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Review

Curing a viral infection by targeting the host: The example of cyclophilin inhibitors

Kai Lin [a,*], Philippe Gallay [b]

[a] Permeon Biologics, Inc., One Kendall Square, Cambridge, MA 02139, USA
[b] Department of Immunology & Microbial Science, The Scripps Research Institute, La Jolla, CA 92037, USA

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ABSTRACT

Every step of the viral life cycle is dependent on the host, which potentially can be explored for antiviral targets. Historically, however, drug discovery has focused mainly on viral targets, because of their perceived specificity. Efforts to pursue host targets have been largely hampered by concern over potential on-target toxicity, the lack of predictive cell culture and animal models, and the complexity of host–virus interactions. On the other hand, there are distinct advantages of targeting the host, such as creating a high barrier to resistance, providing broad coverage of different genotypes/serotypes and possibly even multiple viruses, and expanding the list of potential targets, when druggable viral targets are limited. Taking hepatitis C virus (HCV) as the example, there are more than 20 inhibitors of the viral protease, polymerase and NS5A protein currently in advanced clinical testing. However, resistance has become a main challenge with these direct-acting antivirals, because HCV, an RNA virus, is notoriously prone to mutation, and a single mutation in the viral target may prevent the binding of an inhibitor, and rendering it ineffective. Host cyclophilin inhibitors have shown promising effects both *in vitro* and in patients to prevent the emergence of resistance and to cure HCV infection, either alone or in combination with other agents. They are also capable of blocking the replication of a number of other viral pathogens. While the road to developing host-targeting antivirals has been less traveled, and significant challenges remain, delivering the most effective antiviral regimen, which may comprise inhibitors of both host and viral targets, should be well worth the effort.

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1. Introduction

Viruses, particularly those with small genomes, encode only a few proteins of their own and depend on the host for many essential functions. Natural selection has therefore favored viruses that most efficiently hijack the host machinery. Hepatitis C virus (HCV) is a prime example. It enters cells by binding to the cell surface receptors CD81 (Pileri et al., 1998), human scavenger receptor class B type I (Scarselli et al., 2002), tight junction protein claudin-1 (Evans et al., 2007) and occludin (Ploss et al., 2009). Following decapsidation and release of its positive-strand RNA genome, the

* Corresponding author. Tel.: +1 781 999 4168.
E-mail address: kai.lin@ymail.com (K. Lin).

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viral polyprotein is translated from the internal ribosomal entry site (IRES) at 5′-UTR by recruiting a number of cellular proteins including the eukaryotic initiation factors (Hellen and Pestova, 1999). The polyprotein is subsequently cleaved into individual viral proteins by the host signal peptidase and peptide peptidase, as well as viral proteases (Pène et al., 2009). A replication complex, formed by both viral nonstructural proteins and cellular co-factors on endoplasmic reticulum (ER)-derived membrane, synthesizes both negative- and positive-strand viral RNA (Romero-Brey et al., 2012). Virions containing the positive-strand viral RNA genome are assembled on ER-associated lipid droplets (LD) where the viral core protein is located, then bud through the ER lumen and Golgi to be released (Lindenbach 2013). In summary, essentially every step of the HCV life cycle is dependent on the host.

Given that host interactions are critical for viral replication, one would imagine that many host factors could be potential antiviral targets. However, antiviral drug discovery has historically been focused mainly on viral targets. Almost all currently approved antiviral drugs (excluding immunomodulators) and those under development target viral proteins. For example, all five licensed hepatitis B virus (HBV) inhibitors are nucleos(t)ide polymerase inhibitors (Fung et al., 2011). All herpesvirus drugs target the viral DNA polymerases (Price and Prichard, 2011). Currently there are only two classes of inhibitors for influenza, viral neuraminidase inhibitors and M2 channel blockers (Barik 2012). More than two dozen human immunodeficiency virus type-1 (HIV-1) drugs have been approved targeting the viral reverse transcriptase, protease or integrase (De Clercq 2010). The only host-targeting antiviral that has been successfully developed is maraviroc, a CCR5 inhibitor blocking HIV-1 entry (Dorr et al., 2005).

Hepatitis C has been the most active area for antiviral drug development over the past two decades, with two drugs already approved that inhibit the viral NS3 protease, and many more in development targeting the viral NS5B polymerase and NS5A protein. On the other hand, resistance has become a main challenge with these direct-acting antivirals, because of the high replication and mutation rates of the virus. An alternative and complementary strategy is to target host factors essential for viral replication, which may create a higher genetic barrier to resistance and could be used in combination with viral inhibitors. This review discusses opportunities and challenges in developing host-targeting antivirals, using cyclophilin inhibitors as a specific example.

2. Discovery of host antiviral targets

Both systemic screens and rational approaches have been employed to identify novel host factors that are essential for viral replication. Efforts have been taken to screen for cellular proteins that directly interact with specific proteins of HIV-1 (Jäger et al., 2011), influenza virus (Sharma et al., 2011), or HCV (Taguwa et al., 2008; Wang et al., 2006; Huang et al., 2013). However, methods such as yeast-two hybrid screening often result in hits that do not necessarily have functional relevance. Thus, it is important to confirm the finding through either loss (e.g. siRNA) or gain (e.g. ectopic expression) of function studies.

The most common approach is to screen a host siRNA library for targets that lead to an inhibition of viral replication when knocked down. Multiple screens have been performed for HIV-1 (Brass et al., 2008; Zhou et al., 2008), influenza virus (König et al., 2010), and HCV (Li et al., 2009; Tai et al., 2009). Interestingly, the percentage of overlapping hits from screens done by different groups on the same virus has been surprisingly low, which could result either from different assay conditions and/or a high rate of false positives. There were a few exceptions. For example, PI4Kα was reported as a hit in almost every HCV screen performed using either a subgenomic replicon or full-length infectious virus (Borawski et al., 2009; Tai et al., 2009; Berger et al., 2009; Trotard et al., 2009; Vaillancourt et al., 2009; Reiss et al., 2011). Such a reproducible hit suggests that the target is less likely to be affected by the conditions of the cells or assays, and may thus represent a more attractive candidate for drug discovery.

Whole cell-based phenotypic screens of large compound libraries have also generated a lot of leads, but with unknown targets or mechanisms (Kim et al., 2007; Chockalingam et al., 2010; Gastaminza et al., 2010). Compounds that inhibit viral targets can be easily ruled out by secondary screens; the remaining hits could then be candidates to identify potential host targets. The challenge is to deconvolute the pathway and identify the target, using chemogenetic approaches such as pull-down of the protein target using a labeled inhibitor. The advantage of such an approach is that, once the target is confirmed, the lead compounds have already been identified as potential chemistry starting points. Transcriptional and proteomic profiling comparing virus-infected cells to uninfected cells, or drug-treated vs. untreated cells, could also generate rich information on host–virus interactions (Su et al., 2002; Jacobs et al., 2005; Xu et al., 2012).

Sometimes targets are being pursued based on a specific hypothesis. Ribavirin was known to enhance the antiviral response when being used in combination with IFN-α. While the exact mechanism of ribavirin remains unclear, it is a weak inhibitor of inosine-5′-monophosphate dehydrogenase (IMPDH), which catalyzes the conversion of IMP to XMP, an essential step in the *de novo* biosynthesis of guanine nucleotides. Inhibition of IMPDH leads to a depletion of intracellular GTP pools and thus blocks viral replication. This hypothesis triggered the effort in developing a more potent and specific inhibitor of IMPDH, VX-497 (merimepodib), which indeed blocked HCV replication *in vitro* and showed some antiviral effect in patients (Markland et al., 2000; Marcellin et al., 2007).

A more focused approach is to analyze specific pathways that are known to be involved in viral replication. For example, it has been well characterized that HCV replicates on an ER-associated membrane web structure, and that HCV virions are assembled on ER-associated lipid droplets, both of which can be affected by host lipid biosynthesis (Romero-Brey et al., 2012; Lindenbach 2013). Thus, cellular proteins that are involved in lipid metabolism could be potential antiviral targets. Several studies have demonstrated that statins were able to inhibit HCV replication *in vitro* (Ikeda et al., 2006; Kim et al., 2007). A specific inhibitor of diglyceride acyltransferase-1 (DGAT-1) was reported to block HCV virion assembly and release (Herker et al., 2010). More recently, fatty acid synthase was proposed as another host antiviral target (Evanchik et al., 2012; Huang et al., 2013). Pathways involved in HCV replication, potential host targets and their known inhibitors are summarized in Table 1.

3. Myths and realities of host targets

While many host factors are known to be essential for viral replication, few have exploited for drug development because of the perceived hurdles (Table 2). The most common myth is that host targets are not specific, and thus may cause more toxicity. While inhibition of the normal cellular function of a host protein could potentially lead to target-related toxicities, there are many ways in which such a risk can be mitigated. First, a rapidly multiplying virus likely has a very different threshold for certain protein functions from those of normal cells, as reflected in the fact that many cellular pathways and genes are up-regulated in infected cells. It is therefore possible that knocking down these targets to their “normal” level may be sufficient to block viral replication, while main-

Table 1
Cellular pathways involved in HCV replication, potential antiviral targets, and their known inhibitors.

Pathways	Targets	Inhibitors	References
Nucleotide metabolism	IMPDH	VX-497, MPA	Markland et al., (2000) and Pan et al. (2012)
	DHODH	Leflunomide	Hoffmann et al. (2011)
Lipid biosynthesis & metabolism	HMG–CoA	Statins	Ikeda et al. (2006)
	DGAT-1	PF4620110, LCQ908	Herker et al. (2010)
	NPC1L1	Ezetimibe	Sainz et al. (2012)
	FAS	TVB-2640	Evanchik et al. (2012) and Huang et al. (2013)
Kinases & signaling	EGFR	Erlotinib	Lupberger et al. (2011)
	PKA, PI3K	Wortmannin	Farquhar et al. (2008) and Matsumoto et al. (2009)
Membrane vesicle trafficking	PI4K	PIK93, AL-9	Borawski et al. (2009) and Bianco et al. (2012)
	VAP-A/B, Rab/GAP/ARF	Brefeldin A	Goueslain et al. (2010)
Protein translation & modification	miR122	Miravirsen	Jopling et al. (2005) and Lanford et al. (2010)
	α-glucosidase I	Celgosivir	Durantel (2009)
	HSP90	Geldanamycin	Okamoto et al. (2006)
	eIF2α	Nitazoxanide	Elazar et al. (2009)
Autophagy	Atg5/Atg7/LC3	Chloroquine	Mizui et al. (2010)

Abbreviations: IMPDH = inosine-5′-monophosphate dehydrogenase; DHODH = dihydroorotate dehydrogenase; MPA = mycophenolic acid; HMG-CoA = 3-hydroxy-3-methyl-glutaryl-coenzyme A; DGAT-1 = Diacylglycerol acyltransferase 1; NPC1L1 = Niemann-Pick C1-Like 1; FAS = fatty acid synthase; EGFR = epidermal growth factor receptor; PKA = protein kinase A; PKC = protein kinase C; PI3K = phosphatidylinositol 3-kinase; PI4K = phosphotidylinositol 4-kinase; VAP-A/B = vesicle-associated membrane protein-associated protein subtype A/B; Rab = Rab GTPase; GAP = GTPase-activating proteins; ARF = ADP ribosylation factor; miR122 = microRNA-122; HSP90 = heat shock protein 90; eIF2α = eukaryotic initiation factor 2α; Atg5 = autophagy related protein 5; Atg7 = autophagy related protein 7; LC3 = microtubule-associated protein light chain 3.

Table 2
Potential advantages ("pros") and disadvantages ("cons") of host targets for antiviral therapy.

Pros	Cons
High barrier to resistance	Potential on-target toxicity
Broad genotype/serotype coverage	Poor translation of *in vitro* to *in vivo*
Potential broad-spectrum antivirals	Effect of host polymorphism
Number of druggable targets	Complex mechanism of action
Fast development path for known inhibitors	

taining cellular function. Second, there are often redundant pathways or proteins for a given cellular function. Inhibition of a particular target, unique to the virus, could be compensated by another cellular protein with overlapping function. Third, many cellular genes are actually non-essential, so that their inhibition may be tolerated, depending on the concentration and duration of the treatment. While there is a risk of target-related toxicity, this does not differ from other types of drug discovery. The objective remains the same: to identify the optimal dose of a drug that achieves the desired therapeutic effect, without incurring significant toxicity.

Another "myth" is that host targets are often merely *in vitro* artifacts, with poor translation to *in vivo* or clinical efficacy, mainly because the function of host targets is more likely to be affected by *in vitro* cell culture conditions or the animal models employed. If there is a significant difference in the target or pathway *in vitro* and *in vivo*, one might see a poor correlation of the effect. For example, the IMPDH inhibitor VX-497 potently inhibited HCV replication *in vitro*, but its efficacy in patients was more limited and variable (McHutchison et al., 2005; Rustgi et al., 2009). One possible explanation is that the level and supply of nucleotides, and thus the dependence of viral replication on their *de novo* synthesis, are very different *in vitro* and *in vivo*, which could significantly affect the efficacy of inhibitors modulating nucleotide metabolism. This is also true for compounds modulating pyrimidine biosynthesis, such DHODH (Hoffmann et al., 2011). Statins are another example; they showed strong anti-HCV activity *in vitro* (Ikeda et al., 2006), but gave largely disappointing results in clinical studies (Bader et al., 2008; Sezaki et al., 2009; Forde et al., 2009; O'Leary et al., 2007; Milazzo et al., 2009), probably because the antiviral effect of statins can be significantly affected by cellular levels of cholesterol or lipid, which are quite different *in vitro* and in patients. It is therefore indeed a challenge that host targets are more liable to the lack of predictive *in vitro* models. The impact of host polymorphism should also be examined. The mechanism of action of host-targeting inhibitors is usually much more complex and difficult to determine than inhibitors of viral targets.

On the other hand, there are significant advantages in pursuing host targets, especially the fact that host targets could provide a higher barrier to resistance than viral inhibitors. Taking HCV as the example, despite the success in developing specific inhibitors of viral targets, resistance has become a major challenge, because HCV, an RNA virus, is notoriously prone to mutation and resistance. The viral RNA-dependent RNA polymerase has no proof-reading function, resulting in a high error rate in synthesizing viral RNA of $\sim$1 mutation per viral genome produced (Powdrill et al., 2011). Combined with a high replication rate of $\sim 10^{12}$ virions per day, HCV exists as a large pool of variants or quasispecies in every patient (Ribeiro et al., 2012). Theoretically, this means that all "mutations" are already pre-existing before the start of antiviral treatment. Moreover, for most of the viral inhibitors discovered to date, a single mutation in a viral gene could affect the inhibitor binding site, conferring a high level of resistance. Resistance can therefore develop very quickly, both *in vitro* and in patients. A complementary and arguably better strategy is to target host factors that create a higher genetic barrier to resistance.

Host-targeting inhibitors can also help cover multiple viral genotypes or serotypes, since host factors are less likely to be affected by viral heterogeneity. Furthermore, because many viruses exploit the same cellular pathways for replication, it is not only perceivable, but already demonstrated *in vitro* that some host-targeting inhibitors are active against multiple viruses (Markland et al., 2000; Pfefferle et al., 2011). The concept of developing broad-spectrum antivirals is not only desirable, but probably necessary in situations such as respiratory infections, where the cause is not clear and a quick diagnosis is not available.

Host targeting is also the more feasible approach to tackle agents such as the polyomaviruses, in which druggable viral targets are not available or are very limited. Host factors involved in the pathogenesis of viral infections can also be explored as targets (Pastorino et al., 2010; McLay et al., 2013). Another potential advantage of host targets is that many have already been investigated and pursued for other clinical indications. Thus, known inhibitors are already available to validate the targets, and in some

cases, provide a faster development path. This was certainly the case with cyclophilin inhibitors, which we will now discuss.

4. Cyclophilin inhibitors

4.1. Discovery of cyclophilin as a novel target for HCV

Interest in pursuing cyclophilin as a potential target for HCV was triggered by an unexpected clinical finding. Cyclosporin A (CsA), an approved immunosuppressive drug for preventing graft rejection following solid organ transplantation, also has anti-inflammatory effects, and can be used to treat psoriasis and ulcerative colitis. In a clinical trial originally designed to investigate the benefit of CsA to suppress hepatitis-associated inflammation, it was surprisingly discovered that the combination of CsA and IFN-α2b resulted in a significantly greater antiviral response than IFN-α2b alone (Inoue et al., 2003). The function of cyclophilin in HCV replication and the direct antiviral activity of CsA were then confirmed *in vitro* (Watashi et al., 2003; Nakagawa et al., 2004). Knock-down of cyclophilins in replicon cells using specific siRNAs also led to a strong inhibition of HCV replication (Nakagawa et al., 2005; Yang et al., 2008; Gaither et al., 2010).

Cyclophilins, a family of highly conserved cellular peptidyl–prolyl cis–trans isomerases (PPIase), are involved in many cellular processes such as protein folding and trafficking. There are over 16 human cyclophilins with different subcellular localizations and functions (Wang and Heitman, 2005). Cyclophilin A (CypA), the first to be identified (Handschumacher et al., 1984), is the binding partner of CsA, which blocks T-cell activation and IL-2 production through the inhibition of calcineurin and NF-AT, although the PPIase function of CypA is not involved in these activities (Zydowsky et al., 1992). While CypA is one of the most abundant cytosolic proteins (~0.1% of all proteins), interestingly it is not essential to cells. CypA knock-out mice are generally healthy, with no decrease in life span (Colgan et al., 2004). The main side effect is allergic blepharitis, which has not been observed in humans treated with cyclophilin inhibitors. These findings suggest that a pharmacological inhibition of cyclophilin may be well tolerated.

The role of cyclophilin in HCV replication has been extensively studied since its discovery. The PPIase function of cyclophilin is to convert prolines in the protein from trans- to cis-form, which is the required conformation for trafficking of many proteins and/or the formation of multi-protein complexes; this function is essential for HCV replication (Chatterji et al., 2009; Liu et al., 2009b; Kaul et al., 2009). Furthermore, the domain II of HCV NS5A protein directly interacts with cyclophilins and is a substrate of CypA and CypB PPIase *in vitro* (Hanoulle et al., 2009).

As shown in the model illustrated in Fig. 1, current data suggest that cyclophilin inhibitors block viral replication mainly by disrupting the interaction of CypA and viral proteins, and thus the formation of an active replication complex. On the other hand, this is likely an over-simplified model, as other data suggest that cyclophilins play additional roles in the HCV life cycle. It was shown previously that CypB can also bind to the HCV NS5B protein directly and stimulate its RNA synthesis activity *in vitro* (Watashi et al., 2005; Heck et al., 2009). Another study suggested that CypA is recruited into the HCV replication complex via its interaction with NS5B (Liu et al., 2009b). Cyclophilins may also be involved in HCV polyprotein processing, specifically the self-cleavage of the NS2–NS3 junction (Ciesek et al., 2009) and the cleavage of the NS5A–NS5B junction by the NS3 protease (Kaul et al., 2009). Moreover, inhibition of cyclophilins may alter lipid trafficking and block virion secretion (Anderson et al., 2011). Recently it was reported that treatment with a cyclophilin inhibitor led to an enhanced secretion of type I and type III IFNs and an increased expression of IFN-stimulated genes (ISG), both *in vitro* and in patients, suggesting that cyclophilin inhibitors help restore the host innate immune response to HCV infection (Hopkins et al., 2012). In summary, cyclophilins are probably involved in multiple steps of the HCV life cycle, which remain to be further elucidated.

4.2. Development of the first-generation cyclophilin inhibitor NIM811

Although CsA showed some interesting clinical efficacy in HCV patients, there were obvious concerns about using such a highly immunosuppressive drug to treat a chronic viral infection. Fortunately, following the discovery of CsA, a number of analogs were identified over the years which had very different properties in terms of calcineurin-binding and immunosuppression. Once the anti-HCV activity of CsA was discovered, these "old" compounds were examined for their activities in inhibiting HCV *in vitro*. The anti-HCV activities of these cyclosporin analogs were shown to correlate with their cyclophilin-binding affinity, but not their calcineurin-binding and immunosuppressive activities (Ma et al., 2006). NIM811, a non-immunosuppressive cyclosporin analog with a methyl-isoleucine at position 4 instead of the methyl-leucine in CsA (Fig. 2), emerged as an ideal candidate. It exhibited 2–5-fold higher binding affinity to cyclophilins than CsA, but was completely devoid of calcineurin-mediated immunosuppressive activity (Billich et al., 1995). It inhibited HCV replication *in vitro* with an EC_{50} of 0.12 μM in the absence of human serum and 0.63 μM in the presence of 40% human serum. The pharmacokinetic profile of NIM811 was quite similar to that of CsA, but it did not induce any significant nephrotoxicity like CsA, which can probably be attributed to calcineurin inhibition. Another major advantage of NIM811 was that it can be generated in a large quantity through total biosynthesis (i.e. fermentation), using a genetically modified cyclosporin-producing strain (Weber et al., 1994). Thus, it provided a quick development path.

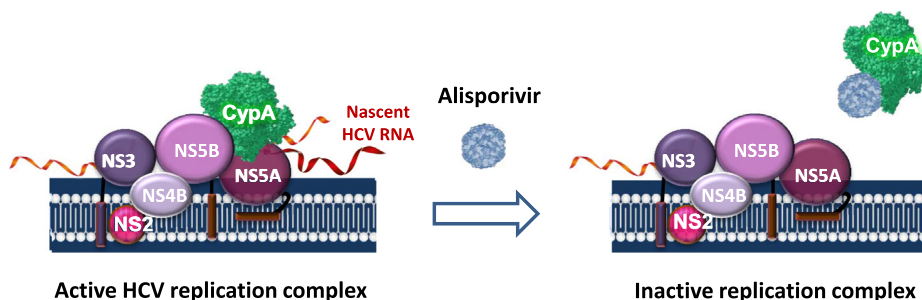

Fig. 1. Mechanism of action of cyclophilin inhibitors in HCV replication. Cyclophilin A (CypA) binds directly to the NS5A protein of HCV, a substrate of CypA PPIase, which is required for the formation of a functional viral replication complex. Cyclophilin inhibitors such as alisporivir inhibit CypA PPIase, blocking its interaction with NS5A, and thus inhibit viral replication.

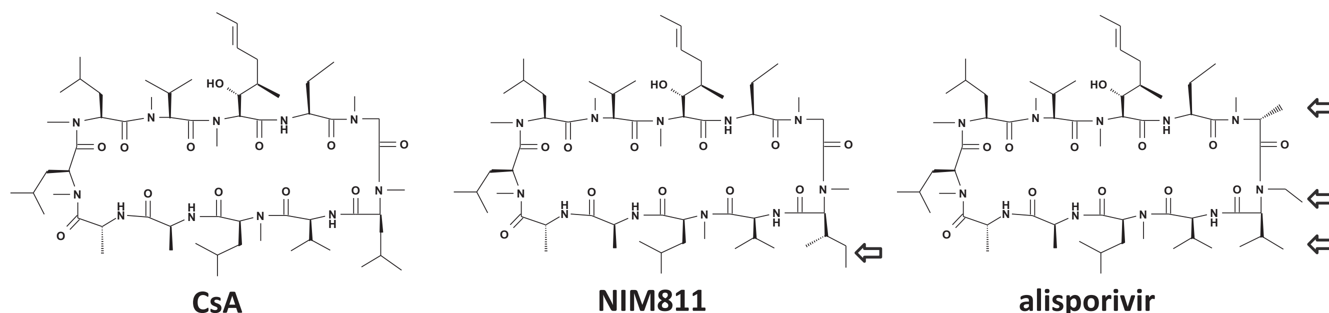

Fig. 2. Chemical structures of cyclophilin inhibitors. From left to right: cyclosporin A (CsA) and two non-immunosuppressive analogs, NIM811 and alisporivir. Arrows indicate modifications in structure.

The pharmacokinetics and safety of NIM811 were first evaluated in a single ascending dose study in healthy volunteers. The compound was administered orally to 40 subjects divided in five different dose groups (50, 150, 400, 800 and 1600 mg). NIM811 was well tolerated. There was no drug-related adverse event in either lab results or clinical evaluation. The compound was rapidly absorbed, with the peak concentration (C_{max}) occurring in blood after 1.5 h of dosing and a terminal elimination half-life ($t_{1/2}$) of around 12–20 h. Drug exposure increased with dose, but was slightly under-proportional.

The compound was subsequently investigated in a double-blind, placebo-controlled study in genotype 1 HCV-infected patients. Multiple ascending doses, 25 and 75 mg once daily (QD) or 100, 200, 400 and 600 mg twice daily (BID) of NIM811 were given orally as monotherapy for 14 days (Lawitz et al., 2011). There was no significant change in C_{max} and a slightly higher (1.2×) AUC at day 13 vs. day 1. The pharmacokinetics of NIM811 was comparable between HCV-infected patients and healthy subjects. All doses were well tolerated, and there was no serious adverse event. The most common side effect was mild to moderate headache, 16% in drug-treated groups vs. 5.3% in placebo. Mild, clinically non-significant elevations of bilirubin and significant declines in platelet counts were observed in the 400 and 600 mg bid groups. Interestingly, a significant reduction of ALT was observed with NIM811 at doses ⩾100 mg BID in the absence of a virological response, suggesting a beneficial biochemical response unrelated to the antiviral effect of the compound. However, no significant viral load reduction was observed with NIM811 monotherapy. At 600 mg BID the C_{trough} of the compound in blood was 0.47 μM, which was still well below its serum-adjusted HCV replicon EC_{50}, ~1.5 μM (extrapolated from EC_{50} in 40% human serum). Thus, a higher dose of the compound would likely be required to achieve antiviral efficacy. However, the exposure of the drug was already under dose-proportional, i.e. only ~30% increase from 200 mg BID to 400 mg BID and ~20% from 400 mg BID to 600 mg BID, so further increasing the dose using the same formulation may not be sufficient.

A different strategy was therefore considered, based on the enhanced antiviral effect observed *in vitro* with the combination of NIM811 and IFN-α (Ma et al., 2006) as well as clinical data demonstrating that CsA plus IFN-α was more effective (Inoue et al., 2003). Rather than further increasing the dose of NIM811 as a monotherapy, the compound was investigated in combination with IFN-α. Twenty genotype 1 HCV patients, who had relapsed during prior IFN-α therapy, were randomized to receive 600 mg NIM811 BID or matching placebo for 14 days and PEG-IFNα-2a on days 1 and 7. The mean reduction in HCV viral load was 2.85 $\log_{10}$ in the NIM811-PEG-IFNα-2a arm compared to 0.56 $\log_{10}$ in the PEG-IFNα-2a alone arm ($p < 0.0001$). Also, ALT decreased from 78.4 U/L at baseline to 26.3 U/L by day 14 in the combination arm, whereas it was unchanged at 66.0 U/L in the PEG-IFNα-2a alone arm. The main safety finding was a decrease of platelet count from $203 \times 10^9\ L^{-1}$ at baseline to $105 \times 10^9\ L^{-1}$ on day 14 in the combination arm, vs. 177×10^9–$139 \times 10^9\ L^{-1}$ in the control arm. There was also a statistically, but not clinically significant increase in serum bilirubin. No serious adverse event was observed in either group.

Despite the promising proof-of-concept efficacy result, there were significant challenges to further develop NIM811, particularly in the absence of a solid formulation for long-term studies. The dose and efficacy of the compound were also likely limited by its under-proportional exposure and significant side effect in decreasing platelet.

4.3. Development of alisporivir

Alisporivir (Debio-025) is a more potent non-immunosuppressive cyclosporin analog with a methyl-alanine at position 3 instead of sarcosine in CsA, and an N-ethyl valine at position 4, instead of N-methyl leucine (Fig. 2). These modifications not only abolish calcineurin binding activity, but also further increase the binding affinity to cyclophilins (Paeshuyse et al., 2006). Thus, the compound is more potent than both CsA and NIM811 in inhibiting HCV with an EC_{50} of 0.045 μM and a serum (100%) adjusted EC_{50} of 0.33 μM. Importantly, it was much more difficult to develop resistance against alisporivir *in vitro* compared to inhibitors of viral targets (Delang et al., 2011; Li et al., 2011).

The compound was initially developed for HIV-1. In a Phase I trial, 23 HIV-1 patients including 19 co-infected with HCV were treated with 1200 mg alisporivir BID for 15 days. While the HIV-1 RNA decreased by ~1 $\log_{10}$, surprisingly there was a much more profound reduction in HCV RNA, 3.63 $\log_{10}$ in alisporivir treated patients vs. 0.73 $\log_{10}$ in the placebo group, which was the first demonstration of clinical efficacy in HCV patients with a cyclophilin inhibitor alone (Flisiak et al., 2008). The most frequent adverse effect was jaundice in 10 patients, including 4 who discontinued the treatment, which was associated with a reversible hyperbilirubinemia. There was also a decrease of $65.3 \times 10^9\ L^{-1}$ in the platelet count in the alisporivir group, compared to a decrease of $11.5 \times 10^9\ L^{-1}$ in the placebo group. In a subsequent Phase IIa study, HCV patients received alisporivir at reduced doses of 200, 600 or 1000 mg BID for the first week and then QD for three more weeks in combination with PEG-IFN-α2a. A mean viral load reduction of 4.6 $\log_{10}$ was achieved in genotypes 1 and 4 patients, and the effect was even more pronounced in genotypes 2 and 3 patients, with a 5.9 $\log_{10}$ viral load reduction after 4 weeks (Flisiak et al., 2009).

Based on these results, 600 mg QD alisporivir (with a loading dose of 600 mg BID during the first week) was selected to be further studied in Phase IIb trials, which gave a plasma C_{trough} slightly above the serum adjusted EC_{50} of the compound. Treatment-naïve,

genotype 1 HCV patients were treated with alisporivir or placebo in combination with PEG-IFN-α2a and ribavirin for 24–48 weeks (Flisiak et al., 2011). Overall, 76% of the patients receiving alisporivir triple therapy for 48 weeks achieved a sustained virological response (SVR), i.e. cure of HCV infection, compared to 55% with PEG-IFN-α and ribavirin only. The efficacy was comparable to the two approved HCV protease inhibitors, while the rate of viral breakthrough was much lower in alisporivir-treated patients, confirming its higher barrier to resistance. In another Phase II trial, the compound was tested in the more difficult to treat IFN non-responders. Remarkably, HCV became undetectable in 75.4% of the patients who received 400 mg BID of alisporivir in combination with PEG-IFN-α and ribavirin for 24 weeks, compared to only 8.9% receiving PEG-IFN-α and ribavirin alone (Alberti et al., 2012).

Since alisporivir had shown more potent antiviral activity in genotype 2/3 HCV patients, a Phase II trial was also carried out to study its effect in an IFN-free regimen (Pawlotsky et al., 2012). First, treatment-naïve, genotypes 2 and 3 patients were given alisporivir 1000 mg QD alone or 600 or 800 mg QD in combination with ribavirin for 6 weeks. Patients achieving rapid virological response (RVR), i.e. with >2 $\log_{10}$ viral load reduction, were then allowed to continue on IFN-free therapy for another 20 weeks, whereas PEG-IFN-α was added to those who did not achieve RVR. In the end, about half of the patients receiving 800 mg QD alisporivir plus ribavirin, and one-third of those who received 1000 mg QD alisporivir only, were cured of HCV infection. This was the first demonstration that a host-targeting antiviral alone can cure a viral infection. Importantly, it has been shown both *in vitro* and in patients that it is much more difficult to develop resistance against the host-targeting cyclophilin inhibitor, which makes it an ideal candidate to be combined with inhibitors of viral targets as an IFN-free, all-oral regimen (Coelmont et al., 2009; Li et al., 2011).

The main side-effect associated with alisporivir treatment was a transient and reversible increase of bilirubin at a total daily dose of above 1000 mg. Mechanistic studies suggested that this was due both to inhibition of the uptake transporters OATP1B1 and OATP1B3, which led to elevated indirect bilirubin, and to inhibition of the efflux transporter MRP2, which led to elevated direct bilirubin (Kovacs et al., 2012). Importantly, none was associated with any increase in ALT, AST or γ-GT, or with hemolysis.

To date, alisporivir has been dosed in almost 2000 subjects with a good overall safety profile. A pivotal Phase III study was initiated in early 2011 to study the combination of alisporivir with PEG-IFN-α2a and ribavirin in treatment-naïve, genotype 1 HCV patients. However, unfortunately, six cases of severe pancreatitis were reported, including one death in early 2012. The compound was subsequently put on clinical hold until the cause and risk of pancreatitis can be assessed. It should be noted that all cases of pancreatitis occurred in the triple-therapy arm including IFN-α and ribavirin. None has been reported to date with alisporivir alone or in an IFN-free regimen, which appears to have a better safety profile (Griffel et al., 2012). Thus, the hope is that alisporivir and/or other cyclophilin inhibitors will be further pursued in combination with other HCV inhibitors.

4.4. Other cyclophilin inhibitors

A number of cyclophilin inhibitors are under development (Table 3). SCY-635 (Hopkins et al., 2010) was the third non-immunosuppressive cyclosporin analog to demonstrate clinical efficacy in HCV patients. HCV genotype 1 patients who received 300 mg TID of SCY-635 alone had a mean viral load reduction of 2.2 $\log_{10}$ after 15 days of treatment (Hopkins et al., 2012). Interestingly, the compound appeared to have a different side-effect profile, compared to those of NIM811 and alisporivir. The most frequently reported adverse events were elevated serum creatinine phosphokinase (CPK) and liver enzymes (ALT/AST), but there has been no report of bilirubin increase or platelet decrease. Some of the new cyclosporin analogs, such as EDP-546, have also shown much reduced inhibition of bilirubin transporters and no significant effect on CYP450, and may thus address the liabilities associated with the first-generation cyclophilin inhibitors, i.e. hyperbilirubinemia and drug–drug interactions (Jiang et al., 2012; Sawada, 2012). Another class of cyclophilin inhibitor, sanglifehrins (Moss et al. 2012), as well as a series of small-molecule inhibitors identified through fragment-based screening, have also shown promising *in vitro* activities (Ahmed-Belkacem et al., 2012).

4.5. Cyclophilin and HIV-1

A potential advantage of targeting the host is that a drug may inhibit multiple viruses that exploit the same cellular pathways. HIV-1 provided the first hint that cyclophilins could play a role in viral replication 20 years ago, when Luban et al. (1993) discovered CypA and CypB directly bind the HIV-1 Gag polyprotein, and that cyclophilin inhibitors blocked these interactions. Subsequently, two independent studies demonstrated that CypA, but not CypB, is specifically incorporated into HIV-1 particles, via contacts with a proline-rich domain of Gag. Preventing CypA–Gag interactions, either by adding cyclophilin inhibitors or by introducing mutations into the proline-rich region of Gag, block both CypA packaging and the infectivity of released virions (Franke et al., 1994; Thali et al., 1994).

The hydrophobic pocket of CypA that encompasses its isomerase activity is critical for HIV-1 Gag binding and particle incorporation (Braaten et al., 1997). More recent work suggests that CypA modulates the uncoating of HIV-1 capsid cores, which are delivered into the cytosol of target cells after fusion of the viral and cellular membranes (Strebel et al., 2009; Ylinen et al., 2009). Capsid core uncoating is a necessary step for penetration of the viral genome into the nucleus of non-dividing cells and subsequent integration of the viral genome into the host chromosomes (Strebel et al., 2009; Ylinen et al., 2009). Since CypA catalyzes the cis/trans isomerization of proline bonds within HIV-1 capsid (Bosco et al., 2002), one can envision that this folding activity orchestrates in a finely tuned, time-dependent manner the disassembly of capsid cores.

Table 3
Non-immunosuppressive cyclophilin inhibitors currently under development for HCV therapy.

Compound	Sponsor	Chemical Class	HCV EC_{50}	Status	Reference
NIM811	Novartis	Cyclosporin	120 nM	Terminated	Lawitz et al. (2011)
Alisporivir	Novartis	Cyclosporin	45 nM	PhIII	Pawlotsky et al. (2012)
SCY-635	Scynexis	Cyclosporin	100 nM	PhIIa	Hopkins et al. (2012)
EDP-546	Enanta	Cyclosporin	67 nM	Preclinical	Jiang et al. (2012)
ASP5286	Astellas	Cyclosporin	45 ng/mL	Preclinical	Sawada (2012)
BC556	Biotica	Sanglifehrin	38 nM	Preclinical	Moss et al. (2012)
F680, F684	INSERM	Small molecule	200 nM	Preclinical	Ahmed-Belkacem et al. (2012)

Supporting this hypothesis, very recent studies provide evidence that CypA enhances HIV-1 infection by coordinating proper uncoating of capsid cores as well as viral genome integration targeting (Schaller et al., 2011; Shah et al., 2013). These data strongly suggest that CypA present in the cytosol of target cells controls the choice of nuclear import machineries that is exploited by HIV-1.

Another function for CypA–capsid interactions was recently proposed. Specifically, Manel et al. (2010) obtained several lines of evidence that CypA–HIV-1–capsid interactions modulate the activation of the innate response. Dendritic cells, which are critical to sensing pathogens and subsequently triggering the host innate response, are normally refractory to HIV-1 infection. However, when this resistance to infection is bypassed, HIV-1 activates both dendritic cell maturation and an antiviral type I interferon response. It was nicely demonstrated that the modulation of this innate response relies on interactions between newly synthesized HIV-1 capsid and CypA and subsequent activation of the transcriptional interferon regulatory factor 3 (IRF3). Cyclophilin inhibitors such as CsA prevent the CypA–capsid interaction-mediated dendritic cell activation. These findings strongly suggest that CypA–capsid contacts modulate a dendritic cell intrinsic sensor for HIV-1 that is important for the antiviral immune response.

The anti-HIV activities of non-immunosuppressive cyclosporin analogs such as NIM811 and alisporivir have been well characterized *in vitro* (Rosenwirth et al., 1994; Billich et al., 1995; Ptak et al., 2008; Daelemans et al., 2010). Moreover, as mentioned above, alisporivir also showed clinical efficacy against HIV-1 (Flisiak et al., 2008). HIV–HCV co-infected patients remain one of the most difficult to treat populations. The dual antiviral activities of cyclophilin inhibitors present an interesting opportunity to treat both viral infections with one drug.

5. Cyclophilin and other viruses

CypA was first identified in the core of the influenza virion in 2008 (Shaw et al., 2008), but its function remained to be elucidated. It was later reported that CypA interacted with the M1 protein of influenza virus, inhibiting its nuclear translocation at an early stage of infection (Liu et al., 2009a). Furthermore, CypA enhanced ubiquitin-mediated degradation of M1 and inhibited the initiation of viral replication (Liu et al., 2012b). Interestingly, it appeared that CsA was capable of inhibiting the replication of influenza virus in both CypA-dependent and independent pathways (Liu et al., 2012a). It enhanced the binding of CypA to M1, but also impaired the nuclear export of viral mRNA in the absence of CypA. Importantly, the non-immunosuppressive cyclosporin analog $MeIle^4$–CsA, i.e. NIM811, showed a similar antiviral effect, raising the possibility of developing cyclophilin inhibitors for influenza.

Bouchard et al. (2003) reported that CsA and NIM811 inhibited HBV replication by blocking mitochondrial transition pore and calcium signaling. It was shown recently that CypA interacted with HBV surface antigen (HBsAg), promoting its secretion both *in vitro* and in patients (Tian et al., 2010). Interestingly, siRNAs specifically targeting CypA not only inhibited HBV replication but also blocked HBsAg secretion *in vitro*. Furthermore, the cyclophilin inhibitor alisporivir was capable of reducing both HBV DNA and secreted HBsAg (Phillips et al., 2012). Given the fact that the only class of specific antiviral drug currently available for HBV, nucleos(t)ide polymerase inhibitors, potently inhibits viral replication, but neither clears viral cccDNA nor induces seroconversion in the majority of the patients, it would be of great interest to develop drugs with novel mechanisms that could lead to better seroconversion and viral clearance.

Cyclophilins also appear to play an important role in the replication of other viruses. Luo et al. (2004) first reported that CypA bound to the nucleocapsid protein of SARS coronavirus. More recently, it was shown that cyclophilins interact with viral nonstructural protein 1 (Nsp1), and that CsA inhibited the replication of multiple coronaviruses (de Wilde et al., 2011; Pfefferle et al., 2011). In addition, cyclophilins may be involved in the replication of herpes simplex virus (Vahlne et al., 1992), human cytomegalovirus (Keyes et al., 2012), vesicular stomatitis virus (Bose et al., 2003), vaccinia virus (Damaso and Keller, 1994), and human papillomavirus (Bienkowska-Haba et al., 2009). The development of cyclophilin inhibitors could therefore potentially benefit the treatment of many different viral infections.

6. Future perspective

The discovery and development of cyclophilin inhibitors exemplify the opportunities and challenges in pursuing host-targeting antivirals. The advantages of a high barrier to resistance, broad genotype coverage, an accelerated development path with known inhibitors and even the possibility of broad-spectrum antivirals have all been demonstrated with cyclophilin inhibitors. Interestingly, the toxicity and side-effect profiles of the inhibitors investigated to date are very different from one another, both in preclinical studies and in patients, and seem to be more related to the individual compound than to the target. While challenges remain to better understand mechanisms of action and toxicity, one could argue that this situation does not differ from developing inhibitors of viral targets. The chance of success is not necessarily lower either, considering the number of viral targets and inhibitors that have been pursued and failed.

With regard to the discovery of novel host targets, the impact of limited cell culture and animal models should not be overlooked. Lessons can be learned from some previous failures in translating *in vitro* findings to clinical efficacy, such as the statins and IMPDH inhibitors. Nevertheless, given the right approach and the right target, blocking virus–host interactions could be a highly effective strategy to treat viral infections.

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