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) were added simultaneously in the culture medium 24 h before the treatment to various compounds. Production of hypodiploid cells was detected by flow cytometry as described in the Materials and methods section. Histogram represents the percentage of hypodiploid cells of 10000 cells/ sample. Data are the mean ± SE of at least three separate experiments and columns not sharing the same superscript differ significantly (P<0.05).*

## 4.5 Effects of Pcy and MDL 72527 on nuclear Histone Deacetylase (HDAC) activity of SW480 cells

We also investigated the effects of Pcy and MDL on nuclear HDAC activity in SW480 cells treated for 48 h. A non significant effect on HDAC activity was observed in figure 31 in cells treated with Pcy single or combined with MDL.

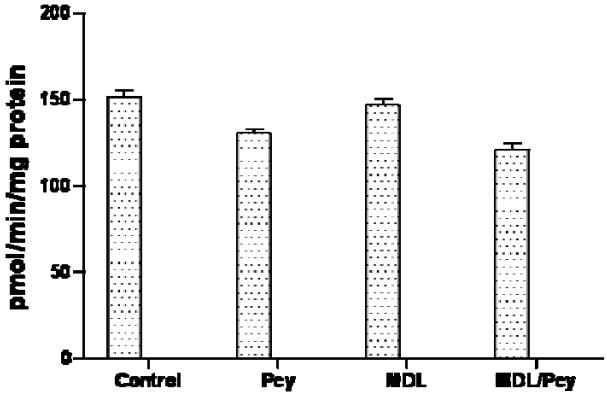



***Figure 31. Activity of nuclear histone deacetylase (HDAC).*** *SW480 cells were treated with 0.1% DMSO (control), 80 µg/ml Pcy, 50 µM MDL for 48 h. They were harvested by scrapping and nuclear extracts were prepared as described in the Materials and methods section. Histogram represents the pmol of deacetylated substrate/mg of total proteins. Data are presented as the mean ± SE of at least three separate experiments.*

## 5. Conclusions

Our findings provide information about the selective potentiating effect of MDL on apoptosis triggered by apple Pcy in human colon cancer derived metastatic SW620 cells, through the activation of other apoptotic mechanisms than those activated by Pcy used alone. In SW620 cells, the Pcy-triggered apoptosis potentiated by MDL is characterized by an inhibition of the intrinsic mitochondrial pathway, depletion of the intracellular polyamine pool leading to the activation of the extrinsic apoptotic pathway through the up-regulated expression of TRAIL-death receptors DR4 and DR5, which was associated with reduced activity of nuclear HDAC. The complementary results obtained by measuring the activity of caspase-8, -3 and 9 confirmed that Pcy-triggered apoptosis enhanced by MDL occured by activation of the extrinsic apoptotic pathway activating of caspase-8 and caspase-3, in parallel a reduced expression of caspase-9 was observed.

On the other hand, in SW480 cells apoptosis triggered by Pcy was not enhanced by MDL. In these cells, it seemed that the extrinsic pathway was the main apoptotic pathway induced by Pcy single or combined with MDL, involving the activation of DR4 and DR5 receptors without significant changes in the activity of nuclear HDAC. These data suggest that MDL did not cause important changes in the intracellular polyamine pool of SW480 cells compared to SW620 cells. The differential effect of MDL may be explained by the differences in polyamine metabolic rate between these cells lines. It has been reported that the key regulatory enzymes ODC and AdoMetDC are 10 fold more active in SW620 cells than in SW480 cells. In addition, the ability to uptake and accumulate polyamines from the environment is also higher in SW620 than in SW480 cells. Similarly, total polyamine content of SW620 cells is significantly higher than in SW480 cells (Duranton et al, 2003).

The increased expression of DR4 and DR5 receptors observed in SW480 cells by Pcy might be regulated by post-transcriptional mechanisms whereas in SW620 cells these death receptors seem to be regulated at transcriptional level. This hypothesis will be confirmed by analyzing the levels of mRNA corresponding to DR4 and DR5 after Pcy treatment in SW480 and SW620 cells. DR4 and DR5 receptors are attractive molecular targets for cancer therapy. Moreover its ligand, TRAIL, is one of the most

promising candidates for cancer therapy. The SW480 cells have been described as TRAIL-sensitive cells (Jin et al, 2004) whereas SW620 cells present a TRAIL-resistant phenotype (Vaculová et al, 2006). To know the effectiveness of apple Pcy in sensitization of SW480 and SW620 cells to TRAIL-induced apoptosis we examined molecular markers of TRAIL-death receptor mediated apoptosis susceptible of being activated by apple Pcy. These studies are presented in the next part of this first chapter.

**Differential Induction of Apoptosis by Apple Procyanidins in TRAIL-Sensitive Human Colon Tumor Cells and Derived TRAIL-Resistant Metastatic Cells**

Maria-Elena MALDONADO-CELIS, Souad BOUSSEROUEL, Francine GOSSE, Carole MINKER, Annelise LOBSTEIN, and Francis RAUL

**Journal of Cancer Molecules (JOCM), 2009, vol. 5, n° 1, pages 21-30**

# 3. Results: PUBLICATION

**Differential Induction of Apoptosis by Apple Procyanidins in TRAIL-Sensitive Human Colon Tumor Cells and Derived TRAIL-Resistant Metastatic Cells**

*Maldonado-Celis ME, Bousserouel S, Gossé F, Minker C, Lobstein A, Raul F.*

***Journal of Cancer Molecules 2009; 5: 21-30.***

# 4. Complementary results

## 4.1 Effect of combined treatment with Pcy and TRAIL on DNA fragmentation

Pro-apoptotic effects of Pcy were characterized by identifying the cells in the SubGo/G1 region that exhibit DNA content lower than 2n. By using the PI that stains DNA and by the flow cytometry we determined the percentage of cells in SubGo/G1 region based on DNA content (Nicoletti et al, 1991). However, there is an assay widely used and highly sensitive for identifying cells dying of apoptosis named TUNEL (Gavrieli et al, 1992) through detection of DNA fragmentation into oligomers of about 180 bp by endonucleases at linker DNA site between nucleosomes that result from apoptotic signaling cascades. In this assay DNA strand breaks are detected with the enzyme terminal deoxynucleotidyl transferase (TdT) that catalyzes the addition of dUTPs that are secondary labeled with FITC (Figure 32).

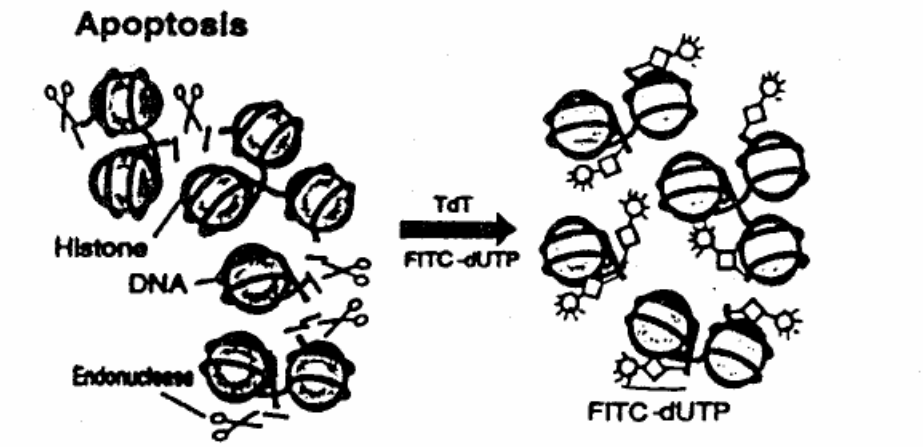
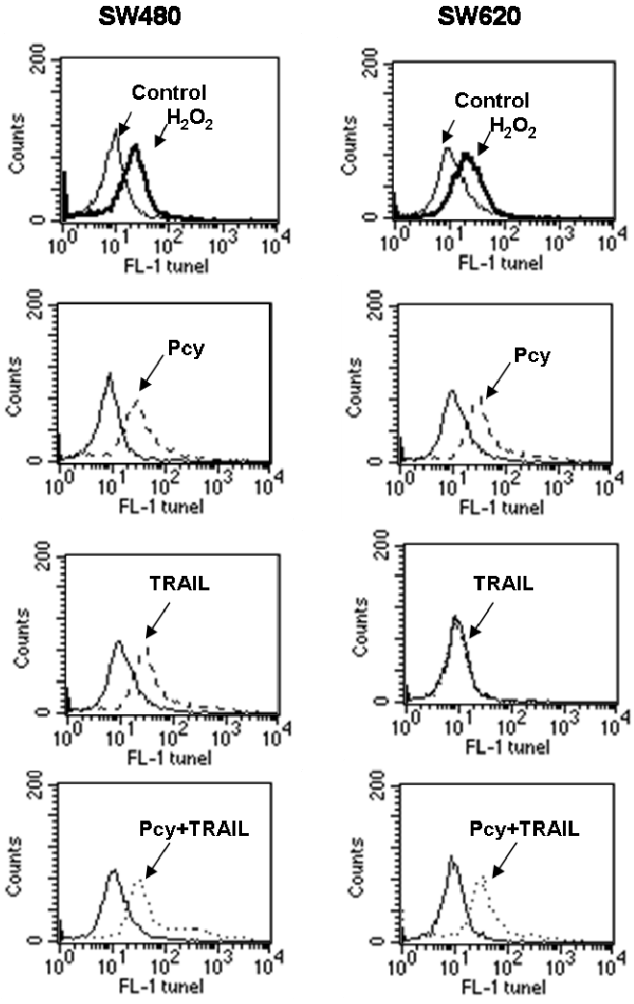



***Figure 32. Principle of TUNEL method (MBL International Corporation, Nagoya, Japan).***

To confirm that Pcy trigger apoptosis single or combined with TRAIL in SW480 and SW620 cells, we used the TUNEL assay following the manufacture's instructions of the Mebstain apoptosis kit (MBL, Nagoya, Japan). Briefly, cells were washed with PBS (2% BSA) two times and fixed with 4% paraformaldehyde for 30 min at 4°C. Cells were permeabilized by adding 200 μl of 0.5% Tween-20 with 0.2% BSA to the cell

pellet. The TdT reaction was carried out in the presence of FITC-dUTP reagent. Data from 10 000 events per sample were collected and analyzed using a FACScan flow cytometer (FACScan, BD Biosciences, Erembodegem, Belgium).

As shown in Figure 33, cells with DNA strand breaks were detected in SW480 cells after treatment with Pcy and/or TRAIL compared to non-treated cells. In SW620 cells, the addition of exogenous TRAIL did not induce nuclear DNA strand breaks, whereas treatments with Pcy alone and combined with TRAIL increased significantly percentage of cells with DNA strand breaks (Figure 33).




**% of TUNEL positive cells**

| | SW480 | SW620 |
|---|---|---|
| Control | 0.9±0.3[a] | 0.3±1.6[a'] |
| $H_2O_2$ | 73±1.3[b] | 72±2.7[b'] |
| Pcy | 80±1.2[c] | 72±2.7[b'] |
| TRAIL | 93±0.4[d] | 1±0.5[a'] |
| Pcy+TRAIL | 96±2.3[d] | 90±1.7[c'] |

***Figure 33. Effects of Pcy and TRAIL nuclear DNA****. Cells were treated with DMSO 0.1% (control), 80 µg/ml Pcy, 30 ng/ml TRAIL, or Pcy + TRAIL for 65 h. The TUNEL assay was used to determine the percentage of cells with nuclear DNA single strand breaks in SW480 and SW620 cells. The strand breaks were detected by flow cytometry with the TdT-reaction in presence of dUTP-FITC. As a positive control hydrogen peroxide ($H_2O_2$) treated cells were included. Columns represent the percentage of cells exhibiting nuclear DNA strand breaks corresponding to the TUNEL positive cells. Data are the mean value ± SE of at the least three independent experiments. For each cell line, columns not sharing the same superscript letter differ significantly: a≠b≠c≠d and a'≠b'≠c', P < 0.05.*

## 5. Conclusions

In this study we showed that Pcy treatment enhanced the TRAIL-death receptors mediated apoptotic pathway. Pcy potentiated the pro-apoptotic effects of TRAIL in human colon adenocarcinoma SW480 cells. Furthermore, Pcy sensitized the adenocarcinoma-derived metastatic SW620 cells which are, under basal conditions, TRAIL-resistant. These results were confirmed by TUNEL assay in the complementary results. Pcy activated mainly the extrinsic apoptotic pathway in SW480 cells involving activation of TRAIL-DR4/DR5 mediated apoptosis via caspase-8 and caspase-3. Neither change in Bid protein, nor changes in Bcl-2 and Bax ratio were detected during the various treatments, as well as cytochrome *c* was not released and caspase 9 was not activated after Pcy treatment.

In contrast, the apoptotic effects of Pcy in SW620 cells were more complex and involve a cross-talk between the extrinsic and the mitochondria (intrinsic) apoptotic pathway. Pcy treatment of SW620 cells led to an important increase number of ROS-producing cells compared to SW480 cells. This event may explain the important involvement of mitochondrial apoptotic pathway in SW620 cells associated with the drop of mitochondrial membrane potential, and the release of cytochrome *c* into the cytosol. Besides, these mitochondrial alterations may be a downstream consequence of the activation of the extrinsic apoptotic pathway because of decreased of Bid protein and progressive increase of Bax protein.

Activation of a caspase- and mitochondrial-dependent apoptosis is not exclusive for TRAIL-pathway. The Fas(CD95)/FasL system, the paradigm for the study of the extrinsic pathway to apoptosis leads to a caspase cascade that is not influenced by Bcl-2 overexpression, whereas a crosstalk with the intrinsic mitochondrial-dependent pathway is required. It is known that SW480 cells present a Fas-sensitive phenotype whereas its derived SW620 cells are Fas-resistant (Bergmann-Leitner and Abrams, 2000), thus it is possible that Fas-pathway could be activated by Pcy in SW480 cells, but not in SW620 cells. However, it is unknown whether Fas/FasL system responds differently to Pcy compared with the TRAIL-pathway. To address these questions, we analyzed in the second chapter the effect of apple Pcy on Fas-mediated apoptosis in both cell lines.

**CHAPTER II**

# FAS-MEDIATED APOPTOSIS TRIGGERED BY APPLE PROCYANIDINS IN HUMAN COLON CANCER SW480 CELLS AND THEIR DERIVED-METASTATIC SW620 CELL LINE

## I. Differential activation of Fas(CD95) apoptotic pathway by procyanidins in human colon cancer cells and their derived metastatic cells (Publication submitted)

# 1. Results: PUBLICATION (Submitted)

**Differential activation of Fas(CD95) apoptotic pathway by procyanidins in human colon cancer cells and their derived metastatic cells**

*Maldonado-Celis M-E, Bousserouel S, Gossé F, Minker C, Lobstein A, Raul F*

***International Journal of Biochemistry and Cell Biology, 2009 (submitted)***

## Original Article

# Differential activation of Fas(CD95) apoptotic pathway by procyanidins in human colon cancer cells and their derived metastatic cells

**Maria-Elena Maldonado-Celis[a,b,c], Souad Bousserouel[a,b], Francine Gossé[a,b], Carole Minker[d], Annelise Lobstein[d], and Francis Raul[a,b*]**

[a] IRCAD, Laboratory of Nutritional Cancer Prevention, 1 Place de l'hôpital 67000 Strasbourg, France

[2b]University of Strasbourg, Unit of Pathophysiology and Translational Research, Faculty of Medicine, 67000 Strasbourg, France

[c] Universidad de Antioquia, Escuela de Nutrición y Dietética, A.A. 1226 Medellín, Colombia

[d] CNRS UMR7081, University of Strasbourg, Faculty of Pharmacy, 67400 Illkirch, France

* Corresponding author at:

IRCAD
Laboratory of Nutritional Cancer Prevention
1, Place de l'Hôpital
67091 Strasbourg-Cedex
France
Tel: +33-3-8811-9023
Fax: +33-3-8811-9097
E-mail: francis.raul@ircad.u-strasbg.fr

**Abstract**

We investigated the effects of apple procyanidins (Pcy) on Fas receptor expression and function in human colon adenocarcinoma SW480 cells and in their FasL-resistant derived metastatic SW620 cells. Pcy up-regulated the expression of Fas receptor at the cell surface of both cell lines but activated Fas gene transcription only in SW620 cells. In SW480 cells, Pcy combined with Fas agonist CH-11 enhanced Fas-mediated apoptosis involving the loss of mitochondrial membrane potential and DNA fragmentation which were abrogated by the antagonist antibody of Fas receptor, the anti-Fas ZB4. On the contrary, in SW620 cells, CH-11 was not able to enhance Pcy-triggered apoptosis indicating that Fas receptor-mediated apoptosis was not activated in these cells despite an up-regulation of Fas receptor gene expression. However, it was observed in SW620 cells that Pcy activated the Fas receptor-mediated apoptotic pathway after a specific blockade of TRAIL-death receptors DR4/DR5. The present data showed that Pcy was able to activate Fas receptor apoptotic pathway in SW480 cells and favoured a cross-talk between TRAIL and Fas receptors in SW620 cells since a specific blocking of TRAIL death receptors favoured the activation of the Fas receptor-mediated apoptosis. These important data may allow the emergence of new therapeutic protocols targeting death receptors against resistant metastatic cells.

**Key words:** Apoptosis, Colorectal Cancer, Flavonoids, Fas, TRAIL, Mitochondria

**Abbreviations:** CCCP, carbonyl cyanide 3-chlorophenylhydrazone; CM-$H_2$DCFDA, 5-(and-6)-chloromethyl-2,7 dichlorodihydrofluorescein diacetate, acetyl ester; $\Delta\Psi m$, mitochondrial membrane potential; $DiOC_2(3)$, 3,3'-dihexyloxacarbocyanine iodide; DCFH, dichloro-dihydro-fluorescein; DCF, dichloro-fluorescein; DR4/DR5, death receptor 4/death receptor 5; FADD, Fas-associated protein with death domain; ITS, insulin-transferrin-selenium; MFI, mean fluorescence intensity; NK, natural killer; Pcy, procyanidins; PI, propidium iodide; ROS, reactive oxygen species; TRAIL, Tumor necrosis factor (TNF)-related apoptosis-inducing ligand; TUNEL, Terminal deoxynucleotide transferase (TdT) d-UTP biotin nick-end labeling

## 1. Introduction

Over the past few years, it has been shown that phytochemicals present in the human diet can prevent the occurrence of degenerative diseases such as cancer (Surh, 2003; van Breda et al., 2008). Apples are a rich source of polyphenol constituents, especially of flavonoids which are distributed in the peel, core and pulp (Thielen et al., 2004; Lata and Tomala, 2007). Flavonoids present in apples are divided into different classes: flavonols like quercetin conjugates (3-galactoside, 3-glucoside, 3-rhamnoside), flavan-3-ols derivatives including oligomers formed by catechin and epicatechin units, the procyanidins (Pcy) (Renard et al., 2007; Auger et al., 2004).

Pcy have recently gained interest because of potential health promoting effects by acting as antioxidant, anticarcinogen, cardiopreventive, antimicrobial, anti-viral, and neuro-protective agents (Gerhauser, 2008; Aron and Kennedy, 2008). We have recently reported that apple Pcy inhibit the growth of human metastatic colon adenocarcinoma-derived SW620 cells, through the inhibition of protein kinase C activity, down-regulation of polyamine biosynthesis and activation of polyamine catabolism, and through Pcy-triggered apoptosis involving TRAIL receptor-mediated pathway (Gossé et al., 2005, 2006; Maldonado-Celis et al., 2008). It was also reported that apple Pcy are able to inhibit the promotion/progression phases of colon carcinogenesis in rats (Gossé et al., 2005). However, the cellular and molecular mechanisms by which Pcy induce apoptosis in cancer cells are not well understood. Previously, we reported that TRAIL-DR4/DR5 blocking antibodies increased Pcy-triggered apoptosis in SW620 cells (Maldonado-Celis et al., 2008). These data suggested the activation of an alternative death pathway. In the present report we investigated on the possible implication of the Fas pathway.

Fas (Apo-1/CD95) is a member of the tumour necrosis factor (TNF) receptor superfamily activated by Fas-ligand (FasL) or certain agonist anti-Fas antibodies (Nagata, 1994; Nagata and Golstein, 1995) that result in the activation of an apoptotic process in sensitive cells. The Fas system is one of the death-pathway activated in tumour cells by cytotoxic T lymphocytes cells in the human body. The signals of Fas ligand (FasL) or agonist antibodies are transduced by intracellular death domains that interact with adapter molecules which are conserved among the TNF receptors superfamily (TRAIL-DR4/DR5, TNFR1). The adapter molecule Fas-associated death domain (FADD) binds directly to the death domains of TNF receptors superfamily to transduce the apoptotic signal leading to the activation of caspase-8 and subsequently to

the downstream activation of effector caspases such as caspase-3. Activation of Fas pathway may also induce a mitochondria-dependent apoptotic pathway and depending on the cell type, either one or both signaling pathways may be activated (Krammer, 2000; Siegel et al., 2000).

Most cancer cells, unlike normal cells, are relatively resistant to FasL-mediated apoptosis allowing immune escape and cell growth of a selective population of malignant cells that consequently may facilitate metastatic spreading (Bergmann-Leitner and Abrams, 2000; Houston et al., 2003; O'Connell et al., 1997; 1999). The loss of sensitivity to Fas-mediated apoptosis may play an important role in the progression of malignancy. This is supported by the observation that tumour cells that display resistance to chemotherapeutic agents, may concomitantly exhibit resistance to functional Fas expression (Bhushan et al., 1998; Fulda et al., 1998; Landwoski et al., 1997). In the present report we investigated the effects of apple Pcy on Fas receptor expression and function in human colon adenocarcinoma SW480 cells and in their FasL-resistant derived metastatic SW620 cells.

## 2. Materials and Methods

*2.1. Isolation and characterization of apple procyanidins.* Polyphenols were purified from a cider apple (*Malus domestica*, variety Antoinette). Apples were reduced into a homogeneous powder which was extracted by water:ethanol:acetic acid (975:1000:25). After filtration, evaporation under vacuum and freeze drying, the crude extract was dissolved in 2.5% acetic acid and separated by preparative HPLC (Lichrospher RP 18, 12 µm, Merck, Darmstadt, Germany) to remove sugars and other non-phenolic polar compounds. Polyphenols were eluted with acetonitrile:water:acetic acid (300:700:25). Fractions containing polyphenols were evaporated and freeze-dried. The polyphenols were fractionated on a Fractogel column by a method adapted from Souquet et al. (2000). Pcy were characterized and quantified by thiolysis coupled with reverse-phase HPLC (Guyot et al., 2001). On a weight basis, the Pcy-fraction contained 78% Pcy, consisting of 95% (-)-epicatechin and 4% (+)-catechin. The mean degree of polymerization was close to seven. The Pcy fraction was almost totally devoid of monomeric flavonoids and other phenols (<2%). Pcy was diluted in dimethylsulfoxide (DMSO) and used at 80 µg/ml final concentration.

***2.2. Cell culture and treatments.*** SW480 and SW620 cells were obtained from the European Collection of Animal Cell Culture (ECACC, Salisbury, UK). They were cultured in 75 $cm^2$ Falcon flasks in Dulbecco's modified Eagle's medium containing 25 mM glucose and 2 mM L-glutamine, 10% heat-inactivated (56°C) horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% non-essential amino acids (Invitrogen Corp., Cergy Pontoise, France). Incubations were carried out at 37°C in a humidified atmosphere with 5% $CO_2$. The culture medium was replaced every 48 h. For all experiments, horse serum was reduced to 3%, and the medium was supplemented with 10 µg/ml insulin, 5 µg/ml transferring and 5 ng/ml selenium and (ITS-defined medium; Gibco, Invitrogen, Cergy-Pontoise, France). Cells were exposed to different compounds 24 h after seeding and incubated for 48 h. DMSO final concentration in culture medium was 0.1% for control and treated cells. Stock solution of human Fas-activating monoclonal antibody (clone CH-11; MBL, Nagoya, Japan) was tested at 50 ng/ml. The human Fas-blocking antibody (clone ZB4; MBL, Nagoya, Japan) was added at 1 µg/ml 1 h before treatment with Pcy. For experiments with DR4/DR5 blocking antibodies, cells were pre-treated with human blocking anti-DR4 and anti-DR5 (250 ng/ml) (Alexis Biochemicals, Switzerland) for 24 h before Pcy treatment.

***2.3. Detection of cell surface expression of Fas receptor (CD95).*** Cells were treated with Pcy (80 µg/ml) for 48h and harvested by trypsinization. Cells pellets were washed with PBS and incubated for 30 min at 4°C in darkness with FITC-conjugated mouse anti-human-CD95 (IgG1κ, clone DX2, 1:50) (BD Pharmingen, San José CA, USA) or FITC-conjugated mouse IgG1 monoclonal isotype control antibody (BD Bioscence, Belgium) for 30 min at 4°C in the dark. After washing with PBS, cells were re-suspended in PBS, and the fluorescence of 10,000 events per sample was analysed with a FACScan flow cytometer and CellQuest Software (BD Biosciences, Belgium).

***2.4. Total RNA extraction and RT-PCR detection of Fas (CD95) mRNA transcripts.*** Total RNA was extracted using an RNeasy Mini kit (QIAGEN, VWR, Denmark) following manufacturer's instructions. RNA was reversed transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City CA, USA). TaqMan Gene Expression assays were used to measure transcription levels of the selected genes

(Fas receptor, Hs00236330 m1; Applied Biosystems). Beta actin was applied as an endogenous control (cat no Hs99999903 m1; Applied Biosystems). Real time RT-PCR was performed by using TaqMan Universal PCR master mix (Applied Biosystems) and ABI Prism 7500 Sequence Detection System (Applied Biosystems Sequence detector) in triplicate wells. Data were analyzed by a comparative threshold cycle ($C_T$) method and statistical analysis was performed as described by Livak and Schmittgen (2001).

***2.5. Cell death analysis by flow cytometry.*** SW480 and SW620 cells were seeded in culture dishes and harvested by trypsinization at 24 and 48 h after treatment with DMSO 0.1% (control), Pcy (80 μg/ml), anti-Fas agonist CH-11, blocking antibodies: anti-Fas ZB4, anti-DR4, anti-DR5. Cells were centrifuged and fixed in 1 ml methanol:PBS (9:1, v/v) at -20°C for at least 30 min, washed twice in phosphate buffer saline (PBS) and resuspended in 200 μl PBS containing 0.25 mg/ml RNAse A and 0.1 mg/ml propidium iodide (PI) (Sigma-Aldrich, Munich, Germany), incubated in the dark at 37°C for 30 min. The fluorescence of 10.000 cells was analyzed by flow cytometry and CellQuest software (FACScan, BD Biosciences, Erembodegem, Belgium).

***2.6. Determination of apoptosis by Terminal deoxynucleotide transferase (TdT) dUTP-biotin nick-end labeling (TUNEL) assay.*** Cell DNA fragmentation was determined according to Gavrieli et al. (1992). For the TUNEL assay, the Mebstain apoptosis kit (MBL, Nagoya, Japan) was used following the manufacturer's instructions. Briefly, cells were washed with PBS (2% BSA) two times and fixed with 4% paraformaldehyde for 30min at 4°C. Cells were permeabilized by adding 200 μl of 0.5% Tween-20 with 0.2% BSA to the cell pellet. The TdT reaction was carried out in the presence of FITC-dUTP reagent. For positive control, $H_2O_2$ (1.05 %, v/v) was added to cell culture medium 30 min before trypsinization. Data from 10,000 events per sample were collected and analyzed using a FACScan flow cytometer (FACScan, BD Biosciences, Erembodegem, Belgium).

***2.7. Mitochondrial membrane permeability changes.*** Changes in mitochondrial membrane permeability were assessed by using the MitoProbe™ $DiOC_2(3)$ assay kit (Invitrogen Corp., France) as described previously (Maldonado-Celis et al., 2008). Cells

were cultured with Pcy and harvested by trypsinization at 48 h. After trypsinization, cells were stained with $DiOC_2(3)$ or for positive control with a mitochondrial membrane-potential disrupter, the carbonyl cyanide 3-chlorophenylhydrazone (CCCP) at 50 µM at 37°C for 30 min in darkness. Cells were washed and re-suspended in PBS for analyze of 10,000 events by flow cytometry with excitation at 488 nm and green (FL-1: 515 nm) or red (FL-3, (600 nm)) emissions filters according to manufacturer's instructions. This method allows to quantify cells with depolarized mitochondrial membrane by flow cytometry analysis using CellQuest Software (FACScan, BD Biosciences, Belgium).

***2.8. Statistical analysis.*** All data were presented as mean ± standard error (SE) from three independent experiments. Significant differences between control and treated groups were evaluated by one-way ANOVA analysis. Student's t test or Tukey's multiple comparisons post-test was used to determine statistical differences between the data. Statistical analysis were performed using GraphPad Prism version 4.0, 2003 (GraphPad Software, San Diego CA, USA).

## 3. Results

### *3.1. Effect of Pcy on cell surface and mRNA expression of Fas receptor in SW480 and SW620 cells*

We measured the effect of Pcy on the cell surface expression of Fas receptor by flow cytometry using a specific antibody, by indicating the percentage of positive cells expressing Fas (Fig. 1A) as well as by measuring the mean fluorescence intensity (MFI), a numerical data used as an indicator of antigen expression level per cell (Fig. 1B). As shown in Fig. 1A and 1B, Fas receptor was already expressed at the surface of untreated SW480 cells, and Pcy-treatment caused an increase by 3 fold of Fas receptor expression. On the contrary, under basal conditions, only a very low percent of SW620 cells expressed Fas receptor at their surface (3%). After Pcy-treatment, a huge increase (+ 65%) in the amount of SW620 cells expressing Fas receptor was observed, this corresponded to a 13 fold increased expression of Fas receptor per cell when compared with untreated controls (Fig. 1B).

Pcy-treatment of SW620 cells caused a 22 fold (after 24 h) and 35 fold (after 48 h) increase of Fas receptor transcripts as measured by real time RT-PCR when compared to untreated cells (Fig. 1C). In contrast, Pcy-treatment did not change the level of Fas gene expression in SW480 cells. These data indicate that Pcy up-regulated Fas receptor at a transcriptional level in SW620 cells whereas in SW480 cell Pcy regulated Fas receptor expression at a post-transcriptional level.

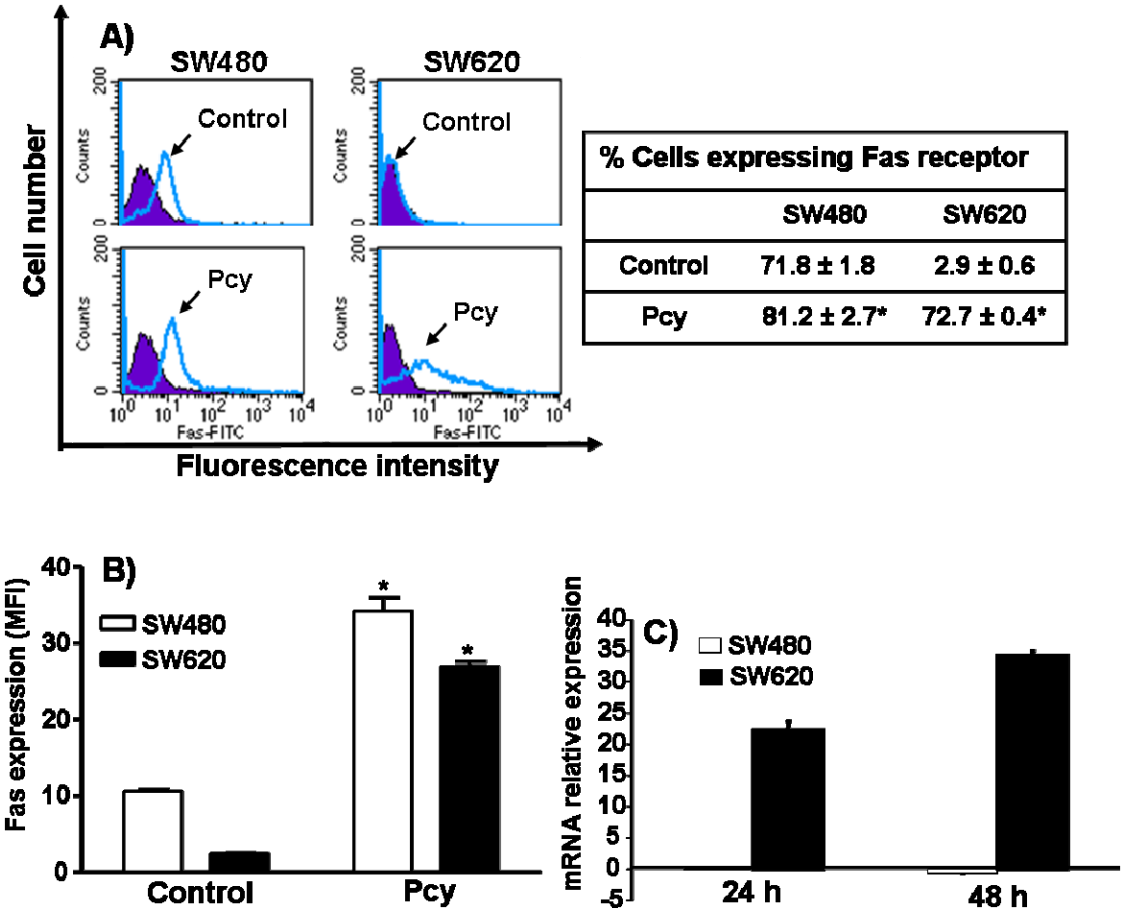



***Figure 1. Effects of Pcy on Fas expression in SW480 and SW620 cells****. (A) Detection of Fas receptor at the cell surface analyzed by flow cytometry in cells exposed to 0.1% DMSO (controls) and to Pcy (80 µg/ml) for 48h. Cells were incubated with FITC-conjugated anti-human CD95. Fluorescence shift to the right is indicative of an increase of cells expressing Fas receptor at their cell surface. Cells stained with isotype control are represented by filled curve and Fas receptor positive cells are represented by open curve (representative experiment). The percentage of cells expressing Fas receptor is given in the table. Data are the mean value ± SE of at least three independent experiments. For each cell line, Pcy treated versus control: *P < 0.01. B) Mean fluorescence intensity (MFI) of Fas receptor cell surface expression analyzed by flow cytometry with anti-CD95-FITC after 48h of Pcy treatment. Data are the mean value ± SE of at least three independent experiments. For each cell line, Pcy treated versus control: *P < 0.01. C) Fas mRNA expression levels analyzed by real time RT-PCR after 24 h and 48 h treatment with Pcy (80 µg/ml). Total RNA was reversed transcribed and analyzed as described in the Materials and methods section. Histogram represents the fold increased mRNA expression over non-treated cells. Data are presented as mean ± SE of three separate experiments.*

### *3.2. Effect of combined treatment with Pcy and agonist anti-Fas CH-11 on cell death*

SW480 cells are known to be FasL-sensitive while their derived metastatic SW620 cells are FasL-resistant (Bergmann-Leitner and Abrams, 2000). As shown in Fig. 2A, Pcy in combination with the Fas agonist CH-11 increased by two fold the amount of hypodiploid SW480 cells (Sub G0/G1 region) when compared to cells treated with Pcy used as a single drug. In contrast, in SW620 cells Pcy in combination with CH-11 did not affect the percentage of dead or dying cells. We used the TUNEL method (Gavrieli et al., 1992) in order to detect single DNA strand breaks and to confirm the apoptotic effects caused by Pcy single or combined with CH-11 (Fig. 2B). Cells with DNA strand breaks were detected in both cell lines after treatment with Pcy single or combined to CH-11. The Fas receptor agonist (CH-11) when used as a single drug caused DNA damage in SW480 cells but not in the metastatic SW620 cells.

During Fas-mediated apoptosis, two different signaling pathways have been identified depending on cancer cell type (Bergmann-Leitner and Abrams, 2000). One of them, is the mitochondrial pathway induced after binding of FasL to its receptor, resulting in the cleavage of Bid protein, the loss of mitochondrial membrane potential ($\Delta\Psi m$), favouring the release of cytochrome c and the activation of caspases-9, and -3. We examined whether Pcy and/or CH-11 had an effect on mitochondrial membrane using flow cytometry after staining cells with $DiOC_2(3)$. This cyanide dye accumulates in the mitochondrial matrix and is released in the cytosol after membrane depolarization (membrane with reduced $\Delta\Psi m$) (Maldonado-Celis et al., 2008). As shown in Fig. 2C, the percentage of SW480 cells with depolarized mitochondrial membrane induced by Pcy single (21%) or CH-11 (21%) was significantly ($p < 0.05$) increased when Pcy was combined with CH-11 (35%). At the opposite, in SW620 cells Pcy induced an increased percentage of cells with loss of $\Delta\Psi m$ (46%), however this effect was not modified after the combined treatment with CH-11 (43%). Taken together these results indicate that Pcy enhanced the response of SW480 cells to Fas-mediated apoptosis, whereas in SW620 cells Pcy did not overcome the cell-resistance to CH-11 (or by extension to FasL).

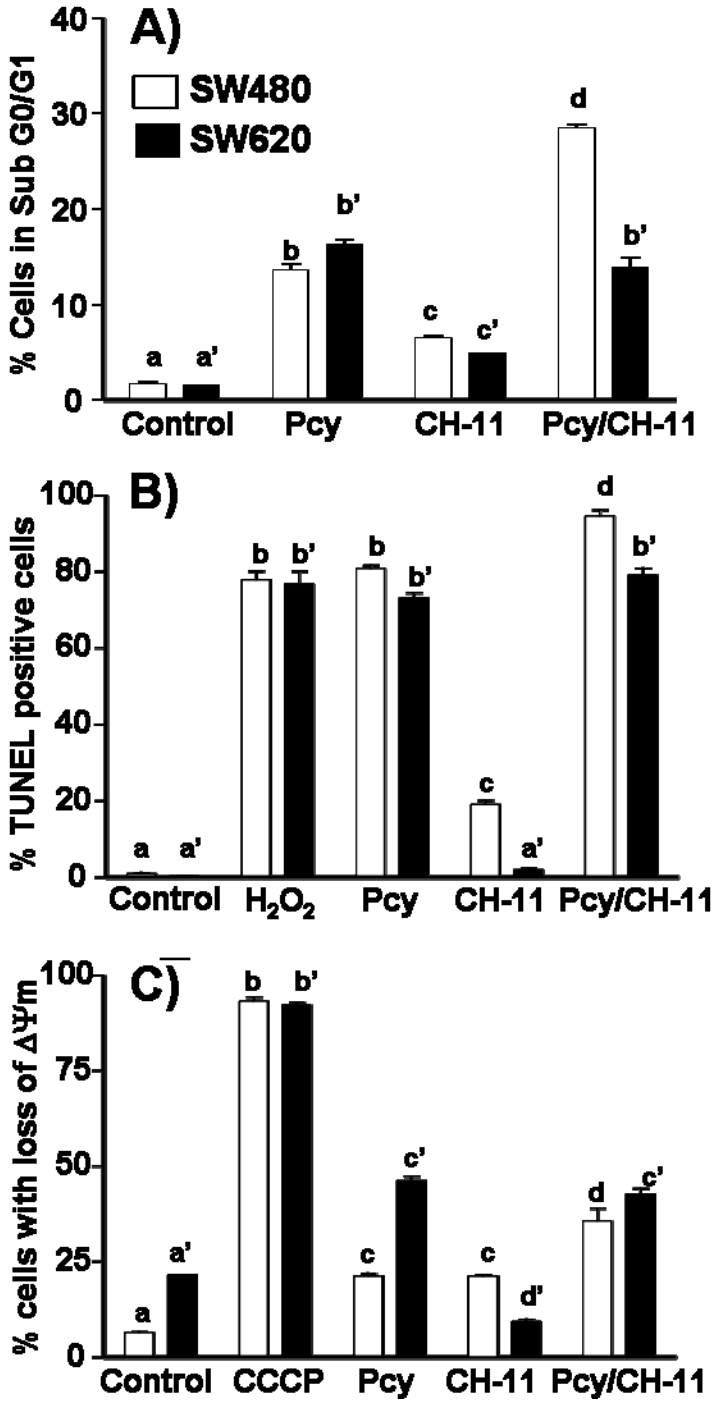



***Figure 2. Effects of Pcy and anti-Fas agonist CH-11 combination on cell death.*** *Cells were treated with DMSO 0.1% (control), Pcy (80 µg/ml), anti-Fas CH-11 (50 ng/ml), or Pcy + anti-Fas CH-11 for 48 h. A) The percentages of hypodiploid cells present in the Sub G0/G1 region after 48 h of treatments were analyzed by PI staining as described in the Materials and methods section and are presented as histograms. Data are mean percentage ± SE of cells in the subG0/G1 region of at least three independent experiments. For each cell line, columns not sharing the same superscript letter differ significantly: a≠b≠c≠d and a'≠b'≠c', P<0.05. B) The TUNEL assay was used to determine the % of cells with nuclear DNA single strand breaks in SW480 and SW620 cells. The strand breaks were detected by flow cytometry with the TdT-reaction in presence of dUTP-FITC. As a positive control hydrogen peroxide ($H_2O_2$) treated cells were included as described in Materials and methods section. Columns represent the percentage of cells exhibiting nuclear DNA strand breaks corresponding to the TUNEL*

*positive cells. Data are the mean value ± SE of at least three independent experiments. For each cell line columns not sharing the same superscript letter differ significantly: a≠b≠c≠d and a'≠b', P < 0.05. C) Effect on mitochondrial membrane potential (Δψm). Cells were harvested and stained with $Di0C_2(3)$ and CCCP was used as a positive control as described in Materials and methods section. Columns represent the percentage of cells with reduced green fluorescence corresponding to the percent of cells with reduced Δψm. Data are the mean value ± SE of at the least three independent experiments. For each cell line, columns not sharing the same superscript letter differ significantly: a≠b≠c≠d and a'≠b'≠c'≠d', P<0.05.*

### *3.3. Effects of Fas receptor-blocking antibody ZB4*

To investigate the correlation between Fas receptor expression and apoptosis, cells were pre-treated with blocking anti-Fas ZB4 for 1 h before Pcy and/or agonist anti-Fas CH-11 treatments. In SW480 cells, blocking Fas receptor caused a significant ($p < 0.05$) reduction in the amount of apoptotic cells after 48 h treatment with Pcy (from 14% to 6%) and Pcy/CH-11 (from 28% to 7%). At the opposite, in SW620 cells, the amount of apoptotic cells was not modified by the blocking antibody (Fig. 3A). These data indicate that in SW480 cells Pcy-triggered death may occur via an activation of Fas receptor, but this effect was not observed for SW620 cells.

### *3.4. Activation of Fas apoptotic pathway after blocking TRAIL death receptors*

In SW480 cells, we showed previously that Pcy-enhanced cell sensitivity to TRAIL-induced apoptosis was associated to an up-regulated expression of DR4/DR5 receptors (Maldonado-Celis et al., 2009). These findings led us to hypothesize that Pcy might activate simultaneously TRAIL-DR4/DR5 and Fas receptor-mediated apoptosis. Fas- and TRAIL-death receptors were blocked simultaneously with specific blocking anti-Fas (ZB-4) and anti-DR4/-DR5 antibodies. As shown in Fig. 3B, the specific inactivation of DR4/DR5 receptors caused a 50% reduction in the amount of hypodiploid cells observed after Pcy treatment (Fig. 3A). When SW480 cells were exposed to the combination of blocking antibodies a further significant reduction ($p < 0.01$) in the percentage of apoptotic cells was observed.

In Pcy-treated SW620 cells, we reported previously that the amount of apoptotic cells was significantly enhanced after the blocking of TRAIL-death receptors DR4 and DR5 (Maldonado-Celis et al., 2008). These receptors as well as Fas are similar in that FADD is recruited directly for activation of the apoptotic cascade (Hewitt et al., 2000). Thus, these results led us to consider that an alternative apoptotic pathway triggered by

Pcy in SW620 cells after the blocking of TRAIL pathway might involve the activation of the Fas receptor-pathway. To test this hypothesis we exposed Pcy-treated SW620 cells to blocking antibodies against Fas (ZB4) and DR4/DR5. As shown in Figure 3B, ZB4 counteracted the pro-apototic effects observed after the inactivation of DR4/DR5 receptors reducing by 80% the amount of apoptotic cells. These data showed that in SW620 cells, Pcy was able to activate Fas-receptor after a blocking of TRAIL-death receptors, explaining therefore the enhanced apoptosis observed when SW620 cells were exposed to the DR4/DR5 blocking antibody.

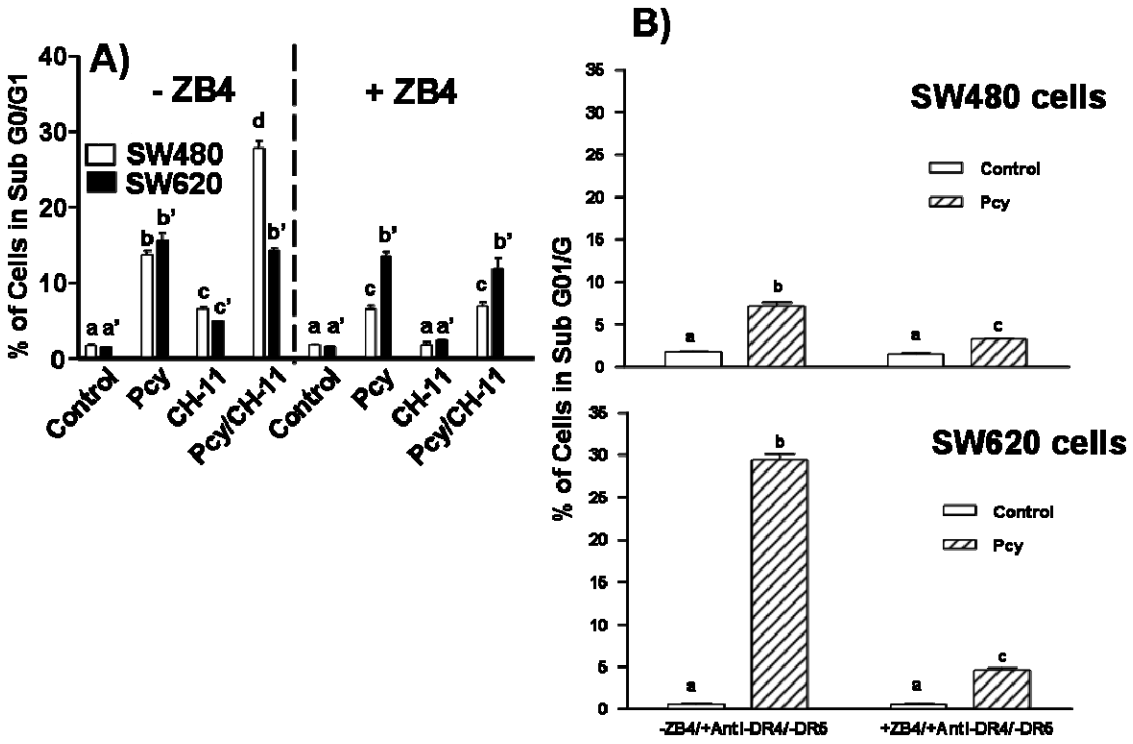



***Figure 3*. Percentage of hypodiploid SW480 and SW620 cells.** *Induction of hypodiploid cells was detected by flow cytometry as described in the Material and methods section. Cells were treated with DMSO 0.1% (control), Pcy (80 µg/ml), anti-Fas CH-11 (50 ng/ml), or Pcy + anti-Fas CH-11 for 48 h. A) Analysis of hypodiploid cells in presence of blocking Anti-Fas ZB4 (1µg/ml). Cells were pre-treated 1h before addition of Pcy (80 µg/ml) for 48 h. B) Analysis of hypodiploid cells in presence of blocking Anti-Fas ZB4 (1µg/ml) and/or Anti-DR4/-DR5 (250 ng/ml). Blocking antibodies were added simultaneously in the culture medium 24 h before Pcy (80 µg/ml) treatment. Histograms represent the percentage of hypodiplold cells of 10.000 cells. Data are the mean ± SE of at least three separate experiments and for each cell line, columns not sharing the same superscript differ significantly ($P < 0.05$).*

## 4. Discussion

We investigated the involvement of Fas receptor-pathway in the Pcy-induced apoptotic response of human colon adenocarcinoma SW480 cells and their derived metastatic SW620 cells. In the present report, we showed that these cell lines responded differently to Pcy-induced apoptosis. Pcy caused a post-transcriptional activation of Fas

receptor-mediated apoptosis in SW480 cells. In contrast, the metastatic SW620 cells exhibited a Fas-resistant phenotype as described previously (Bergmann-Leitner and Abrams, 2000; Hewitt et al., 2000; Huerta et al., 2007) that could not be circumvented by Pcy treatment despite the up-regulation of Fas receptor gene expression. Surprisingly, activation of the Fas receptor-mediated apoptotic pathway by Pcy was observed in SW620 cells only after a blocking of TRAIL-DR4/DR5 receptor functions. This result suggests that Fas-resistant phenotype may be associated with alterations in the downstream events between DR4/DR5 and Fas receptors, a subject that deserves further investigations.

We observed that untreated SW480 cells expressed Fas receptor at a higher level than for SW620 cells in which the expression of Fas receptor was only marginal, in accordance with previous reports (Huerta et al., 2007; Lamy et al., 2007; O'Connell et al., 2000). In metastatic SW620 cells, Pcy caused the up-regulation of Fas gene transcripts concomitantly with a huge expression of receptor at the cell surface. In SW480 cells, Pcy up-regulated Fas receptor expression at a post-transcriptional level with an increased (+10%) percentage of cells expressing the receptor at their surface. This suggest that Pcy may favour the delivery of Fas receptor to the cell membrane. Fas receptor is a glycoprotein that requires N-glycans post-transcriptional modifications for an efficient expression at the cell membrane surface and sensitivity to FasL-signaling which has been confirmed by inhibiting glycosyltranferases leading to an intracellular accumulation of Fas- receptors (Li et al., 2007). Thus, it would be of interest to determine whether Fas glycosilation is involved in the Pcy up-regulated expression of Fas-receptor at the cellular surface.

The apoptotic signaling pathway activated by Fas receptor leading ultimately to caspase-3 activation is mediated through two main pathways: the DISC/caspase-8, and the mitochondria/cytochrome C/Apaf-1/caspase-9 pathways (Russo et al., 1999). The activation of the effector caspase-3, results in the extensive degradation of chromosomal DNA into oligomers of about 180 bp (Gavrieli et al., 1992). Mitochondria play an important role in cell death signaling (Kroemer, 1999), alterations in mitochondrial structure and function occur in early stages of apoptosis. Further investigations on Fas receptor function in SW480 and SW620 cells were assessed by combining Pcy with the agonist anti-Fas CH-11 reproducing the activation by FasL. Under these conditions enhanced apoptosis of SW480 cells was observed as evidenced by the increased amount of hypodiploid cells, the loss of mitochondrial membrane potential, and the increased

DNA fragmentation. Our data showed that in SW480 cells, the Fas receptor-mediated apoptotic pathway is activated by Pcy. Mitochondrial alterations may also be a downstream consequence of the activation of the extrinsic apoptotic pathway mediated by Fas receptor, which may occur simultaneously with an activation of TRAIL-DR4/DR5-mediated apoptosis (Maldonado-Celis et al., 2009). Simultaneous activation of TRAIL-death- and Fas-receptors mediated apoptosis in colon cancer cells may be considered as an important strategy for colon cancer chemoprevention. Indeed, Fas receptor-mediated apoptosis in tumour cells might contribute to the cytotoxic effect of $CD8^+$ T and natural Killer (NK) cells by favouring FasL liberation from these cells leading to the elimination of the tumour cells (O'Connell et al., 1999). These results raise the possibility that a combined treatment with Pcy and FasL or agonist antibodies of Fas receptor might represent a promising approach against tumour growth.

On the other hand, SW620 cells were resistant to Fas-receptor mediated apoptosis when treated by Pcy in presence of Fas receptor agonist (CH-11), which did not enhance the apoptotic response of Pcy-treated SW620 cells despite the up-regulated expression of Fas-receptor. Although Pcy did not overcome the resistance of SW620 cells to FasL, Pcy may activate alternative apoptotic pathways. We have previously shown that Pcy initiated in SW620 cells, a cross-talk between the TRAIL (extrinsic) apoptotic pathway and the mitochondrial (intrinsic) apoptotic pathway involving enhanced expression of TRAIL-DR4/DR5 receptors in SW480 and SW620 cells, and activated the polyamine catabolism leading to ROS production which participated to mitochondria disruption (Maldonado-Celis et al., 2008, 2009).

In the present study we observed that the simultaneous inactivation of Fas and TRAIL-DR4/-DR5 receptors inhibited Pcy-induced apoptosis in SW620 cells, this suggested that Fas resistance in the presence of Pcy may occur at two levels: i) TRAIL-death receptors competes with Fas receptor for binding endogenous FADD when these receptors are activated by the combined treatments Pcy + TRAIL or Pcy + CH-11 respectively. Since the regions of FADD interacting with Fas receptor and DR4/DR5 are very similar (Thomas et al., 2004; 2006), the number of Fas receptors relative to DR4/DR5 receptors might not be sufficient to induce apoptosis; ii) DR4/DR5 and Fas receptors have different C-terminal tails. The corresponding region for DR4 and DR5 positively regulates FADD binding, caspase activation and apoptosis whereas the C-terminal tail of Fas receptor has the opposite effect and inhibits the binding of FADD to the receptor death domain (Thomas et al., 2004). We may hypothesize that the C-

terminal tail of DR4 and DR5 receptors located outside the death domain could represent additional regulatory sites for the activation of Fas receptor in order to overcome an inactivation of DR4/DR5 receptors (Thomas et al., 2004). However, at present there is no evidence showing such a direct interaction between Fas and DR4/DR5 receptors.

In the present report, we demonstrate the ability of Pcy to activate Fas receptor-mediated apoptotic pathway in the SW480 cells (Fig. 4). This supports the view that Pcy may help cancer cells to recover Fas sensitivity during colon carcinogenesis contributing to the elimination of tumour cells by FasL liberated from immune cells. In contrast, the inability of Pcy to sensitize SW620 cells to Fas-mediated apoptosis despite the Pcy-triggered up-regulation of Fas receptor expression suggested a loss of function for Fas that may play an important role in the progression toward malignancy. Indeed, it was shown that tumour cells showing resistance to chemotherapeutic agents may concomitantly exhibit resistance to functional Fas receptor expression (Bhushan et al., 1998; Fulda et al., 1998; Landowski et al., 1997). Our data suggested that Pcy was able to initiate a cross-talk between death receptors, since a specific blocking of TRAIL death receptors favoured the activation of the Fas receptor-mediated apoptosis in metastatic SW620 cells (Fig. 4). These important data may allow the emergence of new therapeutic protocols targeting death receptors in metastatic cells. Further investigations have to be extended to other cancer cells in order to understand and identify the mechanisms by which Pcy might be able to increase cell sensitivity and bypass the resistance to Fas-induced apoptosis.

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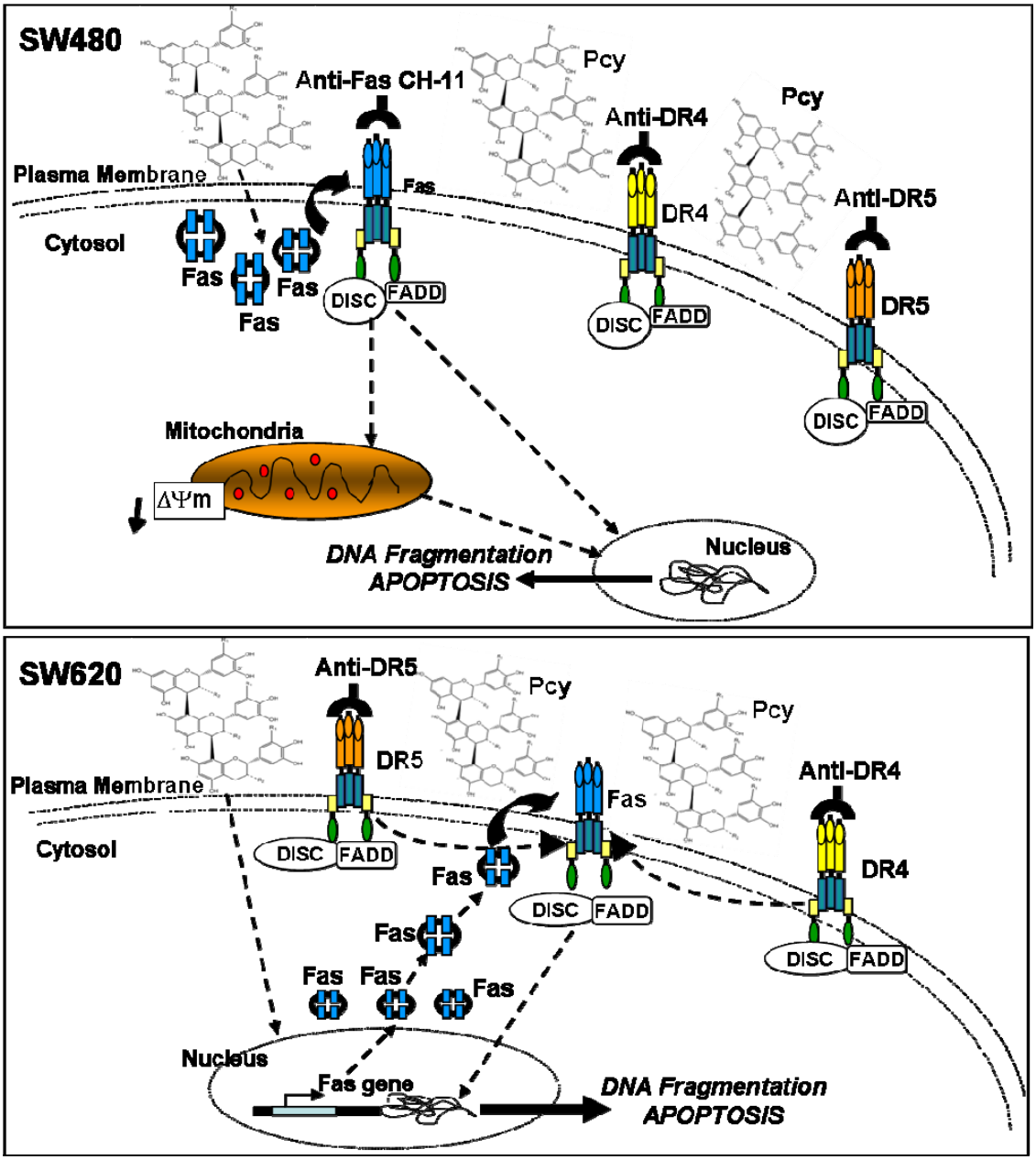


***Figure 4. Scheme of the Fas-receptor mediated apoptotic pathways activated by Pcy in SW480 and SW620 cells****. (A) In SW480 cells, Pcy caused activation of Fas-receptor mediated apoptosis through a post-transcriptional activation of Fas-receptor leading to mitochondrial perturbation and DNA fragmentation. TRAIL-DR4/-DR5 death receptors mediated apoptosis was triggered by Pcy independently of Fas-receptor activation. (B) In Fas-resistant SW620 cells, Pcy caused a cross-talk between death receptors favouring Fas-receptor mediated apoptosis only when TRAIL DR5/DR5 receptors were blocked.*

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## 2. Conclusions

In the present study we reported comparative effects of Pcy on the Fas-receptor expression and function in human colon adenocarcinoma SW480 cells and in their FasL-resistant derived metastatic SW620 cells. We showed that Pcy up-regulated Fas receptor at transcriptional level in SW620 cells whereas in SW480 cell Pcy regulated Fas receptor at post-transcriptional level. However, the Fas-receptor mediated apoptosis was activated in SW480 cells, whereas the metastatic SW620 cells exhibited a Fas-resistant phenotype as described previously (Bergmann-Leitner and Abrams, 2000; Huerta et al, 2007; Hewitt et al, 2000). Although Pcy did not overcome the resistance of SW620 cells to the Fas-receptor agonist anti-CH11, Pcy may activate alternative apoptotic pathways as described before. In addition, we observed that the simultaneous inactivation of Fas and TRAIL-DR4/-DR5 receptors inhibited Pcy-induced apoptosis in SW620 cells as well as in SW480 cells. Results obtained in SW620 cells suggest that Pcy might initiate a cross-talk between Fas and TRAIL death receptors, whereas in SW480 this result indicate that the extrinsic Fas- and TRAIL-mediated apoptosis are the main apoptotic pathways triggered by Pcy in these cells.

These differences may be of interest for the development of new therapeutic strategies targeting death receptors and sensitizing colon cancer cells to FasL produced by cytotoxic immune cells during colon carcinogenesis. However, further investigations are required to understand and identify the mechanisms by which Pcy is able to increase cell sensitivity in colon cancer cells and bypass the resistance to Fas-induced apoptosis in metastatic cells. One strategy could be the study of the effects of Pcy on the transcriptional factors NF-κB and p53, since is known they have consensus sequences in promoters of TRAIL-DR4/-DR5 and Fas receptors (Halaby et al, 2007; Henson et al, 2003; Muller et al, 1998; Wu et al, 1997; Yoshida et al, 2001;). This study is presented in the next chapter.

**CHAPTER III**

# EFFECT OF APPLE PROCYANIDINS ON CELL MEMBRANE OF HUMAN COLON CANCER SW480 CELLS AND THEIR DERIVED-METASTATIC SW620 CELLS

## I. Apple procyanidins activate apoptotic signaling pathway in human colon adenocarcinoma cells by a lipid raft independent mechanism (publication)

**Apple procyanidins activate apoptotic signaling pathway in human colon adenocarcinoma cells by a lipid-raft independent mechanism**

Maria E. MALDONADO-CELIS, Souad BOUSSEROUEL, Francine GOSSE, Annelise LOBSTEIN, Francis RAUL

**Biochemical and Biophysical Research Communications, 2009, vol. 388, n° 2, pages 372-376**

# 3. Results: PUBLICATION

**Apple procyanidins activate apoptotic signaling pathway in human colon adenocarcinoma cells by a lipid raft independent mechanism**

*Maldonado-Celis M-E, Bousserouel S, Gossé F, Lobstein A, Raul F*

***Biochemical and Biophysical Research Communications 2009; 388: 372-376.***

# 4. Complementay results

## 4.1 Effect of cell membrane cholesterol depletion on Pcy-triggered cell death

As it was shown earlier in this study, Pcy treatment of SW620 cells induced significant changes in the expression of TRAIL-DR4 and –DR5 receptors at the cell surface associated to an enhanced sensitivity to TRAIL in these cells. Thus, we aimed to known whether Pcy induce the lipid raft localization of TRAIL-death receptors for their activation. By using the cholesterol-binding compound nystatin in SW620 cells treated with Pcy single, or combined with TRAIL, it was observed in figure 34 that nystatin potentiated the cytotoxic effect of Pcy used as single drug by 2-fold in SW620 cells. Moreover, nystatin increased the effect pro-apoptotic of TRAIL in presence of TRAIL. These findings suggest that rafts-membranes enriched of cholesterol may be not involved in the activation of TRAIL-mediated apoptosis by Pcy.

## 4.2 Effect of nystatin on DR4/DR5 expression in SW620 cells

To know whether the enhanced Pcy-induced apoptosis by nystatin was associated to an increased number of DR4/DR5 receptor present at the cell surface, we evaluated the effect of nystatin on the cell surface expression of DR4/DR5 receptors in Pcy-treated cells by measuring the MFI by flow cytometry using specific antibodies against DR4/DR5 receptors. As shown in Figure 35A, Pcy increased significantly the number of DR4 and DR5 receptors present at the cell surface when compared with untreated control cells. In the presence of nystatin no significant changes for DR5 expression, but for DR4 were observed in Pcy-treated cells. As shown in Figure 35B, the number of DR5 transcripts measured by real time RT-PCR analysis was not modified by nystatin in Pcy-treated cells, but for DR4.

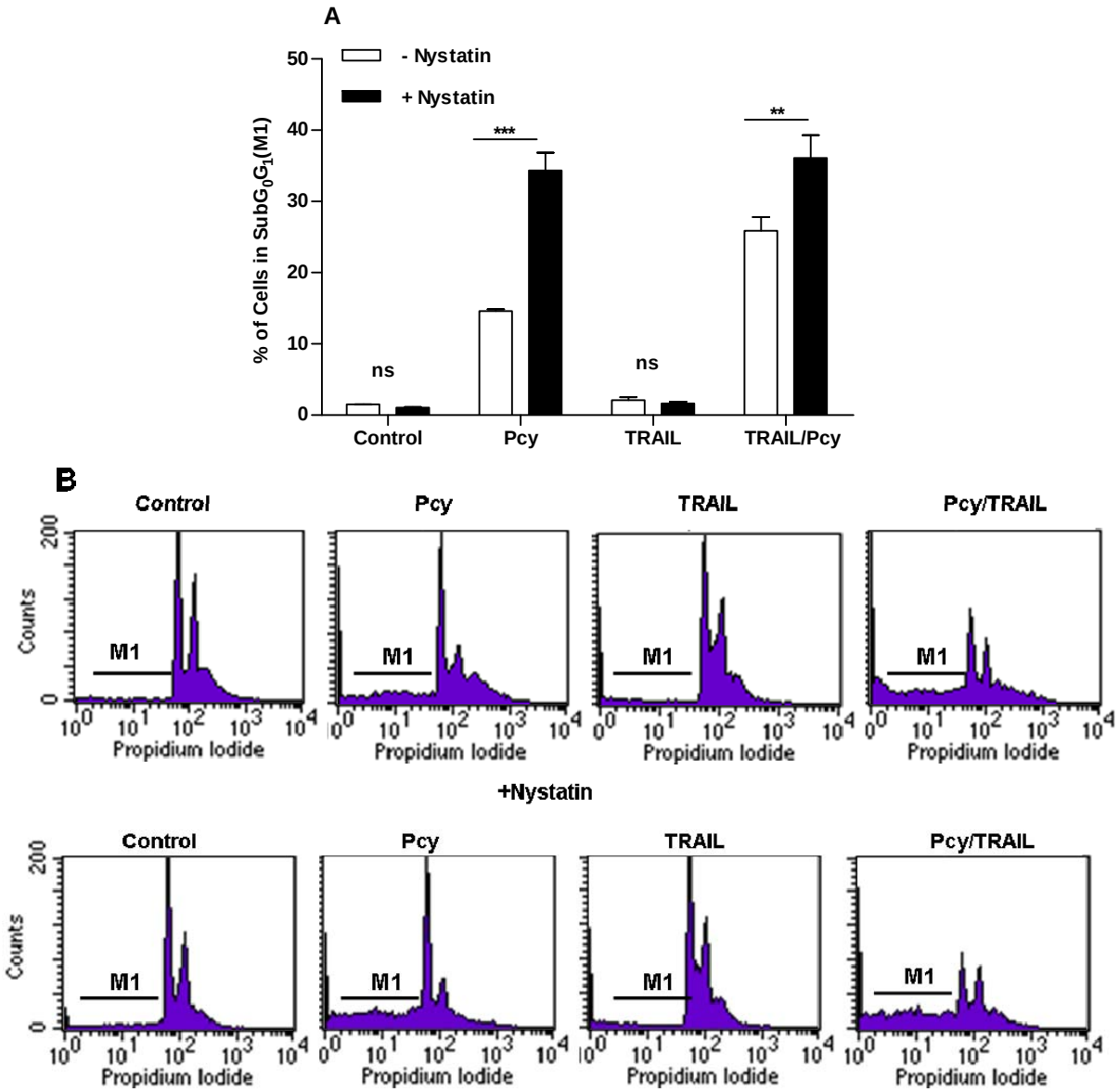


***Figure 34. Analysis of hypodiploid SW620 cells in presence of nystatin****. Cells were pre-treated with 50 µg/ml Nystatin before addition of DMSO 0.1% (control), 80 µg/ml Pcy and/or 30 ng/ml TRAIL for 48 h. (A) Analysis of hypodiploid cell population corresponding to the Sub G0/G1 region was performed by flow cytometry using the PI staining. Data are presented as the mean ± SE of three independent experiments. Columns represent the percentage of hypodiploid cells. Comparison between groups was done by two-way ANOVA. ***P < 0.001; ns: not significant. (B) Representative histograms of at least three independent experiments indicanting the hypodiploid cell population (M1) analyzed by flow cytometry using PI staining.*

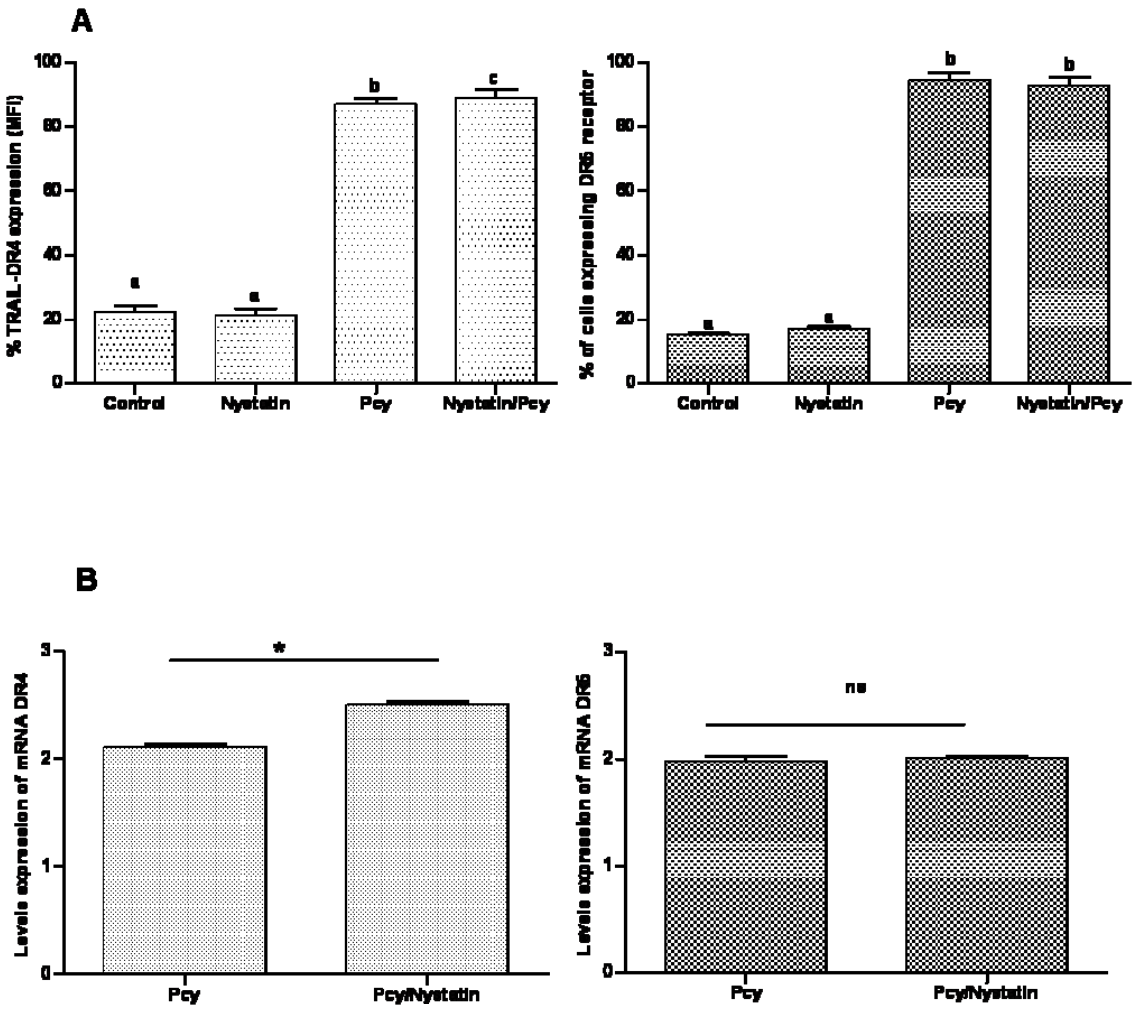


***Figure 35. Effects of Nystatin on the expression of DR4/DR5 in Pcy-treated SW620 cells.*** *(A) Mean intensity fluorescence (MFI) of DR4/DR5 cell surface expression. Control cells were exposed to 0.1% DMSO, Pcy (80 μg/ml) and nystatin (50 μg/ml) for 48 h. Cells were incubated with anti-DR4-FITC/anti-DR5-FITC and analysed by flow cytometry. Comparison between groups was done by one-way ANOVA. For each cell receptor, Columns not sharing the same superscript letter differ significantly: a≠b≠c, P < 0.05. (B) mRNA protein expression levels of DR4/DR5 analyzed by RT-PCR after 48 h treatments with Pcy (80 μg/ml) single or combined with nystatin (50 μg/ml). Total RNA was reversed, transcribed and analyzed as described in materials and methods section. Histograms represent the fold increase expression over the non-treated cells. Data are presented as mean ± SE of three separate Data are presented as mean ± SE of three separate experiments. P< 0.001; calculated by the comparative cycle threshold method. Comparison between Pcy- and Pcy/Nystatin-treated cells was done by two-tailed paired t-test. *P<0.05, ns: not significant.*

### 4. 3 Effect of Pcy on lipid-raft formation in SW620 cell membrane

To confirm that Pcy-trigger extrinsic apoptosis pathway by a mechanism independent of lipid raft-formation in SW620 cells, we aimed to analyze the protein caveolin, a raft-associated protein which is associated with cholesterol and sphingolipids in certains areas of the cell membrane leading to the formation of caveolaes for activating signaling transduction mechanism of membrane receptors (Liu et al, 2002). Caveolin levels protein were analyzed by western blot using a polyclonal antibody anti-human caveolin. In SW620 cells treated or not with Pcy, levels of caveolin were extremely low (data not showed), and did not exceed the levels observed in SW480 cells before, confirming the observations of Bender et al (2000).

## 5. Conclusions

Taken together, our observations suggest that Pcy may activate an extrisnxic death signaling pathway through a cholesterol lipid-raft independent mechanism in SW480 and SW620 cells. The sequestering-cholesterol compound, named nystatin, contributed to potentiate the apoptotic effect of apple Pcy in SW480 without up-regulation of DR4/DR5 receptors expression at post-transcriptional and/or transcriptional level. Moreover, we confirmed that DR4/DR5 receptors were not localized in lipid-raft microdomains as occurred with TRAIL. By other hand, the potentiated effect of apple Pcy by nystatin in SW620 cells was accompanied by the increased expression of DR4 in presence of nystatin, but not for DR5 which suggest that DR4 receptor seems to be more efficient than DR5 for activating apoptotic signaling probably by the formation of more hetero- or homodimer complexes containing a DR4 receptor activated, in this case, by a mechanism clearly independent of lipid raft formation in SW620 cells because of depletion of cholesterol as well as absence of caveolin protein. However, further investigations are required to characterize the membrane events that take place by Pcy in colon cancer cells.

# *GENERAL DISCUSION*
# *&*
# *CONCLUSIONS*

# GENERAL DISCUSION AND CONCLUSIONS

Dietary chemopreventive compounds offer a great potential in the fight against cancer by inhibiting the carcinogenesis process through the activation and regulation of programmed cell death (apoptosis). In this study, by employing an *in vitro* model of colon carcinogenesis that represent the progression from a primary tumor (SW480 cells) to metastatic disease (SW620 cells), we have found that apple Pcy can directly or indirectly influence some important targets involved in apoptosis: polyamines metabolism, TRAIL-death receptor pathway, Fas-receptor pathway, mitochondrial integrity. It is encouraging to know from the results obtained in this work, that a dietary agent such as apple Pcy presents such multipotent anti-cancer properties and may represent a promising agent for the chemoprevention of colon cancer through the induction of apoptosis.

## 1. Relationship between polyamines metabolism and the apoptosis triggered by apple Pcy

The polyamine metabolism that differ in various aspects in SW480 and SW620 cells (Duranton et al, 2002, 2003), has been studied as a potential target of apple Pcy because they are involved in cell proliferation and the maintenance of cell viability. In the first chapter, we showed that apple Pcy-induced apoptosis in SW480 and SW620 cells was potentiated by the use of MDL 72527 (MDL) in the metastatic SW620 cells but not in SW480 cells. The difference in sensitivity observed to MDL in SW480 and SW620 cells was not probably a function of PAO activity, which present similar activity in both cell lines (Duranton et al, 2002). Therefore other aspects could be involved, such as the intracellular pool of polyamines and their acetylated forms.

Here, we have observed that Pcy-induced apoptosis in SW620 cells was accompanied by a decreased intracellular pool of polyamines and a higher accumulation of acetylated polyamines, indicating respectively a reduction of polyamine biosynthesis and an enhanced polyamines catabolism. This is correlated to a previous reports by Gossé et al (2005), showing that Pcy down-regulated ODC and AdoMet DC activities in SW620 cells, the two enzymes of polyamine biosynthesis. These effects of Pcy on

polyamines metabolism were enhanced in presence of MDL 72527, a specific inhibitor of PAO activity. The depletion of the intracellular pool of polyamines in presence of Pcy/MDL led to apoptosis, and the addition of exogenous polyamines to SW620 cell culture inhibited the potentiation by MDL of Pcy-triggered apoptosis which confirmed the importance of polyamines (putrescine, spermidine and spermine) in cell proliferation and maintainance of cell viability (Gerner et al, 2009).

The massive formation of acetylated polyamines may contribute to cell death because of depletion of Acetyl-CoA (Babbar et al, 2006; Kee et al, 2004). In addition, it has been speculated about the possibility that the accumulation of intracellular N-acetylated polyamines affects histone acetylation by competing with the acetyl-CoA: spermidine N8-acetyltransferase, an enzyme that has also histone acetylating properties (Desiderio et al, 1992). However, our findings in SW620 cells suggest that in the presence of Pcy, MDL might favour the hyperacetylation of the promoters of TRAIL-death receptors leading to the up-regulation of DR4/DR5 expression (Inoue et al, 2002; Saunders et al, 2006) which may be controlled at transcriptional level, favoured by an inhibition of 50% nuclear HDAC activity caused by Pcy/MDL. Under these conditions, was induced the activation of the extrinsic apoptotic pathway through the TRAIL-death receptors confirmed by the reduction of Pcy/MDL-induced cell death after a specific inhibition of DR4/DR5 receptors. The SW620 cell line is normally resistant to TRAIL-death receptor mediated apoptosis because they not express DR4 and DR5 receptors at the cell surface (Huerta et al, 2007; Vaculová et al, 2006). Thus the activation by Pcy/MDL of TRAIL-death receptor mediated apoptosis through the modulation of polyamine metabolism can be of great interest in chemoprevention, since this pathway can preferentially activate apoptosis in cancer cells but not in normal non-cancerous cells (Ashkenai et al, 1999). Furthermore, the combination Pcy/MDL could potentiate the effect of TRAIL exogenous and/or the TRAIL produced by cells of innate immune system (Herbeuval et al, 2003).

The apoptotic pathway activated by Pcy alone in SW620 cells was different to that observed in presence of MDL. Pcy activated the intrinsic mitochondrial apoptotic pathway through the alteration of mitochondrial membrane potential involving activation of caspases-9 and -3, effects caused by the Pcy-enhanced ROS production which were prevented by MDL. Indeed, MDL, inhibited ROS generated through the

activation of polyamine catabolism caused by Pcy. This amount of intracellular ROS may be a way to kill cancer cells by activating the mitochondrial permeability transition (Chen et al, 2003; Poli et al 2004; Valko et al, 2006).

Regarding about the SW480 cells, there are no previous studies about the pro-apoptotic properties of apple Pcy in colon cancer cells and its relationship with polyamine metabolism. Here we showed that apple Pcy induced apoptosis in SW480 cells, although this effect was not enhanced by MDL 72527 as observed in SW620 cells, which suggest that Pcy-induced apoptosis in SW480 cells was unrelated to the production of $H_2O_2$ by the oxidation of N1-acetyl derivatives of spermidine and spermine, a reaction catalyzed by the PAO when these acetylated polyamines are excessively formed by the SSAT enzyme (Ignatenko et al, 1996; Gossé et al, 2006). SW480 cells showed to be less sensitive to combined treatment of Pcy and MDL compared to SW620 cells, spite of similar PAO activity in both cell lines under basal conditions (Duranton et al, 2002). This difference on the apoptotic effects observed with Pcy/MDL in SW480 cells may be related to the low polyamine biosynthetic activity and low intracellular content of acetylated forms of polyamines observed in these cells (Duranton et al, 2003). Indeed, if Pcy down-regulate polyamine biosynthesis (Gossé et al, 2005, 2006), which is already significantly low under basal conditions in SW480 cells (Duranton et al, 2002, 2003), probably the levels of non-acetylated forms of polyamines would be lower in these cells.

In SW480 cells, Pcy single or combined with MDL activated an extrinsic apoptotic pathway by up-regulating expression of TRAIL-death receptors DR4/DR5. We investigated whether the activation of TRAIL-death receptor pathway under these conditions was associated to a reduced activity of HDAC. Pcy as well as MDL used as single drugs did not affect the enzyme activity whereas MDL in combination with Pcy reduced by 20% HDAC activity, which could favour hyperacetylation of the promoters of DR4/DR5 receptors. However, no significant difference was observed in the percentage of SW480 cells expressing DR4/DR5 receptors after Pcy single or combined Pcy/MDL treatments, which suggests that the enhanced expression of TRAIL-death receptors at the cell surface of SW480 cells might be regulated at a post-transcriptional level. The alteration of the polyamine metabolism is a factor involved in the Pcy-induced apoptosis of SW620 metastatic cells but not of SW480 colon cancer cells.

**2. Activation of TRAIL-death receptors mediated pathway**

Once we showed that Pcy can induce apoptosis in SW480 and SW620 cells through the activation of TRAIL-death receptors DR4/DR5 receptors, we aimed to know the mechanisms that could be activated downstream DR4/DR5 in both cell lines by Pcy alone or in the presence of exogenous TRAIL. We observed that Pcy enhanced the sensitivity to the apoptotic effects of TRAIL in SW480 cells and overcame TRAIL-resistance in SW620 cells. Co-administration of Pcy and TRAIL enhanced apoptotic signaling leading to nuclear DNA fragmentation in both cell lines compared with TRAIL alone.

In SW480 cells, the increased the expression of DR4/DR5 receptors by Pcy at the cell surface enhanced sensitivity to TRAIL. In these cells, the main apoptotic pathway activated by Pcy single and combined with TRAIL was the extrinsic pathway, involving the activation of caspase-8 and caspase-3. Mitochondrial dysfunctions observed in SW480 cells exposed to Pcy and Pcy/TRAIL were limited and did not cause the release of cytochrome c into cytosol and caspase-9 activation, because neither Bid protein nor Bcl-2/Bax were modified. In contrast in the metastatic SW620 cells, Pcy initiated apoptosis through a crosstalk between TRAIL-death receptor pathway and the intrinsic (mitochondrial) apoptotic pathway via an activation of the caspase-8,a reduction of full length Bid protein, and paralleled to a progressive increase Bax protein and mitochondria membrane permeabilization. Such mitochondrial dysfunction favoured the release of cytochrome c into cytosol leading to the activation of caspase-9 and consequently of caspase-3. In SW620 cells, these events are associated to ROS production induced by Pcy which were also enhanced in the presence of exogenous TRAIL.

The importance to activate TRAIL apoptotic pathway for the treatment of cancer is highlighted by the recent introduction of TRAIL-receptor agonistic antibodies in human phase 1 trials (Marini et al, 2006; Plummer et al, 2007; Reed, 2003). However, most solid tumor cells are relatively resistant to TRAIL-induced apoptosis. Although, numerous studies have shown that TRAIL resistance can be overcome by the combined application of chemotherapeutic drugs. Thus, to find natural phytoconstituents able to enhance the apoptotic effect of TRAIL, which is produced by cells of the immune

system as macrophages or natural killer cells (Herbeuval et al, 2003), and sensitize TRAIL-resistant cancer cells, represents a potential important strategy for cancer therapy.

Pcy, similarly to phytochemicals such as curcumin (Jung et al, 2005), quercetin (Kim et al, 2008; Psahoulia et al, 2007), apigenin (Horinaka et al, 2006), luteolin (Horinaka et al, 2006), and resveratrol (Shankar et al, 2007) augment TRAIL-mediated apoptosis in cancer cells by increasing in death-receptor expression on the cell surface, improvement the transport of DR4/DR5 proteins to the cell surface. Failure in trafficking mechanisms for delivering TRAIL-death receptors may contribute to TRAIL-resistance in colon cancer cells (van Geelen et al, 2004). In SW480 cells, although total amount of DR4 and DR5 transcripts was similar in control and treated cells, Pcy increased DR4 and DR5 expression at cell surface and sensitized SW480 cells to TRAIL. This effect was similar to that observed by Jin et al (2004) using the glycosilation inhibitor tunicamicyn in SW480 TRAIL-resistant clones. By contrast, in TRAIL-resistant SW620 cells, Pcy-increased the levels of DR4/DR5 transcripts. This was were correlated to the enhanced localization of death receptors at the cell surface. Our results suggest that Pcy regulate post-transcriptional mechanisms involved in delivery of death receptors to the cell membrane in SW480 cells, whereas in SW620 cells Pcy overcome TRAIL-resistance by regulating transcription as well as intracellular transport mechanisms leading to increase cell surface expression of TRAIL-death receptors.

It has been proposed that the basic expression level on the cell surface is not enough to determine TRAIL-sensitivity, but also redistribution of TRAIL-death receptors to the cell membrane and formation of lipid rafts. Redistribution of death receptors in lipid rafts, which are plasma membrane microdomains, enriched with cholesterol, glycosphingolipids and caveolar-associated proteins such as caveolin. All of them able to regulate the efficacy of signaling by death receptors (Muppidi et al, 2004; Nachbur et al, 2006; Psahoulia et al, 2007). In colon cancer cells, resveratrol (Delmas et al, 2004) and quercetin (Psahoulia et al, 2007) induced DR4/DR5 receptors redistribution in lipid rafts colocalized with caveolin-1, becoming sensitive to TRAIL, which was prevented by the cholesterol sequestering agent nystatin. Caveolin-1 is an integral membrane protein involved in cellular signal transduction which has been

described as a marker for raft-associated caveolae that interacts with lipids such as cholesterol for the structure of caveolae (Liu et al, 2002).

In our study, it is to highlight that Pcy activated TRAIL-death receptor pathway through a lipid-raft independent mechanism in both cell lines. This was confirmed with the analysis of lipid-raft fractions of Pcy-treated SW480 and SW620 cells, where the levels of DR4, DR5 and caveolin were similar to the basal conditions. Caveolin protein can interacts directly with and inhibits or sequesters the inactive form of many signaling molecules via the scaffolding domain (Liu et al, 2002). Therefore, that Pcy activate directly TRAIL-death receptor pathway independently of lipid-raft formation could be considered an interesting strategy for sensitizing SW480 and SW620 cells to TRAIL-death receptor mediated apoptosis and overcome resistance mechanisms that suppress the signaling activity of TRAIL-death receptors. It has been proposed that antitumoral properties of flavanols and Pcy can be associated to the oligomeric chain length influencing directly the interactions with the lipid bilayer and membrane proteins (Oteiza et al, 2005; Shoji et al, 2005; Tarahovsky et al, 2008).

In response to the interaction of Pcy with the cell membrane DR4 and DR5 are activated, the procaspase-8 is recruited to the DISC complex via binding to FADD (Bodmer et al, 2000; Kischkel et al, 2000) which results in caspase-8 activation. This was supported by the ability of a selective inhibitor of caspase-8 to reduce the induced apoptosis by Pcy single or combined with TRAIL in SW480 cells compared to SW620 cells. Caspase-8 has two important substrates, procaspase-3 and Bid protein. Caspase-3 is the main downstream effector caspase that cleaves the majority of the cellular substrate in apoptotic cells (Porter et al, 1999). Bid is the link in the crosstalk between the extrinsic and the intrinsic apoptotic pathways, disrupts mitochondria and favours the release of pro-apoptotic factors such as cytochrome c (Özoren et al, 2002). Caspase-8 cleaves Bid as efficiently as it cleaves pro-caspase-3 (Timmer et al, 2007). However, although caspase-8 was activated in both cell lines after Pcy and combined treatment with TRAIL, BID was cleaved only in SW620-treated cells. As a consequence a decreased mitochondrial membrane potential, was observed in these cells leading to the release of cytochrome c into cytosol and caspase-9 activation, indicating that the mitochondrial pathway is activated in Pcy-induced apoptosis through the action of TRAIL-death receptors.

The reason for inefficient cleavage of Bid protein in Pcy-treated SW480 cells is not the lack of caspase-8 enzyme activity, but may be influenced by posttranslational modifications of Bid (Desagher et al, 2001; Özoren et al, 2002), mutations to Bid cleavage sites (Riddle-Taylor et al, 2007), or the presence of negative regulator (Sinicrope et al, 2004) in SW480 cells. It has been demonstrated that phosphorylation of Bid prevents caspase-8 cleavage (Desagher et al, 2001). In addition, tBid has been reported to be ubiquitinated and targeted for degradation (Breitschopf et al, 2000). It has also been shown that in the SW480 cells Bcl-2 overexpression blocked TRAIL-death receptor mediated apoptosis by inhibiting Bax translocation into mitochondria and reduced cytochrome c release (Si benicropte et al, 2004). Thus, strategies to overcome this observed Bcl-2-mediated resistance to mitochondrial pathway have the potential to greatly increase treatment efficacy. One approach involves the use of small molecules inhibitor that binds to Bcl-2 and inhibits it function (Sinicrope et al, 2004).

Many studies have shown that cancer preventive agents induced apoptosis through generation of ROS (Kroemer, 1999; Li et al, 1999). In our study, apoptosis induced by Pcy single or combined with TRAIL in SW620 cells also involves generation of ROS, which can result from the increased polyamines catabolism as well as frm the mitochondrial permeabilization caused by tBid and Bax. ROS produced in cytosol may also activate cell intrinsic pathway of apoptosis by perturbing mitochondrial function which is inhibited by MDL the induction of polyamine oxidase as we have shown. Taken together our observations suggest that ROS generation is another mechanism by which Pcy may sensitizes TRAIL-resistant SW620 cells as observed in cancer cells treated with resveratrol (Shankar et al, 2007) and curcumin (Jung et al, 2005). Accumulation of ROS leads to mitochondrial functional changes, such as the increased permeability transition pore opening and loss of $\Delta\psi m$, events associated with cytochrome c release and caspase activation (Chen et al, 2003; Debatin et al, 2002).

**3. Modulation of Fas- receptor mediated apoptosis**

In the second chapter of this study, we showed that in contrast to the effect observed with Pcy in activating the TRAIL-death receptor mediated apoptosis in SW480 and SW620 cells, the sensitivity exhibited to Fas (CD95)-receptor mediated apoptosis in presence of Pcy was different between both cells.

Fas-receptor (CD95) is an integral cell membrane protein and a member of the TNF family of receptors (Itoh et al, 1991). Pcy sensitized SW480 cells to Fas receptor-mediated apoptosis. This wass associated with an up-regulated expression at post-transcriptional level of Fas receptor without significant changes at transcriptional level, which suggest that the sensitizing mechanism of Pcy to Fas implies a favoured delivery of Fas receptor into the cell membrane. On the contrary, the SW620 cells showed a Fas-resistant phenotype as already described (Bergmann-Leitner et al, 2000), despite the up-regulation of Fas transcripts correlated with a huge expression of receptor at the cell surface.

Tumours have developed multiple mechanisms for evading surveillance of the immune system. Most cancer cells are relatively resistant to Fas-mediated apoptosis (Krammer, 1997; Natoli et al, 1995; O'Connel et al, 2000; Shima et al, 1995). This protects tumour cells from FasL expressed as a cytotoxic mediator by T cells (Ju et al, 1995; Stalder et al, 1994) or NK cells (Montel et al, 1995) infiltrated into the tumour. Colon cancer cells have acquired defensive strategies (Fas resistance) against this effect by either down-regulating Fas-receptor (Krueger et al, 2001) or by acquiring Fas-receptor signaling defects (O'Connell et al, 2000). Mutated intracytoplasmic domain of Fas receptor has been reported, precluding establishment of a functional DISC (Cascino et al, 1996). Some cancers express high levels of Fas-associated phosphatase-1 (FAP-1) (Arai et al, 1998; Sato et al, 1995) which interacts with the C-terminal region of Fas leading to inhibition of downstream events. The microinjection of a tripeptide corresponding to the three amino acids of Fas receptor C-terminal region prevented this interaction and restored Fas sensitivity of colon cancer cell line (Yanagisawa et al, 1997).

The activation of Fas-receptor by apple Pcy was confirmed in both cell lines by two strategies: i) use of an antagonist antibody to Fas-receptor, the anti-Fas ZB4; ii) Pcy combination with an agonist antibody of Fas-receptor, the anti-Fas CH-11 reproducing the activation by FasL.

Under these conditions, in SW480 cells an increased in the number of hypodiploid cells, loss of mitochondrial membrane potential and DNA fragmentation consequence of the activation of Fas receptor were observed. As showing by the potentiated pro-apoptotic effects of anti-Fas CH-11 enhanced by Pcy which were abrogated by the anti-Fas ZB4. We also observed in these cells that Pcy combined with anti-Fas CH-11 activated a type II (mitochondrial)-apoptotic pathway, an effect observed for anti-Fas CH-11 in other experimental models (Huang et al, 1999, 2000). Whereas, the use of multimeric forms of Fas ligand induce physiologically relevant apoptotic signaling type I (without mitochondria) pathway through the activation of Fas receptor (Clemons et al, 2005). In any case, this supports the view that Pcy may help cancer cells to recover Fas sensitivity contributing to the elimination of tumour cells by FasL or by agonists of Fas-receptor.

On the contrary, in SW620 cells the anti-Fas CH-11 did not enhance the pro-apoptotic effects observed with Pcy alone. However, in SW620 cells it was not excluded that Fas-receptor might be implicated in the Pcy-induced apoptosis after a blockade of TRAIL-DR4/-DR5 receptors that induced its activation by Pcy, suggesting that Pcy were able to initiate a cross-talk between DR4/DR5 and Fas in metastatic cells. These findings suggest molecular defects at level of Fas signal transduction. Thus, to understand the basis of the resistance of metastatic-derived colon cancer cells to Fas-mediated apoptosis might provide new strategies and treatment modalities for targeting death receptors in cancer cells.

Here, we propose that Pcy may restore Fas-sensitivity in metastatic cells by overcoming several aspects contributing to Fas-resistance:

i) The ratio between DR4/DR5 and Fas receptors in cell membrane may play a role in determining Fas-sensitivity. The number of Fas receptor relative to DR4/DR5 might not be sufficient to induce Fas-mediated apoptosis, since both type of receptors interact similarly with the adaptor protein FADD

(Thomas et al, 2004, 2006) after their activation by the combined treatments Pcy /TRAIL or Pcy / anti-Fas CH-11;

ii) DR4/DR5 and Fas receptors have different C-terminal tails. The corresponding region for DR4 and DR5 positively regulates FADD binding, caspase activation and apoptosis whereas the C-terminal tail of Fas receptor has the opposite effect and inhibits the binding of FADD to the receptor death domain (Thomas et al, 2004). We may hypothesize that the C-terminal tail of DR4 and DR5 receptors located outside the death domain could present additional regulatory sites for the activation of Fas receptor in order to overcome an inactivation of DR4/DR5 receptors (Thomas et al, 2004). However, at present there is no evidence showing such a direct interaction between Fas and DR4/DR5 receptors;

iii) Crosslinking of FasL and TRAIL to their respective receptors activates PKC which in turn triggers anti-apoptotic mechanisms for the mitochondrial pathway (Scaffidi et al, 1999; Trauzold et al, 2001). Thus, one may speculate about the possibility that the simultaneous inhibition with blocking antibodies for DR4/DR5 and Fas affects PKC activity which can also, be inhibited by apple Pcy as described Gossé et al (2005) in SW620 cells. However, all these aspects deserve further investigations.

The different apoptotic signaling pathways triggered by apple Pcy demonstrated in the course of the study are summarized in Figures 36 and 37.

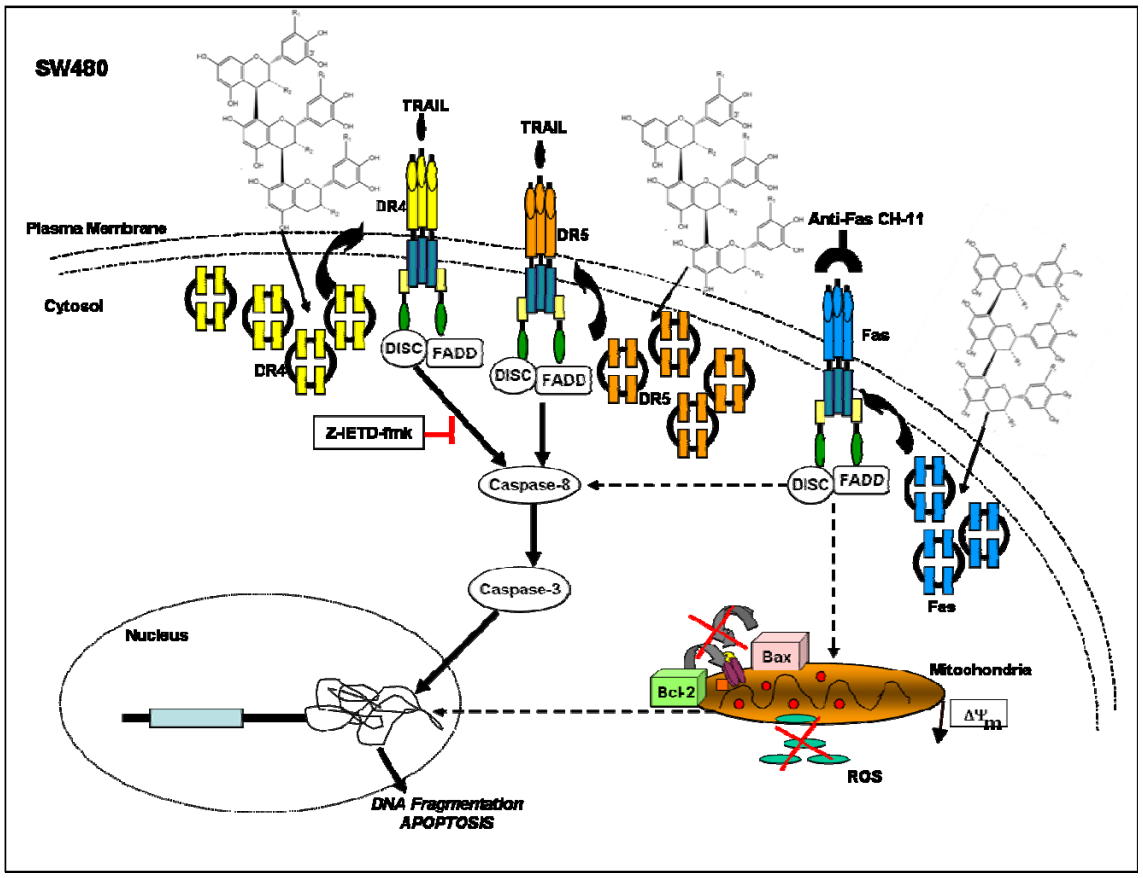


***Figure 36. Apoptotic signaling pathways triggered by apple Pcy in SW480 cells****. Pcy caused mainly an activation of the extrinsic apoptotic pathway involving DR4, DR5 and Fas receptors. Activation of receptors leads to a direct activation of caspase-8 and caspase-3. Despite expression of Bcl-2 and Bax, and ROS production, the loss of mitochondria membrane potential was insufficient to cause cytochrome c release and caspase-9 activation. DISC, Death-induced signaling complex; FADD, Fas-associated death domain;ROS, reactive oxygen species;* $\Delta\psi_m$*,mitochondrial membrane potential.*

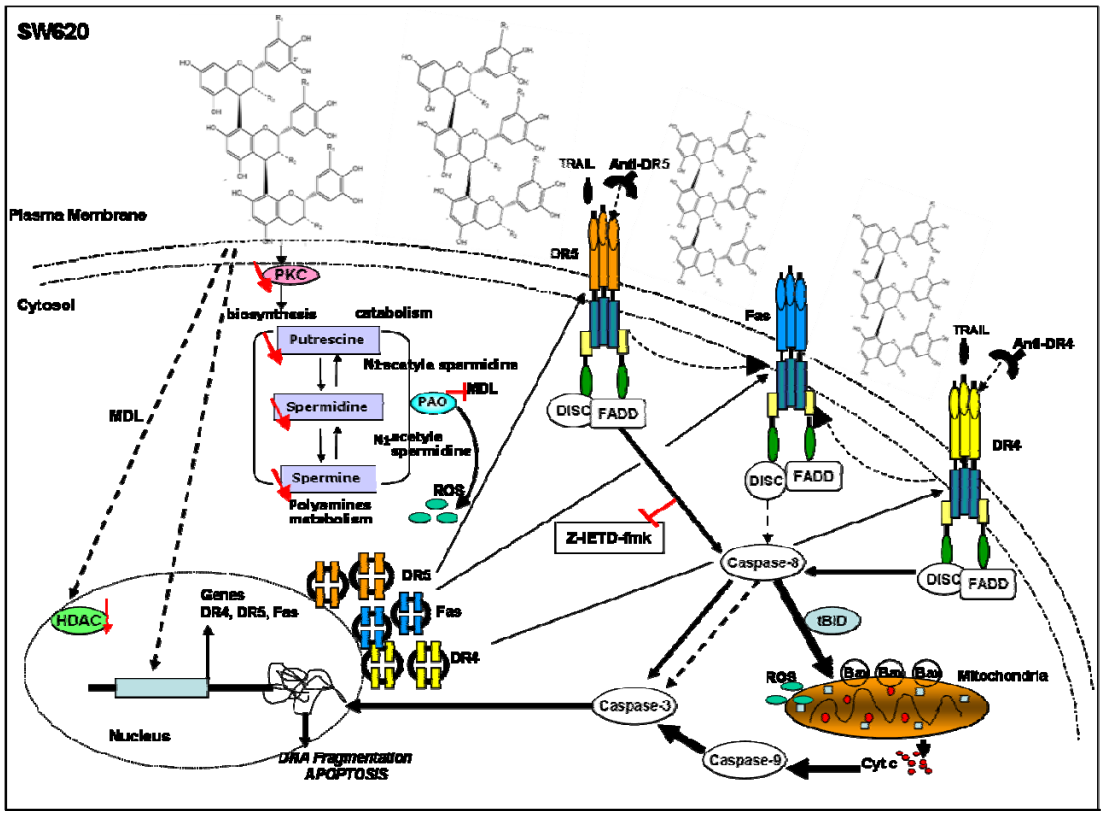


***Figure 37. Apoptotic signaling pathways triggered by apple Pcy in SW620 cells****. Pcy caused inhibition of polyamine biosynthesis and enhanced polyamine polyamine catabolism leading to ROS production. This was associated with the inhibition of HDAC activity which might favour the observed up-regulation of DR4, DR5 and Fas gene expression. Activation of death receptors led to the activation of caspase-8, cleavage of Bid (tBID) protein, progressive increase of Bax protein causing mitochondria dysfunction, release of cytochrome c into cytosol, and caspase-9 and caspase-3 activation.*
*PKC, protein kinaseC, PAO, Polyamine oxidase; MDL, MDL 72527; HDAC, Histone deacetylase;ROS, reactive oxygen species; DISC, death induced signaling complex; FADD, Fas-associated death domain; tBID, truncated BID; Cyt c, Cytochrome c.*

## 4. Perspectives

The knowledge acquired in this study about the apoptotic effects induced by Pcy in colon cancer cells might open new possibilities for alternative therapies that are less toxic than current treatments.

On the other hand, it is clear that our studies using the apple Procyanidins were focused on the pro-apoptotic effects on colon cancer cells. It would therefore be interesting to extend this study to other regulated non-apoptotic pathways to know whether other types of cell death can be activated by the apple Pcy such as autophagy. It might also be interesting to determine whether the effects of Pcy on cell death were similar on other human colon cancer cell lines.

In this study we have observed that the expression of death receptors (DR4/DR5, Fas) was regulated at transcriptional level by Pcy only in SW620 cells. The mechanism involved in the transcriptional activity that leads to up-regulation of the respective apoptotic genes by Pcy is unknown. Therefore, it would be of interest to determine the effects of apple Pcy on transcription factors that have consensus sequences in the promoters of *DR4*, *DR5* and *FAS* genes such as NF-κB and p53. Some reports have highlighted the role of NF-κB as a pro-apoptotic factor as well as for p53 (Ryan et al, 2000; Wu et al., 1997) by up-regulating pro-apoptotic genes such as Bax (Grimm et al, 2005), Fas, FasL (Henson et al, 2003); DR5 (Yoshida et al, 2001; Chen et al, 2008). Indeed, SW620 cells have a mutated form of p53 (R273H; P309S) (Huerta et al., 2007) suggesting that mutated p53 might be responsive than wild-type p53 to chemopreventive agents. However, the mutated form of p53 in SW620 cells has been shown to possess a residual transcriptional activity and is able to bind directly or through protein-protein interactions with other transcriptional factors to its consensus DNA sequence as described for wild-type (Millau et al, 2008). Thus, it remains to know whether Pcy-triggered apoptosis in SW620 cells is dependent or not of their p53 status.

Furthermore, it must never be forgotten that single compounds are invariably only partially effective as anticarcinogens, and that in the field of chemoprevention and chemotherapy, combinations of agents are frequently far more efficient than any single drug (Lamprecht and Lipkin, 2003). Therefore, it would be of high interest to investigate the possible synergistic effects of apple procyanidins used in combination with other phytochemical present in human diet on cell death pathways related to chemoprevention of cancers of the alimentary tract, as a strategy for promoting prevention.

The cell culture studies presented in this work need to be completed with the evaluation of the pro-apoptotic effects of apple Pcy in a preclinical model of colon carcinogenesis through a study of the expression profile of pro-apoptotic genes as well as the of their respective proteins in the adenoma and adenocarcinoma at different stages of their formation.

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# *PARTICIPATIONS TO INTERNATIONAL CONGRESS & OTHER PUBLICATIONS*

# PARTICIPATIONS

- **International Conference on Biogenic Amines: Biological and Clinical Perspectives. Maldonado ME**, Roussi S, Foltzer-Jourdainne C, Gossé F, Chaabi M, Lobstein A, Raul F. **Potentiation of apple procyanidin-triggered apoptosis by the PAO inhibitor MDL 72527 in human colon cancer-derived metastatic SW620 cells (oral presentation)**. October 17-21 2007, Catania, Italy.

- **First International Congress on Nutrition and Cancer M.E. Maldonado-Celis,** C. Foltzer-Jourdainne, F. Gossé, M. Chaabi, A. Lobstein, F. Raul. **Different apoptotic pathways triggered by apple procyanidins in human colon adenoma and derived metastatic cells (oral presentation).** May 19-23 2008, Antalya, Turkye.

- **12$^{th}$ Colombian National Symposium of Human Nutrition. Maldonado Celis ME.** Apple procyanidins: anticarcinogenic actions in colon cancer. October 20-22 2008, Medellin, Colombia.

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- **Maldonado Celis ME.** Influencia de la nutrición en el funcionamiento del sistema inmune. Perspect Nutr Hum 2008; 10: 53-58.

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