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CYCLOPHILIN INHIBITORS: A NOVEL CLASS OF PROMISING HOST TARGETING ANTI-HCV AGENTS

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Abstract

With the approval in 2011 of the protease inhibitors Victrelis and Incivek, direct-acting antivirals (DAA) have begun to revolutionize HCV treatment. Although the addition of Incivek or Victrelis to PEGylated IFN α and ribavirin (pIFN α /RBV) may improve cure rates and shorten the treatment duration of the “old” standard of care (SOC), this triple therapy will not be suitable for patients intolerant to pIFN α or RBV. The efficacy of this triple therapy will also certainly be attenuated in pIFN α /RBV non-responders. As Incivek is inactive against genotype 3 (GT3) combined with the fact that all protease inhibitors and most of the non-nucleoside polymerase inhibitors in development are active primarily against GT1, pIFN α /RBV will remain the SOC for non-GT1 until new classes of inhibitors enter into clinical practice. GT1 patients, who do not respond to this new triple therapy will have developed resistance to protease inhibitors that will limit future treatment options. There is thus an important need for the identification of new potent HCV agents. A novel class of HCV inhibitors that have great potential for the treatment of HCV has recently emerged: the host-targeting antivirals (HTA) cyclophilin (Cyp) inhibitors.

Keywords

HCV; Treatment; Cyclophilins; Cyclophilin inhibitors; Host-targeting antiviral

Introduction

Hepatitis C

HCV is the major causative agent of acute and chronic liver diseases [1]. Primary infection is often asymptomatic or associated with mild symptoms, whereas persistently infected individuals exhibit a high risk for chronic liver diseases, such as hepatocellular carcinoma and cirrhosis [1]. Nearly 200 million people worldwide (3% of the population), including 4 to 5 million in the US, are chronically infected with HCV and 4 million new infections occur every year [2, 3]. In the developed world, HCV accounts for two-thirds of all cases of liver cancer and transplants [4], and, in the US, approximately 12,000 people are estimated to die from HCV each year [5].

The “Old” Standard of Care

The introduction of interferon alpha (IFN α) and the nucleoside analog RBV greatly improved the percentage of chronically HCV-infected patients able to reach a sustained antiviral response (SVR) [6, 7]. SVR is defined by undetectable HCV RNA levels 24 weeks following completion of therapy, and long-lasting viral clearance. Yet, the “old” standard pIFN α /RBV therapy had a low success rate of approximately 50% in patients with GT1 [8, 9] and causes severe side effects (i.e., sustained flu-like symptoms, anemia and depression) [10]. Not only is GT1 the most prevalent HCV genotype in Europe, North and South America, China and Japan, it is also the most difficult to treat [11]. Thus, there is an urgent need for the development of additional anti-HCV agents with novel mechanisms of antiviral action in order to provide alternate treatments for the increasing number of patients who are unresponsive to the current pIFN α /RBV treatment [12, 13].

Direct-Acting Antivirals

Recent discoveries related to the HCV life cycle have led to the development of novel therapies that inhibit viral replication by acting directly on viral components. These compounds, characterized as direct-acting antiviral agents (DAAs), have been investigated in naive as well as previously treated patients, and results from these studies have been encouraging. DAAs targeting the HCV-encoded protease NS3, and the polymerase NS5B and NS5A proteins, are the most advanced and promising small molecule therapies currently under clinical evaluation for HCV [14]. NS3-targeting molecules inhibit the serine protease activity of the virally-encoded protease and suppress the proteolytic processing of newly translated HCV polyproteins into mature viral proteins required to form replication complexes [15]. NS5B is the HCV-encoded, RNA-dependent RNA polymerase that catalyzes the formation of nascent plus stranded RNA genomes as well as the minus stranded replication intermediates [15]. Inhibition of this function (by non- or nucleotide/side analogs) halts HCV genome synthesis. The function of NS5A is not clear, but it appears to play multiple key roles in viral replication, including regulating the activity of the NS5B polymerase, cell signaling pathways, and viral particle release [16].

The “New” Standard of Care

With the approval in 2011 of the HCV protease inhibitors boceprevir (Victrelis) (Merck) and telaprevir (Incivek) (Vertex Pharmaceuticals), DAAs have begun to revolutionize hepatitis C treatment. Incivek and Victrelis were approved for use only in combination with pIFN α /RBV. The addition of either protease inhibitor increased clinical cure rates, known formally as SVR rates, by 30 to 40% over control groups receiving the standard pIFN α /RBV alone. However, this triple therapy (Incivek or Victrelis plus pIFN α /RBV) will not be suitable for patients either intolerant of, or with contraindications to, pIFN α or RBV. This includes patients with decompensated cirrhosis or following solid organ transplantation. Also, the efficacy of this triple therapy will probably be reduced in treatment-experienced patients, especially those who were non-responders to a previous treatment with the “old” SOC. Moreover, although Incivek has similar antiviral activity against GT2, this agent has no effect in patients with GT3 infection [17, 18]. All current protease inhibitors and most non-nucleoside polymerase inhibitors in development are active primarily against GT1. Thus,

pIFN α /RBV will certainly remain in the SOC for non-GT1 infections until new classes of inhibitors enter into clinical practice. Furthermore, the 30–40% of GT1 patients, who do not respond to this new triple therapy will have developed resistance to protease inhibitors, which will limit future treatment options. Although the addition of Incivek or Victrelis to pIFN α /RBV may improve cure and shorten the treatment duration of SOC, this approach will likely not meet the needs of many difficult-to-treat patient groups. Therefore, there is an urgent need to identify newer safer anti-HCV agents, which exert potent suppression of HCV replication in treatment regimens that do not contain IFNs.

DAA in the Pipeline

In the DAA development pipeline are two agents that entered Phase III development this year, including two additional protease inhibitors - TMC435 (Johnson & Johnson's Tibotec unit) and BI201335 (Boehringer Ingelheim) – which are used in addition to the standard pIFN α /RBV. New mechanisms other than protease inhibitors that have entered large Phase IIb studies include another protease inhibitor - asunaprevir (BMS-650032) (Bristol-Myers Squibb), the non-nucleoside polymerase inhibitors – setrobuvir (Anadys), tegobuvir (Gilead Sciences) and filibuvir (Pfizer), the nucleoside/tide polymerase inhibitors – mericitabine (Roche) and PSI-7977 (Pharmasset), and the NS5A inhibitor - daclatasvir (BMS-790052) (Bristol-Myers Squibb). Remarkably, a recent study showed that an IFN-free treatment of PSI-7977 plus RBV produced 100% sustained virological response after 12 weeks of treatment and 12 weeks of follow-up (SVR12) in previously untreated patients with GT2 or GT3 infection in the absence of viral breakthrough [19]. Similarly, 90% of prior null responders with GT1b demonstrated SVR, with undetectable viral load at weeks 12, 24, 36 (12 weeks post-treatment, or SVR12), and 48 (24 weeks post-treatment, or SVR24) after an IFN-free regimen of the NS5A inhibitor daclatasvir (BMS-790052) plus the NS3 protease inhibitor asunaprevir (BMS-650032) [20].

Host-Targeting Antivirals – The Cyclophilin Inhibitors

Another promising therapy strategy consists of targeting host factors, which are absolutely required for HCV replication. Emerging host-targeting antivirals (HTAs) candidates for future anti-HCV therapies include inhibitors of viral entry, internal ribosome entry site, viral RNA replication, and viral assembly and release. A majority of these HTAs are in preclinical or very early clinical development. However, one class of HTAs has showed great promise in HCV patients - the cyclophilin (Cyp) inhibitors. To date, three Cyp inhibitors – alisporivir (Novartis), NIM-811 (Novartis) and SCY-635 (SCYNEXIS) - have demonstrated safety and efficacy in patients with HCV in phase I and II trials [21–27] (Figure 1).

Alisporivir (Novartis).—Alisporivir, a synthetic Cyp inhibitor derived from cyclosporine A (CsA), was the first oral, non-immunosuppressive Cyp inhibitor, to enter into clinical trials. Alisporivir has potent anti-HCV activities in vitro, high affinity to Cyp and low immunosuppressive activity (Figure 1).

Phase I Studies: A 15-day double-blind, placebo controlled Phase I study, with twice daily oral doses (1200 mg) of alisporivir showed rapid absorption, with peak plasma levels reached after 2 h and a terminal half life of 100 h. A maximal HCV RNA level drop of 3.6

$\log_{10}$ was observed in alisporivir-treated patients [28]. In 15 out of 16 subjects treated with alisporivir, HCV viral load decreased by more than 2 $\log_{10}$. In 3 patients, the virus became undetectable after 8 or 15 days. All 3 HCV genotypes (1, 3 and 4) identified in the study, responded well to the dose administered and no patient developed a viral breakthrough during the treatment, suggesting that alisporivir has a pan-genotypic antiviral activity and a high barrier for resistance. However, transient hyperbilirubinemia led to the discontinuation of treatment in some patients [28].

Phase II Studies: The efficacy of alisporivir in combination with pIFN α in chronic HCV patients was evaluated in a Phase II study [29]. The study included 90 participants who received one of the following treatments for 29 days: a) pIFN α + placebo; b) pIFN α + 200 mg/day alisporivir; c) pIFN α + 600 mg/day alisporivir; d) pIFN α + 1000 mg/day alisporivir; and e) 1000 mg/day alisporivir. Among patients with GT1 or GT4, reductions in HCV RNA at day 29 were 4.75 $\log_{10}$ for d); 4.61 $\log_{10}$ for c); 2.49 $\log_{10}$ for a); 2.20 $\log_{10}$ for e); and 1.8 $\log_{10}$ for b). Eight of 12 patients (67%) in d) had undetectable viral load at day 29, compared with 3 of 12 patients (25%) in both a) and e). Among GT2 or GT3 patients, 4 out of 6 (67%) achieved undetectable HCV RNA with alisporivir monotherapy. The reduction of HCV RNA levels in this group was 4.22 $\log_{10}$. At lower doses, the safety profile of alisporivir was comparable to that of placebo. Five of 24 patients (21%), who received 1000 mg of Alisporivir developed reversible indirect hyperbilirubinemia. The efficacy of alisporivir combined with pIFN α /RBV was recently tested in patients with GT1 [30]. Patients received alisporivir/pIFN α /RBV or placebo/pIFN α /RBV treatment for 24 or 48 weeks. SVR was 76 and 55% in the alisporivir and placebo-control arms, respectively. Alisporivir was well tolerated and associated with low viral breakthrough [30]. Among patients taking alisporivir, 7 of 10 cases of viral breakthrough happened after treatment discontinuation or dose reduction. 3% of alisporivir recipients in the 24-week and response-guided arms and 7% in the 48-week arm had severe hyperbilirubinemia ($>5 \times$ upper limit of normal); this was reversible and not associated with elevated amino alanine aminotransferase. These results demonstrate the superiority of alisporivir combined with pIFN α /RBV in achieving SVR24 in GT1 treatment-naïve patients.

Alisporivir was also tested in a Phase II study of treatment-naïve patients with GT2 and GT3. Newly licensed DAAs are not indicated for GT2 and GT3 patients [31, 32]. Phase II data showed oral once daily alisporivir plus RBV provided viral clearance in almost half of GT2 and GT3 patients with HCV [33]. Almost half the patients (49%) in the study on alisporivir plus RBV achieved viral clearance (negative HCV RNA) as early as week 6 [33]. One third of patients (32%) receiving alisporivir alone also achieved viral clearance after 6 weeks [33]. In addition, 97% of patients with viral clearance who continued to receive IFN-free alisporivir plus RBV maintained this viral clearance up to week 12 [34]. Achieving an early viral clearance is considered one of the most important predictors of sustained viral response, also known as viral cure. After GT1, GT2 and GT3 are the second most prevalent forms of HCV worldwide [34, 35]. These results suggest that alisporivir may be a valuable future treatment option either alone or in combination regimens, providing physicians with flexibility in the treatment process along with favorable tolerability and a high barrier to

resistance. Other Phase II studies are ongoing in other patient populations, i.e., GT1 experienced patients.

Phase III Studies: A Phase III study with alisporivir is ongoing to evaluate the efficacy and safety of alisporivir plus pIFN α /RBV in previously untreated GT1 patients.

NIM811 (Novartis).—NIM811 was the first synthesized Cyp inhibitor derived from CsA found to be devoid of immunosuppressive activity. Like Alisporivir, NIM811 exhibits potent anti-HCV activities in vitro and high affinity to CypA (Figure 1).

Phase I Studies: NIM811 was studied in ascending doses in a randomized, double-blind, placebo-controlled 14-day trial in GT1 patients [36]. Doses of 10 to 600 mg were given orally once or twice daily as monotherapy. NIM811 was well tolerated at all doses. Although a normalization of liver transaminases was observed, no antiviral effect was observed in the monotherapy arms [36].

Phase II Studies: In the same study [36], NIM811 (600 mg) or placebo bid for 14 days was co-administered with pIFN α administered on days 1 and 8 to GT1 relapsers. In the combination group, the mean HCV RNA decline was 2.85 log₁₀, compared to a 0.56 log₁₀ in the pIFN α alone arm. There were no severe or serious adverse events (AE). Further clinical studies with NIM811 were halted due to the absence of antiviral effect in the monotherapy study described above.

SCY-635 (SCYNEXIS).—SCY-635 is one of the latest non-immunosuppressive Cyp inhibitor derived from CsA. Like Alisporivir and NIM811, SCY-635 has potent anti-HCV activities in vitro and binds Cyp with a high affinity (Figure 1).

Phase I Studies: SCY-635 was evaluated in a 15-day Phase I randomized, double-blind, placebo-controlled, multi-dose study in GT1 patients [37]. Participants (20) were enrolled into one of 3 ascending-dose cohorts, receiving total daily doses of 300, 600, or 900 mg SCY-635 administered orally in divided doses 3 times daily for 15 days. Within each cohort, patients were randomized to receive SCY-635 or placebo in a 6:1 ratio. Pharmacokinetic assessments and viral load monitoring were performed throughout the treatment period. Treatment with placebo or SCY-635 300 or 600 mg/d was associated with minimal changes in viral load. In contrast, in the 900 mg cohort, all treated patients experienced a reduction in HCV viral load, with a group mean maximum decrease of 2.2 log₁₀ on day 15 [37]. In the 900 mg cohort, mean through plasma concentrations of SCY-635 remained above the replicon-derived EC₉₀ value (90% effective concentration) from day 3 through day 15. One participant achieved undetectable HCV viral load on day 15. SCY-635 was generally well tolerated, with no serious AE or premature treatment discontinuations [37].

Phase II Studies: A Phase IIa study of SCY-635 at a daily dose of 600 mg (300 mg bid) in combination with pIFN α /RBV in treatment-naïve subjects with GT1 was recently completed. This study examined the antiviral efficacy of 28 days of triple combination therapy in more difficult to treat patients with either the CT or TT IL28B genotype.

CYCLOPHILIN INHIBITORS IN THE PIPELINE

Additional Cyp inhibitors recently emerged in preclinical studies. Among them are the Sangamides and EP-CyP546, which exhibit potent anti-HCV activities in vitro without immunosuppressive activities.

Sangamides (BIOTICA):

Sanglifehrin A, a Cyp-binding polyketide natural product, which is structurally distinct from CsA (Figure 1), and its natural analogues are mixed non-ribosomal peptide/polyketides produced by the soil bacterium *Streptomyces* sp. A92–308110 and are amenable to biosynthetic engineering for lead optimization and large-scale production by fermentation. Sanglifehrins A to D are more potent than CsA at blocking CypA isomerase activity (Figure 1) [38]. Sangamides are amide derivatives of sanglifehrin A that have high affinities to CypA and potent cross-genotypic antiviral activity in vitro [39]. Sangamides clear HCV replicons from hepatoma cells and exhibit additive antiviral effects when used in combination with DAAs [39]. Sangamides display in vivo pharmacokinetics potentially suitable for once daily administration. In contrast to CsA, sangamides tested showed no significant inhibition of the multidrug resistance gene 1 protein (MDR1), a major xenobiotic transporter, inhibition of which can lead to drug-drug interactions with concomitant medications, which are MDR1 substrates [39]. Sangamides tested also showed no significant inhibition of Multidrug Resistance Protein 2 (MRP2), the primary transporter for excretion of organic anions and their conjugates that was proposed to be at least partly the cause of transient elevated bilirubin levels in alisporivir-treated patients [39]. BC556 was recently selected by BIOTICA as their lead sangamide compound for development, based on a differentiated preclinical profile, including CypA PPIase K_i of 0.3 nM and average EC_{50} in HCV replicons of 20 nM (personal communication).

EP-CyP546 (ENANTA):

The non-immunosuppressive Cyp inhibitor EP-CyP546 was recently examined in vitro for its anti-HCV activities. EC_{50} values in GT1b and 1a were obtained for EP-CyP546 at 75 nM and 127 nM compared with 84 nM and 151 nM for alisporivir [40]. The combinations of EP-CyP546 with IFN α or DAAs were found to be additive to synergistic [40]. No cross-resistance between reported DAA-resistant mutants and EP-CyP546 was observed. Apparently, EP-CyP546 remained effective against a previously reported CsA-resistant mutation [40]. EP-CyP546, in combination with an NS5A inhibitor, suppresses the emergence of HCV resistance usually observed under NS5A inhibitor selection [40]. The synthesis and structure of EP-CyP546 have not yet been reported.

F680 and F684 (Department of Virology, University of East-Paris, Creteil-France):

Using a fragment-based drug design (FBDD) approach by means of nuclear magnetic resonance (NMR) and X-ray crystallography, small (100–150 Da) non-peptidic Cyp inhibitors were identified [41]. Two hit compounds (F680 and F684) exhibited significant inhibition of CypA, CypB and CypD enzymatic activities [41]. Importantly, the compounds also exerted significant anti-HCV GT1b activities deprived of cytotoxicities: IC_{50} of 3.12 μ M for F680 and 0.87 μ M for F684 [41].

CYCLOPHILINS AS INTRACELLULAR TARGETS OF CYCLOPHILIN INHIBITORS

In 2003, Watashi et al. provided the first link between HCV and cyclophilins (CyPs) by demonstrating that Cyp inhibitors - CsA and NIM811 - suppress HCV replication in vitro [42]. This landmark discovery was confirmed by many laboratories [43–48]. Supporting the notion that CyPs could play a role in HCV replication, transient knockdown expression of CypA, CypB, CypC, CypH, Cyp40 or CypE diminishes HCV replication [49, 50]. More recently, three independent laboratories demonstrated using stable knockdown methodologies that CypA, but not CypB or CypC, is vital for HCV replication [50–52]. These differences in the contribution of Cyp members to HCV replication may originate from distinct knockdown approaches: transient [49, 50] versus stable [50–52] knockdown. Since viral replication is monitored over several days, one can postulate that the stable knockdown approach provides more definitive results. If this is correct, CypA represents the principal Cyp member assisting HCV. Other observations may explain why HCV exploits CypA rather than CypB or CypC. First, both CypB and CypC reside in the lumen of the endoplasmic reticulum [54], a cellular compartment certainly inaccessible for interactions with HCV replication complexes, which reside in the cytosol, not in the lumen [15]. Second, CypA is 10- and 100-fold more abundant than CypB and CypC, respectively [54]. Third, CypA resides in the cytosol, in close contact with the HCV replication complex [54]. Fourth, we and others showed that a subset of CypA molecules, via their isomerase pocket, locate in a protease-resistant compartment similar to that where HCV replicates [55, 56]. Together, these observations support the notion that CypA is the major Cyp member, which governs HCV replication.

CypA was originally isolated from bovine thymocytes using CsA as bait [57]. CypA is an abundant cytosolic protein ubiquitously expressed in eukaryotic cells [57]. Subsequent studies showed that the neutralization of CypA by CsA suppresses tissue rejection by inactivating T lymphocyte subsets [58]. Meanwhile, Fischer and colleagues identified an activity, classified now as peptidyl-prolyl *cis-trans*-isomerase (PPIase) [59], which catalyzes the *cis* to *trans* interconversion of proline-containing peptides [60]. A few years later, the same group discovered that the PPIase activity that they previously identified as an *in vitro* catalyst of peptide bond rotation on the amino side of proline residues is CypA [61]. CsA, by binding to the hydrophobic pocket of CypA, neutralizes its isomerase activity [62, 63]. The existence of CypA knockout mice [64], and knockout human cell lines [65], suggests that CypA is optional for cell growth and survival. It also suggests that the neutralization of CypA by Cyp inhibitors will not lead to unanticipated clinical toxicities or dose-limiting immunosuppression, especially in HCV patients. Importantly, CypA-knockout mice are resistant to immunosuppression by CsA [64], further demonstrating that CypA is a major *in vivo* target for Cyp inhibitors. Interestingly, although CypA was identified 25 years ago, its cellular function remains to be fully elucidated.

MECHANISMS OF ACTION OF CYCLOPHILIN INHIBITORS

Although it is likely that Cyp inhibitors mediate their antiviral effect by binding to the isomerase pocket of intracellular Cyps, primarily CypA, it is poorly understood how the binding of the drug to the host protein stops HCV replication. Yet, recent findings may shed light on the mechanisms of action of Cyp inhibitors. Specifically, several studies including ours, have demonstrated that the nonstructural HCV NS5A protein serves as a direct ligand for CypA [66–73]. This is in accordance with the fact that HCV variants develop mutations mostly in the NS5A gene when cultured under Cyp inhibitor selection [53, 69, 70, 71, 74, 75, 76]. Most importantly, Cyp inhibitors such as CsA, alisporivir, SCY-635 and the sangamides prevent and disrupt CypA-NS5A interactions [38, 39, 66–773, 77]. We showed that the interaction between the host CypA and the viral NS5A protein is conserved among HCV genotypes [69]. This is perfectly in agreement with in vitro as well as in vivo observations that Cyp inhibitors exhibit pan-genotypic anti-HCV activity [21–27]. Altogether these data suggest that preventing the contact between CypA and NS5A is deleterious to the virus.

Recent NMR, isothermal titration microcalorimetry (ITC) and surface plasmon resonance (SPR) studies demonstrated that CypA could directly interact with domains II and III of NS5A [66, 71, 73, 78, 79]. The contact surface on CypA corresponds to its enzymatic pocket, whereas on domains II and III of NS5A, it is distributed over many proline residues [66, 73, 78, 79]. This is accordance with the fact that CypA demonstrates nanomolar binding affinity for exposed proline residues and catalyzes the *cis* to *trans* interconversion of proline-containing peptides [60]. NMR heteronuclear exchange spectroscopy yielded direct evidence that many proline residues in domains II and III of NS5A, but not all, form valid substrates for the isomerase activity of CypA [66, 73, 78, 79]. Further work is required to determine i) which prolines in NS5A serve as true CypA substrates in a physiological cellular context rather than in an in vitro context; ii) whether CypA could isomerize proline peptide bonds in a physiological cellular context; and iii) whether the CypA-mediated isomerization of specific peptidyl-prolyl bonds within NS5A plays any role in HCV replication. A direct contact between CypA and the domain II of NS5A is consistent with the recent finding that CypA stimulates the RNA binding activity of the domain II of NS5A [72]. The addition of CsA or the introduction of mutations in the isomerase pocket of CypA abrogates the CypA-mediated stimulation of NS5A (domain II) RNA-binding [72].

Given that CypA apparently has a higher affinity to domain II than domain III of NS5A [66, 73, 78, 79], one can envision that domain II serves as the major binding site for CypA. Importantly, previous work demonstrated that the domain II of NS5A contains a binding site for the NS5B polymerase [80]. If CypA and NS5B share a similar binding region in the domain II of NS5A, one cannot exclude the possibility that CypA, by interacting with this domain, could affect either i) NS5A functions; ii) NS5B functions; or iii) both. An attractive scenario is that NS5A governs NS5B polymerase activity. On one hand, NS5A acts as a negative regulator of NS5B. Specifically, NS5A, by binding to NS5B, hampers the polymerase activity of NS5B. In this model (Figure 2, Model I), CypA, by binding to the domain II of NS5A, prevents the subsequent binding of NS5A to NS5B, allowing NS5B to freely replicate the viral genome. In this scenario (Figure 2, Model I), Cyp inhibitors, by

preventing CypA-NS5A contacts, permit NS5A to interact with NS5B. This unregulated contact between NS5A and NS5B would lead to an abortive RNA replication. One can envision that the direct contact of NS5A to NS5B decreases either the binding of NS5B to the viral RNA or the enzymatic activity of NS5B. One cannot also exclude the possibility that NS5A, free of CypA, binds strongly to the viral RNA and represents an obstacle for NS5B-mediated RNA synthesis. On the other hand, NS5A acts as a positive regulator of NS5B. In this model (Figure 2, Model II), CypA, by binding to NS5A, promotes a positive function of NS5A on the polymerase activity of NS5B. CypA-NS5A complexes could enhance either the viral RNA binding of NS5B or its enzymatic activity. In this model (Figure 2, Model II), Cyp inhibitors, by disrupting CypA-NS5A complex formation, would inhibit the NS5A-mediated enhancement of the catalytic efficiency of the NS5B polymerase, ultimately leading to an abortive RNA replication.

Previous studies suggested that CypA modulates NS5B polymerase activity by binding directly to NS5B [80]. However, more recent findings indicate that CypA does not bind NS5B directly. Specifically, using an NMR approach, Xanoulle and colleagues convincingly demonstrated that although CypA binds NS5A directly, it fails to bind NS5B [79]. We recently confirmed these results by showing that GST-NS5B efficiently captures NS5A, but not CypA, and conversely that GST-CypA captures NS5A, but not NS5B [Gallay, unpublished data]. Xanoulle and colleagues also obtained evidence that both CypA and NS5B bind the same region of NS5A [79], supporting one of our models above (Model I).

Remarkably, a recent study suggested that the Cyp inhibitor SCY-635 modulates the IFN response in vivo [37]. Specifically, daily administration of SCY-635 to HCV patients triggered rapid (less than 2 h) increases in plasma concentrations of IFN α , λ 1, λ 3, 2'5'OAS1 and neopterin [37]. In contrast, no changes in IFN expression and immune activation were observed in either placebo treated HCV patients or SCY-635-treated non-HCV infected normal healthy volunteers [37]. Together these data suggest that the in vivo neutralization of CypA decreases the viral load and increases the expression of multiple components of the IFN response. Previous studies reported that NS5A has the ability to counteract the mounting cell IFN response against HCV [81]. Thus, one cannot exclude the possibility that Cyp inhibitors, by disrupting CypA-NS5A complexes, exert a dual inhibitory effect: i) interfering with HCV replication; and ii) restoring the IFN response. One could speculate that differences (i.e., structures, pharmacologies, etc.) between endogenous IFNs induced by Cyp inhibitors and exogenous IFN (pIFN α) could lead to more acceptable toxicity profiles and better/higher likelihood of activity in combination with DAAs. Nevertheless, it remains to be determined whether the boost of the innate response observed in SCY-635-treated HCV patients results either from the inhibition of HCV replication, causing the immediate shutdown of expression of viral proteins known to antagonize the IFN response (i.e., NS3, NS5A, core and E2) [81], or from an unknown role of CypA in the IFN response.

Further work is needed to i) determine which activity of CypA (folding, trafficking, or another unknown activity) controls HCV replication; ii) understand at a molecular level how the CypA activity assists HCV replication; and iii) identify which viral or host proteins are the true CypA ligands governing HCV replication. A complete understanding of the

mechanisms that control the antiviral effect of Cyp inhibitors is imperative because it will not only provide new anti-HCV therapies, but it will also help our understanding of the early and late steps of the HCV life cycle.

CYCLOPHILIN INHIBITORS PROVIDE LOW VIRAL BREAKTHROUGH RATE AND HIGH BARRIER TO HCV RESISTANCE

Multiple studies showed that HCV variants resistant to Cyp inhibitors (CsA, NIM811, Alisporivir and SCY-635) emerge in vitro [53, 68, 69, 70, 71, 74, 75, 76]. However, the time needed for Cyp inhibitor resistance selection is extremely long (3–6 months) compared to other DAAs such as protease or polymerase inhibitors (2–3 weeks) [71]. The level of HCV resistance to Cyp inhibitors is relatively low (5–10-fold) compared to other DAAs such as protease or polymerase inhibitors (>100-fold) [46, 71]. As mentioned above, Cyp inhibitor-resistant HCV variants mostly develop mutations in NS5A [52, 67, 68, 69, 70, 73, 74, 75]. The D320E mutation, which locates in the domain II of NS5A, often emerged under Cyp inhibitor selection [69, 70, 71, 82, 83]. Unlike wild-type virus, the D320E mutant virus replicates efficiently in CypA-knockdown cells [70, 71], suggesting that the mutation somehow renders HCV less dependent on CypA to replicate in hepatoma cells in vitro. Importantly, we and others showed that the D320E mutation does not influence NS5A binding to CypA or the sensitivity of CypA-NS5A interactions to Cyp inhibitors [68–71]. Interestingly, comparative NMR studies with NS5A peptides that contain D320 or E320 revealed a shift in population between the major and minor conformers [71]. These data suggest that the D320E mutation confers low-level resistance to Cyp inhibitors (i.e., alisporivir), likely by reducing the need for CypA-dependent isomerisation of NS5A. It is worthy to mention that the RNA binding of the domain II of NS5A that contains the D320E mutation was unaffected by CypA [72].

More recently, the emergence of Cyp inhibitor-resistant variants was examined in vivo. As mentioned above, in a Phase IIb study, GT1 treatment-naïve patients receiving alisporivir in combination with pIFN α /RBV achieved 76% SVR [30]. Importantly, in the same study, viral breakthrough events were examined. A total of 215 intent-to-treat patients (43 GT1a; 172 GT1b) were treated with alisporivir in combination with pIFN α /RBV. While on full-dose of alisporivir, 6/215 (2.8%) patients (1 GT1a; 5 GT1b) experienced viral breakthrough, compared with 4/73 (5.5%) patients in the control (placebo with pIFN α /RBV) arm [82]. No viral breakthrough was observed until treatment week 12. In 3 of 6 alisporivir-treated patients, viral breakthrough occurred after pIFN α /RBV dose adjustment/stoppage; in 2 of the other 3 viral breakthroughs, pharmacokinetics analysis revealed suboptimal alisporivir exposure. Population sequencing of HCV genome did not identify any genotypic change consistently associated with viral breakthrough, confirmed by clonal sequencing of NS5A, the putative in vivo viral target of CypA. Interestingly, the D320E mutation was seen at the time of initial viral breakthrough in 1 patient [84]. However, phenotypic assays demonstrated only a slight (~3-fold) decrease in susceptibility to alisporivir with GT1b replicons bearing D320E alone or the entire NS5A gene of the patient isolate. These data suggest that the emergence of D320E or viral resistance is not the primary cause of viral breakthrough. Importantly, a number of mutations that confer resistance to DAAs including

NS5A inhibitors were seen at baseline for patients, who achieved RVR and subsequently SVR24 with alisporivir [84], supporting the *in vitro* data of lack of cross-resistance between alisporivir and DAAs. Altogether these data strongly suggest a low potential for development of resistance to Cyp inhibitors (exemplified by alisporivir) in treated patients.

THE FUTURE OF CYCLOPHILIN INHIBITORS

Several lines of evidence suggest that Cyp inhibitors represent a promising class of novel and alternate anti-HCV agents. First, CypA has been shown to be optional for cell growth and survival, validating the potential of Cyps as therapeutic targets. Second, Cyp inhibitors provide a high barrier to the development of resistance. To date, very low viral breakthrough was observed, and no genotypic change was constantly associated with viral breakthrough. Third, the AE mediated by Cyp inhibitors such as alisporivir are mild and transient [29, 30]. Fourth, in contrast to protease inhibitors, Cyp inhibitors exhibit pan-genotypic anti-HCV activity.

In order to treat the broadest possible patient populations, the ultimate goal is to develop a pIFN α /RBV-free therapy resulting in a potent antiviral response in the absence of drug-resistance. One possibility to reach this goal is to identify a cocktail of drugs with different mechanisms of action, with non-overlapping resistance profiles, which target both viral and host factors. Therefore, an attractive future combination of an HTA (i.e., a Cyp inhibitor) as part of a cocktail with DAAs (i.e. NS3 and NS5B inhibitors) may constitute the backbone of a new, safe and effective pIFN α /RBV-free regimen.

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REFERENCES

1. Dienstag JL, McHutchison JG. American Gastroenterological Association technical review on the management of hepatitis C. *Gastroenterology* 2006; 130: 231–64 [PubMed: 16401486]
2. Emerging drugs for hepatitis C. Soriano V, Madejon A, Vispo E, Labarga P, Garcia-Samaniego J, Martin-Carbonero L, Sheldon J, Bottecchia M, Tuma P, Barreiro P. *Expert Opin Emerg Drugs*. 2008; 13: 1–19 [PubMed: 18321145]
3. Alter MJ. Epidemiology of hepatitis C virus infection. *World J Gastroenterol*. 2007;13:2436–41. [PubMed: 17552026]
4. Shepard CW, Finelli L, Alter MJ. Global epidemiology of hepatitis C virus infection. *Lancet Infect Dis*. 2005;5:558–67. [PubMed: 16122679]
5. The prevalence of hepatitis C virus infection in the United States, 1999 through 2002. Armstrong GL, Wasley AM, Simard EP, McQuillan GM, Kuhnert WL, Alter MJ *Ann Intern Med*. 2006; 144:705–14. [PubMed: 16702586]
6. Davis GL, Balart LA, Schiff ER, Lindsay K, Bodenheimer HC, Perrillo RP, Carey W, Jacobson IM, Payne J, Dienstag JL, Vanthiel DH et al. Treatment of chronic hepatitis C with recombinant interferon. A multicenter randomized, controlled trial. *N Engl J Med*. 1989; 321:1501–06. [PubMed: 2509916]
7. Sy T, Jamal MM. Epidemiology of hepatitis C virus (HCV) infection. *Int J Med Sci*. 2006; 3:41–6. [PubMed: 16614741]

8. Simmonds P, Bukh J, Combet C, Deleage G, Enomoto N, Feinstone S, Halfon P, Inchauspe G, Kuiken C, Maertens G, Mizokami M et al. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology*. 2005; 42:962–73. [PubMed: 16149085]
9. Cross TJ, Antoniadou CG, Harrison PM. Current and future management of chronic hepatitis C infection. *Postgrad Med J*. 2008; 84:172–6. [PubMed: 18424572]
10. Manns MP, Wedemeyer H, Cornberg M. Treating viral hepatitis C: efficacy, side effects, and complications. *Gut*. 2006; 55:1350–9. [PubMed: 16905701]
11. World Health Organization. Global surveillance and control of hepatitis C. Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium. *J Viral Hepat*. 1999; 6:35–47. [PubMed: 10847128]
12. Davis GL, Albright JE, Cook SF, Rosenberg DM. Projecting future complications of chronic hepatitis C in the United States. *Liver Transplant*. 2003; 9:331–338.
13. Deuffh-Burban S, Wong JB, Valleron AJ, Costagliola D, Delfraissy JF, Poynard T. Comparing the public health burden of chronic hepatitis C and HIV infection in France. *J Hepatol*. 2004; 40:319–326. [PubMed: 14739105]
14. Asselah T, Benhamou Y, Marcellin P. Protease and polymerase inhibitors for the treatment of hepatitis C. *Liver Int*. 2009; 29:57–67. [PubMed: 19207967]
15. Chevaliez S, Pawlotsky JM. Chapter 1: HCV genome and life cycle Hepatitis C Viruses: Genome Mol Biol (Ed: Tan SL) 2006; Horizon Bioscience, Norfolk, UK 5–47.
16. He Y, Staschke KA, Tan SL. In: Tan SL, editor. HCV NS5A: A Multifunctional Regulator of Cellular Pathways and Virus Replication Hepatitis C Viruses: Genomes and Molecular Biology. 2006; Norfolk (UK): Horizon Bioscience: Chapter 9.
17. Foster GR, Hézode C, Bronowicki JP, Carosi G, Weiland O, Verlinden L, van Heeswijk R, van Baelen B, Picchio G, Beumont M. Telaprevir alone or with peginterferon and ribavirin reduces HCV RNA in patients with chronic genotype 2 but not genotype 3 infections. *Gastroenterology*. 2011; 141:881–9.e1. [PubMed: 21699786]
18. Pawlotsky JM. Treatment failure and resistance with direct-acting antiviral drugs against hepatitis C virus. *Hepatology*. 2011; 53:1742–51. doi: 10.1002/hep.24262. [PubMed: 21374691]
19. Gane EJ, Stedman CA, Hyland RH, Sorensen RD, Symonds WT, Hindes RG, Berey MM. Once Daily PSI-7977 plus RBV: Pegylated interferon-Alfa not required for Complete Rapid viral response in Treatment-naïve Patients with HCV GT2 or GT3. 2011; 62nd Annual Meeting of the American Association for the Study of Liver Disease San Francisco Abstract 34.
20. Chayama K, Takahashi S, Kawakami Y, et al. Dual Oral Combination Therapy with the NS5A Inhibitor BMS-790052 and the NS3 Protease Inhibitor BMS-650032 Achieved 90% Sustained Virologic Response (SVR12) in HCV Genotype 1b-Infected Null Responders. 2011; 62nd Annual Meeting of the American Association for the Study of Liver Disease San Francisco Abstract LB-4.
21. Cyclophilin inhibitors for the treatment of HCV infection. Fischer G, Gallay P, Hopkins S CURR OPIN INVESTIG DRUGS 2010 11 8 911–918.
22. Flisiak R, Dumont JM, Crabbé R. Cyclophilin inhibitors in hepatitis C viral infection. 2007; Expert Opin Investig Drugs. 16:1345–54.
23. Gallay PA. Cyclophilin inhibitors. *Clin Liver Dis*. 2009; 13:403–17. [PubMed: 19628157]
24. Pockros PJ. New direct-acting antivirals in the development for hepatitis C virus infection. *Therap Adv Gastroenterol*. 2010; 3:191–202.
25. Lin K Development of novel antiviral therapies for hepatitis C virus. *Virol Sin*. 2010; 25:246–66. [PubMed: 20960299]
26. von Hahn T, Ciesek S, Manns MP. Arrest all accessories--inhibition of hepatitis C virus by compounds that target host factors. *Discov Med*. 2011; 12:237–44. [PubMed: 21955851]
27. Vermehren J, Sarrazin C. New hepatitis C therapies in clinical development. *Eur J Med Res*. 2011; 16:303–14. [PubMed: 21813371]
28. Flisiak R, Horban A, Gallay P, Bobardt M, Selvarajah S, Wiercinska-Drapalo A, Siwak E, Cielniak I, Higersberger J, Kierkus J, Aeschlimann C, Groscurin P, Nicolas-Métral V, Dumont JM, Porchet H, Crabbé R, Scalfaro P. The cyclophilin inhibitor Debio 025 shows potent anti-hepatitis C effect in patients coinfecting with hepatitis C and human immunodeficiency virus. *Hepatology*. 2008; 47:817–26. [PubMed: 18302285]

29. Flisiak R, Feinman SV, Jablkowski M, et al. Efficacy and Safety of Increasing Doses of the Cyclophilin Inhibitor Debio 025 in Combination with Pegylated Interferon Alpha-2a in Treatment Naïve Chronic HCV Patients. 43rd Annual Meeting of the European Association for the Study of the Liver 2008; Milan.
30. Flisiak R, Pawlotsky JM, Crabbé R, Calistru PI, Kryczka W, Haüssinger D, Mazzella G, Romero-Gomez M, Purcea D, Vuagniaux G, Bao W et al. Once daily Alisporivir (DEB025) plus peg-IFN-2a/ribavirin results in superior sustained virologic response (SVR24) in chronic hepatitis c genotype 1 treatment naïve patients. Ann Meet Eur Assoc Study Liver 2011; Abstract 46.
31. Vertex Incivek Prescribing Information. 2011
32. Merck Victrelis Prescribing information. 2011
33. Pawlotsky JM, Flisiak R, Rasenack J, Sarin SK, Piratvisuth T, Chuang WL, Foster G, Wedemeyer H, Hezode C, Zhang W, Avila C, Naoumov NV. Once Daily Alisporivir Interferon (IFN)-free Regimens Achieve High Rates of Early HCV Clearance in Previously Untreated Patients with HCV Genotype (G) 2 or 3. American Association for Study of the Liver Congress, San Francisco 2011; Abstract LB-11.
34. World Health Organization Global Alert and Response, Hepatitis C - Diseases, The Hepatitis C Virus. Available at: www.who.int/csr/disease/hepatitis/whocdscsrlyo2003/en/index2.html. 2003.
35. Germer JJ, Mandrekar JN, Bendel JL, Mitchell PS, Yao JD. Hepatitis C virus genotypes in clinical specimens tested at a national reference testing laboratory in the United States. J Clin Microbiol. 2011; 49:3040–3. [PubMed: 21613437]
36. Lawitz E, Godofsky E, Rouzier R, Marbury T, Nguyen T, Ke J, Huang M, Praestgaard J, Serra D, Evans TG. Safety, pharmacokinetics, and antiviral activity of the cyclophilin inhibitor NIM811 alone or in combination with pegylated interferon in HCV-infected patients receiving 14 days of therapy. Antiviral Res. 2011;89:238–45. [PubMed: 21255610]
37. Hopkins S, Scorneaux B, Sluder A, Ribeill Y. The non-immunosuppressive cyclophilin inhibitor SCY-635 exerts clinical antiviral activity by upregulating the expression of endogenous interferons. American Association for Study of the Liver Congress, San Francisco 2011.
38. Gregory MA, Moss SJ, Coates N, Foster T, Bobardt M, Chatterji U, Gallay PA, Leyssen P, Neyts J, Nur-E-Alam M, Pirae M, Suthar D, Warneck T, Zhang M-Q, and Wilkinson B Preclinical characterization of naturally occurring polyketide cyclophilin inhibitors from the sanglifehrin family. Antimicrobial Agents and Chemotherapy. 2011; 55:1975–81. [PubMed: 21383094]
39. Moss SJ, Bobardt M, Leyssen P, Coates N, Chatterji U, Dejian X, Foster T, Liu J, Nur-e-Alam M, Suthar D, Yongsheng C, Warneck T, Neyts J, Gallay P, Wilkinson B, Gregory MA. Sangamides, a new class of cyclophilin inhibiting host targeted antivirals for treatment of HCV infection. Med. Chem. Commun. 2011; DOI: 10.1039/C1MD00227A.
40. Owens CM, Brasher BB, Polemeropoulos A, Long J, Gao X, Qiu Y-L, Wang G, Jiang L-J, Or YS. EP-CyP546 is a Potent Non-Immunosuppressive Cyclophilin Inhibitor with an Excellent Preclinical Profile. American Association for Study of the Liver Congress, San Francisco 2011; Abstract 367.
41. Ahmed-Belkacem A, Colliandre L, Bessin Y, Barthe P, Bourget W, Douguet D, Ahnou N, Pawlotsky J-M, Guichou J-F. Rational design of a novel family of non-peptidic cyclophilin inhibitors that potently inhibit HCV replication by fragment-based drug design. 17th International Symposium on Hepatitis C Virus & Related Viruses; Yokohama, Japan 2010; Abstract P-45.
42. Watashi K, Hijikata M, Hosaka M, et al. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. Hepatology 2003;38:1282–8. [PubMed: 14578868]
43. Nakagawa M, Sakamoto N, Enomoto N, et al. Specific inhibition of hepatitis C virus replication by cyclosporin A. Biochem Biophys Res Commun 2004;313:42–7. [PubMed: 14672695]
44. Goto K, Watashi K, Murata T, et al. Evaluation of the anti-hepatitis C virus effects of cyclophilin inhibitors, cyclosporin A, and NIM811. Biochem Biophys Res Commun. 2006;343:879–84. [PubMed: 16564500]
45. Ishii N, Watashi K, Hishiki T, et al. Diverse effects of cyclosporine on hepatitis C virus strain replication. J Virol. 2006;80:4510–20. [PubMed: 16611911]

46. Ma S, Boerner JE, TiongYip C, et al. NIM811, a cyclophilin inhibitor, exhibits potent in vitro activity against hepatitis C virus alone or in combination with alpha interferon. *Antimicrob Agents Chemother.* 2006;50:2976–82. [PubMed: 16940091]
47. Paeshuyse J, Kaul A, De Clercq E, et al. The non-immunosuppressive cyclosporin DEBIO 025 is a potent inhibitor of hepatitis C virus replication in vitro. *Hepatology.* 2006;43:761–70. [PubMed: 16557546]
48. Houck DR, Hopkins S preclinical evaluation of SCY-635, a cyclophilin inhibitor with potent anti-HCV activity. *Hepatology.* 2006;44: Suppl. 1, Abstract 934.
49. Suppression of hepatitis C virus replication by cyclosporin A is mediated by blockade of cyclophilins. Nakagawa M, Sakamoto N, Tanabe Y, Koyama T, Itsui Y, Takeda Y, Chen CH, Kakinuma S, Oooka S, Maekawa S, Enomoto N, Watanabe M. *Gastroenterology.* 2005;129:1031–41. [PubMed: 16143140]
50. Multiple cyclophilins involved in different cellular pathways mediate HCV replication. Gaither LA, Borawski J, Anderson LJ, Balabanis KA, Devay P, Joberty G, Rau C, Schirle M, Bouwmeester T, Mickanin C, Zhao S, Vickers C, Lee L, Deng G, Baryza J, Fujimoto RA, Lin K, Compton T, Wiedmann B. *Virology.* 2010;397:43–55. [PubMed: 19932913]
51. Yang F, Robotham JM, Nelson HB, Irsigler A, Kenworthy R, Tang H. Cyclophilin A is an essential cofactor for hepatitis C virus infection and the principal mediator of cyclosporine resistance in vitro. *J Virol.* 2008;82:5269–78. [PubMed: 18385230]
52. Chatterji U, Bobardt M, Selvarajah S, Yang F, Tang H, Sakamoto N, Vuagniaux G, Parkinson T, Gallay P. The isomerase active site of cyclophilin A is critical for hepatitis C virus replication. *J Biol Chem.* 2009;284:16998–7005. [PubMed: 19380579]
53. Kaul A, Stauffer S, Berger C, Pertel T, Schmitt J, Kallis S, Zayas M, Lohmann V, Luban J, Bartenschlager R. Essential role of cyclophilin A for hepatitis C virus replication and virus production and possible link to polyprotein cleavage kinetics. *PLoS Pathog.* 2009;5:e1000546.
54. Wang P, Heitman J. The cyclophilins. *Genome Biol.* 2005;6:226. [PubMed: 15998457]
55. Liu Z, Yang F, Robotham JM, Tang H. Critical role of cyclophilin A and its prolyl-peptidyl isomerase activity in the structure and function of the hepatitis C virus replication complex. *J Virol.* 2009;83:6554–65. [PubMed: 19386705]
56. Chatterji U, Bobardt MD, Lim P, Gallay PA. Cyclophilin A-independent recruitment of NS5A and NS5B into hepatitis C virus replication complexes. *J Gen Virol.* 2010;91:1189–93. [PubMed: 20107018]
57. Handschumacher RE, Harding MW, Rice J, et al. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. *Science.* 1984;226:544–7. [PubMed: 6238408]
58. Flechner SM. Cyclosporine: a new and promising immunosuppressive agent. *Urol Clin North Am.* 1983;10:263–75. [PubMed: 6304967]
59. Lang K, Schmid FX, Fischer G. Catalysis of protein folding by prolyl isomerase. *Nature* 1987;329:268–70. [PubMed: 3306408]
60. Fischer G, Bang H, Berger E, Schellenberger A. Conformational specificity of chymotrypsin toward proline-containing substrates. *Biochim Biophys Acta.* 1984;791:87–97. [PubMed: 6498206]
61. Fischer G, Wittmann-Liebold B, Lang K, et al. Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins. *Nature.* 1989;337:476–8. [PubMed: 2492638]
62. Ke HM, Zydowsky LD, Liu J, et al. Crystal structure of recombinant human T-cell cyclophilin A at 2.5 Å resolution. *Proc Natl Acad Sci U S A.* 1991;88:9483–7. [PubMed: 1946361]
63. Zydowsky LD, Etzkorn FA, Chang HY, Ferguson SB, Stolz LA, Ho SI, Walsh CT. Active site mutants of human cyclophilin A separate peptidyl-prolyl isomerase activity from cyclosporin A binding and calcineurin inhibition. *Protein Sci* 1992;1:1092–9. [PubMed: 1338979]
64. Colgan J, Asmal M, Yu B, Luban J. Cyclophilin A-deficient mice are resistant to immunosuppression by cyclosporine. *J Immunol.* 2005;174:6030–8. [PubMed: 15879096]
65. Braaten D, Luban J. Cyclophilin A regulates HIV-1 infectivity, as demonstrated by gene targeting in human T cells. *EMBO J* 2001;20:1300–9. [PubMed: 11250896]
66. Hanouille X, Badillo A, Wieruszeski JM, Verdegem D, Landrieu I, Bartenschlager R, Penin F, Lippens G. Hepatitis C virus NS5A protein is a substrate for the peptidyl-prolyl cis/trans

- isomerase activity of cyclophilins A and B. *J Biol Chem.* 2009;284:13589–601. [PubMed: 19297321]
67. Waller H, Chatterji U, Gallay P, Parkinson T, Targett-Adams PJ. The use of AlphaLISA technology to detect interaction between hepatitis C virus-encoded NS5A and cyclophilin A. *Virol Methods.* 2010;165:202–10.
 68. Fernandes F, Ansari IU, Striker R. Cyclosporine inhibits a direct interaction between cyclophilins and hepatitis C NS5A. *PLoS One.* 2010;5:e9815. [PubMed: 20352119]
 69. Chatterji U, Lim P, Bobardt MD, Wieland S, Cordek DG, Vuagniaux G, Chisari F, Cameron CE, Targett-Adams P, Parkinson T, Gallay PA. HCV resistance to cyclosporin A does not correlate with a resistance of the NS5A-cyclophilin A interaction to cyclophilin inhibitors. *J Hepatol.* 2010;53:50–6. [PubMed: 20451281]
 70. Yang F, Robotham JM, Grise H, Frausto S, Madan V, Zayas M, Bartenschlager R, Robinson M, Greenstein AE, Nag A, Logan TM, Bienkiewicz E, Tang H. A major determinant of cyclophilin dependence and cyclosporine susceptibility of hepatitis C virus identified by a genetic approach. *PLoS Pathog.* 2010;6:e1001118.
 71. Coelmont L, Hanouille X, Chatterji U, Berger C, Snoeck J, Bobardt M, Lim P, Vliegen I, Paeshuyse J, Vuagniaux G, Vandamme AM, Bartenschlager R, Gallay P, Lippens G, Neyts. DEB025 (Alisporivir) inhibits hepatitis C virus replication by preventing a cyclophilin A induced cis-trans isomerisation in domain II of NS5A. *J. PLoS One.* 2010;5:e13687.
 72. Foster TL, Gallay P, Stonehouse NJ, Harris M. Cyclophilin A interacts with domain II of hepatitis C virus NS5A and stimulates RNA binding in an isomerase-dependent manner. *J Virol.* 2011;85:7460–4. [PubMed: 21593166]
 73. Verdegem D, Badillo A, Wieruszeski JM, Landrieu I, Leroy A, Bartenschlager R, Penin F, Lippens G, Hanouille X. Domain 3 of NS5A protein from the hepatitis C virus has intrinsic alpha-helical propensity and is a substrate of cyclophilin A. *J Biol Chem.* 2011;286:20441–54. [PubMed: 21489988]
 74. Fernandes F, Poole DS, Hoover S, Middleton R, Andrei AC, Gerstner J, Striker R. Sensitivity of hepatitis C virus to cyclosporine A depends on nonstructural proteins NS5A and NS5B. *Hepatology.* 2007;46:1026–33. [PubMed: 17600342]
 75. Robida JM, Nelson HB, Liu Z, Tang H. Characterization of hepatitis C virus subgenomic replicon resistance to cyclosporine in vitro. *J Virol.* 2007;81:5829–40. [PubMed: 17376913]
 76. Hopkins S, Scorneaux B, Huang Z, et al. The Genetic and Biochemical Basis for Resistance to SCY-635. 59th Annual Meeting of the American Association for the Study of Liver Diseases; San Francisco: 2008 Abstract 1814.
 77. Hopkins S, Huang Z, Mosier S, Chatterji U, Gallay P. The Non-Immunosuppressive Cyclophilin Inhibitor SCY-635 Inhibits the Association of NS5A and Cyclophilin A. American Association for Study of the Liver Congress, Boston 2010; Abstract 367.
 78. Badillo A, Receveur-Brechot V, Miron S, Hanouille X, Molle J, Montserret R, Bartenschlager R, Lippens G, Ricard-Blum S, Penin F. Overall structural model of non-structural protein 5A from hepatitis C virus and modulation by cyclophilin A. 18th International Symposium on Hepatitis C Virus & Related Viruses; Seattle, Washington 2011; Abstract 09.14.
 79. Hanouille X, Huvent I, Rosnoblet C, Verdegem D, Dujardin M, Badillo A, Coelmont L, Fritzinger B, Wieruszeski J-M, Landrieu I, Neyts J, Bartenschlager R, Penin F, Lippens G. HCV NS5A: NMR analyses of its interactions with NS5B and cyclophilin A and molecular characterization of a cyclophilin inhibitor-resistance mutation. 18th International Symposium on Hepatitis C Virus & Related Viruses; Seattle, Washington 2011; Abstract 08.02.
 80. Shirota Y, Luo H, Qin W, Kaneko S, Yamashita T, Kobayashi K, Murakami S. Hepatitis C virus (HCV) NS5A binds RNA-dependent RNA polymerase (RdRP) NS5B and modulates RNA-dependent RNA polymerase activity. *J Biol Chem.* 2002;277:11149–55. [PubMed: 11801599]
 81. He Y, Staschke KA, Tan SL. In: Tan SL, editor. HCV NS5A: A Multifunctional Regulator of Cellular Pathways and Virus Replication. *Hepatitis C Viruses: Genomes and Molecular Biology.* Norfolk (UK): Horizon Bioscience 2006; Chapter 9.

82. Puyang X, Poulin DL, Mathy JE, Anderson LJ, Ma S, Fang Z, Zhu S, Lin K, Fujimoto R, Compton T, Wiedmann B. Mechanism of resistance of hepatitis C virus replicons to structurally distinct cyclophilin inhibitors. *Antimicrob Agents Chemother*. 2010;54:1981–7. [PubMed: 20176894]
83. Goto K, Watashi K, Inoue D, Hijikata M, Shimotohno K. Identification of cellular and viral factors related to anti-hepatitis C virus activity of cyclophilin inhibitor. *Cancer Sci*. 2009;100:1943–50. [PubMed: 19659609]
84. Li B, Snoeck J, Tang Y, Jones CT, Tiongyip C, Bao W, Yu J, Vandamme A, Vuagniaux G, Crabbe R, Avila C, Naoumov NV, Lin K. Alisporivir - A Host-targeting Antiviral, Provides Low Viral Breakthrough Rate and High Barrier to Resistance in HCV Genotype 1 Treatment-naïve Patients in the Phase IIb ESSENTIAL Study. American Association for Study of the Liver Congress, San Francisco 2011.

Cyclophilin Inhibitors	Structure	Immuno-suppression Activity (IL-2 inhibition)	CypA PPIase Inhibition (Ki)	Anti-HCV Activity (EC ₅₀)	Phase I	Phase II	Phase III	Adverse Events
Cyclosporine A		0.006 µg/mL	9.79 nM	0.3 µM				No data
NIM811		>10 µg/mL	2.11 nM	0.06 µM			Halted	No data
Alisporivir		21.5 µg/mL	0.34 nM	0.04 µM			Fully enrolled	Transitional hyper bilirubinemia
SCY-635		13 µg/mL	1.8 nM	0.1 µM				No data
Sanglifehrin A			2.4 nM	0.32 µM				No data
Sanglifehrin B			0.32 nM	0.07 µM				No data
Sanglifehrin C			6.8 nM	1.6 µM				No data
Sanglifehrin D			6.9 nM	0.26 µM				No data
1,2-oxezinane amide (Sangamide)			2.7 nM	0.1 µM				No data
Preclinical Studies					Clinical Studies			

Figure 1:
Preclinical and Clinical Properties of Cyp Inhibitors

Figure 2:
Putative Mechanisms of Antiviral Action of Cyp Inhibitors