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Hepatitis C virus entry and cell-cell transmission: implication for viral life cycle and antiviral treatment

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Where there’s a will, there’s a way.

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Abbreviations

BHV	Bat hepacivirus
CARD	Caspase recruitment domain
CC	Cytotoxic concentration
CD81	Cluster of differentiation 81
CI	Combination index
CLDN	Claudin
CTRL	Control
DAA	Direct-acting antiviral
EGFR	Epidermal growth factor receptor
EIA	Enzyme immunoassay
EMCV	Encephalomyocarditis virus
EphA2	Erythropoietin-producing hepatocellular receptor tyrosine kinase class A2
HA	Human albumin
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCVcc	Cell culture-derived HCV
HCVpp	Hepatitis C virus pseudoparticle
HIV	Human immunodeficiency virus
HTA	Host-targeting agent
HTEI	Host-targeting entry inhibitors
IC	Inhibitory concentration
IFN	Interferon
IL	Interleukin
IRES	Internal ribosome entry site
ISG	Interferon-stimulated gene
LOQ	Limit of quantification
LT	Liver transplantation

mAb	Monoclonal antibody
MAV	Mitochondrial antiviral signaling protein
miRNA	microRNA
MLV	Murine leukemia virus
MOA	Mechanism of action
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NANB	Non-A, non-B
NPC1L1	Niemann Pick C1-Like 1
NPHV	Non-primate hepacivirus
NTR	Non translated region
OCLN	Occludin
ORF	Open reading frame
PEG-IFN	Pegylated interferon
PCR	Polymerase chain reaction
PHH	Primary human hepatocyte
PKI	Protein kinase inhibitor
PKR	Protein kinase R
RBV	Ribavirin
RHV	Rodent hepacivirus
RIG-I	Retinoic acid inducing interferon gene I
RLR	RIG-I like receptor
SOC	Standard of care
SR-BI	Scavenger receptor class B type I
SVR	Sustained viral response
TLR	Toll like receptor
UTR	Untranslated region
WHO	World health organization

Résumé

Le virus de l'hépatite C (HCV) est l'une des principales causes d'hépatite chronique, de cirrhose et de carcinome hepatocellulaire dans le monde. En effet, plus de 130 millions de personnes sont chroniquement infectés à travers le monde et l'infection à HCV provoque environ 350 000 décès chaque année. Le traitement standard actuel de l'infection chronique à HCV est constitué d'IFN-α pégylé, de ribavirine (RBV) et d'un antiviral direct ciblant la protéase ou la polymérase virale (Moore et a. Curr Infect Dis Rep 2014). Les nouveaux antiviraux à action directe (DAA), tels que le sofosbuvir et le simeprevir, devraient révolutionner le traitement de l'infection chronique à HCV en permettant de guérir la majorité des patients traités (Chung et al. N Engl J Med 2014). Cependant, certains sous-groupes de patients seront encore difficiles à traiter, comme par exemple les patients avec une maladie du foie avancée, les patients transplantés, les patients immunodéprimés (Liang et al. N Engl J Med 2013) ainsi que les patients co-infectés HIV/HCV. En outre, tous les génotypes du HCV ne répondent pas de la même manière à des combinaisons de DAA et les coûts élevés limitent l'accès au traitement dans les pays à revenu faible, moyen et élevé (Chung et al. N Engl J Med 2014).

Parmi les groupes de patients les plus difficiles à traiter sont des personnes atteintes d'une maladie hépatique au stade terminal nécessitant une transplantation hépatique (TH) (Liang et al. N Engl J Med 2013). En raison de l'échappement viral aux réponses immunitaires de l'hôte et l'absence de stratégies antivirales préventives, la réinfection du greffon est universelle. En outre, les thérapies à base d'IFN ont une efficacité limitée chez les transplantés du foie et ne sont pas bien tolérés par ces patients (Crespo et al. Gastroenterology 2012). L'utilisation des inhibiteurs de protéase de première génération télaprévir et bocéprévir est limitée par des interactions médicamenteuses si elle est combinée à des agents immunosuppresseurs (Garg et al. Hepatology 2011). De plus, l'émergence de souches résistantes sera certainement plus élevée chez ces patients en raison d'une plus faible efficacité du traitement standard et de la nécessité d'ajuster les doses des différents antiviraux. Ainsi de nouvelles stratégies thérapeutiques ciblant idéalement différentes étapes complémentaires du cycle viral et présentant une meilleure efficacité et innocuité sont nécessaires pour pallier aux limites de ces traitements.

Une cible antivirale prometteuse complémentaire aux traitements actuels est l'entrée virale. En effet, l'entrée du HCV dans sa cellule hôte est la première étape

des interactions virus-hôte. Elle est nécessaire pour l'initiation et la persistence de l'infection (Zeisel et al. J Hepatol 2013). L'entrée virale joue un rôle important dans la pathogenèse de l'infection par le HCV, en particulier au cours de la ré-infection du greffon après TH (Fafi-Kremer et al. J Exp Med 2010). Des cibles potentielles pour des inhibiteurs d'entrée comprennent CD81 (Pileri et al. Science 1998), SR-BI (Scarseli et al. EMBO J 2002), les protéines des jonctions serrées claudine 1 (CLDN1) (Evans et al. Nature 2007) et occludine (Ploss et al. Nature 2009), le récepteur du facteur de croissance épidermique (EGFR) (Lupberger et al. Nat Med 2011) et Nieman-Pick C1-like 1 (Sainz et al. Nat Med 2012). Les inhibiteurs d'entrée sont caractérisés par une activité pan-génotypique (Fofana et al. Gastroenterology 2012). En agissant par un mécanisme d'action complémentaire, les inhibiteurs d'entrée pourraient agir en synergy avec les antiviraux actuels. Pour explorer le potentiel des inhibiteurs d'entrée pour des traitements de l'hépatite C chronique associant différents antiviraux, nous avons étudié dans la première partie de ma thèse l'efficacité antivirale des inhibiteurs d'entrée, tels que des anticorps monoclonaux spécifiques de CD81, SR-BI et CLDN1 et les inhibiteurs erlotinib et dasatinib ciblant l'EGFR et EphA2, en combinaison avec d'autres antiviraux dans des modèles de culture cellulaire et dans des souris uPA-SCID avec un foie chimérique.

Nous avons évalué l'effet antiviral de différentes combinaisons d'inhibiteurs d'entrée et l'IFN-α, des antiviraux directs ou des antiviraux ciblant l'hôte. Nous avons identifié de nouvelles combinaisons caractérisées par des synergies marquées in vitro. Nous avons observé une bonne corrélation entre l'activité antivirale des différentes molécules dans les modèles expérimentaux et chez les patients (Gao et al. Nature 2010). Les synergies étaient robustes et significatives à l'IC50, l'IC75 et l'IC90, à toutes les concentrations testées et comparables dans des modèles expérimentaux de prévention et de traitement d'une infection à HCV. En outre, nous avons montré un effet synergique entre un inhibiteur d'entrée et un DAA in vivo en utilisant le modèle des souris uPA-SCID avec un foie chimérique (Xiao et al. Gut 2014).

Compte tenu de l'efficacité, l'innocuité, la pharmacocinétique, le stade de développement et des synergies entre les différents composés testés, nos données suggèrent que des combinaisons entre le simeprevir (Reesink et al. Gastroenterology 2010) ou le sofosbuvir (Lawitz et al. N Engl J Med 2013) et

l'erlotinib pourraient être des premiers nouveaux traitements à évaluer dans les essais cliniques randomisés. Des combinaisons entre le sofosbuvir et des anticorps dirigés contre CLDN1 ou SR-BI pourraient être prometteuses pour la prévention de l'infection du greffon hépatique suite au développement clinique des anticorps monoclonaux. Les nouvelles combinaisons identifiées dans cette étude pourraient élargir l'arsenal thérapeutique contre l'infection par le HCV et permettre la prévention de la reinfection du greffon au moment de la TH, le traitement des patients difficiles à traiter et de proposer des alternatives aux patients présentant des contre-indications à certains antiviraux des traitements standards actuels ou futurs.

Les virus utilisent différentes voies pour leur transmission et leur propagation dans les tissus infectés (Zhong et al. Curr Opin Virol 2013). Le HCV se transmet par l'entrée virale classique utilisant la diffusion acellulaire mais est également capable d'infecter directement les cellules voisines par transfert de cellule à cellule (Meredith et al. J Hepatol 2013). Alors que l'entrée virale classique joue un rôle essentiel dans l'initiation de l'infection par le HCV, la transmission de cellule à cellule semble jouer un rôle important dans la persistance virale et dans le maintien de l'infection (Zeisel et al. J Hepatol 2013). Une caractéristique clé de la transmission de cellule à cellule est sa résistance aux anticorps neutralisants présents chez les personnes infectées par le HCV (Timpe et al. Hépatologie 2008). Dans la seconde partie de ma thèse, nous avons décidé d'évaluer le rôle de la transmission de cellule à cellule dans la résistance aux antiviraux en utilisant le HCV de génotype 2 comme modèle, et d'explorer la transmission de cellule à cellule comme une cible pour prévenir et traiter la résistance aux DAA.

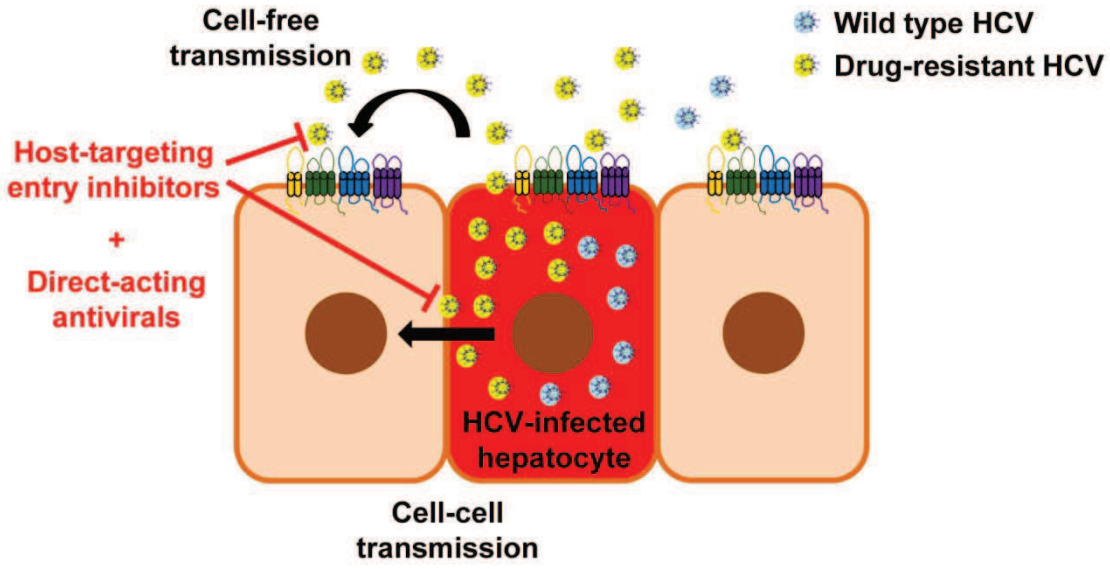
Nos données indiquent que la propagation de souches HCV résistantes aux DAA grâce à la transmission de cellule à cellule peut contribuer à l'échec du traitement et que l'inhibition de la transmission de cellule à cellule améliore l'activité antivirale des DAA dans des modèles de culture cellulaires (Xiao et al. PloS Pathogens 2014). En effet, en interférant avec la transmission de cellule à cellule, des inhibiteurs d'entrée ciblant des facteurs de l'hôte (HTEIs) sont en mesure d'empêcher le développement d'une résistance antivirale comme le montre l'absence d'une résistance fonctionnelle dans les cellules traitées avec une combinaison de DAA et HTEI. Plus important encore, lorsqu'il est ajouté à un DAA, un HTEI permet d'éliminer rapidement et efficacement l'infection à HCV, comme démontré par

l’absence de détection de l'ARN du HCV dans les cultures cellulaires traitées par la combinaison d’antiviraux. Etant donné que nos résultats indiquent que la principale voie de transmission des variantes du HCV résistants aux DAA est la propagation de cellule à cellule directe, nous supposons que l'effet préventif des HTEIs est principalement dû à leur effet sur ce mode de transmission. L’ensemble de ces données indique que l’inhibition de la transmission virale de cellule à cellule augmente l'activité antivirale des DAA et empêche l’apparition de résistance aux DAA dans le modèle HCV de génotype 2 in vitro (Xiao et al. PloS Pathogens 2014).

En outre, nos données montrent que les HTEIs présentent des différences dans leur barrière génétique à la résistance. En effet, alors que le traitement avec l’anticorps monoclonal OM-7D3-B3 spécifique de CLDN1 a permis d’éliminer l’infection à HCV en l’absence de résistance, le traitement avec l’anticorps monoclonal NK-8H5-E3 spécifique de SR-BI a entraîné le développement de variants viraux qui pourraient échapper à l’inhibition par ces anticorps. En effet, des résistances ont été décrites pour un inhibiteur de SR-BI (Zhu et al. J Infect Dis 2012). En outre, une étude récente a démontré élégamment que le HCV peut perdre sa dépendance à SR-BI pour la transmission de cellule à cellule (Catanese et al. J Virol 2013). Nos résultats montrent que les composés ciblant SR-BI semblent avoir une barrière génétique plus faible pour la résistance que des HTEIs ciblant CLDN1. Cela peut être dû au fait que CLDN1 est un facteur essentiel pour la transmission de cellule à cellule tandis que le virus peut s’affranchir de SR-BI dans ce processus (Catanaise et al. J Virol 2013).

Nous avons montré que la combinaison d'un HTEI et d’un DAA était plus efficace que deux DAA pour traiter une infection persistante à HCV de génotype 2a en culture cellulaire. Etant donné qu’une combinaison de deux DAA n'a pas permis d’éliminer une infection à HCV de génotype 2a et 2b dans un modèle animal (Shi et al. Gut 2013) et que des résistances aux DAA ont été observées (Liang et al. N Engl J Med 2013), nos données suggèrent que la stratégie antivirale décrite dans cette étude peut pallier aux limites des DAA en particulier pour les infections à HCV de génotypes autre que le génotype 1. Comme notre étude de preuve de concept est basée sur une souche HCV de génotype 2a, d’autres études sont nécessaires pour étudier l’implication de ces résultats pour les autres génotypes.

Nos résultats permettront d'ouvrir la voie à la conception de futures études cliniques combinant des HTEIs et des DAA dans l'infection par le HCV de génotype 2. De plus, nos résultats ont des implications pour le traitement d'autres infections virales. En effet, étant donné que cibler des facteurs de l'hôte est une nouvelle stratégie pour vaincre la résistance (Nathan et al. Sci Transl Med 2012), l'inhibition de la transmission de cellule à cellule par les HTEIs offre de nouvelles perspectives pour la lutte contre un large éventail d'infections virales, y compris l'infection par le HIV, le virus de la rougeole ou le HTLV-1 où la transmission de cellule à cellule a été suggéré jouer un rôle dans la peristance virale (Sattentau. Nat Rev Microbiol 2008).



Résumé Figure. Combinaisons d'inhibiteurs d'entrée et des antiviraux directs dans l'infection par le HCV

Introduction

Hepatitis C virus (HCV) infection is a global health problem with approximately 130 million people suffering from HCV-induced liver diseases: chronic hepatitis, liver cirrhosis and hepatocelluar carcinoma. 20 years after the discovery of HCV, a treatment allowing to cure all chronic hepatitis C patients does not exist. The combination of pegylated interferon-α (PEG-IFN-α) and ribavirin (RBV) has been the standard of care for treating HCV infection since 1998. However, only 40-50% of patients infected with HCV genotype 1 achieve sustained viral response (SVR) with this combination treatment. Moreover, side effects of PEG-IFN-α and RBV are universal and frequent, leading to discontinuation of treatment in up to 27% of patients (Liang et al. 2013; Sarrazin et al. 2012).

Recently, with the rapid development of direct-acting antivirals (DAAs), a series of new anti-HCV drugs have received FDA approval including telaprevir, boceprevir, sofosbuvir and simeprevir. The triple therapy with PEG-IFN-α and RBV plus a DAA has greatly improved the outcome of patients with chronic hepatitis C. Nevertheless, given the side effects of IFN and its route of administration, an all-oral IFN-free regimen has become the future direction for the treatment of hepatitis C. The recent establishment of HCV cell culture system has enabled researchers to investigate the whole viral life cycle and this lead to the discovery of numerous host factors, which have the potential to be utilized as targets for novel antiviral drugs. In the past couple of years, we have discovered epidermal growth factor receptor (EGFR) and Ephrin receptor A2 (EphA2) as novel HCV entry factors and developed a series of host-targeting entry inhibitors (HTEIs), which exhibit cross-inhibition of HCV from all the major genotypes (Fofana et al. 2010; Zahid et al 2013; Fofana et al. 2013; Lupberger et al 2011).

In the first part of my PhD thesis, we set out to develop novel antiviral strategies based on the newly developed antivirals targeting complementary steps of the viral life cycle. We uncovered novel combinations of DAAs and/or HTEIs and validated their antiviral efficacy in cell culture models and in the human liver uPA-SCID mouse model. In the second part, we investigated the role of virus cell-cell transmission in the development of antiviral resistance. After a brief overview of our current knowledge on HCV, this manuscript presents the original data from our studies followed by a discussion and perspectives.

1. History of HCV discovery

In the 1960's and 1970's hepatitis B virus (HBV) (Bayer et al. 1968) and hepatitis A virus (HAV) (Feinstone et al. 1973) were discovered and blood tests were developed to screen for HBV and HAV. However, there were some patients developing hepatitis after receiving transfusion without evidence of HBV and HAV. Therefore, this posttransfusion hepatitis was classified as "non-A, non-B (NANB) hepatitis". Dr. Harvey Alter infected chimpanzees with human blood material from patients with NANB (Alter et al. 1978) and observed tubules (tubule-forming agent) in the cytoplasm of infected chimpanzee hepatocytes (Shimizu et al. 1979). This agent could be filtered through 50 nM filters, indicating it was a novel type of virus (He et al. 1987). After years of efforts, a team of researchers led by Dr. Daniel W. Bradley and Dr. Michael Houghton identified the virus, called hepatitis C virus (HCV), using cDNA immunoscreening (Choo et al. 1989; Kuo et al. 1989). From then on, significant improvements have been made in diagnosis of HCV through blood test, which greatly reduced the risk of HCV infection by blood products.

2. Epidemiology and transmission of hepatitis C

It's estimated by the world health organization (WHO) that approximately 130 million people are chronically infected with HCV worldwide. The prevalence of HCV varies geographically and is associated with the economic development (Figure 1) (Thomas et al. 2013). Egypt has the highest HCV prevalence with 50% of persons born before 1960 infected with HCV due to unsterile injection of anti-schistomiasis drugs between the 1950s and 1980s.

HCV is mainly transmitted through blood and blood-derived products, but sexual contact (especially heterosexual intercourse) and mother-to-child transmission may also occur. Since HCV screening was established since the 1990s, HCV transmission through blood transmission has significantly decreased nearly to zero. In many developed countries, intravenous drug use is a major cause of HCV transmission, leading to 60-80% of HCV prevalence in intravenous drug users. Tattooing with nonsterile instruments also has high risk of HCV transmission. Although there is still a debate whether sexual contact can transmit HCV, it is believed that anogenital mucosa trauma represents a risk. Vertical transmission rate

is less than 10% and to date there is no measurement to prevent the risk. Other transmission routes include: unsterile hospital equipment, sharing personal item contaminated with HCV, etc (Alter et al. 2007).

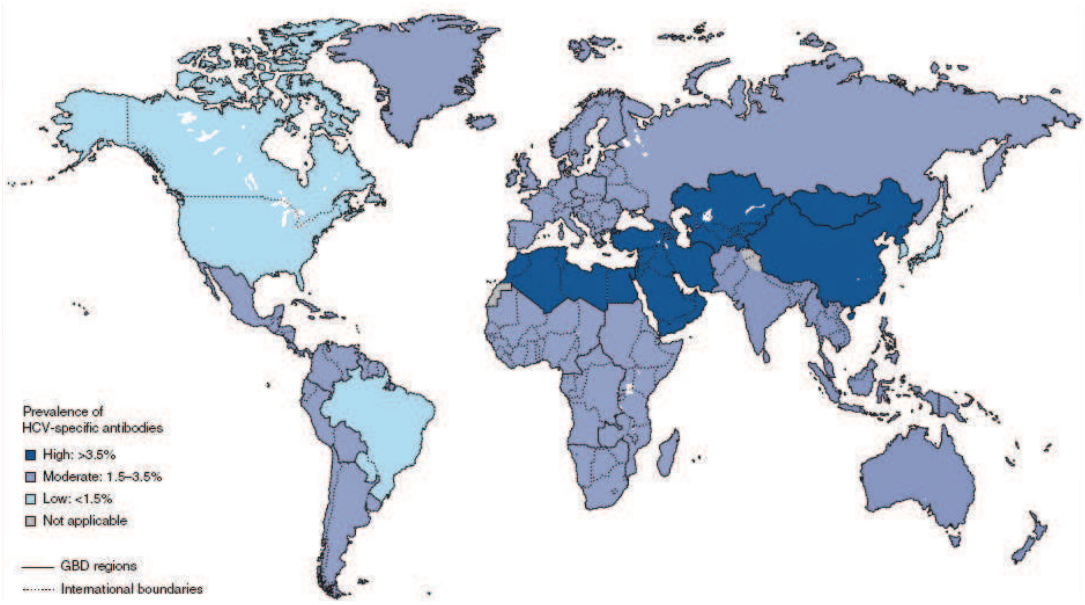


Figure 1. Global prevalence of HCV (Thomas. 2013)

3. Diagnosis of hepatitis C

HCV antibody enzyme immunoassay, recombinant immunoblot assay and RNA polymerase chain reaction (PCR) are used for detection of HCV. Detection of anti-HCV antibodies by enzyme immunoassay (EIA) is used for screen of HCV. PCR test is applied to assess viral load and provide information to evaluate therapy efficacy. HCV RNA becomes detectable (1-4 weeks after infection) in the serum earlier than anti-HCV antibodies (4-14 weeks after infection) (Zeisel et al. 2008). In acute infection phase, only HCV RNA but not anti-HCV antibody can be detected (Figure 2). Chronic hepatitis C is defined by positive HCV RNA 6 months after contamination. Anti-HCV antibodies remain positive in the patients who clear HCV spontaneously or through therapies for many years. In chronic HCV patients, both HCV RNA and anti-HCV antibodies are positive. Positive HCV RNA with negative anti-HCV antibody can be observed in immunosuppressed patients. As an important predictor to current standard of care (SOC) for HCV treatment, determination of the HCV genotype and

the polymorphism of interleukin-28B (IL-28B) are helpful for prediction of therapy efficacy.

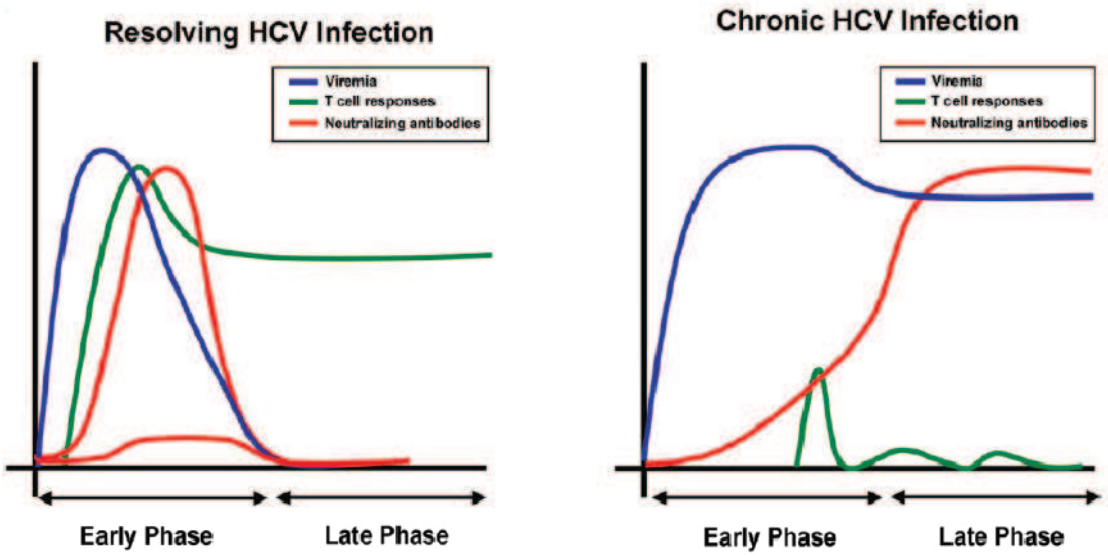


Figure 2. Neutralizing antibody and HCV RNA in resolving and chronic HCV infection (Zeisel et al. 2008)

4. Pathogenesis of hepatitis C

HCV causes hepatitis, which spontaneously resolves in 10-30% of patients. Most patients develop chronic hepatitis C. Although HCV infection causes liver inflammation, the clinical symptoms are not obvious for most of the HCV-infected patients at the early stage. Long-term HCV persistance may cause cirrhosis and hepatocelluar carcinoma, resulting in severe complications and fatality. There are extrahepatic diseases associated with HCV infection, including cryoglobulinemic vasulitis and encephalopathy (Figure 3). It's believed that HCV is almost not cytopathic and the liver damage is mainly caused by immune responses. HCV core protein can result in lipid accumulation in the hepatocytes, leading to liver steatosis, which is the only direct pathogenic effect of HCV. Local immune responses recruit specific T cells to the liver and result in lymphoid infiltration, necrosis and degeneration. Hepatic stellate cells are also activated and produce collagen to form fibrous septa and cirrhosis ultimately. Hepatocellular carcinoma (HCC) occurs in 1-4%

of patients with HCV-related cirrhosis. Studies showed that viral proteins core, NS3, NS4B and NS5A play a role in transforming cells in cooperation with oncogenes (Pawlotsky. 2004).

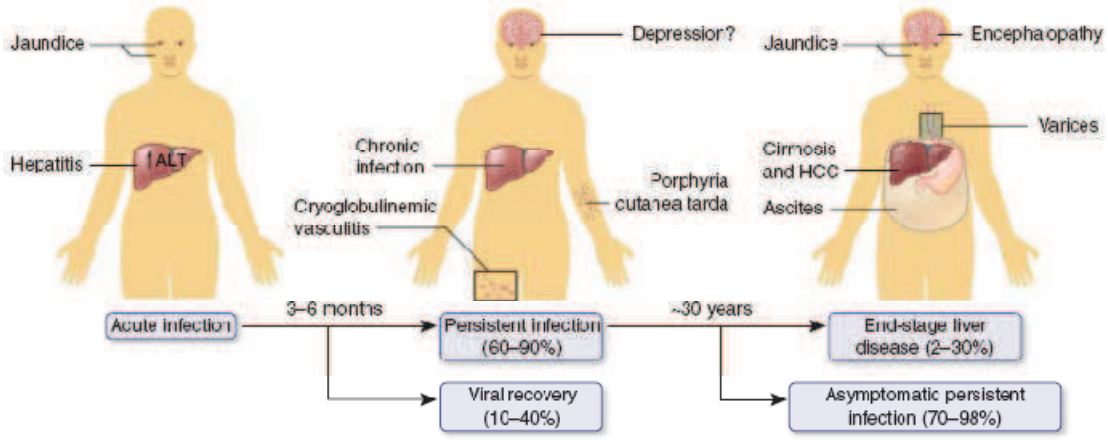


Figure 3. Outcomes associated with HCV infection (Thomas. 2013)

5. Viral genome and genetic diversity

HCV is a positive-sense, single stranded RNA virus, classified as a member of the genus Hepacivirus in the family Flavivirdae (Simmonds et al. 1993) (Figure 4). The HCV 9.6-kb genome is composed of 5' and 3' untranslated regions (UTRs) and an open reading frame (ORF), which is translated into a polyprotein via an internal ribosome entry site (IRES) in the 5' UTR. The polyprotein is then cleaved into structural proteins (core, E1 and E2) and nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) (Figure 5). The liver-specific microRNA miR-122 binds to 5'-UTR and regulates HCV translation and replication.

Researchers used sequence analysis of HCV NS5, core, E1, and 5'UTR to classify HCV into seven major genotypes (Figure 6) with multiple subtypes in each genotype, among which 1a, 1b, 3a and 4a are the most prevalent worldwide. Genetic analysis of genotypes and subtypes from large population sizes of HCV revealed that the worldwide spread of HCV started from Africa decades before HIV spread from the 1980s (Simmonds. 2013).

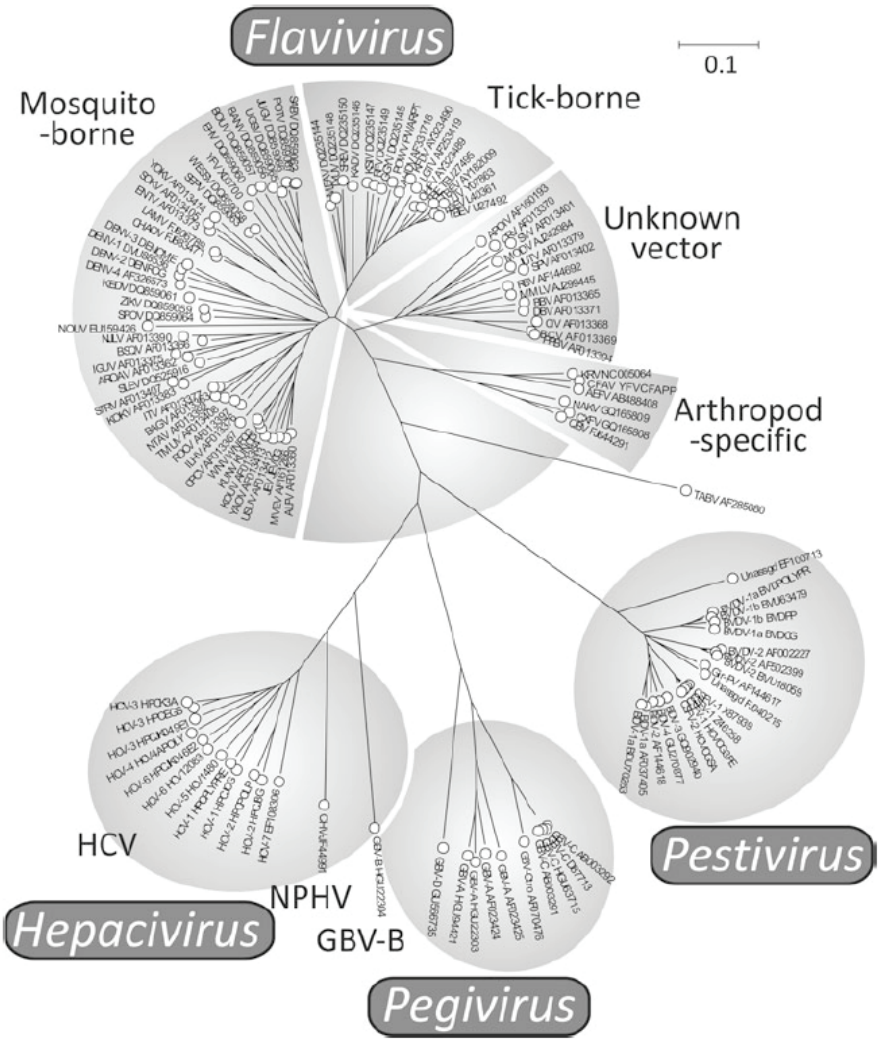


Figure 4. Phylogenetic tree of members of the family Flaviviridae (Simmonds. 2013)

The origin of HCV has been an enigma for a long time. Recently, several viruses (NPHV, RHV and BHV) similar to HCV have been discovered in nonprimate species such as horses, dogs and bats (Kapoor et al. 2011; Kapoor et al. 2013; Quan et al. 2013). Although the new findings shed light on HCV evolution, further investigations are needed to determine whether HCV indeed transferred into humans from bats or rodents thousands of years ago.

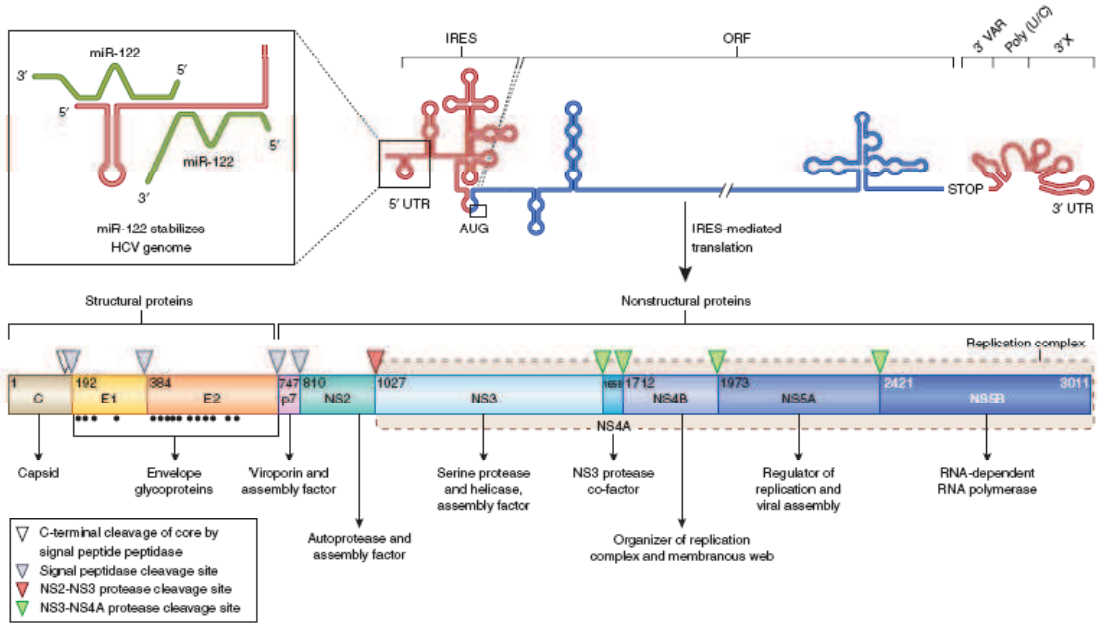


Figure 5. HCV genome (Scheel et al. 2013)

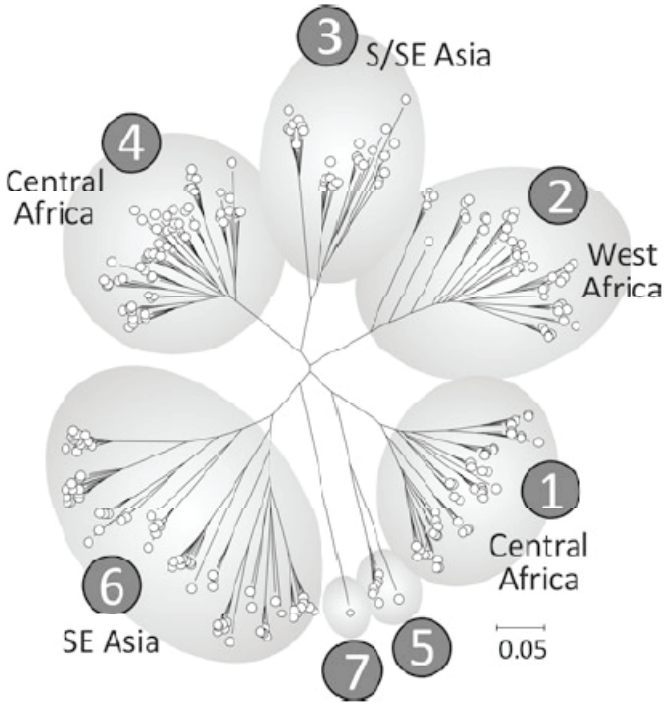


Figure 6. Evolutionary tree of HCV genotypes 1-7 (Simmondes. 2013)

6. HCV cell culture models

Due to the difficulty of infecting cell lines with serum from HCV-infected patients, researchers generated several model systems to study different aspects of the HCV life cycle. Subgenomic viral RNA encoding a selection marker (neomycin resistance gene) under the control of the HCV IRES and HCV NS3-NS5B under the control of the IRES from encephalomyocarditis virus (EMCV) in 1999 (Lohmann et al. 1999) (Figure 7). High HCV replication level was observed after transfecting the subgenomic viral RNA into hepatoma line Huh7. The replicon cell lines maintained HCV RNA for years under selective pressure. The HCV replicon system is mainly used to study HCV replication. The most successful model to investigate HCV entry is the HCV pseudoparticle (HCVpp) model. HCVpp are generated by co-transfecting three plasmids encoding the HCV envelope glycoproteins E1E2, the gag-pol of either murine leukemia virus (MLV) or human immunodeficiency virus (HIV) and a reporter gene (e.g. luciferase) into 293T cells (Bartosch et al. 2003; Hsu et al. PNAS 2003). The pseudoparticles containing HCV E1E2 envelope proteins interact with HCV receptors to enter into the host cells. The viral entry is assessed by the expression of a reporter gene. A series of host entry factors (e.g. claudin-1 (CLDN1), EGFR, EphA2, the Niemann-Pick C1-like cholesterol adsorption receptor (NPC1L1)) have been identified using this pseudoparticle system. However, the fact that these pseudoparticles are not generated in liver-derived cells and lack association with lipoproteins limits the usage of this model to study interactions of lipoproteins and host factors.

In 1999 a genotype 2 HCV strain named “JFH-1” was isolated from a Japanese patient suffering from fulminant hepatitis C, leading to a breakthrough in the attempts to establish an infectious HCV cell culture model. In contrast to other viral strains, JFH-1 subgenomic replicons replicated efficiently without additional adaptive mutations (Kato 2001). Transfecting wild-type full-length JFH1 RNA into hepatoma cells (Huh7) produced infectious viral particles, which closely resembled serum-derived viruses and allowed to infect relevant animal models *in vivo* (chimpanzees and human liver uPA-SCID mice) (Wakita et al. 2005; Lindenbach et al. 2006) (Figure 7). JFH1 chimeras containing JFH1 replicase genes (NS3-NS5B) and non-replicase genes (Core-NS2) from other HCV stains (e.g. J6) replicated more robustly than JFH1 and are, therefore, widely used in HCV cell culture today. HCV

recombinants comprising 5'UTR—NS5A from other genotypes 1—7 and NS5B—3'UTR from JFH1 allowed to broaden the JFH1 model to other HCV genotypes and investigate the antiviral activity against different genotypes of HCV (Li et al. 2014; Steinmann et al 2013). Of note, expressing HCV structural proteins in *trans* in packaging cell lines complemented HCV replicons to produce single-round infectious HCV trans-complementary particles (HCV_{TCP}) (Ishii et al. 2008; Steinmann et al. 2008; Adair et al. 2009), which can be used to study first-round of HCV infection.

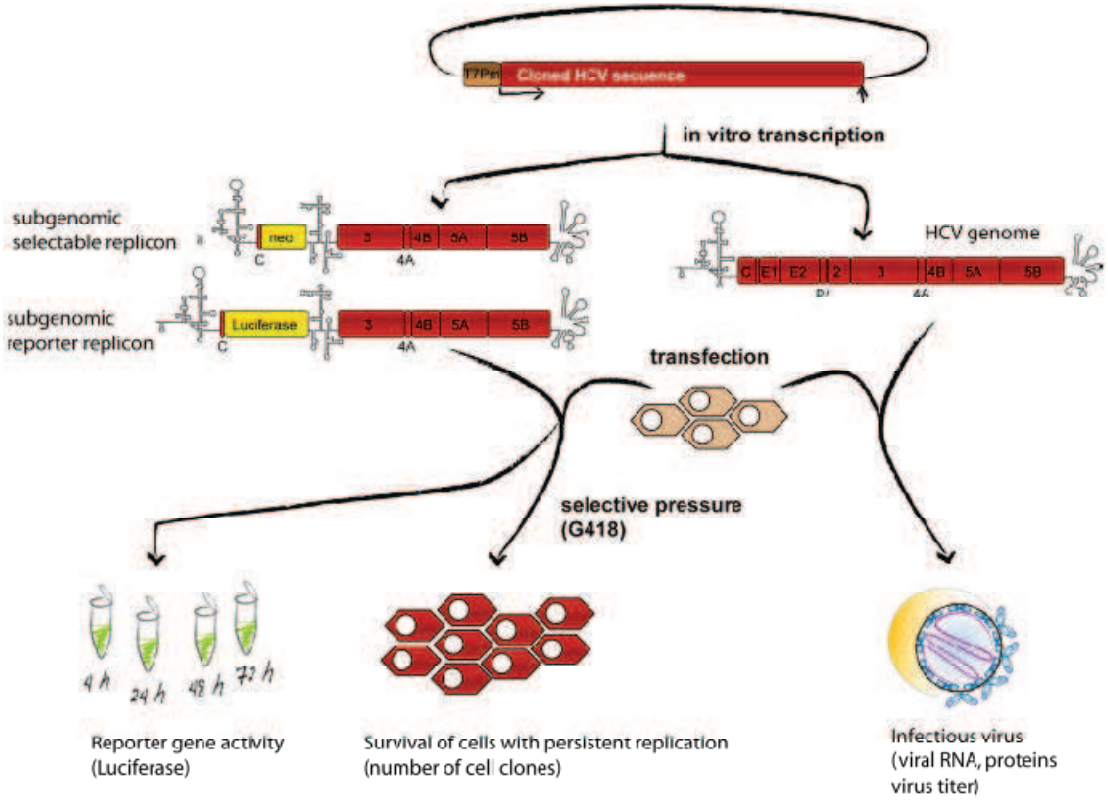


Figure 7. HCV cell culture model (Lohmann et al. 2014).

7. HCV animal models

Being genetically closest to human, chimpanzees (Pan troglodytes) have served as HCV animal model for over 20 years. HCV acute infection in chimpanzee causes necroinflammation in the liver and the infection persists in 60% of chimpanzees after acute infection. The pathogenesis of HCV infection in chimpanzees is milder than that in patients. Many preclinical studies were done with chimpanzees, providing important data to elucidate immune response and evaluate drug efficiency. However, the high costs to handle and the ethical concerns limit the use of chimpanzees in a large scale. Many countries have banned the experiments with chimpanzees (Billerbeck et al. 2013). HCV infection was also observed in a non-rodent small mammal, the tree shrew (Tupaia belangeri). Still, the variable infection rate and the rare animal resource limit the further development of this animal model.

Recently, mouse models supporting HCV infection were established and are now widely used. To overcome the absent susceptibility of mouse hepatoctyes to HCV, human primary hepatocytes were transplanted into uPA-SCID mice, which have liver destruction driven by the uPA transgene and immune deficiency due to the SCID background. The human liver chimeric uPA-SCID mice can be infected with HCV from cell culture or patients, enabling researchers to develop anti-HCV drugs and study HCV pathogenesis *in vivo* (Barth et al. 2008). Another human liver mouse model with another immunodeficiency and inducible liver degeneration background $Fah^{-/-}Rag2^{-/-}$ $IL2rg^{-/-}$[FGR] was also developed. The advantage of this FGR mouse model is that the timing of inducing liver degeneration and engrafting primary human hepatocytes can be controlled and planned. The absence of immune system is the major shortcoming of the human liver immunosuppressed mice model. Generating a mouse model with both human liver and human immune system thus represents the future direction. Furthermore, a transgenic humanized model has also been developed. The expression of human CD81 and OCLN in the mouse liver enabled HCV entry into mouse hepatocytes in vivo (Dorner et al. 2011). Then, abrogating innate immune genes IRF1, IRF7, IFN receptors and STAT1 allowed HCV replication at low level in mice (Dorner et al. 2013). However, the undetectable level of HCV RNA 90 days after infection indicates this transgenic mouse model is still in its infancy and needs further development.

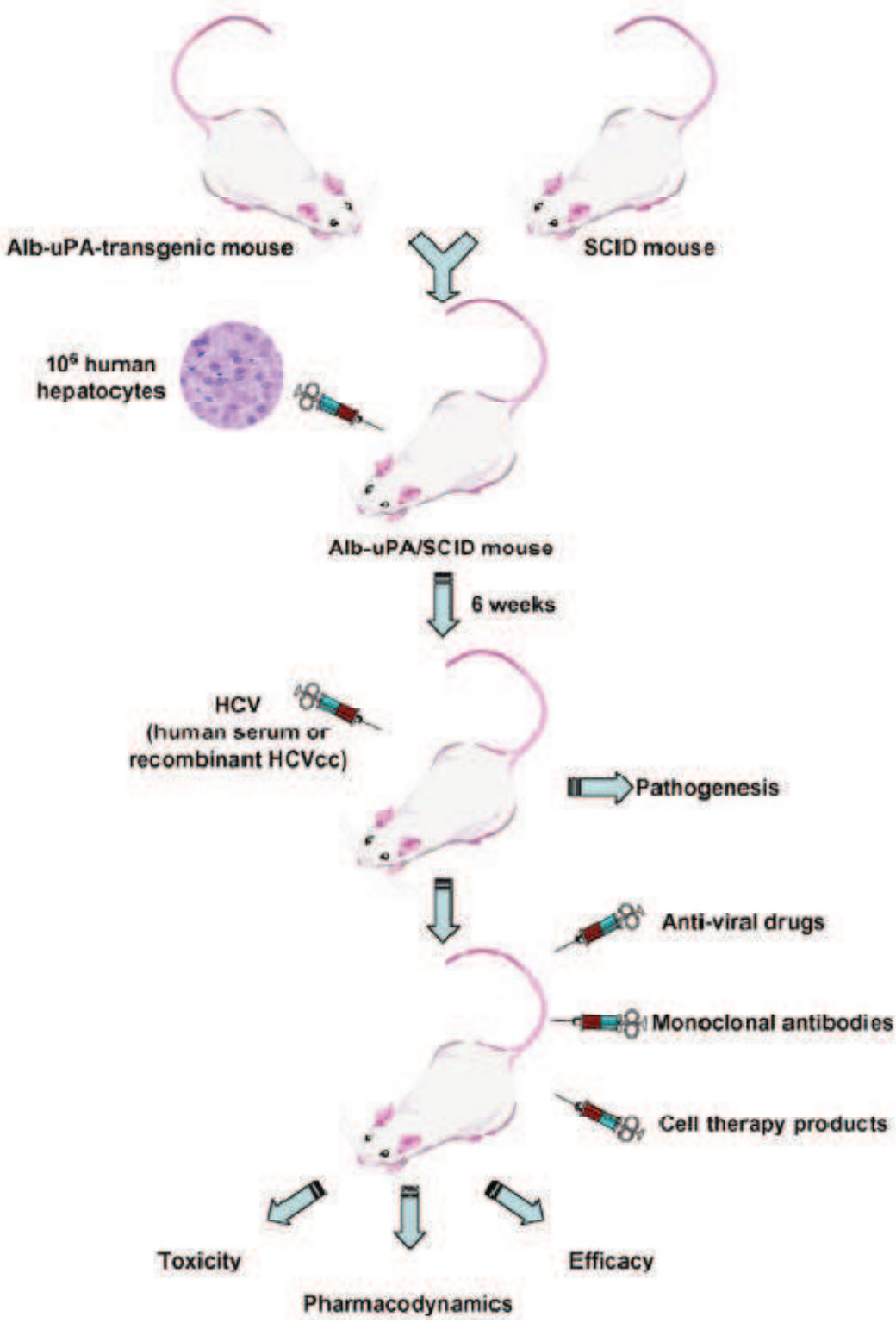


Figure 8. Human liver uPA-SCID mouse model with HCV infection (Barth et al. 2008)

8. HCV entry and cell-cell transmission

HCV entry is the first step of virus-host interactions orchestrated by multiple viral and host factors. The infectious viral particle is composed of a capsid containing the HCV RNA, surrounded by the envelope glycoproteins E1E2 in a lipid envelope derived from host cells (Figure 9). Since HCV has a specific tropism for human hepatocytes, it has been postulated that there are defined molecules expressed on human hepatocytes regulating HCV entry.

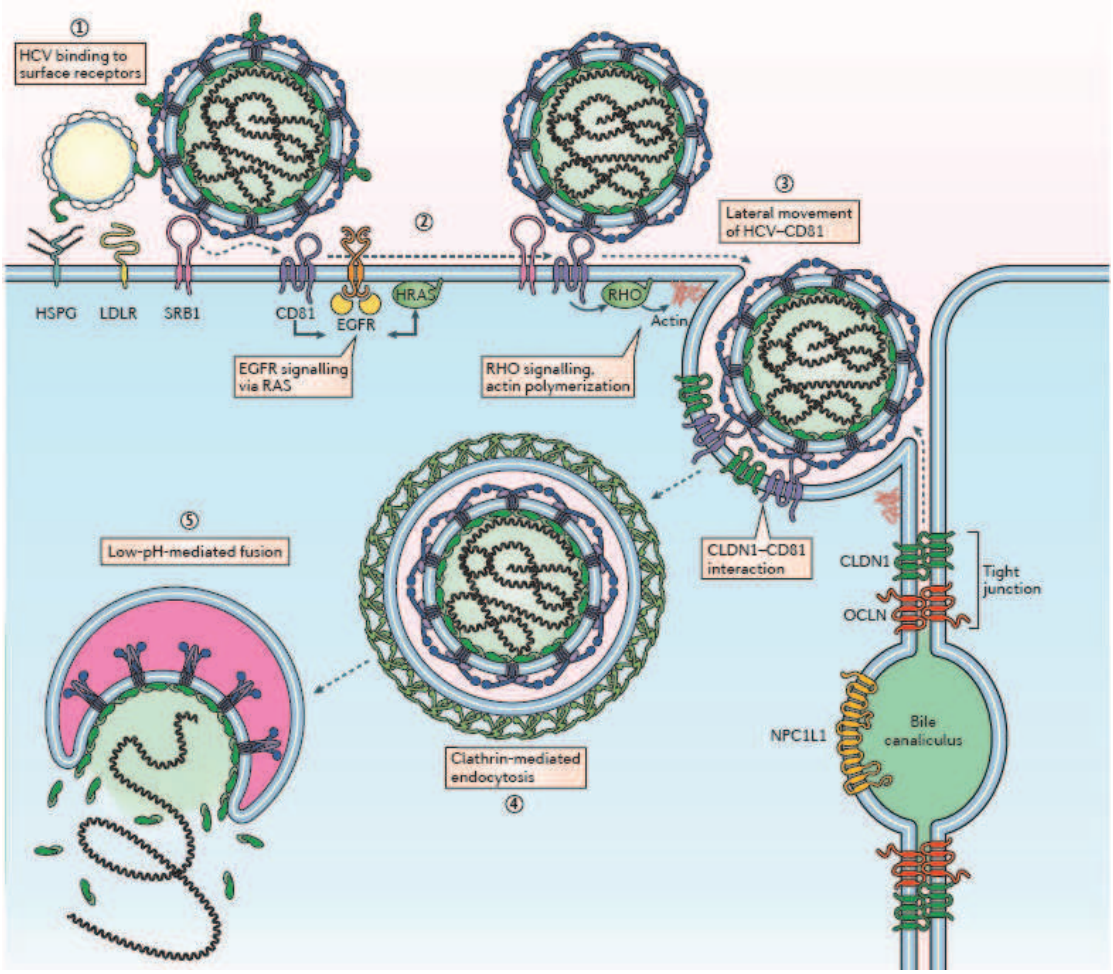


Figure 9. HCV entry (Lindenbach et al. 2013).

Using various models, several host factors have been discovered to mediate HCV entry in the past few years. Low-density-lipoprotein receptor (LDLR) and glycosaminoglycans (GAGs) are believed to first initiate attachment of HCV to the

host cell membrane (Agnello et al. 1999; Germi et al. 2002; Monazahian et al. 1999). LDLR also has a role in cholesterol metabolism and HCV replication (Albecka et al. 2012). Viral attachment is followed by receptor-mediated endocytosis. The first identified HCV receptor was CD81, which was discovered to directly bind to purified recombinant HCV envelope protein E2 (Pileri et al. 1998). Subsequently, scavenger receptor type B class I (SR-BI) was first identified as another E2 binding partner and to also contribute to HCV entry at post-binding steps (Scarselli et al. 2002; Barth et al. 2005; Bartosch et al. 2005; Voisset et al. 2005; Zeisel et al. 2007; Barth et al. 2008; Dao Thi et al. 2012; Zahid et al. 2013). Using the HCVpp system and a cDNA library from an HCV permissive hepatoma cell line (Huh7.5), Evans et al. discovered that CLDN1 conferred HCV permissiveness in human embryonic kidney cells (293T), indicating that CLDN1 is a key host factor for HCV entry (Evans et al. 2007). Different from CD81 and SR-BI, instead of binding to HCV glycoproteins, CLDN1 associates with CD81 to form a CD81-CLDN1 co-receptor complex on the membrane to mediate HCV entry (Harris et al. 2010; Krieger et al. 2010). CLDN1 is a tight junction protein and highly expressed at tight junction sites in polarized cells. Mutagenesis studies demonstrated that mutations delocalizing CLDN1 from the cell membrane impair HCV entry, indicating the localization of CLDN1 is important for HCV entry (Liu et al. 2009). Among the members of the CLDN family of proteins, CLDN6 and CLDN9 also are able to associate with CD81 to support HCV entry. However, the role of CLDN6 and CLDN9 appears not to play a major role in HCV entry due to their low expression in human hepatocytes (Fofana et al. 2013). Using a similar cDNA expression and screening strategy in mouse hepatic cells, another tight junction protein, occludin (OCLN), was identified as an entry factor defining the HCV species tropism together with CD81 (Ploss et al. 2009). Some studies suggested that the HCV E1E2 heterodimer but not E1 or E2 alone may interact with OCLN (Douam et al. 2014). However, further studies need to be done to confirm whether OCLN directly interacts with the HCV envelope or serves as an indirect co-receptor. This HCV-host factor interplay at the plasma membrane ultimately leads to clathrin-mediated endocytosis, during which viruses are internalized in clathrin-coated vesicles and transported into early and late endosomes. The low pH in endosomes induces fusion of the viral envelope and the endosomal membrane (Takikawa et al. 2000).

Disruption of CD81-CLDN1 complexes by protein kinase A inhibitors suggest that specific signaling pathways are necessary for HCV entry (Farquhar et al. 2008; Lupberger et al. 2011; Zona et al. 2013). HCV E2 binding to CD81 has been shown to activate the Rho GTPase family members to assist the HCV-CD81 movement to cell-cell contact through regulating actin filaments (Brazzolli et al. 2008). Screens using a siRNA library targeting host cell kinases identified multiple host factors forming a cell kinase network involved in HCV entry. EGFR and EphA2 were further uncovered as auxiliary co-factors promoting HCV entry by mediating formation of CD81-CLDN1 complex and viral fusion (Lupberger et al. 2011).

Clinical observation of iron accumulation in the liver of HCV patients led to the discovery that transferring receptor 1 (TfR1) responsible for iron uptake is a host factor for HCV entry. Finally, a cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1) was identified as an HCV entry factor probably involving cholesterol uptake. Although a multitude of entry factors have been identified (Figure 10), further studies are needed to understand how these molecules work cooperatively to mediate HCV entry.

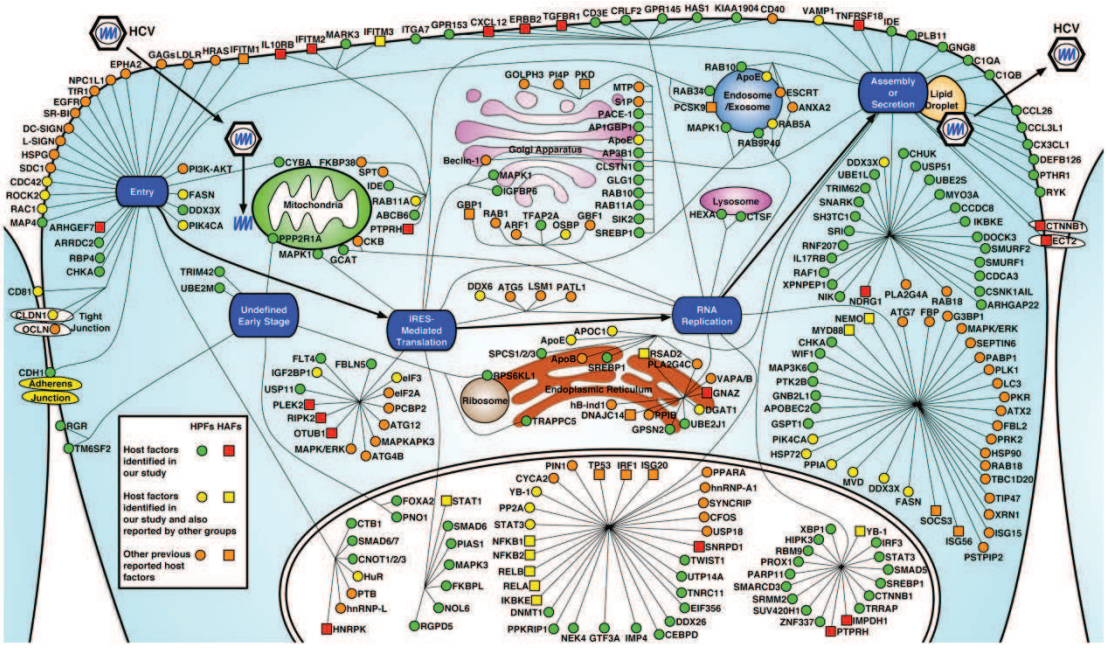


Figure 10. HCV host factors (Li et al. 2014)

Viruses spread within a host through two transmission routes: cell-free transmission and cell-cell transmission (Figure 11). For cell-free transmission, viruses released from the host cells need to diffuse to reach the target cells and can be eliminated by neutralizing antibodies and other immune elements such as complement present in viral traveling routes. Virus-infected cells preferentially deliver virions to adjacent uninfected cells through cell-cell contacts. Cell-cell transmission not only obviates viral diffusion and rate-limiting steps of viral entry (e.g. viral attachment) but also enables the virus to evade direct immune responses. It has been reported that viruses use different mechanisms of cell-cell transmission, such as cell-cell membrane fusion (herpesviruses, paramyxoviruses and retroviruses), crossing tight junctions (herpesviruses); movement across neural synapses (herpesviruses, paramyxoviruses and rhabdoviruses), actin- or tubulin- structures (herpesviruses), formation of nanotubes and virological synapses (HIV-1) (Sattentau. 2008) (Figure 12). HCV spread takes place in the presence of neutralizing antibodies indicating the existence of neutralizing antibodies-resistant HCV cell-cell transmission (Timpe et al. 2008). Most of the entry factors such as CD81, CLDN1, SR-BI are also required for HCV cell-cell transmission, suggesting targeting entry factors can inhibit HCV cell-cell transmission (Timpe et al. 2008; Zahid et al. 2013; Fofana et al. 2013). CD81-independent cell-cell transmission has been reported (Timpe et al. 2008; Jones et al. 2010), however, the efficient inhibition of cell-cell transmission by anti-CD81 mAb argues its role in HCV spread (Fofana et al. 2013). The resistance of HIV cell-cell transmission to antiretroviral therapy indicates the limitations of current treatments in inhibiting virus cell-cell transmission (Sigal et al. 2011). Therefore, developing novel strategies to block cell-cell transmission is important to control viral spread and enhance the efficacy of current antiviral therapies.

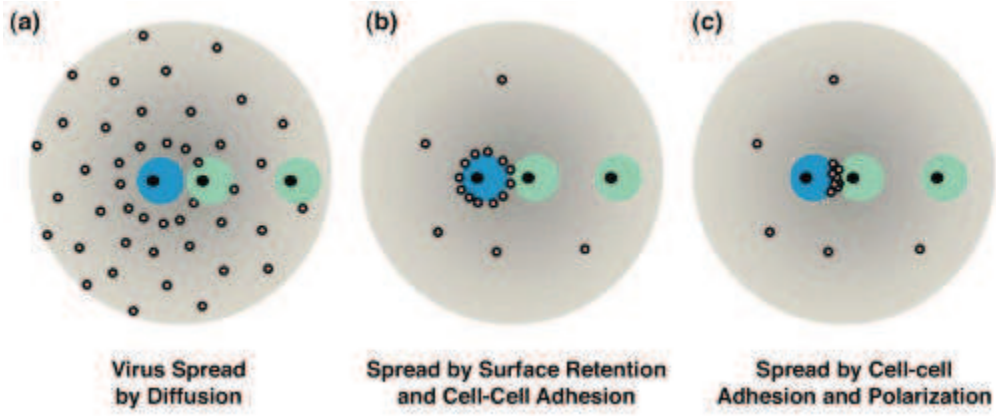


Figure 11. Cell-free transmission and cell-cell transmission (Zhong et al. Curr Opin Virol 2013)

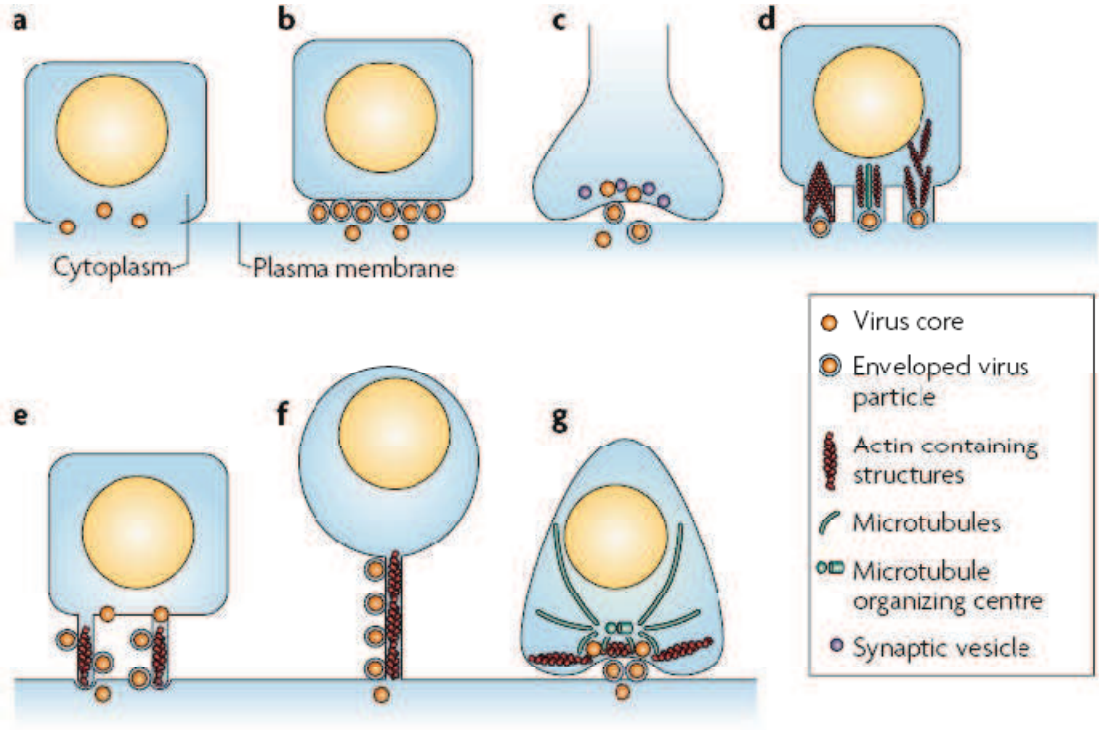


Figure 12. Different mechanisms of virus cell-cell transmission (Sattentau. 2008)

9. HCV translation, replication and assembly

After fusion, the virus is decapsidated and the viral genome is released into the cytoplasm for initiation of viral translation and replication. HCV positive sense RNA serves as a template for direct translation. The IRES in the 5'-UTR directs the cap-independent translation at the ER. The IRES binds to a ribosome 40S unit to form a

48S complex together with the initiation factors (eIF)3, eIF2 and eIF5 and then assemble into a 80S ribosome. The HCV ORF is translated into a polyprotein precursor, which is then processed by cellular and viral proteases into structural and nonstructural proteins (Moradpour et al. 2013) (Figure 13). HCV replicates in NS4B-induced dot-like structures in the cytoplasm, named membranous web, which is thought to originate from ER. Multiple viral determinants and host factors form the replication complex to initiate replication. The 3'-UTR and 5'-UTR have cis-acting RNA elements to initiate synthesis of complementary RNA strands with the catalytic activity of the viral RNA-dependent RNA-polymerase NS5B. Several host factors including the human VAMP-associated protein (hVAP) A, hVAP-B and cyclophilins participate in HCV RNA synthesis (Lohmann. 2013). Recently, the microRNA miR-122 has been discovered as an essential host factor for HCV replication. It binds to the 5'-UTR to prevent RNA degradation by RNAses and evade innate immune responses (Machlin et al. 2011). miR-122 is also involved in forming miR-122-protein complexes to stimulate RNA translation (Niepmann. 2013). After completion of the above steps, viral core protein E1E2 is trafficked to cytosolic lipid droplets and then packages the viral genome to assemble new nascent viral particles, which mature through the secretory pathway (Lindenbach et al. 2013).

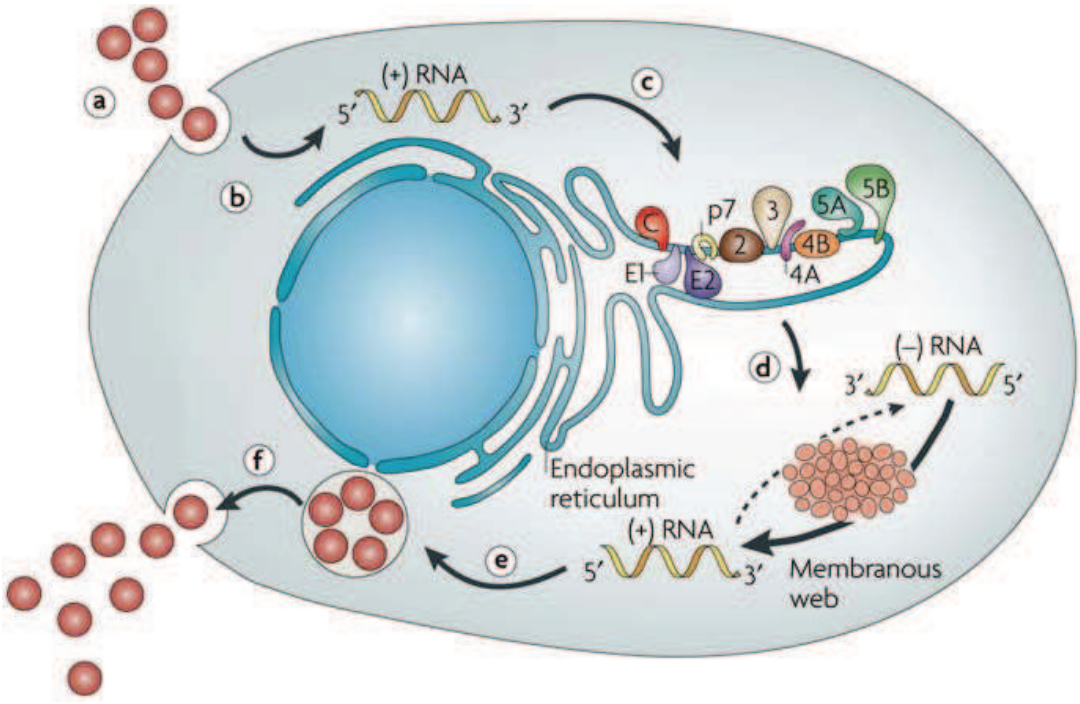


Figure 13. HCV life cycle (Moradpour et al. 2013)

10. Innate and adaptive immune responses to HCV

The innate immune response is the fist line of defense against HCV. Its role is to sense HCV and initiate local antiviral responses in the infected cells and recruit immune cells to mediate adaptive responses. HCV RNA is sensed mainly by RIG-I like receptors (RLRs) including RIG-I, MDA5 and LGP2, protein kinase R (PKR) and Toll-like receptor 3 (TLR3). Recogition of RNA by RIG-I and MDA5 activates caspase recruitment domains (CARDs), which activate mitochondrial antiviral signaling protein (MAVs) and downstream signaling to induce IFN. The virus evolved to evade innate immunity resulting in chronic infection. The viral NS3-NS4A plays a centrol role in the viral evasion process by blocking RIG-I signaling and cleaving MAVS. Moreover, HCV proteins antagonize IFN-induced JAK-STAT signaling and the expression of interferon-stiumlated genes (ISGs) to suppress innate immune responses (Horner et al. 2013).

The adaptive immune responses, consisting of neutralizing antibody as well as HCV-specific CD4+ and CD8+ T cell responses, are important for the outcome of HCV infection. Anti-HCV antibodies targeting different viral proteins are detected at an early phase of infection. However, not all anti-HCV antibodies exhibit a neutralizing capacity. Moreover, the virus is able to evade from the neutralizing antibody responses due to its error-prone replication process which results in high variability in the E1E2 region (von Hahn et al. 2007). Therefore, HCV persists despite of detection of anti-HCV neutralizing antibodies in patients with chronic hepatitis C. A study invesigating a cohort of HCV patients, who were infected with HCV from a single-source outbreak through contaminated immunoglobin injection, indicated that an early development of neutralizing antibodies may contribute to clearance of acute HCV infection and delayed presence of neutralizing antibodies may lead to chronic infection (Pestka et al. 2007). The presence of neutrilizing antibodies was observed to be associated with low viral load, indicating their role in controling HCV viremia (Lavillette et al. 2005). Potent multi-epitope-specific $CD4^+$ and $CD8^+$ T cell responses were observed in patients spontaneously resolving from acut HCV infection, indicating broad and strong T cell responses during acute hepatitis C is associated with viral clearance (Zeisel et al. 2008). HCV-specific $CD4^+$ T cells exert regulatory function by assisting $CD8^+$ T to clear infected hepatocytes and activating B cells to produce neutralizing antibodies. The rapid disappearance of this reponse with loss of

$CD4^+$ T helper cells is associated with chronic infection (Semmo et al. 2005). $CD8^+$ T cells can recognize antigens presented by HLA class I molecules on HCV-infected cells and induce cytotoxicity to the target cells through secreting perforin and secrete antiviral cytokines. In HCV patients, $CD8^+$ T cells appear 6-8 weeks after HCV infection and cause liver injury. Viral mutations at HLA class I binding sites prevent antigen recognition and help viral evasion from $CD8^+$ T cell response.

11. Treatment of chronic hepatitis C

Hepatitis C is a curable infection. The aim of antiviral therapy is to achieve a sustained virological response (SVR), which is defined as continued undetectable HCV RNA 24 weeks after the end of the treatment. According to long-term observations, 99% of patients achieving SVR eradicate HCV infection. IFN has long been the cornerstone of antiviral treatment against HCV. As a potent innate immune therapeutic, IFN-α exerts its antiviral effect by inducing ISGs to inhibit HCV replication. IFN monotherapy was first applied to treat HCV infection in the late 1980s and resulted in a 15-20% SVR rate in chronic hepatitis C patients. Ribavirin (RBV) enhanced IFN-α's antiviral effect and has been used in combination with IFN-α to treat HCV infection since 1998. Clinical trial data showed that 35-45% of patients with chronic hepatitis C achieved SVR with the combination of IFN-α and RBV. Then, pegylated IFN-α (PEG-IFN-α) with a longer half-life and higher efficacy was developed and replaced the standard IFN-α. Combination of PEG-IFN-α and RBV has been used as the SOC for the treatment of chronic hepatitis C for more than 10 years. Polymorphism of IFNL3, a human gene of innate immunity, was identified as a predictor for the outcome of HCV infection and the posibility of response to IFN-based therapy (Suppiah et al. 2009; Tanaka et al. 2009; Thomas et al. 2009; Ge et al. 2009). Patients with different genotypes respond differently to the combination of IFN-α and RBV, which yields SVR in 80% of patients infected with HCV genotype 2-3 but only in 40-50% of patients infected with HCV genotype 1. IFN-α/RBV is also associated with a series of side effects including fatigue, headache, fever, depression and anaemia. More than 10% of patients don't tolerate the side effects and stop the treatment (Sarrazin et al. 2012). Other forms of IFNs, such as IFN-γ, which probably have higher efficacy and fewer side effects than IFN-α, are currently under investigation in clinical trials.

The establishment of HCV cell culture systems has advanced our understanding of the viral life cycle and lead to the development of direct-acting antivirals (DAAs) targeting viral replication. Compounds against the HCV NS3/NS4A protease, the NS5B polymerase and NS5A have been developed. NS3 and NS4A have serine protease activity responsible for cleaving the HCV polyprotein into four non-structural proteins. BILN2016 was the first protease inhibitor targeting NS3/NS4A and its function in inhibiting HCV replication was proved in clinical trials. However, its cardiotoxicity precluded its further development. Telaprevir and boceprevir were the first protease inhibitors licensed by the FDA in 2011 for the treatment of HCV genotype 1 infection. In phase 3 clinical trials, combination of telaprevir, PEG-IFN-α and RBV improved SRV to 75% compared with bitherapy. PEG-IFN-α and RBV in combination with telaprevir or boceprevir has become the new SOC for HCV genotype 1 infection in Europe and the United States. Recently the 2nd wave protease inhibitor simeprevir and the polymerase inhibitor sofosbuvir have received FDA approval for the treatment of HCV genotype 1 infection in combination of PEG-IFN-α and RBV. Of note, sofosbuvir, which has pan-genotypic activity, has also been approved for HCV genotype 2 and 3 infection in combination with RBV. An all-oral IFN-free regiment represents the future direction for HCV therapy. Although the rapid development of DAAs greatly improves the outcome of patients with chronic hepatitis C, there are still obstacles for curing all the patients. First generation protease inhibitors have a low genetic barrier to resistance and therefore select resistant viruses a few days after treatment. Some mutations induce cross resistance to different DAAs. Furthermore, since protease inhibitors telaprevir and boceprevir are metabolized by Cyp3A enzyme, which is responsible for metabolism of many drugs, they are contraindicated with many drugs such as statins, antidepressants and immunosuppressors, restricting the usage of these DAAs in some special subgroups of patients.

Novel HCV replication inhibitors are in the late stage of clinical development. Alispovivir, which targets cyclophilin A, a host factor for HCV replication, has entered phase 3 clinical trial and showed potent antiviral effect with high genetic barrier to resistance. miR-122 antagonist, miravisen, is also being investigated in clinical trials (Pawlotsky. 2013).

The discovery of HCV entry factors enabled the generation of entry inhibitors to inhibit viral entry, the first step of the virus life cycle. Our lab and other groups have generated a series of inhibitors targeting HCV entry. These include anti-CD81 mAbs (Fofana et al. 2013), anti-CLDN1 mAbs (Fofana et al. 2010), anti-SR-BI mAbs (Zahid et al. 2013) and the kinase inhibitors erlotinib and dasatinib (Lupberger et al. 2011). *In vitro* and *in vivo* experiments showed that these entry inhibitors efficiently prevented HCV infection in a genotype-independent manner.

Aims of the study

HCV infecting 170 million people worldwide poses a threat to global health. Although the cure rate has been improved with the recent development of DAAs, there is still a long way to go to cure all the patients. Discovery of HCV entry factors lead to the generation of entry inhibitors blocking HCV entry into host cells. Our lab and other groups have generated a series of entry inhibitors and shown that entry inhibitors prevent HCV infection with pan-genotypic activity. From entering a host cell to producing de novo virions, HCV needs multiple steps to complete its life cycle and to maintain its infection. Targeting complementary steps of the viral life cycle represents an interesting therapeutic strategy as this may enhance the antiviral effect and increase the genetic barrier to viral resistance.

In the first part of my thesis, we set out to generate novel strategies for the prevention and treatment of HCV infection by combining entry inhibitors and DAAs. State-of-the-art cell culture and mouse models were used to evaluate the efficacy of novel combination therapies. By assessing the efficacy of different combinations, we aimed to screen out several potent combinations, which might have clinical implications in the future.

Although DAAs are efficient in inhibiting HCV replication, most of them have low genetic barrier to resistance and result in emergence of DAA-resistant viruses and treatment failure. Viral cell-cell transmission is an important transmission route since it obviates early steps of viral entry and enables the virus to escape neutralizing antibodies. In the second part of my thesis, we thus investigated the role of cell-cell transmission role in the spread of DAA-resistant viruses and explored novel strategies to prevent the emergence of resistant viruses through blocking cell-cell transmission using entry inhibitors.

Results

Part 1:

Synergy of entry inhibitors with direct-acting antivirals uncovers novel combinations for prevention and treatment of hepatitis C

Introduction

The goal of the first part of my PhD was to assess the potential of novel therapies for prevention and treatment of HCV infection. Although triple therapies combining PEG-α, RBV and a DAA have improved cure rate of HCV to more than 90%, there is still an unmet medical need to develop more efficient IFN-free therapies to cure all the patient infected with HCV. As multiple HCV entry factors have been discovered in the past few years, targeting entry factors becomes a novel strategy to prevent and treat HCV infection. Our laboratory has developed and characterized a series of entry inhibitors, which have been shown to have potent efficacy in prevention of HCV infection.

Applying these entry inhibitors and newly developed DAAs, we performed a screening to test combinations of entry inhibitors and DAAs in HCV cell-culture models and used both the calculation of the combination index and the Prichard 3D model to assess the efficiency of each individual combination. The results showed that all the tested combinations of an entry inhibitor and a DAA showed synergistic effect in preventing HCV infection with different potency, while combing two DAAs only resulted in an additive effect, suggesting that targeting complementary steps of the viral life cycle is a promising strategy to prevent HCV infection. Furthermore, we differentiated hepatoma cells with 1% DMSO and established HCV persistent infection in cell culture. Noteworthy, combination of an entry inhibitor and a DAA also demonstrated a synergistic effect to treat persistent HCV infection. Finally, we validated this finding using the human-liver uPA-SCID mouse model.

According to the data from our study, some promising combinations with highest efficacy and safety profile are highlighted for HCV prevention and treatment in future clinical trials. In summary, this study provides novel therapeutic strategies for prevention and treatment of HCV. The results from this study have been published in *Gut*.

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Synergy of entry inhibitors with direct-acting antivirals uncovers novel combinations for prevention and treatment of hepatitis C

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Keywords: host-targeting agents; resistance; transplantation; treatment

Abbreviations

CC: cytotoxic concentration; CI: combination index; CLDN1: claudin-1; CTRL: control; DAA: direct-acting antiviral; EGFR: epidermal growth factor receptor; HA: human albumin; HCV: hepatitis C virus; HCVcc: cell culture-derived HCV; HIV: human immunodeficiency virus; HTA: host-targeting agent; IC: inhibitory concentration; IFN: interferon; LOQ: limit of quantification; LT: liver transplantation; mAb: monoclonal antibody; MOA: mechanism of action; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PHH: primary human hepatocyte; PKI: protein kinase inhibitor; RBV: ribavirin; SOC: standard-of-care; SR-BI: scavenger receptor B type I.

Licence for Publication

ABSTRACT

Objective: Although direct-acting antiviral agents (DAAs) have markedly improved the outcome of treatment in chronic hepatitis C virus (HCV) infection, there continues to be an unmet medical need for improved therapies in difficult-to-treat patients as well as liver graft infection. Viral entry is a promising target for antiviral therapy. **Design:** Aiming to explore the role of entry inhibitors for future clinical development, we investigated the antiviral efficacy and toxicity of entry inhibitors in combination with DAAs or other host-targeting agents (HTAs). Screening a large series of combinations of entry inhibitors with DAAs or other HTAs, we uncovered novel combinations of antivirals for prevention and treatment of HCV infection. **Results:** Combinations of DAAs or HTAs and entry inhibitors including CD81-, scavenger receptor B type I (SR-BI)- or claudin-1 (CLDN1)-specific antibodies or small-molecule inhibitors erlotinib and dasatinib were characterized by a marked and synergistic inhibition of HCV infection over a broad range of concentrations with undetectable toxicity in experimental designs for prevention and treatment both in cell culture models and in human liver-chimeric uPA/SCID mice. **Conclusion:** Our results provide a rationale for the development of antiviral strategies combining entry inhibitors with DAAs or HTAs by taking advantage of synergy. The uncovered combinations provide perspectives for efficient strategies to prevent liver graft infection and novel interferon (IFN)-free regimens.

Significance of this study

What is already known on this subject?

DAAs increase the response to IFN-based antiviral therapy against HCV genotype 1 but also lead to selection of drug-resistant HCV variants.

Given their important side effects and drug-drug interactions, DAAs against HCV are not approved for patients undergoing liver transplantation (LT), HCV/human immunodeficiency virus (HIV) co-infected patients or pediatric patients.

Although early clinical trials have demonstrated impressive outcomes for combinations of DAAs in IFN-free regimens for treatment naïve patients, there will be a need for novel antivirals addressing resistance, treatment of patients with co-morbidity, co-medication or immunosuppression and patients undergoing LT.

HCV entry into target cells is a promising target for preventive and therapeutic antiviral strategies.

What are the new findings?

Given their complementary mechanism of action, entry inhibitors inhibit viral infection in a synergistic manner in combination with DAAs both in cell culture models and in human liver-chimeric uPA/SCID mice.

Synergy between entry inhibitors and DAAs or other HTAs holds promise for a variety of possibilities of combination therapies for prevention of HCV liver graft infection and might be also useful for treatment of hepatitis C.

How might it impact on clinical practice in the foreseeable future?

Novel combinations based on synergy may widen the therapeutic arsenal against HCV infection for prevention of liver graft infection, treatment of difficult-to-treat-patients and provide alternatives for patients with contraindications to particular compounds of standard-of-care (SOC) or future IFN-free regimens.

INTRODUCTION

The current SOC in chronic genotype 1 HCV infection consists of pegylated IFN-α, ribavirin (RBV) and a protease inhibitor - telaprevir or boceprevir[1-3]. Furthermore, direct-acting antivirals (DAAs) sofosbuvir and simeprevir have been licensed very recently. Although expected to revolutionize HCV treatment by offering cure in the very large majority of treated patients[4], DAAs appear to have distinct limitations in certain difficult-to-treat patient subgroups, such as patients with advanced liver disease, transplant, HIV/HCV-coinfected and immune-compromised patients[2]. Hurdles in these groups include resistance and side effects[2, 4]. Furthermore, not all genotypes respond similar to DAA combinations and high costs limit access to therapy in low, middle and high income countries[2, 4].

Among the most difficult-to-treat patient groups are individuals with HCV-induced end-stage liver disease undergoing LT[1, 2]. Due to viral evasion from host immune responses and absence of preventive antiviral strategies, graft re-infection is universal. Moreover, IFN-based therapies have limited efficacy and tolerability in LT recipients[5]. The use of telaprevir and boceprevir is limited by drug-drug interactions if combined with immunosuppressive agents[6, 7]. Furthermore, emergence of resistant strains will certainly be higher in these patients because of lower efficacy of SOC and greater need for dose adjustments[5]. These challenges define a need for additional combinations of therapeutics, ideally targeting complementary steps of the viral life cycle, with improved efficacy and safety.

A promising antiviral target complementary to targets of SOC and DAAs is viral cell entry. HCV entry is the first step of virus-host cell interactions and is required for dissemination and maintenance of infection[8]. Viral entry plays an important role in the pathogenesis of HCV infection, especially during HCV re-infection of the graft after LT[9, 10]. Targets for entry inhibitors include CD81, SR-BI, tight junction proteins CLDN1 and occludin, epidermal growth factor receptor (EGFR)[8, 11] and Nieman-Pick C1-Like 1[12]. Entry inhibitors exhibit a broad pan-genotypic activity[10, 12-21]. By acting through a complementary mechanism of action (MOA), entry inhibitors may synergistically act with SOC. To explore the future application of entry inhibitors for combination therapy in IFN-free regimens, we investigated the antiviral efficacy of entry inhibitors in combination with DAAs or other HTAs in state-of-the-art cell culture models and in human liver-chimeric uPA/SCID mice.

MATERIAL AND METHODS

Cell lines. Huh7.5.1 cells and primary human hepatocyte (PHH) culture have been described[13].

Antibodies and inhibitors. CLDN1- (OM-7D3-B3)[22], SR-BI- (NK-8H5-E3)[14] and CD81- (QV-6A8-F2C4)[16] monoclonal antibodies (mAbs) have been described. Erlotinib and dasatinib were from LC Laboratories, IFN-α2a and IFN-α2b from Roche and Merck. Alisporivir, telaprevir, boceprevir, danoprevir, simeprevir, daclatasvir, mericitabine and sofosbuvir were synthesized by Acme Bioscience.

Analysis of antiviral activity of compounds and combinations on HCV infection. Each compound was tested individually or in combination with a second compound using the cell culture-derived HCV (HCVcc)-Huh7.5.1 model[14, 23]. Production of HCVcc has been described[13]. For prophylactic/prevention combination experiments, Huh7.5.1 cells were pre-incubated with IFN-α, DAAs or HTAs for 1h at 37°C before incubation for 4h at 37°C with HCVcc and both compounds. For therapeutic/treatment combination experiments, Huh7.5.1 cells were infected with HCVcc during 5d to establish chronic persistent HCV infection before adding compounds for additional 5d[23]. Viral infection was analyzed by assessing luciferase activity[10, 13, 22].

Analysis of synergy. Synergy was assessed by two independent methods: the Combination Index (CI)[24] and/or the method of Prichard and Shipman[25]. A CI <0.9, 0.9-1.1, and >1.1 indicates synergy, an additive effect, and antagonism, respectively[24]. For the method of Prichard and Shipman, a surface >20% above the zero plane indicates synergy and a surface <20% below the zero plane indicates antagonism[25]. Validity of the assay and methods were confirmed by non-synergistic or antagonistic combinations.

HCV cell culture persistence assay. HCV RNA (Jc1 or Luc-Jc1) was electroporated into Huh7.5.1 cells to establish persistent HCV infection. Then, 1% DMSO was used to differentiate the cells and maintain them in culture without passage for up to 20d[26]. Medium containing compounds at the indicated concentrations was

replenished every 4-5d until the end of the experiment. Luciferase activity or RT-PCR was used to monitor viral infection and viral load[27].

***In vivo* experimentation.** Human liver-chimeric uPA/SCID mice were transplanted with PHH at 3 weeks of age by intrasplenic injection of 10^6 cells suspended in PBS as described previously[28]. Successful engraftment was determined by measuring the human albumin (HA) concentration in the serum of transplanted mice by specific ELISA (Bethyl, Catalog No. E80-129). Mice with HA levels >1 mg/mL were used for IV inoculation with HCV Jc1-containing infectious mouse serum (6 $x10^3$ IU). Eight weeks later, the mice were allocated to different treatment groups. Mice received telaprevir (300 mg/kg) or vehicle (carboxymethylcellulose 0.5%, tween-80 0.2%) *per os* twice a day and were intraperitoneally injected with 500 µg of control or anti-SR-BI mAb (NK8-H5-E3) twice a week for 2 weeks. Blood was collected by retro-orbital puncture every 5-10d under isoflurane anesthesia for the determination of serum HCV RNA level and HA concentration. Experiments were performed in the Inserm Unit 1110 animal facility according to local laws and ethical committee approval (AL/02/19/08/12 and AL/01/18/08/12).

Toxicity assays. Huh7.5.1 cells and PHH were incubated with compounds for 48h and/or 5d[22, 23]. Cytotoxic effects were analyzed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay[13, 22] or PrestoBlue assay (Invitrogen) with flavopiridol or anti-Fas antibody as positive controls[22]. 50% cytotoxic concentrations (CC_{50}) of entry inhibitors were calculated by regression analysis.

Statistical analysis. Statistical analysis and Combinations Indexes estimations have been run under Bayesian paradigm. Results are given as mean and [95% credible interval]. Data were analyzed by IC (50/75/90). Group comparisons were based on the mean difference. Normality was assessed with a Shapiro-Wilk test. When required, data transformation was used to reach normality. Each data set was analyzed using hierarchical (mixed) model with fixed group effects and random treatment effect as described[29]. The whole dataset was analyzed using a two-stage hierarchical model, with the fixed group effects and two random effects which were treatment and IC (50/75/90), in order to take account of both levels of repeated measurements. Dummy variables, representing the IC studied (50/75/90) had also

been considered as fixed effects to test differences between CI in each case. For all of these models, uninformative priors for coefficients were used: Gaussian distributions with mean 0 and precision 0.001, Gamma distribution with parameters 0.1 and 0.1 for the model precision. Hyperpriors for random effects were also uninformative: Normal with mean 0 and precision 0.001, and a uniform distribution (0,100) for dispersion parameters. Assumption of homogeneous dispersions in random effects was respected. Computations were run with R 3.00 and WinBUGS 1.4. For each analysis, a single MCMC chain with 5000 iterations as burn-in and 100 000 iterations was used to generate the posterior distribution. Convergence was checked and present in every case. Unless otherwise stated, results are shown as means ± SEM from three independent experiments performed in triplicate. For the method of Prichard and Shipman, one representative experiment performed in triplicate is shown.

RESULTS

Synergy of entry inhibitors and DAAs uncovers novel combinations for IFN-free regimens. A major effort of current drug development is to develop IFN-free treatments based on the combination of DAAs with or without RBV[1]. Addressing these concepts, we studied the combined antiviral effect of entry inhibitors with clinically licensed protease inhibitors telaprevir[30, 31], boceprevir[32, 33], simeprevir[34] and danoprevir – a protease inhibitor in late-stage clinical development[35] using the HCVcc cell culture model. The antiviral effect of each molecule was tested alone or in combination to determine the Combination Index (CI). Combination of telaprevir or boceprevir with a sub-IC_{50} concentration of all entry inhibitors tested - which exerts only minimal inhibition on HCV infection - resulted in synergy with CIs of 0.48-0.71 at IC_{90} (figure 1A and supplementary table 1). Calculation of 95% credible intervals indicates that the differences between CI values did not occur by chance (figure 1A and supplementary table 1). Synergy was also observed at IC_{50} and IC_{75} (figure 1A and supplementary table 1). Inversely, combination of telaprevir and boceprevir was additive (CI of 0.94, 95% confidence interval [0.84-1.04]; figure 1A) confirming the validity of the assay. Combination of simeprevir or danoprevir with entry inhibitors resulted in synergy at all inhibitory concentrations (CIs of 0.06-0.65 at $IC_{90;}$ figure 1A and supplementary table 1), demonstrating the relevance of adding an entry inhibitor to improve antiviral efficacy in preventing HCV infection. Highly effective combinations included *inter alia* combinations of telaprevir with erlotinib (figure 2A), and boceprevir or simeprevir with anti-CLDN1 mAb (figure 2B-C).

A number of NS5A and polymerase inhibitors have reached early- to late-stage clinical development including clinical licensing of the first compounds. As the first NS5A inhibitor, daclatasvir[36] has shown potent antiviral activity against HCV genotype 1 in mono- and combination therapy[37, 38]. Marked synergy at all inhibitory concentrations was observed for combination of daclatasvir with entry inhibitors (CIs of 0.27-0.89 at IC_{90}; figure 1B, supplementary table 1C). Effective combinations included *inter alia* combinations of daclatasvir and anti-SR-BI, anti-CLDN1 mAbs or erlotinib decreasing its IC_{50} up to 60-fold (figure 3A-C).

Finally, we investigated synergy between entry inhibitors and the polymerase inhibitor sofosbuvir[39], which recently obtained FDA approval and has the potential

to become the cornerstone for an efficacious, all-oral combination regimen for many HCV-infected patients[40, 41]. Combination of sofosbuvir with entry inhibitors resulted in synergy at all inhibitory concentrations (CIs of 0.41-0.61 at IC_{90}; figure 1C, figure 4A-B, D-E and supplementary table 1). Potent combinations included *inter alia* combinations of sofosbuvir and anti-SR-BI or anti-CLDN1 mAbs, decreasing its IC_{50} up to 18-fold, and combinations of sofosbuvir with erlotinib or dasatinib decreasing its IC_{50} up to 210-fold (figure 4A-B, D-E). These data demonstrate the potential of combining sofosbuvir with entry inhibitors to improve its antiviral activity. Similar results were obtained when combining mericitabine[35] with entry inhibitors (CIs of 0.18-0.68 at IC_{90}; figure 1C and supplementary table 1).

To further confirm synergy over a broad range of concentrations of two compounds, we performed combinations of each DAA with a defined entry inhibitor, testing a full checker-board of compound dose-response curves[25]. Particularly, combinations of low doses of two compounds resulted in an antiviral effect well above the expected value (figure 2D-F, figure 3D-F and figure 4C, F). These results demonstrate that adding an entry inhibitor markedly increases the antiviral activity of different DAAs currently evaluated in IFN-free regimens, thereby defining novel antiviral combinations for further preclinical and clinical development in IFN-free regimens.

Combination of HTAs results in a synergistic antiviral effect. It is still not clear whether DAA-based therapies will be effective and safe in difficult-to-treat populations including patients with co-morbidity, complex co-medication, immunosuppression and patients undergoing LT. Combinations of HTAs are a promising alternative to DAAs for IFN-sparing regimens, allowing increasing the genetic barrier to resistance. The clinically most advanced HTA is cyclophilin A inhibitor alisporivir[42]. Combination of alisporivir with entry inhibitors resulted in a marked synergy (CIs of 0.19-0.69 at IC_{75} and 0.06-0.50 at IC_{50}; figure 5D and supplementary table 2). Effective combinations included *inter alia* alisporivir and erlotinib or anti-CLDN1 mAb (figure 5E). Indeed, very low concentrations of these entry inhibitors markedly increased the antiviral effect of alisporivir by decreasing its IC_{50} up to 3,000-fold. Synergy of alisporivir and anti-CLDN1 mAb was confirmed using the method of Prichard and Shipman (figure 5F). Taken together, these data

demonstrate the promise of combining HTAs acting on complementary steps of the viral life cycle and open perspectives for HTA-based DAA or IFN-free regimens. These combinations may be of particular interest for/to patients who are resistant to SOC, or cannot tolerate SOC or combinations of DAAs. Furthermore, combinations of two entry inhibitors resulted in synergy on HCVcc infection at all inhibitory concentrations (CIs of 0.13-0.68 at IC_{90}; figure 5A-C and supplementary table 2) except for combination of anti-CLDN1 and anti-CD81 mAbs that resulted in an additive effect (CI of 0.95, 95% credible interval [0.85-1.06] at IC_{90}; figure 5A).

Entry inhibitors potentiate the antiviral activity of IFN-α in a synergistic manner. Since IFN-α is the key component of current SOC, we also investigated whether HCV entry inhibitors potentiate the antiviral activity of interferon alfa (IFN-α) by combining an entry inhibitor with IFN-α2a or IFN-α2b. Combination of IFN-α2a or IFN-α2b with a sub-IC_{50} concentration of receptor-specific mAb resulted in a synergistic activity at IC_{90} in inhibiting HCVcc infection (CIs of 0.16-0.53) (supplementary figure 1 and supplementary table 3). Synergy combining IFN-α2a or IFN-α2b with erlotinib has been described elsewhere[23]. Synergy was also observed at IC_{50} and IC_{75} for all combinations tested (supplementary figure 1 and supplementary table 3). Conversely, in line with previously-reported data[43], combination of IFN-α2a or IFN-α2b with sorafenib, a different protein kinase inhibitor (PKI) that inhibits the antiviral effects of interferon, resulted in antagonism (CI of 1.23; 95% credible interval [1.13-1.34] and 1.27, 95% credible interval [1.16-1.38]; supplementary figure 1A-B and supplementary table 3), demonstrating that the observed synergies are specific for the combinations and not related to technical issues of the model. Calculation of 95% credible intervals indicates that the differences between CI values did not occur by chance (supplementary table 3). These data demonstrate that entry inhibitors enhance the antiviral activity of IFN-α.

Synergy in experimental design for treatment of HCV infection. Finally, to explore whether synergy between DAAs or HTAs and entry inhibitors also occurs in experimental approach mimicking treatment of chronic infection, we used a well-established design of persistent infection[23]. Similar to the prevention design shown before, combining DAAs or alisporivir with entry inhibitors also resulted in high

synergy when compounds were added post-infection in chronically infected cells (figure 6) as well as in chronically infected DMSO-differentiated hepatoma cells[26] (figure 7), which are more physiologically relevant than normal hepatoma cells. These data demonstrate the potential of combining DAAs or HTAs with entry inhibitors to improve their antiviral activity for the treatment of chronic hepatitis C.

Entry inhibitors limit viral rebound from DAA therapy. Using the cell culture model system for chronic HCV infection, we studied whether entry inhibitors limit viral rebound following discontinuation of DAA treatment. As shown in figure 8, treatment of persistently HCV-infected cells with a well-characterized and recently FDA-approved protease inhibitor, simeprevir, resulted in a rapid reduction of viral load in a time-dependent manner. However, an increase in viral load was observed following withdrawal of simeprevir. In contrast, the addition of an entry inhibitor (anti-CD81 mAb or erlotinib) at the time of simeprevir withdrawal allowed to further decrease the viral load, indicating that entry inhibitors limit viral rebound from DAA therapy. Cell viability test at the end of the experiment demonstrated that the reduction of viral infection was not due to cytotoxicity (table 1D).

Combination of entry inhibitors and DAAs *in vivo*. To assess the relevance of combining an entry inhibitor and a DAA for treatment of HCV infection and provide experimental evidence for a synergistic effect of entry inhibitors and DAAs *in vivo,* we used human liver-chimeric uPA/SCID mice persistently infected with HCVcc (Jc1). Nine persistently infected mice were grouped randomly to receive either telaprevir monotherapy, anti-SR-BI mAb monotherapy, combination of telaprevir and anti-SR-BI mAb, or the control treatment for 2 weeks as described in Material and Methods. In line with previous reports[44], telaprevir only slightly reduced viral load of HCV genotype 2-infected mice, while anti-SR-BI mAb-treated mice showed a reduction of HCV RNA levels of approximately 1 $\log_{10}$ at the end of the treatment. Interestingly, the combination of telaprevir and anti-SR-BI mAb resulted in a more potent reduction of viral load at each tested time point than the calculated sum of both monotherapies (at d10 HCV load for the control treatment is 6.86±0.06 $\log_{10}$; for telaprevir monotherapy 6.72±0.42 $\log_{10}$; for anti-SR-BI mAb 6.03±0.54 $\log_{10}$; for combination therapy in the responder mouse 3.71 $\log_{10}$), reaching more than 2 $\log_{10}$ in one of the

mice after 10d (figure 9A). One mouse treated with telaprevir and anti-SR-BI mAb did not respond to treatment (figure 9A). Stable levels of human albumin in the blood of mice from different treatment groups (figure 9B) suggested that their liver function was not affected by the treatment, indicating that telaprevir or/and anti-SR-BI mAb combination therapy did not result in major hepatotoxicity *in vivo*.

Absent toxicity of combinations in primary human hepatocytes and hepatoma cells. Noteworthy, the uncovered combinations did not exhibit any detectable toxicity in PHH nor Huh7.5.1 cells, neither in short- or long-term infection experiments (table 1). In contrast, anti-Fas antibody or the well-characterized kinase inhibitor flavopiridol resulted in easily detectable toxicity (table 1). 50% cytotoxic concentrations (CC_{50}) of entry inhibitors in long term experiments were more than 10-fold or 100-fold higher than the therapeutic concentrations used in treatment experiments (table 1C). Taken together, these data demonstrate that the uncovered combinations have a favourable safety profile in cell culture models and primary cells providing the rationale for their further development.

Table 1. Absent toxicity of combinations of compounds in Huh7.5.1 cells and primary human hepatocytes. Cytotoxic effects on Huh7.5.1 cells (A, B, C, D) and PHH (A) using the highest concentrations of each compound used in combination (IFN-α, 10 IU/ml; DAAs, 10 nM or µM; alisporivir, 10 µM; receptor-specific mAbs, 10 µg/ml and PKIs, 10 µM) were assessed by analyzing the ability to metabolize MTT after 48h (A, B), 5d (C), or 20d (D) as described[23-32]. Anti-Fas antibody (10 µg/ml) or flavopiridol (10 µM) was used as a positive control of toxicity. Toxicity analyses of the most efficient combinations are shown. Data are presented as relative cell viability compared to PHH or Huh7.5.1 cells cultured in the absence of compounds or solvent (=100%). Means ± SD from one representative experiment performed in triplicate are shown.

A. Cytotoxic effects of combination therapies on Huh7.5.1 cells and PHH after 48h.

Compound 1	Concentration	Compound 2	Concentration	Relative Huh7.5.1 viability (%)	Relative PHH viability (%)
IFN-α2a	10 IU/ml	anti-CLDN1	10 µg/ml	106±11	101±2
IFN-α2b	10 IU/ml	anti-CLDN1	10 µg/ml	108±15	97±3
telaprevir	10 µM	erlotinib	10 µM	84±21	93±5
boceprevir	10 µM	anti-CLDN1	10 µg/ml	94±8	103±3
simeprevir	10 µM	anti-CLDN1	10 µg/ml	104±5	97±4
danoprevir	10 µM	anti-CLDN1	10 µg/ml	91±12	103±1
daclatasvir	10 nM	anti-SR-BI	10 µg/ml	114±18	101±2
mericitabine	10 µM	anti-CD81	10 µg/ml	131±22	106±5
sofosbuvir	10 µM	anti-SR-BI	10 µg/ml	95±15	110±7
sofosbuvir	10 µM	anti-CLDN1	10 µg/ml	93±8	97±5
sofosbuvir	10 µM	dasatinib	10 µM	89±14	103±8
sofosbuvir	10 µM	erlotinib	10 µM	87±15	101±4
anti-Fas	10 µg/ml				16±2

B. Cytotoxic effects of combination therapies on Huh7.5.1 cells after 5d.

Compound 1	Concentration	Compound 2	Concentration	Relative Huh7.5.1 viability (%)
telaprevir	10 µM	anti-CLDN1	10 µg/ml	92±4
telaprevir	10 µM	erlotinib	10 µM	89±3
daclatasvir	10 nM	anti-SB-BI	10 µg/ml	103±16
daclatasvir	10 nM	anti-CLDN1	10 µg/ml	106±7
sofosbuvir	10 µM	anti-CLDN1	10 µg/ml	93±2
sofosbuvir	10 µM	erlotinib	10 µM	91±4
alisporivir	10 µM	anti-SR-BI	10 µg/ml	97±8
alisporivir	10 µM	anti-CLDN1	10 µg/ml	108±6
flavopiridol	10 µM			20±6

C. 50% cytotoxic concentrations (CC_{50}) of entry inhibitors in Huh7.5.1 cells. Dose-dependent toxicity of entry inhibitors was measured using Prestoblue assay as described in Material and Methods. CC_{50} were calculated by regression analysis.

Compound	CC_{50}
anti-CLDN1*	>1 mg/mL
anti-CD81*	>1 mg/mL
anti-SR-BI*	789±189 µg/mL
erlotinib**	>100 µM
dasatinib**	>100 µM

*The stock concentration of the entry inhibitors was 2 mg/mL. **Erlotinib or dasatinib started to precipitate in the medium at the concentrations higher than 100 µM.

D. Cytotoxic effects of combination therapies as described in figure 8. Cell viability was measured at the end of the experiment using Prestoblue assay as described in Material and Methods.

Combination	**Relative Huh7.5.1 viability (%)**
telaprevir+mock	97±10
telaprevir+anti-CLDN1	91±3
telaprevir+erlotinib	81±4

DISCUSSION

There is an unmet medical need for novel strategies for the prevention of HCV graft infection following LT, and a need to develop more efficient and better tolerated combination therapies for chronic infection for certain patient subgroups and patients with resistance[1, 2]. Many DAAs are restricted to genotype 1 and their use in difficult-to-treat patients could be hindered by significant and potentially harmful drug-drug interactions[7]. Among the most promising DAAs are polymerase and NS5A inhibitors, which demonstrated potent pan-genotypic antiviral activity with a high barrier for resistance when combined with SOC or ribavirin[45, 46]. Nevertheless, *in vivo* emergence of resistant mutants conferring cross-resistance to polymerase inhibitors sofosbuvir and mericitabine has been described[47], and combination of daclatasvir with protease inhibitors was associated with significant viral breakthrough rates[3, 37]. Recent phase III studies indicate that sofosbuvir and RBV had only limited efficacy against genotype 3 when compared with SOC, with lowest efficacy in cirrhotic patients[40, 41]. Thus, strong antiviral efficacy and high genetic barrier to resistance will remain key requirements for IFN-free regimens to avoid early

treatment failure and the optimal combination for defined patient groups and viral genotypes still needs to be defined.

To address the limitations of SOC and DAAs, we evaluated the antiviral effect of combining entry inhibitors with IFN-α, DAAs or HTAs. Our results uncover novel combinations of entry inhibitors and IFN-α, DAAs or HTAs highlighted by marked synergy *in vitro* (figure 1-7, table 2, supplementary figure 1 and supplementary tables 1-3). Antiviral activity of compounds in the HCVcc model correlates well with clinical antiviral activity in patients[36], and synergy was robust and significant at IC_{50}, IC_{75} and IC_{90}, at all concentrations tested and similar in experimental prevention and treatment designs. Furthermore, we also demonstrate evidence for a synergistic effect of the combination of an entry inhibitor with a DAA *in vivo* using the human liver-chimeric uPA/SCID mouse model (figure 9).

A challenge of current and future therapies remains the countering of the development of drug-resistant variants[1-3]. Compared to the high variability of viral proteins targeted by DAAs, the variability of host factors targeted by HTAs is low[8]. Therefore, host-targeting entry inhibitors may impose a higher genetic barrier to resistance than DAAs. Supplementary to this concept, we and others have shown that entry inhibitors potently inhibit highly infectious escape variants of HCV that are resistant to host neutralizing antibodies[9, 10, 13-15, 22]. Interestingly, the disease outcome for HIV-infected individuals has significantly improved with the development of antiretroviral drugs targeting different steps of the viral life cycle including viral entry[48]. Although viral variants resistant to HIV entry inhibitors have been described, there is no evidence of cross-resistance between different classes of antivirals[48]. In contrast to HIV, co-receptor tropism/switch has not been described for HCV as a potential mechanism for viral escape and successful antiviral therapy can definitively eradicate HCV from infected patients. Combination of entry inhibitors with compounds targeting complementary steps of the viral life cycle may open a perspective to overcome antiviral resistance, allow shortening treatment schedules, lower the risk for adverse effects and reduce the doses of the single compounds.

Given that HTAs interfere with host targets, there is theoretically a greater risk of cellular toxicity than with DAAs. Host targets CD81, SR-BI, CLDN1 and EGFR described in this study are expressed in various tissues and play an important role in cell adhesion, lipid metabolism or signalling. Noteworthy, the large majority of

licensed drugs in clinical use (e. g. in cardiovascular, inflammatory disease or oncology) targets host proteins and side effects are not necessarily more pronounced than in drugs targeting the virus[8]. Indeed, the EGFR inhibitor erlotinib, a clinically licensed drug for non-small-cell lung cancer has a favourable safety profile and is well tolerated in lung cancer patients[49]. Moreover, clinical licensed HIV host-targeting entry inhibitors do not necessarily display more adverse events than antiretroviral drugs[48]. Nevertheless, safety is an important issue and needs to be carefully addressed for both DAAs and HTAs. Host-targeting antibodies will need to be humanized prior to clinical development.

A pan-genotypic, highly efficient, IFN-free regimen is the ultimate goal for HCV therapy. Compared to many DAAs which act in genotype-dependent manner, host-targeting entry inhibitors exhibit a broad pan-genotypic activity: examples include CLDN1-[22], SR-BI-[14, 15] and CD81-specific[16, 17] mAbs, ITX-5061[18], erlotinib[13], ezetimibe[12], flavonoids[19, 20], lectins[21], phosphorothioate oligonucleotides[50] and silymarin[51, 52]. Thus, combination of entry inhibitors with DAAs may define novel options for patients with non-genotype 1 infections and simplify treatment regimens.

Entry inhibitors are ideally suited for prevention of HCV liver graft infection. Currently there is no option to protect HCV-negative grafts from re-infection while the clinical use of hepatitis B immune globulin (a well-characterized HBV entry inhibitor) in combination with nucleos(t)ide analogues has essentially eliminated HBV recurrence in LT patients[5]. Since HCV entry is a key mechanism for liver graft infection[9, 10], the observed synergy between DAAs and entry inhibitors uncovers a promising opportunity for prevention of graft infection. The combination of host-targeting entry inhibitors with DAAs may be particularly attractive since the high variability of HCV has so far hampered the development of efficient cross-neutralizing anti-envelope antibodies[53].

Interfering with HCV cell entry offers several targets at different steps of viral entry: blocking virus-target cell interaction during attachment, interfering with post-binding events such as CD81-CLDN1 associations or viral fusion[8]. Interestingly, combination of different entry inhibitors, including CLDN1-specific mAb and kinase inhibitors, also showed synergy on HCV entry and infection (figure 5A). While CLDN1-specific antibodies and PKIs both interfere with CD81-CLDN1 co-receptor

interactions, PKIs also interfere with glycoprotein-dependent viral fusion[13]. This additional and complementary MOA on membrane fusion of PKIs is most likely responsible for the synergistic effect. This concept is confirmed by the finding that combination of anti-CD81 and anti-CLDN1 mAbs, which target a similar step in the viral entry process, only exhibits an additive effect (figure 5A). Thus, these results define novel combinations of entry inhibitors for prevention of graft infection.

Interestingly, two entry inhibitors erlotinib (targeting EGFR and CD81-CLDN1 co-receptor associations) and ITX-5061 (targeting HCV-SR-BI interactions) have reached clinical development to determine the safety and efficacy in patients with HCV infection (ClinicalTrials.gov Identifier NCT01835938 and NCT01292824). Moreover, a significant number of compounds tested in this study are already FDA-approved for treatment of chronic HCV infection: telaprevir, boceprevir, sofosbuvir and simeprevir. Erlotinib and dasatinib have been approved for cancer treatment exhibiting a good safety profile[54]. It will thus be interesting to investigate the synergistic effect of these drugs in patients with chronic hepatitis C. Taken into account the efficacy, safety, pharmacokinetics, stage of development combined with the synergy data of the various compounds (figure 1-7), our data suggest that combinations of simeprevir[34] or sofosbuvir [40] with erlotinib could be a starting point to assess synergy in randomized clinical trials. Combining sofosbuvir and anti-CLDN1 or anti-SR-BI mAb might be promising for prevention of liver graft infection following the clinical development of the monoclonal antibodies. Collectively, novel combinations based on synergy uncovered in this study may widen the therapeutic arsenal against HCV infection for prevention of liver graft infection, treatment of difficult-to-treat-patients and provide alternatives for patients with contraindications to particular compounds of SOC or future IFN-free regimens.

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Footnotes

FX and IF contributed equally to this work.

Contributors

TFB initiated, designed and supervised research. FX, IF, CT, LM, RA, ER, PL, JN, MBZ and TFB performed research. FX, IF, CT, LM, RA, ER, PL, JN, MBZ and TFB analyzed data. NM and MS performed statistical analyses. FH and MD provided reagents. FX, IF, MBZ and TFB wrote the paper.

Competing Interests

The authors declare no conflict of interest. Inserm and the University of Strasbourg have filed patent applications on the synergy of interferons, direct-acting antivirals and entry inhibitors as well as SR-BI-specific antibodies, erlotinib, dasatinib for prevention and treatment of HCV infection. Inserm, University of Strasbourg and Genovac/Aldevron have filed a patent application for claudin-1 specific-antibodies for prevention and treatment of HCV infection.

Ethics approval

All animal experiments in this study were performed in accordance with local laws and ethical committee approval (AL/02/19/08/12 and AL/01/18/08/12).

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FIGURE LEGENDS

Figure 1. Combination of DAAs and entry inhibitors results in a synergistic activity in preventing HCV infection. Huh7.5.1 cells were pre-incubated for 1h with serial concentrations of (A) telaprevir, boceprevir, simeprevir or danoprevir, (B) daclatasvir or (C) mericitabine or sofosbuvir and 0.01 µg/ml of receptor-specific (anti-CD81, anti-SR-BI or anti-CLDN1) mAbs or 0.1 µM PKIs (erlotinib or dasatinib) before incubation with HCVcc Luc-Jc1 in the presence of both compounds. HCVcc infection was analyzed by luciferase activity as described[22]. The CIs at $IC_{50,}$ IC_{75} and IC_{90} are indicated in supplementary table 1. Means ± 95% credible intervals from three independent experiments performed in triplicate are shown.

Figure 2. Antiviral synergy of entry and protease inhibitors. Combination (A) telaprevir and erlotinib, (B) boceprevir or (C) simeprevir and anti-CLDN1 mAb was performed as described in figure 1. (D-F) Synergy was confirmed using the method of Prichard and Shipman[25].

Figure 3. Synergy of entry inhibitors and daclatasvir. Combination of daclatasvir and (A) anti-SR-BI mAb, (B) anti-CLDN1 mAb or (C) erlotinib was performed as described in figure 1. (D-F) Synergy was confirmed using the method of Prichard and Shipman[25].

Figure 4. Entry inhibitors enhance the antiviral activity of sofosbuvir in a synergistic manner. Combination of sofosbuvir and (A) anti-SR-BI mAb, (B) anti-CLDN1 mAb, (D) dasatinib or (E) erlotinib was performed as described in figure 1. (C,F) Synergy was confirmed using the method of Prichard and Shipman[25].

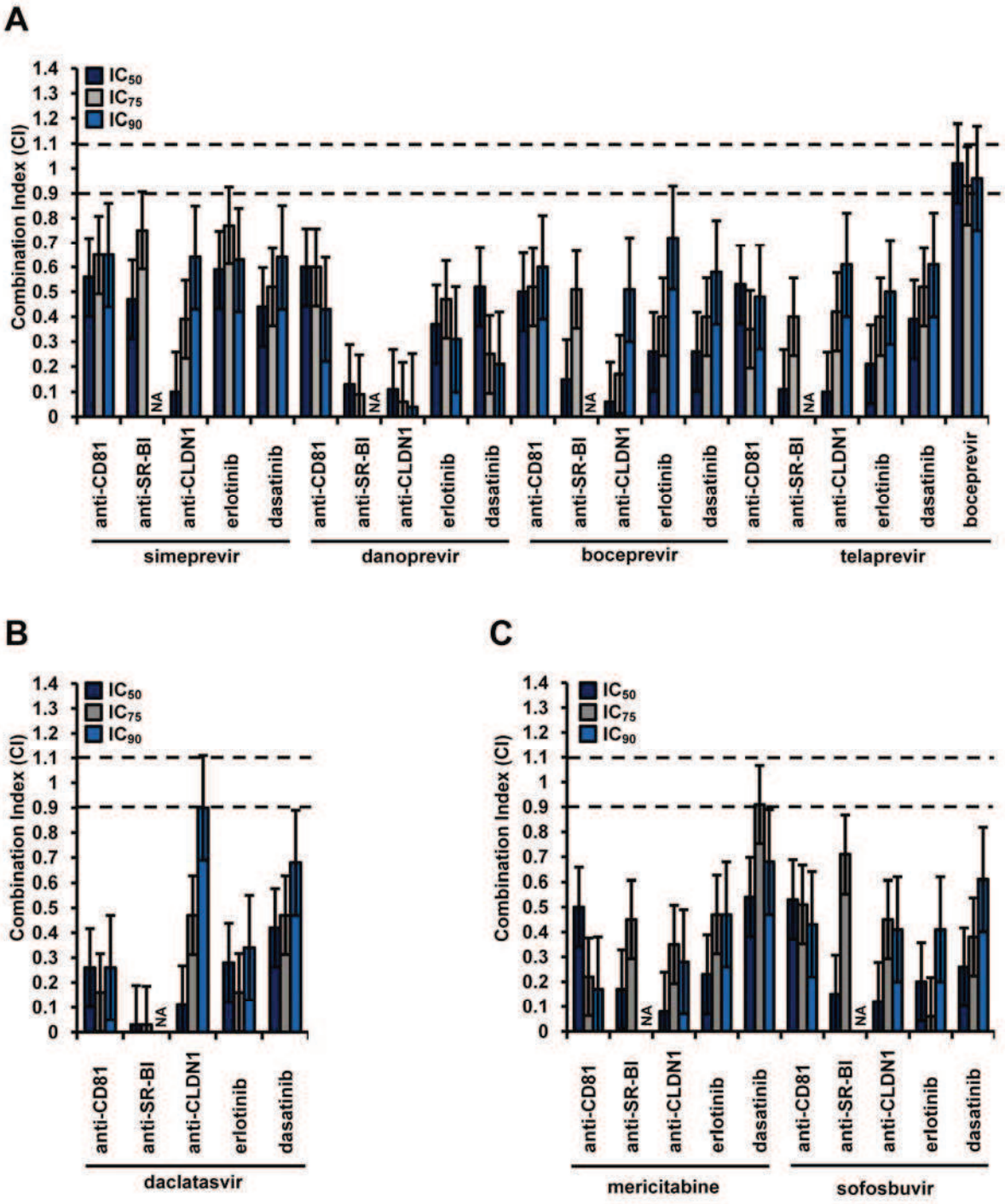
Figure 5. Synergy of HTAs on HCV infection. (A) Combination of receptor-specific (anti-CD81, anti-SR-BI or anti-CLDN1) mAbs and PKIs (erlotinib or dasatinib) was performed as described in figure 1 and the CIs at $IC_{50,}$ IC_{75} and IC_{90} are indicated in supplementary table 2. Means ± 95% credible intervals from three independent experiments performed in triplicate are shown. (B) Combination of anti-CLDN1 mAb and dasatinib (left) or erlotinib (right). (C) Synergy was confirmed using the method of Prichard and Shipman[25]. (D) Combination of alisporivir and entry inhibitors was performed as described in figure 1 and the CIs at $IC_{50,}$ IC_{75} and IC_{90} are indicated in supplementary table 2. Means ± 95% credible intervals from three independent experiments performed in triplicate are shown. (E) Combination of alisporivir with erlotinib (left) or anti-CLDN1 mAb (right). (F) Synergy was confirmed using the method of Prichard and Shipman[25].

Figure 6. Combination of DAAs, HTAs and entry inhibitors results in synergy in treatment of established HCV infection. Huh7.5.1 cells were infected for 5d with HCVcc prior to treatment with DAAs or alisporivir and entry inhibitors as described in Material and Methods. Synergy between (A) telaprevir and anti-CLDN1 mAb (left) or erlotinib (right), (B) daclatasvir and anti-SR-BI mAb (left) or anti-CLDN1 mAb (right), (C) sofosbuvir and anti-CLDN1 mAb (left) or erlotinib (right), (D) alisporivir and anti-SR-BI (left) or anti-CLDN1 mAb (right) was assessed using the method of Prichard and Shipman[25].

Figure 7. Synergistic effect of combining DAAs and entry inhibitors in treatment of HCV infection using persistently infected DMSO-differentiated hepatoma cells. HCV-infected cell culture was established by transfecting RNA encoding wild-type HCV Luc-Jc1 into Huh7.5.1 cells. The cells were then differentiated in the presence of 1% DMSO for 5d before treatment with the combinations of (A) telaprevir + anti-CLDN1 mAb (left) or erlotinib (right), (B) daclatasvir + anti-SR-BI mAb (left) or anti-CLDN1 mAb (right), (C) sofosbuvir + anti-CLDN1 mAb (left) or erlotinib (right), (D) simeprevir + erlotinib (left) or anti-CLDN1 mAb (right). HCVcc infection was assessed by luciferase activity 5d after treatment. Synergy was assessed using the method of Prichard and Shipman[25].

Figure 8. Entry inhibitors limit viral rebound from DAA therapy. Persistently HCV (Jc1) infected and DMSO-differentiated cells described in Material and Methods were first treated with 500 nM simeprevir for 8d followed by incubation with either 1% DMSO control medium (mock), 10 µg/mL anti-CD81 mAb or 10 µM erlotinib sequentially for another 12d. Cells maintained in 1% DMSO throughout the experiment were used as control (CTRL). Viral load was assessed by HCV-specific RT-PCR every 4d. The limit of quantification (LOQ), indicated by a dashed line, was 10^3 copies/ml. Means ± SD from a representative experiment performed in triplicate are shown. An asterisk indicates one out of three samples was HCV RNA negative; two asterisks indicate two samples were HCV RNA negative.

Figure 9. Effect of combination of an entry inhibitor and DAA on HCV infection *in vivo*. Human liver-chimeric uPA/SCID mice engrafted with PHH were inoculated with HCVcc (Jc1) eight weeks before the experiment as described in Material and Methods. Nine mice were grouped randomly into control group (two mice), telaprevir monotherapy group (two mice), SR-BI-specific mAb (NK8-H5-E3) monotherapy group (two mice) and combination therapy group (three mice). Mice received telaprevir monotherapy (300mg/kg PO BID), anti-SR-BI mAb monotherapy (500 µg IP twice a week), combination treatment of telaprevir and anti-SR-BI mAb or control treatments as described in Material and Methods. Indicated by an asterisk, two mice from the group treated with combination of telaprevir and anti-SR-BI mAb had to be sacrificed before the end of the experiment due to bleeding and injury. (A) HCV RNA from mouse sera was measured by RT-PCR every 5-10d. Viral load of each individual mouse is shown. (B) Human albumin (HA) concentration in the blood of human liver-chimeric uPA/SCID mice. HA was measured during the treatment as described in Material and Methods.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B
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IC75
IC90
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anti-CD81
anti-SR-BI
anti-CLDN1
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boceprevir
simeprevir
danoprevir
telaprevir
daclatasvir
mericitabine
sofosbuvir

Figure 1

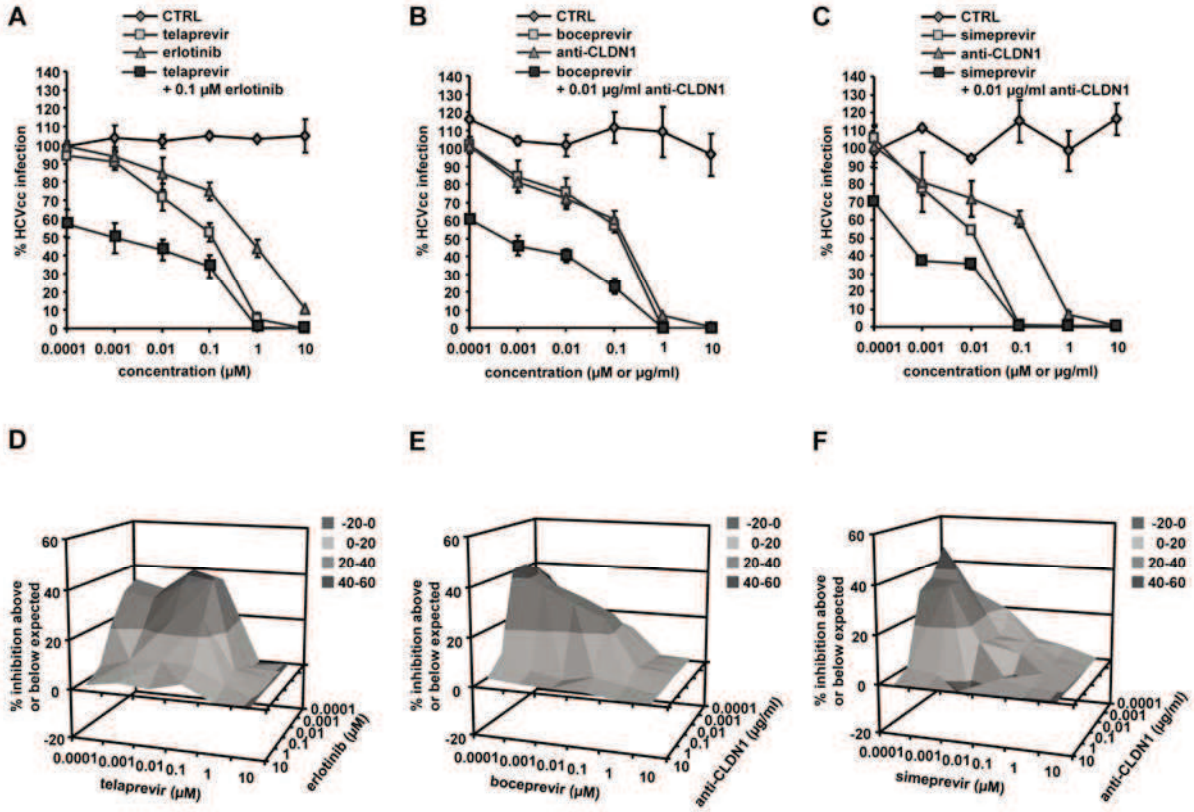


Figure 2

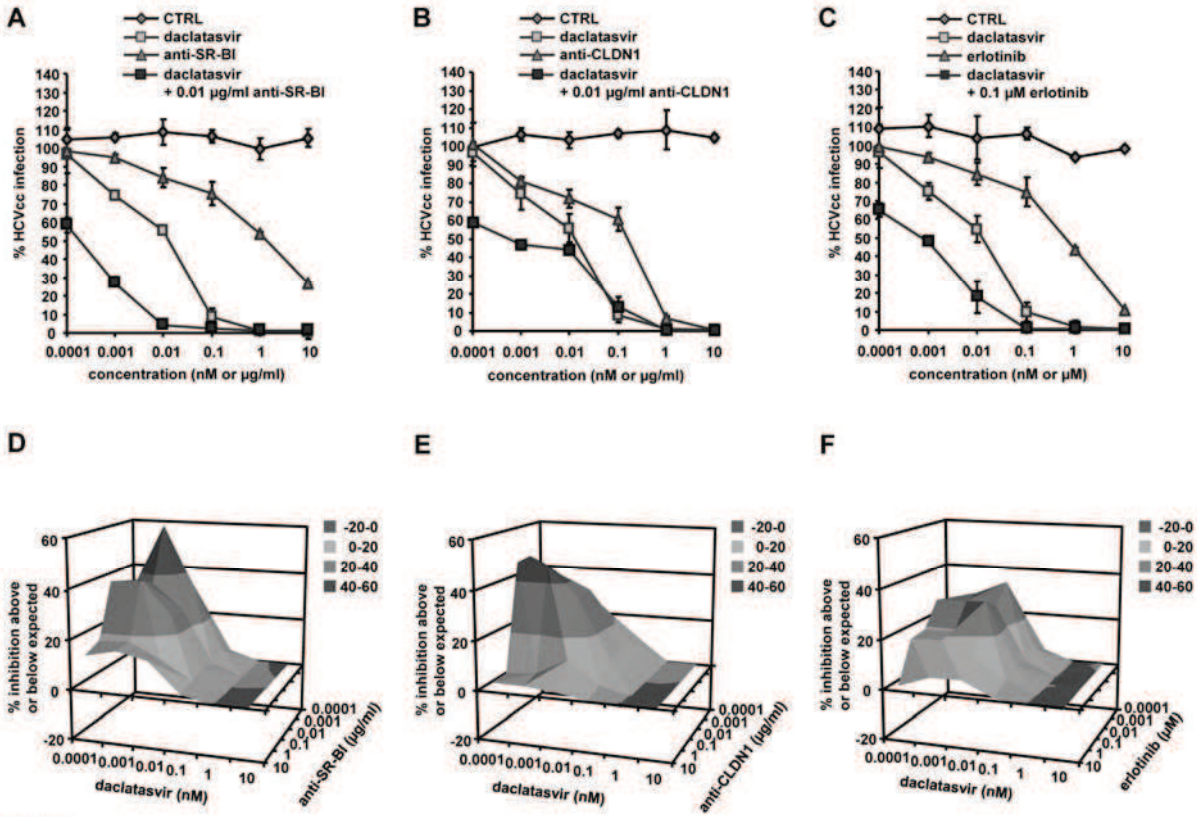
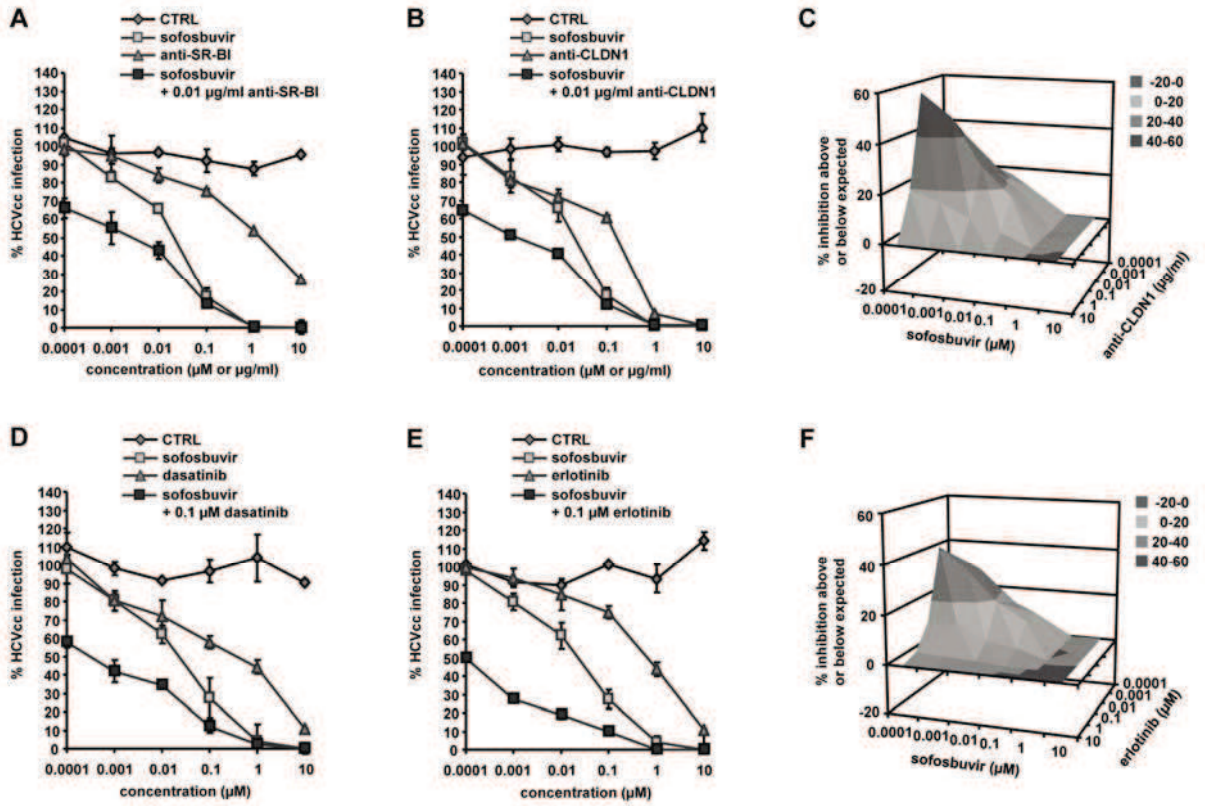


Figure 3



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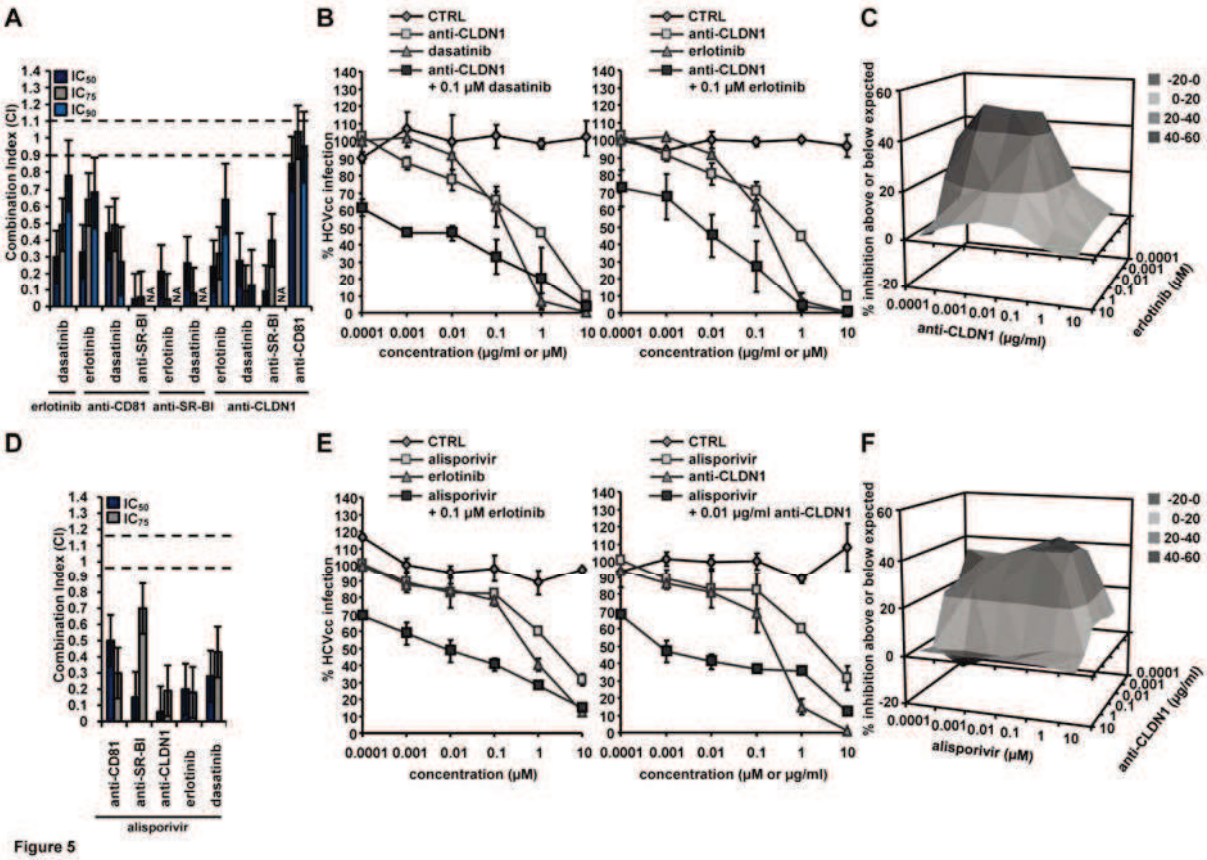

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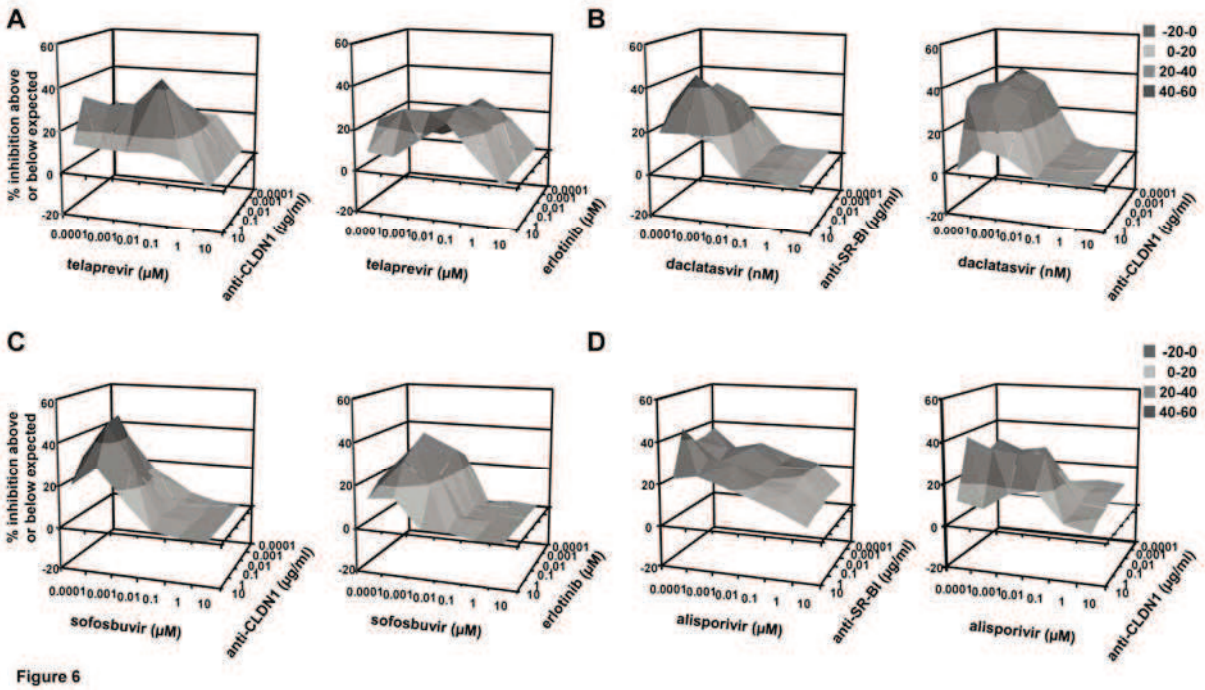

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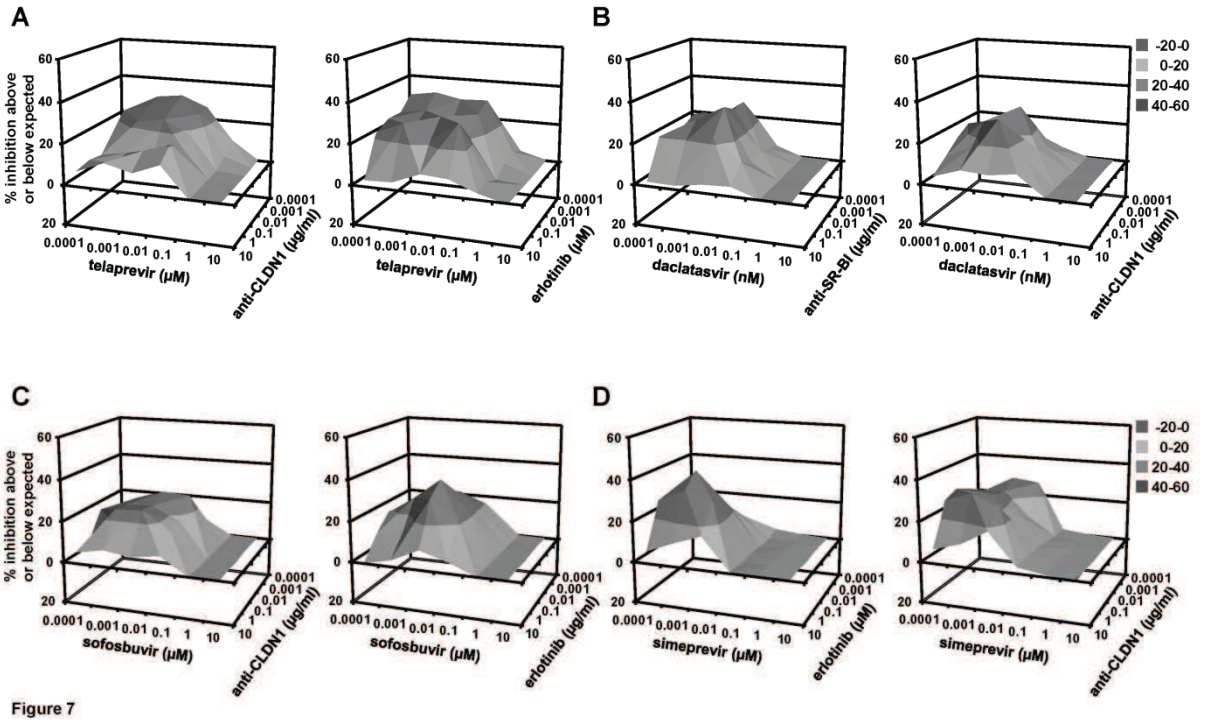
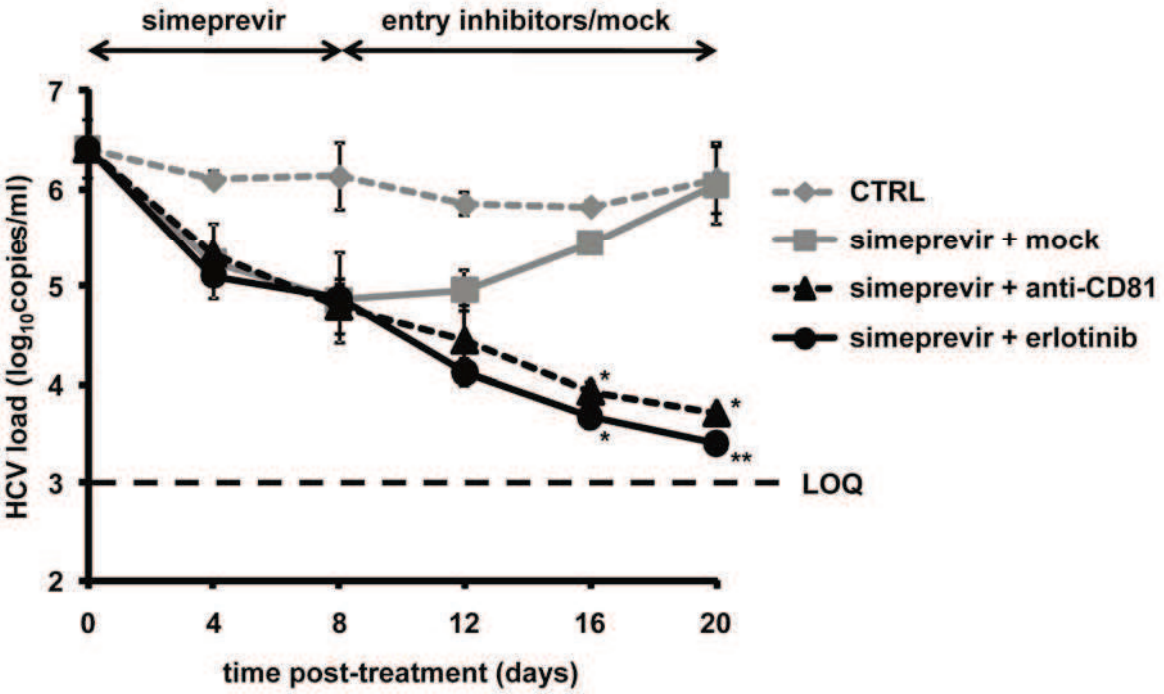


Figure 7


simeprevir
entry inhibitors/mock
HCV load (log10copies/ml)
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simeprevir + anti-CD81
simeprevir + erlotinib
LOQ
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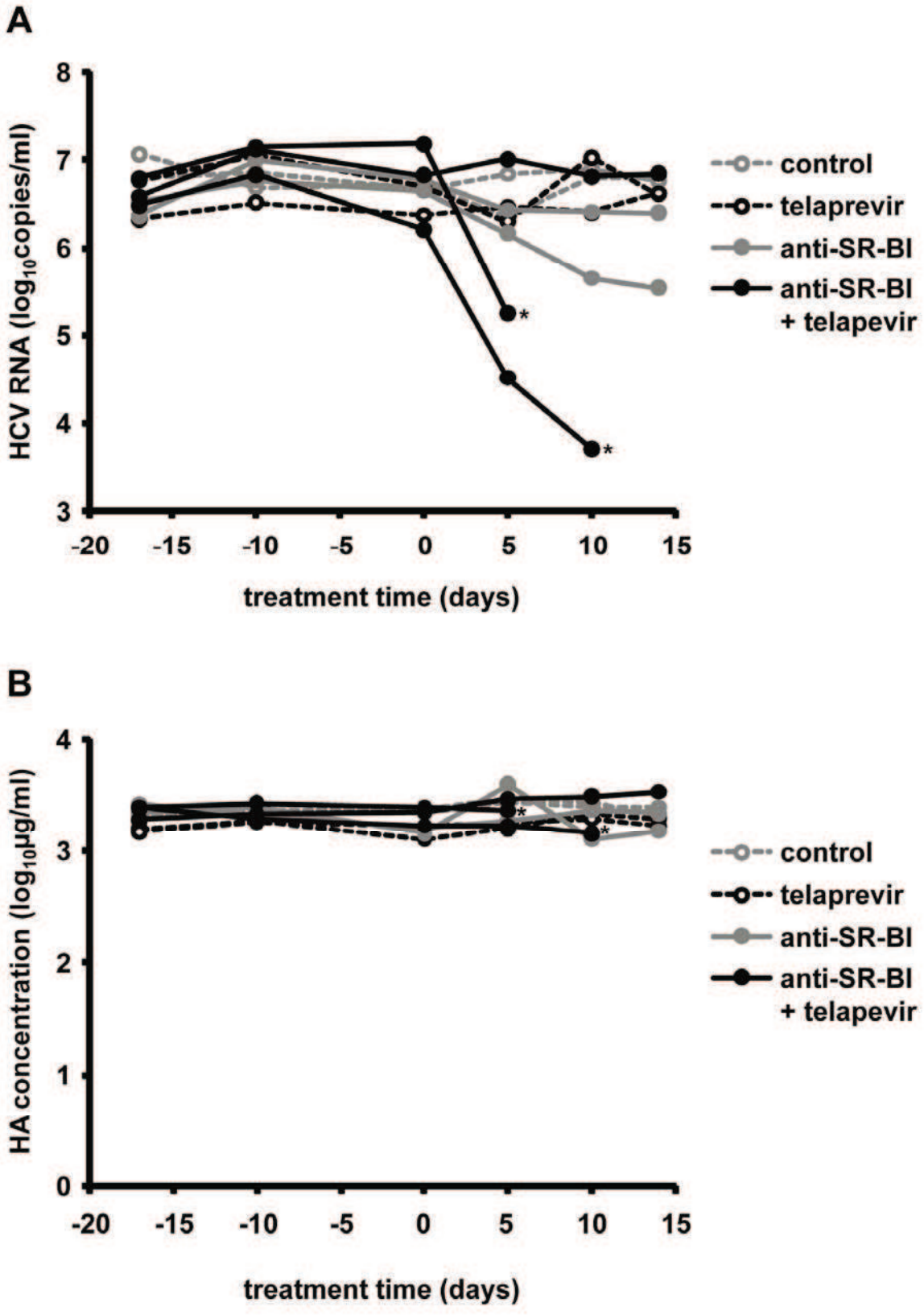
Figure 8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treatment time (days)
control
telaprevir
anti-SR-BI
anti-SR-BI
+ telapevir
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HA concentration (log10µg/ml)
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3
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1
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-20
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-5
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treatment time (days)
control
telaprevir
anti-SR-BI
anti-SR-BI
+ telapevir
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*

Figure 9

SUPPLEMENTARY TABLES

Supplementary Table 1. Synergy of entry inhibitors and direct-acting antivirals on inhibition of HCV infection. Huh7.5.1 cells were pre-incubated with serial concentrations of DAAs (protease inhibitors telaprevir, boceprevir, simeprevir or danoprevir; NS5A inhibitor daclatasvir; or polymerase inhibitors mericitabine or sofosbuvir) and 0.01 µg/ml receptor-specific (anti-CD81, anti-SR-BI or anti-CLDN1) or respective isotype control mAbs or 0.1 µM PKIs (erlotinib or dasatinib) for 1h at 37°C. The mix was removed and Huh7.5.1 cells were incubated for 4h at 37°C with HCVcc Luc-Jc1 in the presence of both compounds. HCVcc infection was analyzed by luciferase reporter gene expression[1]. The Combination Index (CI) at (A) IC_{50}, (B) IC_{75} and (C) IC_{90} was calculated as described[2]. A CI less than 0.9, between 0.9 and 1.1, and more than 1.1 indicates synergy, additivity, and antagonism, respectively. Means [95% credible intervals] from three independent experiments performed in triplicate are shown for the CI.

A.

Compound 1	IC_{50} (µM or nM[1])	Compound 2	IC_{50} (µM or nM[1]) for combination	CI
telaprevir	0.15±0.06	anti-CD81	0.004±0.001	0.53 [0.46-0.61]
		anti-SR-BI	0.015±0.001	0.11 [0.03-0.19]
		anti-CLDN1	0.006±0.003	0.11 [0.03-0.18]
		erlotinib	0.001±0.002	0.22 [0.14-0.30]
		dasatinib	0.02±0.03	0.39 [0.30-0.46]
boceprevir	0.14±0.02	anti-CD81	0.0004±0.0001	0.50 [0.42-0.58]
		anti-SR-BI	0.02±0.002	0.16 [0.08-0.24]
		anti-CLDN1	0.0005±0.001	0.06 [-0.01-0.14]
		erlotinib	0.008±0.004	0.27 [0.19-0.35]
		dasatinib	0.001±0.0005	0.26 [0.18-0.34]
simeprevir	0.013±0.001	anti-CD81	0.0007±0.0001	0.56 [0.48-0.64]
		anti-SR-BI	0.006±0.0007	0.47 [0.39-0.55]
		anti-CLDN1	0.0005±0.0002	0.1 [0.04-0.20]
		erlotinib	0.005±0.002	0.59 [0.50-0.66]
		dasatinib	0.0025±0.002	0.44 [0.36-0.52]
danoprevir	0.006±0.003	anti-CD81	0.0006±0.0004	0.6 [0.53-0.68]
		anti-SR-BI	0.0007±0.001	0.13 [0.05-0.21]
		anti-CLDN1	0.0003±0.0004	0.11 [0.03-0.19]
		erlotinib	0.001±0.0005	0.37 [0.29-0.45]
		dasatinib	0.0016±0.0007	0.52 [0.44-0.60]
daclatasvir	0.012±0.003	anti-CD81	N.A.	N.A.
		anti-SR-BI	0.0002±0.0004	0.03 [-0.04-0.12]
		anti-CLDN1	0.0006±0.0002	0.11 [0.03-0.19]
		erlotinib	0.0009±0.0004	0.28 [0.20-0.36]
		dasatinib	0.002±0.0008	0.42 [0.34-0.49]
mericitabine	0.12±0.03	anti-CD81	0.00015±0.0001	0.50 [0.43-0.59]
		anti-SR-BI	0.019±0.007	0.17 [0.09-0.25]
		anti-CLDN1	0.0028±0.00035	0.08 [0.01-0.17]
		erlotinib	0.003±0.006	0.23 [0.16-0.32]
		dasatinib	0.035±0.01	0.54 [0.46-0.62]
sofosbuvir	0.021±0.002	anti-CD81	0.0006±0.0004	0.53 [0.45-0.61]
		anti-SR-BI	0.003±0.0007	0.15 [0.07-0.23]
		anti-CLDN1	0.0012±0.0003	0.12 [0.04-0.20]
		erlotinib	0.0001±0.00002	0.20 [0.13-0.29]
		dasatinib	0.00029±0.0001	0.26 [0.18-0.34]

[1] Telaprevir, boceprevir, danoprevir, simeprevir, mericitabine, sofosbuvir: µM; daclatasvir: nM

IC_{50} of individual entry inhibitors: anti-CD81, 0.02±0.01 µg/ml; anti-SR-BI, 1.3±0.4 µg/ml; anti-CLDN1, 0.18±0.03 µg/ml; erlotinib, 0.5±0.06 µM; dasatinib, 0.4±0.3 µM. N.A.: not applicable.

B.

Compound 1	IC_{75} (µM or nM[1])	Compound 2	IC_{75} (µM or nM[1]) for combination	CI
telaprevir	0.5±0.15	anti-CD81	0.1±0.09	0.35 [0.27-0.43]
		anti-SR-BI	0.2±0.05	0.4 [0.32-0.48]
		anti-CLDN1	0.2±0.02	0.42 [0.34-0.50]
		erlotinib	0.2±0.05	0.4 [0.32-0.48]
		dasatinib	0.25±0.05	0.52 [0.44-0.60]
boceprevir	0.4±0.04	anti-CD81	0.15±0.04	0.52 [0.44-0.60]
		anti-SR-BI	0.2±0.02	0.51 [0.43-0.59]
		anti-CLDN1	0.06±0.03	0.17 [0.1-0.25]
		erlotinib	0.15±0.09	0.4 [0.32-0.48]
		dasatinib	0.15±0.04	0.4 [0.32-0.48]
simeprevir	0.04±0.01	anti-CD81	0.02±0.002	0.65 [0.57-0.72]
		anti-SR-BI	0.03±0.01	0.74 [0.67-0.82]
		anti-CLDN1	0.015±0.003	0.39 [0.31-0.47]
		erlotinib	0.03±0.005	0.76 [0.69-0.84]
		dasatinib	0.02±0.002	0.52 [0.44-0.60]
danoprevir	0.045±0.003	anti-CD81	0.02±0.006	0.6 [0.52-0.68]
		anti-SR-BI	0.004±0.002	0.1[0.02-0.18]
		anti-CLDN1	0.0018±0.0003	0.07 [-0.01-0.15]
		erlotinib	0.02±0.005	0.47 [0.39-0.55]
		dasatinib	0.01±0.009	0.25 [0.18-0.33]
daclatasvir	0.045±0.01	anti-CD81	0.00025±0.0002	0.17 [0.01-0.24]
		anti-SR-BI	0.001±0.0005	0.04 [-0.04-0.12]
		anti-CLDN1	0.02±0.01	0.47[0.39-0.55]
		erlotinib	0.006±0.004	0.17 [0.09-0.24]
		dasatinib	0.02±0.006	0.47 [0.39-0.55]
mericitabine	0.45±0.05	anti-CD81	0.03±0.01	0.22 [0.15-0.30]
		anti-SR-BI	0.2±0.05	0.45 [0.38-0.53]
		anti-CLDN1	0.15±0.05	0.35 [0.28-0.43]
		erlotinib	0.2±0.04	0.47 [0.39-0.55]
		dasatinib	0.4±0.05	0.90 [0.82-0.98]
sofosbuvir	0.07±0.003	anti-CD81	0.025±0.005	0.51 [0.43-0.59]
		anti-SR-BI	0.05±0.03	0.70 [0.63-0.78]
		anti-CLDN1	0.03±0.005	0.45 [0.37-0.53]
		erlotinib	0.002±0.001	0.07 [-0.01-0.15]
		dasatinib	0.025±0.005	0.38 [0.30-0.46]

[1] Telaprevir, boceprevir, danoprevir, simeprevir, mericitabine, sofosbuvir: µM; daclatasvir: nM

IC_{75} of individual entry inhibitors: anti-CD81, 0.065±0.02 µg/ml; anti-SR-BI, 10±0.3 µg/ml; anti-CLDN1, 0.5±0.06 µg/ml: erlotinib, 3.8±0.2 µM; dasatinib, 4±0.8 µM.

C.

Compound 1	IC_{90} (µM or nM[1])	Compound 2	IC_{90} (µM or nM[1]) for combination	CI
telaprevir	1±0.2	anti-CD81	0.45±0.05	0.48 [0.38-0.59]
		anti-SR-BI	N.A.	N.A.
		anti-CLDN1	0.6±0.09	0.61 [0.50-0.71]
		erlotinib	0.5±0.1	0.5 [0.40-0.61]
		dasatinib	0.6±0.1	0.61 [0.50-0.71]
boceprevir	0.7±0.04	anti-CD81	0.4±0.1	0.6 [0.49-0.70]
		anti-SR-BI	N.A.	N.A.
		anti-CLDN1	0.35±0.05	0.51 [0.41-0.62]
		erlotinib	0.5±0.13	0.71 [0.61-0.82]
		dasatinib	0.4±0.05	0.58 [0.48-0.68]
simeprevir	0.08±0.02	anti-CD81	0.05±0.01	0.65 [0.54-0.75]
		anti-SR-BI	N.A.	N.A.
		anti-CLDN1	0.05±0.01	0.64 [0.53-0.74]
		erlotinib	0.05±0.017	0.63 [0.52-0.73]
		dasatinib	0.05±0.03	0.64 [0.53-0.74]
danoprevir	0.2±0.02	anti-CD81	0.08±0.02	0.43 [0.33-0.54]
		anti-SR-BI	N.A.	N.A.
		anti-CLDN1	0.006±0.001	0.06 [-0.05-0.17]
		erlotinib	0.06±0.01	0.32 [0.21-0.42]
		dasatinib	0.04±0.03	0.22 [0.12-0.33]
daclatasvir	0.09±0.005	anti-CD81	0.02±0.01	0.27 [0.17-0.38]
		anti-SR-BI	N.A.	N.A.
		anti-CLDN1	0.08±0.01	0.89 [0.78-0.99]
		erlotinib	0.03±0.01	0.35 [0.24-0.45]
		dasatinib	0.06±0.01	0.67 [0.57-0.78]
mericitabine	1.5±0.2	anti-CD81	0.2±0.1	0.18 [0.08-0.29]
		anti-SR-BI	N.A.	N.A.
		anti-CLDN1	0.4±0.1	0.28 [0.19-0.40]
		erlotinib	0.7±0.15	0.47 [0.37-0.58]
		dasatinib	1±0.1	0.68 [0.57-0.78]
sofosbuvir	0.25±0.02	anti-CD81	0.1±0.08	0.43 [0.33-0.54]
		anti-SR-BI	N.A.	N.A.
		anti-CLDN1	0.1±0.05	0.41 [0.31-0.52]
		erlotinib	0.1±0.03	0.41 [0.31-0.52]
		dasatinib	0.15±0.06	0.61 [0.50-0.71]

[1] Telaprevir, boceprevir, danoprevir, simeprevir, mericitabine, sofosbuvir: µM; daclatasvir: nM

IC_{90} of individual entry inhibitors: anti-CD81, 0.3±0.2 µg/ml; anti-CLDN1, 0.8±0.05 µg/ml; erlotinib, 10±0.6 µM; dasatinib, 10±0.7 µM. N.A.: not applicable.

Supplementary Table 2. Synergy of host-targeting agents on the inhibition of HCV infection. Huh7.5.1 cells were pre-incubated with (A) serial dilutions of receptor-specific (anti-CD81, anti-SR-BI or anti-CLDN1) mAbs or respective isotype control mAbs and 0.1 µM PKIs (erlotinib or dasatinib); or with (B) serial concentrations of alisporivir and 0.01 µg/ml of receptor-specific (anti-CD81, anti-SR-BI or anti-CLDN1) mAbs or 0.1 µM PKIs (erlotinib or dasatinib) for 1h at 37°C. HCVcc infection was carried out and analyzed and the CI at (A) IC_{50}, (B) IC_{75} and (C) IC_{90} was calculated as described in supplementary table 1. Means [95% credible intervals] from three independent experiments performed in triplicate are shown for the CI. IC_{50} of individual entry inhibitors: anti-CD81, 0.02±0.01 µg/ml; anti-SR-BI, 1.3±0.4 µg/ml; anti-CLDN1, 0.18±0.03 µg/ml; erlotinib, 0.5±0.06 µM; dasatinib, 0.4±0.3 µM.

A.

Compound 1	**IC_{50} (µg/ml or µM)**	**Compound 2**	**IC_{50} (µg/ml) for combination**	**CI**
anti-CD81	0.02±0.01	erlotinib	0.0025±0.007	0.33 [0.25-0.41]
		dasatinib	0.0029±0.002	0.44 [0.36-0.52]
		anti-SR-BI	0.0006±0.0003	0.4 [0.32-0.48]
anti-SR-BI	1.3±0.4	erlotinib	0.002±0.001	0.21 [0.13-0.29]
		dasatinib	0.015±0.007	0.26 [0.18-0.34]
anti-CLDN1	0.18±0.03	erlotinib	0.005±0.03	0.24 [0.16-0.32]
		dasatinib	0.005±0.003	0.28 [0.20-0.36]
		anti-SR-BI	0.014±0.005	0.09 [0.01-0.17]
		anti-CD81	0.086±0.002	0.85 [0.77-0.93]
erlotinib	0.5±0.06	dasatinib	0.027±0.005	0.3 [0.22-0.38]

IC_{50} of individual entry inhibitors: erlotinib, 0.5±0.06 µM; dasatinib, 0.4±0.3 µM.

B.

Compound 1	IC_{75} (µg/ml or µM)	Compound 2	IC_{75} (µg/ml) for combination	CI
anti-CD81	0.065±0.02	erlotinib	0.04±0.009	0.64 [0.56-0.71]
		dasatinib	0.03±0.01	0.49 [0.41-0.57]
		anti-SR-BI	0.004±0.002	0.06 [-0.02-0.14]
anti-SR-BI	10±0.3	erlotinib	0.06±0.01	0.04 [-0.04-0.12]
		dasatinib	0.4±0.09	0.08 [-0.01-0.16]
anti-CLDN1	0.5±0.06	erlotinib	0.15±0.1	0.32 [0.24-0.40]
		dasatinib	0.025±0.015	0.09 [0.01-0.17]
		anti-SR-BI	0.2±0.06	0.4 [0.32-0.48]
		anti-CD81	0.45±0.05	1.04 [0.96-1.12]
erlotinib	3.8±0.2	dasatinib	1.8±0.3	0.78 [0.7-0.86]

Compound 1	IC_{75} (µM)	Compound 2	IC_{75} (µM) for combination	CI
alisporivir	10±2	anti-CD81	1.5±0.5	0.3 [0.23-0.38]
		anti-SR-BI	7±1	0.69 [0.62-0.77]
		anti-CLDN1	1.8±0.4	0.20 [0.12-0.27]
		erlotinib	1.5±0.9	0.19 [0.11-0.27]
		dasatinib	4±1	0.43 [0.35-0.51]

IC_{75} of individual entry inhibitors: erlotinib, 3.8±0.2 µM; dasatinib, 4±0.8 µM.

C.

Compound 1	IC_{90} (µg/ml or µM)	Compound 2	IC_{90} (µg/ml) for combination	CI
anti-CD81	0.3±0.2	erlotinib	0.2±0.09	0.68 [0.57-0.78]
		dasatinib	0.08±0.04	0.27 [0.18-0.38]
		anti-SR-BI	N.A.	N.A.
anti-SR-BI	N.A.	erlotinib	N.A.	N.A.
		dasatinib	N.A.	N.A.
anti-CLDN1	0.8±0.05	erlotinib	0.5±0.09	0.64 [0.53-0.74]
		dasatinib	0.08±0.02	0.13 [0.02-0.23]
		anti-CD81	0.75±0.05	0.95 [0.85-1.06]
		anti-SRBI	N.A.	N.A.
erlotinib	10±0.6	dasatinib	7.7±1.0	0.78 [0.88-0.68]

IC_{90} of individual entry inhibitors: anti-CD81, 0.3±0.2 µg/ml; anti-CLDN1, 0.8±0.05 µg/ml; erlotinib, 10±0.6 µM; dasatinib, 10±0.7 µM. N.A.: not applicable.

Supplementary Table 3. Synergy of entry inhibitors and IFN-α2a or IFN-α2b on inhibition of HCV infection. Huh7.5.1 cells were pre-incubated with serial concentrations of IFN-α2a or IFN-α2b and 0.01 µg/ml of receptor-specific (anti-CD81, anti-SR-BI or anti-CLDN1) or respective isotype control mAbs for 1h at 37°C. Means ± SD from at least three independent experiments performed in triplicate are shown. HCVcc infection was carried out and analyzed and the CI at (A) IC_{50}, (B) IC_{75} and (C) IC_{90} was calculated as described in supplementary table 1. Means [95% credible intervals] from three independent experiments performed in triplicate are shown for the CI.

A.

Compound 1	IC_{50} (UI/ml)	Compound 2	IC_{50} (UI/ml) for combination	CI
IFN-α2a	0.3±0.16	anti-CD81	0.032±0.001	0.60 [0.53-0.69]
		anti-SR-BI	0.08±0.04	0.27 [0.19-0.35]
		anti-CLDN1	0.02±0.01	0.13 [0.06-0.22]
IFN-α2b	0.2±0.05	anti-CD81	0.035±0.01	0.67 [0.59-0.75]
		anti-SR-BI	0.07±0.04	0.36 [0.29-0.45]
		anti-CLDN1	0.02±0.05	0.17 [0.09-0.25]

IC_{50} of individual receptor-specific mAbs: anti-CD81, 0.02±0.01 µg/ml; anti-SR-BI, 1.3±0.4 µg/ml; anti-CLDN1, 0.18±0.03 µg/ml.

B.

Compound 1	IC_{75} (UI/ml)	Compound 2	IC_{75} (UI/ml) for combination	CI
IFN-α2a	1±0.7	anti-CD81	0.5±0.09	0.65 [0.56-0.72]
		anti-SR-BI	0.6±0.05	0.6 [0.52-0.67]
		anti-CLDN1	0.3±0.1	0.32 [0.24-0.40]
IFN-α2b	0.7±0.07	anti-CD81	0.3±0.1	0.58 [0.50-0.66]
		anti-SR-BI	0.4±0.05	0.57 [0.49-0.65]
		anti-CLDN1	0.3±0.05	0.44 [0.36-0.52]

IC_{75} of individual receptor-specific mAbs: anti-CD81, 0.065±0.02 µg/ml; anti-SR-BI, 10±0.3 µg/ml; anti-CLDN1, 0.5±0.06 µg/ml.

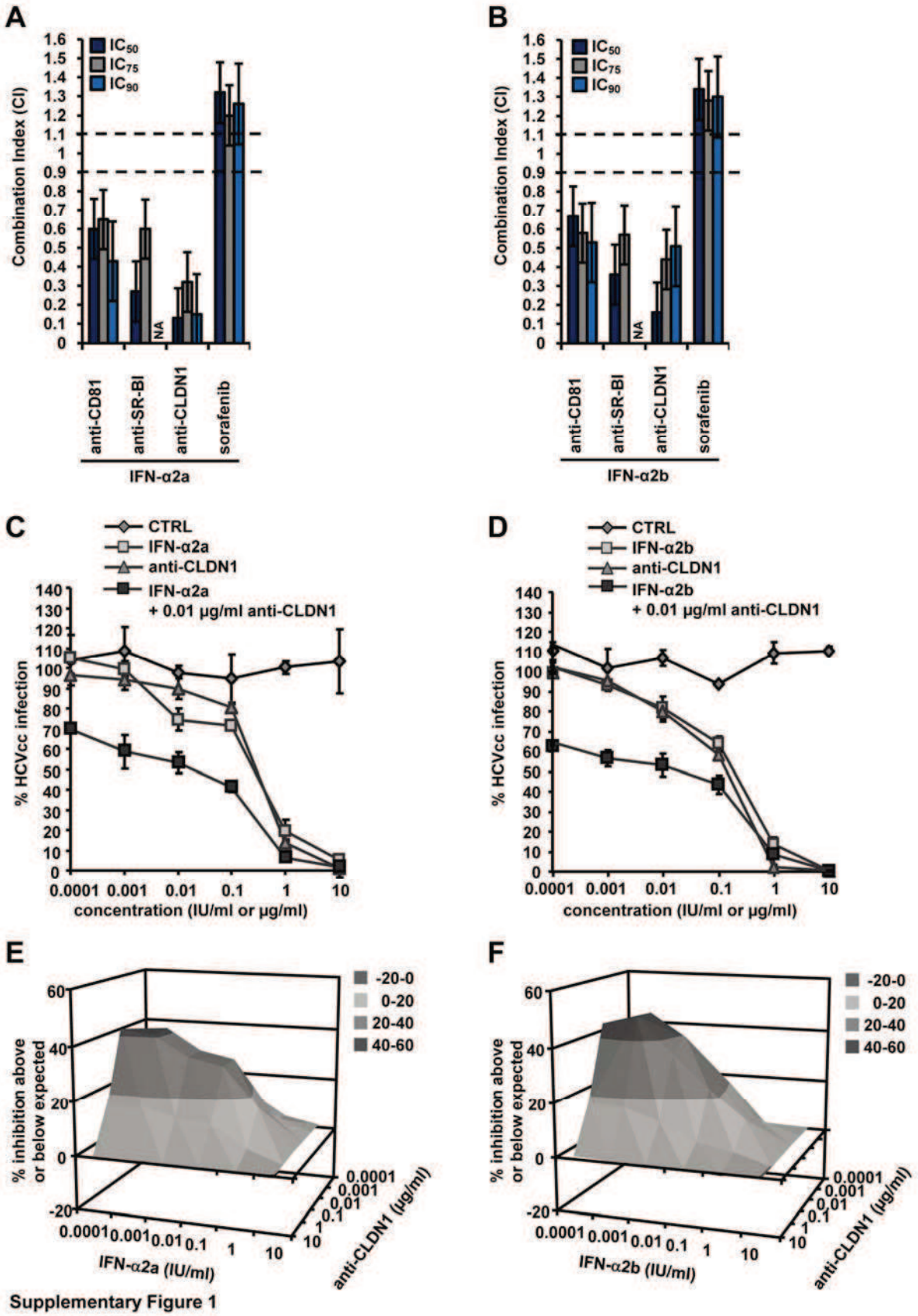
C.

Compound 1	IC_{90} (UI/ml)	Compound 2	IC_{90} (UI/ml) for combination	CI
IFN-α2a	5±09	anti-CD81	2±0.17	0.43 [0.33-0.54]
		anti-SR-BI	N.A.	N.A.
		anti-CLDN1	0.7±0.18	0.16 [0.06-0.27]
IFN-α2b	2±0.2	anti-CD81	1±0.26	0.53 [0.43-0.64]
		anti-SR-BI	N.A.	N.A.
		anti-CLDN1	1±0.43	0.51 [0.41-0.62]

IC_{90} of individual receptor-specific mAbs: anti-CD81, 0.3±0.2 µg/ml; anti-CLDN1, 0.8±0.05 µg/ml. N.A.: not applicable.

SUPPLEMENTARY FIGURE LEGEND

Supplementary Figure 1. Antiviral activity of IFN-α and entry inhibitors in combination. Huh7.5.1 cells were pre-incubated for 1h with serial concentrations of (A) IFN-α2a or (B) IFN-α2b and 0.01 µg/ml of receptor-specific (anti-CD81, anti-SR-BI, anti-CLDN1) mAbs before incubation with HCVcc Luc-Jc1 in the presence of both compounds. HCVcc infection was analyzed by luciferase activity as described[1] (A-B) The CIs at $IC_{50,}$ IC_{75} and IC_{90} are indicated in Supporting Table 3. Dotted lines at combination values of 0.9 and 1.1 indicate the boundaries of additivity. Means ± 95% credible intervals from at least three independent experiments performed in triplicate are shown. (C-D) Combination of IFN-α2a or IFN-α2b with anti-CLDN1 mAb. (E-F) Synergy was confirmed using the method of Prichard and Shipman[3].



Supplementary Figure 1

SUPPLEMENTARY REFERENCES

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Part 2:

Hepatitis C virus cell-cell transmission and resistance to direct-acting antiviral agents

Introduction

The goal of the second part of my PhD was to investigate the role of cell-cell transmission in viral resistance and assess novel strategies to prevent viral resistance. HCV cell-cell transmission plays an important role in HCV spread. The newly developed DAAs improved the outcome of patients infected with HCV, however, the rapid emergence of resistant variants becomes problematic and decrease the efficacy of DAAs. We first made mutations in the HCV NS3 or NS5A region to generate DAA-resistant HCV. Then I set up a spread assay by co-culturing HCV-infected and non-infected Huh7.5.1 cells and monitoring viral infection for two weeks. Using this HCV spread assay, we showed that HCV cell-cell transmission is the dominant transmission route for both wild-type and DAA-resistant HCV in cell culture. Entry inhibitors efficiently blocked cell-cell transmission and the spread of both wild-type and DAA-resistant HCV. I then set up a long-term HCV infection assay in order to investigate the antiviral effects of inhibitors in a relatively long period of time. This assay is based on DMSO-treated differentiated hepatoma cells. In this long-term HCV infection assay, we observed that DAA monotherapy has low genetic barrier to resistance and resulted in rapid viral rebound three weeks after treatment. Sequence analysis revealed that DAA-resistant variants emerged and became the dominant strain. In contrast, blocking cell-cell transmission using entry inhibitors prevented the emergence of DAA-resistant variants and potentiated the efficacy of DAAs. These data indicate that combination of a DAA and an entry inhibitor allows to clear HCV in the persistent HCV infection cell culture.

Taken together, in this study we discovered the important role of virus cell-cell transmission in the emergence of drug-resistance. Blocking cell-cell transmission prevents viral resistance to DAA and contributes to viral clearance. The proof-of-concept will be applied not only in HCV infection but also in many other virus infections, where cell-cell transmission plays a role in their spread. The results of this study have been published in *PLoS Pathogens*.

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Hepatitis C virus cell-cell transmission and resistance to direct-acting antiviral agents

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Abstract

Hepatitis C virus (HCV) is transmitted between hepatocytes via classical cell entry but also uses direct cell-cell transfer to infect neighboring hepatocytes. Viral cell-cell transmission has been shown to play an important role in viral persistence allowing evasion from neutralizing antibodies. In contrast, the role of HCV cell-cell transmission for antiviral resistance is unknown. Aiming to address this question we investigated the phenotype of HCV strains exhibiting resistance to direct-acting antivirals (DAAs) in state-of-the-art model systems for cell-cell transmission and spread. Using HCV genotype 2 as a model virus, we show that cell-cell transmission is the main route of viral spread of DAA-resistant HCV. Cell-cell transmission of DAA-resistant viruses results in viral persistence and thus hampers viral eradication. We also show that blocking cell-cell transmission using host-targeting entry inhibitors (HTEIs) was highly effective in inhibiting viral dissemination of resistant genotype 2 viruses. Combining HTEIs with DAAs prevented antiviral resistance and led to rapid elimination of the virus in cell culture model. In conclusion, our work provides evidence that cell-cell transmission plays an important role in dissemination and maintenance of resistant variants in cell culture models. Blocking virus cell-cell transmission prevents emergence of drug resistance in persistent viral infection including resistance to HCV DAAs.

Author Summary

In spite of the rapid development of antiviral agents, antiviral resistance remains a challenge for the treatment of viral infections including hepatitis B and C virus (HBV, HCV), human immunodeficiency virus (HIV) and influenza. Virus spreads from infected cells to surrounding uninfected host cells to develop infection through cell-free and cell-cell transmission routes. Understanding the spread of resistant virus is important for the development of novel antiviral strategies to prevent and treat antiviral resistance. Here, we characterize the spread of resistant viruses and its impact for emergence and prevention of resistance using HCV as a model system. Our results show that cell-cell transmission is the main transmission route for antiviral resistant HCV strains and is crucial for the maintenance of infection. Monoclonal antibodies or small molecules targeting HCV entry factors are effective in inhibiting the spread of resistant HCV in cell culture models and thus should be evaluated clinically for prevention and treatment of HCV resistance. Combination of inhibitors targeting viral entry and clinically used direct-acting antivirals (DAAs) prevents antiviral resistance and leads to viral eradication in cell culture models. Collectively, the investigation provides a new strategy for prevention of viral resistance to antiviral agents.

Introduction

There is accumulating evidence that viruses use different routes for transmission and spread in infected tissue [1,2]. A well-characterized example is hepatitis C virus (HCV) that is transmitted between hepatocytes via classical cell entry using cell-free diffusion but also uses direct cell-cell transfer to infect neighboring cells [3,4] (Figure 1A). While cell-free entry is most appropriate for the initiation of HCV infection, cell-cell transmission is thought to play an important role in viral persistence and in the maintenance of infection [5]. A key feature of cell-cell transmission is its resistance to neutralizing antibodies present in HCV-infected individuals [4].

Several host factors involved in cell-free viral entry have also been shown to contribute to cell-cell transmission. These include scavenger receptor BI (SR-BI), CD81, tight junction proteins claudin-1 (CLDN1) and occludin (OCLN) as well as host cell kinase epidermal growth factor receptor (EGFR) and its signal transducer HRas [6-12]. HCV envelope glycoproteins are also essential for this process [11]. However, whereas the majority of monoclonal antibodies (mAbs) targeting the viral envelope fails to inhibit cell-cell transmission, several host-targeting entry inhibitors (HTEIs) have been shown to inhibit HCV cell-cell transmission [6-12].

Antiviral resistance remains a major challenge for the treatment of chronic viral infections including HCV, hepatitis B virus (HBV), human immunodeficiency virus (HIV) and influenza infection. Antiviral resistance to nucleos(t)ide analogs is a major cause of treatment failure in chronic HBV-infected patients [13]. Although the combination of antiretroviral drugs has markedly improved the effective control of the progression of HIV disease, the emergence of multidrug-resistant viruses still threatens their long-term efficacy [14]. The recent introduction of a first-generation protease inhibitor to PEG-IFN-α/ribavirin (RBV) therapy has improved the outcome for HCV genotype 1-infected patients [15,16], but a main limitation of these DAAs is their low genetic barrier for resistance [17,18]. Next generation viral protease inhibitors, NS5A and polymerase inhibitors are currently being evaluated in combination with PEG-IFN-α or other DAAs in IFN-free regimens, with or without RBV [19-26] with sofosbuvir and simeprevir having received FDA approval. Although newly developed DAAs are very effective in the majority of previously untreated patients, antiviral resistance as well as differences in virological outcomes for different genotypes and subtypes have been reported [24,27,28]. Furthermore, a

significant number of patients with advanced liver disease and who are null or partial responders to previous therapy still do not achieve a sustained virological response [18,22,26,29].

The functional role of cell-cell transmission and spread in the emergence of antiviral resistance is unknown. The aim of this study was to assess the role of cell-cell transmission in antiviral resistance using HCV genotype 2 infection as a model, and to explore cell-cell transmission as a target to prevent and treat DAA-resistance.

Material and Methods

Cell lines. Culture of Huh7.5.1 [30], Huh7.5-GFP [11] and CD81$^-$ Huh7.5 cells [31] has been described.

Antibodies and inhibitors. CLDN1- (OM-7D3-B3) [32], SR-BI- (NK-8H5-E3) [8] and CD81- (QV-6A8-F2C4) [9] specific mAbs and respective control mAbs have been described. Erlotinib was obtained from LC Laboratories. Anti-HCV neutralizing antibodies (AP33 from Genentech and purified human anti-HCV IgG from our laboratory) have been described [33,34]. Mouse/human IgG was from BD Bioscience and NS5A-specific mouse mAb was from Virostat. The E2-specific human antibody (CBH-23) was a kind gift from Dr. Steven Foung (Stanford University, USA) [35]. Inhibitors of HCV protease (telaprevir, boceprevir and simeprevir) and HCV NS5A (daclatasvir) were synthesized by Acme Bioscience, Inc.

Primers. Primers used to generate NS3 mutations: 5'-GTT GGG CTC TTC CGA TCA GCT GTG TGC TCT C-3' (A156S, sense), 5'-GAG AGC ACA CAG CTG ATC GGA AGA GCC CAA C-3' (A156S, antisense), 5'- CGG GGA AGT CCA AAT CAT GTC CAC AGT CTC TCA-3' (L36M, sense), 5'-TGA GAG ACT GTG GAC ATG ATT TGG ACT TCC CCG-3' (L36M, antisense), 5'-CGT CGT TGG GCT CTT CAA AGC AGC TGT GTG CTC T -3' (R155K, sense) and 5'- AGA GCA CAC AGC TGC TTT GAA GAG CCC AAC GAC G-3' (R155K, antisense). Primers used in nested PCR for direct sequencing of NS3 mutations: NS3 outer forward, 5′-ATC GTC TGG GGA GCG GAG AC-3′; NS3 outer reverse, 5′-AAT TTG CCA TAT GTG GAG TAC GT-3′; NS3 inner forward, 5′-ACG GCT GCA TGT GGG GAC AT-3′; NS3 inner reverse, 5′-GTG CTC TTT CCA CTG GT-3′. Primers used for amplifying and sequencing E1E2 mutations: 5’-TTT GCC GAC CTC ATG GGG TAC AT-3’; reverse, 5’-TCC GCT AAG AAG AGC AGG AAT AAG AGT A-3’. Primers used in nested PCR for amplifying NS5A cDNA fragments: NS5A outer forward, 5’-CTA CGT GAC GGA GTC GGA TG-3’; NS5A outer reverse, 5’-AAC TTT TCC TCT TCG GGG CT; NS5A inner forward, 5’-CAG CGT GTG ACC CAA CTA CT-3’; NS5A inner reverse, 5’-TCG GGG CTA CAG GGA GTT AT-3’. Primers used for sequencing NS5A region: TAA CTG AGG ACT GCC CCA TCC CAT, TTA AGC CCA ACG CAG AAC GA, CGC AGA CGT ATT GAG GTC CAT GCT AA.

Production and infection of DAA-resistant HCVcc. Drug-resistant individual or combined mutations were introduced in the NS3 region of the Luc-Jc1 (genotype 2a/2a) and/or Jc1 plasmid [36-38] using the QuikChange II XL site-directed mutagenesis kit (Stratagene) as previously described [39]. A one-step polymerase chain reaction (PCR) mutagenesis was performed using the primers as described in "**Primers**". Mutations NS3-A156S, NS3-R155K and NS3-L36M/R155K were confirmed by DNA sequence analysis (GATC Biotech) for the desired mutation and for exclusion of unexpected residue changes in the NS3 encoding sequences. HCVcc J4/JFH1 (genotype 1b/2a) and HCVcc J4/JFH1 NS5A-Y93H (Y2065H) have been described [40]. HCVcc ($TCID_{50}$ 10^3/mL to 10^4/mL) were produced as described [6]. Viral infection was analyzed by assessing the intracellular luciferase activity [6,32] or intracellular HCV RNA levels as described [6,32,41].

HCV spread assay. Huh7.5.1 cells transfected with HCVcc Luc-Jc1 or Luc-Jc1 containing the NS3 A156S mutation were cultured with fresh Huh7.5.1 cells (1:1) in 24-well plates. Medium was replenished every 3-4 days until the end of the experiment. Cells were harvested at different time points and HCV infection was assayed in cell lysates by monitoring luciferase activity and the percentage of infected cells was assessed by NS5A immunostaining with flow cytometry over 14 days. To investigate the role of cell-cell transmission for viral spread and dissemination, neutralizing antibodies (nAbs, 25 µg/mL AP33 or 25 µg/mL anti-HCV IgG) potently inhibiting cell-free entry [6,11] were added to block cell-free transmission until the end of the experiment (Figure 1B).

HCV cell-cell transmission assay. Cell-cell transmission was assessed as illustrated in Figure 1C and described previously [6,11]. Producer Huh7.5.1 cells were electroporated with HCV Jc1 or J4/JFH1 RNA with DAA-resistant mutations and co-cultured with naive target Huh7.5-GFP cells in the presence of 1 or 10 µg/mL CLDN1-specific mAb, 10 µM erlotinib, 10 µg/mL SR-BI specific mAb or DMSO solvent/rat IgG control. A well-described pool of anti-HCV nAbs (anti-HCV IgG, 25 µg/mL) [34] was added to block cell-free transmission. After 24 h of co-culture, cells were fixed with 1% paraformaldehyde, stained with an NS5A-specific mouse antibody (0.1 µg/mL) or an E2-specific human antibody (CBH-23, 1 µg/mL) and

analyzed by flow cytometry [6,11]. Dead cells and doublets were excluded by scatter gating and cell doublets were discriminated based on FSC-A and FSC-H parameters as described [42]. Cell-cell transmission was defined as percentage HCV infection of Huh7.5-GFP target cells in the presence of anti-HCV neutralizing Abs.

Long-term HCV infection assay. Huh7.5.1 cells were electroporated with Jc1 RNA and seeded in 12-well plates (10^5 cells/well). Cells were treated with control medium, CLDN1- or SR-BI-specific mAb (10 µg/mL), simeprevir (500 nM), daclatasvir (5 nM), the combination of CLDN1- or SR-BI-specific mAb and simeprevir or the combination of daclatasvir and simeprevir. 1% DMSO medium was used during the whole cultivation process to transition the cells into non-dividing stage as described recently [43]. Media were replenished every 3-4 days and HCV RNA was monitored by RT-PCR [44]. Absent HCV RNA quantification by RT-PCR was confirmed using the Abbott RealTime HCV assay (Abbott) (LOD 48 IU/mL with 250 µL liquid sample).

Sequencing of HCV E1E2 envelope, NS3 protease and NS5A mutations. 5 µL of purified extracellular viral RNA, isolated and purified using QIAamp Viral RNA Mini Kit (Qiagen), was reverse-transcribed into cDNA (Thermo Scientific). HCV E1/E2, NS3 and NS5A cDNA fragments were amplified by nested PCR using the primers as described in "**Primers**". The PCR products were then separated on a 1% agarose gel and purified using Wizard SV Gel and PCR Clean-Up System (Promega). The presence of predominant mutations was analyzed by DNA direct sequence analysis (GATC Biotech) using Chromas Pro Version 1.7.3 software (Technelysium Pty Ltd). To further identify DAA-resistant mutations in the HCV NS3 gene, the purified second round PCR products were ligated into a pGEM-T vector (Promega) and then used to transform JM109 competent cells for clonal selection on LB/ampicillin/IPTG/X-Gal plates according to the manufacturer's protocol. Plasmid DNA from selected clones was amplified and purified using a Qiagen Miniprep Kit (Qiagen) for DNA sequencing (GATC Biotech).

Cell viability assays. Cytotoxic effects on cells were assessed at the end of the long-term HCV infection assay by analyzing the ability of the cells to metabolize 3-

(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described [6].

Statistical analysis. Unless otherwise stated, results are expressed as means ± standard deviation (SD) from at least 3 independent experiments performed in triplicate. Statistical analyses were performed using Student's *t*-test, with a *p*-value of <0.05 being considered statistically significant.

Results

Cell-cell spread is the main route for transmission and dissemination of DAA-resistant viruses. The spread of DAA-resistant viruses has an important impact for the development of antiviral resistance, leading to viral breakthrough and treatment failure. However, the role of viral cell-cell transmission and spread for resistance is unknown. To address this question, we first generated DAA-resistant viruses by introducing well-characterized DAA-resistance mutations in NS3 (NS3-A156S, NS3-R155K and NS3-L36M/R155K) or NS5 region (NS5A-Y93H) [45]. Introduction of mutations increased the IC_{50} of telaprevir, boceprevir and daclastavir up to 10-fold (Figure S1 and Table S1), demonstrating that these DAA-resistant viruses are indeed able to escape inhibition by DAAs.

We then investigated the spread of DAA-resistant viruses using a recently developed state-of-the-art model for viral spread [6,8] which is displayed in Figure 1B and described in Materials and Methods. As shown in Figure 2A-B, both wild-type and DAA-resistant (A156S) viruses efficiently spread during the first 14 days after infection, despite the presence of anti-HCV nAb (AP33) efficiently blocking viral entry through cell-free transmission, with an increase of more than 2 $\log_{10}$ in their viral load. Sequence analysis demonstrated that DAA-resistant virus (A156S) was indeed the predominant variant at day 14 in the experiments displayed in Figure 1B, D and F (data not shown). Thus, given an inhibition of viral entry through cell-free transmission of more than 95% in the presence of nAb (AP33) [6] (Figure 2G), we conclude that cell-cell transmission represents the main transmission route for DAA-resistant viruses. We quantified the percentage of HCV positive cells at the end of the viral spread assay (Figure 3). The majority of the cells became HCV positive (96%/WT, 92%/A156S) after 14 days of viral spread (Figure 3A-B). Although the cell-free HCV in the supernatant was efficiently neutralized by nAb (anti-HCV IgG) (Figure 3C), the spread of wild-type and DAA-resistant HCV was still efficient in the presence of nAb (86%/WT, 82%/A156S) (Figure 3A-B). Given the minor effect of nAbs efficiently inhibiting cell-free transmission, these data confirm that cell-cell transmission is the major route of viral dissemination for both wild-type and DAA-resistant HCV.

HTEIs efficiently block cell-cell spread of DAA-resistant HCV. We next investigated whether HTEIs inhibit total spread of DAA-resistant viruses. As shown in Figure 2C-F and Figure 3A-B, in contrast to telaprevir, which did not inhibit viral spread of the DAA-resistant virus, HTEIs such as CLDN1-specific mAb and erlotinib markedly inhibited viral spread of wild-type and DAA-resistant viruses. Collectively, these data demonstrate that blocking the spread of DAA-resistant viruses by HTEIs is useful to prevent viral breakthrough caused by DAA resistance (Figures 2C-F, 3A-B, S2 and S3).

To confirm that HTEIs inhibit viral spread by inhibition of cell-cell transmission of DAA-resistant HCV, we applied a well-established cell-cell transmission assay (Figure 1C). In this assay HCV producer cells are co-cultured with HCV target cells for 24 h in the presence of broadly nAb (anti-HCV IgG) [6,8] to inhibit cell-free viral entry. Since anti-HCV IgG inhibited up to 95% of HCV cell-free entry (Figure 2G and 3C), viral transmission thus occurs predominantly via cell-cell transfer in this assay. As shown in Figure 4A and 4C (left panels), HCVcc Jc1(2a/2a) NS3-A156S and Jc1(2a/2a) NS3-L36M/R155K are indeed efficiently transmitted through cell-cell transmission, and the extent of their spread through this route was similar to the wild-type strain (data not shown) [6,8], demonstrating that DAA-resistant HCVcc are transmitted through cell-cell transfer and thus escape circulating neutralizing antibodies. CLDN1-specific mAb and erlotinib markedly inhibited cell-cell transmission of protease inhibitor-resistant viruses (Figure 4). J4/JFH1 NS5A (1b/2a) is hundreds of times less infectious than Jc1 [46], resulting in less efficient viral cell-cell transmission than Jc1 NS3-A156S and NS3-L36K/R155K (Figure S4). Although cell-cell transmission for this strain was very low, the HTEIs appeared also to have a potential inhibitory effect on cell-cell transmission of NS5A inhibitor-resistant viruses (Figure S4). These results demonstrate that HTEIs prevent cell-cell transmission of DAA-resistant viruses in cell culture models.

Viral spread through HCV host entry factors is essential for maintenance of persistent viral infection. Interestingly, in Figure 2E-F, the HTEIs (CLDN1-specific mAb and erlotinib) not only inhibited viral spread, but also were capable of decreasing viral load 7 days after treatment with HTEIs, suggesting that blocking HCV cell-cell transmission impairs maintenance of HCV infection. To further test this

hypothesis, we monitored HCV infection in CD81 knock-out hepatoma cells (CD81$^-$ Huh7.5) that are resistant to cell-free entry and only display minimal levels of CD81-independent cell-cell transmission [11,31]. Briefly, we transfected CD81$^-$Huh7.5 cells with HCV RNA (Luc-Jc1) and monitored HCV infection in the viral spread assay over 2 weeks. Consistent with the results shown in Figure 2E-F, HCV load in CD81$^-$ Huh7.5 cells gradually decreased, while it increased over 30 times in control CD81-expressing Huh7.5 cells (Figure 2H). Collectively these results indicate that cell-cell viral spread is essential for maintenance of persistent HCV infection in cell culture models.

Inhibition of cell-cell transmission by HTEIs prevents emergence of DAA-resistant variants and results in viral clearance. To assess whether blocking cell-cell transmission of DAA-resistant variants by HTEIs impairs the emergence of viral resistance in cell culture models we established long-term HCV infection experiments using HCV-Jc1 transfected Huh7.5.1 cells incubated in the presence of DMSO [43,47]. The incubation of cells in the presence of DMSO has been shown to allow studying virus-host interactions during long-term infection and has been suggested to be one of the most physiological HCV cell culture models based on liver-derived cell lines [43,47].

We chose a well-characterized protease inhibitor, simeprevir, which has recently received FDA approval to treat chronic hepatitis C, for further studies. Approximately 60% of the cells stained HCV-positive before initiation of treatment (Figure S5). It has been shown that simeprevir efficiently inhibits HCV replication in HCV cell culture with IC_{50} being below 13 nM [48]. We used a dose of 500 nM (>IC_{90}), which reduced viral load more than 10-fold within 3 days in HCV cell culture confirming that the dose is highly potent and relevant for inhibition of genotype 2 replication (Figure 5A). As shown in Figure 5A, simeprevir treatment resulted in a rapid decline of the viral load initially, reducing the viral load of HCV-infected cells by up to 1.5 $\log_{10}$ within 5-6 days after introducing the DAA. However, viral rebound was observed after 2-3 weeks, with a viral load increasing to the same level as untreated cells in line with previous reports [48].

In contrast, treatment using an HTEI such as CLDN1-specific mAb (OM-7D3-B3), which has been shown to inhibit HCV entry in a pan-genotypic activity without

displaying any cytotoxic effect on hepatic cells [32], led to a slow but steady decrease of the viral load (Figure 5A). No viral rebound was observed during CLDN1-specific mAb treatment, demonstrating that CLDN1 as a target has a high genetic barrier to HCV resistance. Finally, combination of CLDN1-specific mAb and simeprevir resulted in a more rapid, efficient and sustained reduction in viral load than simeprevir monoexposure (Figure 5A). Most interestingly, during combination treatment, HCV RNA became undetectable by qualitative RT-PCR and using the clinically licensed Abbott RealTime HCV assay (Abbott) with a limit of detection of 48 IU/mL (Figure 5A). Viral load remained undetectable after withdrawal of the combination treatment, indicating that viral eradication was sustained and indeed due to the combination of entry and protease inhibitor (Figure 5A). According to our previous study and reports from others, anti-CLDN1 mAb and simeprevir exhibit no toxicity to hepatoma cells *in vitro* at the concentrations used in this study [32,48]. Nevertheless, we performed additional experiments to exclude that toxic effects were responsible for decline in viral load and loss of virus. As shown in Table 2, MTT-based cell viability assays at the end of the long-term experiments showed no differences between treated and untreated cells. These data confirm that the clearance of viral infection is indeed due to HTEI treatment and not related to adverse effects of the compounds during long-term treatment.

To further explore the development of viral resistance, we performed sequence analyses of viral variants at different time points (the start of treatment, 10 and 23 days after treatment). Whereas DAA-monotherapy resulted in the emergence of well-described NS3 resistance mutations 23 days after treatment (Figure 6 and 7), wild-type NS3 HCV remained the predominant strain in CLDN1-specific mAb alone as well as in combination of CLDN1-specific mAb and simeprevir treated cells. Although sequence analyses revealed some rarely occurring variants associated with low DAA resistance (e.g. NS3 I170T) in the presence of combination of CLDN1-specific mAb and simeprevir, these variants were cleared at the end of the treatment as indicated by undetectable viral RNA (Figure 5A). These results demonstrate that the HTEI functionally prevents antiviral resistance to DAA by impairing the spread of resistant viruses in cell culture models.

To assess whether prevention of resistance is universal to HTEIs or compound-dependent, we performed side-by-side experiments using a well-

characterized SR-BI-specific mAb NK-8H5-E3. This antibody has been shown to block efficiently cell-free viral entry and viral dissemination in cell culture models [8], Although the combination of this SR-BI-specific mAb and simeprevir transiently decreased viral load and delayed viral rebound, it did not result in viral clearance as observed in CLDN1-specific mAb/simeprevir combination therapy (Figure 5B). Sequence analysis in cells treated with anti-SR-BI mAb and simeprevir revealed emergence of variants conferring resistance to HCV protease inhibitors (NS3 Y56H) [49] (data now shown) and to SR-BI inhibitors (N415D [39]) (Figure S6). Using direct sequencing we did not detect mutation G451R [50], indicating that G451R is not emerging at high frequency (Figure S6). These data indicate that distinct HTEIs have different genetic barriers for antiviral resistance and that the CLDN1-specific mAb OM-7D3-B3 used in this study appears to have a higher genetic barrier than the SR-BI-specific mAb NK-8H5-E3. This SR-BI-specific antibody was less efficient in inhibiting HCV cell-cell transmission as compared to the CLDN1-specific mAb (Figures S7 and S8), further confirming that an efficient inhibition of HCV cell-cell transmission appears to be required to prevent emergence of DAA-resistant virus in cell culture models.

Finally, we also performed a long-term cell culture infection assay investigating a combination of two DAAs on HCV infection. We tested a highly potent NS5A inhibitor, daclatasvir, which has been shown to have potent pan-genotypic activity against HCV [51], first alone and then in combination with simeprevir in the long-term HCV infection assay. In cell culture, a concentration of 0.1 nM has been shown to alter the subcellular localization and biochemical fractionation of its target NS5A [52]. The concentration of daclatasvir (5 nM) used in the experiment resulted in a more than 10-fold decrease of viral load indicating that the dose is below the IC_{90} in this experimental setting (Figure 5C). However, during long-term treatment the viral load rebounded to the level of the untreated cells at day 31 with emergence of the DAA-resistant NS5A mutation, Y93H (Table 3). Furthermore, in contrast to the combination of an HTEI and a protease inhibitor simeprevir, the combination of daclatasvir and simeprevir (at concentrations > IC_{90}) failed to eradicate HCV genotype 2 infection in Huh7.5.1 cells and HCV load rebounded from day 45 on with emergence of DAA-resistant mutations in both NS3 and NS5A regions (Figure 5C and Table 3).

Taken together, these data indicate that blocking virus cell-cell transmission by an HTEI prevents emergence of drug resistance to DAAs.

Discussion

Although the development of DAAs has greatly improved the outcome of chronic hepatitis C patients, viral resistance to DAAs is still a challenge impeding treatment success. In this study, we demonstrate that HCV strains which are resistant to DAAs predominantly disseminate using cell-cell transmission and show that effective blockade of cell-cell transmission using HTEIs prevents viral resistance resulting in rapid virus elimination.

The ability of a virus to spread within a host is a key determinant of its persistence and virulence. While cell-free entry is important for initiation of infection by virions entering the liver through the bloodstream, HCV dissemination within the liver and establishment of chronic HCV infection may mainly occur by direct cell-cell transmission between adjacent hepatocytes [4]. Although differences in the ability of diverse HCV genotypes to spread via cell-free and cell-cell transmission have been observed [3], cell-cell transmission appears to serve as an important route of transmission for most genotypes [3,12]. While cell-cell transmission has been shown to be relevant for viral evasion from host neutralizing antibodies [6], our data indicate that the spread of DAA-resistant HCV through cell-cell transmission facilitates viral evasion and may contribute to treatment failure. Blocking cell-cell transmission improves antiviral activity of DAAs in cell culture models.

Functional results obtained in cell culture and animal models demonstrate strong evidence that cell-cell transmission also plays a relevant role in dissemination of several viruses including HIV, herpes simplex virus (HSV), measles virus or human T-lymphotropic virus type 1 (HTLV-1) [1,2]. Indeed, cell-cell transmission has been described to spread resistance to antiretroviral drugs in HIV infection [53,54]. Discovery of a novel HBV entry factor [55] will allow to investigate whether cell-cell transmission plays a role in HBV transmission. Thus, this study may provide a novel concept to prevent viral resistance in treatment of other viruses.

By interfering with cell-cell transmission (Figure 4), HTEIs are able to prevent the development of antiviral resistance as shown by the absence of functional resistance in cells treated with a combination of DAA and HTEI (Figure 5A). Most

importantly, when added to a DAA, an HTEI allowed rapid and efficient viral clearance as shown by repeatedly confirmed absence of HCV RNA using a highly sensitive and clinically licensed commercial assay (Figure 5A). Since our data indicate that the main transmission route of DAA-resistant variants is direct cell-cell spread (Figure 2 and Figure 3), we assume that the preventive effect of HTEIs is mainly due to their effect on this mode of transmission. Taken together, these data indicate that blocking viral cell-cell transmission enhances antiviral activity of DAAs and prevents DAA-resistance in cell culture models as shown for HCV genotype 2 infection as an example.

It has been discovered that cell-cell transmission of HIV is resistant to DAAs and may lead to therapy failure [53]. Here, we show that HCV cell-cell spread exists in the presence of DAAs in cell culture models (Figure 3). Furthermore, our data demonstrate evidence that HTEIs have differences in their genetic barrier to resistance. Indeed, whereas treatment with CLDN1-specific OM-7D3-B3 mAb resulted in viral clearance without functional evidence for resistance (Figure 5A), SR-BI-specific NK-8H5-E3 mAb resulted in the development of resistant variants apparently escaping anti-SR-BI treatment (Figure 5B). Resistance has been described for a small molecule SR-BI antagonist [39]. Furthermore, a recent study elegantly demonstrated that HCV can lose SR-BI-dependence for cell-cell spread [10]. Together with the findings observed for SR-BI-specific mAb NK-8H5-E3, our data demonstrate that defined SR-BI-targeting compounds appear to have a lower genetic barrier for resistance than other HTEIs such as CLDN1-targeting compounds. This may be due to the fact that CLDN1 is an essential factor for cell-cell transmission whereas SR-BI may be bypassed by other entry factors [10]. Although accessory receptors CLDN6 and 9 have been suggested to confer partial escape from CLDN1-targeting agents for certain isolates in Huh7.5 cells [56], escape could not be confirmed in primary human hepatocytes where expression of CLDN6 and 9 is very low [57].

A theoretical drawback of using HTEIs instead of DAAs as antivirals is their potential higher toxicity *in vivo* given these molecules target host factors and not viral factors. Nevertheless, it has to be pointed out that the development of several DAAs targeting HCV proteins had to be stopped due to adverse effects [5]. Moreover, it's worth noting that the majority of current drugs widely used for metabolic or

inflammatory diseases or cancer, targets host proteins [5]. The preliminary data obtained in this study suggest that the combination of HTEIs and DAAs does not result in detectable toxicity in cell-based assays (Table 1). Furthermore, HTEIs targeting SR-BI or EGFR have been shown to have an acceptable clinical safety profile in inflammatory disease and cancer [58,59].

Collectively, our findings are not only relevant for the understanding of antiviral resistance but may also be of interest for the development of future HCV therapies. For null or partial responders and difficult-to-treat patients with co-morbidity or defined genotypes, there is an unmet medical need for improved antiviral regimens [20]. Compared to the various combinations of DAAs of different classes which are currently evaluated in late stage clinical development and expected to receive regulatory approval soon, the combination of DAAs with a HTEI with a high genetic barrier provides a novel strategy for prevention of antiviral resistance in difficult-to-treat patients where viral breakthrough drive therapy failure [18,26] or future patients exhibiting multiresistance to various DAA combination therapies [18,26].

Indeed, this hypothesis is supported by our results of long-term experiments in cell culture showing that the combination of an HTEI and a DAA cured persistent HCV genotype 2a infection. Since a similar NS3 protease/NS5A inhibitor DAA combination failed to clear HCV genotype 2a and 2b infection in an HCV animal model *in vivo* [60] and viral resistance has been observed for DAAs in particular for genotype 2 and 3 in randomized clinical trials (for review see [26]), our data suggest that the antiviral strategy described in this study may address limitations of DAAs in particular for non genotype 1 infections. Since our proof-of-concept study is based on an HCV genotype 2a viral construct, future studies are needed to investigate its relevance for other genotypes.

In this regard it is of interest to note that small molecule HTEIs are currently investigated as monotherapy in randomized clinical trials [61] (erlotinib: University of Strasbourg Hospitals, ClinicalTrials.gov Identifier NCT01835938) and novel inhibitors of HCV cell-cell transmission are also in preclinical development [62]. Our study provides evidence and directions for their future application in HCV treatment.

Finally, our results have implications for the treatment of other viral infections. As targeting the host is an emerging strategy to overcome resistance [63,64],

blocking cell-cell transmission by HTEIs provides a novel perspective for fighting a wide range of viral infections including HIV, measles virus or HTLV-1 infection where cell-cell transmission has been suggested to play a role in transmission [1,2,53].

Accession numbers/ID numbers

The amino acid sequence of HCV polyprotein [recombinant Hepatitis C virus J6/JFH1] has been previously deposited in NCBI under access number AEB71614.1. The access numbers of human CD81, CLDN1, SR-BI, OCLN, EGFR, HRas and IFN-α are NP_004347.1, claudin-1 NP_066924, NP_005496.4, AAB00195.1, AAB19486.2 and CAG47067.1 and AAA52724.1, respectively. The nucleotide sequence of complete genome of recombinant hepatitis C virus J6/JFH1 has been previously deposited in GenBank under access number JF343793.1

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Figure Legends

Figure 1. Cell culture model systems for analysis of HCV dissemination and cell-cell transmission. (A) Transmission pathways of HCV. HCV can disseminate through two routes of transmission: cell-free entry and cell-cell transmission. Cell-free entry is the classical pathway for initiation of HCV infection and requires a well-defined panel of host entry factors including SR-BI, CD81, CLDN1, OCLN and the EGFR signaling pathway [6-10,12]. Cell-free entry does not require cell-cell contact and is efficiently inhibited by neutralizing antibodies (nAbs). Cell-cell transmission requires direct cell-cell contact and is resistant to nAbs present in HCV-infected patients. Cell-cell transmission is considered to play a key role for viral spread and maintenance of infection [4,31]. (B) HCV spread and dissemination assay. The HCV spread assay monitors viral spread in cell cultures using a low number of Huh7.5.1 cells containing replicating HCVcc, which expand over a time period of 14 days [6,8]. In the absence of nAb or presence of control antibody the virus is transmitted by cell-

cell and cell-free spread (upper panel [6,8]). Cell-free transmission can be blocked by anti-HCV nAbs allowing to study viral spread mediated by cell-cell transmission only (lower panel). (C) *HCV cell-cell transmission assay [6,7,11]*. The cell-cell transmission assay allows the investigation of cell-cell transmission and its inhibition by HTEIs. In this assay HCV^+ Huh7.5.1 producer cells containing replicating HCVcc (stained by an anti-NS5A antibody and indicated in red) are co-cultured with HCV^- Huh7.5 GFP target cells (indicated in green) for 24 h in the presence of nAb blocking cell-free transmission. Flow cytometry is used to quantitate infected HCV^+ Huh7.5 GFP target cells (indicated by red and green), which are infected via cell-cell transmission and stained with anti-NS5A antibody.

Figure 2. The spread of DAA-resistant viruses is resistant to neutralizing antibodies against HCV but is efficiently inhibited by HTEIs. DAA-resistant variant (Luc-Jc1 A156S in (B), (D) and (F)) or wild-type HCVcc (Luc-Jc1 in (A), (C) and (E)) transfected Huh7.5.1 cells and uninfected Huh7.5.1 cells were incubated in the presence of isotype control antibody (mouse IgG, 25 µg/mL) (total transmission) (A-B), anti-HCV neutralizing antibody (anti-E2 mAb, AP33, 25 µg/mL) to block cell-free transmission (cell-cell transmission) (A-B), 1 µM of telaprevir (C-D), 10 µg/mL of anti-CLDN1 mAb (E-F) or 10 µM of erlotinib (E-F) 2-3 days after transfection. Different media were replenished every 3-4 days. HCV infection was measured by luciferase reporter gene expression in cell lysates at indicated time points as described [36]. (G) HCVcc (Luc-Jc1) was preincubated with cell culture medium (mock control) or control IgG (25 µg/ml) or nAb (AP33 or anti-HCV IgG, 25 µg/ml) for 1 h and then incubated with Huh7.5.1 cells for 4 h at 37°C. HCV load was analyzed 72 h post-infection by luciferase activity. (H) $CD81^-$Huh7.5 cells or Huh7.5 cells were transfected with HCV Luc-Jc1 and maintained in cell culture over 2 weeks. The intracellular viral load was monitored by measuring luciferase activity every 2-4 days. Means ± SD from at least three independent experiments performed in triplicate are shown.

Figure 3. Cell-cell transmission is the main transmission route for DAA-resistant viruses. The spread assay was performed as shown in Figure 2 with or without 25 µg/mL anti-HCV IgG to neutralize cell-free transmission of virus. The relative percentage HCV-positive cells/total cells was determined by immunostaining

for NS5A and flow cytometry. Huh7.5.1 uninfected cells were used as a negative control ("uninfected"). Percentage of wild-type (WT) (A) or DAA-resistant HCV (A156S) (B)-infected cells without treatment (mock) or in the presence of 25 µg/mL anti-HCV IgG (nAb), 10 µg/mL anti-CLDN1 mAb or 1 µM telaprevir treatment as described in Figure 2 is shown. The supernatant (SN) with or without anti-HCV IgG containing cell-free wild-type or A156S HCV was used to infect fresh Huh7.5.1 cells (C). The cell culture medium was taken as a negative control (C). The data are represented of three experiments.

Figure 4. HTEIs effectively block cell-cell transmission of DAA-resistant viruses. The experimental setup is shown in Figure 1C. $NS5A^+$ HCV producer cells (Pi) were transfected with HCV RNA encoding for HCV Jc1 NS3-A156S (A-B) or Jc1 NS3-L36M/R155K (C-D). NS5A+ HCV producer cells and GFP-expressing target cells (T) were co-cultivated with nAb (anti-HCV IgG, 25 µg/mL) to block cell-free transmission as described [6]. Cell-cell transmission of wild-type or drug-resistant strains was determined by quantification of GFP^+ $NS5A^+$ target cells (Ti) by flow cytometry. Protease or NS5A inhibitor-resistant HCV variant producer cells (Pi) cultured with uninfected target cells (T) were then incubated with 1 µg/mL of CLDN1-specific mAb or 10 µM of erlotinib or control medium. HCV-infected target cells (GFP^+NS5A^+) were quantified by flow cytometry (A and C). Percentage of infected target cells is shown as histograms (B and D) and is represented as means ± SD from three experiments performed in triplicate. *p<0.005.

Figure 5. Addition of HTEIs to DAA prevents the emergence of DAA-resistant variants. (A) Huh7.5.1 cells were transfected with RNA encoding wild-type HCV Jc1 and plated in the presence of 1% DMSO and treated with anti-CLDN1 mAb (10 µg/mL), simeprevir (500 nM) alone or in combination with anti-CLDN1 mAb (10 µg/mL) or in the absence of treatment (CTRL). The combination treatment was stopped on day 51 while anti-CLDN1 mAb and simeprevir in monotherapy continued until day 58. Viral load was assessed by RT-PCR every 3-4 days. The limit of quantification (LOQ), indicated by a dashed line, was 10^3 copies/mL. The experiment was performed in triplicate and repeated twice. Among the detected triplicate samples, one out of three was HCV RNA negative on day 27 (empty circle under the

LOQ), two out of three negative on day 30 (empty circles under the LOQ), three out of three negative from day 37 on. The undetectable HCV load from day 40 was confirmed by a clinically licensed HCV RNA detection assay, the Abbott RealTime HCV assay (Abbott), and is indicated by a star (LOD of Abbott qRT-PCR is 48 IU/mL with 250 µL liquid sample). (B) A similarly designed experiment was performed using anti-SR-BI mAb NK-8H5-E3 instead of anti-CLDN1 mAb. (C) Combination of daclatasvir and simeprevir fails to clear HCV genotype 2 infection. Using the same assay as described above, the cells were treated with 5 nM daclatasvir, 500 nM simeprevir, combination of 5 nM daclatasvir and 500 nM simeprevir or mock (CTRL). Viral load was assessed by RT-PCR every 3-4 days. Means ± SD from a representative experiment performed in triplicate are shown.

Figure 6. Analysis of DAA-resistant mutations emerged during treatment protocols by direct sequencing. In the long-term HCV infection assay shown in Figure 5A, HCV RNA in the supernatants from different treatments was purified on day 23. Direct sequencing was performed to identify predominant viral mutations in HCV NS3 protease region as described in Material and Methods. Data are displayed as NS3 amino acid sequence in treated cells.

Figure 7. Mutational analysis of viral variants with treatment of protease inhibitors or/and HTEIs in the long-term HCV infection assay. Clonal sequencing of HCV NS3 mutations in simeprevir monotherapy and the combination of simeprevir and anti-CLDN1 mAb. To further identify simeprevir induced DAA-resistance mutations, HCV RNA from (A) mock (CTRL), (B) 10 µg/mL anti-CLDN1 mAb, (C) 500 nM simeprevir or (D) 500 nM simeprevir + 10 µg/mL anti-CLDN1 mAb treatment on day 23 as shown in Figure 5A and 6 was isolated and amplified as described in Material and Methods. Following cloning and sequencing of the NS3 protease region, the relative distribution of viral variants (wild-type (WT) and NS3 mutations listed in a clockwise order beside the pie charts) was analyzed and is indicated in different shades of grey in the pie charts. The major DAA-resistance mutation, D168V, is highlighted in blue and WT is in light grey. For each variant, the number of detected clones is indicated in the parenthesis.

Table. 1. **The CLDN1-specific antibody is efficient in inhibiting HCV spread.** The spread assay was performed as shown in Figure 3 and Figure S3. The data are pooled and represented as mean±SD form three experiments performed in triplicate.

Treatment	Percentage of HCV positive cells
mock	96 ± 2%
anti-CLDN1	10 ± 2%
simeprevir	20 ± 3%
daclatasvir	15 ± 3%

Table 2. Absent toxicity in Huh7.5.1 cells treated with a HTEI and/or a DAA or 2 DAAs. Cytotoxic effect on Huh7.5.1 cells in the long-term HCV infection assay (Figure 5) were assessed by analyzing the ability to metabolize MTT as described in Material and Methods. 10 µM flavopiridol was used as a positive control. Data are presented as relative cell viability compared to cells cultured in the absence of compounds. Mean ± SD from one representative experiment performed in triplicate are shown.

Treatment	Treatment duration (days)	Concentration	Relative cell viability (%)
mock	60	N/A	100 ± 9
anti-CLDN1	60	10 µg/ml	123 ± 7
anti-SR-BI	60	10 µg/ml	93 ± 1
simeprevir	60	500 nM	99 ± 5
daclatasvir	50	5 nM	102 ± 8
anti-CLDN1+ simeprevir	60	10 µg/ml, 500 nM	133 ± 6
anti-SR-BI+ simeprevir	60	10 µg/ml, 500 nM	92 ± 9
daclatasvir + simeprevir	50	5 nM, 500 nM	107 ± 11
flavopiridol	3	10 µM	20 ± 6*

Table 3. Analysis of NS3A and NS5A mutations during DAA monotherapy or treatment with a combination of DAAs. In the long-term HCV infection experiment shown in Figure 5C, HCV RNA in the supernatants from different treatments was purified on day 31 and day 45. Direct sequencing was performed to identify predominant viral mutations in HCV NS3 and HCV NS5A regions as described in Material and Methods.

Treatment	Treatment duration (days)	NS3A sequence	NS5A sequence
mock	31	WT	WT
daclatasvir	31	WT	Y93H
simeprevir	31	Q29H, D168A/V	WT
simeprevir+ daclatasvir	45	L36V, E79A, Y56H, D168A/E/V	C92S, Y93H

Supporting Information Legends

Figure S1. Functional characterization of protease inhibitor-resistant viruses in HCV infection and their sensitivity to DAAs and HTEIs. Huh7.5.1 cells were pre-incubated for 1h with serial concentrations of (A) telaprevir, (B) boceprevir, (C) CLDN1-specific mAb, (D) CD81-specific mAb, (E) erlotinib, (F) SR-BI-specific mAb (NK-8H5-E3), (G) daclatasvir, (H) CLDN1-specific mAb or respective control reagents before incubation with HCVcc-Jc1-Luc containing the DAA-resistant mutations NS3-A156S (A, C and E), NS3-R155K (B, D and F) or NS5-Y93H (G and H), respectively in the presence of each compound. HCV infection was analyzed 72 h post-infection by luciferase reporter gene expression in cell lysates as described in Material and Methods. Means ± standard error of the means (SEM) from at least three independent experiments performed in triplicate are shown.

Figure S2. Reduction of HCV load by the CLDN1-specific antibody and daclatasvir in viral spread assay. Daclatasvir (0.5 nM) or anti-CLDN1 mAb (10 µg/mL) was used in HCV spread assay as described in Material and Methods as well as in Figure 2. The intracellular viral load was monitored by measuring luciferase activity every 3-4 days. Means ± SD from one representative experiment performed in triplicate are shown.

Figure S3. Control of HCV spread by the CLDN1-specific antibody and daclatasvir. As described in Material and Methods as well as in Figure 3, the relative percentage HCV-positive cells/total cells at day 14 from the experiments shown in Figure S2 was determined by immunostaining for NS5A and flow cytometry. Uninfected Huh7.5.1 cells were used as a negative control ("uninfected") (A). Percentage of wild-type HCV-infected cells without treatment (mock) (B) or in the presence of anti-CLDN1 mAb (10 µg/mL) (C) or daclatasvir (0.5 nM) (D) was shown. One representative experiment out of three independent experiments is shown.

Figure S4. Cell-cell transmission of NS5A inhibitor-resistant viruses and effect of HTEIs. 1 µg/mL of CLDN1-specific mAb or 10 µM of erlotinib was used in the cell-cell transmission assay established with HCV RNA encoding for HCV J4/JFH1 NS5A-Y93H as described in Material and Methods as well as in Figure 4. (A) HCV-

infected target cells (GFP^+NS5A^+) were quantified by flow cytometry. (B) Percentage of infected target cells is shown as histograms and is represented as means ± SD from three experiments performed in triplicate. *p<0.005.

Figure S5. Percentage of HCV-positive cells at the initiation of treatment in the long-term HCV infection assay. (A) The uninfected Huh7.5.1 cells were taken as a negative control. (B) The relative percentage of HCV (Jc1)-positive cells/total cells was determined as described in Material and Methods as well as in Figure 3.

Figure S6. Analysis of E1E2 mutations emerging during treatment with the SR-BI-specific antibody. In Figure 5B, HCV RNA in the supernatants from the SR-BI-specific antibody-treated or mock-treated cells was purified on day 47. Direct sequencing was performed to identify viral mutation(s) in HCV E1E2 region and the sequence of Jc1 construct as described in Material and Methods. The sequence of HCV core, E1 and E2 was shown. Mutation N415D is indicated with a star.

Figure S7. The CLDN1-specific antibody is more effective than the SR-BI-specific antibody in controlling HCV spread. Anti-CLDN1 mAb (10 µg/mL) or SR-BI mAb (10 µg/mL) was used in the spread assay as described in Material and Methods as well as in Figure 2. The intracellular viral load was monitored by measuring luciferase activity every 3-4 days. Means ± SD from one representative experiment performed in triplicate are shown.

Figure S8. The CLDN1-specific antibody is more effective than the SR-BI-specific antibody in inhibiting HCV cell-cell transmission. Cell-cell transmission assay is described in the Material and Methods as well as in Figure 4. An anti-E2 human antibody (CBH-23) was used to stain HCV-positive cells in the presence of anti-SR-BI mAb. (A) Rat or (B) mouse IgG was used as control for (C) the CLDN1-specific antibody (10 µg/mL) or (D) the SR-BI-specific antibody (10 µg/mL), respectively. (E) Percentage of infected target cells is shown as histograms and is represented as mean ± SD from three experiments performed in triplicate. *p<0.005.

Table S1. Functional characterization of DAA-resistant viruses in HCV infection and their sensitivity to HTEIs. In the experiment shown in Figure S1, IC_{50} was

calculated. Means ± SD from at least three independent experiments performed in triplicate are shown.

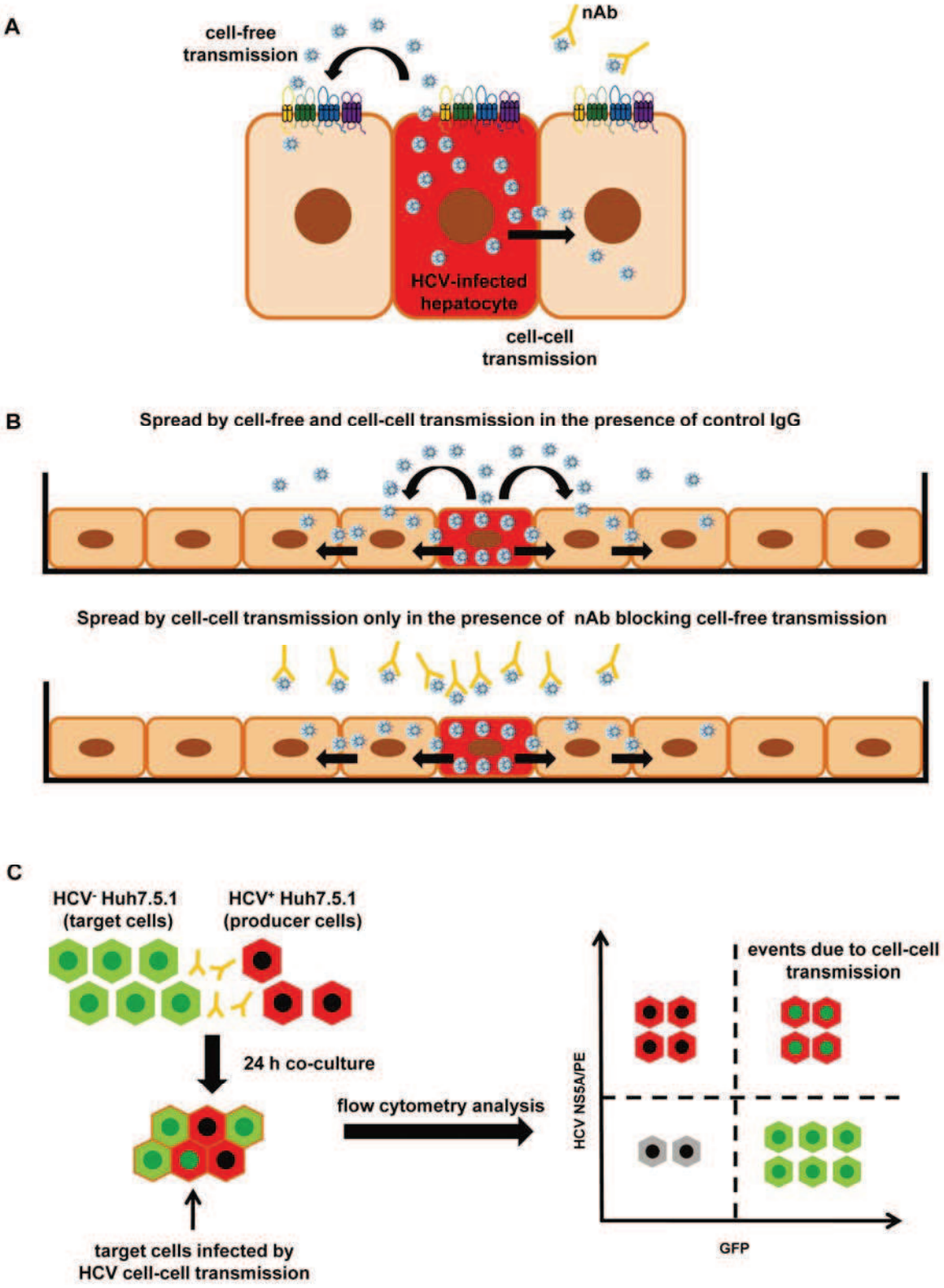
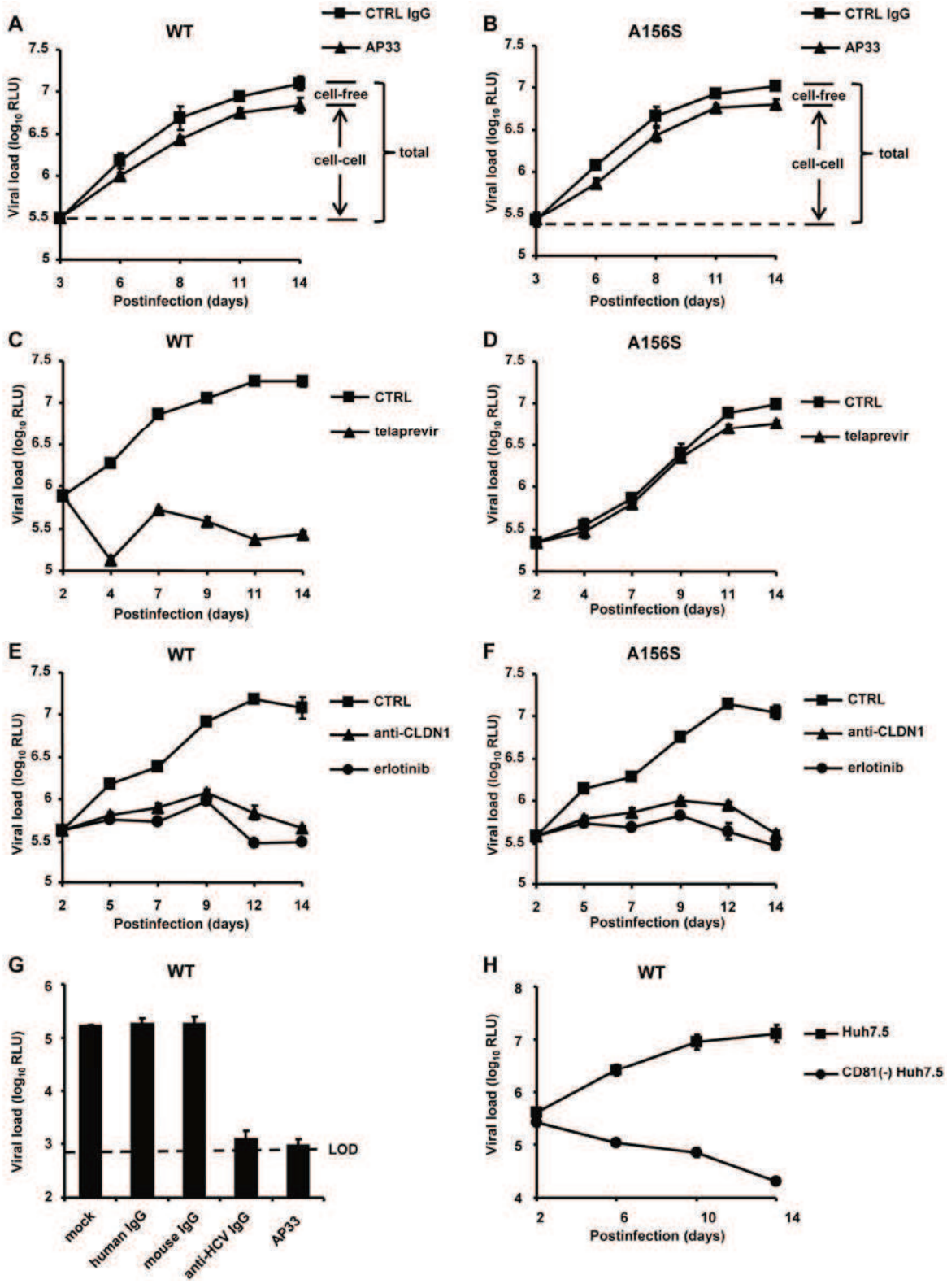

A
cell-free
transmission
nAb
HCV-infected
hepatocyte
cell-cell
transmission
B
Spread by cell-free and cell-cell transmission in the presence of control IgG
Spread by cell-cell transmission only in the presence of nAb blocking cell-free transmission
C
HCV- Huh7.5.1
(target cells)
HCV+ Huh7.5.1
(producer cells)
24 h co-culture
target cells infected by
HCV cell-cell transmission
flow cytometry analysis
events due to cell-cell
transmission
HCV NS5A/PE
GFP

Figure 1


A
WT
CTRL IgG
AP33
cell-free
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total
Viral load (log10 RLU)
Postinfection (days)
B
A156S
C
WT
CTRL
telaprevir
D
A156S
E
WT
anti-CLDN1
erlotinib
F
A156S
G
WT
LOD
mock
human IgG
mouse IgG
anti-HCV IgG
AP33
H
WT
Huh7.5
CD81(-) Huh7.5

Figure 2

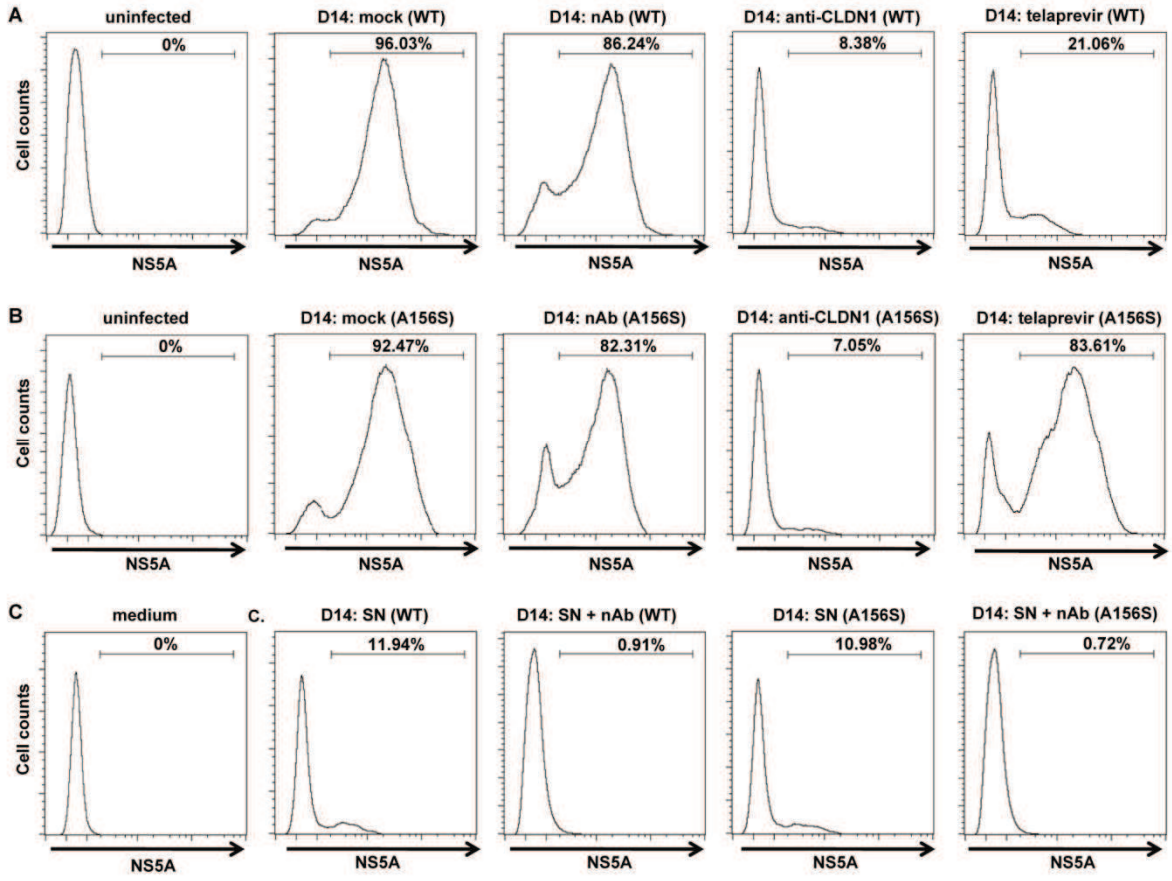
A
uninfected
0%
D14: mock (WT)
96.03%
D14: nAb (WT)
86.24%
D14: anti-CLDN1 (WT)
8.38%
D14: telaprevir (WT)
21.06%
Cell counts
NS5A
B
uninfected
0%
D14: mock (A156S)
92.47%
D14: nAb (A156S)
82.31%
D14: anti-CLDN1 (A156S)
7.05%
D14: telaprevir (A156S)
83.61%
C
medium
0%
C.
D14: SN (WT)
11.94%
D14: SN + nAb (WT)
0.91%
D14: SN (A156S)
10.98%
D14: SN + nAb (A156S)
0.72%

Figure 3

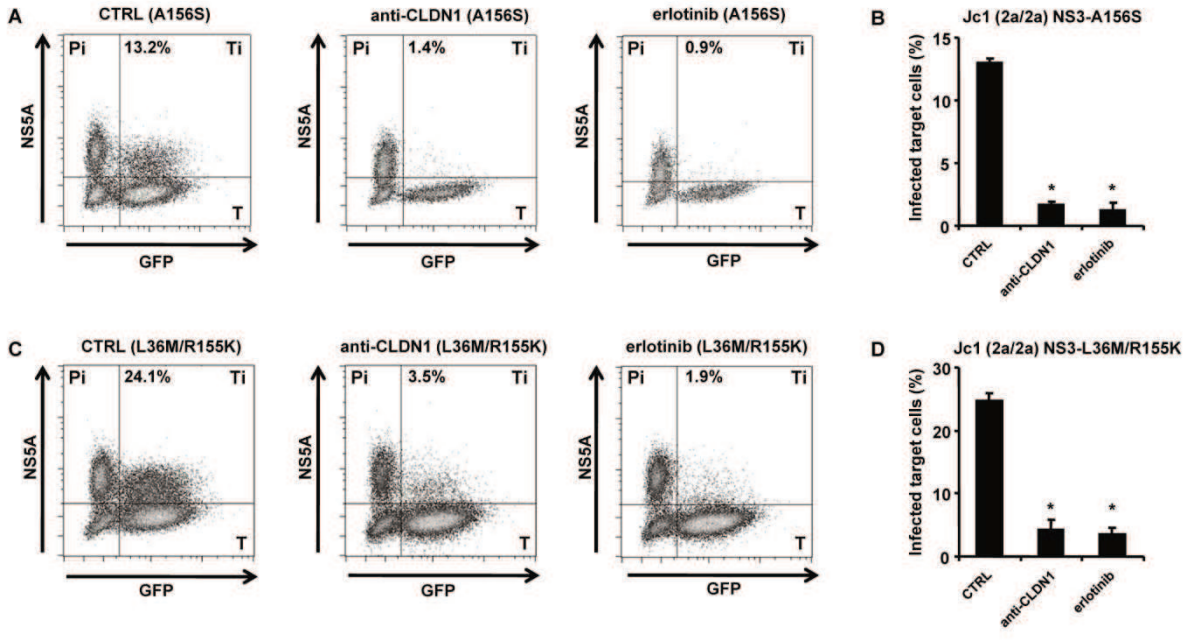
A
CTRL (A156S)
Pi
13.2%
Ti
T
NS5A
GFP
anti-CLDN1 (A156S)
1.4%
erlotinib (A156S)
0.9%
B
Jc1 (2a/2a) NS3-A156S
Infected target cells (%)
CTRL
anti-CLDN1
erlotinib
C
CTRL (L36M/R155K)
24.1%
anti-CLDN1 (L36M/R155K)
3.5%
erlotinib (L36M/R155K)
1.9%
D
Jc1 (2a/2a) NS3-L36M/R155K

Figure 4

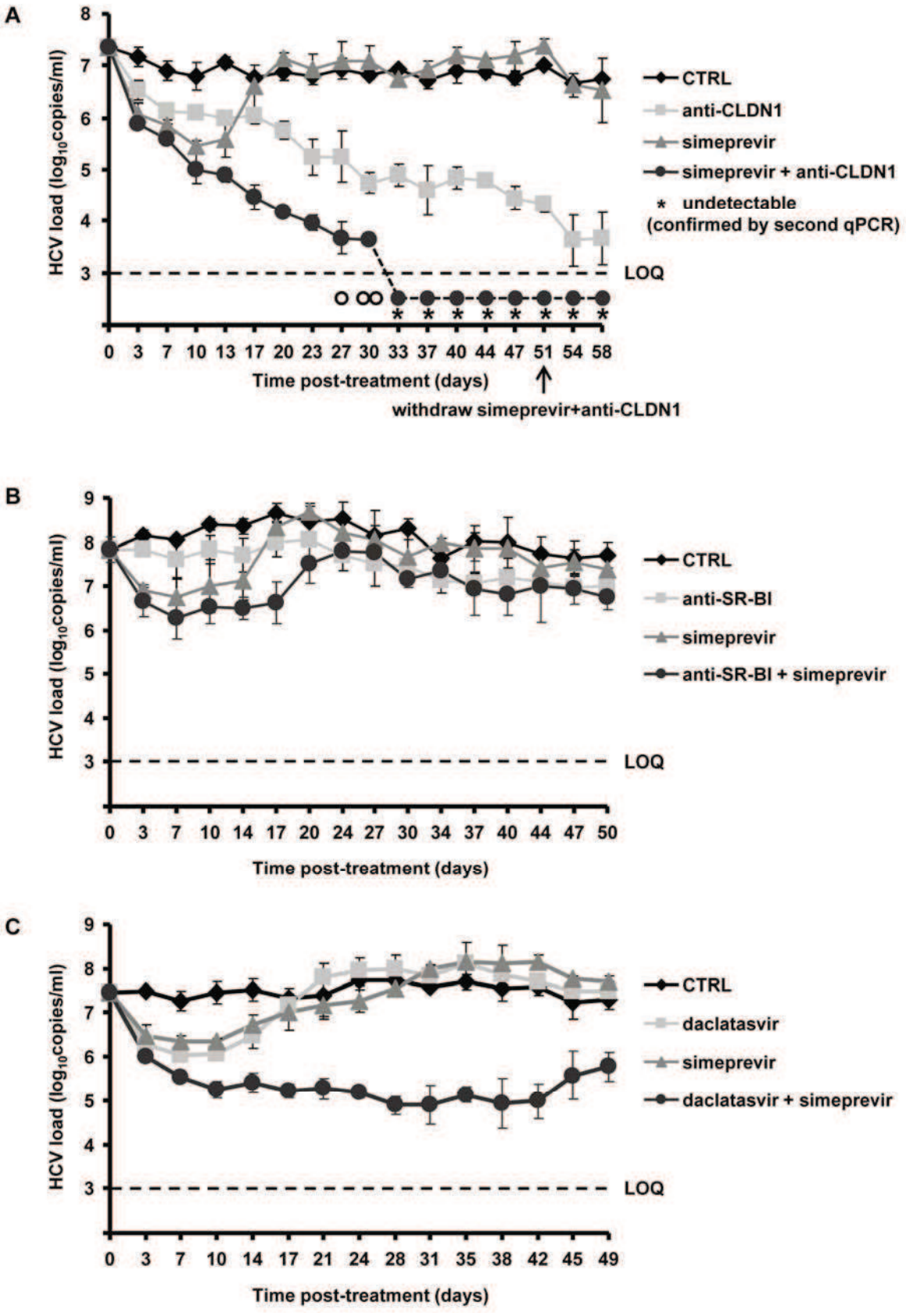
A
HCV load (log_{10}copies/ml)
CTRL
anti-CLDN1
simeprevir
simeprevir + anti-CLDN1
* undetectable
(confirmed by second qPCR)
LOQ
Time post-treatment (days)
withdraw simeprevir+anti-CLDN1
B
HCV load (log_{10}copies/ml)
CTRL
anti-SR-BI
simeprevir
anti-SR-BI + simeprevir
LOQ
Time post-treatment (days)
C
HCV load (log_{10}copies/ml)
CTRL
daclatasvir
simeprevir
daclatasvir + simeprevir
LOQ
Time post-treatment (days)

Figure 5

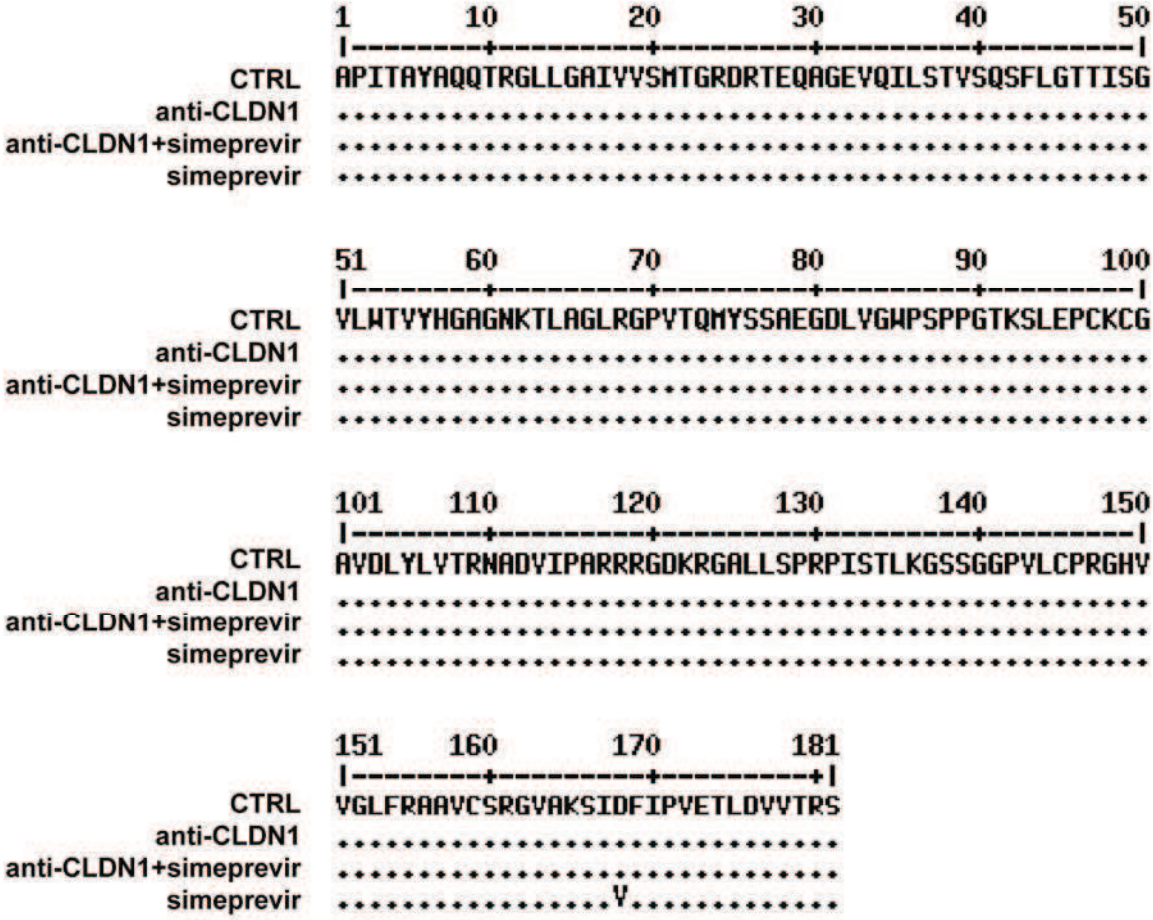

Figure 6

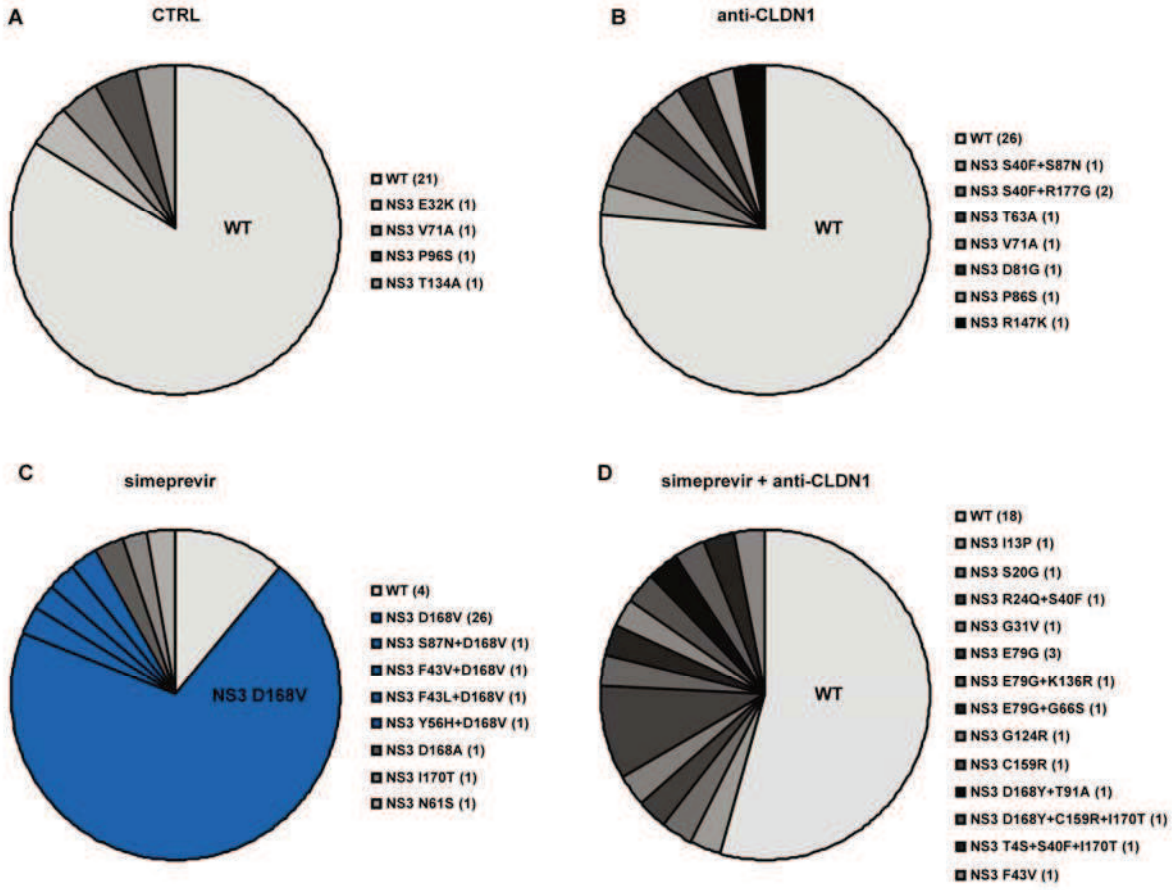
A
CTRL
WT
WT (21)
NS3 E32K (1)
NS3 V71A (1)
NS3 P96S (1)
NS3 T134A (1)
B
anti-CLDN1
WT
WT (26)
NS3 S40F+S87N (1)
NS3 S40F+R177G (2)
NS3 T63A (1)
NS3 V71A (1)
NS3 D81G (1)
NS3 P86S (1)
NS3 R147K (1)
C
simeprevir
NS3 D168V
WT (4)
NS3 D168V (26)
NS3 S87N+D168V (1)
NS3 F43V+D168V (1)
NS3 F43L+D168V (1)
NS3 Y56H+D168V (1)
NS3 D168A (1)
NS3 I170T (1)
NS3 N61S (1)
D
simeprevir + anti-CLDN1
WT
WT (18)
NS3 I13P (1)
NS3 S20G (1)
NS3 R24Q+S40F (1)
NS3 G31V (1)
NS3 E79G (3)
NS3 E79G+K136R (1)
NS3 E79G+G66S (1)
NS3 G124R (1)
NS3 C159R (1)
NS3 D168Y+T91A (1)
NS3 D168Y+C159R+I170T (1)
NS3 T4S+S40F+I170T (1)
NS3 F43V (1)

Figure 7

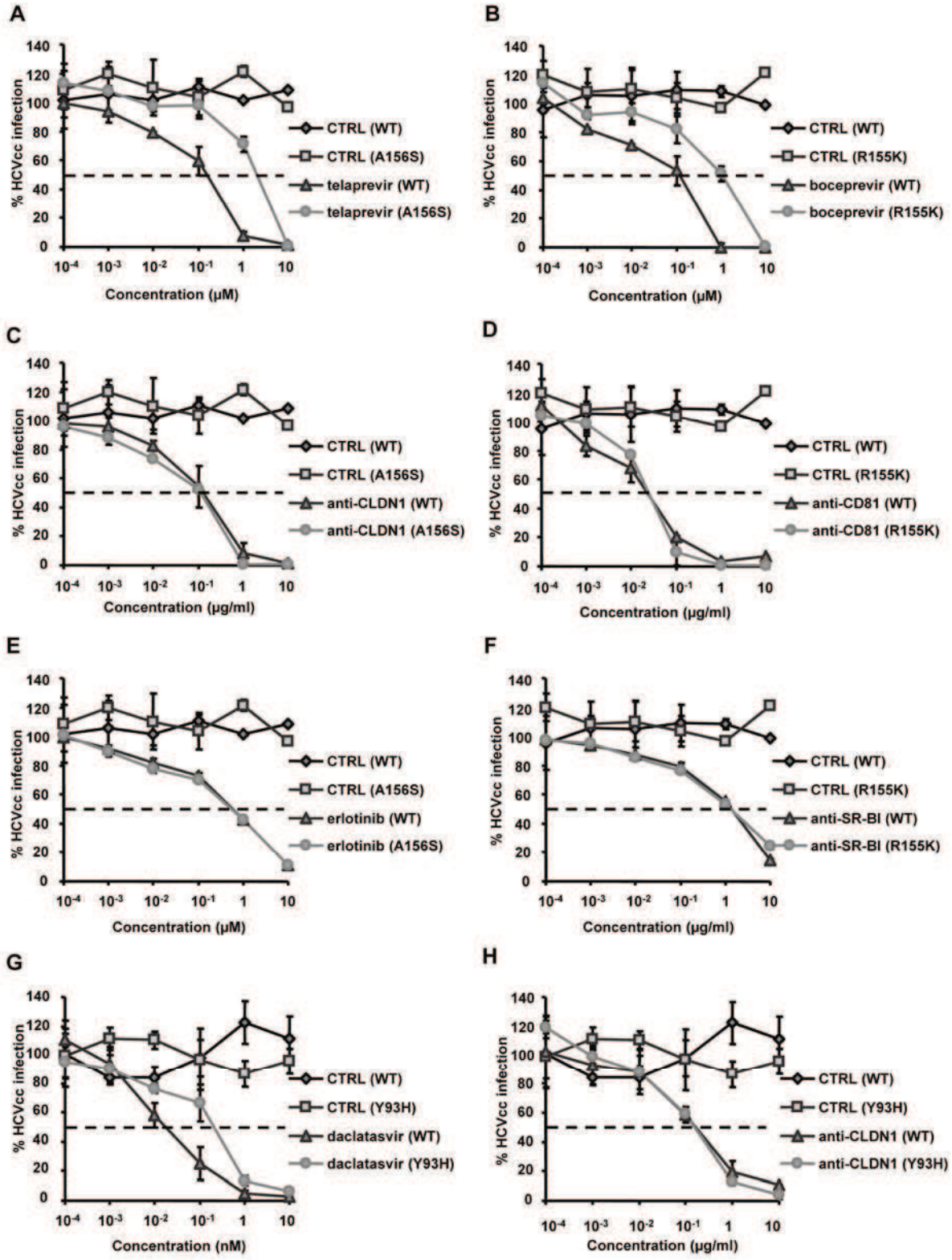
A
% HCVcc infection
Concentration (µM)
CTRL (WT)
CTRL (A156S)
telaprevir (WT)
telaprevir (A156S)
B
% HCVcc infection
Concentration (µM)
CTRL (WT)
CTRL (R155K)
boceprevir (WT)
boceprevir (R155K)
C
% HCVcc infection
Concentration (µg/ml)
CTRL (WT)
CTRL (A156S)
anti-CLDN1 (WT)
anti-CLDN1 (A156S)
D
% HCVcc infection
Concentration (µg/ml)
CTRL (WT)
CTRL (R155K)
anti-CD81 (WT)
anti-CD81 (R155K)
E
% HCVcc infection
Concentration (µM)
CTRL (WT)
CTRL (A156S)
erlotinib (WT)
erlotinib (A156S)
F
% HCVcc infection
Concentration (µg/ml)
CTRL (WT)
CTRL (R155K)
anti-SR-BI (WT)
anti-SR-BI (R155K)
G
% HCVcc infection
Concentration (nM)
CTRL (WT)
CTRL (Y93H)
daclatasvir (WT)
daclatasvir (Y93H)
H
% HCVcc infection
Concentration (µg/ml)
CTRL (WT)
CTRL (Y93H)
anti-CLDN1 (WT)
anti-CLDN1 (Y93H)

Figure S1

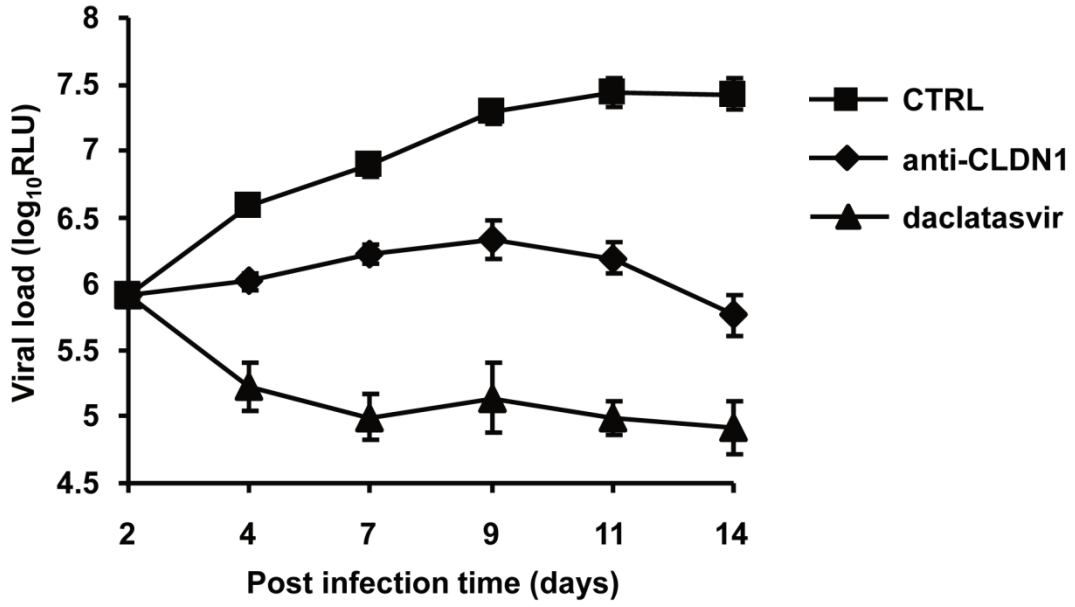

Figure S2

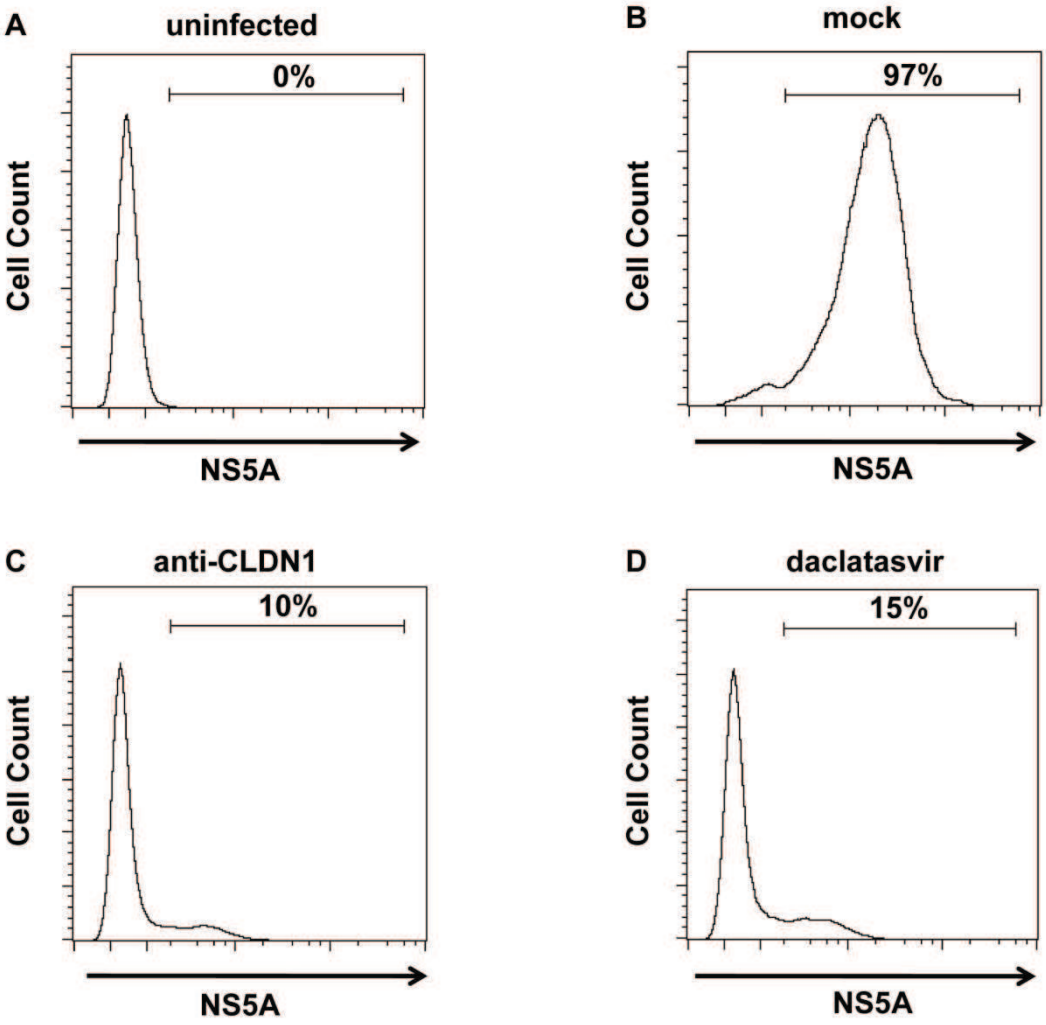

Figure S3

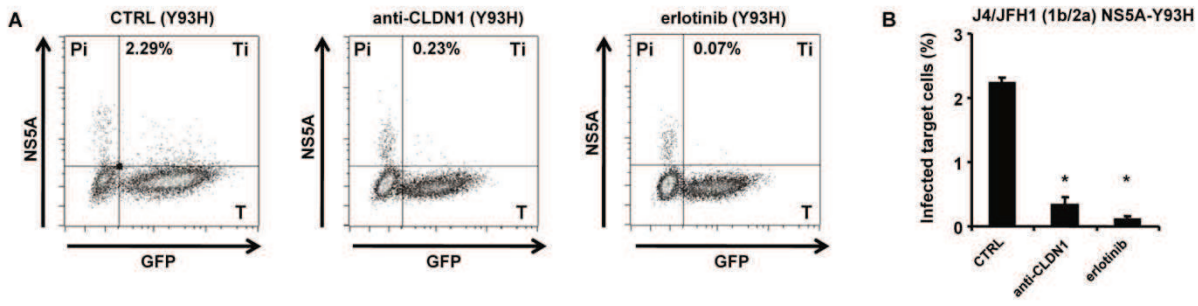

Figure S4

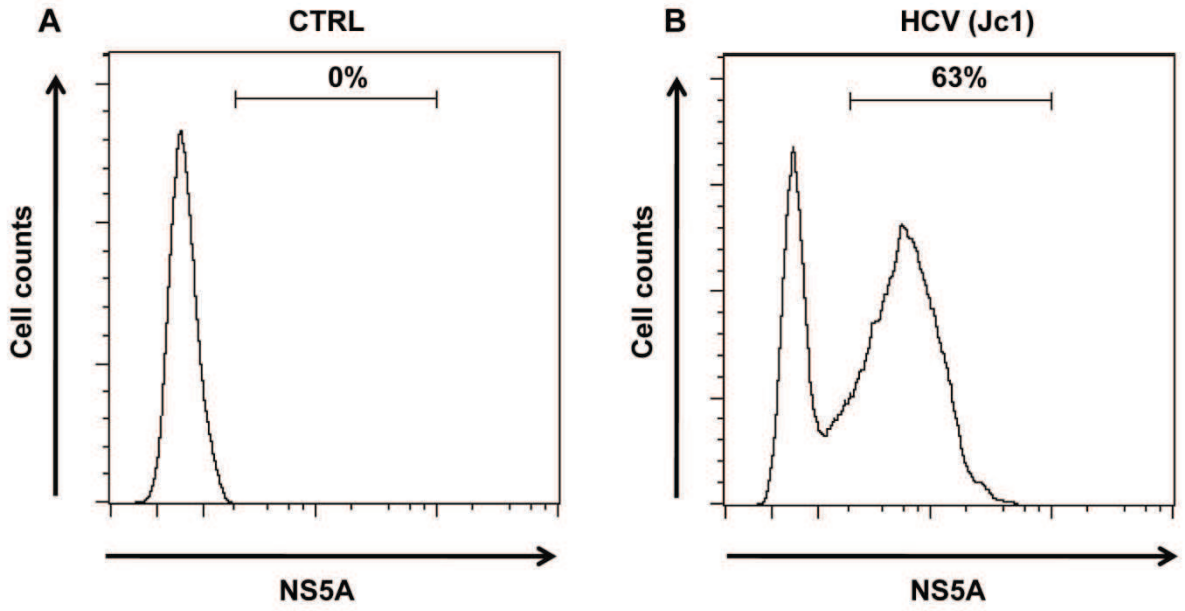

Figure S5

```
                1       10        20        30        40        50        60        70        80        90       100
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
Jc1 (mock)      MSTNPKPQRKTKRNTNRRPQDVKFPGGGQIVGGVYLLPRRGPRLGVRATRKTSERSQPRGRRQPIPKDRRSTGKSWGKPGYPWPLYGNEGLGWAGWLLSP
Jc1 (anti-SR-BI) MSTNPKPQRKTKRNTNRRPQDVKFPGGGQIVGGVYLLPRRGPRLGVRATRKTSERSQPRGRRQPIPKDRRSTGKSWGKPGYPWPLYGNEGLGWAGWLLSP

               101       110       120       130       140       150       160       170       180       190       200
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
Jc1 (mock)      RGSRPSWGPNDPRHRSRNVGKVIDTLTCGFADLMGYIPVVGAPLGGVARALAHGVRVLEDGVNFATGNLPGCSFSIFLLALLSCITTPVSAAEVKNISTG
Jc1 (anti-SR-BI) RGSRPSWGPNDPRHRSRNVGKVIDTLTCGFADLMGYIPVVGAPLGGVARALAHGVRVLEDGVNFATGNLPGCSFSIFLLALLSCITTPVSAAEVKNISTG

               201       210       220       230       240       250       260       270       280       290       300
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
Jc1 (mock)      YMVTNDCTNDSITWQLQAAVLHVPGCVPCEKVGNASQCWIPVSPNVAVQRPGALTQGLRTHIDMVVMSATLCSALYVGDLCGGVMLAAQMFIVSPQHHWF
Jc1 (anti-SR-BI) YMVTNDCTNDSITWQLQAAVLHVPGCVPCEKVGNASQCWIPVSPNVAVQRPGALTQGLRTHIDMVVMSATLCSALYVGDLCGGVMLAAQMFIVSPQHHWF

               301       310       320       330       340       350       360       370       380       390       400
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
Jc1 (mock)      VQDCNCSIYPGTITGHRMAWDMMMNWSPTATMILAYAMRVPEVIIDIISGAHWGVMFGLAYFSMQGAWAKVVVILLLAAGVDARTHTVGGSAAQTTGRLT
Jc1 (anti-SR-BI) VQDCNCSIYPGTITGHRMAWDMMMNWSPTATMILAYAMRVPEVIIDIISGAHWGVMFGLAYFSMQGAWAKVVVILLLAAGVDARTHTVGGSAAQTTGRLT

               401       410       420       430       440       450       460       470       480       490       500
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
Jc1 (mock)      SLFDMGPRQKIQLVNTNGSWHINRTALNCNDSLHTGFIASLFYTHSFNSSGCPERMSACRSIEAFRVGWGALQYEDNVTNPEDMRPYCWHYPPRQCGVVS
Jc1 (anti-SR-BI) SLFDMGPRQKIQLVDTNGSWHINRTALNCNDSLHTGFIASLFYTHSFNSSGCPERMSACRSIEAFRVGWGALQYEDNVTNPEDMRPYCWHYPPRQCGVVS
                              *

               501       510       520       530       540       550       560       570       580       590       600
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
Jc1 (mock)      AKTVCGPVYCFTPSPVVVGTTDRLGAPTYTWGENETDVFLLNSTRPPLGSWFGCTWMNSSGYTKTCGAPPCRTRADFNASTDLLCPTDCFRKHPDTTYLK
Jc1 (anti-SR-BI) AKTVCGPVYCFTPSPVVVGTTDRLGAPTYTWGENETDVFLLNSTRPPLGSWFGCTWMNSSGYTKTCGAPPCRTRADFNASTDLLCPTDCFRKHPDTTYLK

               601       610       620       630       640       650       660       670       680       690       700
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
Jc1 (mock)      CGSGPWLTPRCLIDYPYRLWHYPCTVNYTIFKIRMYVGGVEHRLTAACNFTRGDRCNLEDRDRSQLSPLLHSTTEWAILPCSYSDLPALSTGLLHLHQNI
Jc1 (anti-SR-BI) CGSGPWLTPRCLIDYPYRLWHYPCTVNYTIFKIRMYVGGVEHRLTAACNFTRGDRCNLEDRDRSQLSPLLHSTTEWAILPCSYSDLPALSTGLLHLHQNI

               701       710       720       730       740       750
                |--------+---------+---------+---------+---------|
Jc1 (mock)      VDVQFMYGLSPALTKYIVRWEWVILLFLLLADARVCACLWMLILLGQAEA
Jc1 (anti-SR-BI) VDVQFMYGLSPALTKYIVRWEWVILLFLLLADARVCACLWMLILLGQAEA
```

Figure S6

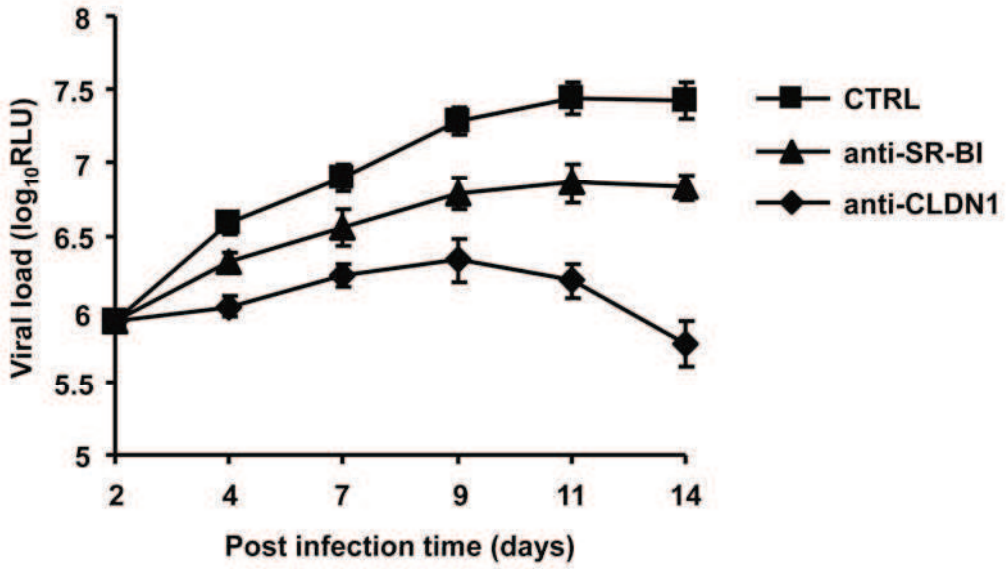

Figure S7

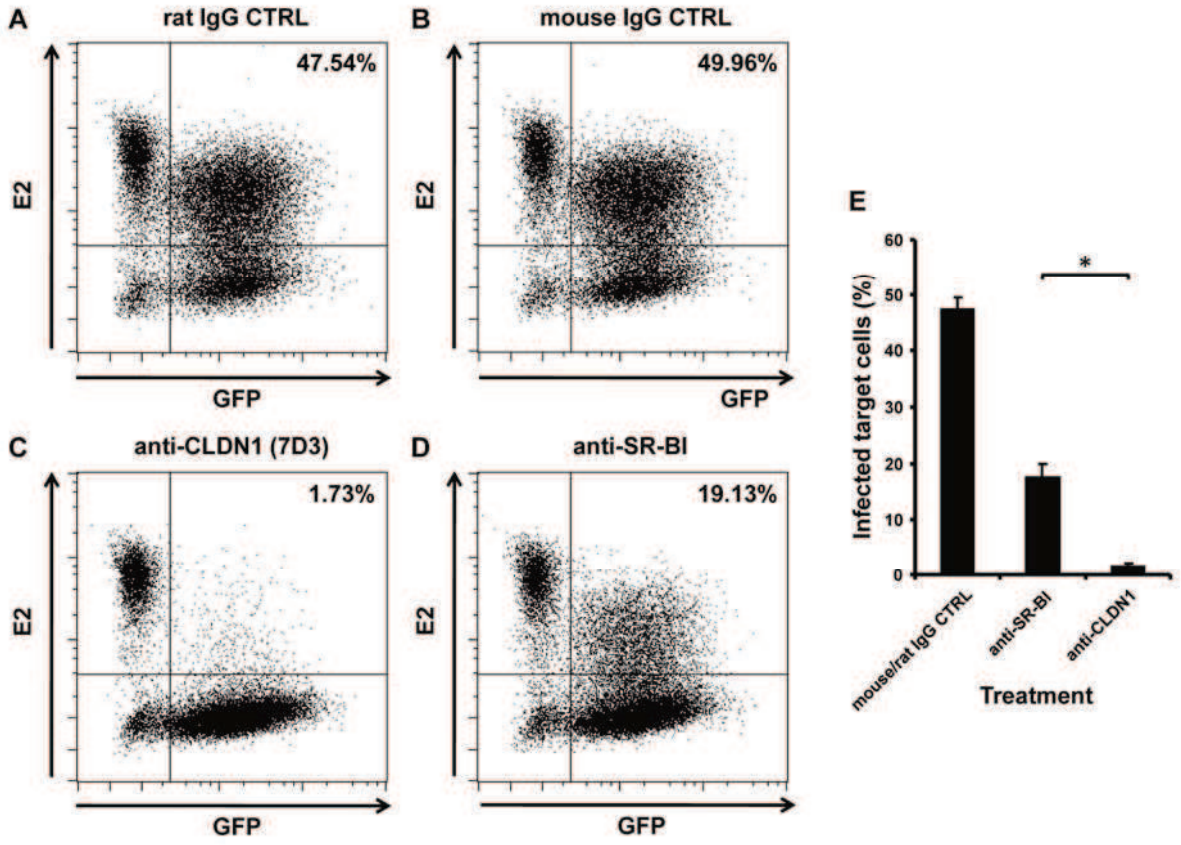
A
rat IgG CTRL
47.54%
E2
GFP
B
mouse IgG CTRL
49.96%
E2
GFP
C
anti-CLDN1 (7D3)
1.73%
E2
GFP
D
anti-SR-BI
19.13%
E2
GFP
E
Infected target cells (%)
60
50
40
30
20
10
0
*
mouse/rat IgG CTRL
anti-SR-BI
anti-CLDN1
Treatment

Figure S8

Compound	IC_{50} for WT	IC_{50} for R155K	IC_{50} for A156S	IC_{50} for Y93H
anti-CD81	0.022±0.08 µg/mL	0.023±0.05 µg/mL		
anti-CLDN1	0.12±0.05 µg/mL		0.11±0.07 µg/mL	0.13±0.06 µg/mL
anti-SRBI	1.3±0.3 µg/mL	1.3±0.4 µg/mL		
erlotinib	0.43±0.5 µM		0.42±0.4 µM	
telaprevir	0.13±0.04 µM		1.2±0.3 µM	
boceprevir	0.11±0.03 µM	1.0±0.04 µM		
daclatasvir	0.016±0.009 nM			0.2±0.05 nM

Table S1

Conclusions and perspectives

As more and more DAAs are being approved for the treatment of chronic hepatitis C, an all-oral IFN-free regimen has become the trend of future HCV therapy. A series of inhibitors targeting entry factors have been reported to be efficient in preventing HCV entry in a pan-genotypic activity. To develop novel therapies for the prevention and treatment of HCV, we combined entry inhibitors and DAAs in HCV cell culture models and a mouse model of HCV infection. Our data demonstrate that entry inhibitors and DAAs act synergistically to prevent and treat HCV infection, while two DAAs only result in an additive effect. These data indicate that targeting complementary steps of viral life cycle is an efficient antiviral strategy. The combination of an entry inhibitor and a DAA was no toxic even at the highest doses used in cell culture as well as in the mouse model. Indeed, our toxicity studies showed that entry inhibitors have a good safety profile and the combination of an entry inhibitor and a DAA did not exhibit toxicity at high doses in vitro and in vivo. Considering the antiviral efficacy, safety profile and the development stage of different combinations, we suggest combinations of sofosbuvir and anti-CLDN1 or anti-SR-BI mAbs for prevention of HCV infection and combinations of simeprevir or sofosbuvir with erlotinib for treatment of HCV infection in future clinical trials. This study provides a novel strategy for prevention and treatment of HCV infection.

With the broad administration of DAAs in clinical practice, viral resistance becomes an important issue. Indeed, the low genetic barrier of some DAAs leads to emergence of resistant viruses and treatment failure. In our study, we showed that cell-cell transmission is the major transmission route in cell culture and DAA-resistant viruses exploit this transmission route to develop viral resistance. Entry inhibitors are able to block the cell-cell transmission of both wild-type and DAA-resistant viruses. DAAs are not efficient in inhibiting HCV due to the rapid emergence of DAA-resistant viruses leading to viral rebound in the long-term HCV cell culture. Blocking HCV cell-cell transmission by entry inhibitors prevents the emergence of DAA-resistant viruses and potentiates DAAs to clear HCV infection. This provides a novel strategy to clear HCV infection by blocking the cell-cell transmission of DAA-resistant HCV.

Entry inhibitors have perspective for prevention and treatment of HCV infection. Since HCV from different genotypes need a set of receptors to enter the host cell, inhibitors targeting entry factors will have pan-genotype activity, a concept which is supported by previous studies from our laboratory showing that anti-CLDN1

and anti-SR-BI mAbs as well as the small molecule kinase inhibitors erlotinib and dasatinib cross-inhibit HCV from all the major genotypes using HCVcc and HCVpp systems (Fofana et al. 2010; Zahid et al. 2013). A lot of entry inhibitors have been developed or discovered using cell culture models and mouse models. These include antibodies or peptides targeting host entry factors (anti-CD81, anti-CLDN1, anti-SRBI, anti-ApoE, CLDN1 peptides and ApoE peptides), protein kinase inhibitors (erlotinib and dasatinib), ezetimibe, ITX5061, flavonoids, lectins, phosphorothioate oligonucleotides, silymarin, etc (Krieger et al. 2010; Fofana et al. 2010; Zahid et al. 2013; Fofana et al. 2013; Jiang et al. 2013; Si et al. 2012; Liu et al. 2012; Lupberger et al. 2011; Syder et al. 2011; Haid et al. 2012; Calland et al. 2012; Meuleman et al. 2011). Among these, erlotinib and ITX5061 have entered clinical trials (ClinicalTrials.gov identifier NCT01835938 and NCT01292824). It is believed that entry inhibitors might have higher genetic barrier to resistance than DAAs. In HIV field, enfuvirtide blocking HCV fusion was the first entry inhibitor receiving FDA approval for HIV treatment in 2003. Then, more and more entry inhibitors blocking the gp120-CD4 or gp120-coreceptor or gp41-mediated membrane fusion have entered clinical stage and shown good efficacy and safety profile in combination with non-nucleoside reverse transcriptase inhibitors to treat HIV patients in the long-term (Haqqani et al. 2013; Nozza et al. 2014), providing successful experience for developing novel treatment for HCV.

Since entry inhibitors target the host, toxicity and side effects are major concerns. HCV entry inhibitor anti-CD81 mA caused elevated transaminases in uPA-SCID mouse model, indicating its liver toxicity in some extent (Zona et al. 2014). Inhibition of SR-BI lipid transfer by anti-SR-BI mAbs raises concern over whether it influences lipid metabolism *in vivo* (Zahid et al. 2013). Nevertheless, in erlotinib and ITX5061 clinical trials, no serious side effects have been observed till now. Most of the HIV entry inhibitors have tolerable side effects (Haqqani et al. 2013; Nazza et al. 2014), indicating that targeting the host doesn't necessarily result in serious side effects. Nevertheless, toxicity and side effects are important in developing novel entry inhibitors. Thus, targeting specific epitopes important for viral entry but not influencing the physiological function of the host factors is the future direction.

The antiviral's genetic barrier to resistance is important for its long-term efficacy. Entry factors are required for viral entry. However, it has been observed that the virus

was able to decrease its dependence on some receptors to circumvent defined host factors targeted by an inhibitor (Catanese et al. 2013). Indeed, the SR-BI inhibitor ITX5061 selected resistant virus with a mutation in E2 region (N415D). In our study, we also found that anti-SR-BI mAb NK-8H5-E3 was not effective in inhibiting HCV infection with emergence of viral variants containing E2 mutations in long-term. These evidences indicate that inhibitors targeting SR-BI might not have high genetic barrier to resistance. Anti-CLDN1 mAb OM-7D3-B3 has relatively high efficacy in inhibition HCV infection in the long-term cell culture without emergence of resistant variants, suggesting its high genetic barrier to resistance. Therefore, different entry inhibitors might have different genetic barriers depending on the importance of the targets for viral entry. Since entry inhibitors have different host targets, the occurrence of cross-resistance should be rare. Therefore, the mutant viruses resistant to one entry inhibitor can still be inhibited by another one. Developing entry inhibitor with high efficacy and high genetic barrier to resistance will be the future goal. Combining entry inhibitors with IFN-α or DAAs not only resulted in synergistic effects but also increase genetic barrier to resistance, providing a promising strategy for HCV treatment.

HCV propagates with cell-free and cell-cell transmission. In our study, cell-cell transmission was the dominant route of transmission. It has also been suggested that HCV spreads *in vivo* by cell-cell transmission. Indeed, this mode of viral spread avoids surveillance of the immune system. In our study, we discovered that cell-cell transmission of mutant HCV results in treatment failure *in vitro*. Due to lack of appropriate methods and model systems, studying cell-cell transmission *in vivo* is still a challenge. It has been reported that mutations in E1E2 region can change the preference of viral transmission route (Catanese et al. 2013), indicating both pathways can be utilized as the main route by the virus depending on different virus-host interactions. Therefore, inhibitors with high efficacy of both inhibiting cell-free and cell-cell transmission should be prioritized in the drug development.

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Fei XIAO

Hepatitis C virus entry and cell-cell transmission: implication for viral life cycle and antiviral treatment

Résumé

Le virus de l'hépatite C (HCV) représente un problème de santé publique à l'échelle mondiale. Les thérapies actuelles ne permettent pas de guérir tous les patients infectés par le HCV et certains antiviraux ont des effets secondaires importants. Dans la première partie de ma thèse, nous avons identifié des combinaisons d'antiviraux à action directe (DAA) et d'inhibiteurs d'entrée caractérisés par un effet synergique dans la prévention et le traitement du HCV dans des modèles de culture cellulaire et les souris uPA-SCID avec un foie chimérique. Ceci représente une nouvelle stratégies de lutte contre l'infection par le HCV. Dans la seconde partie de ma thèse, nous avons démontré que le mode de transmission du HCV de cellule à cellule est la voie de transmission dominante dans les modèles de culture cellulaire. De plus, les virus résistant aux DAA se progagent efficacement grâce à la transmission de cellule à cellule. L'inhibition de la transmission de cellule à cellule en utilisant des inhibiteurs d'entrée est un moyen efficace pour empêcher l'émergence de virus résistant aux DAA et pour potentialiser l'efficacité antivirale des DAA pour éradiquer l'infection par le HCV.

Mots clés: virus de l'hépatite C, entrée virale, transmission de cellule à cellule, antiviral à action directe, inhibiteur d'entrée

Résumé en anglais

Hepatitis C virus (HCV) poses a threat to global health with infecting about 170 million people. Current therapies cannot cure all the patients infected with HCV and have obvious side effects. In the first part of my thesis, we uncovered combinations of direct-acting antivirals (DAAs) and entry inhibitors caracterized by a synergistic effect in prevention and treatment of HCV infection using HCV cell culture models and human liver chimeric uPA-SCID mice, thereby providing a new strategy to control HCV infection. In the second part of my thesis, we demonstrated that HCV cell-cell transmission is the dominant transmission route in cell culture models and that DAA-resistant HCV spread efficiently through cell-cell transmission to develop viral resistance. Blocking cell-cell transmission using entry inhibitors allows to prevent the emergence of DAA-resistant virus and potentiates the antiviral efficacy of DAAs to clear HCV infection. In summary, we provide novel strategies to enhance antiviral efficacy by combining entry inhibitors and DAAs and to prevent viral resistance by blocking viral cell-cell transmission.

Key words: hepatitis C virus, viral entry, cell-cell transmission, direct-acting antiviral, entry inhibitor