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Commentary

Entry inhibitors and future treatment of hepatitis C

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ABSTRACT

Hepatitis C virus (HCV) is a major cause of liver cirrhosis and hepatocellular carcinoma. Furthermore, HCV-induced liver disease is the leading indication for liver transplantation. The recent introduction of direct-acting antivirals (DAAs) has revolutionized HCV treatment by making possible the cure of the majority of patients. However, their efficacy and safety in difficult-to-treat patients such as patients receiving immunosuppression, those with advanced liver disease, co-morbidity and HIV/HCV-co-infection remain to be determined. Furthermore, prevention of liver graft infection remains a pressing issue. HCV entry inhibitors target the very first step of the HCV life cycle and efficiently inhibit cell–cell transmission – a key prerequisite for viral spread. Because of their unique mechanism of action on cell–cell transmission they may provide a promising and simple perspective for prevention of liver graft infection. A high genetic barrier to resistance and complementary mechanism of action compared to DAAs makes entry inhibitors attractive as a new strategy for treatment of multi-resistant or difficult-to-treat patients. Clinical studies are needed to determine the future role of entry inhibitors in the arsenal of antivirals to combat HCV infection. This article forms part of a symposium in *Antiviral Research* on "Hepatitis C: next steps toward global eradication."

The addition of direct-acting antivirals (DAAs) to the armamentarium for treatment of hepatitis C virus (HCV) infection now makes possible treatment regimens that could produce cure in the large majority of patients. However, monotherapy with a DAA has been shown to rapidly select for drug resistance and combination therapies are therefore necessary when using these drugs. Furthermore, recent clinical studies indicate that a significant subset of patients may still need alternative approaches to achieve viral cure. HCV entry inhibitors target the very first step of the HCV life cycle and efficiently inhibit cell–cell transmission – a key prerequisite for viral spread and persistence. In this review, we discuss unmet medical needs following the licensing of DAAs, highlight host-targeting entry inhibitors as antivirals, review their stage of development and finally discuss how entry inhibitors may address future unmet medical needs in HCV therapy.

1. Unmet medical needs in antiviral therapy in the era of interferon treatment

Hepatitis C virus (HCV)-induced liver cirrhosis and hepatocellular carcinoma (HCC) pose major challenges for healthcare systems worldwide and are leading indications for liver transplantation (LT) (Liang and Ghany, 2013). For many years, pegylated interferon-α (PEG-IFN-α) and ribavirin (RBV) have been the backbone of antiviral therapy in hepatitis C. The addition of an HCV protease inhibitor (either telaprevir or boceprevir) to PEG-IFN-α/RBV has markedly improved sustained virological response (SVR) rates in genotype-1 infected patients with cure rates in excess of 70% (Jacobson et al., 2011; McHutchison et al., 2010; Poordad et al., 2011). These triple combination treatment regimens, however, not only have the side effects and limitations of the previous standard of care (SOC) therapy with PEG-IFN-α/RBV but are burdened with additional adverse reactions specific to the two approved protease inhibitors (Liang and Ghany, 2013). A major limitation of telaprevir and boceprevir is their low genetic barrier for resistance with drug-resistant variants emerging rapidly (Pawlotsky, 2011; Sarrazin and Zeuzem, 2010). Furthermore, these DAAs are not approved for liver transplantation (Liang and Ghany, 2013) where IFN-α-based antiviral therapies have been shown to be associated

Abbreviations: CLDN1, claudin-1; DAA, direct-acting antiviral; HCV, hepatitis C virus; OCLN, occludin; SR-BI, scavenger receptor class B type I; TfR1, transferrin receptor; PS-ON, phosphorothioate oligonucleotides; PEG-IFN-α, pegylated interferon-α; RBV, ribavirin.

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with limited efficacy and tolerability. Due to viral evasion from host immune responses and the absence of preventive antiviral strategies, re-infection of the graft is currently universal (Crespo et al., 2012). Importantly, no licensed options exist for graft recipients to prevent re-infection of the grafted liver (Liang and Ghany, 2013).

Other DAAs have been evaluated in combination with PEG-IFN-α or in IFN-free regimens, with or without RBV, including second-wave first generation and second-generation viral protease inhibitors, NS5A and polymerase inhibitors (Gane et al., 2013b; Jacobson et al., 2013; Kowdley et al., 2013; Lawitz et al., 2013a,b; Lok et al., 2012; Manns et al., 2012; Pawlotsky, 2013; Pockros et al., 2013; Sulkowski et al., 2013a,b; Zeuzem et al., 2013a). These DAAs show higher potency and pan-genotypic activity than first-generation protease inhibitors (Schinazi et al., 2014). Furthermore, clinical trials investigating the combinations of several DAAs have shown that eradication of HCV can be achieved in IFN-free regimens, oftentimes allowing for shorter duration of treatment (Chayama et al., 2012; Gane et al., 2013a, 2010; Jacobson et al., 2013; Lawitz et al., 2013b, 2014; Lok et al., 2012; Osinusi et al., 2013; Poordad et al., 2013; Zeuzem et al., 2013b). Interestingly, second generation protease inhibitor simeprevir recently obtained FDA-approval for chronic hepatitis C treatment (press release Johnson & Johnson, November 2013).

Nevertheless, breakthroughs due to selection of DAA-resistant HCV variants as well as differences in virological outcomes for different genotypes and subtypes have already been reported (Gane et al., 2013a; Jacobson et al., 2013; Poordad et al., 2013; Soriano et al., 2012; Tong et al., 2014). Indeed, it has been shown that HCV mutations emerge *in vivo* conferring cross-resistance to polymerase inhibitors mericitabine and sofosbuvir (Tong et al., 2014). Moreover, although demonstrating potent pan-genotypic activity, NS5A inhibitor daclatasvir has been associated with viral breakthroughs when combined with protease inhibitors (Lok et al., 2012; Sarrazin et al., 2012). Furthermore, there is evidence for DAA-based therapies being less effective in difficult-to-treat patients. These patients comprise partial non-responders or null-responders to prior PEG-IFN-α/RBV therapy, patients with advanced liver disease, and transplanted, HIV/HCV-coinfected, hemodialyzed, or immune-compromised patients (Heim, 2013; Liang and Ghany, 2013). The potential limitation of DAAs in advanced liver disease and certain genotypes is illustrated by the example of a SVR of only 37% in patients with liver cirrhosis and genotype 3 infection treated with RBV and sofosbuvir (Kowdley et al., 2013; Lawitz et al., 2013a).

A major challenge for management are individuals with HCV-induced end-stage liver disease undergoing LT (Ciesek and Wedemeyer, 2012; Crespo et al., 2012; Fink and Jacobson, 2011). Liver graft re-infection is universal which is considered to be the result of viral evasion from host immune responses. Licensed preventive antiviral strategies are absent. IFN-based therapies are limited by reduced efficacy and tolerability in LT recipients (Crespo et al., 2012). Furthermore, emergence of resistant strains will certainly be higher in these patients because of lower efficacy of SOC and greater need for dose adjustments (Crespo et al., 2012). Finally, clinical trials suggest that use of telaprevir or boceprevir in transplantation may be limited by drug–drug interactions with concomitant use of immunosuppressive agents (Liang and Ghany, 2013). Taken together, these clinical challenges demonstrate that there is an unmet medical need to address the limitations of DAA-based therapies.

2. Entry inhibitors – a unique and complementary mechanism of action compared to DAAs

Viral cell entry is emerging as a novel target of hepatitis C therapy (von Hahn et al., 2011; Zeisel et al., 2011, 2013b). This process is the first step of HCV-host cell interactions involving the HCV envelope glycoproteins E1 and E2 as well as several host factors (Farquhar et al., 2011; Ploss and Evans, 2012; Zeisel et al., 2013a). HCV cell entry provides several attractive opportunities for interference with the viral life cycle: blocking virus-target cell interaction during attachment, interfering with post-binding events or viral fusion (Zeisel et al., 2013b). Although compounds targeting the viral envelope are considered to be entry inhibitors (such as anti-envelope antibodies), in this review we will focus on host targeting-entry inhibitors. *In vivo*, HCV most likely first interacts with the basolateral surfaces of hepatocytes. Highly sulfated heparan sulfate proteoglycans (Barth et al., 2003) represent likely first attachment sites allowing viral concentration on the basolateral hepatocyte membrane. The virus then interacts with several entry factors including scavenger receptor class B type I (SR-BI) (Lacek et al., 2012; Scarselli et al., 2002), CD81 (Farquhar et al., 2012; Harris et al., 2010; Pileri et al., 1998). Other host cell factors to be shown to mediate entry are tight junction proteins claudin-1 (CLDN1) (Evans et al., 2007) and occludin (OCLN) (Ploss et al., 2009), NPC1L1 (Sainz et al., 2012) and transferrin receptor 1 (TfR1) (Martin and Uprichard, 2013). Epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2) (Lupberger et al., 2011), HRas (Zona et al., 2013) have been shown to be regulatory molecules in HCV entry.

These host factors offer multiple targets for antiviral therapy. CLDNs are critical components of tight junctions (TJ) regulating paracellular permeability and polarity. CLDN1 may localize to TJ of hepatocytes but also to basolateral surfaces (Reynolds et al., 2008). It has been suggested that non-junctional CLDN1 may be involved in HCV entry (Cukierman et al., 2009; Evans et al., 2007). Studies in Huh7.5 based cell lines have suggested that CLDN6 and CLDN9 can serve as entry factors and may play a role as targets for antiviral therapy (Haid et al., 2014; Meertens et al., 2008; Zheng et al., 2007), however their functional relevance could not be confirmed in primary human hepatocytes where expression is very low (Fofana et al., 2013b). Targeting viral entry offers the advantage of combating viral infection at its initial steps and before the virus starts to produce genomic material that will persist in infected cells (Zeisel et al., 2011, 2013b). Furthermore, since entry is required for dissemination and maintenance of infection this approach may also treat persistent infection (Zeisel et al., 2011, 2013b). Indeed, the development of antiretroviral drugs targeting different steps of the viral life cycle including viral entry was shown to improve the outcome for HIV infected individuals (Henrich and Kuritzkes, 2013; Sharma et al., 2013).

Compared to the high variability of viral proteins targeted by DAAs, the variability of the required host entry factors appears to be low (Ciesek et al., 2011; Deest et al., 2014; Zeisel et al., 2011, 2013b). By imposing a higher genetic barrier to resistance than DAAs, entry inhibitors may reduce emergence of viral escape variants. Indeed, entry inhibitors have been demonstrated to potently inhibit highly infectious escape variants of HCV that are resistant to host neutralizing antibodies (Fafi-Kremer et al., 2010; Fofana et al., 2012, 2010; Lupberger et al., 2011; Zahid et al., 2013). Nevertheless, viral escape has also been described for anti-SR-BI antibodies (Catanese et al., 2013). Interestingly, it has been shown that viral escape variants to anti-SR-BI antibodies are more susceptible to neutralizing antibodies (Bankwitz et al., 2010; Prentoe et al., 2011).

Furthermore, in contrast to compounds targeting the viral particle, entry inhibitors are also less likely to be genotype-dependent, since so far the major HCV genotypes have been shown to enter into the cell using the same cellular pathways. Examples for pangenotypic action include CLDN1-(Fofana et al., 2010), SR-BI-(Lacek et al., 2012; Zahid et al., 2013) and CD81-specific (Fofana et al., 2013a; Meuleman et al., 2008) monoclonal antibodies, ITX-5061

Table 1
Examples of host-targeting entry inhibitors against hepatitis C virus infection.

Target	Examples of compounds	Stage of development	References
CD81	Anti-CD81 mAbs	Mouse model	Meuleman et al. (2008)
	Imidazole based compounds	Cell culture	VanCompernolle et al. (2003)
SR-BI	ITX5061	Phase I/IIa	ClinicalTrials.gov Identifier: NCT01165359
	Anti-SR-BI mAbs	Mouse model	Lacek et al. (2012), Meuleman et al. (2012)
	Serum amyloid A	Cell culture	Lavie et al. (2006)
CLDN1	Anti-CLDN1 mAbs	Cell culture	Krieger et al. (2010), Fofana et al. (2010))
EGFR	Erlotinib	Phase I/IIa	ClinicalTrials.gov Identifier: NCT01835938
NPC1L1	Ezetimibe	Mouse model	Sainz et al. (2012)
TfR1	Anti-TfR1 mAbs	Cell culture	Martin and Uprichard (2013)
Post-attachment	Flavonoids	Cell culture	Calland et al. (2012), Haid et al. (2012)
Fusion/internalization	PS-ON	Mouse model	Matsumura et al. (2009)
	Arbidol	Cell culture	Boriskin et al. (2008, 2006)
	Chloroquine	Cell culture	Blanchard et al. (2006), Tscherne et al. (2006)
	Silymarin	Cell culture	Marino et al. (2013), Wagoner et al. (2010)

(Syder et al., 2011), erlotinib (Diao et al., 2012; Lupberger et al., 2011), ezetimibe (Sainz et al., 2012), flavonoids (Calland et al., 2012; Haid et al., 2012), phosphorothioate oligonucleotides (Matsumura et al., 2009) and silymarin (Marino et al., 2013; Wagoner et al., 2010) where proof-of-concept studies of entry inhibitors have been conducted *in vitro* and *in vivo* using the chimeric uPA-SCID mouse model (Table 1).

While cell-free entry is most relevant for initiation of HCV infection, HCV neutralizing antibody-resistant cell–cell transmission is thought to play an important role for viral evasion and persistence (Timpe et al., 2008; Zeisel et al., 2013b). Most host factors including CD81, SR-BI, CLDN1, OCLN, EGFR, EphA2, HRas and NPC1L1 have been shown to be involved in direct cell–cell transmission (Brimacombe et al., 2011; Lupberger et al., 2011; Sainz et al., 2012; Timpe et al., 2008). Interestingly, CD81-independent routes as well as differential requirements for SR-BI have been described (Catanese et al., 2013;Timpe et al., 2008; Witteveldt et al., 2009). The HCV envelope glycoproteins are also essential for this process (Witteveldt et al., 2009). With the exception of an alpaca anti-envelope monoclonal antibody (Tarr et al., 2013), monoclonal or polyclonal antibodies targeting the viral envelope glycoproteins fail to abrogate this process (Brimacombe et al., 2011). By acting through a different mechanism of action host-targeting entry inhibitors act as potent inhibitors of HCV cell–cell transmission (Lupberger et al., 2011; Sainz et al., 2012; Zahid et al., 2013; Zeisel et al., 2013b).

The crucial role of host cell entry factors for both *de novo* infection and viral spread also suggest that targeting viral entry may have a therapeutic effect in chronic infection: Indeed, recent studies have shown that an anti-SR-BI antibody can at least partially and temporary inhibit viral spread when added postinfection (Meuleman et al., 2012). Furthermore, an HBV entry inhibitor has been shown to impair intrahepatic virus spreading in humanized mice previously infected with hepatitis B virus *in vivo* (Volz et al., 2013). These data suggest that constant re-infection of non-infected or cured hepatocytes is most likely required for viral spread *in vivo*. Immune necrosis-mediated liver regeneration may be another mechanism where *de novo* infection of previously uninfected hepatocytes is required to maintain a chronic infection. Since entry inhibitors are expected to block the *de novo* infection of hepatocytes, it is conceivable that a potent entry inhibitor may also have a therapeutic effect on chronic infection.

3. Preclinical and clinical development of entry inhibitors

In contrast to DAAs, the development of entry inhibitors has long been hampered by the lack of a small animal model and robust cell culture systems to study HCV entry. While the development of replicons (Blight et al., 2000; Lohmann et al., 1999) were a breakthrough for the development of DAAs, the establishment of HCV pseudo-particles (HCVpp) (Bartosch et al., 2003; Hsu et al., 2003) and recombinant infectious HCV (HCVcc) (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005) model systems has enabled screening and identification of entry inhibitors. The development of transgenic and knock-out immunodeficient mice with hepatocyte-lethal phenotype (Alb-uPA/SCID and Fah/Rag2/IL2rγ mice) (Bissig et al., 2010; Mercer et al., 2001) that can be successfully transplanted with primary human hepatocytes allowed study of the efficacy and safety of entry inhibitors *in vivo*. Humanized transgenic mouse models (Dorner et al., 2013, 2011) provide further tools for the preclinical development of entry inhibitors.

Today, most entry inhibitors are in preclinical development: examples include antibodies targeting CD81, SR-BI and CLDN1, synthetic small molecules such as ezetimib or plant-derived derivatives. An overview of host-targeting entry inhibitors is shown in Table 1. The most advanced compound of an HCV entry inhibitor is ITX5061, a small molecule inhibitor targeting the HCV entry factor SR-BI (Syder et al., 2011): A Phase 1b study assessing the safety of ITX5061 in HCV-treatment naive patients is ongoing (ClinicalTrials.gov Identifier NCT01165359) and an open-label, proof-of-concept Phase 1b study assessing the safety and tolerability of ITX-5061 in LT patients has been initiated (ClinicalTrials.gov Identifier NCT01292824). Recent data revealed ITX5061 being less efficient in chronically infected patient (Sulkowski et al., 2014) suggesting further investigation for alternative strategies such as combining entry inhibitors with DAAs. Furthermore, a randomized clinical trial assessing the virologic response and short-term safety of erlotinib in patients infected with HCV genotype 1b has recently been initiated (ClinicalTrials.gov Identifier NCT01835938). The clinical potential of kinase inhibitors such as erlotinib (targeting EGFR) has been emphasized by a recent case report describing rapid virologic response (RVR) in an HCV-positive HCC patient after LT (Bardou-Jacquet et al., 2011).

A theoretical drawback of using host-targeting entry inhibitors is a potentially greater risk of cellular toxicity given that they interfere with host targets (Zeisel et al., 2013b). However, the large majority of licensed drugs in clinical use (e.g. in cardiovascular, inflammatory disease or oncology) target host proteins and side effect profiles of these approved drugs are not necessarily worse than those of drugs that directly target the virus (Zeisel et al., 2013b). Of note, one entry inhibitor targeting a host protein has already been approved for the treatment of HIV infection: maraviroc is a competitive inhibitor of the CD4 co-receptor and HIV entry factor CCR5 and is currently in clinical use in combination therapies both as a reserve drug, if resistance to the direct antiretroviral substances has developed, and as a first line agent (Saag et al., 2009). Other CCR 5 inhibitors are being studied. Interestingly, the

rationale behind the successful treatment of the famous "Berlin Patient" was to introduce a mutated version of the host protein CCR 5, rendering functional resistance to HIV (Hutter et al., 2009). The patient has been taken off antiretroviral therapy and HIV has become undetectable, suggesting long-term control and a possible functional cure (Didigu and Doms, 2012).

Maraviroc and the experimental CD4 receptor antibody ibalizumab have generally not been found to cause more severe or more frequent adverse events than direct antiretroviral drugs (Henrich and Kuritzkes, 2013; Sharma et al., 2013). Furthermore, the pharmacologic properties of the two HCV entry inhibitors described above (erlotinib and ITX5061) have already been characterized and studied extensively and both drugs are currently approved in non-small cell lung cancer and pancreatic cancer; besides, they have been used for the treatment of malignant diseases in numerous therapeutic trials and have therefore a known and predictable side-effect profile (Shepherd et al., 2005; Syder et al., 2011) greatly facilitating their use in clinical trials on HCV infected patients. Nevertheless, a thorough investigation of safety is a key issue in preclinical and clinical development of host-targeting entry inhibitors.

4. Perspective of entry inhibitors to address challenges of DAAs: liver graft infection and difficult-to-treat patients

Viral entry has been demonstrated to play an essential role in the pathogenesis of HCV infection, especially during re-infection of the graft after LT where both viral entry and escape from antibody-mediated neutralization have been shown to play a key role for the selection of viral variants in the early phase of liver transplantation (Fafi-Kremer et al., 2010; Fofana et al., 2012). Given the importance of host entry factors for re-infection of the graft during LT and their unique mechanism of action, entry inhibitors appear ideal for the prevention of HCV re-infection in the transplantation setting where currently no approved therapy exists to protect HCV-negative transplanted livers from re-infection. This is supported by the experience in prevention of HBV graft infection, where the use of hepatitis B immune globulin (a well-characterized HBV entry inhibitor) in combination with nucleos(t)ide analogues has essentially eliminated HBV recurrence in LT patients (Crespo et al., 2012).

Approved DAA-based regimens are problematic in this group of patients because of drug-drug interactions and severe side effects (Liang and Ghany, 2013). Two studies with limited patient numbers have evaluated the combination of sofosbuvir and ribavirin in the setting of liver transplantation (Charlton et al., 2013; Curry et al., 2013). The first trial demonstrated SVR in 64% of patients after LT when the treatment was given prior to transplantation, and the latter revealed early SVR four weeks after treatment (SVR4) in 77% when given for recurrence of HCV infection after OLT. While promising, a significant portion of patients in the first study still experienced recurrence and the data in the second study is preliminary since only SVR4 and not SVR was provided. The study of second generation DAAs for prevention of graft infection is ongoing.

The high variability of HCV has so far hampered the development of efficient cross-neutralizing antibodies (Chung et al., 2013; Sarrazin et al., 2012; Schweitzer and Liang, 2013), although the development of next-generation monoclonal antibodies is ongoing (Giang et al., 2012). Another interesting concept is the use of patient-derived immunoglobulin for prevention of liver graft infection (ClinicalTrials.gov Identifier NCT01804829). Since host cell entry factors play a key role for viral evasion in liver graft infection and provide a genetic barrier to resistance (Fofana et al., 2012), entry inhibitors may therefore provide a simple and targeted strategy to prevent liver graft infection. Indeed, antibodies directed against CLDN1 have been shown to be efficient against transplant escape variants (Fofana et al., 2010) and antibodies directed against CD81 and SR-BI have demonstrated potent antiviral effect in prophylactic and post-exposure *in vivo* treatment studies (Lacek et al., 2012; Meuleman et al., 2012, 2008). Clinical trials are needed to evaluate this concept in HCV-infected patients.

Drug resistance remains a challenge in current and future therapies for hepatitis C (Liang and Ghany, 2013; Pawlotsky, 2011). The ultimate objective for HCV therapy is an IFN-free regimen that is pan-genotypic and highly efficient. Host-targeting entry inhibitors, by acting on cellular targets that are less prone to mutations, may impose a higher genetic barrier for resistance (Zeisel et al., 2011, 2013b).

Finally, by acting through a differential mechanism of action, combining entry inhibitors that have different targets of action with DAAs should result in an additive or synergistic antiviral effect (Zhu et al., 2012). Therefore, combining compounds targeting entry factors and complementary steps of the viral life cycle such as replication is a promising approach for prevention of infection in LT and cure of chronic HCV infection. Furthermore, this may considerably increase the barrier to resistance due to the potential absence of cross-resistance between entry inhibitors and DAAs (Zhu et al., 2012). Compared to many DAAs, entry inhibitors act in a pan-genotypic manner, thereby suggesting novel options for patients with non-genotype 1 infections. Analogous to the currently available antiretroviral treatment regimens for HIV infection, approval of several different drug combinations for hepatitis C may help to individualize therapy in difficult-to-treat patients and for multi-resistant virus strains. Furthermore, as targeting the host is an emerging strategy to overcome resistance (Nathan, 2012), the development of entry inhibitors may also represent a promising approach for other viral infections. Taken together, novel combinations based on entry inhibitors represent a promising approach against HCV infection for prevention of liver graft infection, treatment of difficult-to-treat-patients and may offer alternatives for patients with contraindications to current IFN-based or future IFN-free regimens. Clinical studies are warranted to evaluate the future role of entry inhibitors in the management of HCV infection.

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