Identification of PTC725, an Orally Bioavailable Small Molecule That Selectively Targets the Hepatitis C Virus NS4B Protein

Zhengxian Gu,^a Jason D. Graci,^a Frederick C. Lahser,^b Jamie J. Breslin,^a Stephen P. Jung,^a James H. Crona,^a Patricia McMonagle,^b Ellen Xia,^b Shaotang Liu,^b Gary Karp,^a Jin Zhu,^a Song Huang,^a Amin Nomeir,^b Marla Weetall,^a Neil G. Almstead,^a Stuart W. Peltz,^a Xiao Tong,^{b,*} Robert Ralston,^{b,*} Joseph M. Colacino^a

PTC Therapeutics, Inc., South Plainfield, New Jersey, USA^a; Merck Research Laboratories, Kenilworth, New Jersey, USA^b

While new direct-acting antiviral agents for the treatment of chronic hepatitis C virus (HCV) infection have been approved, there is a continued need for novel antiviral agents that act on new targets and can be used in combination with current therapies to enhance efficacy and to restrict the emergence of drug-resistant viral variants. To this end, we have identified a novel class of small molecules, exemplified by PTC725, that target the nonstructural protein 4B (NS4B). PTC725 inhibited HCV 1b (Con1) replicons with a 50% effective concentration (EC₅₀) of 1.7 nM and an EC₉₀ of 9.6 nM and demonstrated a >1,000-fold selectivity window with respect to cytotoxicity. The compounds were fully active against HCV replicon mutants that are resistant to inhibitors of NS3 protease and NS5B polymerase. Replicons selected for resistance to PTC725 harbored amino acid substitutions F98L/C and V105M in NS4B. Anti-replicon activity of PTC725 was additive to synergistic in combination with alpha interferon or with inhibitors of HCV protease and polymerase. Immunofluorescence microscopy demonstrated that neither the HCV inhibitors nor the F98C substitution altered the subcellular localization of NS4B or NS5A in replicon cells. Oral dosing of PTC725 showed a favorable pharmacokinetic profile with high liver and plasma exposure in mice and rats. Modeling of dosing regimens in humans indicates that a once-per-day or twice-per-day oral dosing regimen is feasible. Overall, the preclinical data support the development of PTC725 for use in the treatment of chronic HCV infection.

Chronic hepatitis C virus (HCV) infection is a worldwide epidemic disease with an estimate of over 170 million people chronically infected worldwide (1). Approximately 60 to 85% of HCV infections result in chronic hepatitis that can lead to liver fibrosis, cirrhosis, and hepatocellular carcinoma (2). The current standard of care (SOC) for chronic hepatitis C infection, pegylated alpha interferon in combination with ribavirin, has serious side effects and limited efficacy, especially for infection with HCV genotype 1, which is the most prevalent HCV genotype (3, 4). Two HCV protease inhibitors, boceprevir (Victrelis) and telaprevir (Incivek), for the therapy of HCV genotype 1 infection in combination with the SOC were approved for use nearly 2 years ago. In addition, a number of other direct-acting antivirals (DAAs) in clinical trials have demonstrated encouraging efficacy in combination therapies (5). Currently, the HCV antivirals in preclinical and clinical development are inhibitors of the viral protease, polymerase, or nonstructural protein 5A (NS5A) (6). Due to the emergence of viral variants resistant to the DAAs, even in combination therapy with the SOC (7–9) and the potential for viral rebound after cessation of antiviral therapy, it is essential to discover and develop novel HCV inhibitors that act on new targets and can be used in combination with the SOC and/or DAAs to enhance efficacy and to delay or possibly prevent the emergence of drug-resistant variants. We have identified a novel class of small molecules that inhibit HCV RNA replication by targeting the viral nonstructural protein 4B (NS4B).

The HCV RNA genome is approximately 9.6 kb, containing a single open reading frame that encodes a polyprotein precursor that is processed to individual structural (C-E1-E2-p7) and nonstructural (NS2-NS3-NS4A-NS4B-NS5A-NS5B) proteins by host and viral proteases. The virus genome encodes a serine protease (NS3) and an RNA-dependent RNA polymerase (NS5B). Efforts to discover and develop inhibitors of these viral enzymes, as well as

NS5A, have constituted the most productive approach to chemotherapeutic intervention in chronic HCV infection.

NS4B is another potential target for novel antivirals to treat chronic HCV infection. NS4B is a 27-kDa integral membrane protein that plays an essential role in HCV replication (for a review, see references 10 and 11). Although its functions are not yet fully understood, a number of roles have been postulated for HCV NS4B. NS4B is thought to act as a scaffold with which viral proteins and RNAs, as well as other key cellular factors, interact to form viral replication complexes in the endoplasmic reticulum (ER) that are required to allow viral RNA replication (12–23).

NS4B possesses NTPase and adenylate kinase activities (24, 25), binds viral RNA (26), and contributes to the process of virus assembly and release (27–29). Disruption of the GTPase or RNA-binding activity leads to impairment of HCV replication. NS4B has also been reported to interact with many HCV proteins (30–36), as well as with several cellular proteins (28, 37, 38). NS4B has been suggested to regulate viral protein translation (39–41). The finding that NS4B is essential in the replication cycle of HCV

Received 25 March 2013 Returned for modification 16 April 2013

Accepted 23 April 2013

Published ahead of print 29 April 2013

Address correspondence to Joseph M. Colacino, jcolacino@ptcbio.com.

* Present address: Xiao Tong, Hoffman-La Roche, Nutley, New Jersey, USA; Robert Ralston, Department of Pharmacology, Toxicology and Therapeutics, The University of Kansas Medical Center, Kansas City, Kansas, USA.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AAC.00527-13

The authors have paid a fee to allow immediate free access to this article.

makes it an attractive and compelling antiviral target for new therapeutics.

A new target within the HCV NS4B protein that is essential for viral genome replication, a conserved amphipathic helix termed 4BAH2, has been reported (42). This amphipathic helix displays a potential for self-oligomerization as well as the ability to promote the aggregation of lipid vesicles into macromolecular assemblies resembling key features of membranous webs, called the HCV replication platform. The 4BAH2 vesicle aggregation-promoting activity was used to screen for candidate pharmacologic inhibitors. Several such inhibitors were reported to alter HCV genome replication in a dose-dependent manner (42).

NS4B remains an underexploited target for the inhibition of HCV replication in clinical practice (43). Here, we report PTC725, a member of a new class of highly selective inhibitors of HCV RNA replication that target NS4B. PTC725 is a clinical development candidate with low-nanomolar activity against HCV replicon RNA replication in cell culture assays and attractive pharmaceutical properties.

MATERIALS AND METHODS

Compounds. PTC725, or (S)-6-[3-cyano-6-ethyl-5-fluoro-1-(pyrimidin-2-yl)-1H-indol-2-yl]-N-(1,1,1-trifluoropropan-2-yl)pyridine-3-sulfonamide (44), and its analogs were synthesized at PTC Therapeutics, Inc. Figure 1A shows the chemical structure of PTC725.

Reagents. Alpha interferon was purchased from Invitrogen (Carlsbad, CA). Mouse monoclonal anti-NS4B (2-H1; ab24283) and anti-NS5A (C65388) antibodies were purchased from Abcam (Cambridge, MA) and Meridian (Saco, ME), respectively. Rabbit polyclonal anti-protein disulfide isomerase (PDI) (H-160; sc-20132) antibody was obtained from Santa Cruz (Santa Cruz, CA). Alexa Fluor 488 dye-labeled goat anti-mouse IgG (A11001) and ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (P36931) were purchased from Invitrogen (Carlsbad, CA). The CellTiter 96 aqueous nonradioactive cell proliferation assay (MTS [3-(4,5-dimethyl-2-thiazolyl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] kit; G5421) was obtained from Promega (Madison, WI). Boceprevir was obtained from Schering-Plough Research Institute (Kenilworth, NJ). VX-222, a nonnucleoside HCV NS5B polymerase inhibitor in clinical development (<http://www.clinicaltrials.gov/ct2/show/NCT01080222>), was purchased from Selleckchem (Houston, TX). [^{14} C]thymidine, [^{14} C]uridine, and [^{14} C]leucine were purchased from GE Healthcare (Piscataway, NJ).

Cells. Huh-7 (hepatocarcinoma) cells harboring the subgenomic HCV genotype 1b (Con1) replicon (45) (referred to as 1b replicon cells) were obtained from ReBLikon GmbH (Gau-Odernheim, Germany). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1% nonessential amino acids (Invitrogen, Carlsbad, CA), 1% penicillin-streptomycin, and 250 μ g/ml G418 (Invitrogen, Carlsbad, CA).

HCV replicon inhibition assay. The ability of compounds to inhibit HCV RNA replication was assessed in the genotype 1b replicon cells. Briefly, the replicon cells were plated at a density of 5,000 cells per well in 96-well plates in DMEM containing 10% fetal bovine serum (FBS) in the absence of G418. Serial dilutions of test compounds in dimethylsulfoxide (DMSO) were added to wells, and cells were incubated at 37°C and 5% CO₂ for 3 days prior to determining the extent of inhibition of HCV replicon RNA replication and the effect of the compound on cell proliferation. The final concentration of DMSO in the medium was 0.5%. In some experiments, 1b replicon cells were treated with compound for up to 8 days to assess the extended effect of compounds on replicon replication. In some experiments, the activity of compounds was also tested in Huh-7 cells harboring HCV genotype 1a (H77S) genome (46). Inhibition of replicon or genome RNA replication was quantified by real-time qRT-PCR

FIG 1 (A) Chemical structure of PTC725, (S)-6-[3-cyano-6-ethyl-5-fluoro-1-(pyrimidin-2-yl)-1H-indol-2-yl]-N-(1,1,1-trifluoropropan-2-yl)pyridine-3-sulfonamide. (B) Inhibition of HCV 1b replicon replication by PTC725. HCV 1b replicon cells were treated with PTC725 for 3 days. Inhibition of replicon replication was measured by reduction of replicon RNA as determined by qRT-PCR. The median ($\pm$ SEM) values of the inhibition of the replicon RNA from quadruplicate measurements were plotted against the compound concentrations. The host cell GAPDH mRNA was used as an indicator of the effect of PTC725 on cell proliferation. (C) Dose-response and time-dependent inhibition of HCV replicon replication by PTC725. HCV 1b replicon cells were treated with PTC725 at concentrations above the EC₉₀, as indicated. Medium containing freshly diluted PTC725 was replenished every 3 days, and the cells were splitting prior to reaching confluence. Inhibition of replicon replication was measured at days 3, 6, and 8 by reduction of replicon RNA levels as determined by qRT-PCR. The averages of the inhibition of the replicon RNA level from duplicate measurements were plotted against the duration of the compound treatment.

(quantitative reverse transcription-PCR). At the end of compound treatment, the cells were lysed with Cell-to-cDNA lysis buffer (Ambion, Austin, TX). RT-PCR was carried out using TaqMan one-step RT-PCR master mix reagent kits (Applied Biosystems) with HCV primers (sense [S66], ACGCAGAAAGCGTCTAGCCAT; antisense [A165], TACTACCGGT TCCGCAGA) and probe (5'-6-carboxyfluorescein-TCCTGGAGGCTGC ACGACACTCAT-6-carboxytetramethylrhodamine-3') at a concentration of 100 μ M. The effect of the compound on the abundance of the housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) mRNA as a control for cytotoxicity was determined by using predeveloped TaqMan assay reagents (Applied Biosystems, Foster City, CA). Both HCV replicon and GAPDH RNAs were amplified in the same well

using an ABI 7900HT (Applied Biosystems). The quantity of HCV replicon or GAPDH RNA from each sample was determined by applying PCR cycle-time values to a standard curve. The percent reduction of HCV RNA in the presence of test compound was calculated using the formula $[1 - (\text{compound treatment sample} - \text{background control}) / (\text{non-compound-treated control} - \text{background control})] \times 100$. EC_{50} and EC_{90} (50 and 90% effective concentrations, respectively) values were calculated by non-linear regression using Prism software (GraphPad, San Diego, CA).

The activity of PTC725 in combination with other anti-HCV agents was assessed in 1b replicon cells over a 3-day treatment. The combination experiments were designed using a checkerboard cross pattern of drug concentrations, including each drug alone. The inhibitory effect of each combination or single agent was determined by monitoring replicon RNA replication using qRT-PCR, with GAPDH RNA as an endogenous control. The data were analyzed according to the method described by Chou and Talalay (47). The combination indices (CIs) of PTC725 with other anti-HCV agents were calculated by using CalcuSyn software (Biosoft, Cambridge, United Kingdom). A CI value of 1 indicates an additive effect, a CI value of >1 indicates antagonism, and a CI value of <1 indicates synergism.

Emergence of resistance. HCV 1b replicon cells were plated in 60-mm dishes at a density of 2×10^5 cells per dish. The following day, compounds were added at the indicated concentrations. All wells contained DMSO at a final concentration of 0.2%. Three days later, the cells were split 1:20 into new plates and the medium with compound was changed twice per week. After a total of 30 days, colonies of resistant cells were stained using an aqueous solution of 50% methanol, 1% formaldehyde, and 1% crystal violet.

Cytotoxicity assays. The cytotoxicity of compounds was assessed by assaying their effects on cell proliferation (MTS assay), levels of GAPDH mRNA, and incorporation of ^{14}C -labeled precursors. The 1b replicon cells were plated at a density of 5,000 cells per well in a 96-well plate and cultured for 1 day, and then they were treated with test compound in duplicate for 3 days in the presence of 10% FBS and 0.5% DMSO. In the MTS assay, cell viability was assessed using the Cell-Titer aqueous reagent kit (Promega) in accordance with the protocol provided by the supplier. GAPDH mRNA was quantified by qRT-PCR as described above. In the MTS and GAPDH experiments, the effect of PTC725 on the proliferation of an additional 12 different human tumor cell lines was assessed. These cells were treated with compound for 3 days. Camptothecin (Sigma, St. Louis, MO), a cytotoxic quinoline alkaloid which inhibits DNA topoisomerase I, was used as a positive control. The effect of PTC725 on the synthesis of cellular DNA, RNA, or protein was tested using [^{14}C]thymidine, [^{14}C]uridine, or [^{14}C]leucine, respectively. The incorporated intracellular radioactivity was counted on a TopCount NXT (Packard, Meriden, CT).

De novo selection of HCV replicon resistance. To select replicons that are resistant to PTC725, 1b replicon cells were cultured at subconfluence with fixed concentrations (4, 40, and 120 nM) of compound in the presence of G418. In parallel, 1b replicon cells cultured in the absence of PTC725 were used as a mock selection control for the appearance of nonspecific mutations. Cell culture medium was replenished with fresh medium containing the appropriate concentration of PTC725 every 3 to 4 days. Replicon cells were split (1:4) when they reached approximately 80% confluence. Cell growth rate was monitored as an indicator of replicon resistance. After approximately 4 weeks of selection (6 to 7 passages) with PTC725, the selected replicon cells regained a normal growth rate in the presence of G418, indicating replicon resistance to PTC725. Susceptibility of the selected replicons to PTC725 was evaluated by quantification of replicon RNA as described above.

cDNA cloning. To identify mutations in the replicon which conferred resistance to PTC725, total cellular RNA from the selected replicon cells was extracted and cDNA of replicon RNA was prepared using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) or a Superscript III first-strand synthesis system (Invitrogen). The genes for NS4B and NS5A and

the IRES (internal ribosomal entry site) region of the replicon were amplified from the cDNA by PCR using appropriate primers and cloned into pCR2.1-TOPO vector (Invitrogen). *E. coli* DH5 α -T1^R or TOP10 (Invitrogen) was transformed with plasmid DNA. Plasmid DNAs from 17 to 20 clones were sequenced at GENEWIZ (South Plainfield, NJ). Sequencing data were analyzed using the Sequencher software program (Gene Codes Corporation, Ann Arbor, MI). The mutations observed in the selected replicons were introduced into wild-type Con1 replicon DNA carrying the adaptive mutation S1179I (48) by site-directed mutagenesis to demonstrate that these mutations indeed conferred resistance to PTC725. The presence of mutations in all DNA constructs was confirmed by sequencing.

Establishment of cells stably transfected with a recombinant replicon. Stable cell lines carrying mutated recombinant replicons were established by transfecting the recombinant replicon RNA into Huh-7 cells and maintained in 500 $\mu\text{g}/\text{ml}$ of G418. All mutations in the recombinant replicons were confirmed by sequencing. The susceptibility of the stable recombinant replicon cells to PTC725 was assessed as described above.

Evaluation of replication capacity of replicons carrying drug resistance mutations. In order to examine the effect of drug resistance mutations on the replication capacity of the HCV replicon, we conducted replicon cell colony formation assays (49, 50). A total of 1×10^6 Huh-7 cells were transfected with 4 μg of *in vitro*-transcribed replicon RNA by electroporation and then cultured with G418 at a concentration of 250 $\mu\text{g}/\text{ml}$ for approximately 3 weeks. Replicon cell colonies were detected by staining with 0.2% crystal violet in 2% ethanol.

Indirect IF microscopy. In order to study effects of the NS4B inhibitors and drug resistance mutations on subcellular localization of viral proteins, we performed indirect immunofluorescence (IF) assays to detect HCV NS4B and NS5A proteins in wild-type and NS4B mutated 1b replicon cells. Replicon cells were cultured on sterile German glass coverslips. Cells were fixed in 4% paraformaldehyde, washed with phosphate-buffered saline (PBS), permeabilized with 0.05% Triton X-100 for 5 min, washed again, and blocked in 10% FBS in PBS for 1 h. The cells were then incubated with the primary monoclonal antibody against NS4B or NS5A diluted in 10% FBS in PBS for 2 h at room temperature, followed by washes in PBS. The cells were then incubated with the secondary antibody for 1 h. After incubation with the secondary antibody, cells were washed with PBS and coverslips were then mounted onto slides with ProLong Gold antifade reagent with DAPI. Images were captured using a Zeiss Axiovert 200 fluorescence microscope and associated software (Zeiss, Germany). Digital images were taken with a Retiga 1300 camera (QImaging, Surrey, Canada).

Quantification of replicon proteins. HCV NS4B and NS5A proteins in the replicon cells were quantified by immunoblotting. Total cellular protein of replicon cells was prepared in a 10% SDS solution and subjected to conventional SDS-PAGE followed by immunoblotting. The HCV NS4B and NS5A proteins were detected using the same antibodies as described above and used for IF microscopy. PDI, an endoplasmic reticulum (ER) membrane protein, was used as an internal control. The blotted target bands were quantified with an Odyssey infrared imager (Li-Cor, Lincoln, NE).

Pharmacokinetic studies in animals. All procedures were performed in a laboratory certified by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) with approval from the Institutional Care and Animal Use Committee. The intravenous and oral pharmacokinetics of PTC725 were evaluated in mice, rats, dogs, and monkeys. Male CD-1 mice (18 to 20 g) and male CRL-CD-BR rats (180 to 220 g) were purchased from Charles River Laboratories (Wilmington, MA). Male beagle dogs (7 to 15 kg of body weight) and male cynomolgus monkeys (3 to 9 kg) were obtained from the Schering-Plough Research Institute colony. Animals were kept in temperature-, humidity-, and light cycle-controlled rooms. Animals were randomly assigned to each treatment group and were subjected to fasting (water allowed) for 18 h prior to dosing, unless otherwise indicated. For oral dosing, PTC725 was sus-

pended in 0.4% hydroxypropylmethyl cellulose (HPMC) at doses of 10 mg/kg for mice, 10 mg/kg for rats, and 5 mg/kg for dogs and monkeys. For intravenous (i.v.) dosing, PTC725 was solubilized in 10% N-methyl 2-pyrrolidone, 40% polyethylene glycol 300 (PEG 300), and 50% propylene glycol (by volume) for mice and rats, and the same components were used for dogs and monkeys at concentrations of 5, 50, and 45%, respectively. PTC725 was administered as an intravenous (2- to 3-min push) dose of 5 mg/kg to mice, 6 mg/kg to rats, and 2.5 mg/kg to dogs and monkeys. After oral dosing of mice and rats, blood was collected (at 0.25, 0.5, 1, 2, 4, 8, 24, and 48 h). After oral dosing of dogs and monkeys, blood was collected at prespecified time points (0.25, 0.5, 1, 2, 4, 8, 24, 48, 72, and 96 h). After intravenous dosing of mice and rats, blood was collected at prespecified time points (0.125, 0.25, 0.5, 1, 2, 4, 8, 24, and 48 h). After intravenous dosing of dogs and monkeys, blood was collected (0.125, 0.25, 0.5, 1, 2, 4, 8, 24, 48, 72, and 96 h). Blood was centrifuged to harvest plasma, and the levels of PTC725 in plasma were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Briefly, PTC725 was serially diluted into plasma from undosed animals of the respective species and used as calibration standards. Internal standard (a close analog of PTC725) was added to plasma samples from dosed animals or calibration standard and then were acidified, and protein was precipitated by the addition of acetonitrile-methanol mixture using a 96-well format. Plasma was centrifuged to precipitate the protein, and the supernatant was analyzed for PTC725 and the internal standard. Also analyzed with each analytical run are two sets of quality control samples (at three concentrations) prepared in plasma from undosed animals of the respective species from a separate weighting, one before the samples and the other after the samples. Analyte concentrations were determined by turbo ion spray LC-MS/MS in the positive-ion mode. The analytical methods were qualified prior to use by analyzing standard spiked samples at different times (inter- and intraday). The precision and accuracy of the analytical methods were satisfactory. Standard noncompartmental analysis methods, using WinNonlin (Pharsight Corporation, Mountain View, CA), were used to calculate pharmacokinetic parameters. The following parameters were determined: $C_{\max}$ (maximal concentration of drug in plasma), $AUC_{0-\infty}$ (total area under the plasma concentration versus the time curve from time zero extrapolated to infinity), $t_{1/2}$ (half-life of elimination), $T_{\max}$ (time to maximum concentration of drug in serum), CL (total body clearance or systemic clearance), V_{ss} (volume of distribution at steady-state), and F (oral bioavailability).

Evaluation of liver-to-plasma ratio of PTC725 in mice and rats. All procedures were performed in an AAALAC-certified laboratory with approval from the Institutional Care and Animal Use Committee. The liver-to-plasma ratio of PTC725 was evaluated in mice and rats following oral administration of PTC725 at 10 mg/kg in 0.4% HPMC. Male CD-1 mice or Sprague Dawley rats (three animals per time point) were sacrificed at 0.15 (mice only), 0.5, 1, 2, 4, 8, 24, and 48 h after dosing. A blood sample was drawn from each animal, and plasma samples were then obtained by centrifugation. The whole liver was removed from each animal and perfused with phosphate-buffered saline to remove traces of blood. After weighing, the liver was cut into small pieces and homogenized with an equal volume of water. The plasma and liver samples were stored at -70°C until analysis using LC-MS/MS. Liver homogenates were treated in the same manner as plasma samples (protein precipitation using acetonitrile-methanol mixture), and the supernatant was analyzed by LC-MS/MS using calibration standards prepared in the same matrices from undosed animals.

RESULTS

PTC725 selectively inhibits HCV replicon replication. A high-throughput screen was initially performed using gene expression modulation by small molecules (GEMS) technology, developed at PTC (51), utilizing the HCV IRES. Hits identified in this screen were subsequently chemically optimized for compound potency and selectivity using the HCV replicon system, as well as for phar-

maceutical properties. Over 10,000 compounds were synthesized and tested. The results of this effort led to the identification of PTC725 (Fig. 1A), a small molecule with selective activity for the inhibition of HCV genotype 1 RNA replication.

The inhibitory activity of PTC725 and that of a subset of analogs was evaluated in HCV genotype 1b (Con1) replicon cells. Table 1 summarizes the inhibition activity of the lead compound PTC725 and its analogs. PTC725 had a mean EC_{50} of 1.7 ± 0.78 nM and EC_{90} of 9.6 ± 3.1 nM (Fig. 1B) in a 3-day treatment. PTC725 also inhibited the replication of the genotype 1a H77S full-length genome (46) with an EC_{50} of 7 nM and an EC_{90} of 19 nM. PTC725 had considerably lower and less selective activity (EC_{50} , ~ 2.2 μM) against genotype 2a infectious JFH-1 virus in Huh-7 cells. PTC725 exhibited dose-response and time-dependent inhibition of HCV replicon replication with maximal reductions of replicon RNA levels by 2 and 3 $\log_{10}$ at $1\times$ and $5\times$ EC_{90} concentrations (Fig. 1C), respectively, in an 8-day treatment.

PTC725 and its analogs have selective activity against HCV RNA replication. PTC725 was tested against a panel of DNA and RNA viruses in cell culture antiviral assays that included adenovirus, herpes simplex virus type 1, influenza virus A, parainfluenza virus type 3, respiratory syncytial virus, yellow fever virus, bovine viral diarrhea virus, and human immunodeficiency virus type 1. PTC725, at concentrations of up to 10 μM , was not active against any of these viruses (data not shown). In cell-free enzymatic assays, PTC725 and its analogs had no activity against HCV NS3/4A protease and helicase or NS5B RNA-dependent RNA polymerase up to 10 μM , the highest concentration tested (data not shown).

The HCV replicon inhibitory activity of PTC725 was selective with respect to cytotoxicity. The cytotoxicity of PTC725 and related compounds was assessed in Huh-7 1b replicon cells in parallel with their anti-replicon activity. Cytotoxicity was also assessed using multiple assays that monitored cell proliferation in multiple cell lines and the macromolecular synthesis of RNA, DNA, and protein. PTC725 had greater than 5,000-fold selectivity with respect to cytotoxicity (CC_{50}/EC_{50}) in 1b replicon cells using MTS cell proliferation assays (Table 1). Furthermore, PTC725 did not have an effect on the levels of GAPDH mRNA, even with a greater than 6,000-fold selectivity window. PTC725 also had a greater than 4,000-fold selectivity window with respect to reduction in total cellular DNA, RNA, and protein synthesis in 1b replicon cells with 3-day compound treatment as determined by ^{14}C radiolabeling incorporation assays. In addition, PTC725 was further assessed for its effect on cell proliferation in various types of tumor cell lines. PTC725 had greater than 1,500- to 5,000-fold selectivity with respect to cytotoxicity as assessed in 12 different tumor cell lines by MTS cell proliferation assays and/or its effect on the level of GAPDH mRNA (Table 2). These results demonstrate that PTC725 and its analogs selectively inhibit the replication of HCV replicons.

Mutations in NS4B confer replicon resistance to PTC725. Resistance to PTC725 was selected by treating genotype 1b replicon-containing Huh-7 cells with compound at concentrations of 4- and 12-fold the EC_{90} value (40 and 120 nM, respectively). Replicons that were approximately 80-fold resistant to PTC725 were generated and sequenced. PTC725-resistant replicons contained mutations encoding amino acid substitutions F98L/C and V105M in NS4B (Table 3). In addition, H94R in NS4B, previously reported to encode resistance to a carboxamide NS4B inhibitor (known as anguizole) (52), was observed in 1 out of 58 colonies of

TABLE 1 Replicon inhibitory activity and effect on cell proliferation of HCV NS4B inhibitors

Compound	EC (nM) for HCV 1b replicon ^b		IC ₅₀ (nM) in HCV 1b replicon cells ^a			
	EC ₅₀	EC ₉₀	GAPDH RNA	MTS	Protein	RNA
PTC725	1.7 ± 0.78 (n = 53)	9.6 ± 3.1 (n = 53)	11,600 ± 6,000 (n = 3)	9,900 ± 4,100 (n = 12)	11,300 ± 2,900 (n = 8)	10,500 ± 3,300 (n = 9)
PTC-971	9.2 ± 3.1 (n = 19)	42 ± 14 (n = 19)	15,700 ± 4,900 (n = 5)	≥ 14,100 ± 6,300 (n = 11)	10,900 ± 3,000 (n = 5)	15,400 ± 3,500 (n = 5)
PTC-113	30 ± 14 (n = 39)	163 ± 59 (n = 39)	13,600 ± 1,000 (n = 5)	16,700 ± 4,700 (n = 14)	8,600 ± 1,600 (n = 8)	13,300 ± 4,900 (n = 8)
PTC-332	5.7 ± 2.8 (n = 20)	25 ± 9.7 (n = 20)	> 10,000 (n = 1)	> 20,000 (n = 5)	≥ 15,400 ± 4,800 (n = 4)	≥ 19,600 ± 4,500 (n = 4)

^a IC₅₀ (50% inhibitory concentration) values for inhibition of synthesis of cellular protein, RNA, and DNA were determined by monitoring the incorporation of [¹⁴C]leucine, [¹⁴C]uridine and [¹⁴C]thymidine, respectively. The number of determinations is given in parentheses.

^b EC₅₀ and EC₉₀ values are presented means ± SEM. The number of determinations is given in parentheses.

TABLE 2 Effect of PTC725 on cellular proliferation

Cell line	Origin of cell line	CC ₅₀ (μM) according to ^a :	
		MTS	GAPDH mRNA
1GROV1	Ovarian cancer	>10	>10
A375	Melanoma	4.5	>10
A2058	Melanoma	2.7	3.4
COLO205	Colon cancer	9.6	>10
H1299	Lung cancer	>10	>10
HCT116	Colon cancer	>10	NT ^b
HT29	Colon cancer	>10	NT
Huh-7	Hepatoma	>10	>10
MB468	Breast cancer	9.1	>10
MCF7	Breast cancer	>10	>10
PC3	Prostate cancer	>10	NT
U87-CCR5	Glioma	>10 (CTB) ^c	NT

^a Cells were treated with compound for 3 days. Values for each of the cell lines were obtained from a single determination using the 3-(4,5-dimethyl-2-thiazolyl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. GAPDH mRNA was quantified using real-time PCR as described in Materials and Methods.

^b NT, not tested.

^c CC₅₀ determined using cell titer blue (CTB).

plasmid DNA of PTC725-selected replicons sequenced. None of these mutations were observed in the mock-selected replicons. Resistance mutations described for the NS4B-targeting compound clemizole hydrochloride (W55R and R214Q in NS4B) (26) were not identified among PTC725-selected replicons. A series of imidazo[1,2-*a*]pyridines was previously reported to interact with HCV NS4B (53). These compounds selected for a similar array of resistance mutations encoding the amino acid substitutions H94N/R, F98L, and V105L/M (53).

To confirm that these mutations confer HCV replicon resistance to PTC725, the mutations encoding the amino acid substitution F98L, F98C, or V105M were engineered into the wild-type 1b HCV replicon by site-directed mutagenesis. The engineered replicons were stably transfected into Huh-7 cells. Replicons containing the F98C, F98L, and V105M amino acid substitutions were 300-, 140-, and 60-fold resistant, respectively, to PTC725 (Fig. 2 and Table 3). In addition, replicons containing both F98C and V105M amino acid substitutions were not significantly more resistant to PTC725 than were replicons carrying each mutation alone (results not shown). The NS4B mutated replicons had a

TABLE 3 Frequency of identified mutations in NS4B among replicons selected for resistance to PTC725

PTC725 selection concn	Frequency of nucleotide mutation (amino acid substitution)			
	A281→G (H94R)	T292→C (F98L)	T293→G (F98C)	G313→A (V105M)
Mock ^a (17)	0 ^b	0	0	0
4 nM (20)	0	2	0	0
40 nM (19)	1	5	3	6
120 nM (19)	0	13	1	5
Fold resistance to PTC725 ^c	16	140	300	60

^a Numbers in parentheses are the number of replicons in which NS4B was sequenced.

^b Numbers in the table represent the number of clones containing the indicated mutation.

^c Fold resistance values were obtained using replicons engineered to contain the mutation identified in the replicons selected for resistance.

FIG 2 Amino acid substitutions in NS4B confer resistance to PTC725. Replicon cells containing replicons with the amino acid substitution F98C, F98L, or V105M in NS4B were treated with PTC725 for 3 days. Replicon RNA levels were quantified by qRT-PCR. The mean inhibition ($\pm$ SEM) values of the replicon RNA level determined from triplicate measurements were plotted against the compound concentration.

similar pattern of resistance against the analogs of PTC725 shown in Table 1.

Consistent with PTC725 targeting NS4B, PTC725-resistant replicons remained fully susceptible to alpha interferon or boceprevir (results not shown). A number of representative replicon mutants carrying substitutions encoding resistance to inhibitors of NS3 protease or NS5B polymerase was shown to remain fully susceptible to PTC725 (Table 4). These results are consistent with the activity of PTC725 being specific for NS4B and not selecting for cross-resistance to alpha interferon or to inhibitors of HCV NS3/NS4A protease or NS5B polymerase. Taken together, these findings demonstrate that PTC725 targets the viral NS4B protein.

The fitness of the NS4B-resistant replicons compared to that of the wild-type replicon was analyzed. A colony formation assay with F98C and V105M replicon cells was conducted to monitor the replication capacity of the HCV replicon (49, 50). Huh-7 cells were transfected with mutated or wild-type replicon RNA and cultured under G418 selection for 3 weeks, and colonies of replicon cells were subsequently detected by staining with crystal violet. The results demonstrated that the F98C and V105M mutations have a slight effect on replicon fitness, with a 37 and 29% reduction in replication capacity, respectively, compared to that of the wild-type replicon cells.

The activity of PTC725 in combination with other antiviral agents is additive/synergistic and suppresses the emergence of replicon resistance. The inhibitory activity of PTC725 was investigated in 1b replicon-containing cells in combination with alpha interferon or HCV protease and polymerase inhibitors. Replicon cells were treated with PTC725 in combination with other HCV agents at various molar ratios for 3 days. Inhibition of replicon replication was determined by quantifying replicon RNA using qRT-PCR. Combination effects on the inhibition of replicon RNA replication are expressed as combination indices (CIs). PTC725 had average mutually nonexclusive CIs of 0.57 and 1.07 when combined with alpha interferon or the marketed HCV protease inhibitor boceprevir, respectively (Fig. 3). When combined with the nonnucleoside HCV polymerase inhibitor VX-222 at various molar ratios, PTC725 had CIs of 0.86 to 1.7, 0.53 to 1.2, and 0.31 to

0.79 at EC_{50} , EC_{75} , and EC_{90} , respectively (Table 5). These results indicate that PTC725 acts synergistically with alpha interferon, additively to synergistically with the HCV polymerase inhibitor VX-222, and additively with the HCV protease inhibitor boceprevir to inhibit HCV replicon RNA replication in cell culture.

The emergence of replicon resistance was assessed in 1b replicon-containing cells. HCV 1b replicon-containing cells under G418 selection were cultured in the presence of PTC725 alone, boceprevir alone, or these agents in combination, and the emergence of replicon resistance was determined. The frequency of emergence of drug-resistant colonies of replicon cells was 0.1 or 0.2% with a 5-fold EC_{90} concentration of PTC725 or boceprevir, respectively (Fig. 4). The combination of PTC725 and boceprevir, however, reduced the frequency of emergence of replicon-resistant colonies to 0.001%. This frequency was at least 100-fold lower than that in the presence of each compound alone and approximately 20-fold lower than would be predicted for the additive combination of PTC725 and boceprevir. These data indicate that PTC725, in combination with boceprevir, synergistically suppresses the emergence of HCV replicon resistance.

The NS4B inhibitors do not alter intracellular localization of HCV proteins to the ER. NS4B residues F98 and V105 are located in the proposed transmembrane domain 1 (TM1) of NS4B (Fig. 5A). Analysis of the predicted positions of residues H94, F98, and V105 in the TM1 region (amino acids 93 to 109) using the Java applet helical wheel model (E. K. O'Neil and C. M. Grisham, University of Virginia; <http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html>) showed that H94, F98, and V105 are predicted to lie on the same face of the transmembrane helix (Fig. 5B). This suggests that these residues are involved in the intramolecular interactions of NS4B and/or in intermolecular interactions between NS4B and other components of the replication complexes. Therefore, it was hypothesized that either binding of the compounds to NS4B or the drug-resistant NS4B mutations would perturb the localization of NS4B in intracellular membranes. To test this hypothesis, we

TABLE 4 Activity of PTC725 against replicons engineered for resistance to inhibitors of HCV protease or polymerase^a

Replicon and amino acid substitution	EC_{90} (nM)	Fold change from wild-type EC_{90}
Con1		
NA	27	1
NS3/4A protease inhibitor-resistant replicons		
V36M	33	1.2
T54A	24	0.9
R155K	33	1.2
NS5B polymerase inhibitor-resistant replicons		
S282T	19	0.7
C316Y	22	0.8
M414T	28	1.0
M423T	25	0.9
P495L	15	0.6
G554D	10	0.4

^a Con1 refers to the HCV 1b replicon carrying the adaptive mutation S1179I. Replicon cells were treated with PTC725 for 3 days. The data were obtained from a single experiment performed in duplicate. NA, not applicable.

FIG 3 Activity of PTC725 in combination with boceprevir or alpha interferon against HCV replicon replication. HCV 1b replicon cells were treated for 3 days as indicated. Inhibition of replicon replication was determined by reduction of replicon RNA as quantified by qRT-PCR. The data were obtained from a single experiment performed in duplicate. Mutually nonexclusive combination indices were calculated using CalcuSyn software.

investigated the effect of the HCV inhibitors on replicons with an NS4B protein containing the F98C substitution, which encodes the highest resistance to PTC725, on the intracellular distribution of NS4B and NS5A in replicon cells by IF microscopy.

HCV NS4B and NS5A proteins in the wild-type and F98C mutant 1b replicon-containing cells were detected with mouse monoclonal antibodies by indirect IF microscopy. First, the effect of PTC-332 (Table 1) on HCV proteins in the replicon cells was assessed. PTC-332 is a structurally close analog of PTC725 in which the only difference is the replacement of the 6-ethyl of PTC725 with a 6-methyl group. As expected, IF detection of both NS4B and NS5A in the wild-type replicon cells, but not in the F98C mutated replicon cells, was abrogated after PTC-332 treatment for 72 h at a concentration of 5-fold the EC₉₀ against the wild-type 1b replicon (results not shown).

We next assessed the effect of PTC-332 on the subcellular localization of viral proteins in replicon cells. Genotype 1b replicon-containing cells were treated with PTC-332 at a range of concentrations for 6 h. The localization of neither NS4B (Fig. 6A) nor NS5A protein (results not shown) in the ER was obviously altered in the wild-type replicon cells treated with PTC-332. Interestingly, the IF detection signals for both NS4B (Fig. 6A) and NS5A proteins (results not shown) were brighter and more punctate in appearance in wild-type replicon cells treated with PTC-332 at concentrations from 5- to 80-fold, but not <5-fold (results not

shown), the replicon EC₉₀ compared to the non-compound-treated control. It should be noted that the concentrations of PTC-332 tested were well below the CC₅₀ (Table 1). Importantly, the brightness of signal for both NS4B and NS5A proteins in the F98C mutated replicon cells was not enhanced (results not shown).

To determine whether the enhanced brightness of the NS4B and NS5A protein IF staining in wild-type replicon cells was due to an increased abundance of the proteins (for example, if binding of the compound to NS4B can protect the viral proteins or protein complex from degradation), we quantified the viral proteins in the wild-type replicon cells treated with PTC-332 by immunoblotting. The lysates of the replicon cells were subjected to conventional SDS-PAGE followed by immunoblotting using the same antibodies to NS4B or NS5A that were used for IF microscopy. ER

TABLE 5 Activity of PTC725 in combination with VX-222 against HCV replicon RNA replication^a

VX-222/PTC725 molar ratio	CI at:		
	EC ₅₀	EC ₇₅	EC ₉₀
1:3	1.0	0.53	0.31
1:1	0.97	0.60	0.41
3:1	0.86	0.60	0.44
9:1	1.7	1.2	0.79

^a HCV 1b replicon cells were treated for 3 days with PTC725 and VX-222 alone or in combination at various molar ratios. Inhibition of replicon replication was determined by reduction of replicon RNA as measured by qRT-PCR. The data were obtained from a single experiment performed in duplicate. The combination index (CI) was calculated using CalcuSyn software.

FIG 4 PTC725 and boceprevir in combination suppress the emergence of replicon resistance. HCV 1b replicon cells were treated with PTC725 or boceprevir alone or in combination under G418 selection. The frequency (%) of drug-resistant replicons was calculated using the following formula: (number of colonies of replicon cells in the presence of inhibitor[s]/number of replicon cells plated) × 100. TMTC, too many to count. The experiment was conducted twice independently with similar results in each case.

FIG 5 Locations of the PTC725-resistant substitutions in NS4B. (A) Diagram of the HCV NS4B topology based on the model proposed by Boletti et al. (56). The PTC725-resistant substitutions are located in the putative TM1 domain. (B) Helical wheel representation of the HCV NS4B TM1 domain using the amino acid sequence for genotype 1b, isolate Con1 (GenBank accession number AJ238799). The TM1 region in NS4B is defined as amino acids 93 to 109 (56). The glutamine (Gln) at amino acid position 93 is indicated as 1 on the helical wheel. The figure was generated using a Java applet (E. K. O'Neil and C. M. Grisham, University of Virginia; <http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html>). Amino acid substitutions that confer resistance to PTC725 are indicated in the diamonds on the helical wheel.

membrane protein PDI was used as an internal control. As shown in Fig. 6B, neither the NS4B nor NS5A protein in the wild-type replicon cells treated with PTC-332 was obviously increased in abundance, as determined using an Odyssey infrared imager. Therefore, further studies are needed to understand the basis for the increased IF brightness of the viral proteins in the wild-type replicon as a result of treatment with PTC-332.

The amino acid substitution F98C in NS4B does not alter the localization of HCV proteins to the ER. We investigated whether the drug resistance substitutions affect the localization of the NS4B protein to the ER and its interaction with the components of the replication complexes. The NS4B F98C substitution was used, since it confers the highest level of resistance to PTC725. The IF staining pattern of the NS4B protein in the F98C mutated replicon cells is similar to that of the NS4B protein in the wild-type replicon

cells (Fig. 7). However, when the F98C mutated replicon was exposed to PTC725 at a concentration 80-fold above the EC_{90} for the wild-type replicon, no increase in brightness or punctation of the IF staining pattern was observed (data not shown). This observation provides further phenotypic evidence for NS4B as the target of PTC725. Similarly, the staining pattern of NS5A was not affected by the presence of this amino acid substitution (results not shown). Furthermore, the levels of NS4B and NS5A were not increased by the F98C mutated replicon (results not shown). These results indicate that the F98C substitution does not alter the localization of NS4B or NS5A to the ER.

Orally administered PTC725 has excellent exposure across multiple species. The summary of pharmacokinetic parameters for PTC725 in mice, rats, dogs, and monkeys following intravenous and oral administration is shown in Table 6. PTC725 showed

FIG 6 Subcellular localization and quantification of viral proteins in HCV replicon cells. The wild-type replicon cells were cultured in the absence or presence of PTC-332 at fold concentrations above the replicon EC_{90} , as indicated, for 6 h. PTC-332 is a structurally close analog of PTC725 in which the only difference is the replacement of the 6-ethyl of PTC725 with a 6-methyl group. (A) Subcellular localization of NS4B protein was examined by indirect IF microscopy. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Representative IF images are shown. Concentrations of PTC-332 above the replicon EC_{90} are labeled. (B) Viral proteins from the replicon cells were quantified by immunoblotting. Lysates of naive Huh7 cells were used as a negative control for the viral proteins. Intensity of the viral protein and PDI blots were analyzed with an Odyssey infrared imager. The ratios of the targeted viral protein blots were calculated by dividing the intensity of the blot from the PTC-332-treated sample by the intensity of the blot from the non-compound-treated sample.

FIG 7 Subcellular localization of NS4B in cells containing wild-type replicons or replicons with the F98C amino acid substitution. NS4B proteins were detected by indirect IF.

moderate clearance after i.v. administration in mice, rats, dogs, and monkeys (4.6 to 15 ml/min/kg, approximately 12 to 25% of hepatic blood flow), with an effective half-life range of 1.4 to 5.4 h and a mean residence time range of 2.0 to 7.8 h. PTC725 showed moderate to high oral bioavailability in mice, rats, dogs, and monkeys (18 to 78%). The lowest oral bioavailability of 18% was observed in monkeys. This is consistent with our results showing that PTC725 was more highly metabolized in isolated liver microsomes from monkeys than from the other species (data not shown). Furthermore, we have seen that PTC725 has higher i.v. clearance in monkeys than in the other species. The lower oral bioavailability and the higher clearance of PTC725 in monkeys help to explain that drug plasma concentrations in this species are lower than the serum-shifted EC_{90} .

Distribution of PTC725 in the liver and brain was evaluated in mice and rats following oral administration of PTC725 at a dose of 10 mg/kg. The liver/plasma AUC ratio was 15 and 23 for mice and rats, respectively, indicating that PTC725 is well distributed to liver, the target organ. In mice and rats, the brain/plasma AUC ratios were 0.16 and 0.3, respectively, indicating limited brain uptake and reducing the risk of central nervous system toxicity.

As shown in Fig. 8A, after oral administration of PTC725 to rats, plasma exposures were above the replicon EC_{90} (measured in the presence of 40% human serum; 600 nM) for ~12 h after a single dose of 10 mg/kg and for 24 h after a single dose of 30 mg/kg. As shown in Fig. 8B, after oral administration of PTC725 to dogs, plasma exposures were above the serum-shifted EC_{90} for ~12 h after a single dose of 5 or 10 mg/kg and for 24 h after a single dose of 20 mg/kg.

Projection of human pharmacokinetics was carried out using fixed-exponent allometry using rat, dog, and monkey pharmacokinetics (54). The projected systemic clearance was in the range of

2.7 to 6.2 ml/min/kg with a geometric mean of 3.6 ml/min/kg. The projected volume of distribution at steady state (V_{ss}), based on allometry, was in the range of 2.1 to 3.9 liters/kg with a geometric mean of 3.1 liters/kg. The projected half-life range was 7 to 17 h with a geometric mean of 10 h, suitable for once- or twice-daily administration. The EC_{90} of PTC725 in the replicon assay is 9.7 nM, with a 60-fold increase in the EC_{90} in the presence of 40% human serum. A target efficacious dose was defined as the dose necessary to maintain $5\times$ the EC_{90} in the presence of human serum. Based on these predicted human pharmacokinetic parameters, efficacious total daily doses of PTC725 are predicted for once-, twice-, and thrice-daily administration to be about 970, 570, and 480 mg, respectively.

DISCUSSION

We report the identification of PTC725, a member of a novel class of small molecules that selectively target NS4B to inhibit HCV replicon RNA replication in cell culture. PTC725 has low nanomolar activity against HCV genotype 1a and 1b replicons and exhibited both dose-response and time-dependent activity in the HCV replicon assay. An 8-day treatment with PTC725 resulted in a 3- $\log_{10}$ reduction of HCV replicon RNA at a concentration of 5-fold above the EC_{90} value, a reduction which is equal to that resulting from treatment of the protease inhibitor boceprevir at 5-fold the EC_{90} . Importantly, PTC725 has high selectivity with respect to cytotoxicity, as assessed in a number of cell lines derived from different human tissues using various endpoints.

Combination therapy has proven to be an effective and necessary approach for chronic HCV infection therapy. A combination of treatment modalities enhances therapeutic efficiency and delays or prevents the emergence of drug-resistant viral variants in both *in vitro* antiviral assays and in the clinical setting (5). PTC725

TABLE 6 Mean pharmacokinetic parameters of PTC725 in multiple species

Species	Pharmacokinetic parameters for:					Oral dosing				
	i.v. dosing									
	Dose (mg/kg)	AUC _(0-∞) (μM · h)	Half-life (h)	Clearance (ml/min/kg)	V _{ss} (liter/kg)	Dose (mg/kg)	AUC _(0-∞) (μM · h)	C _{max} (μM)	T _{max} (h)	F (%)
Mouse	5	11	1.4	15	1.8	10	7	1.9	0.5	33
Rat	6	19	4.6	10	3.9	10	20	2.4	1.3	62
Dog	2.5	18	5.4	4.6	2.1	5	29	4.0	1.0	78
Monkey	2.5	8.7	4.5	11	3.8	5	3.1	0.54	1.8	18

FIG 8 PTC725 plasma concentrations are above the serum-shifted EC_{90} after oral administration to rats and dogs. Rats (A) and dogs (B) were dosed by oral gavage with PTC725 suspended in 0.4% HPMC. Animals were serially bled at specified time points, and the levels of PTC725 were quantified. The dotted line shows the EC_{90} (600 nM) measured in the presence of 40% human serum. In both panels A and B, the error bars represent the SEM. $n =$ three animals in each case.

exhibited synergistic activity in combination with alpha interferon or the NS5B nonnucleoside polymerase inhibitor VX-222 and additive activity in combination with the NS3 protease inhibitor boceprevir against HCV replicon replication in cell culture (Fig. 3, Table 5). Furthermore, PTC725 did not select for cross-resistance to alpha interferon or to inhibitors of the HCV protease and polymerase. These results suggest that the combination of PTC725 with anti-HCV agents in the treatment of HCV infection will have clinical utility.

As drug-resistant viral variants have been observed following the administration of DAAs in mono- or combination therapies (7–9), resistance studies of PTC725 were conducted. Selection and characterization of PTC725-resistant HCV replicons demonstrated that mutations encoding amino acid substitutions in the HCV NS4B confer replicon resistance to these compounds, consistent with acting on the viral NS4B protein. *De novo* selection of resistant HCV replicons identified amino acid substitutions F98C/L and V105M in NS4B, which were shown to confer a high level (>50-fold) of replicon resistance to PTC725. Analysis of HCV NS4B sequences collected in the Los Alamos database (55) found that both F98 and V105 are highly conserved in genotypes 1 (>97% in 1013 sequences) and 3 (100% in 118 sequences) HCV. Interestingly, L98 is present in 61 of 61 sequences analyzed in HCV genotype 2, which explains why PTC725 had lower activity against this virus (data not shown).

The time and frequency required for the selection of replicons resistant to PTC725 were similar to those for selection against the

HCV protease inhibitor boceprevir (Fig. 4). However, the combination of PTC725 and boceprevir resulted in an approximately 100-fold greater reduction in the emergence of drug-resistant replicons compared to each compound alone (Fig. 4). These results indicate that PTC725 has a synergistic effect on the inhibition of the emergence of HCV resistance in combination with other DAAs. Therefore, the results from these studies on drug resistance anticipate that PTC725, as a component of combination therapies, will have clinical benefits for controlling drug-resistant HCV variants.

NS4B localizes in the ER and acts primarily as a scaffold for the assembly of the viral components to form complexes for the translation and replication of viral genomic RNA (12–23). At least four TM domains in NS4B have been proposed to anchor into the ER membrane. Based on the topology model of NS4B (20), the amino acid substitutions that confer resistance to PTC725, at F98 and V105 in NS4B, are localized in the TM1 domain of the protein (Fig. 5A). Furthermore, F98 and V105, as well as H94, lie on the same face of the transmembrane region as predicted in a helical wheel model (Fig. 5B), indicating that these residues are involved in intramolecular interactions or interactions between NS4B and other components of the replication complexes.

Based on the observations described above, we determined whether the NS4B inhibitors and the drug-resistant mutations affect the localization of NS4B at the ER and its interaction with other components of the replication complexes, such as NS5A. PTC-332, a structurally close analog of PTC725 with similar properties that inhibit replicon replication, was tested and did not alter the subcellular distribution of NS4B protein at the ER in both wild-type and F98C substituted replicons (Fig. 6A). These results indicate that the NS4B inhibitors impair NS4B function related to viral replication without interrupting its subcellular localization. Interestingly, the NS4B inhibitor anguizole, shown to interact with the AH2 domain of NS4B, was reported to alter the subcellular distribution of NS4B-green fluorescent protein (GFP) fusion protein (52). The difference in results for our NS4B inhibitors compared to those for anguizole suggests that these agents have different mechanisms of action in affecting NS4B function and subcellular localization. Alternatively, the difference in results may be due to the use of an expressed NS4B-GFP protein for the studies involving anguizole.

HCV NS4B was reported to interact with nearly all HCV proteins (30–36). Therefore, we investigated whether the NS4B inhibitors or their drug-resistant mutations interrupt the interaction of NS4B with other viral proteins. To this end, we assessed the effect of PTC-332 or the amino acid substitution F98C on the subcellular localization of NS5A in the replicon cells by IF. Neither PTC-332 nor the F98C substitution altered the ER localization of NS5A protein in replicon cells. These results indicate that neither NS4B inhibitors nor the drug-resistant mutations disrupt the interaction between NS4B and NS5A at the ER membrane. However, further studies are needed to understand more fully the effect of the NS4B inhibitors on the interaction of NS4B with other viral and cellular proteins.

Interestingly, although the viral proteins in the wild-type replicon cells were abrogated after PTC-332 treatment for 72 h, the intensity and punctate nature of the IF staining for both NS4B and NS5A was enhanced in a dose-dependent manner in wild-type replicon cells treated for only 6 h (Fig. 6A). However, the abundance of the viral proteins was not increased as determined by

immunoblot assay (Fig. 6B). While it is unclear why treatment of wild-type replicon cells with the NS4B inhibitor for a short period of time altered the pattern of IF staining for NS5A and NS4B, potential reasons include (i) increased aggregation of the viral proteins in the ER membrane in the presence of compound; (ii) an effect of the compound on NS4B oligomerization, resulting in a conformational change and/or disruption of the replication complexes; or (iii) interaction of the compound with NS4B (which may be in complex with NS5A) may alter the conformation of the protein or protein complex to increase the binding of the antibody, possibly as a result of the increased exposure of the recognized epitope. Further investigations are needed to elucidate this issue and may help define the mechanism of action of PTC725 and its analogs.

PTC725 has excellent oral exposure and high liver-plasma distribution ratios in multiple species. A single oral dose of 10 mg/kg given to rats or a dose of 5 mg/kg given to dogs provides plasma exposures at or above the *in vitro* replicon EC₉₀ for 12 h, suggesting clinical utility. PTC725 has low potential for drug-drug interactions, as evidenced by lack of inhibition or induction of a panel of human cytochrome P450 enzymes *in vitro* at anticipated efficacious plasma concentrations, and it has low potential for cardiovascular effects as evaluated in an *in vitro* hERG assay. Furthermore, we evaluated PTC725 for its potential cardiovascular effects and found no significant test article-related changes in systolic, diastolic, mean blood pressure, heart rate, electrocardiogram (ECG) intervals (RR, PR, QRS, QT, and QTc Fridericia correction), and ECG morphology in telemetered dogs given PTC725 orally at doses of 2 and 5 mg/kg. Plasma samples taken at 4 h postdosing confirmed the exposure (data not shown). Additionally, PTC725 was well tolerated in rats at up to 2,000 mg/kg/day in a 14-day oral toxicity study and in dogs at up to 1,000 mg/kg in a single-escalation-dose toxicity study as determined by the lack of effect on body weight and the overall good health of the animals (data not shown).

NS4B is an underexploited target for the discovery of clinically useful anti-HCV agents. Here, we have identified PTC725 as a novel anti-HCV compound that targets NS4B. PTC725 has nanomolar activity and a high degree of selectivity with respect to cytotoxicity, as well as favorable combination effects in enhancing antiviral activity and in restricting the appearance of drug-resistant HCV replicon variants in cell culture. Furthermore, PTC725 is orally bioavailable and well tolerated in multiple species. Further development of this molecule as a treatment for chronic HCV infection is warranted.

ACKNOWLEDGMENTS

We thank the following individuals for their scientific and technical contributions: John Babiak, Guangming Chen, Valerie Clausen, Takashi Komatsu, Susanne Kramer, John Piwinski, George Njoroge, Janet Petruska, Panayiota Trifillis, Christopher Trotta, Sophie Zhang, and Nanjing Zhang, as well as Sony Agrawal, Christine Espiritu, Connie Freund, Leo Ouchanov, Nicole Risher, and Yingcong Zheng.

We have received employee compensation from our respective companies.

REFERENCES

- Alter MJ. 2007. Epidemiology of hepatitis C virus infection. *World J. Gastroenterol.* 13:2436–2441.
- Ghobrial RM, Steadman R, Gornbein J, Lassman C, Holt CD, Chen P, Farmer DG, Danino N, Collisson E, Baquarizo A, Han SS, Saab S,

- Donovan JA, Esrason K, Busuttil RW. 2001. A 10-year experience of liver transplantation for hepatitis C: analysis of factors determining outcome in over 500 patients. *Ann. Surg.* 234:384–393.
- Manns MP, Cornberg M, Wedemeyer H. 2001. Current and future treatment of hepatitis C. *Indian J. Gastroenterol.* 20(Suppl. 1):C47–C51.
- Nainan OV, Alter MJ, Kruszon-Moran D, Gao FX, Xia G, McQuillan G, Margolis HS. 2006. Hepatitis C virus genotypes and viral concentrations in participants of a general population survey in the United States. *Gastroenterology* 131:478–484.
- Asselah T, Marcellin P. 2011. New direct-acting antivirals' combination for the treatment of chronic hepatitis C. *Liver Int.* 31:68–77.
- Melnikova I. 2011. Hepatitis C—pipeline update. *Nat. Rev. Drug Discov.* 10:93–94.
- Halfon P, Locarnini S. 2011. Hepatitis C virus resistance to protease inhibitors. *J. Hepatol.* 55:192–206.
- Legrand-Abravanel F, Nicot F, Izopet J. 2010. New NS5B polymerase inhibitors for hepatitis C. *Expert. Opin. Investig. Drugs* 19:963–975.
- Sarrazin C, Zeuzem S. 2010. Resistance to direct antiviral agents in patients with hepatitis C virus infection. *Gastroenterology* 138:447–462.
- Dvory-Sobol H, Pang PS, Glenn JS. 2010. The future of HCV therapy: NS4B as an antiviral target. *Viruses* 2:2481–2492.
- Gouttenoire J, Penin F, Moradpour D. 2010. Hepatitis C virus nonstructural protein 4B: a journey into unexplored territory. *Rev. Med. Virol.* 20:117–129.
- De Vos R, Verslype C, Depla E, Fevery J, Van DB, Desmet V, Roskams T. 2002. Ultrastructural visualization of hepatitis C virus components in human and primate liver biopsies. *J. Hepatol.* 37:370–379.
- Egger D, Wolk B, Gosert R, Bianchi L, Blum HE, Moradpour D, Bienz K. 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J. Virol.* 76:5974–5984.
- El-Hage N, Luo G. 2003. Replication of hepatitis C virus RNA occurs in a membrane-bound replication complex containing nonstructural viral proteins and RNA. *J. Gen. Virol.* 84:2761–2769.
- Elazar M, Liu P, Rice CM, Glenn JS. 2004. An N-terminal amphipathic helix in hepatitis C virus (HCV) NS4B mediates membrane association, correct localization of replication complex proteins, and HCV RNA replication. *J. Virol.* 78:11393–11400.
- Gosert R, Egger D, Lohmann V, Bartenschlager R, Blum HE, Bienz K, Moradpour D. 2003. Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. *J. Virol.* 77:5487–5492.
- Gretton SN, Taylor AI, McLauchlan J. 2005. Mobility of the hepatitis C virus NS4B protein on the endoplasmic reticulum membrane and membrane-associated foci. *J. Gen. Virol.* 86:1415–1421.
- Konan KV, Giddings TH, Jr, Ikeda M, Li K, Lemon SM, Kirkegaard K. 2003. Nonstructural protein precursor NS4A/B from hepatitis C virus alters function and ultrastructure of host secretory apparatus. *J. Virol.* 77:7843–7855.
- Lundin M, Monne M, Widell A, Von HG, Persson MA. 2003. Topology of the membrane-associated hepatitis C virus protein NS4B. *J. Virol.* 77:5428–5438.
- Lundin M, Lindstrom H, Gronwall C, Persson MA. 2006. Dual topology of the processed hepatitis C virus protein NS4B is influenced by the NS5A protein. *J. Gen. Virol.* 87:3263–3272.
- Moradpour D, Gosert R, Egger D, Penin F, Blum HE, Bienz K. 2003. Membrane association of hepatitis C virus nonstructural proteins and identification of the membrane alteration that harbors the viral replication complex. *Antiviral Res.* 60:103–109.
- Pfeifer U, Thomssen R, Legler K, Böttcher U, Gerlich W, Weinmann E, Klinge O. 1980. Experimental non-A, non-B hepatitis: four types of cytoplasmic alteration in hepatocytes of infected chimpanzees. *Virchows Arch. B Cell Pathol. Incl. Mol. Pathol.* 33:233–243.
- Targett-Adams P, Boulant S, McLauchlan J. 2008. Visualization of double-stranded RNA in cells supporting hepatitis C virus RNA replication. *J. Virol.* 82:2182–2195.
- Einav S, Elazar M, Danieli T, Glenn JS. 2004. A nucleotide binding motif in hepatitis C virus (HCV) NS4B mediates HCV RNA replication. *J. Virol.* 78:11288–11295.
- Thompson AA, Zou A, Yan J, Duggal R, Hao W, Molina D, Cronin CN, Wells PA. 2009. Biochemical characterization of recombinant hepatitis C virus nonstructural protein 4B: evidence for ATP/GTP hydrolysis and adenylate kinase activity. *Biochemistry* 48:906–916.

26. Einav S, Gerber D, Bryson PD, Sklan EH, Elazar M, Maerkl SJ, Glenn JS, Quake SR. 2008. Discovery of a hepatitis C target and its pharmacological inhibitors by microfluidic affinity analysis. *Nat. Biotechnol.* 26: 1019–1027.
27. Jones DM, Patel AH, Targett-Adams P, McLauchlan J. 2009. The hepatitis C virus NS4B protein can trans-complement viral RNA replication and modulates production of infectious virus. *J. Virol.* 83:2163–2177.
28. Welker MW, Welsch C, Meyer A, Antes I, Albrecht M, Forestier N, Kronenberger B, Lengauer T, Piiper A, Zeuzem S, Sarrazin C. 2010. Dimerization of the hepatitis C virus nonstructural protein 4B depends on the integrity of an aminoterminal basic leucine zipper. *Protein Sci.* 19: 1327–1336.
29. Welsch C, Albrecht M, Maydt J, Herrmann E, Welker MW, Sarrazin C, Scheidig A, Lengauer T, Zeuzem S. 2007. Structural and functional comparison of the non-structural protein 4B in flaviviridae. *J. Mol. Graph. Model.* 26:546–557.
30. Benureau Y, Warter L, Malcolm BA, Martin A. 2010. A comparative analysis of the substrate permissiveness of HCV and GBV-B NS3/4A proteases reveals genetic evidence for an interaction with NS4B protein during genome replication. *Virology* 406:228–240.
31. Dimitrova M, Imbert I, Kieny MP, Schuster C. 2003. Protein-protein interactions between hepatitis C virus nonstructural proteins. *J. Virol.* 77:5401–5414.
32. Koch JO, Bartenschlager R. 1999. Modulation of hepatitis C virus NS5A hyperphosphorylation by nonstructural proteins NS3, NS4A, and NS4B. *J. Virol.* 73:7138–7146.
33. Neddermann P, Clementi A, De FR. 1999. Hyperphosphorylation of the hepatitis C virus NS5A protein requires an active NS3 protease, NS4A, NS4B, and NS5A encoded on the same polyprotein. *J. Virol.* 73:9984–9991.
34. Paredes AM, Blight KJ. 2008. A genetic interaction between hepatitis C virus NS4B and NS3 is important for RNA replication. *J. Virol.* 82:10671–10683.
35. Piccininni S, Varaklioti A, Nardelli M, Dave B, Raney KD, McCarthy JE. 2002. Modulation of the hepatitis C virus RNA-dependent RNA polymerase activity by the non-structural (NS) 3 helicase and the NS4B membrane protein. *J. Biol. Chem.* 277:45670–45679.
36. Tripathi LP, Kataoka C, Taguwa S, Moriishi K, Mori Y, Matsuura Y, Mizuguchi K. 2010. Network based analysis of hepatitis C virus core and NS4B protein interactions. *Mol. Biosyst.* 6:2539–2553.
37. Manna D, Aligo J, Xu C, Park WS, Koc H, Heo WD, Konan KV. 2010. Endocytic Rab proteins are required for hepatitis C virus replication complex formation. *Virology* 398:21–37.
38. Tong WY, Nagano-Fujii M, Hidajat R, Deng L, Takigawa Y, Hotta H. 2002. Physical interaction between hepatitis C virus NS4B protein and CREB-RP/ATF6beta. *Biochem. Biophys. Res. Commun.* 299:366–372.
39. Florese RH, Nagano-Fujii M, Iwanaga Y, Hidajat R, Hotta H. 2002. Inhibition of protein synthesis by the nonstructural proteins NS4A and NS4B of hepatitis C virus. *Virus Res.* 90:119–131.
40. He Y, Yan W, Coito C, Li Y, Gale M, Jr, Katze MG. 2003. The regulation of hepatitis C virus (HCV) internal ribosome-entry site-mediated translation by HCV replicons and nonstructural proteins. *J. Gen. Virol.* 84: 535–543.
41. Kato J, Kato N, Yoshida H, Ono-Nita SK, Shiratori Y, Omata M. 2002. Hepatitis C virus NS4A and NS4B proteins suppress translation in vivo. *J. Med. Virol.* 66:187–199.
42. Cho NJ, Dvory-Sobol H, Lee C, Cho SJ, Bryson P, Masek M, Elazar M, Frank CW, Glenn JS. 2010. Identification of a class of HCV inhibitors directed against the nonstructural protein NS4B. *Sci. Transl. Med.* 2:15ra6. doi:10.1126/scitranslmed.3000331.
43. Rai R, Deval J. 2011. New Opportunities in anti-hepatitis C virus drug discovery: targeting NS4B. *Antiviral Res.* 90:93–101.
44. PTC Therapeutics Inc. 2010. Indole derivatives and methods for antiviral treatment. WO 2012/117932 A1.
45. Lohmann V, Körner F, Koch J, Theilmann L, Bartenschlager R. 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 285:110–113.
46. Yi M, Villanueva RA, Thomas DL, Wakita T, Lemon SM. 2006. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proc. Natl. Acad. Sci. U. S. A.* 103:2310–2315.
47. Chou TC, Talalay P. 1984. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* 22:27–55.
48. Blight KJ, Kolykhalov AA, Rice CM. 2000. Efficient initiation of HCV RNA replication in cell culture. *Science* 290:1972–1974.
49. Graham EJ, Hunt R, Shaw SM, Pickford C, Hammond J, Westby M, Targett-Adams P. 2011. Colony-forming assays reveal enhanced suppression of hepatitis C virus replication using combinations of direct-acting antivirals. *J. Virol. Methods* 174:153–157.
50. Tong X, Chase R, Skelton A, Chen T, Wright-Minogue J, Malcolm BA. 2006. Identification and analysis of fitness of resistance mutations against the HCV protease inhibitor SCH 503034. *Antiviral Res.* 70:28–38.
51. Bhattacharyya A, Trotta CR, Peltz SW. 2007. Mining the GEMS—a novel platform technology targeting post-transcriptional control mechanisms. *Drug Discov. Today* 12:553–560.
52. Bryson PD, Cho NJ, Einav S, Lee C, Tai V, Bechtel J, Sivaraja M, Roberts C, Schmitz U, Glenn JS. 2010. A small molecule inhibits HCV replication and alters NS4B's subcellular distribution. *Antiviral Res.* 87: 1–8.
53. Shotwell JB, Baskaran S, Chong P, Creech KL, Crosby RM, Dickson H, Fang J, Garrido D, Mathis A, Maung J, Parks DJ, Pouliot JJ, Price DJ, Rai R, Seal JW, Schmitz U, Tai VWF, Thomson M, Xie M, Xiong ZZ, Peat AJ. 2012. Imidazo[1,2-a]pyridines that directly interact with hepatitis C NS4B: initial preclinical characterization. *ACS Med. Chem. Lett.* 3:565–569.
54. Hosea NA, Collard WT, Cole S, Maurer TS, Fang RX, Jones H, Kakar SM, Nakai Y, Smith BJ, Webster R, Beaumont K. 2009. Prediction of human pharmacokinetics from preclinical information: comparative accuracy of quantitative prediction approaches. *J. Clin. Pharmacol.* 49:513–533.
55. Kuiken C, Yusim K, Boykin L, Richardson R. 2005. The Los Alamos hepatitis C sequence database. *Bioinformatics* 21:379–384.
56. Boleti H, Smirlis D, Dalagiorgou G, Meurs EF, Christoforidis S, Mavromara P. 2010. ER targeting and retention of the HCV NS4B protein relies on the concerted action of multiple structural features including its transmembrane domains. *Mol. Membr. Biol.* 27:50–74.