Cyclophilin involvement in the replication of hepatitis C virus and other viruses

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Abstract
In recent months, there has been a wealth of promising clinical data suggesting that a more effective treatment regimen, and potentially a cure, for hepatitis C virus (HCV) infection is close at hand. Leading this push are direct-acting antivirals (DAAs), currently comprising inhibitors that target the HCV protease NS3, the viral polymerase NS5B, and the non-structural protein NS5A. In combination with one another, along with the traditional standard-of-care ribavirin and PEGylated-IFNα, these compounds have proven to afford tremendous efficacy to treatment-naïve patients, as well as to prior non-responders. Nevertheless, by targeting viral components, the possibility of selecting for breakthrough and treatment-resistant virus strains remains a concern. Host-targeting antivirals are a distinct class of anti-HCV compounds that is emerging as a complementary set of tools to combat the disease. Cyclophilin (Cyp) inhibitors are one such group in this category. In contrast to DAAs, Cyp inhibitors target a host protein, CypA, and have also demonstrated remarkable antiviral efficiency in clinical trials, without the generation of viral escape mutants. This review serves to summarize the current literature on Cyps and their relation to the HCV viral life cycle, as well as other viruses.

Keywords: cyclophilin; hepatitis C virus; viruses.

Introduction
Hepatitis C virus (HCV) is the major causative agent of acute and chronic liver diseases (Dienstag and McHutchison, 2006). 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 (Dienstag and McHutchison, 2006). Nearly 200 million people worldwide (3% of the population), including 4 to 5 million in the United States, are chronically infected with HCV, and 4 million new infections occur every year (Alter, 2007; Soriano et al., 2008). In the developed world, HCV accounts for two-thirds of all cases of liver cancer and transplants (Shepard et al., 2005), and, in the United States, approximately 12 000 people are estimated to die from HCV each year (Armstrong et al., 2006). There is no vaccine for HCV, and current new therapeutics have focused on inhibition of viral enzymes and proteins; however, the high rate of mutation associated with the virus-encoded RNA polymerase has been shown to afford escape mutants that are resistant to these compounds. Thus, efficient treatment and eradication of HCV will likely require the concerted targeting and disruption of a number of critical virus-host interactions, coupled with the suppression of viral enzymatic activities.

In humans, there are three distinct families of peptidyl-prolyl isomerases (PPIases), also referred to as immunophilins: cyclophilins (Cyps), FK506-binding proteins (FKBPs), which bind the immunosuppressant FK506, and the parvulin family (Galat, 2004). The three PPIase families are unrelated in sequence and three-dimensional structure; however, all catalyze the cis/trans isomerization of the peptide bond on the N-terminal side of proline residues in proteins (Fischer and Aumuller, 2003). Typically, this PPIase activity serves as a chaperone to drive proper protein folding, as well as conformational changes necessary for functionality, evidenced by a number of early studies that looked at the role of cis/trans isomerization of the petidyl-prolyl bond during variable protein refolding conditions (Schmid, 1993).

CypA is an abundant, cytosolic 18-kDa protein that is found in all human tissues; it was also the first Cyp to be identified by its ability to bind to cyclosporin A (CsA) (Handscharcht et al., 1984). Around the same time, PPIase activity was identified by Fischer et al. (1984); however, it was another 5 years before the connection between CypA and PPIase activity was made (Fischer et al., 1989; Takahashi et al., 1989). CypA is the prototypical member of the Cyp family as it essentially comprises the defining characteristic of this group of enzymes, a Cyp-like domain (CLD), which encompasses the entire region of PPIase activity. Homologs of CypA and other Cyp family members are highly conserved and can be found in all domains of life from archaea and bacteria, all the way up to eukaryotes (Galat, 2004; Wang and Heitman, 2005).

Twenty distinct Cyp open-reading frames (ORFs) have been detected in humans, nearly half of which encode single-domain proteins that are primarily composed of the CLD (Galat and Bua, 2010). The remaining ORFs are multidomain Cyp family members that encode variable domains surrounding the CLD, which serve to confer a diverse array
of activities such as intracellular targeting, RNA recognition, and protein-protein interactions (Galat and Bua, 2010). The diversity of functional activity within the Cyp family is partially attributable to the localization of human Cyps, as they are localized in the cytoplasm, nucleus, endoplasmic reticulum (ER), mitochondria, and attached to various membranes (Galat, 2004; Wang and Heitman, 2005). For example, a number of nuclear Cyps – CypE, CypG, CypH, CypJ, and Cyp60 – remain within the nucleus where they are engaged in spliceosome formation and function (Mesa et al., 2008), whereas RanBP2, the largest member of the Cyp family (~360 kDa), functions in the nuclear membrane and acts as a nucleoporin (Galat and Bua, 2010). In the mitochondria, CypD has been shown to be a major component of the mitochondrial permeabilization transition pore, where it plays an intricate role in mitochondrial-mediated cell death (Nakagawa et al., 2005; Schinzel et al., 2005).

A link between PPIase function and modern medicine arose before the discovery of PPIase activity, as it was discovered in the 1970s that the introduction of the chemical inhibitors CsA and FK506 dramatically decreased immune-mediated tissue graft rejection, thereby revolutionizing organ transplantation (Calne et al., 1978; Starzl et al., 1981). The immunosuppressive activity associated with these compounds was later linked to the ability of Cyp-CsA and FKBP-FK506 complexes to bind and block the phosphatase activity of calcineurin, which in turn prevents the dephosphorylation of nuclear factor of activating T cells (NF-AT), thereby preventing nuclear translocation of NF-AT and subsequent cytokine transcriptional activation (Gothel and Marahiel, 1999). Modern medical Cyp research has largely focused on the role of Cyps in viral infection, reviewed below. It is important to note that Cyps have also been found to play a role in a wide range of diseases and cellular dysfunctions such as cancer, angiogenesis, atherosclerosis, ER stress, and neurodegeneration (Lee and Kim, 2010).

**Cyps and HCV**

In 2003 a groundbreaking study by Watashi et al. (2003) revealed that HCV replication in a cell culture model was severely impeded by the introduction of the chemical Cyp inhibitors CsA and NIM811. With the growing health burden and lack of viable treatment options for HCV, the pharmaceutical industry took notice and spent considerable time and energy in further developing non-immunosuppressive Cyp inhibitors that would be more readily tolerated in prospective patients. A number of these compounds have reached variable phases of clinical trials, the most successful of which is alisporivir (Gallay, 2011). Importantly, clinical trials have recently shown that Cyp inhibitors are capable of suppressing HCV in as little as 2 weeks when combined with ribavirin and pegylated-IFNα (Lawitz et al., 2011), while monotherapy of alisporivir alone was effective at combatting HCV genotype 3 (Sarin and Kumar, 2012). To date, the exact mechanism of action in which these compounds affect HCV replication remains unclear, despite the fact that a number of Cyp-HCV protein interactions have been elucidated. It is also known that chemical Cyp inhibitors are capable of acting on many of the Cyp family members that are present throughout the cell, demonstrated in Figure 1. Generally, it could be reasoned that because many of the single-domain Cyps function as chaperones that serve to monitor polypeptide folding fidelity, subsequent disruption of this activity through Cyp binding to the chemical inhibitor could elicit a state of distress within the ER, a cellular machine to which the HCV life cycle is critically linked. The tolerance of healthy tissues and overall patient health in response to large doses of these compounds suggest that any distress created by Cyp inhibition is minimal or temporary to non-infected cells.

The role of Cyps in the HCV life cycle has been intensively investigated for the better part of the last decade. The majority of this research has focused on evaluating what Cyp family members are involved in HCV replication, which viral proteins interact directly with Cyps, and what viral escape mutations arise during selection for drug resistance. From a number of studies, it is clear that CypA is required for HCV replication and likely the primary target for chemical inhibitors, as evidenced through knockdown assays performed by a number of groups (Yang et al., 2008; Chatterji et al., 2009; Hanouelle et al., 2009; Kaul et al., 2009; Gaither et al., 2010). Importantly, it was also shown that a functional hydrophobic pocket in CypA is necessary for HCV replication, which strongly suggested that the chaperone function or isomerase activity of CypA is essential for HCV genotype 1b (GT-1b) amplification in a cell culture (Chatterji et al., 2009; Kaul et al., 2009; Liu et al., 2009b). The existence of CypA knock-out mice and stable CypA knockdown cell lines indicates that CypA is not required for cell growth and survival, which in turn suggests that the treatment of HCV-infected patients with Cyp inhibitors should not lead to major drug complications (Braaten and Luban, 2001; Colgan et al., 2005).

Multiple studies have shown that CypA interacts directly with NS5A of multiple genotypes, and importantly this binding is abrogated by the presence of a variety of chemical Cyp inhibitors (Hanouelle et al., 2009; Chatterji et al., 2010; Coelmont et al., 2010; Fernandes et al., 2010; Yang et al., 2010; Foster et al., 2011; Gregory et al., 2011; Verdegem et al., 2011). Recently, elaborate nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopy studies have revealed that CypA binds directly to a number of proline residues within domains II and III of the NS5A protein of multiple HCV genotypes (Hanouelle et al., 2009; Coelmont et al., 2010; Yang et al., 2010; Verdegem et al., 2011). These studies showed that CypA bound to these proline residues through the enzymatic pocket of the CLD, and was capable of inducing a cis/trans isomerization in many of these sites (Hanouelle et al., 2009; Coelmont et al., 2010; Yang et al., 2010; Verdegem et al., 2011). A number of studies have identified mutations in the NS5A region of HCV that arise during selection for Cyp-inhibitor escape mutants in replicon cell lines (Fernandes et al., 2007; Goto et al., 2009; Kaul et al., 2009; Chatterji et al., 2010; Puyang et al., 2010). The most common mutation is the D320E amino acid substitution within the NS5A protein; this alteration has been identified by many groups and shown to
confer moderate drug resistance when substituted back into a wild-type background (Goto et al., 2009; Chatterji et al., 2010; Puyang et al., 2010; Yang et al., 2010). Curiously, NS5A carrying the D320E mutation is still capable of binding CypA, and this interaction remains sensitive to chemical disruption, suggesting that HCV resistance to Cyp inhibitors is not dependent on the affinity of CypA for NS5A (Chatterji et al., 2010; Coelmont et al., 2010). Of interest, NMR analysis of GT-1b NS5A peptides that contain the D320E mutation displayed a markedly distinct cis/trans configuration when compared with the wild type (Coelmont et al., 2010). Similarly, CD analysis of peptides with the common Cyp-inhibitor-resistant mutations found in the GT-2a NS5A of HCV genotype 2A, D316E/Y317N, were also found to induce a conformational change, further implicating a role for alternative native protein folding as a means for Cyp inhibitor resistance (Yang et al., 2010). It has also been recently shown that the interaction of domain II of NS5A with functional CypA stimulated NS5A RNA binding capacity when compared with a CypA isomerase-deficient mutant, suggesting a potential role for the PPIase activity of CypA in the folding and functionality of NS5A (Foster et al., 2011).

A number of studies have shown that both CypA and CypB are present in HCV replication complexes, as each is capable of binding to the HCV-encoded RNA polymerase NS5B (Wataishi et al., 2005; Yang et al., 2008; Abe et al., 2009; Chatterji et al., 2009). Importantly, a number of groups have found mutations that arise in NS5B when subgenomic GT-1b HCV replicon cell lines are selected for Cyp inhibitor resistance, with some mutations conferring drug resistance when introduced back into the parental replicon (Fernandes et al., 2007; Robida et al., 2007; Liu et al., 2009b). Nonetheless, the importance and biological function of Cyp-NS5B interactions remain unclear and are likely being intensively studied. What is known is that knockdown of cellular CypB alone failed to significantly affect HCV replication, suggesting an ancillary role for CypB or the presence of a cellular redundancy that is capable of overcoming this defect, e.g., another Cyp (Yang et al., 2008; Chatterji et al., 2009; Kaul et al., 2009). Notably, the Sugawara group recently identified a novel helicase that binds directly to CsA and facilitates the association of HCV NS5B with CypB, offering another link between Cyps and NS5B (Morohashi et al., 2011). Chemical Cyp inhibitors were also shown to dramatically affect full-length genomic HCV replication at the level of NS2 protease activity, and this too was found to be dependent on CypA (Ciesek et al., 2009). In support of this finding, HCV polyprotein cleavage and processing kinetics were shown to be altered in the presence of

![Figure 1](Unauthenticated Download Date | 6/30/17 9:46 PM)
Cyp inhibitors, revealing a general protease dysfunction upon dampening of Cyp activity in an infected cell (Kaul et al., 2009). Further work with Cyps and NS2 are likely ongoing and should clarify the exact role of Cyps in the proteolytic processing of the HCV polyprotein.

Aside from the importance of CypA, other Cyps have been shown to play a role in HCV pathogenesis. The Weidman group was able to demonstrate the diversity of Cyps contributing to the HCV life cycle through siRNA, proteomic, and miRNA analysis of Cyp inhibition in HCV replicon cell lines, unveiling important binding partners of CypA, CypB, CypD, CypH, and Cyp40 that contribute to both cellular function and viral replication (Gaither et al., 2010). Additional studies have also revealed that Cyp inhibition, in the context of HCV replication, is capable of having global effects on the cellular protein landscape and subsequent biological functionalities of the cell. Specifically, the Weidman group demonstrated that NIM811 treatment of a subgenomic Con1 cell line or JFH1-infected cells resulted in a dramatic doubling in size and decrease in the overall number of lipid droplets (LDs) (Anderson et al., 2011). It was also shown that in the presence of HCV and NIM811, apoB accumulates in defined regions of these enlarged LDs and fails to be secreted, which also correlated to a decrease in viral particle release (Anderson et al., 2011). These changes to lipid and apoB trafficking were then shown to be Cyp dependent, with silencing of both CypA and Cyp40 resulting in a similar phenotype with respect to LD size and apoB localization (Anderson et al., 2011). Recently, the Piccoli group has shown that alisporivir is capable of preventing many HCV-induced mitochondrial dysfunctions, including apoptosis (Quarato et al., 2011). Through the use of inducible cell lines, the same group was able to demonstrate that alisporivir treatment prevented the HCV polyprotein-mediated decrease in cell respiration, collapse of mitochondrial membrane potential, overproduction of reactive oxygen species, and mitochondrial calcium overload (Quarato et al., 2011). While it has been known for some time that Cyp inhibitors are capable of binding CypD, thereby preventing the mitochondrial permeability transition and the subsequent pro-apoptotic cascade, this study serves to highlight a hitherto underappreciated, positive, side effect of Cyp inhibition on the HCV-infected cell’s survival (Quarato et al., 2011). Collectively, all of these studies reveal the complexity and breadth of analysis required to evaluate the intricate connection of Cyps to the HCV life cycle and it is readily apparent that while many questions have been addressed, many more remain unanswered.

**Cyps and other viruses**

The first link between Cyps and viral infection came nearly two decades ago when the Goff group showed that both CypA and CypB were able to bind to the HIV-1 Gag protein; importantly, these interactions could be disrupted by the addition of CsA (Luban et al., 1993). The field gained physiological traction the following year when two groups collectively demonstrated that (i) CypA binds to a proline-rich region within the capsid (CA) domain of Gag; (ii) CypA is specifically incorporated into HIV-1 virions; (iii) the CypA-Gag interaction is required for HIV-1 replication *in vitro*; (iv) CsA and the non-immunosuppressive CsA analog NIM811 are capable of inhibiting HIV-1 replication; and (v) CypA virion incorporation could be abrogated by CsA and NIM811 (Franke et al., 1994; Thali et al., 1994). Mutagenesis of both CypA and CypB revealed that the hydrophobic pocket and isomerase activities of each were necessary for HIV-1 CA binding and virion incorporation (Braaten et al., 1997). While the exact contribution, if any, of CypB to HIV-1 pathogenesis has failed to evolve, the importance of CypA has been expanded further. For example, NMR was elegantly used to show that CypA catalyzes cis/trans isomerization of the native HIV-1 CA (Bosco et al., 2002). Follow-up studies have suggested that CypA participates in the uncoating of the HIV-1 CA core, thereby enabling successful integration of the viral genome (Strebel et al., 2009; Yilinen et al., 2009). CypA has also been shown to interact with HIV-1 viral protein R (Vpr) and to be required for *de novo* Vpr synthesis (Zander et al., 2003). NMR and surface plasmon resonance (SPR) spectroscopy revealed CypA catalysis of cis/trans isomerization of the N-terminal of Vpr (Solbak et al., 2010).

Interestingly, the Littman group has revealed a role for the CypA-HIV-1 CA interaction in the activation of the innate immune response, as CsA treatment of HIV-1 during infection of dendritic cells (DCs) triggered interferon regulatory factor 3 (IRF3) transcriptional activation (Manel et al., 2010). As DCs are typically resistant to HIV-1, this work strongly suggests the existence of a CypA- or CA-triggered innate immune sensor, which could not only explain cell permissivity, but could also potentially be exploited as a means to combat HIV-1 (Manel et al., 2010). Recently, the Towers group demonstrated that an additional member of the Cyp family, RanBP2, also interacts with HIV-1 CA through its Cyp-like domain (Schaller et al., 2011). Uniquely, this pairing is impervious to disruption through CsA inhibition (Schaller et al., 2011). In this elegant study, the group was able to demonstrate a concerted role for CypA and RanBP2 in HIV-1 nuclear import, as both mutation of CA as well chemical inhibition of CypA by Cyp inhibitors was shown to separately and distinctly alter nuclear import machinery usage and affect subsequent HIV-1 integration site targeting (Schaller et al., 2011).

An additional link between Cyps and viral capsid proteins was recently discovered when the nucleocapsid protein of the severe acute respiratory syndrome coronavirus (SARS-CoV) was found to also interact directly with CypA: bioinformatics, SPR, and structural modeling resulted in nanomolar dissociation constants as well as putative binding sites (Luo et al., 2004). A recent report has linked Cyps to general CoV replication, as CsA was found to inhibit the replication of SARS-CoV, human CoV 229E, and mouse hepatitis virus in cell culture (de Wilde et al., 2011). Of note, CsA inhibited the SARS-CoV replication cycle at an early step that was not disrupted or affected by the knockdown of either CypA or CypB (de Wilde et al., 2011). An additional group identified a number of immunophilins as binding partners to non-structural protein 1 (Nsp1) of SARS-CoV, and then demonstrated...
physiological relevance as the SARS-CoV infection and Nsp1 overexpression were shown to specifically induce the IL-2 and calcineurin/NFAT transcriptional pathways (Pfefferle et al., 2011). The same group then went on to show that all genera of CoVs were CsA sensitive, corroborating the work of the van Hemert group, illustrating a potential role for Cyps in the CoV life cycle (Favreau et al., 2012). Interestingly, Cyp homologs in yeast have been shown does so remains to be determined (Xia et al., 2005; Tian et al., 2010). Another connection between CypA and viral replication was demonstrated for a serotype of vesicular stomatitis virus (VSV), where CsA treatment was shown to inhibit virus replication, while depletion of endogenous CypA increased influenza virus infectivity (Liu et al., 2009a). This group has recently shown that CypE is also inhibitory to influenza A replication and transcription through its interaction with nucleoprotein, which correlated to disruption of the viral ribonucleoprotein complex (Wang et al., 2011). In contrast, Cyps facilitate infectivity of human papillomavirus (HPV) types 16 and 18 at two distinct steps – one entails CypB and viral CA association at the point of internalization, while the other is downstream (Bienkowska-Haba et al., 2009). CypB is also involved in the replication of Japanese encephalitis virus (JEV), where CypB knockdown and introduction of an isomerase-deficient CypB reduced the ability of JEV to propagate; notably, this effect did not occur at the point of internalization (Kambara et al., 2011). Work with other flaviviruses revealed a general sensitivity of the group to CsA at the point of viral RNA replication, which correlated with the interaction of CypA to non-structural protein 5, as well as to the viral RNA itself (Qing et al., 2009). Another connection between CypA and viral replication was demonstrated for a serotype of vesicular stomatitis virus (VSV), where CsA treatment was shown to inhibit virus replication (Bose et al., 2003). Cytomegalovirus replication has also proven to be sensitive to CsA, in yet another example of a viral requirement for Cyps (Kawasaki et al., 2007; Keyes et al., 2012). Data also suggest that Cyp inhibition affects hepatitis B virus replication, although the manner in which it does so remains to be determined (Xia et al., 2005; Tian et al., 2010). Interestingly, Cyp homologs in yeast have been shown not to assist but to inhibit the replication of plant viruses in the Tombusvirus genus (Mendu et al., 2010; Lin et al., 2012).

Taken together, these studies demonstrate that multiple Cyps are capable of influencing and affecting numerous steps of highly divergent virus life cycles (Figure 1). The reliance of these virus families on Cyp activity for such diverse RNA replication pathways, viral entry, internalization, and maturation processes, further highlights the potential impact of Cyp inhibitor therapeutics. Given the apparent broad-spectrum potential of these compounds, the case can be made that Cyp
inhibitors could be used as a first line of defense for new and emerging viruses.

Moving forward

The ability of Cyp inhibitors to afford pan-genotypic anti-HCV activity, coupled with a low incidence of viral breakthrough, makes them an extremely attractive treatment alternative (Flisiak et al., 2012). One can envision cases when the patient presents with a high viral load, where viral breakthrough could readily occur using DAs alone, such cases would greatly benefit from the addition of a host-targeting antiviral (HTA) such as a Cyp inhibitor. To date, a number of Cyp-virus interactions have been teased apart, yet collectively they fail to fully explain why and how exactly Cyp inhibitors work. Again, the primary function of a Cyp is to serve as a molecular chaperone, presumably to facilitate polypeptide folding and post-translational modification. It is therefore not surprising that Cyps have been found to interact with numerous proteins and subsequently implicated in a wide range of cellular functions. On the basis of this global activity, one would predict transient, lower-affinity interactions between Cyps and target proteins to account for a generalized specificity; however, many high-affinity Cyp-ligand interactions have been described. This curious anomaly suggests that perhaps a tightly bound Cyp represents a distinct or regulatable functionality for the molecule. The importance of Cyps in the viral life cycle of other pathogens further implicates a global requirement of complete cellular functionality for optimal viral fitness. Further insight into the changes that occur upon the cellular landscape once a Cyp inhibitor is introduced to a virally infected cell is clearly warranted, and could serve to elucidate a precise mechanism of action. It is clear that Cyp inhibitors remain a viable treatment alternative for the elimination of HCV, and possibly other virus families, and the continued basic research on their molecular mechanism of action should prove to be enlightening in the years to come.

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