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Exploration of new uracil-based compounds as novel inhibitors of

Hepatitis C Virus replication

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INTRODUCTION

Hepatitis C virus (HCV):

HCV is a member of the *Hepacivirus* genus, along with the pestiviruses (i.e. bovine viral diarrhea virus or BVDV) and flavivirus (including yellow fever virus, dengue and Zika); they constitute the *Flaviviridae* family[1].

Physical and chemical properties of HCV particles

HCV particles from serum of infected patients are characterised by a diameter ranging between 30 and 80 nm[2-4]; surprisingly, *in vitro* produced HCVcc particles showed a diameter between 60 and 75 nm[5,6]. Analysing by density gradients the physical properties of HCV particles, emerged that in patient serum-derived particles the HCV RNA is distributed on a rage of densities from 1,03 g/ml to 1,20 g/ml and the peak of RNA is between 1,04 g/ml and 1,12 g/ml[7,8]. This heterogeneity is dependent on the association of viral particles with different classes of lipoproteins (VLDL, LDL and HDL) and/or immunoglobulins[7,9-12].

HCV particles with higher density are less infectious, confirming how the association of HCV with lipoproteins is crucial for infectivity. Moreover, virus particles isolated from serum of infected chimpanzee have been found to have densities between 1,03 and 1,10 g/ml [9,13,14]. Noteworthy, the peak of infectivity has been described at low density < 1.10 g/ml[15-17], whereas HCV particles, produced in *in vitro* model, showed a higher density, of approximately 1,15 g/ml. This difference has also been shown in-patient sera, exhibiting lower values compared to *in vitro* densities, probably because the hepatoma cell line currently used to produce HCV has deficient lipoprotein metabolism[18]. To support the hypothesis that this low buoyant density of infectious HCV particles is the result of the association of the virions with serum lipoproteins[7,10,12,19], ApoAI, ApoB, ApoC1, and ApoE have been demonstrated to be associated with serum-derived HCV particles[6,20-23]. Based on these results, it has been hypothesized that the virus is produced and secreted into the

serum as a hybrid lipoviral particles (LVPs) following VLDL (very low density lipoprotein) production and secretion pathway[24]. Although the exact structure of the infectious lipo-viral particles and their interactions with HCV has been debated for many years, initially, two models were proposed (Fig. 1): in the first one viral particles interact transiently with lipoproteins, while in the second one they contribute into forming an integrated particle. However, recent studies, using electron microscopy, demonstrated that HCV is part of a bigger structure containing lipoprotein (figure 1B) [25]. This configuration of the LVPs may provide a shield, protecting virus from neutralizing antibodies[19,26,27].

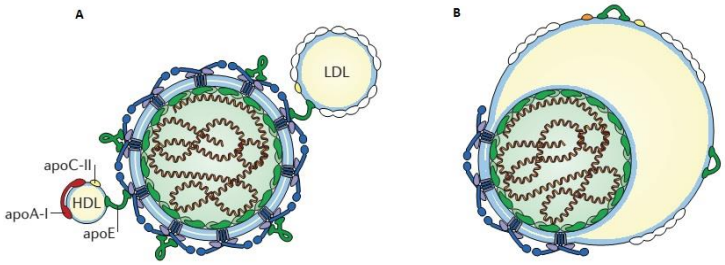


Figure 1. Two models for lipo-viral particle (LVP). In the first the two-particle model (A), HCV particles and serum lipoproteins transiently interact. In the second one (B), the single-particle model for LVP structure, a low-density lipoprotein (LDL) particle is integrated in an HCV particle[24].

Molecular virology of HCV

HCV particles contains one molecule of 9.6 kb single stranded positive RNA genome. It is composed of the 5' non-translated region (NTR), characterized by the presence of an IRES that drives the translation of a single open reading frame (ORF), coding for structural and non-structural proteins, and the 3' NTR[25]. Structural proteins have been shown to form the capsid of the viral particles (core protein) and to be expressed on the envelope as glycoproteins, specifically E1 and E2. The non-structural proteins (NS), which are necessary for viral replication and assembly of infectious virus particles, comprised the ion channel p7,

the NS2 protease, the helicase/protease NS3-4A complex, the cofactor NS4B, the phosphoprotein NS5A and the NS5B RNA-dependent RNA polymerase (RdRp)[28].

IRES-mediated translation leads to the synthesis of a polyprotein of approximately 3000 aminoacids (aa) residing on endoplasm reticulum (ER) that undergoes a processing by cellular and viral proteases. Specifically, core protein is cleaved sequentially by the cellular signal peptidase (SP) and the cellular signal peptide peptidase (SPP), while glycoproteins E1, E2 and p7 are cleaved by SP; non-structural proteins are processed by NS2 and NS3/NS4 proteases[29,30] (Fig. 2). Interestingly, the HCV proteins showed multifunctional roles on different viral stages; in particular the NS3-4A complex, that has been shown to be involved in the HCV replication and pathogenesis[31-36]. In addition, the function of HCV proteins may be influenced by interaction with other viral or cellular molecules, which may induce different structural conformations[37].

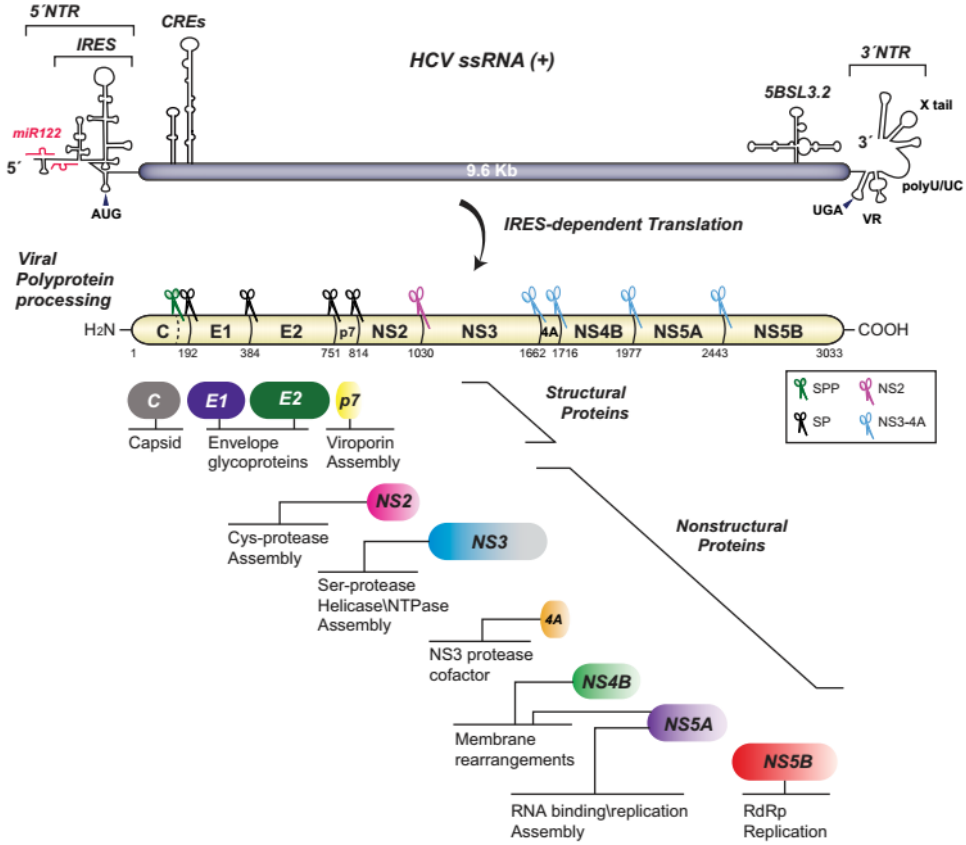


Figure 2. HCV genome organization and polyprotein processing[38]. HCV RNA is translated into a single polyprotein that is processed to obtain mature proteins.

5' NTR

The 5′ NTR extends between nucleotides 1 to 342, is highly conserved through HCV genotypes and it is characterised by the presence of 4 major stem loops; furthermore, it has been shown playing two key functions in the HCV replication cycle. First, in the positive strand it contains a specific internal ribosome entry site (IRES), necessary to drive RNA translation, and thus polyprotein synthesis. Second, it has been proposed that the 5'NTR is essential to direct the synthesis of progeny positive-strand RNA using as template the negative strand RNA. The hypothesis has been supported by the finding that the 5'NTR adopts very different secondary structures between positive and negative strands[39,40].

3' NTR

The 3′NTR is mainly involved in RNA replication[41], presumably playing a key role in the initiation and regulation of negative-strand synthesis[42]. It is constituted by three main regions: a variable region, a polyU/UC tract of variable length (ranging between 30 and 90 base pair depending on HCV isolates), whom function still needs to be clarified, and a highly conserved element known as X-tail or 3′X (98 nts) [43,44]. Deletions in the variable region impair viral replication but they do not determine its abrogation, suggesting that this sequence is not essential[41,45]. The X-tail has been found to be relatively conserved among HCV isolates and has been propose to contain the main regulatory elements required for the negative-strand synthesis[43,44]. Since mutations in this region are affect viral replication, both its conserved sequence and structure are critical[45,46].

Core protein

The core protein is the first structural protein encoded by HCV genome and it is responsible for the nucleocapsid formation. A short region comprising the C-terminus of the core protein acts as a signal sequence that targets the nascent polyprotein to translocate into the ER membrane. In addition, the core signal sequence has been reported to be recognised and

cleaved by the host signal peptidase (SP) with production of an immature, 191 aa core. An additional cleavage is required at the C terminus of immature core, by the signal peptide peptidase (SPP), to obtain the mature core protein (21 kDa) [47,48]. Although the C-terminal (aa177-191) domain D3 is immediately cleaved after translation, and thus is absent from the mature form of HCV core, it has been shown to be important for core stability and correct function. The mature core is a dimeric membrane protein and may be stabilised by an intramolecular disulphide bonds[49,50].

The mature core protein is composed of two domains: the N-terminal hydrophilic D1 domain and the C-terminal hydrophobic domain D2. The D1 has been shown to be involved in RNA binding and homo-oligomerization. Indeed, core D1 possess RNA chaperone activity, which has been reported to be required for the structural remodelling and packaging of the viral RNA[51]. Moreover, it has also been described that D1 can interact with several host factors and thus altering cellular functions upon HCV infection[37,52]. D2 is more hydrophobic and it is thought to interact with phospholipids on lipid droplets (LDs) through amphipathic regions[53].

The HCV core protein, besides its role in formation of the virus nucleocapsid, has been reported to be involved in the modulation of different host pathways and to interact with a variety of cellular factors. Those include numerous transcription factors, such as heterogeneous nuclear ribonucleoprotein K[54], leucine zipper transcription factor (LZIP)[55], 14-3-3 protein[56], NF-kB[57] and RNA helicases, such as DDX3 protein[58], involved in HCV RNA replication[59].

It has also been suggested that core protein might be involved in apoptosis and cell cycle regulation, contributing to the pathogenesis of HCC[37]. For this purpose, HCV core protein is considered as a major viral factor inducing development of hepatocellular carcinoma during HCV infection. To support this hypothesis, it has been demonstrated that HCV core can directly modulate the expression tumor-associated genes, such as the cyclin-dependent

inhibitor p21 [60], involved in cell-cycle control and tumor formation, and it can also interfere with Wnt/β-catenin pathway, which play a major role in the initiation of carcinogenesis[61].

In addition, it has been shown that HCV core protein expression might induce development of hepatic steatosis, particularly in genotype 3 infected patients[62]. Indeed, it has been proved that HCV core enhances the transcriptional activity of sterol regulatory element binding protein 1 (SREBP1) and peroxisome proliferators-activated receptor gamma (PPARγ) [63].

Although it has been largely reported that the core protein predominantly resides in the cell cytoplasm, associated with lipid droplets, which represent the site of virus morphogenesis, it has also been detected in the nucleus[64,65] and mitochondria[66,67]. However, whereas the presence of core on the LDs is mandatory for the virus assembly, the significance of its presence in the nucleus and mitochondria for the virus life cycle or pathogenesis is still not clear.

Alternative open reading frame protein (Core+1; ARFP)

Normally, the correct HCV translation leads to the synthesis of a polyprotein of 3000 aa; however, an overlapping ORF has been reported, which may generate an alternate reading frame product[68]. Indeed, a novel HCV protein (named protein F, core +1 or alternative reading frame protein) has been shown in *in vitro* models, encoded by an alternative open reading frame in the core region[69]. The existence of T-cell response[70] or antibody production[71] against core+1 in HCV infected individuals suggests that this protein is also produced in natural HCV infection. Nevertheless, ARFP has not yet been detected either in patients' sera or in infected tissues and its biological role remains unknown[72].

Moreover, it has been suggested that HCV core+1 synthesis might negatively regulate production of the canonical HCV core protein; furthermore, Core+1 protein seems to not be necessary for HCV life cycle[72], but the presence of specific antibodies in HCV patients suggest that its production *in vivo* and its potential role in the pathogenesis.

E1E2 envelope glycoproteins

HCV encodes for two glycoproteins: E1 and E2. They are classified as type I membrane proteins, with a molecular weight of approximately 35 kDa and 70 kDa, respectively. Both contain a large N-terminal ectodomains (of 160 and 334 aa in E1 and E2, respectively) and a short C-terminal transmembrane domain. The transmembrane domain (TMD) is responsible for anchoring E1 and E2 in the membrane and for their localization into the endoplasmic reticulum (ER). The TMD is characterised by two stretches of hydrophobic residues separated by a short segment containing at least one positively charged amino acid. These positively charged residues have an important role alongside with the N-terminal part of E1 TMD in E1-E2 heterodimerization and retention in the ER, whereas the entire TMD sequence seems to be necessary for anchoring to the membrane[73-75]. Six and seven ectodomains have been described in E1 and E2, respectively, which undergo an extensive N-glycosylation during the translocation into the endoplasmic reticulum lumen. These sites, highly conserved among HCV genotypes, are involved in glycoprotein folding and have been reported to play a specific role in HCV entry[76]. Moreover, a segment containing the N-terminal 27 aa residues of E2, defined as hyper variable region 1 (HVR1), is characterized by a high content of basic aminoacids known to influence HCV pseudo particles (HCVpp see below) infectivity[77,78]. HVR1 has been shown to be partially responsible for HCV escape from the immune response[79]; furthermore, viruses lacking HVR1 have been described as less infectious and exhibited impaired fusion, showing an enhanced neutralization and precipitation by E2 specific antibodies and soluble CD81[80], one of the main factors involved in HCV entry, as described below. Initially, newly-synthesized E1 and E2 are assembled as a non-covalent heterodimer on the ER membrane, then the HCV glycoproteins are subjected

to post-translational changes. Interestingly, on the viral particles they exist as large covalent complexes stabilised by disulfide bridges[81].

HCV glycoproteins E1 and E2 are present in tandem and this organization has also been found in alphaviruses and flaviviruses that encode class II fusion proteins, although for HCV the prediction of which glycoprotein acts as main fusion protein, remains unclear. In fact, over the last 10 years many studies, supported by contrasting evidences, led to the generation of many hypothesis, trying to explain fusogenic activity. Interestingly, to support the idea that E1 is the main protein involved in fusion, sequence analysis revealed that the E1 ectodomain contains a fusion peptide-like motif similar to the homologous peptide of paramyxoviruses and flaviviruses[82]. In addition, has been demonstrated that the structure of this motif is not required to mediate cell fusion, while the presence of specific residues is essential for viral entry[83]. However, comparing the E2 structure with other members of the *Flaviviridae* family, led the authors hypothesized that fusion is mediated by E2, suggesting that E2 belongs to the class II fusion protein[84,85]. To support this idea, Lavillette *et al* described at least three putative regions involved in virus-cell fusion by a mutagenesis approach, suggesting that E2 may contribute to the fusion step, either by direct interaction with the lipid membrane or by induction of conformational change of E1E2 complex[86]. Moreover, several residues responsible for CD81-E2 interaction have been described, supporting the idea that E2 plays a major role in the fusion step[87,88]. However, very recently, Kong *et al* succeeded in obtaining a crystal structure of E2 [89]. Surprisingly, the authors described E2 as a globular structure that contains many regions with no regular secondary structure despite the presence of eight disulfide bonds. Comparing E2 structure with other class II fusion proteins of different Flaviviruses, it emerged that the only shared element is the Ig-fold beta sandwich and that E2 does not adopt the putative three-domain fold described for other class II fusion proteins[88,89]. Furthermore, data obtained allowed to visualize the previous findings regarding the putative CD81 binding sites on E2[87,89,90].

Despite these controversial data concerning fusion properties, observing the nature of E1 and E2 glycoproteins and their capacity to form E1-E2 heterodimer seems to suggest that they may have a synergic role in regulating HCV entry and fusion. Indeed, it has been shown that the E1E2 heterodimer can bind to CD81 stronger than E2 alone indicating that E1 may modulate E2 activity[91,92].

p7 Protein

p7 is a 63 aa, integral trans-membrane protein that is usually listed as NS proteins even though there is not a concrete evidence whether it can be directly associated to viral particle. Its structure is characterised by two transmembrane α-helices (named M1 and M2) linked by a positively charged cytosolic loop with the N and C termini both oriented towards the ER lumen[93]. The first experiments, conducted with electron microscopy, showed that p7 monomers assemble into hexamer or heptamer complexes in artificial membranes[94]. Furthermore, another study, based on electron microscopy, revealed the presence of a hexameric complex characterized by a flower-shaped architecture and six protruding petals oriented toward the ER-lumen[95]. Subsequently, a third α-helix, located upstream of M1, has been identified[96]. A more recent model, developed using similar experimental strategies, suggested that hexameric and heptameric complexes might coexist; in this situation they may form a functional ion channels[97]. Interestingly, analysing p7 structure, some analogies have been found with viral proteins of other viruses such as 6k of alphaviruses, M2 of influenza A virus, *vpu* of HIV-1 which all belong to the viroporin family[97]. Typically, viroporins are not essential for viral RNA replication but they are involved in assembly and release of virus particles. Surprisingly, some recent data seem to suggest that p7 might also have a role on HCV replication[98], even though this eventuality has still to be discussed.

Other studies, conducted *in vitro* and *in vivo*, confirmed that p7 is essential for assembly and release of HCV particles[99-101], moreover, p7 acts in the assembly stage through the interaction with NS2. During viral release, p7 acts mainly to form ion channels that are essential to equilibrate pH gradients within the endo-lysosomal compartments, in order to protect HCV particles from pH-dependent uncoating during egress[95,96,101-104].

NS2 protein

NS2 is a transmembrane protein, containing three putative transmembrane domains at its N-terminal, and one cysteine protease domain at C-terminal. Interestingly, It has been shown that homo-dimerisation of NS2 induces folding of the catalytic domain and generation of two active sites at dimer interface[105]. To date, only one substrate, the junction NS2/NS3, has been identifies for the NS2 protease activity. NS2 is necessary for polyprotein processing and full-length HCV genome replication but it is not required for RNA replication of the subgenomic replicon[106,107].

Interestingly, NS2 has been recently identified also as a key factor for the assembly of new infectious HCV particles and this function has been demonstrated that does not depend on its protease activity[108,109]. Specifically, NS2 has been shown to act in combination with E1-E2 glycoproteins, p7, and the NS3–4A enzyme complex at an early stage during virus assembly[109-114]. Furthermore, Counihan *et al* demonstrated that the recruitment of the core protein, from lipid droplets (LDs) to the putative sites of virus assembly, is strictly dependent on the NS2 and NS3–4A interaction[115]. In particular, it has been shown that this interaction is required to localize NS2 and core-associated LDs to putative sites of the virus assembly[109,111,113].

NS3-NS4A complex

NS3-4A is a non-covalent protein complex composed of NS3 and its cofactor NS4A. NS3 is a 70 kDa protein characterised by two well defined enzymatic activities. NS3/4A structure

has been solved by Yao[116] et al. in 1999, showing that the N-terminal contains the serine protease activity responsible for the processing of the HCV polyprotein to generate the NS proteins, whereas the C-terminus has been reported as the NTPase/RNA helicase catalytic site[117-119]. The N-terminal domain encodes for the viral protease, which belongs to the trypsin/chymotrypsin protease superfamily[120,121]. The protease activity is exerted by the NS3 catalytic triad, and the NS4A cofactor has been shown to stabilise the interaction between the catalytic site inside the NS3 and its substrates, enhancing the efficacy and specificity[122]. The process of NS3-substrate recognition is dependent on the presence of specific multiple aminoacid residues; however, recent experiments in order to investigate potential NS3-4A substrates, has not been able to solve this enigma. In fact, only few of a large number of cellular proteins, that contain the specific consensus sequence, are effectively cleaved by the NS3-4A. On the other hand, several cellular substrates have been identified as effective substrates, although they present some differences in the recognition sequence compared to the canonical one[119].

The NS3 helicase belongs to the RNA helicase super family 2, and shares six helicase motifs with proteins belonging to the same family[123]. Furthermore, the NS3 exerts its helicase activity, nucleic acid unwinding, through ATP consumption; indeed, an APT-binding domain has been well described[124]. However, in experiments were NS3 protein was expressed alone, it showed an impaired activity compared to the entire NS3-NS4A complex. Hence, although the NS3 helicase is essential for HCV RNA replication, more recently it has also been proposed that NS3-4A can play a role in the virus assembly and immune response[125-130] .

Interestingly, an “in-plane amphipathic α-helix” at the N terminus of NS3, and the transmembrane α-helix harboured in the NS4A N-terminal have been shown to be the key factors in the association of NS3-4A with membranes and the structural organization[131].

Surprisingly, the authors described that the NS3-NS4A complex can be found on mitochondrial or mitochondria-associated membranes. It has been hypothesized that this atypical localisation is responsible for interfering with immune response, inactivating MAVS[132,133].

NS4B protein

NS4B is a hydrophobic integral membrane protein of approximately 27 kDa. NS4B has been characterised by its effect to induce the formation of specific membrane alterations, forming a characteristic formation called membranous web; it consists of locally confined membranes in which HCV NS proteins accumulate, interact and drive viral replication[134,135]. NS4B has been demonstrated to be involved in several distinct functions: NTPase activity, interactions with other HCV NS proteins, direct binding to RNA and finally playing a role in the assembly of viral particles[34,136-138]. As already shown for other HCV NS proteins, NS4B can associate to form oligomers[139,140]. Furthermore, NS4B is essential to stabilise the functional replication complex: in fact, mutations affecting its oligomerization impair the membranous web formation and, as consequence, they affect HCV replication. It has also been suggested that the NS4B expression induces membrane curvature and vesicle formation[141].

NS5A protein

NS5A is a membrane-associated phosphoprotein of 447 aa that is involved in modulation of HCV RNA replication and particle formation during assembly. It is characterised by a membrane anchor in the N-terminal and three main domains, which are separated by two low complexity sequence (LCS) [142]. The first two domains, named D1 and D2 have been shown to be involved in RNA replication whereas D3 is required for virus assembly[31,33]. Interestingly, HCV NS5A has been described in two different states: basally phosphorylated (56 kDa) and hyper-phosphorylated (58 kDa). While basal phosphorylation is mainly focused

on NS5A C-terminal and central residues, the hyper-phosphorylation has been described in a specific residue in the LCS. Based on the evidence showing that cell-culture adaptive mutations mostly affect these residues and data obtained with kinase inhibitors, it has been hypothesized that NS5A regulates HCV RNA replication, probably interacting with specific host factors[143-145]. NS5A is anchored to the ER membrane through an N-terminal amphipathic alpha helix that is located into the cytosolic face of the membrane. NS5A has been shown to associate with phospholipid monolayer allowing its interaction with the core protein located on LDs or on the LDs-ER interface. Analysing the crystal structure of D1, it has been demonstrated that the dimerization, characterised by an extended shape, is able to form a channel capable to host both ssRNA and dsRNA[142]. Moreover, the NS5A D1 domain has been found capable to bind RNA as a dimer[146]. Hence, it has been proposed that NS5A dimers might bind RNA and drive it through the HCV replication[147]. Interestingly, D2 and D3 domains have been found natively unfolded suggesting that to reach their stable conformation (mainly alpha helical), several interactions with specific cellular or viral proteins are required. Furthermore, D2 and D3 have been reported as substrate of Cyclophilin A (CypA)[148,149]; CypA, an isomerase enzyme, which have been shown essential for HCV replication; moreover, CypA can be inhibited by cyclosporine A (CsA)[150]. In particular, it has been reported that D2 and D3 can directly bind to the active site of CypA and specifically to proline residues, which have been shown to be substrates for the isomerase activity [148,149]. Finally, it is important to highlight that most of the known mutations conferring CsA resistance, have been found in the NS5A D2 domain[151].

NS5B protein

NS5B is the RNA-dependent RNA polymerase (RdRp) that has been isolated more than 20 years ago; for this reason, it has been largely studied and crystalised[152-155]. The NS5B, a protein of 68 kDa, is characterised by the N-terminal catalytic domain spanning from aa 1 to

530, a linker domain of 40 aa and a short C-terminal membrane anchor (CMA) of 21 aa. This anchor has been reported to be essential for viral replication *in vivo* but, in *in vitro* experiments, it is dispensable for RNA synthesis[156]. *In vivo* the CMA has been reported to be responsible for cytosolic orientation of the RdRp catalytic domain[157]. The N-terminal catalytic domain, is characterised by a well-defined right hand shape, which can be found in many other RNA-dependent RNA polymerases. In this hand-like folding, different structural regions were identified, referred as fingers, thumb and palm[154,158,159]. In addition, authors have identified a beta-flap on the thumb region that has been shown also in other *Flaviviridae* RdRp[160]. Hence, two different conformations have been reported: a closed conformation has been described during the *de novo* initiation step, while the open folding has been reported during the elongation step. Interestingly, the *closed* conformation is able to use ssRNA as template recognising the two initiating nucleotides[155]. Interestingly, on *de novo* replication, the RNA template and nucleotides are surrounded by an encircled active site, which is closed from one side by fingers and on the other by interaction between linker and beta-flap. Once the RNA synthesis is started, the NS5B undergoes a significant change during which the linker and the beta-flap are removed and the RdRp might begin the elongation. Interestingly, from the recent structural models, it has not been completely clarified whether the newly synthesized RNA can emerge as double stranded, paired to the template, or if it is forced to unwind in order to leave the active site[161].

Lifecycle

The HCV life cycle is a complicated process based on different well-distinguished steps: entry, translation, replication, assembly and release. Below, all of these steps have been described individually.

Attachment and entry

The HCV entry is a multistep process that involves several factors and co-factors sequentially interacting with the virus that promote initiation of productive infection. Below are described all known factors involved in viral attachment.

Glycosaminoglycans (GAGs)

GAGs are long unbranched polysaccharides, characterised by repeated disaccharide units that show several degrees of heterogeneity including molecular weight, disaccharide construction, and sulfation. Based on core disaccharide features, GAGs can be classified into four groups: heparin/heparan sulfate, chondroitin/dermatan sulfate, keratan sulfate and hyaluronic acid GAGs. They are commonly associated with cellular proteins to form a panel of molecules exposed on cell surfaces, known as proteoglycans (PG). Interestingly, it has been reported that many viruses use PG for their attachment to host cells. Specifically, a heparan sulfate proteoglycan (HSPG) has been shown to be crucial for the early steps of HCV infection. The direct interaction between the HCV E2 glycoprotein and cell surface GAGs, via the positively charged residues at the N terminus, has been demonstrated and extensively studied using HCV-like particles produced in insect cell lines and recombinant E2 glycoprotein[162]. Nevertheless, it is generally accepted that the primary site of interaction, between HCV and the liver, is the heparan sulphate, especially Syndecan 1 [163]. This interaction is mediated by lipoproteins associated to LVPs and especially Apolipoprotein E (ApoE). For this reason, the role of Apo E exposed on the HCV surface in virus attachment to heparin/heparin sulfate PG has been demonstrated by showing that antibodies targeting ApoE and synthetic peptides derived from ApoE were able to inhibit HCV cell binding[164]. Moreover, Shi *et al* demonstrated the role of different heparin/heparin sulfate proteoglycan (HSPG) core proteins in the HCV binding process, showing that Syndecan 1 plays a major

role in virus attachment compared to other member of the Syndecan family and others HSPG[163].

DC-SIGN and L-SIGN

Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) and the related protein liver/lymph node-specific (L-SIGN or DC-SIGNR) are calcium-dependent lectins expressed on dendritic and endothelial cells in the liver and lymph nodes, respectively. DC-SIGN and L-SIGN have been initially investigated due to their role in mediating human immunodeficiency virus (HIV) binding and internalisation. It has also been shown that the interaction between these molecules and HIV is dependent on the presence of mannose N-linked chains in the HIV envelope protein (Env). Based on these observations, it has been reported that glycosylation of HCV E1-E2 glycoproteins is comparable to that of HIV Env, and, moreover, L-SIGN is expressed in the liver. Interestingly, results obtained from early studies, conducted with soluble E2 (sE2) and HCVpp, confirmed that virus-associated E1 and E2 were able to bind DC-SIGN and L-SIGN[165]. These data have been subsequently confirmed with the HCV cell-cultured (HCVcc, see below) system[166]. Based on these data, it has been hypothesized that L-SIGN, expressed on sinusoidal endothelium cells, might be used as a docking site for circulating HCV within sinusoids, facilitating the virus transfer to hepatocytes. Furthermore, the absence of DC-SIGN and L-SIGN on hepatocyte membranes suggests that they do not play a role as direct virus entry factors; rather, these molecules could enhance infection by promoting virus attachment. Finally, Pöhlmann *et al* suggested that sinusoidal endothelial cells might be involved in capturing and concentrating circulating virions in the liver, allowing their presentation to the hepatocyte[167].

Several human proteins have been identified as factors or co-factors involved in HCV entry; they are briefly described below.

Low-density lipoprotein receptor (LDLR).

LDLR, a member of the low-density lipoprotein family[168], is a cell-surface receptor involved in cholesterol homeostasis. It has been shown to recognise ApoB100 embedded in LDL and ApoE in intermediate lipoprotein (IDL), and thus, mediate endocytosis of these lipoproteins[169]. The presence of lipoprotein components on the virus surface and the role of LDLR in HCV entry were first suggested by V. Agnello[170]. Several studies confirmed that indeed ApoB100 and ApoE are present on the HCV surface[23,26,171]. This hypothesis was also strengthened by the finding that cell entry of patient-derived HCV strains required LDLR[172]. Although several studies clearly demonstrated the role of LDLR in HCV cell entry[170,172-174], in one particular study the role of LDL-R in HCV RNA replication has been suggested in addition to virus entry process[175].

Cluster of Differentiation 81 (CD81).

CD81 is a 26 KDa ubiquitously expressed transmembrane protein belonging to the *tetraspanin* family. It is involved in regulation of cell morphology, motility and signalling[176]. CD81 is a type III membrane glycoprotein characterised by four transmembrane domains producing two extracellular loops and one short intracellular domain. CD-81 was initially identified as an HCV entry factor by Pileri *et al*, based on its interaction with E2 protein[177] and subsequently confirmed using HCVpp model[178]. Several experiments confirmed that disulphide bonds between cysteine residues in the large extracellular loop (LEL) are responsible for the stabilization and integrity of CD81 enabling its interaction with E2[90,179]. Specifically, the LEL has been demonstrated to be crucial for E2 binding[180-182]; moreover, the residues on CD81, involved in the interaction with E2, have been identified[87,90,177]. HCV E1E2 heterodimers have been shown to have a stronger interaction with CD81 than soluble

E2 alone[91] and thus suggesting that CD81 might induce a conformational change in E1E2 heterodimers to promote low pH-dependent fusion and endocytosis[183].

It has also been shown, thanks to new high-resolution fluorescence microscopy techniques, that CD81 is present on the cell surface in particular features, known as dot-like tetraspanin-enriched microdomains (TEMs), in which tetraspanins are present at higher concentrations[184,185]. These areas, which have been equally reported in experiments with other viruses[186], suggested that CD81 clustering on the cell membrane might be linked to susceptibility to HCV infection[187].

Moreover, a new regulatory ligand of CD81 has been described, EWI-2wint (EWI-2 without its N-terminus), whose expression could inhibit HCV entry in cells non-susceptible to infection[188]. Furthermore, silencing CD81 expression by small interfering RNA can efficiently inhibit HCVpp and HCVcc entry into hepatoma cell lines[189], while, on the other hand, CD81 expression in non-hepatic cells conferred susceptibility to HCV infection[178,190]. Specific anti-CD81 antibodies effectively prevent HCV entry but not its binding, confirming the role of CD81 as co-receptor required for the virus cell entry after attachment step [190-192].

Importantly, CD81 has been found to be present in cholesterol rich-microdomains (lipid rafts) and in cell junctions, where it co-localises with SR-BI and CLDN1, respectively[193].

Scavenger Receptor Class B type I (SR-BI).

SR-BI is a 509 amino acids glycoprotein of 82 kDa, which is expressed on the membrane of several cell types including hepatocytes. It is involved in the bidirectional transport of cholesterol and it has been characterised as a receptor for various classes of lipoproteins[194]. It has a large extracellular loop anchored at the membrane at both N- and C- termini[195]. SR-BI has been identified as a potential receptor for viral entry based on its interaction with soluble E2 protein[196]. Furthermore, it has been proposed that it could interact with the hypervariable region 1 (HVR1) [197]. To confirm this hypothesis, the role of SR-BI during HCV

binding has been largely investigated, identifying a region on SR-BI between amino acids 70-87 necessary for E2 recognition[198]. However, the experiments conducted with serum-derived (thus lipoprotein-associated) HCV have demonstrated that the effective interaction between SR-BI and virus-associated ApoB-containing lipoproteins is mediated by virus-associated VLDL[26].

Interestingly, the endogenous function of SR-BI as the lipid transporter has been investigated to determine whether it could confer susceptibility to HCV infection: in fact, using HCVpp and HCVcc, it has been shown that high-density lipoproteins (HDL) enhanced HCV infection[197,199], while oxidized LDLs reduced HCV entry[200,201]. Moreover, Ploss *et al* have demonstrated that human CD81 and Occludin (OCLN) expression is essential to render mouse cells susceptible to infection, whereas the expression of murine SR-BI and CLDN1 homologues may function similarly to the human proteins in promoting HCV entry[202]. Finally, inhibition experiments have shown that anti-SR-BI antibodies block HCV infection of hepatic cell lines[203] and of chimeric mice with transplanted human hepatocytes[204], confirming that SR-BI is an essential factor for HCV infection.

Recent studies have validated the hypothesis that SR-BI is involved in HCV cell entry at binding and post-binding steps[205] as well as in the cell-to-cell virus transmission[198].

Claudin 1 (CLDN1).

CLDN1 is a 211 amino acid, transmembrane protein of 23 KDa. It is an important component of tight junctions and is involved in cellular permeability and polarity[206]. It is expressed in all epithelial tissues and especially in the liver but it can also be found at the basolateral membrane of hepatocytes, as non-junctional CLDN1[207]. This molecule has been identified as a novel entry factor for HCV infection by screening a library of cellular proteins with HCVpp[208]. Although there is no homology between CLDN1 and tetraspanins[209], CLDN1 has been shown to interact directly with HCV virions[210]. Most importantly, CLDN1 has been

shown to complex with CD81, forming a well characterised complex that play a role in the post-binding step[211,212]. The critical region of CLDN1 for HCV entry is the extracellular loop 1[208] and especially the domain containing the highly conserved motif W30-GLW51-C54-C64 [213]. Moreover, some studies have demonstrated that lateral diffusion of CD81-CLDN1 complexes is crucial for HCV entry in vitro[211,214,215]; however, it has not been completely clarified if these complexes are pre-existing in the cells or are induced by HCV infection[216]. It has also been hypothesized that CLDN1 expression in the tight junctions might be related with HCV permissiveness[217], thus suggesting that tight junctions play a critical role for HCV entry. In fact, in infected cells CLDN1 is generally down regulated to avoid superinfection[218]. Moreover, the role of the cell polarisation in the HCV cell entry has been largely investigated: the polarisation affected tight junction formation interfering with the correct localisation of CLDN1 and other proteins[219]. As consequence, a non-junctional CLDN1 pool is produced, with impaired capacity of binding CD81 and thus, reduced HCV entry[219]. Equally, it has been proposed that HCV entry might be mediated by other proteins belonging to the Claudin family, such as CLDN6 and CLDN9, although with different efficiency[220,221].

Occludin (OCLN)

OCLN is a 65 KDa transmembrane protein highly expressed in the tight junctions of polarised cells[222]. This protein has been shown to have an important function in cell-cell adhesion and in anchoring the junctional complex to the cytoskeleton[223,224]. After that this protein has been identified as a new cellular receptor for HCV entry[202], it has been demonstrated that OCLN is required for late, post-binding entry events[218,225]. According to the most recent findings, OCLN and CD81 are considered the two critical cellular factors responsible for human HCV tropism. In fact, the expression of these human proteins confers susceptibility to HCV infection of mouse cells[202]. It has also been demonstrated that glucocorticosteroids affect OCLN expression increasing its level and thus stimulating HCV

entry, as already shown for SR-BI[226]. However, it has not been clarified whether OCLN has a direct interaction with viral proteins and further studies are necessary to fully understand the role of OCLN in this process[209].

EGFR and EphA2.

The epidermal growth factor receptor (EGFR) is a 170 kDa transmembrane glycoprotein characterised by an intracellular domain with tyrosine kinase activity. EGFR overexpression has been detected in a large proportion of hepatocellular carcinoma cases (40-70%) [227]. Recently, EGFR has been identified as a co-factor for HCV entry by RNA interference kinase screening[228]. Moreover, a second entry co-factor, the ephrin type A receptor 2 (EphA2), has been reported; EphA2 is a transmembrane tyrosine kinase protein involved in cell positioning, cell morphology, polarity and motility[229].

From several experiments, performed with HCVpp, HCVcc and replicon systems on several lines of hepatic origin and primary hepatocytes, it emerged that EGFR and EphA2 have no a direct interaction with HCV particles, but that they modulate CD81-claudin-1 association, affecting viral glycoprotein–dependent membrane fusion and facilitating virus entry[228]. This mechanism seems to be dependent on the synergistic expression and the activity of both EGFR and EphA2. Binding of HCV with CD81 but not with CLDN1 activates EGFR, triggering the internalisation of the HCV-CLDN1-CD81 complex.

HCV entry is apparently mediated by EGFR activation but seems to not be related to its kinase activity[230]. On contrary, the complex HCV-CD81-CLDN1 has been shown to associate with two other proteins: CD81-associated protein ITGB1 and Rap2B, which have been reported as putative cofactors for HCV entry. This complex activates a GTPase protein, HRas that represents the link between the HCV entry complex and the signalling pathway of EGFR. EGFR, trough HRas activation, is thought to promote HCV entry via the MAPK pathway, regulating CD81-CLDN1 complex assembly[231]. Furthermore, it has been

suggested that Rap2B, another GTPase protein, acts by regulating tetraspanin-enriched microdomains formation promoting CD81 and ITGB1 clustering[231].

Niemann Pick C1-like 1 receptor (NPC1L1).

NPC1L1 is a glycoprotein with a molecular mass of 170 to 200 kDa, which is supposed containing 13 transmembrane domains with three large extracellular loops (LEL); between them, LEL1 has been shown to bind cholesterol. NPC1L1 is a protein naturally involved in cellular cholesterol absorption and, in humans, it is highly expressed in the liver and in the gastrointestinal tract. In hepatocytes, during steady state NPC1L1 has been found mainly in the endocytic recycling compartment, whereas, in the case of cholesterol depletion, it is translocated to the canalicular membrane. Once exposed on the plasma membrane, NPC1L1 activity is controlling the uptake of biliary cholesterol into the cells. Recently, a model on NPC1L1 function has been proposed[232]; the authors suggested that NPC1L1 can bind to cholesterol present on bile micelles and transfer it to form a NPC1L1-flotillin-cholesterol microdomain that subsequently undergoes endocytosed via clathtrin coated vesicles. This mechanism and its role in cholesterol homeostasis suggests that this cell surface cholesterol-sensing receptor might be involved in HCV entry, based on the evidence that cholesterol is present on virus particles[233]. It has also been shown that knock down of NPC1L1, through the inhibition of pharmacological endocytosis or blockage of its LEL1 by antibodies, dramatically reduced HCV entry[234]. Furthermore, it has been reported that NPC1L1 is likely to be a HCV-specific entry cofactor, since no effects on vesicular stomatitis virus G protein pseudotyped particles were detected. Finally, it has been suggested that cholesterol content in HCV particles might highly influence viral entry via NPC1L1. It has not been completely clarified whether NPC1L1 may interact directly with HCV by removing cholesterol associated with virions, thus revealing a binding site on E1-E2, or conferring required conformational changes for optimal fusion. It has been proposed that NCPC1L1

might play a role similar to that suggested for EGFR, i.e. the cholesterol triggered endocytosis of NPC1L1 and consequently entry of HCV particles.

Transferrin receptor 1 (TfR1).

TfR1 is involved is iron homeostasis, mediating the iron-transferrin complex uptake; it is widely expressed in most of the human tissues. TfR1 is a 760 amino acid single pass type II membrane protein that undergoes endocytosis in a clathrin-dependent way. Once iron is delivered inside the cells, TfR1 is recycled and return to the cell membrane to collect more iron[235]. TfR1 has been proposed as entry factor for several arenaviruses including Machupo virus, Guanarito virus and Sabiá virus[236,237]. A possible correlation between iron metabolism and HCV infection has been proposed recently, supported by several evidences: first, a significant proportion of HCV patients have altered iron levels, suggesting iron overload[238]; in addition, microarray analysis revealed that changes in genes involved in iron metabolism may occur during HCV infection. Based on these observations, TfR1 has been suggested as putative factor in HCV entry. Interestingly, preliminary studies showed that TfR1 might interact with viral envelope glycoproteins; the authors also proposed that such interaction may take place after virus binding to CD81[239].

Cluster of differentiation 63 (CD63).

CD63 is a member of the tetraspanin superfamily, but it does not belong to the CD subfamily. In fact, CD63 constitutes its own subfamily because it has been shown that it originated before other CD molecules[240]. CD63 is ubiquitously expressed and it resides either on the cell surface or in the endosomal system. This protein is characterised by a lysosome-targeting motif that is recognised by AP-2 and AP-3, which are adaptor proteins involved in processing of clathrin-coated vesicles, and respectively mediate endocytosis from the plasma membrane and redistribution from endosomes to lysosomes[241].

Recently CD63 has been identified as a new entry factor by a novel approach based on computational prediction and data integration[242]. The authors further analysed CD63 and discovered that it is able to bind directly to HCV E2. In addition, HCV infection can be efficiently inhibited by anti-CD63 antibody and, in particular, by a polypeptide corresponding to the extracellular domain 2 of CD63[243].

HCV entry: a model

As represented in figure 3, HCV present in the blood stream might interact with endothelial cells of the liver sinusoids, where molecules of the lectin family, in particular L-SIGN, might act as capture receptors for the transmission of the viral particles to the hepatocytes[244]. Subsequently, virions are supposed to be trafficked to the basolateral membrane of the hepatocytes[162,245]. The first site of attachment is believed to be the HSPG[192], especially Syndecan-1 [163]. This might be due to the capacity of the LVPs to interact with both GAGs and LDLR through the virus associated lipoproteins allowing the attachment of HCV virions to the hepatocytes[170]. Post-binding events have not been completely determined, but it is supposed that, after the primary attachment, the viral particles interact with SR-BI that is able to bind HCV indirectly via VLDL associated to the virus[26] and then facilitate their uptake due to its cholesterol transfer function[246]. In sequence, the entry process probably involves the virus interaction with CD81 through E1E2 heterodimers[91]. Then, CD81 forms a complex with proteins of the CLDN family[211], though it has not been completely understood if these interactions are pre-existing or induced by HCV binding. After these early steps, the HCV entry may be altered by the presence of molecules circulating in the blood such as HDL and LDL [247], VLDL [26], or Lipoprotein Lipase (LPL) [248], facilitating (HDL) or inhibiting (VLDL, LDL, LPL) HCV infectivity. The role of other factors is still unclear, although, according to the most recent data, HCV entry co-factors such EGFR and EphA2, once activated by their ligands, are responsible for HCV-CD81-CLDN1 complex modulation and transport into the tight

junctions[220]. This step is supposed to trigger viral glycoprotein–dependent membrane fusion and endocytosis by an actin-dependent mechanism[249,250]. The role of TfR1 is not completely clear; however, it could play a role during endocytosis[239]. The tight junctions have been identified as necessary for protein localisation and virion internalization[202,219,251]; at this step of the entry process the complexes constituted by virion-CD81-CLDN most probably interact with other co-factors such as OCLN and NPC1L1[234] leading to a clathrin-mediated endocytosis[208,220], a common internalization mechanism for different viruses[252-255].

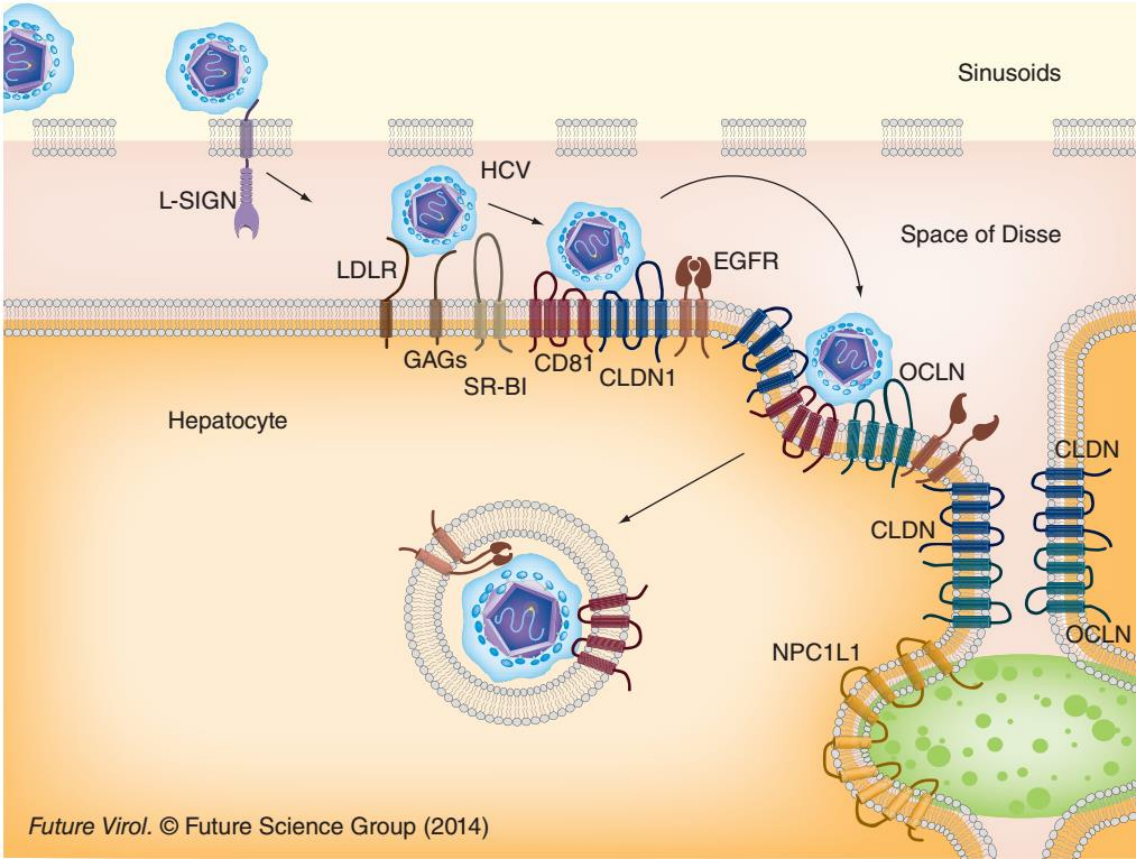


Figure 3. Putative mechanism of HCV entry showing the interactions between the virus and entry factors[256]. Viral particles interact with L-SIGN on sinusoids to be translocated in the Space of Disse where they directly face hepatocytes. Entry process involves several host receptors, such as LDLR, Gags, SR-BI, CD81, CLND1, EGFR. These interactions allow the transport of viral particles to tight junctions, where other proteins like OCLN and NPC1L1 are responsible to mediate clathrin-mediated endocytosis. After the entry, the fusion step that leads to HCV uncoating (not shown).

Fusion mechanism

The HCV fusion is a process that has not been completely described; for many years it has been suggested that it was mediated by the presence of a class II fusion protein, similar to those of other viruses possessing this class of protein[257]. Class II fusion proteins have been shown to induce membrane fusion through a clathrin-mediated endocytosis in a process that is highly dependent on the acidic environment[208,220,252]. For viruses belonging to *Flaviviridae* family, it has been demonstrated that low pH induces conformational changes in the glycoproteins and in the heterodimer dissolution, resulting in the formation of a fusion-competent homo-trimer[258,259].

Moreover, cholesterol has been shown to facilitates HCV-mediated fusion, depending on the presence of functional E1 and E2 proteins[260]. The fusion protein has been reported to act synergistically with lipid and cholesterol during the virus-cell fusion step. Noteworthy, the level of virion-associated cholesterol is significantly important; indeed, the most fusion-competent HCV particles showed the same density as the predominant cholesterol rich lipoprotein LDL [261,262]. Depletion of cholesterol from the virus almost completely abolished HCV infectivity, affecting internalization but not attachment[263].

Interestingly, it has been reported that other cellular lipids, in particular glycerophospholipids, sphingolipids and sterols, might be involved in HCV fusion thank to their physical, mechanical and/or chemical properties, whereas it has been demonstrated that cholesterol might bind to certain viral envelope protein after its organisation in cholesterol-rich microdomains, which have been shown to be implicated in the entry of many virus species such as Ebola and Marburg viruses, Vaccinia virus, murine Hepatitis virus, lymphocytic choriomeningitis virus and Herpes Simplex virus[264,265].

Translation

The HCV positive-strand RNA genome is directly used as a template for translation in the cytosol, immediately after infection and uncoating. This process is driven by an IRES-mediated translation, whom presence has been largely documented in the 5'-NTR of the viral RNA; its presence has been shown to be crucial to start the translation process, bypassing the cellular mRNA processing events and recruiting all the translation factors to the viral RNA.

The HCV IRES is characterized by the presence of several stem-loops (I, IIa, IIb, IIIa, IIIb,, IIIc, IIId, IIIe, IIIf, IV, V, VI). Interestingly, stem-loops IIId, IIIe,IIIf and IV have been shown to constitute the IRES core, forming a double pseudoknot structure, with a transfer RNA (tRNA) like structure[266,267]. This structure has been reported to bind strongly to the small ribosomal 40S subunit thank to multiple contacts. These contacts are responsible for the IRES binding to the 40S without any contribution from other initiation factors[268-271]. It has been suggested that the interaction between the pseudoknot and the 40S ribosomal subunit contribute to the positioning of the AUG codon in the mRNA binding cleft of the 40S ribosome[272]. Interestingly, the stem-loop II has been shown to be required for translation, while the stem-loop I presence is not necessary[273,274]; moreover, the stem-loop II, through the interaction with stem-loop IV, is thought to be responsible for placing the region with AUG into the 40S channel[275]. Moreover, it has been demonstrated that only three eukaryotic initiation factors (eIF) are required to form the 48S ribosomal complex and subsequently the 80S, triggering the IRES-mediated translation. The eIF3 interacts with 40S by binding to the apical part of stem-loop III [276-278]; furthermore, eIF2, in combination with the initiator tRNA (tRNAi) and the guanosine-5'-triphosphate (GTP), forms the eIF2-GTP-Methionine (Met)-tRNAiMet, that is responsible for transferring the Met-tRNAi to 40S in a GTP dependent way. Noteworthy, it has been shown that the recognition of the ORF start codon, driven by the eIF5, is supported by the aforementioned complex and is able to induce GTP, eIF2-

mediated, hydrolysis. Finally, the addition of the 60S to the already established large complex leads to the formation of the translation-competent 80S [279-281]. Probably, the core sequence and the secondary structures, present in the protein, might positively affect translation[282]. Moreover, the 3' NTR has been reported to enhance the translation, especially through elements mapped in its variable region, including poly U/C tract and the 3'-X region[283]. Usually, eIF2 activity is suppressed in case of a host cell-response to virus infections and, in this condition, an alternative eIF has been found, able to sustain the IRES-mediated translation, In particular, eIF2, eIF5 or the eIF2a can be replaced by eIF5b that is able to promote delivery of tRNA in a GTP-independent way[281]. Several other factors, including the La protein have been suggested to contribute to the efficient translation but their mechanisms of action need to be clarified[284,285]. Recent studies have identified the expression of miR-122 as a novel key factor in hepatitis C virus translation[286]. miR-122 has been shown to regulate HCV by binding directly to two adjacent sites close to the 5' end of HCV RNA[287]. Although these experiments were performed using genotype 1a and 1b HCV RNA, the miR-122 binding sites are located in a highly conserved region; moreover, it has been reported that miR-122 is required for replication of infectious type 2a HCV[288]. As miRNAs generally function to repress gene expression by binding to 3'UTR sites, this positive regulation of viral replication via a 5'UTR represents a novel function for miR-122. The mechanism of regulation is not yet known. miR-122 stimulates translation of HCV RNA, but not to a sufficient extent to explain its effects on viral replication, indicating that a second stage of the viral replication cycle must also be regulated[289]. HCV RNA synthesis is not affected by miR-122, suggesting that regulation of other processes such as RNA stability may occur[285].

Replication

Immediately after translation and the subsequent processing of the polyprotein, the NS proteins, comprised between NS3 and NS5B, rapidly constitute the replication complex on the ER membrane. Interestingly, the establishment of the replication complex leads to membrane alterations[135,290]; altered membranes have been reported since the first studies on the human and chimpanzee liver tissues[291-293]. Expressing the NSs comprised in the replication complex, in particular NS4B, leads to a massive vesicles modifications and induces their accumulations, forming a characteristic feature, reported as membranous web[134]. According to several studies, it has been reported that the HCV RNA replication sites are protected by membranes[294-296]. Based on other findings, it has been hypothesized that these vesicles are membrane invagination with a pore that allows the exchange of hydrophilic molecules like nucleotides[297]. Aside single vesicles, more complex structures have been described in HCV infected cells. As shown in figure 4, these structures are defined as double-membrane vesicles (DMVs), massively predominant, together with multiple-membrane vesicles (MMVs) [290,298]; however, their functions have not been completely clarified[38,299]. The morphology of membranous web is not affected by RNA synthesis, although it depends on the expression of NS3-N5B module that is likely to interact with host factors[298-300]. NS4B plays also an important role in the formation of replication complexes; indeed, its expression alone is sufficient to generate a membranous web resembling to the one generated by NS3 to NS5 protein expression[134]. Interestingly, the expression of NS3/4A or NS5B alone has been reported to induce membranous web morphogenesis; moreover, the NS5A expression occasionally produces vesicles, which present the same morphology of DMVs [300]. In addition, HCV is known to alter the expression of genes involved in lipid metabolism; as consequence, an intracellular lipid accumulation has been observed, that is crucial for optimal replication[301-303]. Specifically, it has been suggested that lipids might be involved in two distinct ways: contributing to form the

replication site through membrane proliferation and inducing protein modifications like geranyl-geranylation and palmitoylation[304]; however, very recently has been shown that in replicating cells the majority of NS4B is not palmitoylated[305].

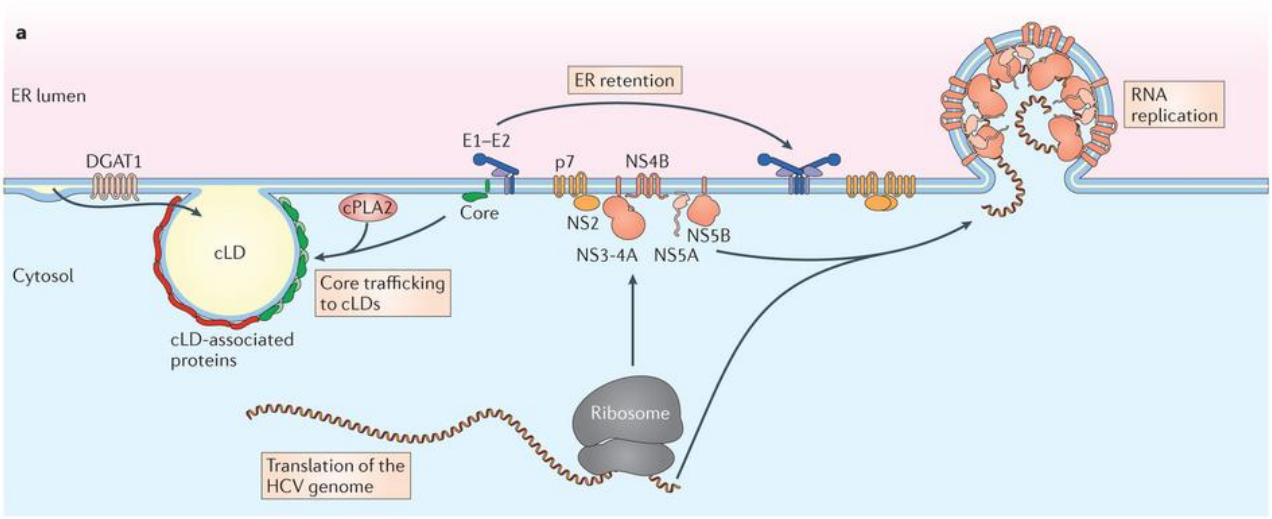


Figure 4. (A) RNA translation and formation of the membranous web[24]. Viral RNA is immediately translated to generate a polyprotein, which is processed to obtain viral proteins. HCV proteins are expressed on the ER membrane, where they form double-membrane vesicles, sites in which RNA replication takes place.

Once membranous web is completely established, RNA replication takes place. This step is a complicated process that has not been yet completely elucidated. Almost all data have been collected from *in vitro* experiments, thus the mechanism observed *in vivo* remains unclear, mainly due to lack of appropriate model systems. Initially, from studies conducted with purified NS5B, it has been proposed that RNA synthesis may initiate in two ways: by a primer-dependent mechanism or by the *de novo* synthesis[152,153,306-308]; however, recent studies seemed to confirm that *de novo* synthesis is the physiological mode of RNA synthesis in HCV infected cells[309].

The first step of HCV replication is the synthesis of the intermediate negative-strand RNA, in a ratio to positive RNA comprised between 10-100 [310,311], which is then used as template

for the synthesis of the positive-strand RNA; the newly synthesized RNA is either packaged into virions or re-used for negative strand synthesis[312]. Interestingly, negative-strand synthesis appears to be rate limiting, suggesting it may represent a mechanism to control replication efficiency[38].

The 3'NTR on the positive strand has been shown to be crucial for viral RNA replication[41], probably for its role in the initiation and regulation of negative-strand synthesis[313]. Moreover, the 3' of the HCV negative strand has been shown to represent the template for RNA synthesis initiation, whereas the 3' end of the positive strand is part of a stable structure that cannot be accessed by the NS5B in the closed conformation[42,314]. These observations suggest that the synthesis of the intermediate negative strand, starting from the positive strand, requires other factors, such as the NS3 helicase.

RNA synthesis *in* vitro can be divided in four different steps: RNA binding, initiation, elongation and termination. NS5B polymerase has been shown to bind to a various number of RNA template, in a slow and inefficient process[315]. Interestingly, the NS5B enzymatic core is able to bind with high affinity to single strand RNAs characterised by more than seven nucleotides[316]. Following the binding of a single-stranded template, a dinucleotide primer is synthesized; for this process, a high concentration of GTP nucleotide is required[317]. Noteworthy, the synthesis of the dinucleotide can generate an accumulation of it *in vitro,* probably due to the closed conformation of NS5B, suggesting that it dissociate rapidly from the NS5B-template complex[155]. Furthermore, it has been hypothesized that the dinucleotide primer is subsequently addressed in a "platform" where the addition of the third base takes place. It has been proposed that the C-terminal linker or the beta flap might represent the site for this "platform" as already shown for other pestiviruses[160,318]. Switching to the elongation requires a high concentration of the third base incorporated and it is facilitated by high GTP concentration[319,320]. Moreover, the switch to elongation requires a conformational change, during which the NS5B C-terminal is removed to hold the egressing

dsRNA and “fingers” shift adapting contacts with the “thumb” [321,322]. Interestingly, it has been demonstrated that the amino acid in position 405 in the thumb is crucial to switch from initiation to elongation, stabilising the close conformation first, and then facilitating the transition to the open conformation[323]. During elongation, it has been estimated that NS5B can incorporate between 100-400 nucleotides per minute[324,325]; interestingly it has been reported that NS5B is able to replicate the full HCV genome *in vitro*, suggesting that the NS3 helicase is not required at this stage[155,324,325]. In this process, the NS5B polymerase is strictly associated to its template and surprisingly, low nucleotide concentrations are required compared to initiation stage[326]. Termination step of RNA synthesis is almost unknown, although it has been suggested that the polymerase might dissociated when reach the end of the template. NS5B-mediated synthesis has been largely documented as error prone, providing evidences for the high variability of HCV isolates. Powdrill *et al* showed that the NS5B error rate is approximately of 10^{-3} per site, with a strong bias toward G:U/U:G mismatches[327].

Viral assembly, maturation and release

The exact mechanisms underneath the assembly of infectious virus particles remain poorly understood but it has been demonstrated that these events are driven by a complex interaction between viral and cellular factors. HCV core protein, thanks to the presence of amphipathic regions, acts like a membrane protein and this feature allows its association to the surface of cytosolic LDs (cLD), which derive from the outer leaflet of the ER[53]. Interestingly, specific mutations in Domain 2 of core protein seemed to impair interaction with cLDs, reducing virus production, presumably affecting the assembly stage[15,49,328]. In particular, a specific mutation in D2, phenylalanine 130, heavily compromises the protein stability and blocks the interaction between core and LDs [49]. The trafficking of core to cLD has been shown to be dependent on the cytosolic phospholipase A2 G4A (PLA2GA4) and

this process can be enhanced by the diacylglycerol acyltransferase 1 (DGAT-1), highlighting the importance of lipid metabolism in HCV life cycle[329,330]. The first study supporting the hypothesis that LDs play a key role in the mechanisms of HCV assembly was provided by Miyanari et al., who proposed the first model with HCV-induced morphogenesis[15]. In this study, the authors described the core protein as the main element responsible for the recruitment of glycoprotein E1E2 to replication complexes and viral genome to LDs. According to this hypothesis, LDs represent the sub-cellular microenvironment where all factors are localised, allowing the initiation of virus assembly (figure 5).

Moreover, it has also been shown (figure 5) that NS2 plays a crucial role in the early stage of assembly: according to authors, NS2 is essential to stabilize the replication complex that includes E1E2, p7 and NS3-4A[109,110,114]. In addition, it has been shown that NS2 can interact directly with NS3-4A, a step fundamental to recruit core-cLDs into the assembly site and with p7, which is also required to localize NS2 to the site of the virus assembly[111,113,115].

Mutations in NS3 and NS5A result in significantly reduction of the viral assembly, suggesting that they may contribute to the replication/assembly transition[331]. During the assembly of viral particles, it has been reported that NS5A is recruited to LDs where it interacts with core and probably also with ApoE, a critical factor for this step[15,31,332]. In fact, interaction between ApoE and NS5A is considered essential to stabilize the viral assembly platform[332].

Furthermore, it has been proposed that HCV particles might be formed through budding into ER, as exhibited by other members of the Flaviviridae family. In fact, pharmacological inhibition of ER-Golgi transport leads to an accumulation of HCV particles inside the cells[333]. From this observation, it has been hypothesized that HCV assembly starts in a site close to intracellular LD structures and that the nucleocapsid formation may take place simultaneously with budding from ER (figure 5)[38,334].

After assembly, it has been proposed that HCV particles undergo some complex modifications that have been described in the post-ER compartment. To support this idea,

several experiments confirmed that maturation and release of HCV virions share features with the pathway of VLDLs production[23]. VLDL synthesis, in hepatocytes, is a complex pathway that can be briefly described as a two-step process requiring several factors. In the first step, ApoB is located with lipids, in a co-translation mechanism, by the microsomal triglyceride transfer protein (MTP) to form the pre-beta VLDL in the ER. In the second step, pre-beta VLDL are subject to a further lipidation, but this mechanism is still unclear and two models have been proposed[335,336]: in the first hypothesis, lipids are included into the VLDL precursor through the fusion with LDs facing the ER lumen, which are known to be associated with ApoE and ApoC. In the second one, it has been suggested that the addition of lipids to VLDL might happen in a post-ER compartment, likely in the Golgi[337].

Considering that ApoB, ApoE and MTP are key components required for VLDL formation, they have been investigated to determine their role in viral particle production. Blocking the VLDL production, interfering with ApoB or ApoE synthesis or through a pharmacological inhibitions of MTP or the acyl-CoA-synthase 3, resulted in massive reduction of HCV production, although no effect was detected on viral replication[21,27,333,338-340]. In contrast, others suggested that ApoB and MTP were not involved, whereas depletion of ApoE alone resulted in a massive inhibition on HCVcc production[21,341]. Based on their results, the authors suggested that HCV maturation is dependent on fusion with ApoE-associated LDs rather than the VLDL pathways. Has to be considered, though, that most of these results were obtained using Huh7 cells, which have been described for having deficient lipoprotein synthesis pathways, and thus an impaired production of ApoB. However, in the last few years, ultrastructural analysis of HCV particles, produced in primary human hepatocytes, confirmed the association of ApoB with secreted viral particles[342]; moreover, it has been demonstrated that knocking down ApoE level significantly reduces virus production and promotes viral-evasion from neutralising antibodies[27].

Finally, during egress the viral glycoproteins undergo an extended glycosylation, and their disulphide bonds are rearranged[81]; furthermore, mature particles are secreted in vesicles, in order to prevent the exposure of the viral progeny to low pH, which would trigger, in a p7-dependent mechanism, premature uncoating[101].

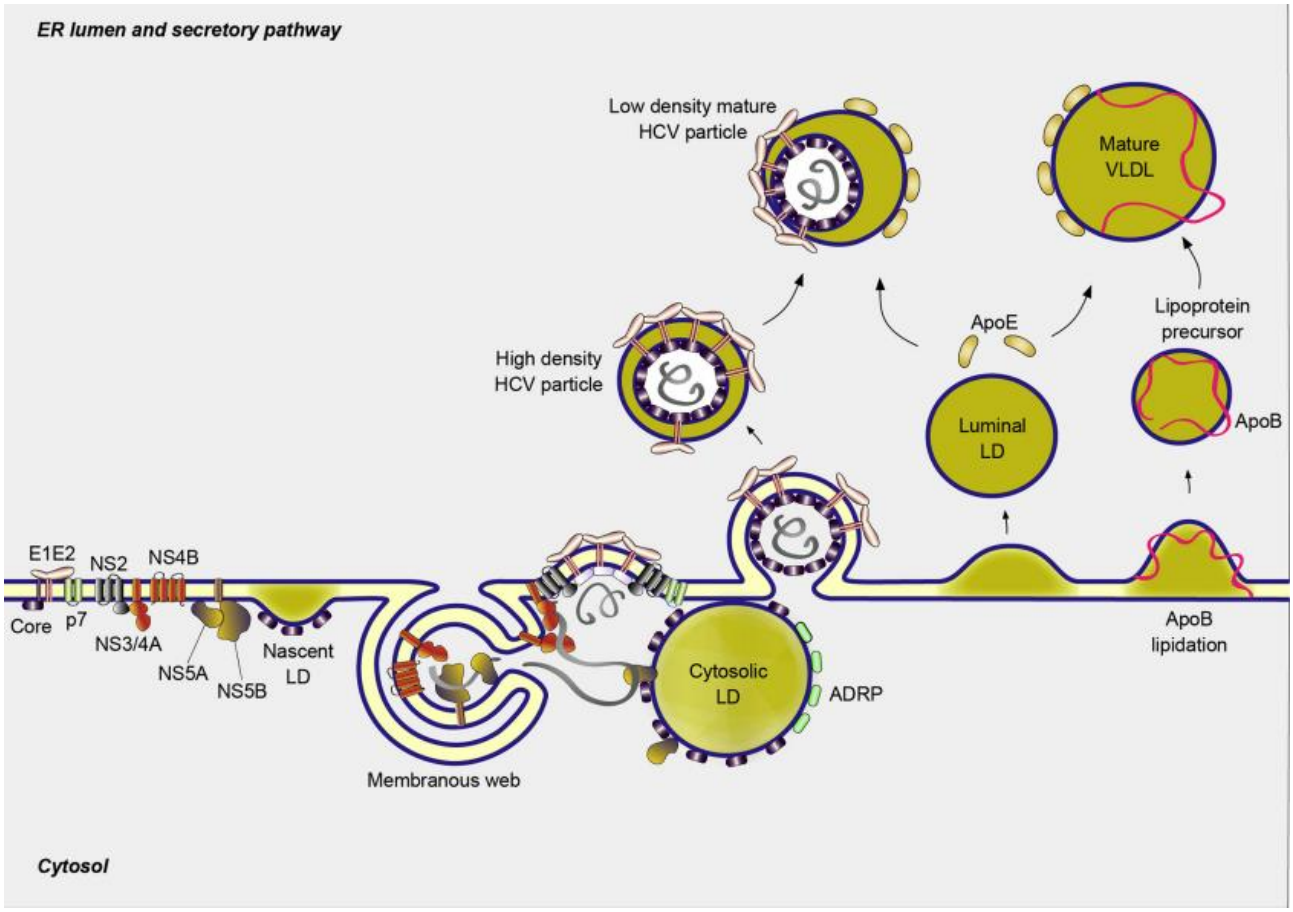


Figure 5. RNA assembly and release process through ER lumen[25]. After RNA replication inside DMVs, viral proteins cooperate with cellular factors to initiate viral assembly. In particular core protein, responsible for recruiting lipid droplets to DMVs and driving progeny positive ssRNA inside nascent particles. Once particles are correctly assembled they are associated with lipoproteins, such as ApoB and ApoE, and released following LDL pathway.

In vitro models to study HCV cell cycle

Since the discovery of HCV, in 1989, many unsuccessful attempts have been done trying to establish efficient *in vitro* systems to culture the virus. In early experiments, human and chimpanzee primary hepatocytes were infected with sera obtained from HCV patients or transfected with cloned viral RNA; all these trials failed to establish a robust replication model[343]. In primary human foetal hepatocytes, infected with serum-derived HCV, the viral RNA was detectable but the replication rate was very low[344]. Furthermore, stable cultures of primary hepatocytes are still very difficult to establish. For these reasons, several human hepatoma cell lines were tested. Human hepatoma cell lines 7721, PLC/PRF5, Hep3B and Huh7 appeared susceptible to infection with HCV from patient sera but the system was still inefficient[345].

Transfection of hepatoma cell lines with cloned viral RNA gave poor results due to non-functional sequences or mutations introduced by RT-PCR. This problem has been circumvented by the isolation and cloning of particular strains, like H77 or JFH1, able to sustain viral replication. Based on these replicative-competent clones and thanks to new molecular biology techniques, different efficient *in vitro* cell culture systems to study HCV were established (Figure 6) [346-348].

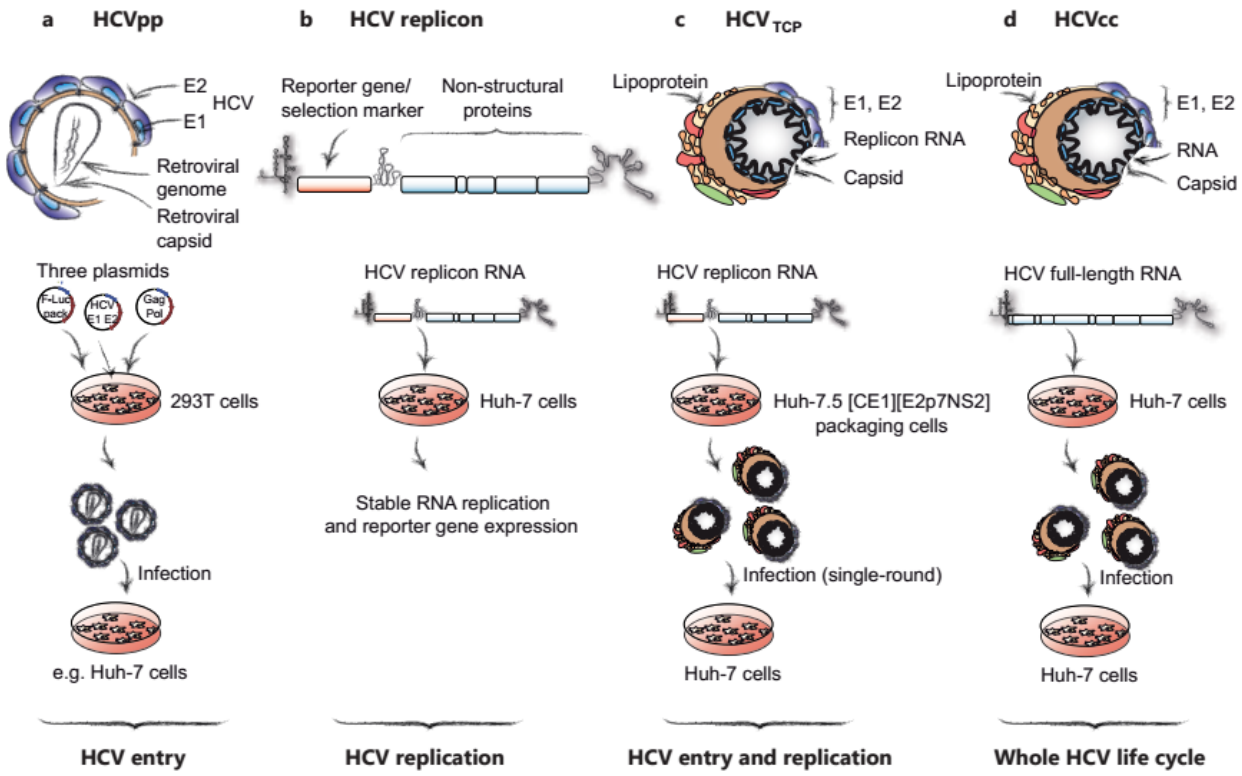


Figure 6. In vitro models developed to study HCV life cycle. Systems are listed accordingly to the HCV life cycle step(s) that they allow to study[347].

HCV pseudo-typed particles (HCVpp).

HCVpp were introduced as the first HCV *in vitro* infection system[178]. HCVpp consist of a retro- or lentiviral nucleocapsid surrounded with HCV enveloped glycoproteins E1 and E2. HCVpp are obtained by co-transfection of the human embryo kidney cells (HEK293T) with three plasmids. The packaging vector encodes the genes for retroviral structural proteins, Gag and Pol, the transfer vector contains a sequence required for encapsidation and a reporter gene (typically Luciferase), which are flanked at 5' and 3' by sequences required for its integration in the cellular DNA; finally, the vector that encodes for HCV envelope glycoproteins E1 and E2. Viral pseudo-particles, produced in HEK293T cells, can be used on Huh7 cells to evaluate viral infectivity, which can be quantified by determining luciferase activity. Infection of primary hepatocytes with HCVpp is possible, although the infection

levels are usually not comparable with those in Huh7 cells[178]. HCVpp are largely used to investigate viral entry, which include viral binding, entry process and fusion; furthermore, they have been described for evaluating neutralization of HCV with anti-envelope antibodies. In fact, HCVpp entry can be neutralized with sera of HCV infected patients containing antibodies targeting E1, or E2 protein[349-351]. In addition, they are often used to identify and characterise molecules able to block HCV entry and to investigate virus-cell-fusion mechanisms[352].

Importantly, due to the non-hepatic origin of HCVpp, these virus pseudotyped particles are not associated with lipoproteins (unlike infectious HCV virions, see below), and thus the entry mechanisms does not consider lipoprotein-associated virus.

HCV replicons

HCV replicons are genetic elements consisting of a portion or the entire genome of HCV; they are able to replicate autonomously and were developed to study HCV replication[317]. Up to date, a large number of replicon system has been established[353,354]. The replicons may be sub-genomic, containing only the non-structural HCV proteins required for RNA replication or full-length, which are characterised by the presence of structural and non-structural proteins, although no viral particles are released. In the first generation, most of them were bicistronic construct and contain the HCV 5'-NTR, the neomycin phosphotransferase gene for selection, an encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) driving translation of the HCV non-structural genes which are followed by the HCV 3'-NTR. The second generation of replicons consist of a monocistronic or bicistronic construct usually encoding a reporter gene (luciferase or GFP) and a selection

gene (puromycin or neomycin resistance) under the control of HCV or EMCV IRES[355]. Moreover, several replicons have been developed from different genotypes[356].

All the replicon constructs have the T7 promoter, upstream of the viral genome cDNA, which drives transcription of RNA. Following *in vitro* transcription with T7 RNA polymerase, replicon RNA is transfected into human Huh7 hepatoma cell line, usually by electroporation to achieve a high efficiency[357].

Several studies have reported that replicons usually may acquire adaptive mutations, which are generally located in the NS3, NS5A and NS5B proteins, within few weeks[358,359]. Importantly, adaptive mutations in NS5A region have been identified as responsible for interferon resistance[360]. In addition, a replicon carrying three adaptive mutations (two in NS3 and one in NS5A) has shown a strong increase of RNA replication[361]. The replicon system has been generated to study the host and viral signalling, adaptive mutations necessary for viral replication and, most importantly, screening of candidates for anti-viral molecules[357,361,362].

However, due to intrinsic limitations, this system could not be used to investigate viral entry or assembly process. Indeed, the replicon cells are not able to produce viral particles[363].

HCV trans-complemented particles (HCV_{TCP})

HCV_{TCP} are authentic viral particles that contain a subgenomic replicon RNA, mostly JFH-1-based, instead of the full-length genome. These particles are generated in Huh7 cell line by transfection with replicon RNA and envelope proteins. However, several packaging cell lines have been obtained through transduction with vectors encoding HCV structural proteins and thus the sequences are stably integrated into the cellular genome[364]. HCV_{TCP}, which are assembled in hepatic cells, show association with lipoproteins, and consequently they resemble more to HCV than HCVpp[365]. Moreover, HCV_{TCP} contain as genome a sub-genomic replicon, as such lacking of sequences encoding structural proteins; hence, the

infection of naïve cells with these particles allows investigating a single cycle infection, evaluating potential effects only one viral entry and RNA replication.

Cell culture derived HCV (HCVcc).

In 2001 it was reported that a sub-genomic replicon, obtained from a clone termed Japanese Fulminant Hepatitis 1 (JFH-1), was able to replicate very efficiently in Huh7 cells without the addition of adaptive mutations[366-368]. However, the real turning came a few years later, when three different groups reported that the full-length JFH-1 genome was able to replicate and to produce virus particles in Huh7 cells; interestingly, these particles have been shown to be infectious for these cells as well as in animal models such as humanized mice and chimpanzees[369-371].

These virus particles were then termed HCV cell-cultured (HCVcc), and they boosted the studies of all steps of the viral life cycle, including also assembly, release and multiple cycles of infection. Considering that the JFH-1 is a genotype 2a based system, many groups focused their studies to obtain clones from other genotypes able to produce infectious virus particles in cell culture[17]. However, these new systems seemed not be able to establish a robust production of virus particles. Only in the last few years, it has been reported that these strains, of different genotypes, can support robust viral culturing when mutated to introduce specific adaptive mutations able to restore high level of viral replication[372-374].

To partially overcome this problem, a wide panel of intra- and inter-genotypic chimeras has been developed to obtain infectious systems of different genotypes[375,376]. Most of the reported chimeras have been produced by replacing the region comprised from core to NS2 protein in the JFH-1 backbone with the same region of other genotypes. Noteworthy, it has been reported the development of chimeras in which JFH-1 sequences encoding NS3/4A or NS5A proteins were replaced with homologous sequences of other genotypes[374,377,378]

These new chimeras might allow to screen new antiviral compounds (especially those targeting NS3/4A and NS5A) and study the drug-resistance mutations developed by all HCV genotypes.

The disease

HCV variability and worldwide distribution.

Hepatitis C is a virus-related liver disease caused by Hepatitis C Virus. The World Health Organization (WHO) estimates that a 170 million people are currently infected with HCV[379]. HCV is divided into 7 different genotypes, which can differ between them up to 31-34% for the nucleotide sequence and up to 30% of the amino acid sequence. Interestingly, the variability in the nucleotide sequence has been observed in the whole viral genome, although some regions show a higher degree of diversity than others; in particular, the region encoding envelope proteins is characterised by a high variability, whereas the 5' NTR is the most conserved region[380-382]. These genotypes (1, 2, 3, 4, 5, 6 and 7) have been characterised for a different worldwide distribution, transmission rates and disease progression[383]. Interestingly, among genotypes has been possible to define about 100 different subtypes (a, b, c, d, etc.), the most frequent of which are HCV 1a, 1b, 2a and 2b[384]. Moreover, HCV variability is so high, due to the polymerase error-prone activity that it circulates in infected patients as a population of different but closely related variants known as "quasispecies" [385].

Their geographical distribution is not homogeneous[386]: in particular, as shown in figure 7, genotypes 1 and 2 are better represented in Europe and the United States; the HCV 3 in India, Australia and Far East, even though is now rapidly increasing in Europe. The genotype 4 is predominant in the Middle East and Africa, whereas HCV 5 in South Africa and HCV 6 in Hong Kong[379,386-388].

Moreover, there is a significant difference between genotypes in the response to therapy and progression of disease. In fact, patients infected with genotypes 1a and 1b respond less well to therapy with interferon[389], as well as genotype 3 patients, that develop more frequently steatosis and have a more rapid disease progression to chronic liver disease[390].

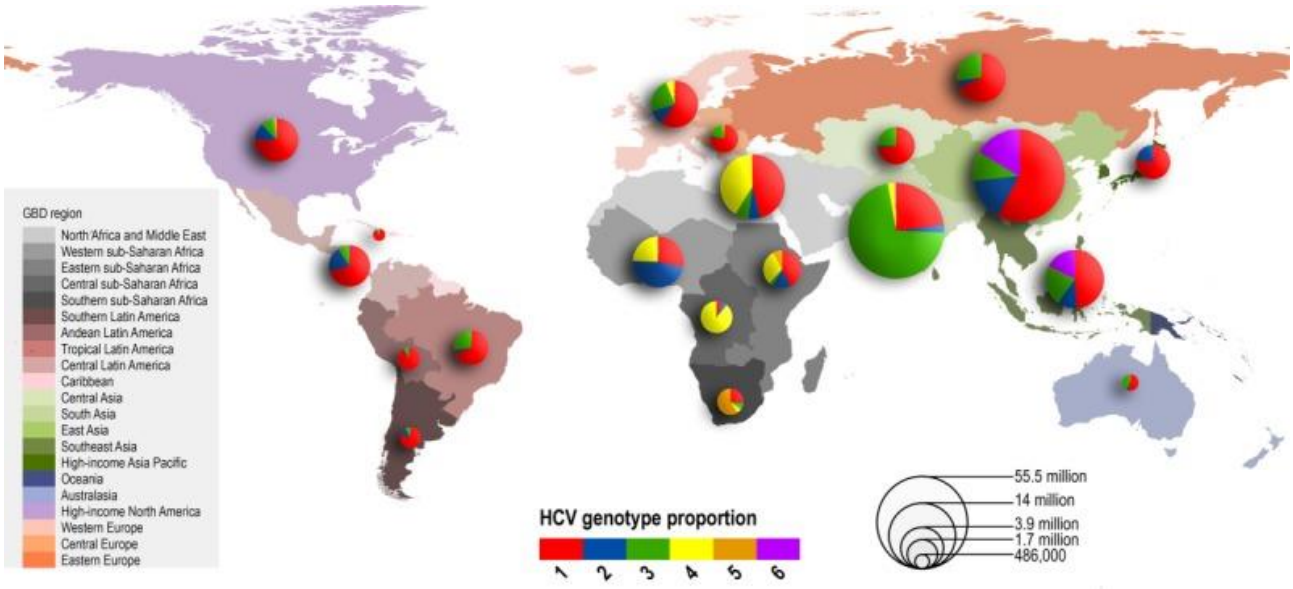


Figure 7: Worldwide distribution of hepatitis C virus in 2015 [386]. Genotype 1 is predominant in Europe, America and China, while genotype 4 in Africa and genotype 3 in Asia. However, due to drug resistance, genotype 3 is rapidly spreading in Europe.

Modes of transmission

The HCV transmission occurs mostly by parental diffusion, mainly using intravenous drugs or by transfusion of infected blood, although the latter has undergone a dramatic decrease following the introduction of screening test for blood donors since 1990; moreover, sexual and parental transmissions, although reported, are rare[391,392].

In fact, the efficiency of sexual transmission is low, and it has been observed mostly in people with multiple partners and homosexual; hence, the risk of transmission to partners is

the same in these two groups[393]. It is important to highlight, however, that the risk increase in HIV co-infected patients[394].

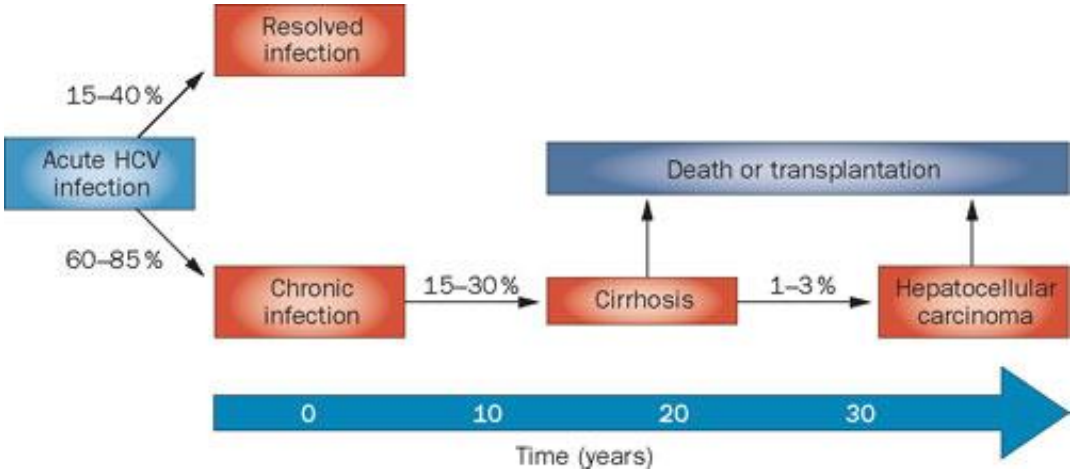


Figure 8: The natural history of HCV infection[395]. 15 to 40% of infected people can spontaneously clear the virus, whereas the remaining 60-85% develops chronic infection. Viral persistence is responsible of a chronic liver damage that can lead to liver cirrhosis (15-30%) and eventually to Hepatocellular carcinoma (HCC).

Natural History

As reported in figure 8, typical HCV infection is characterised by an initial acute phase that is usually cleared in 15-40% of the patients, while remains persistent in 60-85%. Of those patients, with a persistent liver damage, 15-30% develops liver cirrhosis, which could end in hepatocellular carcinoma in 1-3% of the cases[379,384,386]. Moreover, both acute and chronic infections are commonly either asymptomatic or characterized by nonspecific symptoms, making difficult to determine when infection occurred. Noteworthy, HCV patients can be co-infected by HIV and/or HBV; this situation makes it extremely difficult to dissect the relative role played by each virus in the development of liver disease. Furthermore, it has to be considered that estimating the correct alcohol consumption is often complicated in those patients, although alcohol is known to be a very important factor for liver fibrosis progression[396].

The Acute Phase of HCV infection

Typically, the HCV RNA can be detected in the serum between 7-21 days after infection, although is not completely clear the time of incubation, probably depending on the mode of transmission[397,398]. Usually, after 4-12 weeks from infection, a significant increase in the level of alanine aminotransferase (ALT) is observed, representative of liver injury[399]. Indeed, ALT levels might reach concentrations ten times or more higher than the upper normal level, and may be also followed by a rise in serum bilirubin concentration[400,401]. In addition, some patients have been shown to develop symptoms up to 4-12 weeks after exposure, although the majority remain asymptomatic[402,403].

Symptoms commonly reported include nausea, fatigue, abdominal pain, loss of appetite, and mild fever. Although these symptoms are nonspecific, it has been estimated that between 16-33% of patients develop jaundice, in particular if associated with carriage of a single nucleotide polymorphism upstream the IL28B locus; moreover, it has been reported that patients with acute hepatitis, C who become jaundiced, have more probability to clear the infection[404].

Although is a very rare event, several cases of fulminant hepatitis C have been reported[405]; it is characterized by massive liver cells necrosis, and it develops earlier, typically within 2-8 weeks post infection[406].

Interestingly, in a minority of cases (15-40%), HCV infection can undergo spontaneous clearance, usually within 3-4 months from infection, but it is estimated that 60-85% of HCV infected patients establish a persistent infection[407,408]. It has been reported that some favourable alleles, especially in the IL28B polymorphic locus, are associated with spontaneous clearance[409,410]; moreover, the presence of neutralising antibodies has been described in patients that cleared the infection[411,412].

The chronic phase of HCV infection

The current definition of chronic HCV infection can be resumed as the presence of HCV RNA in the patient blood longer than six months after transmission[413]. As consequence, the persistent presence of viral replication is responsible for liver damage, which can frequently cause fibrosis deposition, determining in a low number of patients the onset of liver cirrhosis and finally the development of hepatocellular carcinoma (HCC). The course of the disease is characterised by high variability and patients report different symptoms including nausea, myalgia, loss of weight, right abdominal pain, and fatigue. All these symptoms are non-specific and often are not recognised until advanced a state of fibrosis/cirrhosis is established. Liver cirrhosis is defined histologically as a diffuse process characterized by replacement of the normal liver parenchyma with fibrous tissue and the conversion of normal liver architecture into structurally abnormal regenerative nodules. This process results in the loss of functional liver cells and the establishment of portal hypertension, to which most of liver-related mortality via liver decompensation and/or HCC development is associated. Data from a meta-analysis indicate that cirrhosis develops in 16% of patients within 20 years from infection[414]. It is important to highlight that HCV-associated cirrhosis has a very high variability, between 14-62%, probably due to regional difference and other environmental factors[415]. One of the key point for personalised medicine is trying to differentiate individuals with low or high risk of developing cirrhosis; for this purpose, several risk factors for fibrosis progression in hepatitis C have been identified, also including age[416]. Those data are easily confirmed by the fact that longer is the duration of infection higher is the degree of the reparative processes that are responsible to induce fibrosis. However, it has been proposed that cirrhosis generate in a dynamic and complicated process that could be accelerated in parallel with age increase. Different studies on cohorts including young subjects showed a rather low prevalence of cirrhosis, suggesting that in the young people fibrosis development occurs slowly[416]. Moreover, it has been demonstrated that people exposed to HCV infection

at an age older than 40 years have an increased risk to fibrosis progression in a shorter period of time[417]. On this purpose, it has been demonstrated that infection during childhood lead to milder course, but on the other hand, it has been reported that most of HCV patients develop fibrosis at about 65 years, independently on the age of infection[418]. Gender has also been shown to be an important predictive factor; In fact, the ability to spontaneously clear hepatitis C virus infection is greater in premenopausal women than in men and, among patients with chronic infection, histologic progression occurs rarely in pre-menopausal women[419], confirming data suggesting the involvement of sex hormones[420]. On this purpose, a key role of 17β-estradiol has been largely documented[421,422].

Interestingly, a potential role of viral factors have also been largely investigated, leading at the conclusion that they probably play a minor role[423]. In fact, it has been demonstrated that viral load is not associated to the increased level of liver fibrosis or liver damage[420,424]. However, some exceptions have been described in different studies, showing that genotype 3 is possibly linked to an accelerated and severe course of the disease[425-427]. To confirm these findings, it has also been reported that genotype 3 is strongly associated to the insurgence of liver steatosis; steatosis is one of the factors responsible for boosting liver fibrosis and liver disease[390,428-430].

Finally, another important risk factor, among HCV infected patients, for liver disease progression is excessive alcohol consumption, a largely recognised cause of liver cirrhosis on its own: during a persistent HCV infection, a chronic intake of more than 50 g per day is responsible for a dramatic increase in the progression of the disease[431].

Current and novel HCV therapies

Preventive and therapeutic Vaccination

Despite all the efforts since its discovery, to date no preventive or therapeutic vaccine is available for HCV, mostly due to the high virus variability and viral escape mechanisms[79]. Moreover, recovery from infection, either spontaneously or pharmacologically, does not provide protection to a second exposure to the virus[432].

However, several promising vaccine candidates have been developed to provide preventive protection or as a therapeutic approach. However, the results obtained using therapeutic vaccines have been discouraging, showing a reduced reductions in viral loads; for this reason, a prophylactic vaccine may be a better strategy. In fact, trying to prevent the chronicity of HCV infection might be a result easier to be achieved in uninfected subjects, using a prophylactic vaccine.

Initially, the HCV vaccine research has been largely focused on a prophylactic B-cell vaccine; for this purpose, the potential of the envelope glycoproteins E1 and E2 has been investigated[433]. Several candidates, based on the recombinant E1E2 heterodimer, were tested on healthy volunteers, showing a cross-reactive neutralising antibody response to genotypes 1a, 1b, and 2a. However, the main issues related to this approach are the difficulty to produce intact recombinant heterodimer[434,435] and the viral evasion, based on the same strategies that contribute to establish the chronic infection[79,436-438]. Based on these findings, a new strategy, based on E1-only vaccination, showed in a phase-I clinical trial a low Ab responses; however the authors reported a T-helper 1-mediated response in almost all participants[439].

Interestingly, two therapeutic DNA vaccines have also entered clinical trials: CIGB-230 and chronVac-C. CIGB-230 is based on the mixture of pIDKE2, a plasmid expressing HCV structural antigens, with a recombinant HCV core protein[440]; ChronVac-C consists of a

plasmid coding for optimized NS3and NS4a genes[441,442]. However, one of major difficulties to use DNA vaccines is represented by the method of delivery; in fact, the classical delivery through injection does not allow cells to capture the naked DNA. To overcome this problem, developers coupled the injection with *in vivo* electroporation, a short electrical pulse causing permeabilisation of cellular membranes resulting in the induction of a local inflammatory response[442]. Reported data show that the vaccine gives rise to an evident T cell response after the second booster dose, although its convenience has still to be clarified[443,444].

Finally, in the last years, many HCV vaccines have been developed trying to generate a T-cell response[445-450]; noteworthy, some of them entered into phase-II or I clinical trials as therapeutic or prophylactic vaccine[450-452]. One of the most promising approach is characterised by the use of adenoviral particles to induce the expression of a conserved epitope located in the NS3–NS5 region[452]. In this study, the authors used a novel strategy based on the adenoviral vectors human adenovirus 6 (Ad6) and chimpanzee adenovirus 3 (ChAd3), both containing DNA encoding the same region of the HCV genome. Initially, the immune system has been primed with an initial inoculation of Ad6, and subsequently boosted with several inoculations of ChAd3. These particular adenoviruses were selected as serotypes inducing low anti-vector immune responses. The authors described that the T-cell responses were mostly cytotoxic, although helper T-cells were reported. T-cell responses were triggered by peptides from genotypes 1b, 1a, and to a lesser extent 3a, suggesting a potentially pan-genotypic treatment profile[453]. The authors, using a modified vaccinia Ankara (MVA) vector as boosting agent, showed an increased T-cell response profile. Moreover, they demonstrated the presence of long-term memory T-cells, associated to high levels of CD4+ and CD8+ cell responses[452,453]. In summary, the above vaccine strategies using viral vectors have great potential. However, formulating a vaccine with two different components may be expensive, and repeated boosting may not be practical in the developing world and for some patient groups.

HCV therapy with Interferon

The very first treatment reported to be effective against HCV infection was based on the systematic administration of interferon (INF) α in combination therapy with ribavirin, a purine-analogue. INFs are protein naturally produced by host's immune system in response to a viral infection. Through the years, HCV treatment has been improved with the development of modified version of interferons, like the pegylation (pegylated-interferon or peg-INF), which confers longer biological half-life[454]. INFs are known to activate several direct and indirect antiviral mechanisms (such as viral RNA degradation, stop viral translation) [455,456]. The goal of HCV therapy is to achieve the sustained virological response (SVR), defined as undetectable HCV RNA (<15 IU/ml) after 24 weeks of treatment[457,458]. The success was highly dependent on several host factors, like gender, age, ethnicity, single nucleotide polymorphisms at the *IL28B* gene locus as well as on viral factors, like viral load and mainly, genotype. In fact, peg-IFNα plus ribavirin eradicate the infection in approximately 80% of patients infected by genotype 2 but the rate drops to approximately 40% in the case of genotype 1 [389,459]. Unfortunately, both these compounds are toxic and their administration can cause severe side effects such as headache, fever, severe depression, myalgia, arthralgia and haemolytic anaemia[460].

HCV therapy and DAAs

Progress in the knowledge of HCV life cycle allowed the development of novel direct anti-HCV agents (DAAs), many of which are still being developed. Initially, in 2011 two NS3-4A protease inhibitors, telaprevir and boceprevir, have been approved for triple therapy, still in association with peg-IFNα and ribavirin, for the treatment of patients chronically infected with genotype 1 [461,462]. Although this therapy greatly improved response against genotype 1, marginal effects have been reported on the other genotypes, and as consequence –

considering the high costs - the standard of care for non-HCV1 genotypes remained based on pegIFNα and ribavirin[459]. Moreover, almost all the genotypes, particularly genotype 3, exhibited an immediate and effective resistance to the new antiviral compounds, highlighting the importance of new drugs[463,464].

Very recently, the second generation of new DAAs has been approved (figure 9). Noteworthy, FDA approved Sofosbuvir in 2013 as a highly active inhibitor of HCV NS5B RNA-dependent RNA polymerase[465]. It is the first drug to be used in combination with Ribavirin for treatment of hepatitis C genotypes 2 and 3 in absence of PEGylated interferon[466]. After Sofosbuvir, a new NS3/4A inhibitor, Simeprevir was approved by FDA in 2013. Interestingly, these new compounds, in combination with Ribavirin showed success rates of around 90% for all viral genotypes[467]. Within the last two years, more NS5A and NS3/4A inhibitors were approved, to treat different genotypes. Specifically, combinations that contain HCV NS5A inhibitors, such as Daclatasvir and Ledipasvir, show even higher success rates of 93 to 100% depending on viral genotype1 [468]. In the end of 2014 a new cocktail was approved called Viekira Pak that includes Ombitasvir, Paritaprevir, Ritonavir and Dasabuvir[469]. Viekira Pak showed very promising results in the treatment of chronic hepatitis C [470]. However, access to directly acting anti-HCV therapies is severely limited since these regimes are very expensive[471,472]; moreover, these drugs did not entered in all the regional market, such as Russia, and the treatment of adults is not covered by national health system. In addition, their clinical usage faces development of drug resistant HCV strains[473,474].

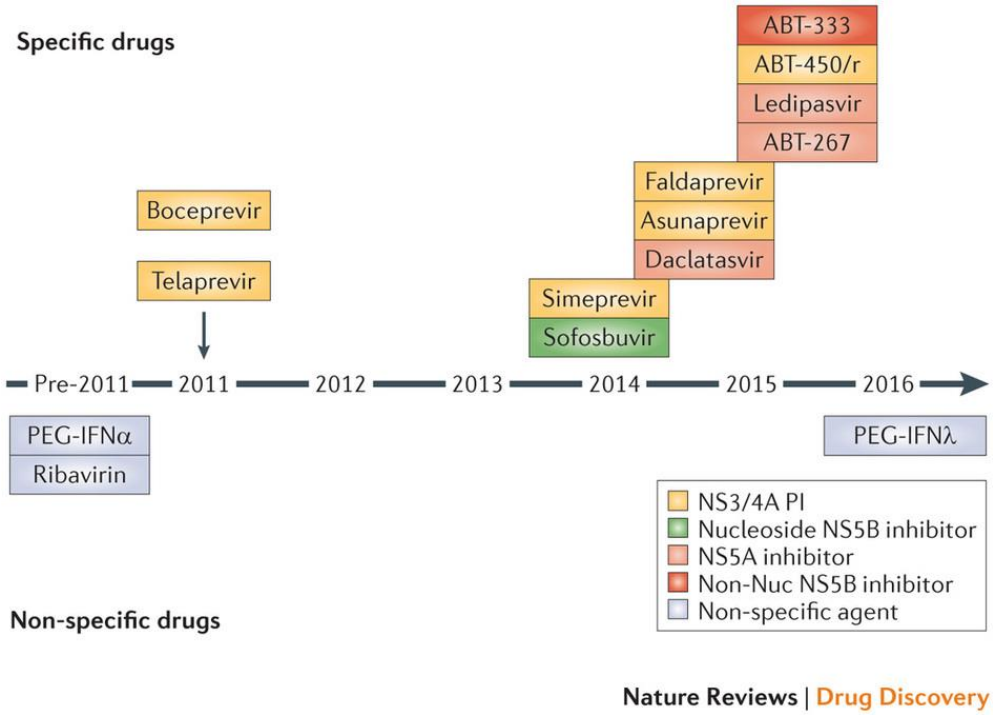


Figure 9: Time-line of HCV therapy[475]. N.B. Peg-IFNλ never reached the market.

For many years the standard therapy has been based on interferon in combination with ribavirin, achieving a sustained virological response (SVR) of approximately 50%. In 2011 the first generation of direct–acting agents has been approved, showing different SVR in relation to viral genotype. In 2013 the second generation of DAAs reached the market, exhibiting SVR in 90% of the cases.

Aim of the study

Hepatitis C Virus (HCV) is a major public health problem worldwide. It has estimated that 170 million people are currently infected. The HCV infection remains persistent in 60-85% of the cases and, as consequence, this can lead to develop advanced liver fibrosis, cirrhosis and, in a small number of cases, hepatocellular carcinoma. Viral strains can be divided into seven epidemiologically relevant genotypes that differ from each other by more than 30% at the nucleotide level. Moreover, 100 or more subtypes have been described and, within an infected individual, a large number of viral variants, called “quasispecies”, have been reported. This high variability of HCV is responsible for immune evasion, facilitates persistence, and represent a big issue in the development of specific antiviral therapies effective across all HCV genotypes. No effective vaccine exists and the therapy based on pegylated IFN-α (pegIFN-α) and ribavirin has been the standard of care for over two decades, showing SVR in approximately 50% of cases. In the last years, new highly efficacious directly-acting antiviral agents have been developed, achieving SVR in approximately 90-100% of the cases. However, their high costs and relative inaccessibility make their use limited. Hence, the development of novel anti-HCV agents to increase effectiveness, shorten treatment periods and widen availability (and affordability) is an urgent task for the modern healthcare. Therefore, the aim of the study presented in this thesis has been to investigate the antiviral effect of new uracil-based antiviral compounds, trying to define their potential mechanism of action. To achieve this goal we tested the inhibition of these molecules on HCVcc evaluating different conditions of viral infection. Furthermore, we focused on each viral stage using different surrogate HCV particles to establish which viral stage is target by our compounds.

To better understand the efficacy and mechanism of these compounds, several issues have been addressed:

- First, we evaluated whether these agents could block HCV infection by itself in Huh7 cells, using JFH1 virus with different models of infection.

- Secondly, we determined for each compound efficacy and tolerability profiles, evaluating several concentrations on infected Huh7 cells.

- We also investigated in which stage of the virus life cycle these drugs exert their action; for this purpose, they were tested on various steps of HCV cell cycle, such as virus cell entry, RNA replication or assembly/secretion; the experiments were carried out using HCVpp, HCV replicons and HCV_{TCP}.

- Since preliminary results showed an inhibitory effect of some drugs on HCV replication, we developed a new assay, based on a specific RT-qPCR, to quantitate the intermediate negative strand RNA.

- We finally evaluated the antiviral activity of these new compounds only on viral replication, trying to dissect where they interfere with HCV normal replication.

We described the antiviral properties of new uracil-based antiviral compounds, showing how they can block viral replication. We attempted to evaluate whether a single candidate might be proposed for future development as a new direct-acting anti-HCV drug.

MATERIAL

AND

METHODS

Materials

Chemicals

Chemical/Reagent	Supplier
Absolute Ethanol	Bamford Laboratories, UK
Agarose	Sigma-Aldrich
Ampicillin (Amp)	Melford
Chloroform	Sigma-Aldrich
Isopropanol	Fisher Scientific
MluI	NEB
Mung Bean nuclease	NEB
Proteinase K	Invitrogen
Restriction Enzymes	NEB
TriReagent	Sigma-Aldrich
Tween-20	Bio-Rad Laboratories
XbaI	NEB

Kits

Kit	Source
Qiagen Plasmid Maxi Kit	Qiagen
RNeasy Plus Mini Kit	Quiagen
Taqman Reverse Transcription Kit	Applied Biosystems
MEGAscript High Yield Transcription Kit	Ambion
Calcium Phosphate Transfection Kit	Sigma-Aldrich

Cells

Cells	Description	Source
Huh-7	Human Hepatoma cell line	A kind gift from Jean Dubuisson (CNRS, Institut de Biologie de Lille, Lille, France)
Huh-7 J20 (J20)	Huh-7 stably transduced with a lentiviral vector encoding for SEAP reporter gene.	Home-made. Iro *et al.* (2009)
Huh-7 J17 (J17)	Huh-7 electroporated with N17 RNA and selected with puromycin.	Home-made. Angus *et al.* (2012)
Huh7-Lunet-CD81	An Huh-7 subclone lacking cellular receptor CD81 expression	Witteveldt *et al*. (2009): a kind gift from Thomas Pietschmann
Huh7L-H/EF	Huh7-Lunet-CD81 cells stably transduced with a vector to overexpress CD81	Witteveldt *et al*. (2009)
A549	Human Lung Carcinoma cell line	American Type Culture Collection
HEK-293T	Human Embryonic Kidney cell line	American Type Culture Collection
MDCK	Canine Epithelial cell line	American Type Culture Collection

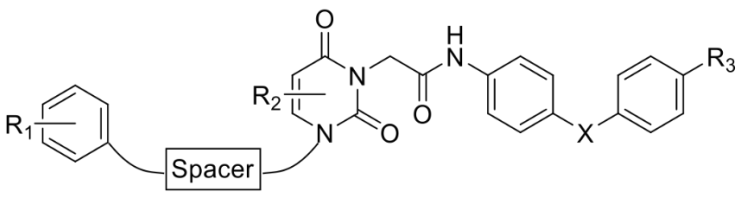
Cell Culture Growth Medium

All cell culture media components were supplied by Life Technologies. Huh-7 cells, Huh7-Lunet-CD81, Huh7L-H/EF, Hek-293T and A549 cells were grown in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mM non-essential amino acids (NEA) and 2 mM glutamine. J20 and J17 cells were propagated as above, but in the presence of 2 µg/ml puromycin (Sigma) [355,476].

Drugs

Compound library was synthesized by Dr. Mikhail Novikov, from Volgograd State Medical University, Volgograd (Russia) and delivered to MRC-University of Glasgow Centre for Virus Research, Glasgow (United Kingdom) for evaluating their activity against HCV. Briefly, compounds were obtained starting from a core molecule, shown below, introducing modifications at X, R1, R2 and R3 sites or modifying the length of the spacer as described previously[477-479]. The panel of compounds generated is listed in the table below.

All drugs were evaluated as potential HCV inhibitors at the concentration of 10 µM, if not indicated elsewhere.



Compound	Spacer	R_1	R_2	R_3	X
Z263	—$O(CH_2)_5$—	4-Br	H	H	O
Z421	—$O(CH_2)_5$—	3-Br	H	H	O
Z434	—$O(CH_2)_5$—	4-CN	H	H	O
Z436	—$O(CH_2)_5$—	H	H	H	O
Z438	—$O(CH_2)_5$—	4-Ph	H	H	O
Z397	—$O(CH_2)_4$—	4-Br	H	H	O
Z400	—$O(CH_2)_6$—	4-Br	H	H	O
Z401	—$O(CH_2)_8$—	4-Br	H	H	O
Z432	—$CH_2O(CH_2)_4$—	4-Br	H	H	O
Z422	—$CH_2O(CH_2)_2OCH_2$—	4-Br	H	H	O
Z433	—$CH_2O(CH_2)_2OCH_2$—	4-Br	5-Et-6-Me	H	O
Z437	—$(CH_2)_6$—	H	H	H	O
Z376	—$O(CH_2)_5$—	4-Br	5-Me	H	O
Z439	—$O(CH_2)_5$—	4-Br	I	H	O
Z385	—$O(CH_2)_5$—	4-Br	H	Cl	O
Z413	—$O(CH_2)_5$—	4-Br	H	Me	O
Z414	—$O(CH_2)_5$—	4-Br	H	F	O
Z377	—$O(CH_2)_5$—	4-Br	H	H	OCH_2
Z387	—$O(CH_2)_5$—	4-Br	H	H	CH_2
Z430	—$O(CH_2)_5$—	4-Br	H	H	C=O
Z431	—$O(CH_2)_5$—	4-Br	H	H	O
Z176	—CH_2—	H	H	H	O

Clones

Name	Virus	Details	Source
pJFH1	HCV	Full-length JFH1 cDNA downstream of the T7 RNA polymerase promoter.	A kind gift from Takaji Wakita[370].
pJFH1$_{GND}$	HCV	As pJFH1, except carries a mutation in the NS5B GDD motif, downstream of the T7 RNA polymerase promoter.	A kind gift from Takaji Wakita[370].
AM7/1	HCV	As pJFH1, except it carries 8 point mutations to improve replication efficiency.	Based on Zhou *et al*. (2014)[480]
pSGR-Luc-JFH1	HCV	Bicistronic replicon encoding Firefly luciferase downstream of the HCV IRES and HCV NS3-3' after ECMV IRES, downstream of the T7 RNA polymerase promoter.	A kind gift from Takaji Wakita[481].
pSGR-Luc-JFH1$_{GND}$	HCV	As pSGR-Luc-JFH1, except carries a mutation in the NS5B GDD motif, downstream of the T7 RNA polymerase promoter.	A kind gift from Takaji Wakita[481].
N17/JFH1$_{\Delta E1E2}$	HCV	Monocistronic replicon, driven by T7 RNA polymerase promoter, encoding Firefly luciferase and puromycin resistance downstream of the HCV IRES, with the deletion of HCV E1 and E2.	Angus *et al.* (2012)[355]
N17/JFH1$_{\Delta E1E2-GND}$	HCV	As N17/JFH1$_{\Delta E1E2}$, except carries a mutation in the NS5B GDD motif, downstream of the T7 RNA polymerase promoter.	Angus *et al.* (2012)[355]
PR8-GFP	Flu	Influenza virus based on a rescued-system expressing green fluorescent protein.	Kind gift from Ben Hale[482].

Antibodies

Name	Description	Source
Anti-NS5A (9E10)	Mouse monoclonal Primary Antibody	Kind gift from Charles Rice[369].
Anti-E2 (AP33)	Mouse monoclonal Primary Antibody	Tarr *et al.* (2006)[349]
Donkey anti-mouse IgG	Alexa Fluor® 488 dye-conjugated secondary Antibody	Invitrogen
Anti-mouse HRP	HRP-conjugated secondary Antibody	Invitrogen

Bacterial Strains

Plasmids were manipulated and growth in the *Escherichia Coli* strain DH5-α.

Solutions

Bacterial Expression

Solution	Components
L-Broth (LB)*	170 mM NaCl, 10 g/l Bactopeptone, 5 g/l yeast extract
LB-agar*	LB plus 1.5 (w/v) agar

* Prepared in-house by media department

DNA manipulation

Solution	Components
DNA loading dye	30% glycerol; 0.25% Bromophenol Blue; 0.25% Xylen Cyanol
TBE (10X)	8.9 M Tris-borate, 8.9 M boric acid, 0.02 M EDTA (pH 8.0)

Tissue Culture

Solution	Components
Trypsin Solution	0.25% Difco trypsin dissolved in PBS, 0.002 (w/v) phenol red (Life Technologies).
Versene	0.6 mM EDTA in PBS, 0.002% (w/v) phenol red (Life Technologies).

Oligonucleotides

Oligonucleotides were ordered from Sigma.

Primer	Sequence (5' → 3')	Position	Product Size
F1	TCACTCCCCTGTGAGGAACT	HCV-5'UTR	82 bp
R1	CCTGGAGGCTGTACGACACT	HCV-5'UTR	
F2	GTCGCCCAGAAGACGTTAAG	HCV-5'UTR	113 bp
R2	CTCCGAAGTTTTCCTTGTCG	HCV-5'UTR	
F3	GCCTTGTGGTACTGCCTGAT	HCV-5'UTR	114 bp
R3	CGGTTGGTGTTTCTTTTGGT	HCV-5'UTR	
F4	ACCACCTATTGCCTCACTGG	HCV-NS2	114 bp
R4	CAACAAACCCACGCCTATCT	HCV-NS2	
F5	AGATCGTTGGCGGAGTATAC	HCV-CORE	299 bp
R5	ACACGTTAGGGTGTCGATGACTT	HCV-CORE	
F6	TCCCGGGAGAGCCATAGTG	HCV-5'UTR	76 bp
R6	TCCAAGAAAGGACCCAGTC	HCV-5'UTR	
RpL_F	GCAATGTTGCCAGTGTCTG	Rplp0-Ex7-8	142 bp
RpL_R	GCCTTGACCTTTTCAGCAA	Rplp0-Ex7	

Methods

DNA Manipulation

Quantitation of DNA

DNA aliquots were quantified by measuring the optical density (OD) absorbance at 260/280 nm using Nanodrop® 2000 (Thermo Scientific).

Nucleotide Sequencing

Nucleotide sequencing of plasmid and amplified DNA was performed using selected primers by GATC biotech (Germany). A minimum of 15 µl of DNA and primers (10 µM) were required for each reaction. Completed sequences were analysed using Chromas Lite (Technelysium) and NCBI alignment software.

Transformation of Competent *E. Coli* Cells

Plasmid was added to 50 µl competent *E. Coli* (NEB) and incubated 30 minutes. For the heat-shock, cells were incubated at 42°C for 90 s and then chilled on ice for other 3 min. The bacteria were resuspended in 500 µl of LB broth and incubated 1 h at 37°C before being plated on LB-agar plates with ampicillin, final concentration 100 µg/ml. Finally, plates were incubated overnight at 37°C.

Large Scale Plasmid Preparation from Transformed Bacteria

A single colony from a streaked selective agar plate was picked and used to inoculate a 3 ml starter culture of LB with Ampicillin (LB-Amp). Following 8 h incubation at 37°C, the starter culture was inoculated in 200 ml of LB-Amp and incubated overnight at 37°C with vigorous shaking (200 rpm). The bacteria were then pelleted by centrifugation at 3000 rpm for 10 min. A large scale DNA extraction was performed using the HiSpeed plasmid Maxi kit (Qiagen) according to the manufacturer's protocol.

Restriction Digestion of Plasmids for *In Vitro* Transcription

JFH-based plasmids were linearised by *XbaI* digestion in a 100 µl reaction for 3h at 37°C, followed by treatment with Mung Bean nuclease to remove the single stranded overhangs (30°C for 30 min). For N17 plasmids a digestion with *MluI* for 3 h at 37°C was performed. Proteinase K (final concentration 100 µg/ml) and SDS (final concentration 0.5%) were added and incubated at 50°C for 30 min to remove proteins. DNA purification was carried out adding 100 µl of neutral phenol-chloroform (25 parts saturated neutral phenol: 24 parts chloroform: 1 part isoamyl alcohol) before centrifugating at 13000 rpm for 2 min. The aqueous layer was placed in a fresh tube and 0.1 volumes 5 M sodium acetate added along with 3 volumes of 100% ethanol. Samples were stored at -20°C for 30 min before being centrifuged at 13000 rpm for 15 min to pellet precipitated DNA. The ethanol was carefully removed and the pellet air-dried at room temperature before being resuspended in 30 µl nuclease-free distilled water (dH_2O). The concentration of linear DNA templates was determined as previously described.

Polymerase Chain Reaction (PCR) Amplification of DNA

The primer pairs, designed for strand-specific assay and listed in the table above, were firstly tested in a PCR on pJFH-1 at the concentration of 500 nM. Samples were amplified in duplicate using FastStart Taq DNA Polymerase (Roche Life Science) in a Veriti Thermal Cycler (Life Technologies) at the conditions expressed in table X. Amplification products were visualised on a 1.5% agarose gel. Subsequently, same primers were tested at the temperature of 65°C to optimise amplification settings.

Stage 1	**Stage 2**			**Stage 3**	**Stage 4**
95°C **5 min**	95°C 30 s	65°C 30 s	72°C 30s	72°C 7 min	**4°C** **hold**
	x 35 cycles				

RNA Manipulation

In Vitro Transcription

In Vitro transcription was carried out using a T7 Megascript kit (Ambion) following the manufacturer's instructions using 1 µg of linear DNA template. Briefly, the reaction mix (containing DNA template, buffer, ribonucleotides and enxyme) was incubated at 37°C overnight and then digested with 1 µl of Turbo DNase at 37°C for 30 min. The RNA was then purified using RNeasy Kit (Qiagen) to remove nucleotides, short oligonucleotides, proteins and salts from the RNA. The RNA concentrations were obtained as described above; typically, the RNA yields obtained were 70-100 µg.

Total RNA extraction

RNA was extracted from cells grown on 12-well dishes using TriReagent (Sigma) according to the manufacturer's protocol. Briefly, cell lysis was performed in 500 µl of TriReagent before adding 100 µl of chloroform. After 10 min of incubation, samples were centrifuged at 13000 rpm for 15 min. The aqueous phase was placed in a new tube and RNA precipitated with 200 µl of isopropyl alcohol before being pelleted by centrifugation at 13000 rpm for 15 min. After a washing step with 70% ethanol, RNA pellets were air-dried and resuspended in 50 µl of nuclease-free double-distilled water (ddH_2O). RNA samples were quantified as previously described and stored at -70°C.

Preparation of Extracellular RNA

RNA extraction from the released viral particles was performed on infected cell supernatants using the RNAEasy kit (Qiagen) in order to minimise RNA loss. Briefly, 300 µl of infected cell medium were digested with RNAse A at 37°C for 2 h to remove all the input RNA prior to RNA purification. All the samples were then eluted in 30 µl of nuclease-free ddH_2O and stored at -70°C.

Electroporation of RNA

Following trypsin treatment and counting, aliquots of 4 x 10^6 cells were centrifuged at 1000 rpm for 5 min at room temperature. Media was discarded and pelleted cells were washed twice by resuspension in 15 ml of PBS, and centrifuged as before. PBS was decanted and cell pellets were resuspended in a total volume of 500 µl PBS and added to a 4 mm gap cuvette (Bio-Rad) along with 10 µg of *in vitro*-transcribed viral RNA. Electroporation was performed using a BioRad GenePulser Xcell (250 V, 950 µF), following the manufacturer's instructions. Cells were then diluted and resuspended in the indicated amount of complete DMEM and seeded into the appropriate tissue culture flash or plate.

Reverse Transcription

First-Strand cDNA Synthesis

Reverse transcription of viral and cellular RNAs was performed using the TaqMan Reverse Transcription Reagents kit. cDNA synthesis from cell pellets or supernatants was performed in the same way. Briefly, for each reaction 250 ng of RNA or 1/3 of the eluted RNA were reverse transcribed using the following reaction mix and temperature cycles:

Component	Volume (µl)
10x RT Buffer	2
$MgCl_2$	2.2
dNTPs	2
Random Hexamers	0.5
RNase Inhibitors	0.2
Multiscribe RT	0.25
ddH_2O	Up to 20

Reaction cycle:

Stage 1	Stage 2	Stage 3	Stage 4
25°C	48°C	95°C	**4°C**
10 min	30 min	5 min	**hold**

Strand-Specific cDNA Synthesis

Total RNA was reverse transcribed with TaqMan Reverse Transcription Reagents (Life Technologies) in a 15 µl reaction using a forward primer for HCV negative strand and a reverse primer for positive strand. To optimise cDNA synthesis, reducing non-specific products, several primer sets were tested; moreover, different concentrations were tested for RT: precisely, 1 µM and 100 nM. Subsequently, RT reaction was performed using different amount of RNA, in order to determine the best quantity for the assay, avoiding background. The quantities tested were 500, 50, 5 and 0.5 ng.

Component	Volume (µl)
10x RT Buffer	1.5
$MgCl_2$	2.2
dNTPs	2
Specific Primer (1 µM)	1.5
RNase Inhibitors	0.2
Multiscribe RT	0.25
ddH_2O	Up to 15

Reaction cycle:

Stage 1	Stage 2	Stage 3
48°C	95°C	**4°C**
30 min	5 min	**hold**

Real-Time PCR

Total HCV quantification

Total viral quantification of RNA from electroporated or infected cells was determined by qPCR. The cDNA obtained from RT reaction with random hexamers was amplified in a 15 µl reaction by a TaqMan assay using 300 nM HCV specific primers (F6 and R6) in the presence of 300 nM FAM™-labelled probe (Life Technologies). All the values were normalised to the selected reference gene: Ribosomal Large Protein 0 (Rplp0). Relative quantifications were carried out using linear regression on plasmid serial dilutions. The reaction mix and the reaction cycle conditions, performed on an Applied Biosystem 7500 Fast Real-Time PCR system, are listed below:

Component	Volume (µl)
2X TaqMan Fast Master Mix	7.5
10 µM Forward Primer	0.45
10 µM Reverse Primer	0.45
5 µM FAM probe	0.9
cDNA	1.5
ddH_2O	Up to 15

Thermal Cycler Protocol:

Stage 1	**Stage 2**	
95°C	95°C	**65°C**
5 min	3 s	**30 s**
	x 40 cycles	

Viral particles quantification

Viral extracellular RNA was analysed by Real Time PCR and the viral amount was determined by absolute quantificative qPCR. Typically, 2 µl of cDNA, obtained from reverse transcription, were amplified using the HCV-specific primer (F6-R6) and FAM probe combination described for Total RNA. The pJFH1 genomic sequence of known concentration was used as a standard to calculate the copy number per µl. Serial dilutions were performed to obtain a complete standard curve.

Quantification of HCV positive/negative strand

Specific-strand quantification was performed by Real Time PCR. Briefly, 1.5 µl of cDNA for each positive and negative strand samples, obtained with specific primers as described above, were amplified in a 15 µl reaction using Fast Sybr Green Master Mix (Life Technologies) with different concentrations of each selected primers; the concentrations tested were: 500, 250, 100, 75 and 50 nM. Absolute quantification was carried out using linear regression on a standard curve based on pJFH-1 serial dilutions.

Component	Volume (µl)
2X Fast Sybr Master Mix	7.5
10 µM Forward Primer	0.075
10 µM Reverse Primer	0.075
cDNA	1.5
ddH_2O	Up to 15

Thermal Cycler Protocol:

Stage 1	**Stage 2**		**Stage 3**
95°C **5 min**	95°C 3 s	65°C 30 s	**Melting** **Curve**
	x 40 cycles		

Tissue Culture Maintenance

Cell Passaging

All the cell types were propagated at 37°C in complete DMEM in an atmosphere of 5% CO_2. Cell lines were typically grown in 80 cm2 or 175 cm2 tissue culture flasks (Nunc). Passage of cells was carried out when cells reached 95% confluence by first gently washing cells in versene followed by their removal with trypsin diluted 1:100 in versene. Cells were resuspended in 10 ml of complete DMEM before re-seeding or use in experiments.

Long Term Storage of cells

Aliquoted cells were stored in complete DMEM medium supplemented with 25% FCS and containing 10% DMSO. Aliquots were left overnight at -70°C before being transferred to liquid nitrogen container for long-term storage.

Measuring cellular viability

Cell viability was measured to evaluate a potential toxic effect of the compounds tested using the WST-1 reagent (Roche, Applied Biosystem). Huh7, Huh7-J20 and Huh7-J17 cells were tested for viability in the same conditions described for antiviral assays. Cells grown in a 96-well tissue culture plate in the presence of the drugs or DMSO control were incubated with the WST-1 reagent for 3 h as per the manufacturer's protocol. Cell viability was obtained reading absorbance at 450 nm with PheraStar (BMG Labtech).

Cell culture of infectious HCV (HCVcc)

Generation of JFH1 Virus and Adaptive Virus

In Vitro synthesized RNA (10 µg) was electroporated into Huh-7 cells. The transfected cells were immediately recovered with fresh medium and seeded into the indicated tissue culture flasks. Following incubation at 37°C for 72 h, the medium containing the infection progeny

was harvested, the cells replenished with fresh medium and incubated overnight before the second collection of viral particles. The viral stock collected thus was filtered through a 0.45 µm pore-sized membrane and stored at 4°C for further experiments.

Measuring Virus Infectivity

Limiting dilution assays were used to quantify the amount of virus infectivity using the focus forming unit (FFU) assay, as described by Zhong *et al.* (2005)[371]. To determine the virus titer by FFU assay, Huh-7 target cells were seeded at the concentration of 4x10^4 per well of a 96-well plate in a total volume of 100 µl complete DMEM. Twenty-four hours later, serial 5-fold dilutions of virus stock were added, with 3 wells per dilution. Seventy-two hours later, the medium was removed and the cells were fixed with ice-cold methanol and incubated at -20°C for 1 h. The cells were then washed three times with PBS-T and probed with anti-NS5A mAb 9E10 (Apath, Rockfeller University) at the dilution of 1:20000 in PBS-T for 1 h at room temperature. Cells were washed again three times with PBS-T and incubated with the anti-mouse FITC-conjugate secondary antibody at 1:500 dilutions in PBS-T for 1 h at room temperature. Finally, after three washes with PBS-T, the cells were overlaid with 100 µl of ddH_2O and visualised under a fluorescent microscope (Nikon Eclipse TS100). The viral titre was calculated as FFU/ml by the average number of NS5A-positive foci detected at the highest dilution.

Drug Screening

The Huh7-J20 reporter cells were seeded into 96-well tissue culture dish at the concentration of 4x10^4 per well of a 96-well plate in a total volume of 100 µl complete DMEM. Twenty-four hours later, cells were infected with HCVcc in the presence or absence (i.e. DMSO control) of compounds and the levels of virus infectivity and replication were determined by measuring the secreted alkaline phosphatase (SEAP) activity in the culture medium at indicated time post-infection as described below. Antiviral screenings were

performed using 3 different infection models. In the first one, cells were pre-treated with drugs for 1 h and then infected in the presence of the drugs. After 3 h, viral inoculum was replaced, and then cells were washed and incubated with fresh medium without drug for 72. In the second model drugs were added at 3 h post-infection and cells incubated for 72 h. The third one is the combination of the other two: fresh drugs were added before, during and after infection to have the compounds always present throughout the course of infection. In all the three models, the antiviral activity was determined by measuring SEAP levels in the infected cell medium using the PhosphaLite kit (Applied Biosystem) as previously described[476]. Typically, 90 µl of culture medium were collected and mixed with 10 µl of 10x lysis buffer to a final concentration of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM NaCl and 0.5% Tritox X-100 to inactivate the virus. To inactivate the endogenous alkaline phosphatase, in a 96-well plate, 30 µl of lysate were mixed 1:1 with 1x dilution buffer and incubated at 65°C for 30 min. The plate was then cooled on ice and before 50 µl of the cooled reaction was mixed with 50 µl assay buffer in a white 96-well microplateplate. Finally, 50 µl of reaction buffer (prepared by diluting CSPD chemiluminescent substrate 1:20 with reaction buffer diluent) were added to this plate before luminescence counting. The SEAP activity was measured by using a Plate Chameleon plate reader (Hidex, Finland). All the RLU values were normalised to DMSO-treated value, which was defined as 100%.

Dose Response Scales

In order to obtain IC_{50} values, 4x10^4 Huh7-J20 cells per well were plated in a 96-well plate and incubated at 37°C overnight. Then, cells were pretreated with drugs for 1 h starting at the concentration of 30 µM and following 3-fold dilutions before being infected with JFH-1 in the presence of drugs in 50 µl of volume. After 3 h of incubation at 37°C, viral inoculum was removed, cells were washed and 110 µl of fresh medium containing drugs were added.

Seventy-two hours post infection cell supernatants were collected and tested for SEAP activity as described above.

For CC_{50} values, same number of Huh7-J20 cells was incubated with serial dilutions of each compound for 72 h before measuring cell viability.

RNA Inhibition

To quantify the inhibitory effect on RNA synthesis, 4x10^^{6} Huh7-Lunet-CD81 or Huh7L-H/EF cells were electroporated with 5 µg of JFH-1 RNA and seeded in the presence of each compound at the concentration of 10 µM. RNA inhibition was also evaluated on AM7/1 adaptive virus, infecting 10^5 Huh7 cells. After 72 h viral RNA quantification was performed from supernatants and on infected cells by RT-qPCR as described above.

Negative Strand Kinetic

Specific-strand viral RNA was evaluated at different time points after transfection: briefly, 4 x 10^6 Huh-7 were electroporated with 10 µg of either JFH1 or $JFH1_{GND}$ RNA and seeded in a 12-well plate for 24, 48 or 72 h. Total RNA was extracted as described at X.x.x.x and negative and positive strand quantification was performed by RT-qPCR.

Negative Strand Inhibition

To evaluate a possible effect on negative strand synthesis, 4 x 10^6 either Huh7-Lunet-CD81 or Huh7L-H/EF cells were electroporated with 5 µg of JFH1 RNA as described previously. Transfected cells were recovered in fresh medium, plated in a 12-well plate in the presence of each drug at the concentration of 10 µM and incubated for 72 h before RNA extraction. Moreover, specific strand RNA was investigated also in Huh7 cells infected with the adaptive virus AM7/1. Negative and positive RNA quantification was performed by RT-qPCR as described above.

HCV pseudo-typed particles (HCVpp)

Generation of Pseudo-typed particles

HCVpp were generated as descried previously[178,483,484] in HEK-293T cells. Typically, 1,4 x 10^6 cells were seeded into 100 mm tissue culture dishes 24 h before transfection. After 24 h cells were then co-transfected with the retrovirus packaging vector pMLV gag-pol (8 µg), the transfer vector pMLV-Luciferase (8 µg) and the HCV JFH-1 E1E2-expressing vector phCMV E1E2 (3 µg) as described previously[178] using the Calcium Phosphate Transfection Kit (Sigma-Aldrich). Briefly, HEK-293T cells were seeded into 100 mm tissue culture dishes in 15 ml complete DMEM 24 h before transfection. For transfection, plasmid DNA was mixed with ddH_2O and 100 µl 2.5 M $CaCl_2$ in a total volume of 500 µl in a sterile 1.5 ml tube. In a second tube, 500 µl of 2X HEPES-Buffered Saline (HeBS) pH 7.05 were added. To form the precipitate, the HeBS solution containing sodium phosphate was slowly mixed with the calcium solution containing DNA. For this procedure, the HeBS was gently bubbled using an automatic pipette pump, during which time the $CaCl_2$/DNA solution was added dropwise. The mix was incubated for 20 min at room temperature to allow the formation of salts/DNA complexes and then distributed dropwise over the cells, followed by gentle mixing by agitation. The DNA/salts co-precipitates adhere to the cell surface and are then taken up by the cells. At 24 h post-transfection, medium was replaced with 8 ml of fresh DMEM. Culture media, containing HCVpp, were collected 72 h post transfection and filtered through a 0.45 µm pore-sized filter. To obtain the vesicular stomatitis virus (VSV) and influenza virus (FLU) pseudoparticles, the same protocol was performed as above in HEK-293T cells, co-transfecting with plasmid encoding the VSV-G or influenza haemagglutinin glycoproteins (3 µg) together with the pMLV gag-pol and pMLV-Luc.

Measuring Viral Entry Inhibition by Luciferase Assay

To evaluate the inhibitory effect on HCV entry, 4 x 10^3 Huh7 cells were plated in a 96-well plate and incubated overnight at 37°C. The following day the medium was removed and cells were incubated with 50 µl of 10 µM of each compound for 1 h. Cells were then infected with HCVpp in the volume of 40 µl in the presence of the drugs for 3 h. Finally, the inoculum was removed, cells washed and 150 µl of fresh medium added. After 72 h post-infection, luciferase assay was performed. Typically, the cell medium was removed and cell lysed in 50 µl of Lysis Buffer (Promega) for 10 min. The lysates were then moved into a white 96-well plate, 50 µl of Bright-Glo reagent (Promega) were added and luciferase activity determined by measuring the Relative Light Unit (RLU) using the Chameleon multiwall plate reader. For VSV and Flu pseudoparticles experiments, the same protocol was executes, except for using virus-specific particles, obtained previously.

HCV replicons

Testing drugs on JFH1-luc replicon

Initially, to investigate a possible antiviral activity at the replication stage, Huh7 cells were electroporated with 10 µg of JFH-luc RNA, seeded in a 96-well plate in the presence of each compound at the concentration of 10 µM and incubated at 37°C for 24 h. After 1 d, cells were lysed and RLU readings taken using a Plate Chameleon plate reader (Hidex, Finland) as described above. All the RLU values were normalised to DMSO-treated value, which was defined as 100%.

Generation of HCV replicon cell line

To generate a stable replicon cell line, persistently harbouring HCV RNA, 4x10^6 Huh7 cells were electroporated with 10 µg of N17/JFH1 and plated in a 100 mm cell-culture dish. After 48 h cells were fed with medium containing 3 µg/ml of puromycin to select positively-

transfected cells. After 1 week, the puromycin-resistance cells were trypsinized, pooled and seeded in a 75 cm^2 cell-culture flask. These cells, named Huh7-J17 or simply J17, were then passaged twice in the presence of 2 µg/ml of puromycin and then stored and used for further experiments.

Antiviral effects on J17 Replicon Cell line

All the compounds were tested as replicon inhibitors on J17 cells. Briefly, $5x10^3$ J17 cells were plated in 96-well plate and treated with each drug at the concentration of 10 µM. RLU readings were taken after 24, 48 and 72 h as described above.

Drug Resistant Mutants

In order to determine whether these compounds could trigger a spontaneous onset of resistant mutations, J17 cells were seeded in a 12-well plate in the presence of each compound at the concentration of 3 µM and cultured for 21 days. Typically, every 3 to 4 days cells were trypsinized, counted and $2x10^4$ cells were lysed to measure luciferase, while $8x10^4$ cells were seeded in a 12-well plate to maintain the cell cultures.

Inhibition of HCV IRES-mediated translation

To test an antiviral effect on HCV translation, $4x10^6$ Huh7 cells were electroporated with SGR-Luc-JFH1$_{GND}$ RNA, able to efficiently translate viral RNA but not to replicate it; then, transfected cells were seeded in a 96-well plate in presence of each compound and incubated for 2, 4 and 8 h. At each time point, cells were lysed and luciferase measured as previously described.

Evaluating an early antiviral effect

$4x10^6$ Huh7 cells were electroporated with subgenomic SGR-Luc-JFH1 RNA and seeded in a 96-well plate in the presence of each compound for a small amount of time, spanning from 1 to 5 hours. Afterwards, cells were washed, re-incubated with fresh medium and incubated

for 24 or 48h. At the appropriate time point, cells were lysed and luciferase measured as described above.

HCV trans-complemented particles (HCV_{TCP})

Generation of HCV_{TCP}

Trans-complemented particles consisted of viral particles containing the replicon N17/JFH RNA as transgene, HCV Core protein that forms capsid and VSV-G as glycoprotein expressed on the envelope. Briefly, $2x10^6$ J17 cells, harbouring N17 sub-genomic RNA, were transfected with 10 µg of phCMV-VSVg DNA using Viafect (promega) and incubated for 3 d. Supernatant-containing particles was then harvested and filtered through 0,45 µm filter. HCV_{TCP} were concentrated using Peg-IT (Biosciences) by overnight incubation at 4°C and then centrifuged at 1500xg for 30 min. The pellet containing particles was resuspended in PBS, titrated, aliquoted and stored at -70°C.

Titration of HCV_{TCP}

HCV trans-complemented particles were titrated using FFU assay with 1:5 limiting dilutions. Briefly, Huh7 cells were infected with TCP-containing medium following serial dilution. After 3 h, cells were washed and re-fed with fresh medium. After 3 days, cells were fixed with ice-cold methanol and then were stained using an NS5A antibody as described above for JFH1. The particle titre was calculated as average of FFU number per millilitre.

Measuring viral inhibition on HCV_{TCP}

$4x10^3$ Huh7 cells, plated in a 96-well tissue culture plate, were pretreated for 1 h with the 4 selected compounds and DMSO and then infected with HCV_{TCP} in presence of drugs. After 3 hours, particle inoculum was removed and replaced with fresh medium containing fresh drugs. After 3 days, cells were lysed and RLU readings taken.

Evaluation on Influenza Virus

Propagation of influenza virus (Flu) strain PR8/GFP

A 75 cm^2 tissue culture flask containing approximately $1x10^7$ MDCK cells, corresponding at 80-90% confluence, was infected with PR8/GFP Flu virus (kindly provided by Dr. Ben Hale) at a MOI of 0.001 and incubated at 37°C. After 1 h the inoculum was removed, cells were washed three times with PBS and finally re-fed with complete medium. After 48-72 h, when approximately 10% was left, the particle-containing medium was harvested, filtered and stored at -70°C and titrate by plaque assay as described[485].

Testing antiviral activity on Flu virus

$2x10^4$ A549 cells were seeded in a 96-well plate the day prior to infection. The next day, cells were pre-treated with decreasing concentration of Z401, as stronger candidate for viral inhibition, or DMSO as reported for HCV. After 1 h cells were infected with PR8/GFP virus and incubated for 1 h at 37°C. Finally, the inoculum was removed and cells re-fed with fresh medium containing drug. After 24 and 48 h viral inhibition was evaluated by fluorescence measurement using PheraStar (BMG Labtech).

Statistical Analysis

All experiments were conducted at least in duplicate and repeated at least 3 times. Multiple-group comparison was performed by one-way analysis of variance. Data are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism 6 software. Statistical significance was defined as $P<0.05$.

RESULTS

Antiviral effect on HCVcc

To assess the effect of these compounds on HCV infection, we used three different protocols: Protocol 1 where Huh7-J20^{476} cells were exposed to drugs or equivalent DMSO (as a vehicle control) before and during HCV infection. The cells were then washed and re-fed with fresh medium (without drug) for 72 h. Thus, this model allows investigating a possible effect on HCV entry. In the second protocol Huh7-J20 cells were infected with HCVcc and then the inoculum was replaced with fresh medium containing the drug or DMSO to determine if the effect of the drug is exerted post-viral entry (e.g. RNA replication and/or virus assembly). In the third protocol, Huh7-J20 cells were pre-treated, infected and exposed in the presence of the compounds for all the time of experiment; this model is commonly used to test effect of compounds on full viral life cycle. Cell viability assays were performed in parallel. Several compounds showed a good inhibitory effect on virus entry, particularly the compound Z401 which inhibited virus infection by 80% relative to the DMSO control (Fig. 7A). Most compounds, however, showed a strong antiviral activity on both the post-entry or full life cycle model, with inhibitory values up to 90% for many of them, with no or little adverse effect on cell viability except compound Z431 (Fig. 7B). Together, these data suggest that most of the compounds affect virus genome replication.

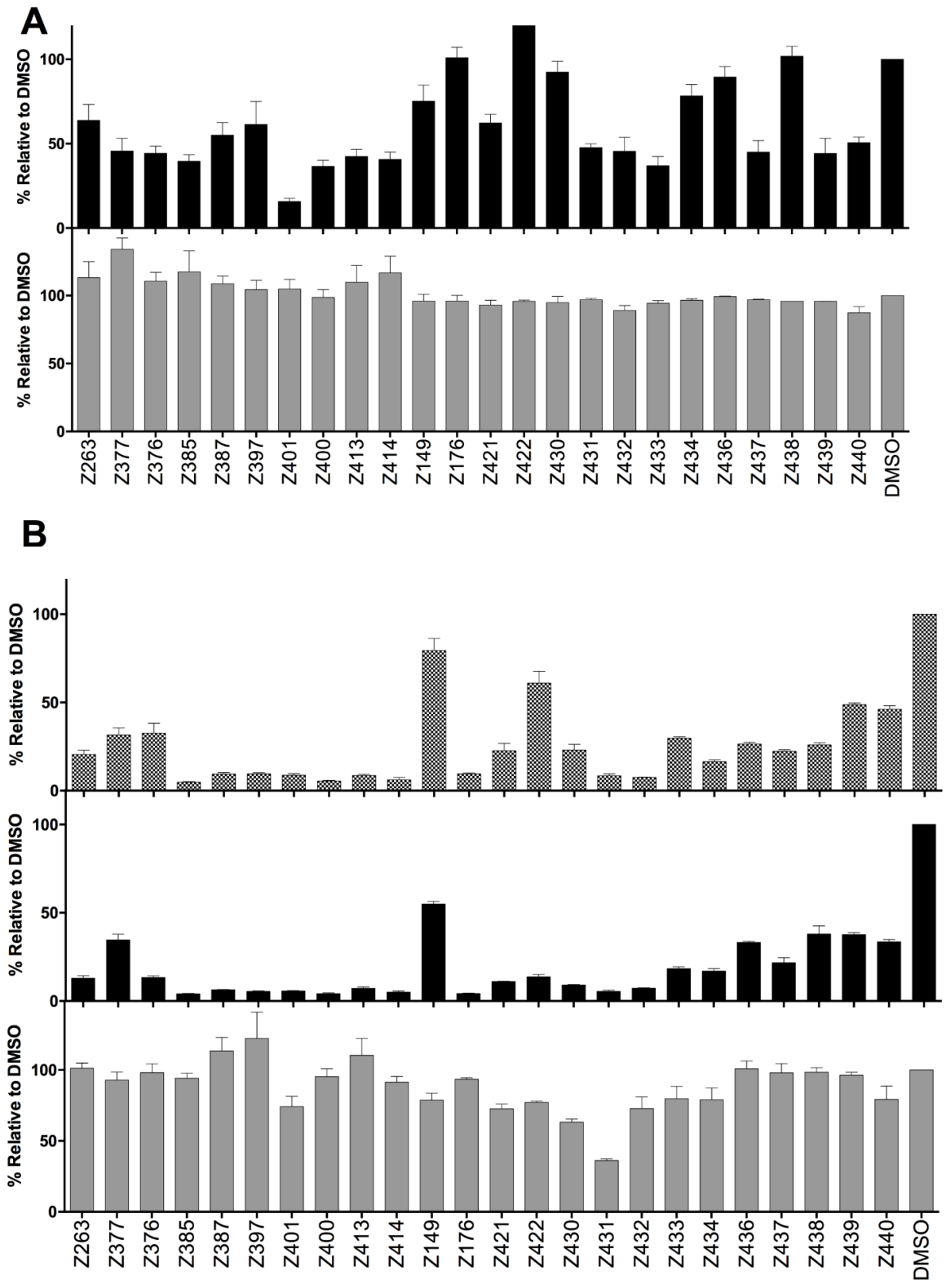


Figure 10. Antiviral properties of new antiviral compounds on HCVcc. Huh7-J20 cells infected and exposed to drugs according to different models: cells were pretreated and infected in the presence of compounds (A), treated after infection or during all time (B); viability was also measured (grey bars).

Inhibition of HCV entry with HCVpp

To further test the effect on HCV entry, we used the surrogate retrovirus-based pseudoparticle (HCVpp) model. HCVpp consist of retroviral particles, expressing HCV glycoproteins E1 and E2 on their surface and with firefly luciferase as transgene. The compounds were tested following the protocol 1 described above for HCVcc. The results showed that just 2 drugs were able to inhibit HCVpp by more than 50%; Z431 strongly blocked HCV entry (84% inhibition), while Z432 showed a moderate effect (62%) (Fig. 10). While Z401 was a potent inhibitor of HCVcc entry (80% inhibition) (Fig. 10A), it had a moderate effect on HCVpp infection (50%) (Fig. 11).

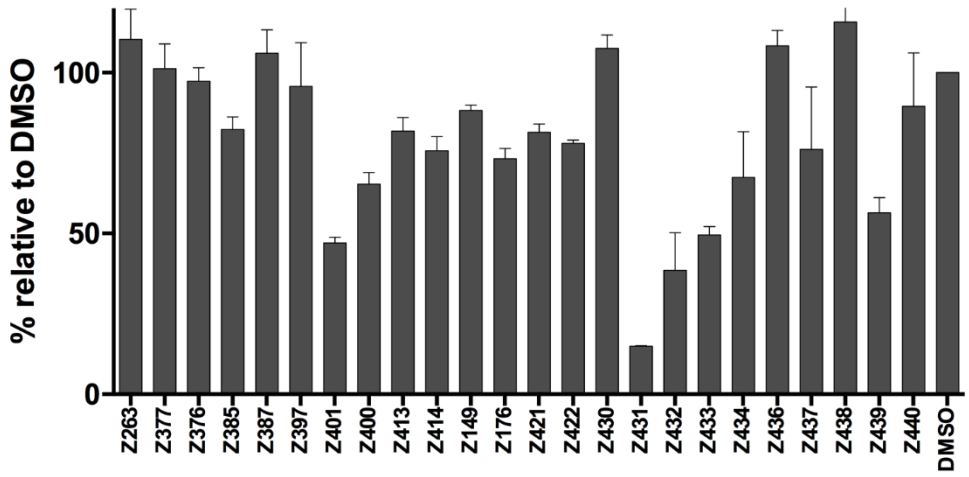


Figure 11. Inhibitory effect on cell entry. Huh7 cells were pre-treated for 1 h and then infected in the presence of compounds. Luciferase signal was measured after 72h.

Entry inhibition of different virus using pseudo-particles

Based on the previous results, we evaluated the specificity of Z431. For this purpose, this compound was evaluated on pseudo-particles of different viruses. Specifically, it was tested as entry inhibitor of Vesicular Stomatitis Virus (VSV) and Influenza virus. Interestingly, as

shown in fig. 12, no effects were detected on VSV of Flu virus (inhibitory effect <50%), while a significant reduction was seen in HCV (80%).

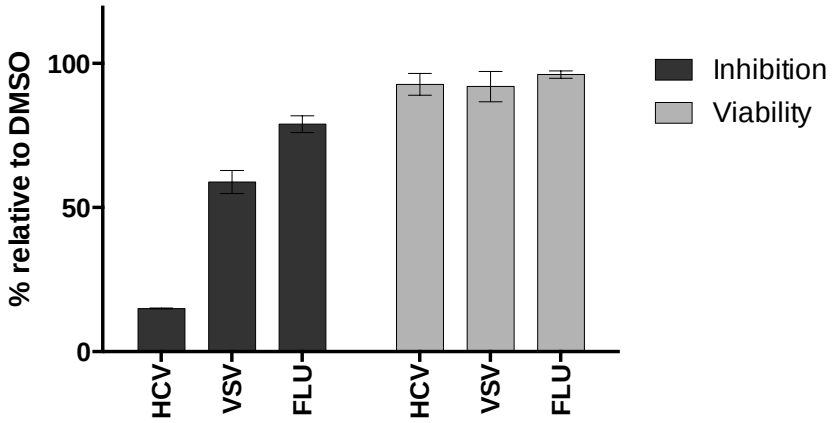


Figure 12. Z431 was tested to evaluate its viral-specificity; for this purpose, its antiviral effect was determined infecting target cells, exposed to Z431, with pseudoparticles obtained from HCV, influenza or VSV. Results were collected 72 h post infection.

Inhibitory effect on HCV replication

Transient Replicon

Our results above indicate that most of our compounds target virus replication. To confirm this, all the drugs were tested for their ability to inhibit replication of a transient sub-genomic replicon. Huh7 cells, electroporated with JFH-luc replicon RNA, were seeded and incubated for 24 h in the presence of the drugs before measuring luciferase signal. Interestingly, almost all the compounds showed a good inhibition of viral RNA replication, with some compounds being able to block up to 95% of replication (Fig. 13). These data confirm the hypothesis that these compounds are able to inhibit HCV replication as previously observed in HCVcc experiments (Fig. 10B). In this model, the cell viabilities were up to 10% less compared to those in previous experiments, possibly due to an increase in drug uptake resulting from electroporation.

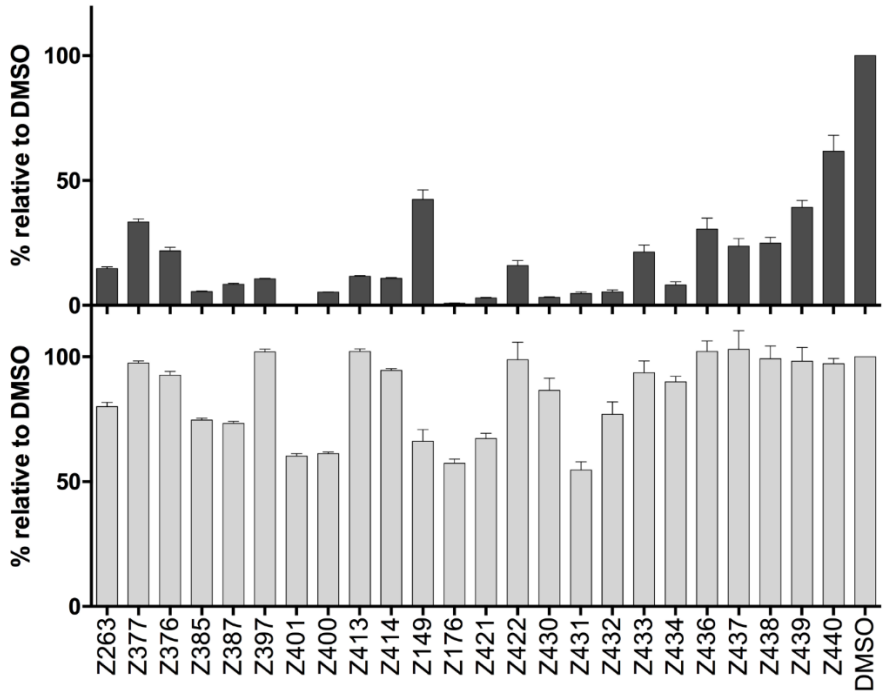


Figure 13. Inhibitory effect on the HCV replicon. Huh7 cells were electroporated with SGR-JFH-luc, seeded and immediately exposed to drugs. After 24 h luciferase readings were taken.

Generation of a replicon cell line

Huh7 cells, electroporated with N17/JFH1 RNA, were plated and selected with puromycin. After 1 week all the clones were pooled together and maintained in presence of puromycin. RNA replication was monitored by RLU readings, confirming stable replication levels (10^6 RLU/sec) for up to 60 days. Moreover, that result was confirmed by RT-qPCR at two different time points, day 17 and 59, showing a constant level of HCV RNA (Fig. 14).

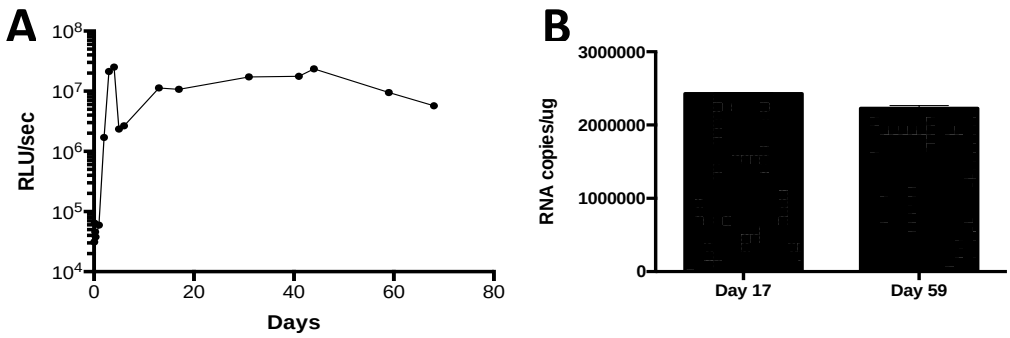


Figure 14. Electroporated Huh7 cells were cultured in the presence of puromycin to generate a stable replicon cell line (Huh7J17). The persistence of viral replication was confirmed by RLU measurement (A) and RNA quantitation by RT-qPCR (B).

Replicon cell line inhibition

The inhibitory effect above, observed on Huh7 cells transiently transfected with the HCV sub-genomic replicon, was then confirmed using the above generated replicon cell line Huh7-J17. Cells were plated in the presence of the drugs and incubated for 24, 48 or 72 h. Results confirmed the previous observation, with a good number of drugs showing high inhibition of the viral replicon RNA, up to 80%, in a time-dependent fashion (Fig. 15). Interestingly, after 24 h almost all the compounds showed an antiviral effect of approximately 50%, while an enhanced effect was seen after 48 h, that remained constant after 72 h. Noteworthy, 9 drugs (Z385, Z387, Z401, Z400, Z176, Z421, Z430, Z431, Z432) showed a strong inhibition, up to 95% compared to DMSO-treated cells at 72 h after seeding. Interestingly, all the compounds induced toxicity effects similar to those observed on electroporated cells.

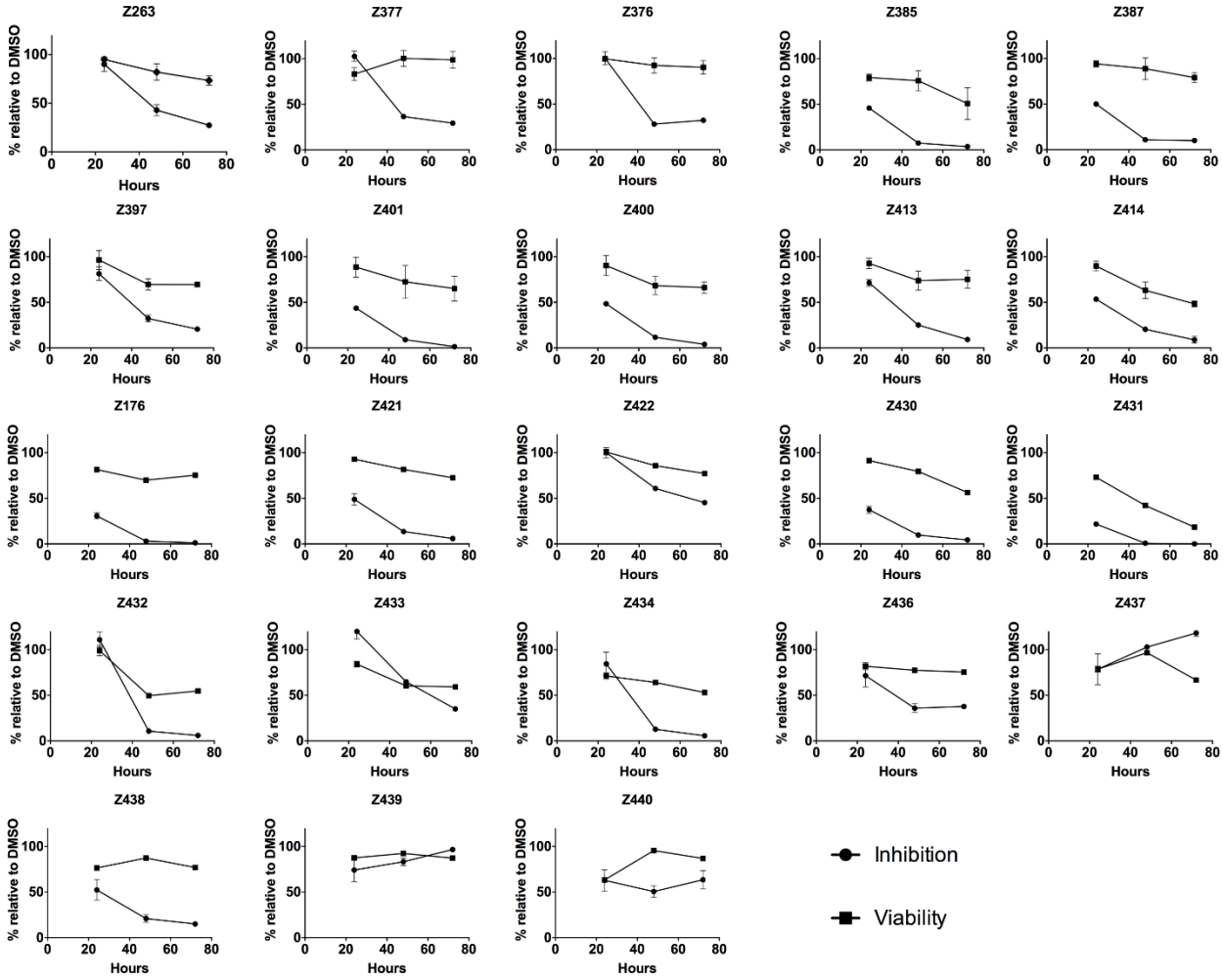


Figure 15. Huh7-J17 cells were exposed to drugs for different time points. For each drug, inhibition and viability were measured after 24, 48 and 72 h.

Determining IC_{50} and CC_{50} values

We next obtained dose-response profiles for each compound to determine their IC50 and CC_{50} values on HCVcc. These experiments were conducted testing all the compounds with concentrations starting at 30 µM and decreasing with 3-fold dilutions. Huh7-J20 cells were treated and infected following the second protocol described above, to investigate effects on all the virus life cycle. IC_{50} and CC_{50} values were determined on SEAP results, collected 3 d post-infection as described above, using a non-linear regression function on normalised samples (Fig. 16). Interestingly, all the compounds showed good IC50 values, in the micromolar range (<5 µM). It is important to highlight that some drugs showed values in the nM scale (9 compounds, Table I). It is also noteworthy that the CC50 values remained approximately at 10 µM for all compounds. We then calculated Selectivity Indexes, which represents the ratio CC_{50}/IC_{50}. Considering a SI cut-off of 3, almost all the compounds passed this limit, with many of them showing extremely higher values. Notably, 4 compound (Z385, Z387, Z401, Z400) exhibited SI values higher than 100, and 2 of them (Z401, Z400) even higher than 200.

Compound	IC_{50}, µM	CC_{50}, µM	SI
Z263	3.1	56.3	18.174
Z421	1.16	44.6	38,548
Z434	1.85	10.5	5,661
Z436	3.62	13.0	3,595
Z438	1.74	9.60	5,534
Z397	2.95	61.1	20,715
Z400	0.243	66.5	273,569
Z401	0.0924	22.6	244,238
Z432	1.29	19.2	14,942
Z422	20	24.9	1,247
Z433	0.991	23.7	23,917
Z437	2.93	14.7	5,029
Z376	2,18	69.2	31.761
Z439	3.72	29.7	7,977
Z385	0.585	59.2	101,145
Z413	0.315	27.4	87,166
Z414	0.627	30.5	48,612
Z377	2.98	18.4	6.196
Z387	0.712	132	185,482
Z430	0.941	11.5	12,173
Z431	0.5	2.67	5,348
Z176	1.34	17.6	13,174

Table I. IC_{50}, CC_{50} and Selectivity Index values calculated for each compound using HCVcc.

The 4 compounds (Z385, Z387, Z400 and Z401) with SI >100, showed interesting inhibition and viability profiles (Fig. 16) and for these reasons were selected for further evaluation as inhibitors of virus RNA replication.

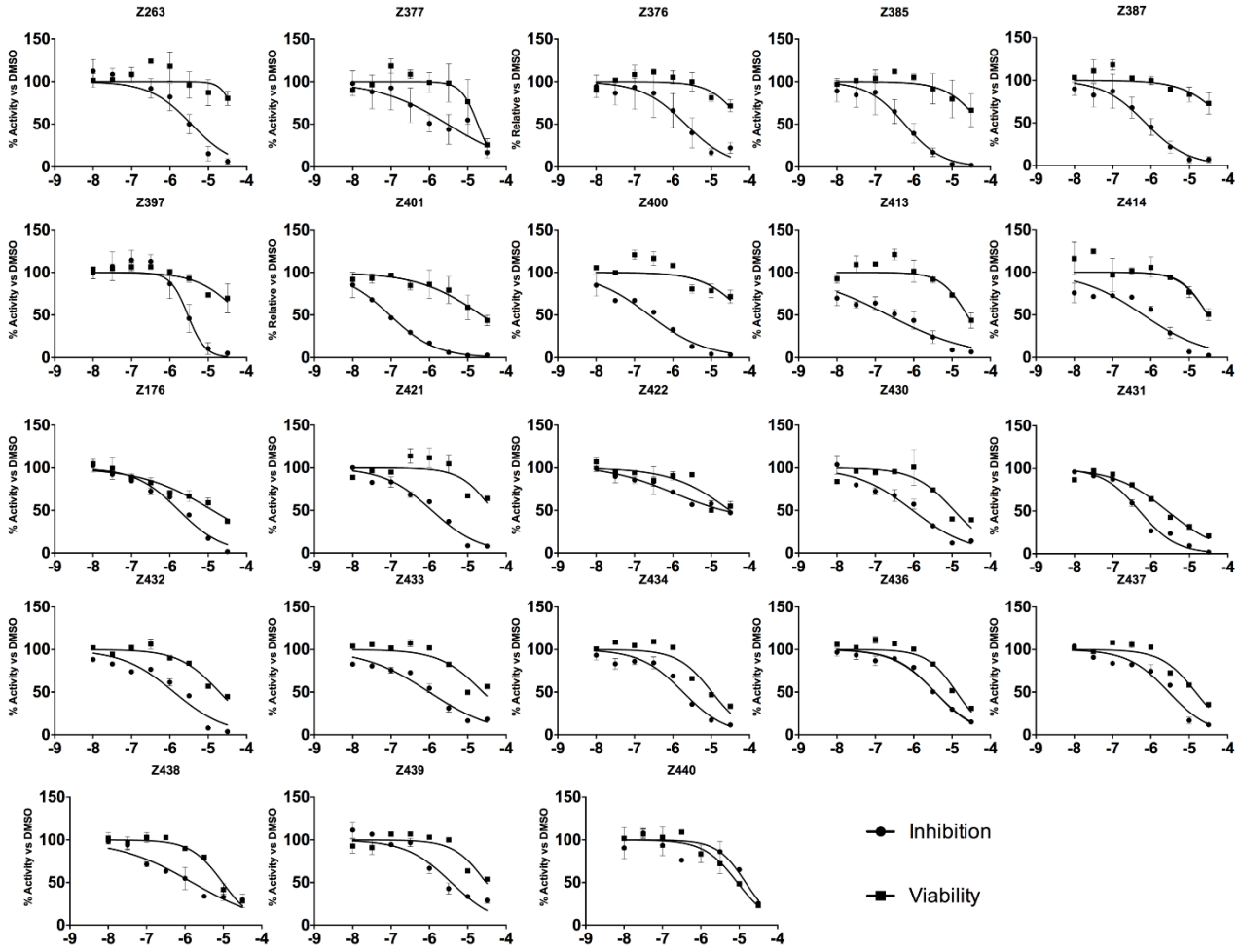


Figure 16. Huh7-J20 cells were infected with JFH1 virus to determine IC_{50} and CC_{50} values performing dose-response scales. Cells were infected according to model 3 previously described and exposed to compounds at the concentration of 30 µM following 3-fold dilutions. Viability and inhibition profiles were determined as described above.

Evaluation of drug resistance mutations

To assess whether our compounds could trigger viral escape, they were evaluated in a long period on the replicon cell line using a concentration of 3 µM, in order to reduce the cytotoxic effect. Huh7-J17 cells were treated with Z385, Z387, Z400, Z401 or DMSO and cultured for 20 days. Replication levels were measured at different time point on the same number of cells. Noteworthy, all the compounds were able to inhibit HCV replication. Specifically, Z385, Z387 and Z400 showed a significant level of inhibition, of approximately 70-80%. Interestingly, Z401 was able to massively block RNA replication, with a decrease in luciferase signal up to 90% (Fig. 17). Moreover, the antiviral activity was evident through the entire period of the experiment, maintaining constant levels of antiviral inhibition. Noteworthy, no rebounds were observed for all the compounds.

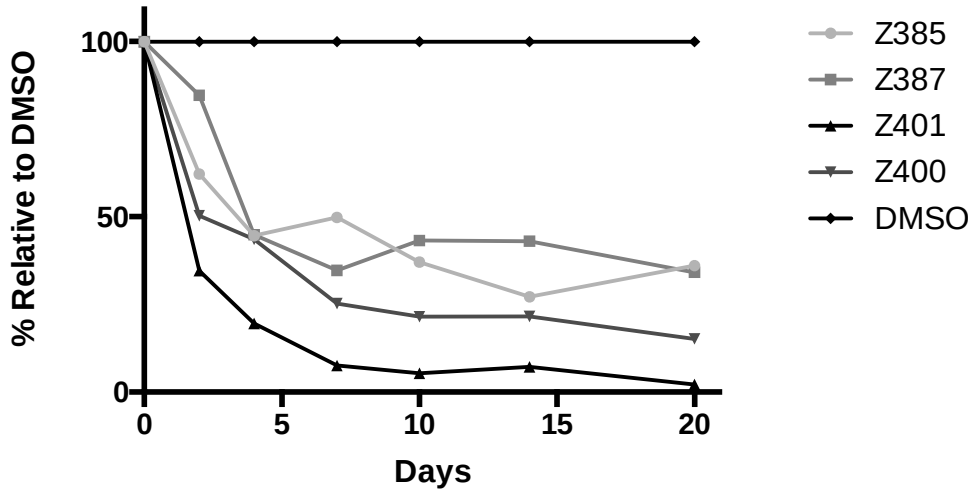


Figure 17. Huh7-J17 cells were exposed to drugs for 20 days to investigate the potential development of drug-resistance mutations. Every 2/3 days cells were lysed and RLU measured.

RNA inhibition on HCVcc

To further confirm antiviral activity, we directly evaluated RNA inhibition, testing Z385, Z387, Z400, and Z401 on a cell culture adaptive virus, called AM7/1 that contains 8 point mutations into HCV JFH-1 genome and grows to high titres (unpublished), showing a consistent replication to high titres. Huh7 cells were infected with HCVcc AM7/1 in the presence of drugs as per Protocol 2 described above and RNA was collected 72 h post-infection. Total RNA was quantified as described above. As shown in Fig. 15, compounds Z385, Z387, Z400 and Z401 reduced the total viral RNA levels over 95%. Similar results were obtained with the parent HCVcc JFH1 strain where a concurrent decrease in virus replication levels was seen in our reporter cell line, showing a significant correlation (Fig. 18).

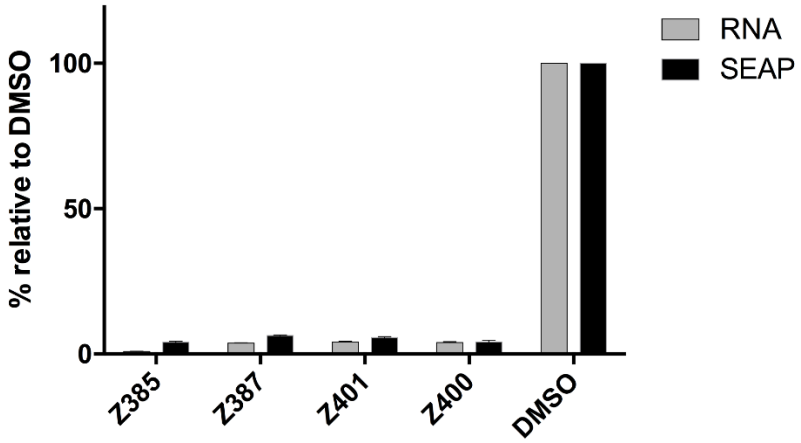


Figure 18. Huh7-J20 cells were infected with AM7/1 adaptive virus according to model 3 described above. RNA inhibition and SEAP reported gene were evaluated 3 days post infection.

Analysis of single-cycle infection on HCVcc

For the next experiments, we chose a cell line called Huh7-Lunet CD81 to mitigate the possible effect of secondary infection on our results. Huh7-Lunet-CD81 cells do not express the cellular receptor CD81, a host factor essential for virus entry but not for cell-to-cell spread[486]. While these cells remain competent for viral RNA replication, assembly and release upon direct introduction of its RNA genome into the cells, they are refractory to virus entry and spread of infection. As such, these cells can be used to investigate the inhibitory effect of antiviral compounds in a single-cycle infection assay. As control, we used Huh7L-H/EF, a Huh7 Lunet CD81-derived cell line over-expressing human CD81[486]. Both cell lines were electroporated with HCVcc JFH-1 RNA, seeded in the presence of the drugs and incubated for 72 h before measuring intracellular RNA levels, and those associated with the viral progeny secreted into the medium of electroporated cells. The latter was performed following digestion with RNAse A to remove any residual untransfected viral RNA. We found that compounds Z385, Z387, Z400 and Z401 exhibited strong inhibition of virus replication. The levels observed on CD81 over-expressing Huh7L-H/EF cells were up to 80-95%, significantly more profound than CD81 negative cells, which showed a mild effect comprised between 50-80% (Fig. 19A); this difference is probably due to an effect on secondary infection, resulting in an increased antiviral effect. In parallel, we also measured the progeny virus release by quantifying viral RNA into the medium, observing a significant reduction for all the compounds in both cell lines, between 70 and 95% (Fig. 19B). In keeping with the results obtained in Fig. 15A, there was a drastic reduction in virus progeny levels in the medium of cells infected in the presence of compounds Z385, Z387, Z400 and Z401 (Fig. 19B). Collectively, these results indicate that compounds Z385, Z387, Z400 and Z401 affect virus RNA replication.

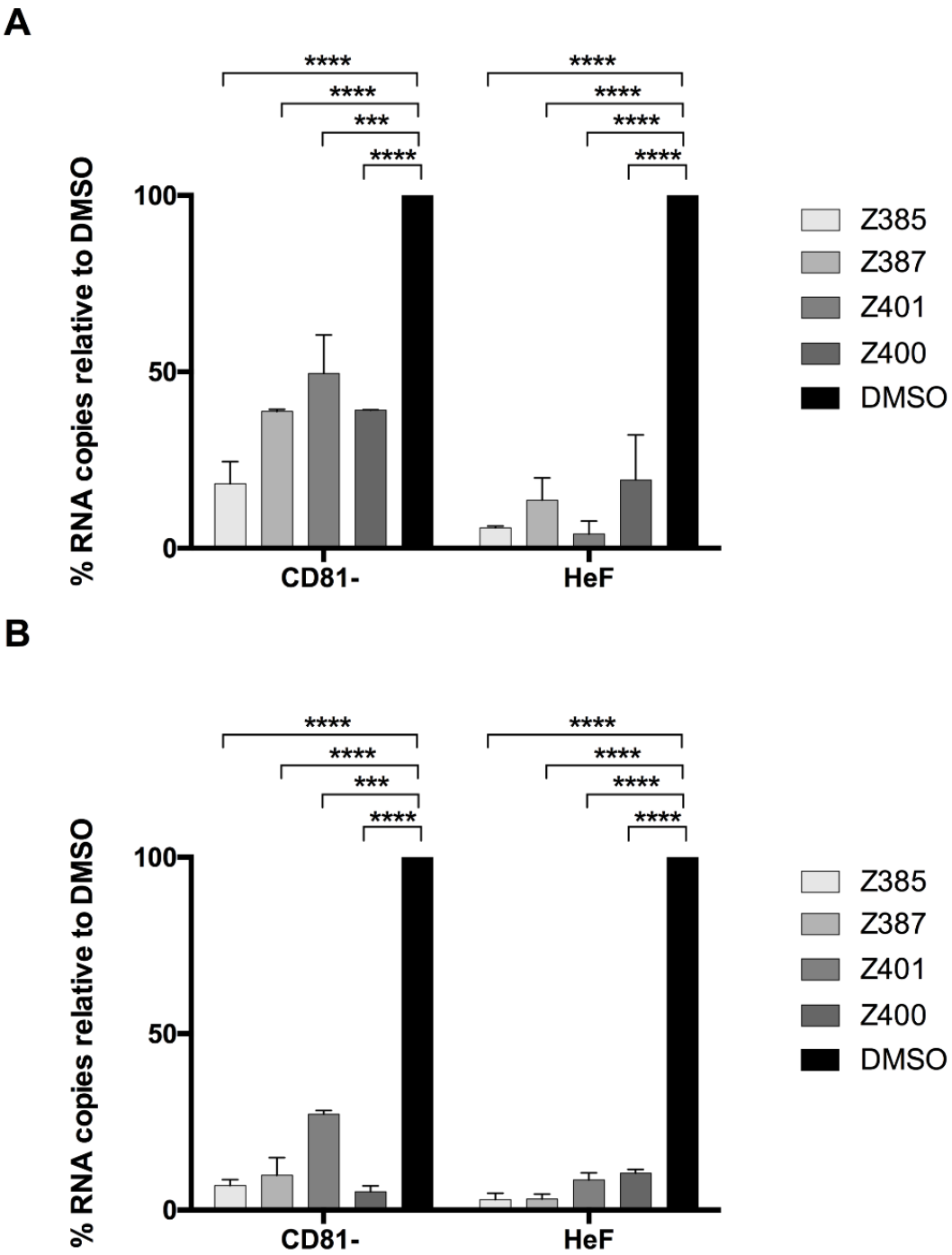


Figure 19. CD81 negative cells and the control HeF cells were electroporated with HCVcc and treated with drugs. After 3 days, inhibition in intracellular RNA (A) and release particles (B) was measured by RT-qPCR. .

Single-cycle infection inhibition on HCV_{TCP}

To further validate our findings, we tested these 4 compounds using trans-complemented pseudo-typed HCV replicon particles (TCP). Based on a system described previously[487], we generated TCP, encapsidating our N17/JFH1 subgenomic replicon[355], in the medium of Huh7-J17 replicon cells that had been transfected with a plasmid construct expressing the VSV glycoprotein protein (VSV-g). The TCPs produced thus are capable of infecting and delivering replication-competent N17 replicon RNA into naïve Huh7 cells without generating progeny virus. Thus, TCPs represent a good model to investigate single-cycle infection allowing analyses of antiviral compounds in the absence of secondary infection or defective cell lines. As shown in Fig. 20, all 4 compounds efficiently inhibited the replication of the N17 replicon in cells infected with the VSV-G pseudotyped TCPs, with an antiviral activity up to 85-95%.

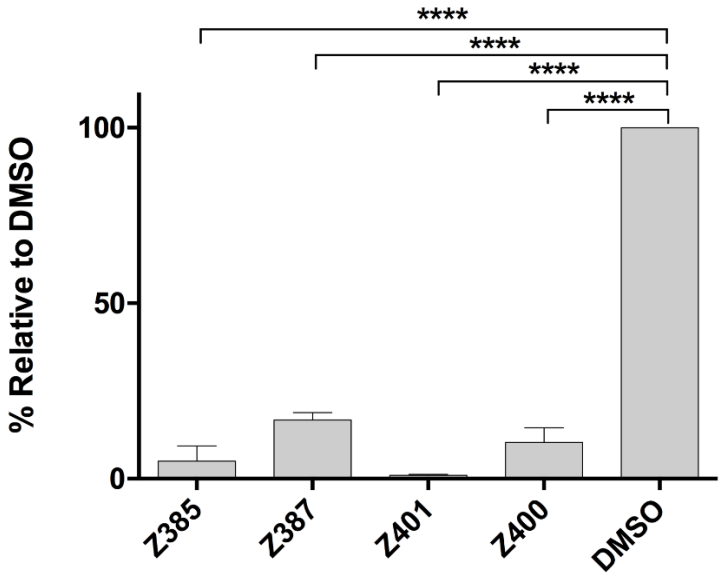


Figure 20. Inhibitory effect on single cycle infection. Huh7 cells were infected with HCV_{TCP} and exposed to compounds for 3 days. Antiviral effect was evaluated measuring luciferase signal.

Effect on an early step of viral replication

Analysing our data presented in Figs. 10B, 13 and 17, we reasoned that these compounds may be acting at an early stage of viral replication. Compound Z401, in particular, showed a partial effect (Fig. 10A) that could be explained by a rapid inhibition of viral replication. To explore this idea, we tested the ability of 4 selected compounds (Z385, Z387, Z400 and Z401) to inhibit viral replication under a slightly modified condition in which cells were treated only during the first hours after RNA transfection. Briefly, immediately after electroporation with SGR-JFH-luc RNA Huh7 cells were treated with drugs for a time ranging from 1 to 5 h. The cells were then washed, and incubated in fresh medium in the absence of drugs 24 or 48 h. The results at 24 h post transfection, shown in Fig. 21A, confirmed a significant block of approximately 50% to 60% for Z385, Z387 and Z400 and 80% for Z401. However, data obtained 48 h post transfection showed a massive rebound of viral replication, with no effects observed for all the compounds except a small inhibition in 5 h treatment; only Z401, exhibited a persistent antiviral activity of 50% (Fig. 21B).

Effect on HCV translation

These compounds where also tested as potential translation inhibitors. For this purpose, Huh7 cells were electroporated with the viral NS5B-defective SGR-JFH-GND-luc RNA, a sub-genomic replicon with a mutation in the GDD domain that blocks the viral NS5B polymerase activity and hence RNA replication. However, this mutant RNA is expected to be translation-competent, at least up to 8 h post-transfection. As such, Huh7 cells, electroporated with JFH-GND-luc RNA, were plated in the presence of the compounds and the antiviral effect was observed after 2, 4 or 8 hours. Interestingly, showed no significant effects on the levels of luciferase, indicating that viral RNA translation was not affected (Fig. 21C). Moreover, when the compounds were tested on the stable replicon cell line treating

the cells for 5 h (data not shown), no inhibitory effects were observed from all the compounds. Together, these results indicate that the compounds tested exert their antiviral effect mainly during the initial stage of viral replication, showing a significant effect on RNA levels. To evaluate whether the effects observed are on RNA synthesis, we developed a new qPCR-based assay to detect and quantitate the replicative-intermediate RNA negative strand.

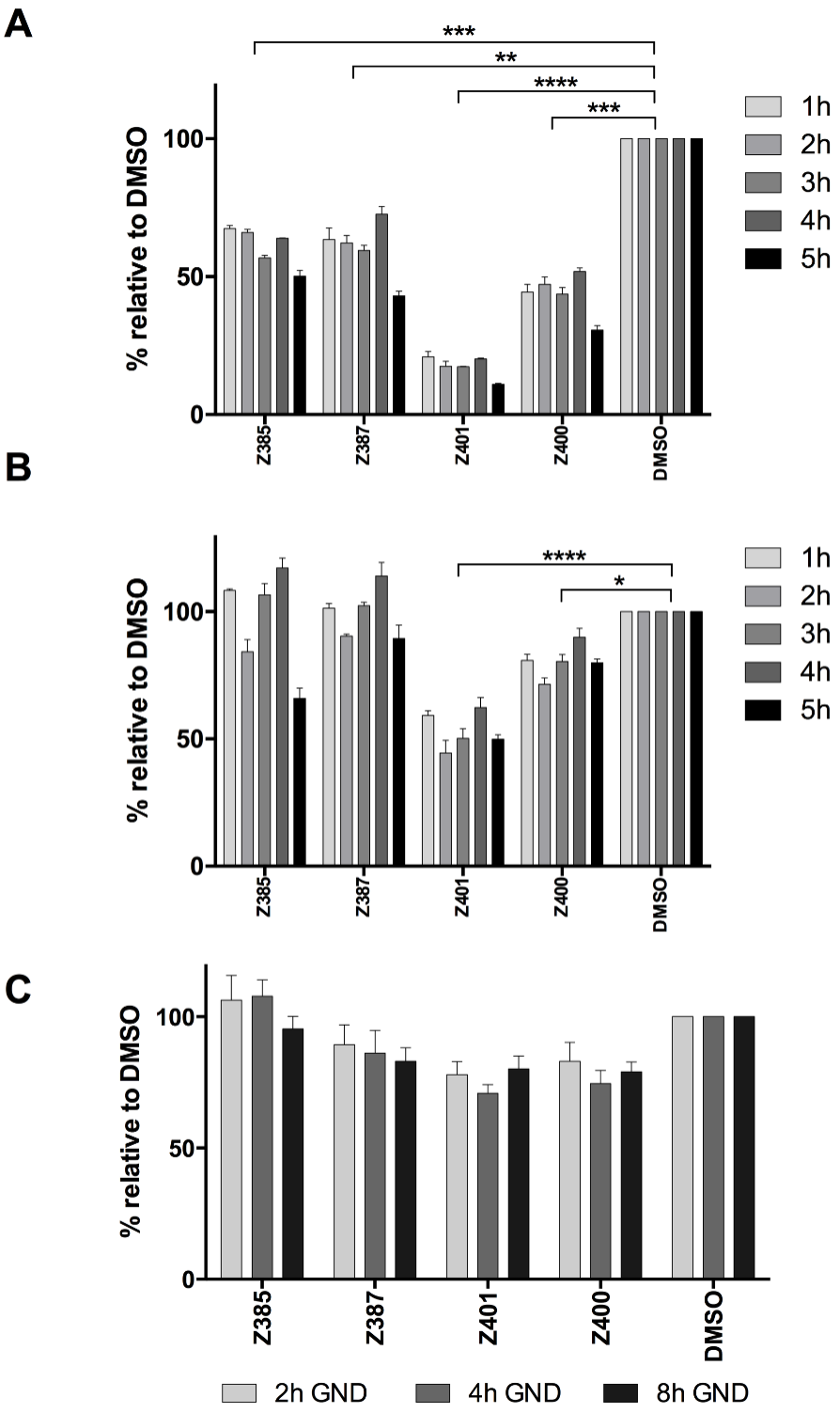


Figure 21. Huh7 cells were electroporated with SGR-JFH-luc RNA and then seeded immediately in the presence of each compound for a short amount of time (1-5 h). Luciferase readings were collected after 24 h (A) or 48 h (B). A potential effect on viral translation was evaluated electroporating Huh7 cells with SGR-JFH-luc$_{GND}$ (C); RLU readings were taken after 2, 4 or 8 h.

Antiviral effect on Influenza Virus

Based on the previous data, we decided to investigate the potential inhibitory effect of Z401, as strongest inhibitor on HCV, on the influenza reporter virus PR8/GFP. Flu virus, belonging to orthomyxoviridae, is a segmented negative strand RNA virus, and, as such, it is a good tool to determine the specificity of our compound. A459 cells were infected with PR8/GFP and exposed to compounds using 3-fold dilution concentrations, following the second model described for HCV, in order to determine the IC_{50} value. Interestingly, we found a moderate inhibition after 24 h, up to 40% at the highest concentration, with an estimated IC_{50} value of 100 μM. However, analysing the antiviral effect after 48 h, a boost in the viral inhibition was detected, with an IC_{50} value of 22 μM (Fig. 22).

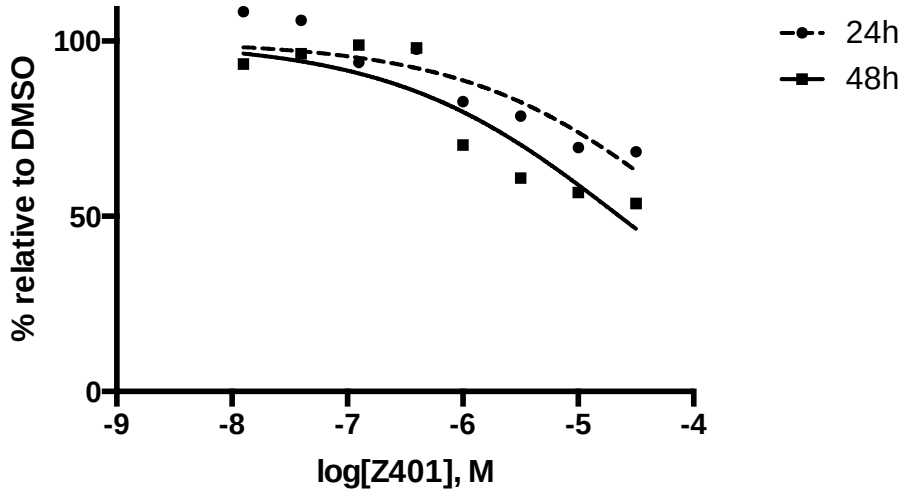


Figure 22. A549 cells were infected with influenza reporter virus PR8/GFP according to model 3 and exposed to Z401 for 24 or 48 h. Inhibition was evaluated measuring reporter GFP signal.

Development of an PCR-based assay for negative strand quantification

Based on the previous results, we demonstrated that our compounds can efficiently inhibit HCV replication and that this effect is exerted interfering with RNA synthesis at an early stage of RNA replication. To validate these findings, we established a new real-time based assay to specifically detect positive or negative strand of HCV and evaluate then the mechanism of these drugs.

Primer Evaluation

Initially, we identified 6 pairs of primers, named 1 to 6 and listed above, that were selected on Vector NTI using as a cut off a score >170. Of those, 4 pairs are located in the 5'UTR, one pair in the NS2-encoding region and one pair in the Core coding sequence, as reported above. All the pairs were tested in an end-point PCR in order to evaluate functionality. Results showed a strong amplification with an annealing temperature of 60°C (Fig. 23) for the entire set, although some non-specific products were observed for pairs 1, 3 and 5. However, when the annealing temperature was increased to 65°C, a best amplification was observed, reducing non-specific amplification, although the pairs 1 and 6 showed a lower amplification; the final temperature selected was 65°C.

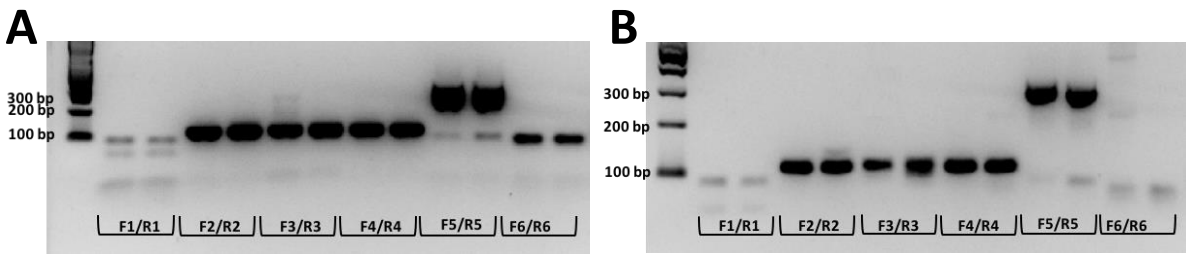


Figure 23. Screening of primer pairs by end-point PCR, using as annealing temperature 60 (A) or 65 (B) degrees.

Real Time Testing

Next, the utility of these primers in the detection and quantitation of HCV genomic negative and positive strands were evaluated. Huh7 cells were electroporated with in vitro-synthesized JFH-1 or JFH-GND RNA. Untransfected Huh7 cells were used as negative control. At 24 or 72 h post-transfection (Fig. 24 A and B, respectively), total RNA was prepared and used as a template to generate viral strand-specific cDNA by reverse transcription using forward and reverse primers previously described. The cDNA was then amplified by Real Time PCR to determine the relative amounts of negative and positive strands using an absolute quantification. As expected, no negative RNA was detected in the control untransfected Huh7 cells at 24 or 72 h, while a partial amplification of positive strand was observed in negative cells for pairs 2 and 4 at both time points. A good amplification of positive strand was observed in both GND RNA- and WT RNA-electroporated cells, except primer pair 1 and 6, which showed a very low amplification, confirming the results obtained with end point PCR. Interestingly, the negative strand of JFH-1 was nicely amplified, especially for the pairs 3, 4 and 5. The ratio observed between quantities of positive and negative strand in our assay for primer pairs 3, 4 and 5, with values ranging between 3 and 7 (Fig. 24). Negative strand was detected in JFH-GND samples (which represented the internal negative control) with primer pairs 2 to 5 for both time points. However, the quantity observed was up to 100-fold lower compared to WT samples, especially for primer pairs 3 and 5, indicating that these species are a product of non-specific amplication or reverse transcription. Based on these results, primer pairs 2 and 4 were excluded due to a non-specific effect on un-infected cells and primer pairs 1 and 6 were discarded considering the poor amplification profiles observed in infected cells, indicating their unsuitability in this assay (Fig. 24).

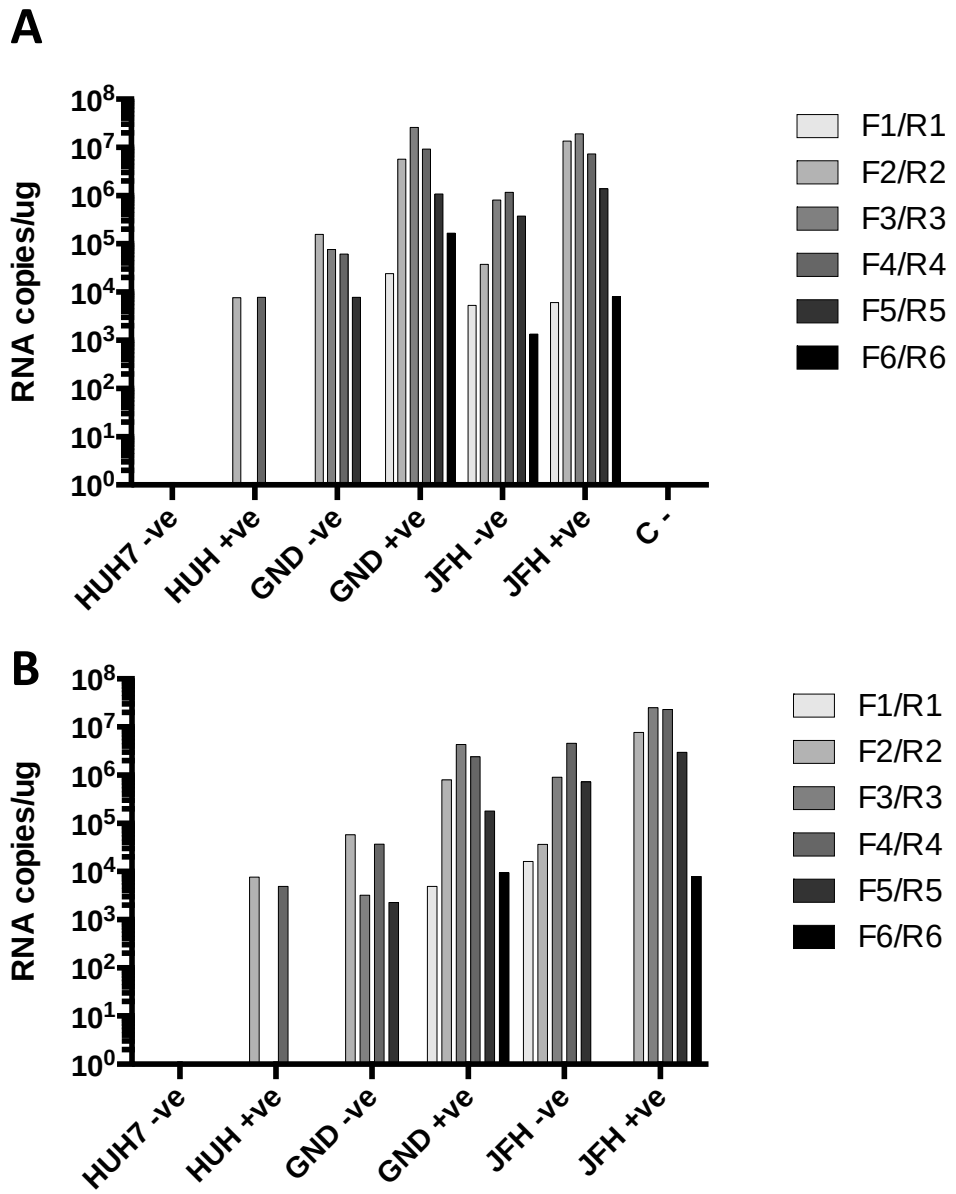


Figure 24. Quantification of positive and negative strand HCV RNA. Huh7 cells were electroporated with WT or GND JFH1 and cultured for 24 h (A) or 72 h (B). Strand-specific quantification was carried out by strand specific RT-qPCR.

Detection of positive RNA in viral particles

The selected pairs 3 and 5 were then evaluated for their specificity to detect positive strand RNA, but not negative strand, in particle-containing medium. It represents a good control to investigate the specificity of the strand-specific primers; as expected, we found a good

amplification of positive strand, correlating with viral titre obtained by FFU staining, while no amplification was detected for negative strand (Fig. 25).

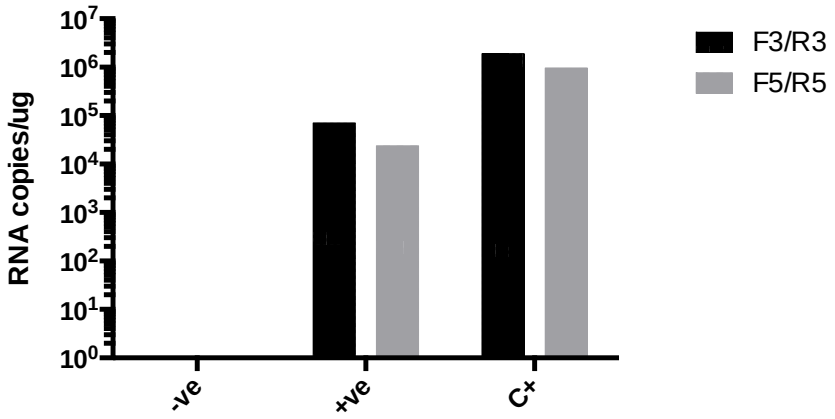


Figure 25. Quantification of positive strand RNA in viral particles. Viral RNA has been extracted from particle-containing medium and negative and positive strand RNA were quantified by RT-qPCR.

Optimisation of RNA amount in the Reverse Transcription

To optimise the RT reaction, we considered if the amount of total RNA could affect the specificity of reverse transcription. To explore this idea, different amounts of input total RNA were assayed to test amplification profiles and investigate the sensibility limit; specifically, we evaluated 1000, 100, 10 and 1 ng. The RT reaction was performed using either pair 3 and 5, on RNA obtained from Huh7 24 hours post electroporation with JFH or JFH-GND. For primer set 3, results showed no significant differences between all the samples tested for positive strand, while reduction in RNA quantity led to a low amplification for negative strand, with 10 or 1 ng showing no amplification. Analysing data obtained with primer pair 5, we found no differences between 1000, 100 or 10 ng for both strand, while a moderate reduction was observed using 1 ng. This experiment confirmed that a small amount of total RNA (~ 100 ng) was enough for a solid quantification, comparable to what observed with increased RNA amounts (Fig. 26A). Moreover, to determine background levels, obtained by negative strand amplification on defective GND virus, we determined the positive/negative

ratio, that has been reported to be variable between 10-100[295]. Considering our results (Fig. 26B), we found that primer pair 5 showed interesting ratio values, of approximately 60 for wt virus and >200 for GND, especially for 100 ng of total RNA, while higher background levels could be detected with different RNA quantities. Interestingly, results obtained from pair 3 showed inconsistent ratio values. To confirm these findings a melting analysis was performed, that showed a non-specific peak in the primer set 3 (Fig. 27A), while a specific amplification was observed in pair 5 (Fig 27B). Based on these results, we outlined the primer set 5 as more specific in combination with the RNA amount of 100 ng for each strand.

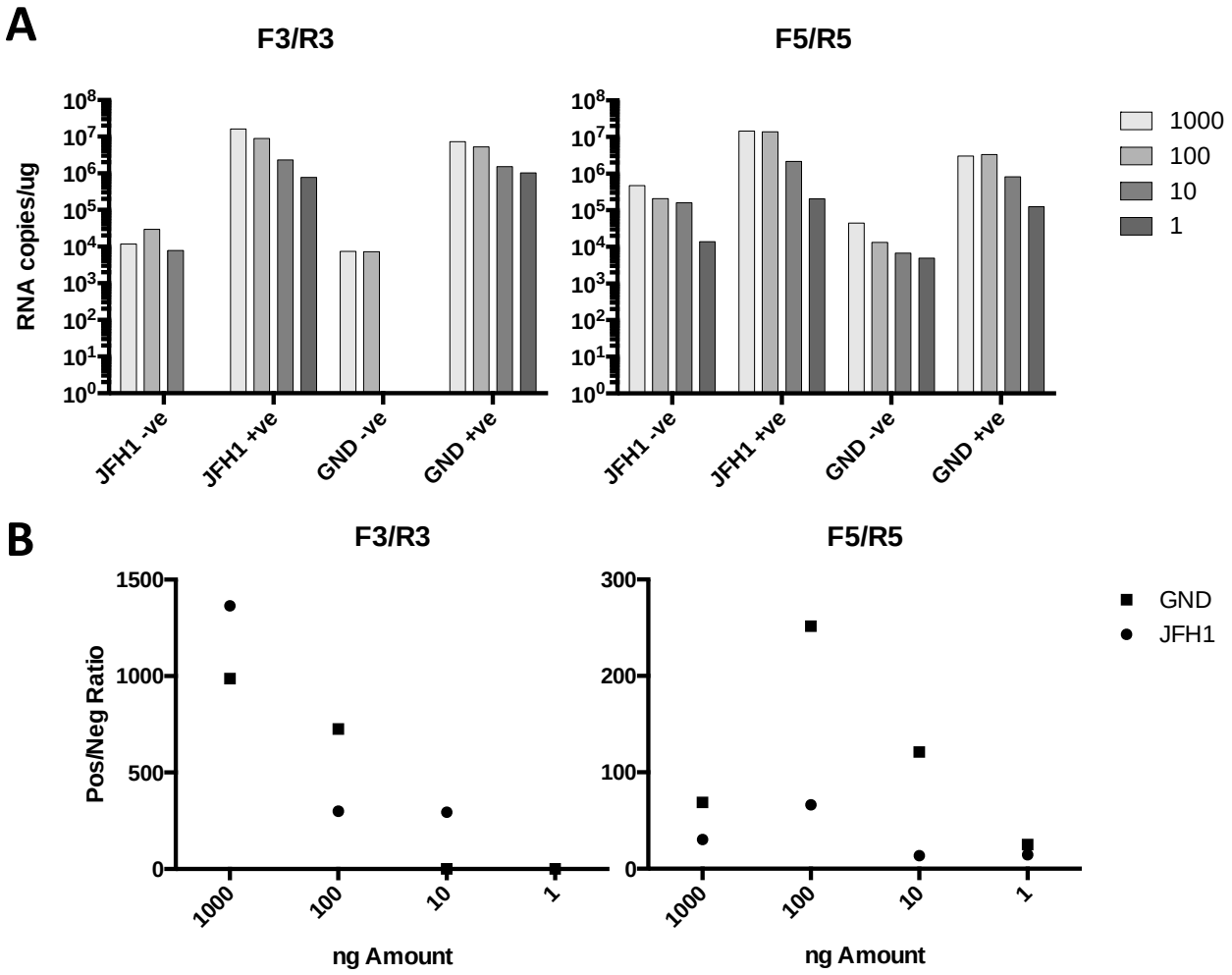


Figure 26. (A) Huh7 cells were electroporated with WT or GND JFH1 and incubated for 24 h. Negative and positive strand HCV RNA were quantified by RT-qPCR using different amount of viral RNA. (B) Ratio between positive and negative strand for both primer sets.

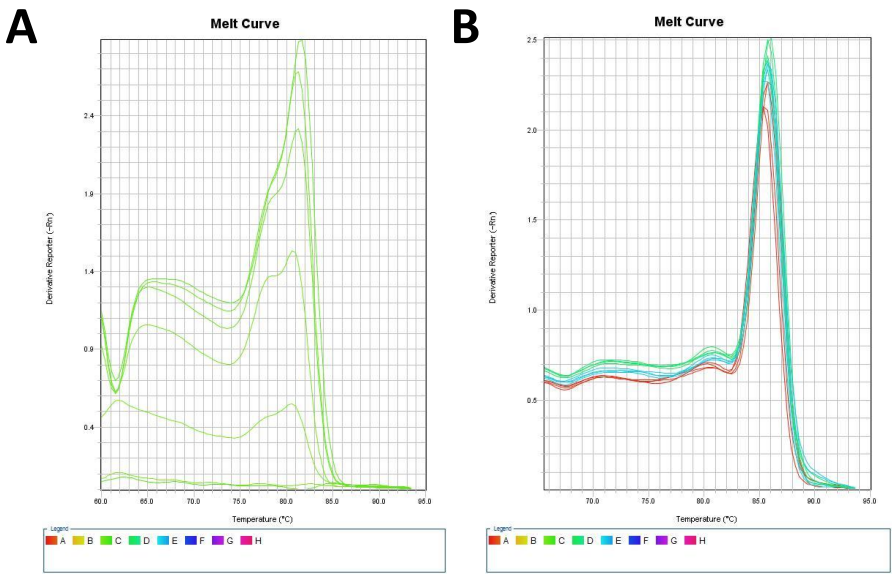


Figure 27. Melting curve analysis performed on qPCR products for primer pair 3 (A) and 5 (B).

Optimising RT Primer Concentration

To optimise cDNA synthesis, RNA obtained from Huh7 24 hours post electroporation with JFH-GND, was reverse transcribed testing 2 different primer concentrations: 1 µM and 0.1 µM. No significant differences were observed for positive strand quantification, while a good variation was observed for negative strand, particularly in the GND virus with a 3-fold decrease, demonstrating the importance of the primer concentration in the RT reaction to avoid non-specific products (Fig. 28). Based on these evidences, all the subsequent RT reactions were conducted using 0.1 µM as final primer concentration for each strand.

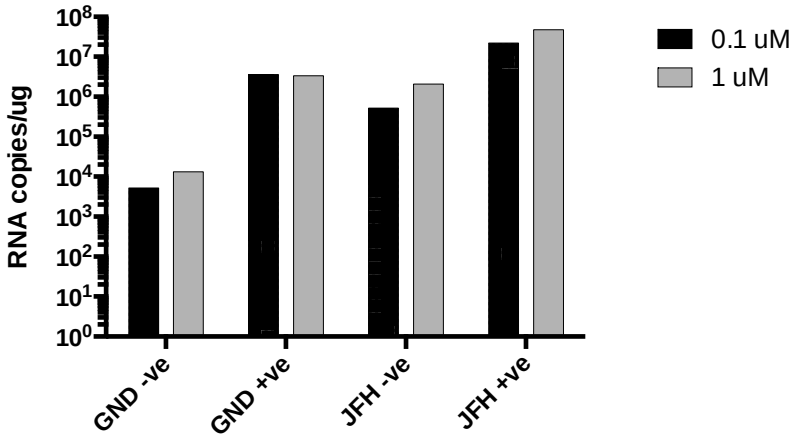


Figure 28. Huh7 cells were transfected with WT or GND JFH1. After 24 h, quantification of strand-specific HCV RNA was carried out using different concentrations of primers during reverse transcription.

Viral kinetic based on negative strand synthesis

Once the assay had been optimised, as described above, we determined negative and positive strand kinetic by means of Huh7 electroporation with WT and defective virus. Negative and positive strand RNA were evaluated after 24, 48, 72; as shown in Fig. 29, similar amounts of positive RNA were detected after 24 h for WT and GND virus, while a significant difference was observed in negative strand quantification. After 48 h, positive and negative strand RNA amounts rapidly increased for WT virus, whereas a massive drop was observed from RNA quantity in GND control. Interestingly, after 72 h a further increase was detected for positive and negative strands in WT virus, while a significant decrease was observed in the defective GND.

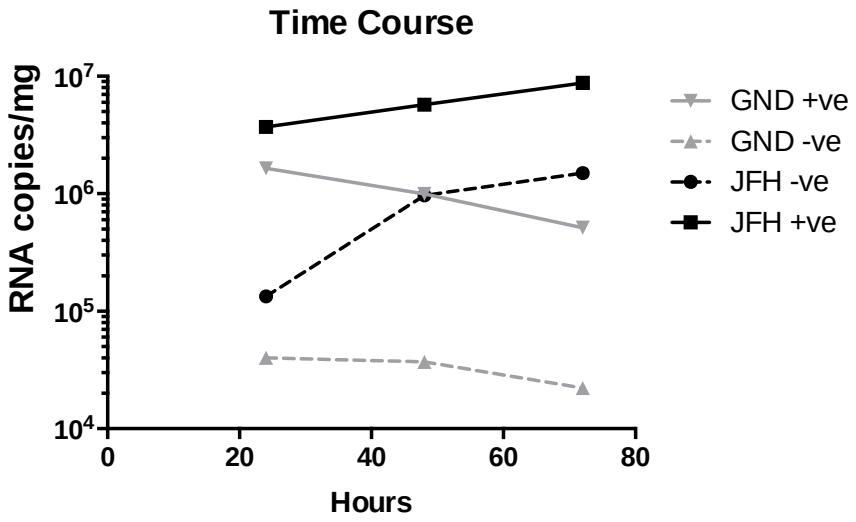


Figure 29. Time course of positive-negative HCV RNA. Huh7 cells were electroporated with WT or GND virus and cultured for 24, 48 or 72 h. Positive and negative strand RNA quantification was performed by RT-qPCR.

Positive and negative strand RNA inhibition

Evaluation on single-cycle infection

To evaluate whether these compounds were able to interfere directly with RNA synthesis, negative and positive strands were evaluated on the single life-cycle model and on control cell line after electroporation; this system allows to investigate negative strand synthesis in conditions were positive strand is over-represented. As reported in Fig. 30 a massive inhibition was detected in negative strand synthesis, up to 90% for both cell lines tested. Noteworthy, positive strand RNA synthesis was impaired up to 60% for CD81 negative cells and up to 40% for HeF cells, showing an important difference between the cell lines; this effect is related to viral spread through secondary infections present in control HeF cells but not in CD81 negative cells.

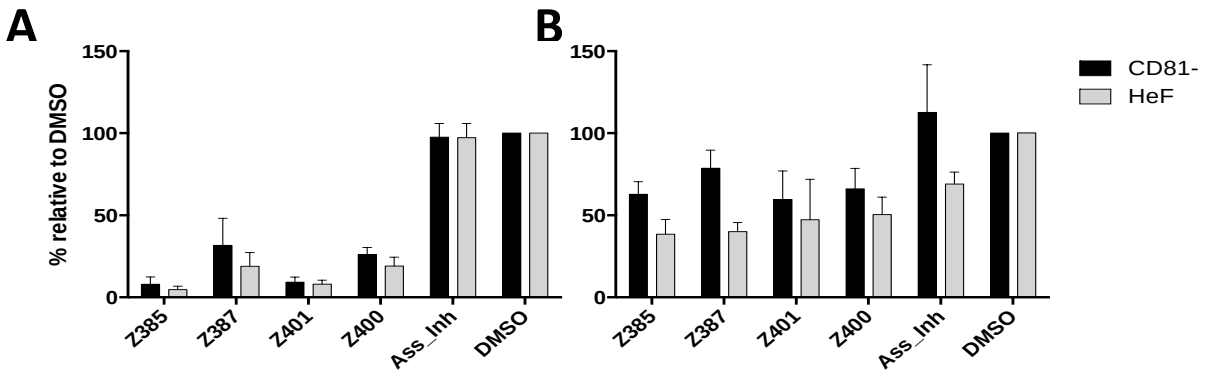


Fig. 30. RNA quantification by RT-qPCR in CD81 negative cells and control HeF cells electroporated with WT virus and incubated for 72 h. Specifically, (A) Negative and (B) Positive strand were evaluated in the presence of a known assembly inhibitor.

Determining positive and negative strand RNA on infection

Finally, to confirm our findings, we evaluated negative strand synthesis after infection with the adaptive virus AM7/1. As expected, an impressive inhibition was detected for both positive and negative strand for all the compounds, up to 95% (Fig. 31). Interestingly, a higher inhibition of negative strand synthesis was observed as compared to the positive strand.

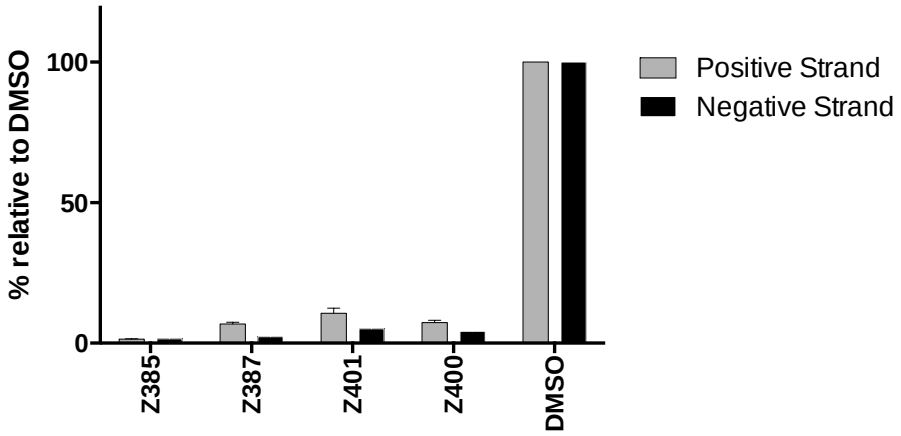


Fig. 31. Quantitation of positive and negative strand in Huh7 cells infected with HCVcc. Huh7 cells were infected with AM7/1 virus and exposed to drugs for 3 days. Positive and negative strand were quantitated by RT-qPCR.

DISCUSSION

Hepatitis C virus (HCV) infection affects 3% of the world population and is major cause of chronic liver disease with severe hepatic consequences such as fibrosis, cirrhosis and hepatocarcinoma. Recently, numerous direct acting anti-viral drugs (DAA) have been introduced, targeting essential viral functions. These new treatments represent a significant step forward as compared to the Pegylated IFN-α-ribavirin therapy. Up to date, DAA are mainly inhibitors of NS3/NS4 HCV protease, some inhibitor of NS5A co-factor and only one, Sofosbuvir, is able to target the NS5B polymerase[488]. However, high costs and limited availability suggest that new antiviral drugs that are more affordable and readily accessible, are needed. Moreover, given the high heterogeneity of HCV, highly variable, it is likely that within few years new drug-resistance variants will arise, making the research for new inhibitory molecules extremely urgent. In our study, presented in this thesis, we demonstrated that our new uracil-based antiviral compounds can massively reduce HCV life cycle by interfering with viral RNA replication. In particular, we demonstrated that these agents are active on an early stage of viral replication, likely blocking the synthesis of negative strand RNA, resulting in a significant decrease in its infectivity.

Initially, using cell-based assays involving three different protocols, we show that most of our compounds inhibit virus genome replication. Our initial data indicated that they had a moderate effect on HCVcc entry. However, a further analysis using HCVpp, which is a well-established virus entry model, identified only Z431 as an HCV-specific entry inhibitor. Treatment of cells post-infection showed that most of the compounds inhibited viral genome replication by up to 95%. That most of the compounds targeted viral genome replication was unequivocally confirmed using the viral sub-genomic replicon system. Interestingly, we found a significant difference in the antiviral activity of the compounds when tested in the stably established replicon cell lines as opposed to in cells that had been freshly electroporated with the replicon RNA. In the latter case, a stronger inhibitory activity was detected, indicating that these compounds likely act on *de novo* RNA synthesis thus

affecting early stages of HCV replication. Our analysis of the effects of the selected compounds in the surrogate viral TCP system further reinforces this hypothesis. Moreover, we proved that culturing replicon cells up to 20 days did not lead to the emergence of any resistance mutation, confirming that these compounds have a very high resistance barrier.

Most of the compounds exhibited IC_{50} values in the micromolar scale and a good cell viability profile. The antiviral activity of the compound Z401 was in the nanomolar range. The Selectivity Index, expressed as the ratio of CC_{50} on $IC_{50,}$ indicated that most of the compounds have high values (>10) and are therefore good candidates for further studies. Here, we selected four compounds for further studies based on an SI cut-off of 100.

To further validate the mechanism of action of the selected compounds, we evaluated their effect on viral RNA levels in infected cells. We used well-established methods to quantitate both total from infected cells and the positive-strand viral RNA from the released virion progeny. We used a cell line defective in the virus entry factor, CD81, thus excluding the possible effect of secondary infection and allowing analyses in a single cycle infection setting. As expected, compounds Z385, Z387, Z401 and Z400 efficiently inhibited both the total viral RNA, and this inhibition corresponded to the reduced levels of viral genomic RNA in the cell medium. In keeping with our data above, the compound Z398 had no effect on total viral RNA levels. Moreover, in the HeF cells in which CD81 expression was restored a better inhibition was observed for all the compounds, probably due their antiviral activity on secondary infection. To confirm these findings, we investigate the release of new viral particles by measuring viral RNA in the cell supernatant; the results showed a reduced release for all the compounds. Our 5 drugs exhibited a strong inhibition of negative strand synthesis, in particular Z385 and Z401, up to 90%. The same effects were then observed infecting cells with an adapted version of JFH1 virus, with strong inhibition of negative strand. These data confirmed that our compounds act in an early stage of viral replication.

To confirm this idea, we treated the electroporated cells for a short time (1-5 h) and then waited 24 or 48 h before evaluate viral replication. All the compounds showed viral inhibition, particularly Z401, able to inhibit up to 80%. However, a rebound in viral replication, especially after 48 h, was observed for all the compounds except Z401, able to maintain 50% of inhibition. We excluded their possible effect on the translation of viral RNA by testing them during the translation phase of a replication-defective subgenomic viral replicon post-electorporation. These data proved that our compounds are highly efficient in blocking viral replication in an early stage and that their effects are stable.

To evaluate species-specificity, Z401, our best candidate, was tested on Influenza virus. Interestingly, good inhibitory profiles were observed, and thus, confirming the effect of Z401 as a strong antiviral with potential pan-viral inhibitory properties.

Finally, we investigate whether these compounds could interfere with viral RNA synthesis. For this purpose, we developed a qRT-PCR assay to quantitate the levels of the replication intermediate negative-strand RNA in infected cells. Initially we evaluated negative and positive RNA synthesis on the CD81 negative cells and their control to compare results in single and multiple cycles of infection. Interestingly, a massive inhibition in the negative strand synthesis in both cell lines was observed, confirming the role of these compounds in blocking RNA synthesis, whereas a moderate inhibition was detected in positive strand, although this effect can be related to the experimental design based on electroporation. To confirm these findings, we measured positive and negative strand inhibition after cell infection, showing a massive inhibition of negative strand RNA, and as consequence, an effective reduction in positive strand RNA confirming that these compounds act impairing viral RNA synthesis.

Based on these results, we are currently investigating if these antiviral properties can be confirmed on HCV strains of a different genotype, to determine if they can be proposed as

candidates for further development. Moreover, different *in vitro* assays have been developed to properly investigate the NS5B activity and its inhibition.

Summarizing the results obtained from all the studies conducted, it is clear that most of the screened compounds exhibit pronounced inhibitory properties (Table I) on viral replication. Z401 is proved to be the most active compound blocking the replication of HCV at IC_{50} 0.0924 μM with a selectivity index of 244 and showing a moderate effect also on Influenza virus.

During my PhD program, I also participated in several projects focused on: a) different aspects of HCV-mediated immunity and b) drug discovery. In particular, I contributed to studies on:

1) Apolipoprotein E Mediates Evasion From Hepatitis C Virus Neutralizing Antibodies

Here, I generated cell culture infectious viruses bearing glycoproteins from different viral genotypes and tested them against several neutralising antibodies, in a setting where the ApoE expression in the host cells was silenced by RNA interference. The aim of this study was to evaluate the role of ApoE in antibody-mediated virus neutralisation[27].

2) Rethinking the old antiviral drug moroxydine: Discovery of novel analogues as anti-hepatitis C virus (HCV) agents

In this project, I tested several new moroxydine-based antiviral compounds, evaluating them on HCVcc and HCV replicons, showing that they can inhibit HCV replication when added constantly to infected cells, suggesting that these agents may have a reduced uptake or a fast metabolism[357].

3) Recent advances in HCV entry (Review)

During my experience at the MRC-University of Glasgow Centre for Virus Research, I acquired a huge knowledge on HCV entry, in particular on the factors involved in HCV attachment and entry that allowed me to write this review[256].

In addition, I also contributed to other collaborative projects on a) pathophysiological mechanisms involved in the progression of liver diseases and b) HCV-mediated innate immunity.

A) In the first project, we investigated the antiviral properties of sex hormones on HCV virus, trying to underline where these hormones can interfering with HCV life cycle conferring protection (manuscript in preparation).

B) I also collaborated on a project to investigate the role of a conserved epitope in natural killer cell mediated host immune response. In this project I generated several mutants evaluating the phenotypic effects induced by these alterations (manuscript submitted). Moreover, we investigate whether the virus can adapt introducing adaptive mutations. Interestingly, we demonstrated that HCV was able to partially restore its replicative fitness introducing an adaptive mutation to compensate the aminoacid changes (manuscript in preparation).

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