A novel class of small molecule compounds that inhibit hepatitis C virus infection by targeting the prohibitin-CRaf pathway

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Hepatitis C virus (HCV) is an important human pathogen that primarily infects human hepatocytes and causes chronic liver diseases (Kontorinis et al., 2004). It is undeniable that recently approved direct-acting antiviral (DAA)-containing regimens have radically changed the paradigm of chronic HCV treatment. However, it remains unclear whether DAAs fully prevent pathology or restore normal immunity. Moreover, with continuous and expanded usage of DAAs, HCV is expected to become progressively more drug resistant, thereby eroding the efficacy of DAAs (Pawlotsky et al., 2015). Lastly, most DAAs are hardly affordable to patients in resource-limited countries. For these reasons, druggable host targets and new lead compounds are highly desirable.

HCV entry is a multifaceted target for intervention. HCV encodes ten viral proteins to complete its life cycle. Viral glycoproteins E1 and E2 together form spikes on the viral envelope, which then engage with cell surface molecules (Lindenbach et al., 2005; Kato et al., 2005; Zhang et al., 2004; Cormier et al., 2004; Bartosch et al., 2003; Petraccia et al., 2000) and trigger the endocytosis of the viral particle (Meertens et al., 2006; Hsu et al., 2003). In addition, E2 interacts with HCV nonstructural protein 2 (NS2) and plays an important role in virus morphogenesis (Selby et al., 1994). Recent advances have suggested that HCV enters hepatocytes in a step-wise fashion by utilizing multiple cellular membrane proteins, including CD81 (Piliero et al., 1998), scavenger receptor BI (SR-BI) (Scarselli et al., 2002), claudin-1 (CLDN1) (Evans et al., 2007), occludin (OCLN) (Liu et al., 2009; Ploss et al., 2009), epidermal growth factor receptor (EGFR) (Lupberger et al., 2011), and cholesterol-uptake receptor Niemann-Pick C1-like 1 (NPC1L1) (Sainz et al., 2012). Zona and coworkers further reported that the GTPase HRas acts as a signal transducer for EGFR-mediated HCV entry by regulating lateral membrane diffusion of CD81 which then enables tetraspanin receptor complex assembly (Zona et al., 2013).

In an attempt to identify novel cellular targets for antiviral development, we found that the cellular proteins prohibitin (PHB) 1 and 2 associate with HCV E2 in infected human hepatoma cells. We further
demonstrated that PHBs mediate HCV entry at a post-binding step through their interaction with the signaling molecule CRaf. Blocking PHB-CRaf interactions with the natural product rocablidade (Roc-A) potently inhibited HCV entry, and the biosynthetically-related rocalglade natural product aglaroxin C displayed both improved potency and therapeutic index.

2. Materials and Methods

Full experimental methods are provided in detail in the Supplemental Information.

2.1. Immunochemistry Staining and Confocal Microscopy

Detailed procedures have been published (Yang et al., 2008). In brief, Huh7.5.1 cells were transfected with si-CTRL (control siRNA), or si-PHB, or si-PHB2, and Stop-Luc construct which contains a firefly luciferase reporter gene whose transcription is prevented by a Stop cassette flanked by LoxP sites. 48 h post-transfection, these recipient cells were mixed at a 1:1 ratio with 293T-CLDN1 cells expressing Cre and HCV E1E2 (H77, genotype 1a) (donor cells) to initiate cell–cell fusion. Luciferase activity was measured 24 h thereafter.

2.2. HCV E1E2-Mediated Cell–Cell Fusion Assay

Detailed protocols have been published (Si et al., 2012). In brief, Huh7.5.1 cells were transfected with si-CTRL (control siRNA), or si-PHB1, or si-PHB2, and Stop-Luc construct which contains a firefly luciferase reporter gene whose transcription is prevented by a Stop cassette flanked by LoxP sites. 48 h post-transfection, these recipient cells were mixed at a 1:1 ratio with 293T-CLDN1 cells expressing Cre and HCV E1E2 (H77, genotype 1a) (donor cells) to initiate cell–cell fusion. Luciferase activity was measured 24 h thereafter.

2.3. Cell Surface Biotinylation Assay

Huh7.5.1 cells from four 150 mm plates were treated with DMSO, Roc-A (20 nM), or infected by HCVcc. 48 h post-transfection, cells were washed three times with ice-cold PBS and resuspended in PBS at a density of 25 × 10^6 cells/mL. Freshly prepared Sulfo-NHS-SS-biotin (Pierce) was added to the cells (final concentration 0.5 μg/mL) and allowed to incubate at 4 °C for 30 min. Cells were then washed three times with ice-cold PBS. 25 mM Tris (pH 8.0) was added in the initial wash to quench any non-reacted biotin reagent. Following cell lysis in RIPA buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 2 mM Na3VO4 and Pierce protease inhibitor cocktail), lysates were cleared by centrifugation at 13,000 × g for 15 min at 4 °C. The cleared lysates were used for immunoprecipitation using a 1:1 mixture of Streptavidin beads (Pierce). Beads were washed three times with RIPA buffer, and bound proteins were eluted by boiling the samples in SDS-PAGE sample buffer and then resolved on 9% SDS-PAGE. Biotinylated proteins were detected by anti-PHB1 and anti-PHB2 antibodies.

2.4. Cytotoxicity/Cell Viability Assay

PHHs (10^5 per well) were treated with Roc-A or DMSO at various concentrations for 48 h in 48-well plates. The numbers of viable cells in culture were determined using the CellTiter-Glo Cell Viability Luminescent Assay kit according to the manufacturer’s instruction (Promega).

2.5. Statistical Analysis

Bar graphs were plotted to show mean ± standard deviation (SD) of at least two independent experiments. Statistical analyses were performed using Graphpad Prism 5. A p value of <0.05 in the Student’s t-test was considered statistically significant.

2.6. Chemical Synthesis

Synthetic rocaglates and derivatives were obtained from the chemical collection at the BU Center for Molecular Discovery (BU-CMD). Chiral, racemic rocaglates (Roche et al., 2010a, 2010b) and rocaglate hydroxamates (Rodrigo et al., 2012) were synthesized using the reported procedures. Chiral, non-racemic (−)-aglaroxin C and (+)-aglaroxin C were synthesized using biomimetic kinetic resolution of chiral, racemic agalin ketone precursors according to our published protocol (Stone et al., 2015) followed by further chemical transformations. (−)-Roc-A, and (+)-Roc-A were synthesized using the same protocol followed by amide formation (Gerard et al., 2006).

3. Results

3.1. PHB1 and 2 Interact with HCV E2

We have previously conducted a comparative proteomics analysis of the HCV-infected human hepatoma cell line Huh7.5.1 in order to identify HCV E2-interacting proteins. PHB1 and 2 were found to be the most abundant proteins in the E2 complex as detected by mass spectrometry. To validate the result, we performed immunoprecipitation using lysates from cells infected with the Flag-E2 JFH1 virus and confirmed that PHB1 and 2 co-precipitated with HCV E2 (Fig. S1A). The PHB-E2 association does not require the presence of other viral components as demonstrated in co-immunoprecipitation (Co-IP) studies (Fig. S1B).

3.2. PHB1 and 2 are Required for HCV Entry

PHB1 is a ubiquitously expressed protein displaying antiproliferative activity (McClung et al., 1989). PHB2, also named repressor of estrogen receptor activity (REA), suppresses estrogen receptor (ER)-dependent gene activation (Montano et al., 1999). Interestingly, PHB has been implicated in the entry process of dengue and chikungunya virus (CHIKV) and also binds to HIV-1 glycoprotein and envelope proteins of white spot syndrome virus (Lan et al., 2013; Wintachai et al., 2012; Kuadkitkan et al., 2010; Emerson et al., 2010). To explore the role of PHB in modulating HCV infection, we transfected Huh7.5.1 cells with siRNA targeting PHB1 and PHB2, respectively. Reduction of endogenous PHB1 or 2 significantly inhibited cell culture grown HCV (HCVcc) as measured by either luciferase assays or real-time PCR quantification of viral RNA (Fig. 1A and B). By contrast, PHB knockdown had no effect at viral RNA levels if the infection took place first (Fig. S1C), suggesting that PHBs are required at an early stage of HCV infection. Notably, PHB1 and PHB2 knockdown also decreased the protein levels of each other (Fig. S1D).

We then found that PHB1 and 2 are required for HCV entry, as silencing PHB1/2 in Huh7.5.1 cells or primary human hepatocytes (PHHs) ablated HCV–HCV pseudotype (HCVpp) infection while having no effect on pseudotyped virus displaying vesicular stomatitis virus G protein (VSV-Gpp) (Fig. 1C). Furthermore, PHB knockdown significantly reduced the infection of HCVpp-bearing glycoproteins from various HCV genotypes (Fig. 1D). When tested in a HCV replicon cell line, however, knockdown of endogenous PHB1 and 2 did not decrease viral RNA replication or protein translation (Fig. S1E & F).

To strengthen the above findings and to exclude the off-target effects of siRNAs, we generated Huh7.5.1 stable clones in which the infection took place first (Fig. S1C), suggesting that PHBs are required at an early stage of HCV infection. Notably, PHB1 and PHB2 knockdown also decreased the protein levels of each other (Fig. S1D).
3.3. PHB1 and 2 can be Found on the Cell Surface and are Required for a Post-Binding Step in HCV Infection

To gain insights into the roles of PHBs in HCV entry, we first examined the cellular distribution of PHB1/2. Typically found in the inner membrane of mitochondria, PHB1 and 2 form a large multimeric complex to stabilize newly synthesized mitochondrial proteins (Back et al., 2002). In addition, PHB1 and 2 are reportedly present on the plasma membrane, cytosol, and nucleus (Mishra et al., 2005; Theiss and Sitaraman, 2011; Kim do et al., 2013; Kolonin et al., 2004). Confocal microscopy study of endogenous PHB1 and 2 in Huh7.5 cells revealed a typical mitochondria localization pattern, although a small portion of PHB2 appeared to traffic to the cell surface (Fig. S2A). To definitively examine if PHB1 and 2 traffic to plasma membrane, cell surface proteins were biotinylated and then purified using streptavidin-agarose beads. Both PHB1 and 2 were detected in the pull-down product, confirming their presence on the plasma membrane (Fig. 2A). Of note, HCV infection did not change the amount of PHB1 and 2 that were found in the precipitates (Fig. 2A, lane 3).
PHBs contain several functional domains, as illustrated in Fig. 2B. Mutational studies revealed that only the removal of the transmembrane domain (TMD) of PHBs led to the loss of cell surface localization (Fig. 2C). Interestingly, upon removal of their C-terminal domains, PHBs preserved both their cell surface and mitochondrial location, yet were unable to interact with HCV E2 (Fig. 2D & E).

To further investigate the mechanistic action of PHB, we directly evaluated the role of PHB on binding of HCV to the cell surface. To this end, HCV virions were incubated with PHB knockdown cells at 4 °C for 2 h to allow binding but not penetration. After extensive wash, surface-bound virions were quantified by measuring the abundance of viral RNA. Shown in Fig. 2F, the amount of viral RNA bound to PHB-knockdown cells was comparable to that of si-CTRL cells, indicating that PHB silencing has no effect on binding of HCV to cells. Similarly, PHB knockdown did not have a significant effect on CD81, SR-BI, or OCLN expression (Fig. S2B), nor did PHBs interact with any of these known entry factors (Fig. S2B). Lastly, we measured the HCV E1E2-dependent fusion and found that PHB knockdown cells were impaired in fusing with 293T cells expressing HCV E1E2 (Fig. 2G). Altogether, these results suggest that PHB1 and 2 are involved in a late stage during virus entry.

3.4. PHB1 and 2 Interact with CRaf

Co-IP studies showed that PHB1 and 2 interact with EGFR and CRaf (Fig. S2C & D). Removal of the entire C-terminal domains of PHB1 and 2 abolished the interaction between PHB and CRaf. In contrast to a recent publication which reported that a Raf inhibitor (10 μM) failed to block HCV infection (Diao et al., 2012), silencing CRaf expression in Huh7.5.1 cells specifically suppressed HCV entry (Fig. S2E). Correctively, our data suggest that the PHB-CRaf pathway is critical for HCV infection.

3.5. A PHB Inhibitor, Rocaglamide A (Roc-A), Inhibits HCV Entry

Next, we asked whether targeting the PHB-CRaf pathway would be a viable approach to block HCV infection. The natural product rocaglamide A (Roc-A) has been shown to directly bind to PHB1 and 2 and blocks PHB-CRaf-MEK-ERK signaling (Polier et al., 2012). Addition of Roc-A at 20 nM did not inhibit HCV RNA replication or protein translation (Fig. 3A). Rather, it significantly reduced HCVcc, HCVpp, CHIKVpp, and dengue virus infection (Fig. S3C–G) at low nanomolar concentrations. Moreover, pretreatment of PHHs with Roc-A at non-cytotoxic concentrations also suppressed HCVpp entry (Fig. 3A). The therapeutic index (TI) of Roc-A is greater than 80 when tested on primary human hepatocytes. Notably, Roc-A treatment decreased the cell surface expression of PHB1 and 2 (Fig. 2A) and also decreased PHB1, 2, Ras, and CRaf at the total protein level (Fig. 3B). Roc-A treatment disrupted the PHB-CRaf interaction in a co-IP experiment (Fig. 3C and D). Finally, Roc-A displayed a half-life of 37 min in a human liver microsomal stability assay (Fig. S4), suggesting that the compound has good metabolic stability.

3.6. Roc-A Derivatives Display Improved Therapeutic Index

To obtain compounds that display more favorable therapeutic index towards HCV, we evaluated a set of 32 additional rocaglate derivatives to ascertain structure-activity relationships (SAR) (Table S1). Interestingly, the natural (+) enantiomer of Roc-A displayed stronger inhibition on HCV entry than the (−) enantiomer (Fig. 3E). Moreover, the chiral, racemic version of the natural product aglaroxin C (Udom Kokpol, 1994) exhibited picomolar half-maximum effective concentrations (EC₅₀) towards the JFH-1 genotype 2a infectious virus in cell culture and a therapeutic index of over 100 (Table 1). Further analysis indicated that (−)-aglaroxin C is the active enantiomer for
HCV viral entry inhibition (Table 1). Moreover, these compounds, even when incubated at 2 μM for 3 h, did not inhibit protein translation (Fig. 3F). Results from a time-of-addition experiment further showed that chiral, racemic aglaroxin C displayed maximal anti-HCV activity when added together with the virus but lost its activity when added 3 h after the infection was initiated (Fig. 3G). This finding bolstered the notion that aglaroxin C is specifically inhibiting viral entry.

Table 1

| Compound       | Structure | EC50  | CC50  | TI (CC50/EC50) | Translation inhibition at 2 μM |
|----------------|-----------|-------|-------|----------------|-------------------------------|
| (+) Roc-A      |           | >200 μM | 300 μM | N/A            | No                            |
| (−) Roc-A      |           | 1 μM  | 50 μM | 50%            | No                            |
| (±) Aglaroxin C | (C10)     | 100 nM | 10 μM | 100%           | No                            |
| (+) Aglaroxin C |           | 20 μM | 100 μM | 5%             | No                            |
| (−) Aglaroxin C |           | 200 nM | 12 μM | 60%            | No                            |

a Compounds were incubated with cells for a total of 3 h on Huh7.5.1 cells.
b Compounds were incubated with cells for a total of 48 h on Huh7.5.1 cells.
c Measured using a luciferase reporter gene that was transfected into Huh7.5.1 cells. The compound was added for 3 h.
d Name appearing in Fig. 3G.
4. Discussion

The identification of PHB1 and 2 as HCV entry factors is somewhat surprising because these two proteins are typically recognized as molecular chaperones that stabilize other mitochondrial proteins. A fraction of PHB1 and 2 can be found on the surface of Huh7.5.1 cells, although they are not required for the initial binding of virions. Given that PHB-CrAF interaction is necessary for CrAF activation by Ras (Rajalingam and Rudel, 2005; Polier et al., 2012), and that HRas is a key host signal transducer for EGFR-mediated HCV entry (Zona et al., 2013), a plausible role of PHBs in HCV entry is to link HRas to CrAF-mediated signaling. In support of this notion, we found that CrAF knockdown reduced HCV infection, as did disruption of PHB-CrAF interaction by Roc-A. PHBs have been implicated in facilitating signal transduction. For example, a recent study showed that PHB1, normally stored in mast cell granules, translocates to plasma membrane lipid rafts upon antigen stimulation in order to activate the tyrosine kinase Syk-dependent signaling that stimulates mast cell degranulation and the secretion of cytokines (28).

Exactly when and how HCV activates the PHB1-mediated signaling remains to be investigated. PHB1/2 do not precipitate with CD81, SR-BI, CLDN1, or OCLN, but are associated with HCV E2 in both infected and co-transfected cells. PHBs are anchored to the plasma membrane or mitochondrial inner membrane by their transmembrane domains with carboxyl termini facing cytoplasm or the intermembrane space of mitochondria. The C-termini of PHBs recruit CrAF to the inner plasma membrane (Mishra et al., 2005). Given that HCV E2 does not traffic to the intermembrane space of mitochondria, the interaction between PHBs and E2 likely takes place in close proximity to the inner plasma membrane although evidence for direct PHB–HCV E2 interaction is still lacking. Since the removal of either the transmembrane domain or C-terminal domain of PHBs abolishes PHB–E2 interaction, HCV E2 may form a signaling complex with membrane-bound PHB–CrAF at some point during entry. The PHB–HCV E2 associations are not mediated by cell membranes, as C-terminal deletion of PHB1 did not alter their membrane localization. C-terminal deletion did, however, abolish PHB–E2 association. Plasma membrane-bound PHB1 is indispensable for the activation of CrAF by Ras (Rajalingam and Rudel, 2005; Rajalingam et al., 2005), and interaction between PHB1 and CrAF requires phosphorylation of PHB1 at Thr 258 and Tyr 259 (Chiu et al., 2013). Further investigation is needed to understand the topology, the phosphorylation status of plasma membrane-bound PHBs, and the likely signaling pathways that PHBs mediate during HCV entry.

An exciting finding of our study is that Roc-A, which binds PHB and inhibits its interaction with CrAF (Polier et al., 2012), potently inhibited HCV entry. Roc-A was first reported as an immunosuppressant and inhibitor of NF-kappa B activity (31). Roc-A and related rocaglates are also recognized as potent anticanicancer compounds (Kim et al., 2006; Ebada et al., 2011) by inhibiting translation initiation through inhibition of the RNA helicase elf4a (Roche et al., 2010a, 2010b; Rodrigo et al., 2012; Chowdhury et al., 2014; Cencic et al., 2009). Roc-A has also been shown to indirectly target heat shock factor 1 (HSF1), a multifacetted transcriptional regulator of the heat-shock response and numerous other cellular processes essential for anabolic metabolism, cellular proliferation, and tumorigenesis (Santagata et al., 2013). In the current study, Roc-A treatment significantly reduced the protein levels of cell surface-bound PHB1 and PHB2 and disrupted PHB–CrAF interaction, indicating that it blocks HCV entry by targeting this pathway. The observation that a racemic, synthetic sample of the natural product agalaroxin C displays improved therapeutic index relative to enantiopure Roc-A suggests the possibility for synergy of enantiomers (Zhuang et al., 2014; Daniëls et al., 2011). In our studies, racemic agalaroxin C did not inhibit protein translation even at 2 μM over 3 h in a translation inhibition assay (Fig. 3F). Future investigations will be needed to understand whether it is possible to selectively target the PHB–CrAF pathway using appropriately functionalized rocaglates (flavaglines) and to what extent there may be synergy between translation inhibition or other mechanisms and HCV viral entry effects via PHB's (Cencic et al., 2010; Rozelle et al., 2014).

In conclusion, the identification of PHB1 and 2 adds additional targets to the repertoire of HCV entry factors. In contrast to most small molecule inhibitors that have advanced to the clinic targeting viral components, Roc-A, a PHB inhibitor, represents a promising drug lead that targets a host factor and hence reduces the likelihood that resistance will be developed. By virtue of its distinct mechanism of inhibition, Roc-A and its derivatives may also be used in combination with other anti-HCV drugs for potential synergistic effects in treating HCV infections, especially in settings where liver cancer is present. The observation that rocaglates (flavaglines) block CHIKVpp entry (Wintchail et al., 2015) (Fig. S3F) and dengue virus infection (Fig. S3G) also raises the hope that Roc-A or an optimized combiner may be developed into a drug curbing infections by the two viruses.

Author contributions

S.L., T.Z., J.A.P., Jr., and T.W. designed the overall project and analyzed data. S.L., C.Q., J.Z., and T.W. performed most cellular and molecular experiments. W.W., N.L. and L.E.B. synthesized and curated rocaglate derivatives. S.L., J.A.P., Jr., and T.W. wrote the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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