A Small-Molecule Inhibitor of Hepatitis C Virus Infectivity


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One of the most challenging goals of hepatitis C virus (HCV) research is to develop well-tolerated regimens with high cure rates across a variety of patient populations. Such a regimen will likely require a combination of at least two distinct direct-acting antivirals (DAAs). Combining two or more DAAs with different resistance profiles increases the number of mutations required for viral breakthrough. Currently, most DAAs inhibit HCV replication. We recently reported that the combination of two distinct classes of HCV inhibitors, entry inhibitors and replication inhibitors, prolonged reductions in extracellular HCV in persistently infected cells. We therefore sought to identify new inhibitors targeting aspects of the HCV replication cycle other than RNA replication. We report here the discovery of the first small-molecule HCV infectivity inhibitor, GS-563253, also called HCV infectivity inhibitor 1 (HCV II-1). HCV II-1 is a substituted tetrahydroquinoline that selectively inhibits genotype 1 and 2 HCVs with low-nanomolar 50% effective concentrations. It was identified through a high-throughput screen and subsequent chemical optimization. HCV II-1 only permits the production and release of noninfectious HCV particles from cells. Moreover, infectious HCV is rapidly inactivated in its presence. HCV II-1 resistance mutations map to HCV E2. In addition, HCV-II prevents HCV endosomal fusion, suggesting that it either locks the viral envelope in its prefusion state or promotes a viral envelope conformation change incapable of fusion. Importantly, the discovery of HCV II-1 opens up a new class of HCV inhibitors that prolong viral suppression by HCV replication inhibitors in persistently infected cell cultures.

One of the most challenging goals of hepatitis C virus (HCV) research is to develop well-tolerated regimens with high cure rates. The standard of care for HCV patients over the past decade has been to treat with pegylated interferon combined with ribavirin. Relatively recently, the HCV NS3-4A protease inhibitors telaprevir and boceprevir were added to this standard of care and have improved the sustained virologic response (1). However, poor tolerability to treatments containing pegylated interferon has motivated researchers to attempt to develop a variety of other interferon-free treatments (2). An interferon-free cure will likely require a combination of at least two antivirals with differing modes of action (3, 4). Combining multiple antivirals with different resistance profiles increases the number of resistance mutations required for viral breakthrough (5). Studies to determine optimal antiviral combinations which take into account the probability of emergence of specific resistance mutations as well as the subsequent viral fitness (3, 6, 7) are under way.

We recently demonstrated that the in vitro combination of HCV entry inhibitors with HCV replication inhibitors could prolong the efficacy compared to a single treatment with either inhibitor class. Specifically, we studied the entry inhibitor anti-CD81 antibody (Ab), which blocks virion binding to the host CD81 receptor (8), and entry inhibitor 1 (EI-1) (9), which blocks viral fusion during entry. When we combined anti-CD81 Ab or EI-1 with the NS3-4A protease inhibitor BILN-2061 (10) or with the NS5A inhibitor daclatasvir (11), we observed prolonged viral suppression for at least 3 weeks in persistently infected Huh7 cells (5). In contrast, monotherapy with these inhibitors only suppressed viral levels for 7 to 10 days.

Many groups are actively studying multiple classes of HCV inhibitors that target stages of the HCV replication cycle outside of RNA replication (i.e., entry, assembly, egress, and infectivity). There are several recent reports highlighting new HCV entry and assembly inhibitors. The entry inhibitors ITX-4520 (12) and anti-SR-BI antibodies (13) are examples that block the SR-BI receptor involved in HCV entry. Further examples of HCV entry inhibitors being studied are the flavonoid BJ486K (14), green-tea polyphenol epigallocatechin-3-gallate (15, 16), EI-1 (9), and related triazines (17), as well as anti-CD81 Ab (5, 8). Some examples of HCV assembly inhibitors discovered in recent years include the grapefruit flavonoid naringenin (18), the core dimerization inhibitor SL209 (19), and the imino sugars PBDNJ0804 (20) and deoxynojirimycin (21). Some imino sugars appear to act as HCV egress inhibitors as well (20).

We report here the discovery of the first small-molecule HCV infectivity inhibitor, HCV infectivity inhibitor 1 (HCV II-1). HCV II-1 interacts directly with GT1a and GT2a HCV particles, likely with E2, and inhibits infectivity by blocking endosomal fusion. HCV II-1 represents a new class of HCV inhibitors that could be added to treatment regimens since we observed that it prolongs...
viral suppression by HCV replication inhibitors in persistently infected cell cultures.

MATERIALS AND METHODS

Cell culture. Huh7-Lunet-CD81 (22) cells were propagated in Dulbecco modified Eagle medium (DMEM) with GlutaMAX-1 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 0.1 mM nonessential amino acids (Invitrogen). Cells were maintained in humidified incubators at 37°C and 5% CO₂.

Antiviral compounds and antibodies. The HCV NS3-4A protease inhibitor BILN-2061 was purchased from Acme Bioscience (Belmont, CA). The HCV NS3A inhibitor daclatasvir was purchased from Selleck Chemicals (Houston, TX). HCV entry inhibitor 1 (EI-1) was purchased from ChemBridge Corp. (San Diego, CA). The mouse monoclonal anti-human CD81 antibody JS-81 was purchased from BD Biosciences (San Jose, CA). Anti-NS5A antibody 9E10 was purchased from Apath (Brooklyn, NY). Bafilomycin A1 was purchased from Sigma-Aldrich (St. Louis, MO). HCV II-1 (G5-563253) was synthesized by Gilead Sciences (Foster City, CA). The full name of this compound is (4S,7S,22)-2,2,2-trifluoroethyl 2-methyl-5-oxo-7-phenyl-1,4,5,6,7,8-hexahydro-[4,5^22]carboline.

Intracellular NS3-4A protease assays. NS3-4A protease activity was used to monitor intracellular HCV replication levels and was measured by using a europium-labeled NS3-4A protease substrate, as described previously (23), with slight modifications. In brief, medium was removed from virus-infected cells and replaced with 50 μL of a lysis/NS3-4A substrate solution containing 1 × lysis buffer (Promega, Madison, WI), 150 mM NaCl, and 150 nM NS3-4A europium substrate (AnaSpec, Fremont, CA) in deionized water. Time-resolved fluorescence was measured for 10 cycles by using a Victor 2 multilabel counter (Perkin-Elmer, Waltham, MA).

High-throughput antiviral and cytotoxicity assays. A library of 450,000 compounds was screened against both infectious HCV (GT2a) (16/HH11) (24, 25) and the HCV (GT2a) replicon (26). In brief, Huh7-Lunet-CD81 cells (22) or HCV/GT2a stable replicon cells were plated on 384-well plates using a MicroFlo dispenser (BioTek, Winooski, VT) and incubated overnight. Subsequently, compounds were added at a concentration of 10 μM to both cell types. Huh7-Lunet-CD81 cells were then infected with HCV at a multiplicity of infection (MOI) of 5 by using a BioMek FX liquid handler (Beckman Coulter, Brea, CA). After 3 days of incubation, intracellular NS3-4A protease activity was used to quantify intracellular HCV replication. “Hits” were defined as compounds that inhibited HCV cell culture (HCVcc), but not the HCV replicon. Dose-response antiviral assays were performed for 5,000 of these hits using the methods described above, except that the compounds were plated at a concentration range of 50 μM to 2.5 mM. The resulting data were fit to the Hill equation using Pipeline Pilot software (Accelrys, San Diego, CA) to calculate the 50% effective concentrations (EC₅₀).

Manual antiviral and cytotoxicity assays. Huh7-Lunet-CD81 cells (22) were seeded in white clear-bottom 96-well plates at a density of 5,000 cells per well. After overnight incubation, 3-fold serial compound dilutions were prepared in dimethyl sulfoxide (DMSO), diluted 250-fold into a DMEM viral stock, and added to the cells in 96-well plates in a final volume of 100 μL. Final compound concentrations typically ranged from 2.5 to 50,000 nM, and the MOI was ≥0.3. Anti-CD81 monoclonal antibody was serially diluted in DMEM, yielding final concentrations from 0.25 to 5,000 ng/mL. After 3 days of incubation, NS3-4A protease activity was used to quantify intracellular HCV replication levels as described below. The resulting data were fit to the Hill equation using SigmaPlot (Systat) to calculate the EC₅₀. For cytotoxicity assays, cells were incubated with compounds as described for antiviral assays, with the exception that no virus was added. After 3 days of incubation, the intracellular ATP levels were measured using a Cell-Titer Glo kit according to the manufacturer’s instructions (Promega, Madison, WI). The resulting data were fit to the Hill equation by using SigmaPlot to calculate CC₅₀ values.

HCV entry inhibition assays. HCV entry into cells was monitored during the attachment stage, the postattachment stage, or during both stages (continuous treatment) similarly to previously published methods (9). In the attachment cases, inhibitor and wet ice-chilled GT2a HCV expressing a Renilla luciferase reporter were added to naive Huh7-Lunet-CD81 cells growing on a 96-well plate at a concentration of 5,000 cells/well. In the postattachment cases, only ice-chilled virus was added to the wells with naive cells. In the continuous-treatment cases, inhibitor and ice-chilled virus were added to the wells with naive cells. Subsequently, the 96-well plate was gently shaken for 1 min at room temperature and then incubated at 4°C for 2 h without shaking. At 4°C, HCV can attach to glycosaminoglycans (GAGs) on cell surfaces but cannot enter cells (9, 27). Next, each well of cells was washed twice with ice-chilled cell culture medium to remove any unadsorbed virus. In the attachment cases the wells were refilled with virus only, in the postattachment cases the wells were refilled with virus only, and, in the continuous treatment cases the wells were refilled with inhibitor and virus. Finally, the plates were incubated for 3 days at 37°C. Viral replication was measured using a Renilla luciferase kit (Promega) according to the manufacturer’s instructions.

For kinetic entry assays, published methods were followed with slight modification (28). Briefly, cold GT2a HCV expressing a Renilla luciferase reporter was incubated with naive cells at 4°C as described above for 2 h. Subsequently, the plate of cells was washed twice with cold, fresh medium and shifted to 37°C, and 5× EC₅₀ of various inhibitors were added at 0, 15, 30, 60, 120, and 240 min after the temperature shift. After ~18 h of 37°C incubation, the inhibitor-containing medium was replaced by fresh medium. The results were quantified after 3 days of 37°C incubation using a Renilla luciferase kit (Promega, Madison, WI) according to the manufacturer’s instructions. The resulting data were fit to a sigmoidal curve using SigmaPlot (Systat, Chicago, IL) to calculate the time of half-maximal entry inhibition.

Equilibrium density gradient analysis. Huh7-Lunet-CD81 cells (22) were plated on a 12-well plate at a concentration of 30,000 cells/well and incubated overnight. The next day, each well was infected with a high-titer DMEM stock of HCV (2a) Min3 virus (24) at an MOI of 5. The infection was allowed to spread for 96 h. At this time, ~95% of the cultures were infected, as determined by NNA staining and quantification using an ImageExpress Micro (Molecular Dynamics, Sunnyvale, CA) (24). Inhibitors of interest were added to the cell cultures at a final concentration of 5× EC₅₀ and the cultures were subsequently incubated for 72 h with one inhibitor-medium change after the first 36 h. From the wells on the plate, the extracellular medium was saved, and the intracellular virus was collected by freeze-thaw lysing the trypsinized cultures in a dry ice-ethanol bath, followed by a room-temperature water bath, a total of four times as previously described (29). A Small-Molecule Inhibitor of HCV Infectivity

A aliquots of extracellular or intracellular virus were separated by using equilibrium density gradient ultracentrifugation according to published methods (25). Briefly, 1 ml of extracellular medium was loaded at the top of a 10-mL 10 to 40% iodixanol gradient. Iodixanol gradients were prepared by using 10 and 40% iodixanol solutions that each contained 10 mM HEPES (pH 7.5) and 0.02% bovine serum albumin. In addition, the 10% iodixanol solution contained 125 mM NaCl, and the 40% iodixanol solution contained 50 mM NaCl to maintain iso-osmolarity. Samples were centrifuged through the gradients at 40,000 rpm (120,000 × g) for 16 h at 4°C in an SW41 Ti swinging-bucket rotor (Beckman, Brea, CA). Fractions (0.4 mL) from each gradient were collected from the top. Each fraction was assayed for relative infectivity using the NS3-4A protease assay described above. Each sample was diluted 50-fold before infecting
naive cells, such that the inhibitor concentration would be too low to prevent infection. The fractions were also weighed in microcentrifuge tubes after three separate runs. Very little variation in the buoyant density of the fractions was observed between experiments, and the average buoyant density was plotted on each graph shown in Fig. 2, 3, and 5.

**HCV infectious stability assays.** HCV infectious stability was measured in cell culture medium by adding various concentrations of inhibitor to 500 μl of DMEM viral stocks, followed by incubation at 37°C. Subsequently, 20-μl aliquots were removed from the tubes and immediately frozen and stored at −80°C. The aliquots were typically frozen at 0, 1, 2, 4, 6, 8, or 24 h. Once all of the time point samples had been collected, they were thawed and used to infect naive Huh7-Lunet-CD81 cells growing on a 96-well plate at a concentration of 5,000 cells/well. Each sample was diluted 50-fold before infecting naive cells such that the inhibitor concentration would be too low to block infection. HCV infectivity was measured after 72 h of incubation by using the NS3-4A protease assay described above.

**Resistance selection with HCV II-1.** Resistance selections were performed using a modified version of a previously published 96-well plate method (30). Costar black 96-well plates (Thermo Fisher Scientific, Waltham, MA) were seeded with 100 μl of a mixture of 50,000 Huh7-Lunet-CD81 cells/ml. The seeded plates were incubated overnight before continuing. The cell culture medium was subsequently removed, and 100 μl of a 1:4 50% tissue culture infective dose(s) (TCID50) of HCV(1b/2a) (con1/1FH1)/ml (22) or 100 μl of a 1:6 TCID50/ml HCV(2a-Min3) (24, 25) stock was added to each well on the appropriate plate. The plates were incubated for 4 to 6 h overnight to permit infection. Next, the medium volume was raised to 200 μl total in all wells, and inhibitor was added to columns 1 to 10 at a final concentration of 50 μM, 25 μM, 12.5 μM, 6.25 μM, 3.1 μM, 1.56 μM, 781 nM, 391 nM, 195 nM, or 98 nM, respectively. To columns 11 and 12, only DMSO was added to the medium (no inhibitor). The plates were incubated for 3 days. Afterward, 100 μl of extracellular medium was removed from the infected plates and added to the corresponding wells on new black 96-well plates that had been seeded with naive Huh7-Lunet-CD81 cells. In addition, 50 μl of medium from column 10 of the original plate was added to column 9 of the new plate, 50 μl of medium from column 9 of the original plate was added to column 8 of the new plate, etc., until column 11 was reached on the new plate. After 4 to 6 h of infection, the medium was replaced with fresh drug medium at the set concentration for each column. In this manner, the virus from each column was challenged to grow at a higher inhibitor concentration in the neighboring column. The remaining 50 μl of extracellular medium from each well on the original plate was saved on an empty 96-well plate at −80°C for subsequent studies. Each black 96-well plate was stained with anti-NS5A Ab (24), and the progression of the HCV infection to cells at increasing inhibitor concentrations was monitored using an ImageXpress Micro apparatus (Molecular Dynamics, Sunnyvale, CA). After passing the extracellular medium to new plates 10 times, infection had spread to the 3.1 μM inhibitor column for four of the eight wells at that concentration for both genotypes. Extracellular medium taken from this concentration was used to infect naive Huh7-Lunet-CD81 cells on 24-well plates in the presence of inhibitor and then subsequently used to infect cells on 12-well plates in the presence of inhibitor. The virus grown on 12-well plates was used to infect naive cells growing in T-75 flasks in the presence of inhibitor and eventually to infect cells growing in a T-175 flask in the presence of inhibitor. Before collecting the extracellular virus from the T-175 flasks, the inhibitor-containing medium was removed, and fresh medium without inhibitor was added. After 24 h of incubation without inhibitor, the extracellular medium was collected to create a viral stock for antiviral assays. The viral RNA was extracted from the extracellular medium using a QIAamp viral RNA kit (Qiagen, Valencia, CA), and the viral genomes were sequenced in each case by Tacgen (San Pablo, CA).

**Assaying for reversibility of HCV II-1 infectivity inhibition.** HCV was treated with either DMSO or 5× EC50 (200 nM) HCV II-1 for 1 h at 37°C. Subsequently, the samples were divided such that one part could be assayed for infectivity without further handling. This part was diluted 50-fold to reduce inhibitor concentration to negligible amounts and then used to infect naive Huh7-Lunet-CD81 cells, followed by an NS3-4A protease assay 72 h later. The remaining part of each sample was subjected to either dialysis or ultrafiltration. Samples were dialyzed in Pierce Slide-A-Lyzer dialysis cassettes (20K MWCO; Rockford, IL) overnight at 4°C into either 1× phosphate-buffered saline (PBS) or cell culture medium. Alternatively, the samples were filtered in a Pierce concentrator (7 ml, 150K MWCO) for 30 min at 2,000 rpm (1,000 × g) 4°C in a Beckman-Coulter Allegra 6R centrifuge (Brea, CA) and then resuspended to the original volume in fresh cell culture medium. Finally, the samples were assayed for infectivity by infecting naive Huh7-Lunet-CD81 cells, followed by an NS3-4A protease assay 72 h later.

**HCV persistently infected cultures.** HCV persistently infected cultures were established using previously described methods (5, 31, 32). Briefly, Huh7-Lunet-CD81 cells (22) were seeded in 12-well plates at a density of 50,000 cells/well. The plates were incubated overnight at 37°C and subsequently DMSO was added to 1% final concentration. After 3 days, monolayers formed, and infectious HCV was added to each well. Infectious GT2a HCV (J6/JFH1) with three adaptive mutations (the Min3 virus was previously described (24)) or infectious GT1b/2a HCV (con1/1JFH1); a chimeric virus expressing the genotype 1b structural genes and the genotype 2a nonstructural genes and six adaptive mutations as previously described (22) was then added to each well at an MOI of 5. Infection was permitted to spread for 7 days until the cultures were ∼95% infected (as determined by NS5A immunofluorescence (24) quantified on an ImageXpress Micro system [Molecular Devices]). Subsequently, compounds of interest were added at concentrations equal to 5 × EC50. Aliquots of 500 μl of extracellular medium were saved at various days after drug addition and stored at −80°C for future analyses (see below). Medium containing compounds were refreshed after taking each time point. Typically, medium samples were collected and compound medium was refreshed on days 0, 2, 4, 7, 10, 14, 18, and 20. On the final day of the time courses, the cell cultures were fixed with ice-cold methanol for 15 min for subsequent indirect immunofluorescence as previously described. The percentages of HCV-infected cells in each culture were estimated by viewing them through a fluorescence microscope (Zeiss, Thornwood, NY) (3). Because the inhibitor-treated confluent cultures had cells growing on top of cells, the absolute accuracy of the automated infectivity quantification was limited. Thus, we present estimated differences in the percentages of infected cells by using a series of pluses to show the relative differences in the percentages of infected cells. Examples of the differences in the numbers of infected cells for “+++,” “++++,” and “+++++” cultures are shown in Fig. 8B.

**RNA purification and quantification.** Stored aliquots of medium collected from infected cells were thawed, and extracellular viral RNA was isolated. Extracellular viral RNA isolation was performed using a QIAamp viral RNA minikit (Qiagen) according to the manufacturer’s protocol. A Quantifast probe RT-PCR kit (Qiagen) was used according to the manufacturer’s protocol to quantify the RNA levels in a 96-well format on a 7300 Real-Time PCR system (Applied Biosystems, Carlsbad, CA). The DNA primers used were synthesized by Integrated DNA Technologies (Coralville, IA). These primers amplified the HCV(1a)/NS3 gene and were designated NS3 2a+ [5′-CGG TCG GAG TAC ATC TCC GGT AGC(FAM) G-3′] and NS3 2a− [5′-CAC GGA GCT GCC AAC AAG ACT-3′].

**Clonal sequencing.** Clonal sequencing was performed to identify resistance mutations at the end of the HCV persistently infected time course experiments. Briefly, extracellular viral RNA were isolated from samples collected on the final day of the time courses as described above. Viral RNA were reverse transcribed using Superscript III (Life Technologies) according to the manufacturer’s protocol. The resulting cDNAs were amplified by PCR using primers specific to the HCV E2(2a), NS3(2a), or NS5A(2a) genes. The DNA primers used were E2-2a start (5′-CGC ACC CAT ACT GGT GGG GGT-3′), E2-2a end (5′-TTG GCC TGC GCC CAA CAA GAT-3′), NS3-2a start (5′-CTC GCT CCC ATC ACT GCT TAT-3′),

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FIG 1 Chemical structure of HCV infectivity inhibitor 1 (HCV II-1), also known as GS-563255 or \((4R,75)-2,2,2\text{-trifluoroethyl} 2\text{-methyl}-5\text{-oxo-7-phenyl-1,4,5,6,7,8-hexahydro-}[4,5\text{-biquinoline}]\text{-3-carboxylate}.\)

NS3-2a end (5′-CAT GAC CTC AAG GTC AGC TTG-3′), NS5A-2a start (5′-TGC TCC GGA TCC TGG CTC C-3′), and NS5A-2a end (5′-GCA CAC GGT GGT ATC GTC CTC-3′). PCRs were performed using Ex Taq polymerase (Thermo Fisher Scientific). PCR products were purified using a Qiagen PCR clean-up kit and subsequently ligated into a TOPO TA pCR4 cloning vector (Life Technologies) according to the manufacturers’ protocols. Ligation products were transformed into TOP10 frozen competent cells (Life Technologies), and clones were selected on Luria broth-carbenicillin plates according to the manufacturer’s protocol. Plasmid DNA was prepared from the selected clones by using a Qiagen miniprep kit according to the manufacturer’s protocol, and DNA sequencing was performed by Elim BioPharm (Hayward, CA).

RESULTS

Discovery and optimization of a small-molecule HCV infectivity inhibitor. Our aim was to find small-molecule inhibitors of HCV replication cycle stages other than RNA replication. Thus, we performed a high-throughput screen of 450,000 compounds against infectious GT2a HCV. To distinguish compounds that inhibited HCV at the stage of RNA replication from those that acted at other stages of the HCV replication cycle, we also performed a parallel screen of the same compounds against a GT2a HCV replicon.

Fifty compounds from the screen exhibited HCVcc EC50 of ≤1 μM with no effect on the HCV replicon. A substituted tetrahydroquinoline (THQ) stood out as the strongest and most selective inhibitor. This THQ had an EC50 of 0.6 μM against infectious HCV but had no effect on HCV replicon replication (EC50 > 50 μM) or cell viability (CC50 > 50 μM). Based on these properties, this compound was selected for optimization of antiviral potency and other properties through medicinal chemistry. A representative compound from the medicinal chemistry effort was substantially improved antiviral activity, referred to as HCV II-1 (Fig. 1), was used to investigate the mechanism of HCV inhibition of this compound class. This compound exhibited an EC50 of 30 ± 4 nM (average of four assays) against GT1b/2a (J6/JFH1) infectious HCV (chimeric GT 2a HCV encoding 1b HCV envelope proteins), an EC50 of 40 ± 4 nM (average of four assays) against GT2a infectious HCV (J6/JFH1), low cytotoxicity (CC50 ≥ 13,065 nM [average of four assays]), an EC50 of 20 ± 5 nM (average of three assays) against GT2a HCV pseudoparticles, and no antiviral effect against other viruses (bovine viral diarrhea virus, HIV, respiratory syncytial virus, and hepatitis B virus) (data not shown). The selective index in GT1b/2a HCV (CC50/EC50) was 933, and in GT2a HCV it was 700.

HCV II-1 inhibits infectivity of intracellular and secreted HCV particles without preventing particle formation or altering particle density. To investigate how HCV II-1 inhibits HCV, we first tested whether it inhibits HCV assembly. We infected Huh7-Lunet-CD81 cells with GT2a HCV and allowed the infection to spread until ~95% of the cell cultures were infected. DMSO (solvent control), the NS3-4A protease inhibitor BILN-2061, or HCV II-1 were added to the infected cultures at 5 × EC50 (530 nM BILN-2061 [5] and 200 nM HCV II-1) for 72 h. We examined viral assembly and egress during inhibitor treatment by measuring viral RNA buoyant density and infectivity in both intracellular extracts and extracellular medium using density-gradient analyses.

In untreated controls, density-gradient analysis of the intracellular extracts revealed an RNA peak that corresponded roughly with a viral infectivity peak around a density of 1.15 g/ml (Fig. 2A).
There was also a less dense RNA peak of ~1.05 g/ml in untreated cells, which likely corresponds to unpackaged or partially packaged viral RNA (Fig. 2A). Treatment with the protease inhibitor BILN-2061 reduced levels of viral RNA and infectivity to nearly background levels (Fig. 2B). This was expected, since this inhibitor prevents HCV RNA replication (10). Treatment with HCV II-1 greatly reduced intracellular infectivity (Fig. 2C) but did not alter the intracellular viral RNA density profile (Fig. 2C). This suggested that HCV particles were produced during HCV II-1 treatment but were not infectious.

When the same analysis was performed on extracellular medium, we again observed an RNA peak that roughly corresponded to an infectivity peak at ~1.15 g/ml in the untreated controls (Fig. 3A). Similar to the intracellular findings, treatment with BILN-2061 nearly eliminated both viral RNA and infectivity (Fig. 3B). Treatment with HCV II-1 also showed a pattern similar to the intracellular fraction with an RNA peak similar to the untreated control case but substantially reduced infectivity (Fig. 3C). Together, these density-gradient analyses suggested that RNA replication and the assembly of HCV particles was occurring in HCV II-1-treated cells but that the assembled HCV particles were non-infectious.

**HCVII-1 acts directly on HCV particles to inhibit infectivity.** The fact that HCV II-1 treatment permitted production of HCV particles with normal buoyant density (Fig. 2 and 3) but without infectivity led us to test whether HCV II-1 inactivates mature infectious HCV particles. Thus, we incubated an infectious viral stock in the presence of various concentrations of HCV II-1 at 37°C and monitored infectivity over time. Interestingly, we observed that HCV II-1 treatment at as little as 0.5× EC_{50} (20 nM) dramatically reduced viral infectivity over time relative to an DMSO-treated control. Viral infectivity at 37°C exhibited a half-life of only 1.2 ± 0.1 h when treated with 0.5× EC_{50} HCV II-1 as opposed to 5.0 ± 0.3 h when left untreated (Fig. 4). At 1.0× EC_{50} (40 nM) HCV II-1, the infectious half-life was reduced to 0.6 ± 0.1 h (Fig. 4). These results suggested that HCV II-1 directly inhibits the infectivity of mature HCV particles.

**HCV II-1 inhibits HCV infectivity without disruption of particle integrity but in an irreversible manner.** We next tested whether HCV II-1 inhibits HCV infectivity by disrupting viral particle integrity. We treated infectious HCV stocks with DMSO (control), HCV II-1 (20× EC_{50}), or with Triton X-100 (5%) and incubated each treated sample with 50 μg of RNase A/ml for 1 h. Equilibrium density gradient centrifugation was then used to fractionate the samples, and fractions were assayed for HCV RNA levels and infectivity (Fig. 5). The viral RNA genome was clearly exposed to degradation by RNase A when 5% Triton X-100 was added to the viral stock (Fig. 5B). In the cases where DMSO or HCV II-1 were added to the viral samples, the viral RNA genome remained protected from RNase A degradation (Fig. 5A and C). We also noted that when viral particles were treated with 20× EC_{50} HCV II-1 for 4 days at 37°C, the rate of viral RNA decay was very similar to a DMSO-treated control (t_{1/2} = 2.4 ± 0.5 days for 0.5× EC_{50} t_{1/2} = 5.0 ± 0.3 hours, 0.5× EC_{50} t_{1/2} = 1.2 ± 0.1 hours, and 1.0× EC_{50} t_{1/2} = 0.6 ± 0.1 hours).

**FIG 3** Characterization of extracellular HCV particles during HCV II-1 treatment. Equilibrium density gradient centrifugation was performed as indicated in Materials and Methods. In all graphs, the solid red line represents HCV infectivity, the blue line represents HCV RNA levels, and the black line represents the average buoyant density. The data shown represent the averages of three assays, and the error bars represent the standard deviations. (A) Equilibrium density gradient centrifugation of the extracellular material from an HCV-infected cell culture treated with 0.2% DMSO for 72 h. (B) Equilibrium density gradient centrifugation of the extracellular material from an HCV-infected cell culture treated with 5× EC_{50} (530 nM) of the NS3-4A protease inhibitor BILN-2061 for 72 h. (C) Equilibrium density gradient centrifugation of the extracellular material from an HCV-infected cell culture treated with 5× EC_{50} (200 nM) of HCV II-1 for 72 h.

**FIG 4** Inhibition of HCV infectivity by HCV II-1 in a cell-free assay. HCV infectious stability in cell culture medium at 37°C during treatment with various concentrations of HCV II-1 (0.2% DMSO [solid circles and solid line], 0.5× EC_{50} = 20 nM [solid squares and dashed line], and 1.0× EC_{50} = 40 nM [solid diamonds and solid line]). The data shown represent the averages of three assays, and the error bars represent the standard deviations.
Inhibition of HCV infectivity by HCV II-1 is not reversible by dialysis or ultrafiltration. (A) HCV infectivity after 1 h of incubation with either DMSO or 5% Triton X-100 plus 50 μg of RNase A/ml for 1 h at 37°C before equilibrium density gradient centrifugation. (B) HCV infectivity after 1 h of incubation with the DMSO control and 5% Triton X-100, plus 50 μg of RNase A/ml for 1 h at 37°C before equilibrium density gradient centrifugation. (C) HCV was treated with final concentrations of 800 nM (20× EC50) HCV II-1 plus 50 μg of RNase A/ml for 1 h at 37°C before equilibrium density gradient centrifugation.

FIG 6 Inhibition of HCV infectivity by HCV II-1 is not reversible by dialysis or ultrafiltration. (A) HCV infectivity after 1 h of incubation with either DMSO or 5× EC50 HCV II-1. (B) HCV infectivity after 1 h of incubation with either DMSO or 5× EC50 HCV II-1, followed by 24 h of dialysis or 1 h of ultrafiltration. The data shown represent the averages of three assays, and the error bars represent standard deviations.

HCV II-1 inhibits HCV entry into cells during both the attachment and the postattachment stages of entry. Since HCV II-1 inhibits HCV infectivity, we tested whether it prevents HCV entry into cells. We treated Huh7-Lunet-CD81 cells with HCV GT2a, as well as with control inhibitors, during (i) only the attachment stage of viral entry when HCV binds nonspecifically to GAGs and other cell surface molecules (incubation performed at 4°C to prevent viral uptake), (ii) only the postattachment stage of viral entry when HCV binds specifically to host receptors such as CD81 and brefeldin A1 (BafA1), (iii) both stages of entry. We treated cultures with DMSO or 5× EC50 concentrations of HCV II-1 (200 nM), anti-CD81 Ab (2.5 μg/ml), and the endosomal fusion inhibitor baflomycin A1 (BafA1) (15 nM). Anti-CD81 Ab and BafA1 did not inhibit viral infection when added during the attachment stage, in agreement with previous reports (9) (Fig. 7A). However, both inhibited viral infection when added during the postattachment stage or when added continuously, as has also been reported (9) (Fig. 7A). In contrast, HCV II-1 inhibited viral entry whether it was added during the attachment stage, the postattachment stage, or continuously (Fig. 7A).

In order to compare HCV II-1 with the entry inhibitor EI-1, we performed a separate set of experiments with GT1b/2a HCV since this compound is GT1b specific. We observed that EI-1 could only prevent HCV entry when added during the postattachment phase or added continuously but not when added during the attachment phase. On the other hand, HCV II-1 could prevent GT1b HCV entry when added during the attachment phase, as well as during postattachment or when added continuously (Fig. 7B).

Furthermore, we examined the effect of adding inhibitors at various times during the attachment stage (i.e., after a temperature shift to 37°C). We observed that anti-CD81 Ab permitted entry sooner than the endosomal fusion inhibitor BafA1, as others have reported (28). Anti-CD81 Ab exhibited a time of half-maximal inhibition of entry (t1/2) of 63 ± 12 min, whereas for BafA1 the t1/2 was 158 ± 20 min. Interestingly, HCV II-1 blocked HCV entry similarly to BafA1 with a t1/2 of 150 ± 24 min (Fig. 7C).
These results suggested that HCV II-1 prevents HCV from fusing with the endosome.

**HCV II-1 resistance maps to HCV envelope protein 2.** To gain further insight into the mechanism of action of HCV II-1, we performed resistance selections using GT1b/2a HCV and GT2a HCV. We performed the resistance selections by passing infectious particles in extracellular medium from low inhibitor concentrations to higher concentrations. The selected viruses exhibited roughly a 20-fold increase in EC50 relative to their respective wild-type values (EC50/H11005 30 nM for wild-type GT1b/2a and 40 nM for wild-type GT2a HCV). In both cases, we sequenced the entire genome and identified putative resistance mutations in the gene encoding envelope protein 2 (E2). In GT1b/2a HCV, the amino acid change observed was E2 P687S, located on the flexible stem that connects E2 domain III to the E2 transmembrane domain (34). This is a fairly uncommon amino acid change found once out of 581 GT1b sequences in the EU database. A codon encoding P687S was engineered into the wild-type GT1b/2a HCV plasmid, the resulting mutant genome was transcribed into RNA and transfected into Huh7-Lunet-CD81 cells. P687S variant GT1b/2a virus exhibited a 16-fold increase in EC50 relative to the wild type (EC50 = 30 nM for wild-type GT1b/2a HCV and EC50 = 480 nM for GT1b/2a HCV P687S), confirming that it confers resistance. The putative GT2 amino acid changes that provided resistance were E2 R643G/K, also located within E2 domain III (34). These amino acid changes are uncommon in that R643 only was observed in 52 GT2a sequences analyzed from the EU database. When a codon encoding E2 R643G was engineered into the GT2a virus, it conferred 18-fold resistance to HCV II-1 (EC50/H11005 40 nM for wild-type GT2 HCV and EC50/H11005 737 nM for R643G GT2a HCV). The E2 R643G variant of GT2a HCV grew to the same titer as its parent (1e6 TCID50/ml), suggesting that the amino acid change did not significantly alter growth. Taken together, the above results with both genotypes suggested that HCV II-1 may interact with the HCV envelope.

**HCV II-1 promoted a prolonged viral suppression by replication inhibitors in persistently infected cell cultures.** Finally, we sought to test the efficacy of the infectivity inhibitor HCV II-1 as a monotherapy and in combination with HCV replication inhibitors in a persistently infected *in vitro* setting. Previously, we have demonstrated that HCV entry inhibitors such as EI-1 and anti-CD81 Ab only slowly reduced extracellular viral RNA levels as monotherapies *in vitro* (3). However, when we combined either
EI-1 or anti-CD81 Ab with an HCV replication inhibitor, they prolonged *in vitro* viral suppression by limiting resistance (5). Thus, we established cultures persistently infected with GT2a HCV and treated with HCV II-1 alone or in combination with the NS3-4A protease inhibitor BILN-2061 or the NS5A inhibitor daclatasvir.

We observed that HCV II-1 transiently reduced extracellular HCV RNA levels by $\sim 1 \log_{10}$ RNA copies/ml by day 10, but the RNA levels rebounded to steady-state levels by day 20 (Fig. 8A). Clonal sequencing identified that the resistance mutations encoding the amino acid changes E2 R643G/K were detectable by the end of the time course. The resistance substitutions, determined based on clonal sequencing HCV(2a) after 3 weeks of HCV inhibitor treatment, were as follows (inhibitor, substitution): HCV II-1, E2 R643G/K; BILN-2061, NS3 D168N; and daclatasvir, NS5A Y93H. Resistance mutations encoding the indicated amino acid changes were observed in one of five clones. Treatment with a single replication inhibitor such as BILN-2061 or daclatasvir caused a greater and more rapid reduction in extracellular HCV RNA levels (decreases of 2.0 to 2.5 $\log_{10}$ RNA copies/ml) (Fig. 8A) before rebounding due to the acquisition of resistance mutations (leading to previously published amino acid changes NS3 D168N [35] and NS5A Y93H [36], respectively) (Table 1).

In contrast to the single-drug treatments, the combination of HCV II-1 with daclatasvir or BILN-2061 caused prolonged reductions of $\sim 2.5$ and $\sim 3.0 \log_{10}$ RNA copies/ml, respectively (Fig. 8A). The combination of the two replication inhibitors (BILN-2061/daclatasvir) elicited a prolonged reduction of $\sim 3.0 \log_{10}$ RNA copies/ml (Fig. 8A). In addition to measuring extracellular virus levels, we also stained the cultures with anti-NS5A Ab on day 20 to examine the relative levels of infected cells. The number of NS5A-positive cells correlated well with the viral levels in the culture supernatants in all cases (Fig. 8B). Together, these results indicated that HCV II-1 prolongs viral suppression by replication inhibitors in persistently infected cell cultures.

**DISCUSSION**

Using a high-throughput screening paradigm where we screened both infectious HCV and a replicon system, we were able to identify compounds that specifically inhibited HCV at stages other than genome replication. Subsequent optimization of one chemical series led to the prototype compound HCV II-1, which has nanomolar activity against both infectious GT1b/2a and GT2a HCV and no inhibitory effect on other tested viruses. HCV II-1 inactivates both intracellular and extracellular HCV, despite allowing the assembly and egress of HCV particles exhibiting normal density. In addition, HCV II-1 rapidly diminishes HCV infectivity in cell-free assays in an irreversible manner and resistance
mutations map to the gene E2. These data collectively suggest that HCV II-1 interacts directly with the HCV envelope.

Although HCV II-1 likely interacts with the HCV envelope, we can only speculate on the details of this mechanism of action. Biochemical evidence for an HCV II-1/E2 interaction is still lacking since radiolabeled-HCV II-1 binding experiments were inconclusive due to insufficient quantities of viral particles (data not shown). This remains an important area for future investigation. However, some insight toward understanding the HCV II-1 mechanism of action may be derived from the cell-free experiments and the entry inhibition experiments. The fact that HCV II-1 rapidly deactivates HCV particles (Fig. 4) and prevents HCV from fusing with the endosome (Fig. 7C) suggests that HCV II-1 either locks the viral envelope in its prefusion state or promotes an envelope conformation change that is incapable of fusion. Along these lines, it has been observed that the Dengue2 entry inhibitor 1662G07 blocks viral fusion by stabilizing an intermediate conformation of the Dengue2 viral envelope that cannot complete the fusion-promoting conformational change (37). Further studies will be necessary to elucidate how HCV II-1 prevents HCV particles from fusing with the endosome whether through a direct interaction with E2 or other means. Moreover, HCV II-1 may prove to be a useful tool for furthering our understanding of HCV endosomal fusion.

It should be noted that to date HCV II-1 has only been tested with cell culture-derived HCV. In the future, it will be important to determine whether HCV II-1 and related compounds can inhibit the infectivity of viral particles in human patients, since these exhibit lower density than viral particles produced in cell culture due to differences in lipoprotein binding (38, 39).

An HCV infectivity inhibitor as part of an HCV cure. An HCV infectivity inhibitor could be complementary to a replication inhibitor treatment regimen. There are no viral dynamic models or clinical data as yet for HCV infectivity inhibitors. However, based on viral dynamic models for HCV entry and replication inhibitors (40), entry inhibitors are predicted to reduce viral load in a monophasic manner reflecting the slow death rate of infected hepatocytes (t1/2 = 2 to 70 days) and the protection of naive, uninfected cells from HCV infection. In contrast, replication inhibitors are predicted to reduce viral load in a biphasic manner. The initial rapid reduction phase is due to the inhibition of virus production and elimination of plasma virus (t1/2 ~ 3 h). The second, slower reduction phase results from the elimination of infected hepatocytes. Our results with persistently infected cells (Fig. 8A) demonstrated that HCV II-1 could only mildly reduce extracellular HCV levels over time (~1 log10 RNA copies/ml) until resistance mutations were acquired. This small reduction in extracellular HCV RNA during HCV II-1 treatment likely occurred because infected cells were dying, and no new cells were being infected despite continued cell growth in the monolayers (5). These results with HCV II-1 monotherapy in persistently infected cultures are similar to our previous observations with the HCV entry inhibitors anti-CD81 Ab and EI-1 in persistently infected cultures (5). This makes sense given that HCV II-1 is essentially an HCV entry inhibitor that acts directly on the virus rather than blocking a cellular receptor such as CD81. In addition, these HCV II-1 monotherapy in vitro results are consistent with recent clinical trial results with the HCV entry inhibitors. Specifically, the HCV entry inhibitors JTK-652 and ITX-5061 exhibited no effect on serum HCV RNA levels when either was used as a monotherapy on chronically infected patients (41, 42). Thus, HCV II-1 monotherapy would be predicted to have little effect on serum HCV RNA levels in patients. However, the combination of HCV II-1 with HCV replication inhibitors led to a prolonged reduction in extracellular HCV RNA levels and HCV-infected cells over a 3-week time course (Fig. 8A and B). Only the combination of two replication inhibitors (BILN-2061/daclatasvir) provided a slightly greater reduction in extracellular HCV RNA levels at day 20 than the HCV II-1/replication inhibitor combinations (Fig. 8A), suggesting that a higher resistance barrier was achieved than with HCV II-1 combined with either replication inhibitor. Presumably, the prolonged reductions in extracellular HCV RNA levels achieved by all of the inhibitor combination cases is due to a higher resistance barrier compared to monotherapy (two resistance mutations had to arise to overcome two classes of inhibitors [Table 1]). Based on these data, an infectivity inhibitor could be a useful component in a treatment regimen also containing a robust replication inhibitor. Finally, since some HCV inhibitors do not interact well with immunosuppressants during liver transplants (43), HCV II-1 or a derivative thereof might be useful both during a liver transplant and after such a transplant to prevent the new liver from being infected.

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