Discovery and Characterization of Substituted Diphenyl Heterocyclic Compounds as Potent and Selective Inhibitors of Hepatitis C Virus Replication

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ABSTRACT

A novel small molecule inhibitor, referred to here as R706, was discovered in a high-throughput screen of chemical libraries against Huh-7-derived replicon cells carrying autonomously replicating subgenomic RNA of hepatitis C virus (HCV). R706 was highly potent in blocking HCV RNA replication, as measured by real time RT-PCR and Western blot of R706-treated replicon cells. Structure activity iterations of the R706 series yielded a lead compound, R803, which was more potent and highly specific for HCV replication with no significant inhibitory activity against a panel of HCV-related positive stranded RNA viruses. Furthermore, HCV genotype 1 replicons displayed markedly higher sensitivity to R803 treatment than a genotype 2a-derived replicon. In addition, R803 was tested in a panel of biochemical and cell based assays for on target and off target activities, and the data suggested the compound had a close to 100-fold therapeutic window while its exact mechanism of action remained elusive. We found that R803 was more effective than IFN-α in blocking HCV RNA replicon in the replicon model. In combination studies, R803 showed weak synergistic effect with IFN-α/ribavirin, but showed only additive effect with a protease inhibitor and an allosteric RdRp (RNA-dependent RNA polymerase) inhibitor (20). We conclude that R803 and related heterocyclic compounds are a new class of HCV-specific inhibitors that could potentially be developed as a treatment for HCV infection.
INTRODUCTION

Hepatitis C virus (HCV) infection is one of the major causes of viral hepatitis, with a great propensity of inducing chronicity (22). Liver inflammation can persist for decades in chronic HCV infection, which eventually leads to cirrhosis, end-stage liver disease, and hepatocellular carcinoma. HCV infection is a significant healthcare problem: it is estimated that approximately 170 million individuals are chronically infected with HCV worldwide, with ~30,000 cases of new infection each year in the United States alone (1-3). No vaccine is currently available to prevent HCV infection. The standard treatment for HCV infection, a combination of pegylated interferon-α (IFN-α) and ribavirin (RBV), is limited by its suboptimal response rate in a significant patient population, side effects, and affordability (12). Thus, it is critical to discover highly effective, safer therapies to improve the clinical management of HCV infection.

HCV is an enveloped RNA virus belonging to the viral family Flaviviridae (10). HCV clinical isolates display high heterogeneity in their genomic RNA and amino acid sequences, and are classified into six genotypes and numerous subtypes (49). It is documented that infections by different genotypes may produce different clinical outcomes, and may respond differently to IFN-α-based antiviral treatment (for review, see (12)). Significantly, patients infected with genotype 1 virus, which accounts for approximately 70% of HCV infections in the USA, exhibit poor response rates to the IFN-α-based treatment. An ideal antiviral should, therefore, be effective against the majority, if not all, of HCV genotypes.

Upon entering the host cell, HCV releases its 9.6-kilobase long genomic RNA into the cytoplasm, where it directs the translation of a single polyprotein of about 3000 amino acids. The giant polyprotein is co-translationally processed by host and viral proteases into structural
proteins (Core, E1, and E2) and non-structural proteins (P7, NS2, NS3, NS4a, NS4b, NS5a, and NS5b). The mature non-structural proteins (except P7 and NS2) and host factors assemble into membrane associated RNA replication complexes, where a vast quantity of progeny viral RNA molecules are amplified from the incoming HCV genomic RNA (15, 19, 36). Although all the steps in the HCV viral life cycle can be targeted for drug discovery against HCV, the viral non-structural proteins, specifically NS3 and NS5b which encode well-defined enzymatic activities crucial for viral replication, are the major targets for antiviral discovery (11, 53).

However, the replication of HCV viral RNA by the viral replication complex is quickly becoming another focus for drug discovery with the development of the HCV replicon system. The analysis of HCV replication was previously hampered due to the lack of a robust HCV cell culture system until the establishment of HCV replicons (39, 6). The first generation HCV replicons are human hepatoma Huh-7 cell lines carrying engineered genotype 1b subgenomic RNA with a genome organization of HCV 5’ NTR-neomycin phosphotransferase (NPT or Neo')-encephalomyocarditis virus (EMCV) IRES-HCV NS3-4a-4b-5a-5b-HCV 3’NTR. Subsequent studies show that the efficiency of replicon establishment can be enhanced substantially by incorporating cell culture adaptive mutations, especially by those in NS3 and NS5a (39, 38, 6, 27). The HCV replicon system has been an effective tool for studying viral RNA replication and viral-host interactions. It also serves as an important cell-based system to evaluate antiviral drugs, and to reveal drug-resistance mechanisms (for review, see (5)). Moreover, the HCV replicon presents a unique drug screening system, allowing for the screening of compounds inhibiting the viral enzymes as well as other targets of the HCV RNA replication process in a cellular environment. Such screens would perhaps facilitate the discovery of inhibitors that block the functions of NS4b and NS5a or interrupt viral-host
interactions, which cannot be readily achieved with biochemical screens. Several efforts have already been made to screen small molecule compound libraries against different versions of the HCV replicon system (18, 47, 50, 55). Here we describe the development of an HCV replicon assay for high throughput screening and characterization of one of the heterocyclic hits from screens of a 230,000-member chemical library. We show that the lead compound of this hit scaffold is effective in inhibiting HCV replicons of different genotypes, and can enhance the inhibitory activity of IFN-α in the replicon model.

MATERIALS AND METHODS

Cell lines and cell culture maintenance. Huh-7, Replicons 9-13 and Huh-mono were obtained from Ralf Bartenschlager through ReBLikon GmbH. The Huh-7 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 1X nonessential amino acids, 2 mM L-glutamine, penicillin-streptomycin (100 U/ml and 100 µg/ml, respectively). G418 (500-1,000 µg/ml) and hygromycin (25 µg/ml) were added to the Huh-7 medium for 9-13 and Huh-mono, respectively (39, 23). Replicons derived from genotype 1a (30) and 1b/3a (29) were provided by Robert Lanford at Southwest Foundation for Biomedical Research and were cultured under the conditions according to the cited references. Human primary hepatocytes were purchased from BD Gentest (Bedford, MA) and maintained in hepatocyte medium Hepato-STIM from BD Gentest. Human Umbilical Vein Endothelial Cells (HUVEC) and Human Mammary Epithelial Cells (HMEC) were purchased from Cambrex (East Rutherford, NJ), and cultured in media suggested by the provider. A549, BJAB,
HEK293, and Jurkat E6.1 were obtained from ATCC (Manassas, VA) and grown in culture media recommended by the vendor.

Modification of HCV replicons. Total RNA was extracted from replicon 5-2Luc (27), and was used as a template for RT-PCR of the replicon genome using primers GAATTGGAATCGATATTGTTACAAC CCCACCC and GTGGTCTGTAAAACGCGGCCGCTCAGAAGAAC. The RT-PCR product was cleaned and digested with restriction enzymes ClaI and NotI. The resulting 1.3kb fragment, which contains part of the firefly luciferase gene (Luc), ubiquitin (Ubi) and the Neomycin Phosphotransferase gene (NPT or Neo'), was gel purified. Meanwhile, plasmid pFK I341PI Luc NS3-3'/ET (37) was also digested with the restriction enzymes ClaI and NotI, and the 12.2kb fragment was gel purified. The two DNA fragments were ligated together to generate replicon plasmid pFK I341-pI-Luc-ubi-NPT-EI-NS3-3'-UTR (hereafter referred to as PLN). Using a similar procedure, the NS2 coding sequence was inserted between EI and NS3 of plasmid PLN to generate PLNCP, a NS2-containing replicon construct. The PLN or PLNCP plasmid was linearized with Scal restriction enzyme and subjected to in vitro transcription using RiboMax Large Scale RNA Production System–T7 (Promega, Madison, WI). After DNaseI digestion and purification, the transcribed RNA (5 µg) was electroporated into Huh-7 cells as reported previously (39). The cells were then transferred to a 15-cm culture dish with Huh-7 medium (40 ml) containing 500 µg/ml G418 for selection. Colonies of surviving cells were isolated and tested for luciferase expression and viral replication. Replicon cell clones PLN5 (derived from PLN) and PLNCP54 (derived from PLNCP) expressed high luciferase activity and were expanded and used in the experiments. In addition, replicons 9-13 (39) and Huh-mono (23) from Ralf Bartenschlager were also used throughout this study.
**High throughput replicon assay.** PLN5 or PLNCP54 replicon cells were plated at a density of 3000 cells/45 µl Huh-7 medium/well into 384 well plates, and were incubated at 37°C and 5% CO₂ for 24 hrs. Compounds (5 µl of 0.1 mM stock) were transferred into each well, and following 24 hrs of compound incubation at 37°C and 5% CO₂, the culture medium and the compounds were removed, and 50% Bright-Glo solution (Promega, Madison, WI) (25 µl) was added to each well. The plates were placed on a shaker at room temperature for 5 minutes, and luciferase activity was measured with a Fluostar plate reader. The inhibitory effect of a hit compound was scored by comparing the luciferase activity of a compound-treated well with that of DMSO-treated control wells.
**TaqMan RT-PCR.** HCV primers and probe were designed with Primer Express software (PE Applied Biosystems) and covered highly conserved 5’-untranslated region (UTR) sequences (sense, 5’-TGCGGAACCGGTGAGTACA-3’; antisense, 5’-CGGGTTGATCCAAAGGA-3’; probe, 5’-FAM-CGGAATTGCCAGGACGACCGG-TAMRA-3’). Replicon 9-13 or PLNCP54 cells were plated at a density of approximately 5,000 cells per 90 µl per well onto a 96-well plate, and a mixture containing 90% of the Huh-7 medium, 7.2% PBS, 1.8% methanol, 1% DMSO, and varying concentrations of R803 or R706 were added to the cells 24 hrs after plating (each concentration in triplicate). 48 hours after compound addition, total RNA was isolated from each sample using RNeasy 96 kit (Qiagen, Valencia, CA). Replicon RNA and cellular GAPDH mRNA were amplified by a protocol modified after a published method (26) using ABI PRIZM 7700 System (PE Applied Biosystems, Foster City, CA). EC\textsubscript{50} of the test compound was calculated by plotting HCV RNA titer (normalized to the endogenous GAPDH mRNA levels) against the compound concentrations.

**Western blot.** Replicon 9-13 or PLNCP54 cells, plated onto 6-well plates, were treated with DMSO or a test compound for 48 hrs. The cells were washed with PBS and lysed in 1X SDS sample buffer, and the lysates were analyzed with the NuPage gel system (Invitrogen, Carlsbad, CA). The gel was blotted onto a nitrocellulose membrane and probed with a monoclonal anti-HCV NS3 antibody (Rigel, South San Francisco, CA) and a monoclonal anti-tubulin antibody (Sigma-Aldrich, St. Louis, MO), and then incubated with HRP-conjugated secondary antibody. NS3 and the cellular β-tubulin were detected by developing the membrane with SuperSignal WestDura kit (Pierce, Rockford, IL).
**BVDV and YFV assays.** The activity of antiviral compounds was measured by their ability to inhibit bovine viral diarrhea virus (BVDV)- or yellow fever virus (YFV)-induced cytopathic effects (CPE). BVDV (strain NADL, ATCC) was grown in Madin-Darby Bovine Kidney (MDBK) cells, and YFV (strain 17D, ATCC) was grown in Vero cells. Cells were plated at 1X10^4/well in 96-well plates. After an overnight incubation at 37 °C and 5% CO_2, the culture medium was removed and a pre-titered aliquot of virus (sufficient to cause complete cell killing at the time of maximal CPE development, or TCID_{100}) was added to each well. Immediately following the virus infection, a DMSO control or increasing doses of R803 were added. At the end of the infection assay, cell viability was determined using CellTiter 96® Reagent (Promega, Madison, WI) on a Vmax plate reader (Molecular Devices, Sunnyvale, CA). The drug concentration that reduced the virus-induced CPE by 50% (EC_{50}) and the concentration that caused the reduction of cell numbers (in the absence of the virus infection) by 50% (CC_{50}) were calculated.

**PV-Luc replicon assay.** PV-Luc plasmid (33) was linearized with PvuI restriction enzyme and subjected to *in vitro* transcription using RiboMax Large Scale RNA Production System–T7 (Promega, Madison, WI). After DNaseI digestion and purification, the transcribed RNA (5ug) was electroporated into Huh-7 cells at 270V, 950uF on a Gene Pulser II according to manufacturer’s descriptions (Bio-Rad). The cells were then dispensed into white 96-well plates at a density of 10,000 cells/90 µl/well. R803 was serially diluted in compound diluent (10% DMSO, 20% Methanol) and added to each well in a volume of 10 µl. The plates were incubated for 18 hrs at 37 °C and 5% CO_2 before luciferase activity was determined using Bright-Glo Luciferase Assay kit (Promega, Madison, WI).
**Compound and/or IFN-α treatment.** Replicon 9-13 cells were plated onto 6-well plates 24 hours prior to the treatment. Serial dilutions of R803 were made into a mixture containing 90% of the culture medium, 7.2% 1X PBS, 1.8% methanol, 1% DMSO, 20 µM RBV, and varying concentrations of IFN-α for a fixed ratio dose-response study. The cells were treated with the designated combinations of R803 (0-80 nM concentrations), IFN-α (0-4 IU/ml), plus 20 µM concentration of RBV for 72 hrs, washed with PBS, lysed in SDS-loading buffer, and analyzed by Western blot as described above.

**HCV IRES in vitro translation (IVT) assay.** A plasmid, pT7-RL-HCV IRES-FL, was linearized via restriction enzyme and transcribed using the Message Machine kit (Ambion, Inc., Austin, TX) to produce dicistronic RNAs that translate Renilla luciferase in a cap-dependent manner, and firefly luciferase under the control of the HCV IRES (genotype 1b). In vitro translation reactions (25 µL) were assembled by mixing TnT kit components as per manufacturer’s instructions (Promega, Madison, WI) with the addition of 2 µL of 0.01 mg/ml dicistronic RNA and 1 µl of R803 at various concentrations dissolved in DMSO. Reactions were incubated for 1 hr at 30 °C, and analyzed using the Dual Luciferase Reporter assay kit (Promega, Madison, WI). Luciferase activities were measured on a Luminoskan Ascent (Thermo Systems, Waltham, MA) and reported as the ratio of firefly (HCV IRES):Renilla (cap) luciferase counts.

**NS2-3 IVT assay.** A pET-24b vector encoding NS2-3 (amino acids 94-217 of NS2 and 1-181 of NS3, genotype 1a, generously provided by Charlie Rice) was used to program 25 µL rabbit reticulolysate reactions (TnT kit, Promega, Madison, WI). Reaction conditions of kit components were as recommended by the manufacturer, but further supplemented with 20 µM ZnCl₂, 9 µCi Redivue [³⁵S]-methionine (GE Healthsciences, Chicago, IL) and 1 µl of R803 at
various concentrations dissolved in DMSO. Reactions were incubated for 3.5 hrs at 30 °C, halted by addition of four volumes of SDS-loading buffer, and subjected to NuPage gel (10%) analysis. The gel was then dried and exposed to a phosphorimaging plate, imaged using a Typhoon 9410 (Molecular Dynamics, Sunnyvale, CA), and bands corresponding to NS2-3, NS3, and NS2 were quantified by using ImageQuant 5.2 software to calculate the extent of proteolytic processing as a percentage of the total translated NS2-3.

**Biochemical assays for NS3-4a**

Biochemical assays for NS3-4a and NS3 NTPase, NS5b RdRp. His-tagged viral enzymes were cloned into the pET vector system (EMD Chemicals, San Diego, CA), expressed, and purified according to the manufacturer’s instructions. The NS3-4a pro was assayed by its ability of cleaving a fluorogenic substrate (Bachem cat# M-2235, Torrance, CA) using a reaction condition modified after a published method (24). The NTPase assay was performed in 96 well plates using a malachite green reagent as previously described (31).

Briefly, in a 25-µl assay volume, 5 µl of R803 dissolved in 20% DMSO was mixed with 5 pmol of NS3, 2.5 mM polyuridylic acid, 200 mM Hepes (pH 7.4), 3 mM MgCl₂, 2 mM DTT, and 100 µg/ml BSA for 5 min at 25°C. The reaction was initiated by adding 2.5 mM ATP to the mixture, followed by a 30-min incubation at 25°C. The reaction was terminated with the addition of 100 µl of Malachite Green solution (0.034% malachite green, 1.05% ammonium molybdate and 0.04% Tween 20) and 100 µl of 34% Sodium Citrate. Color development was allowed for 15 min at 25°C and A₆₃₀ was measured on a plate reader. The amount of NTP hydrolyzed was then calculated from an inorganic phosphate standard curve. For NS3 helicase unwinding assay, the DNA “trap” oligomer (5’-GCCTCGCTGCCGTCGCCA-3’OH) was radiolabeled using [γ-³²P]ATP and polynucleotide kinase (New England Biolabs, Beverly, MA), and then annealed to the unlabeled DNA (“long”) oligomer (5’-
TGGCGACGGCAGCGAGGCTTTTTTTTTTTTTTTTTTTT-3’OH) to generate heteroduplex unwinding substrate. A 20 µL reactions containing 1 nM heteroduplex DNA, 50-200 nM NS3 helicase (encoding amino acids 166-632 of NS3), 5 mM HEPES (pH 7.4), 3.75 mM MgCl₂, 125 µg/ml BSA, 1.25 mM DTT, and 1 µL of varying concentrations of R803 diluted in DMSO, were pre-incubated for 15 minutes at room temperature. The unwinding reaction was initiated by the addition of 5 µL of 40 mM ATP, mixing, and incubating the reactions for 10 min at 37 °C. Reactions were terminated using 6.3 µl 5X stopping buffer (250 mM Tris-HCl pH 7.5, 20 mM EDTA, 0.5% SDS, 0.1% NP-40, 0.1% bromophenol blue, 0.1 % xylene cyanol, 50% glycerol, 250 nM unlabeled “trap” oligomer). Analysis of reaction samples consisted of electrophoresis through a 20% polyacrylamide 1X TBE gel at 250 V for 60 min, exposure to a phosphorimaging plate and visualization using a Typhoon 9410. The resulting image was quantified with ImageQuant 5.2 software. To assay NS5b\textsubscript{RdRp}, a 427-base long HCV 3’-NTR RNA was generated by \textit{in vitro} transcription and used as RNA template. 3-fold serial dilutions of R803 were prepared in DMSO, and 1 µl of each dilution was added to a 12.5 µl mixture containing 40 mM Tris-Cl, pH 7.5, 50 mM KCl, 5 mM MgCl₂, and 20 nM NS5b followed by a 5-min pre-incubation. The reaction was then initiated by adding an RNA template/NTPs mixture (5 nM RNA, 5 mM each of ATP, GTP, and CTP, 2 µM UTP, and 0.1 µl [α-\textsuperscript{33}P]-UTP (GE Healthsciences, Chicago, IL). The reaction mix was incubated at 25 °C for 2 hrs before being terminated with 25 µl of 2X TBE-Urea Sample buffer and heated at 70 °C for 5 min. One-fifth of the RNA products were separated by electrophoresis on a 6% TBE Urea gel (Invitrogen, Carlsbad, CA). The labeled RNA products were analyzed using a Typhoon 9410 as described above.
Transient transfection and VTF-T7 infection (TTI) assay. HEK 293 cells were seeded at a density of $4 \times 10^5$ cells/wells in 2 ml of DMEM supplemented with 10% fetal calf serum and 2 mM L-glutamine. The following day, 25 µM concentration of chloroquine was added to the culture and cells were transfected with 2 µg of pPLNCP or pHuh-mono plasmid (both contained a T7 promoter) using the calcium phosphate ‘bubble’ precipitation method. Briefly, a transfection cocktail was prepared by mixing 2 µg of DNA, 130 µl of H$_2$O, and 20 µl of 2 M CaCl$_2$. A 150-µl aliquot of 2X HBS (pH 7.0–7.1) was added to the cocktail by bubbling with a pipette immediately for 5-10 sec, and the cells were transfected by gently agitating the solution with the medium. Approx. 3 hrs after the transfection, the medium was replaced by fresh DMEM, and VTF-T7 virus (ATCC) was added to the cells at an MOI of 1. One hour after the VTF-T7 infection, the medium–virus mixture was replaced with fresh Huh-7 media containing DMSO or test compound. Cells were harvested 48 hrs after compound addition and lysed by adding 1X SDS-loading buffer (Invitrogen, Carlsbad, CA). The expression and processing of NS2, NS3 and NS5b were detected by Western blot.

**Determination of drug interaction in vitro.** *In vitro* drug interactions were assessed using a modified fixed-ratio isobologram method (13). Briefly, predetermined 50% effective concentration (EC$_{50}$) values were used to decide the top concentrations of the individual drugs to ensure that the EC$_{50}$ fell near the midpoint of a six-point serial dilution. Top concentrations used were 0.08 µM for R803, 4 IU/ml for IFN-α, 0.008 µM for BILN 2061 and 4 µM for RdRp inhibitor (20). The top concentrations were used to prepare fixed-ratio solutions at ratios of 4:0, 3:1, 2:2, 1:3 and 0:4 of R803 and the partner drug. The mixed drugs solutions were then serially diluted and tested on replicon cells. EC$_{50}$ values were calculated for each drug combination and FIC$_{50}$ (Fractional IC$_{50}$) values were calculated (13). A mean FIC$_{50}$ value of
greater than 1 indicates antagonistic effect, a mean FIC$_{50}$ value equals to 1 indicates additive effect, while a mean FIC$_{50}$ value of less than 1 indicates synergistic effect between the tested drug combinations.

RESULTS

Development of a replicon high throughput screening assay. In order to establish HCV replicon cell lines suitable for high throughput screens, plasmids PLN and PLNCP were constructed by modifying the original replicon 9-13 plasmid (Fig. 1) (39). Resembling 9-13, both PLN and PLNCP harbored 3 cell adaptive mutations in NS3 and NS5a coding regions that facilitated replicon colony formation (27). In addition, both of the plasmids carried a firefly luciferase gene under the control of the poliovirus IRES, which significantly increased luciferase signal (4). The replication of PLNCP, because of its expression of NS2 in addition to the other non structural proteins, relies not only on the viral RNA replication complex but also the efficient processing at the NS2/3 junction (52). Upon RNA transfection followed by G418 selection, surviving cell colonies were screened for robust luciferase signal, viral RNA replication, and responsiveness to IFN-α or other known HCV inhibitors. Amongst the colonies screened replicons PLN5 and PLNCP54 showed high level HCV RNA replication and a superior luciferase signal suitable for compound screening in a 384-well format. We anticipated that such screens could identify a wide range of inhibitors, targeting either viral or cellular proteins essential for HCV RNA replication. In addition, compounds inhibiting NS2/3 cleavage could be identified by differential screens using PLN5 and PLNCP54 replicons. A high throughput screen format (see Materials and Methods) was developed by optimizing cell
density, compound dilution and addition procedures, duration of compound treatment, and
luciferase reporter measurement. The initial hits from the screen were then confirmed and
evaluated for potency using serially diluted compounds. In the mean time, the cytotoxicity
indexes of the hit compounds were also evaluated over the same range of compound
concentrations to determine the therapeutic windows of the hit compounds.

Identification of R706 as a replicon hit. Several replicon hit scaffolds were
discovered from the screens of approx. 230,000 compounds using the luciferase-based PLN5
and PLNCP54 assays. The total hit rate for these screens are 0.60%, with hits being defined as
compounds that inhibit equal to or more than 50% of replicon luciferase activity at 10 µM.

Hits were further confirmed on the replicon cells using a 3-fold serial dilution together with a
cytotoxicity assay. The confirmed hit rate is 0.27%, with hits being defined as compounds
with EC\(_50\) (a compound concentration that inhibits HCV replication by 50%) below 10 µM and
a CC\(_{50}\) (a compound concentration that inhibits cell viability by 50%) of 10 fold over EC\(_{50}\).

R706, a diphenyl heterocyclic compound containing a dichloroacetamide moiety (Fig. 2A),
was the most potent hit with an EC\(_{50}\) at 90.56±15.99 nM by luciferase assay (Fig. 2B). R706
series was chosen for further characterization because of its outstanding potency among all the
scaffolds. Western blot analysis showed that R706 dose-dependently inhibited replicon
PLNCP54, with an estimated EC\(_{50}\) at 110 nM and a CC\(_{50}\) value (the compound concentration
that inhibits cell viability by 50%) at ~3 µM as estimated by the levels of the HCV NS3 and
the cellular β-tubulin, respectively (Fig. 3C). Similar EC\(_{50}\) and CC\(_{50}\) values were obtained
using replicon Huh-mono (Fig. 1) by Western blot (data not shown), suggesting that R706 did
not inhibit the neomycin phosphotransferase (NPT or Neo\(^r\)), or the EMCV IRES, or the
luciferase reporter. TaqMan RT-PCR analysis further confirmed that R706 treatment resulted
in a dose-dependent reduction of HCV RNA in the replicon with an EC$_{50}$ at 114.93±10.64 nM, as normalized by the endogenous GAPDH mRNA (Fig. 2C). The EC$_{50}$ values from the 3 different assays were in the same range, and the difference might be attributed, at least in part, to the differences in the decay rate of the respective assay indicators, since the half lives (T$_{1/2}$) of the firefly luciferase, HCV NS3 and HCV RNA are 3.68 hrs (32), 11.5~17.5 hrs (Bartenschlager, personal communications), and 12 hrs (17), respectively. Taken together, these results demonstrated that R706 was a potent HCV replicon inhibitor, effective in reducing viral RNA and protein productions.

**Biological characterization of R803, a lead compound of the R706 scaffold.**

Structure-activity iterations of the R706 scaffold (D. A. Goff et al. manuscript in preparation) led to the discovery of R803 (Fig. 3A), a lead compound with improved biological properties. The anti-viral activity of R803 had been determined by reporter replicon assay with multiple repeats to be 29.88±8.05 nM, an improvement of ~3-fold compared to the parent compound R706. The potency of R803 against the replicon is also confirmed by both Western blot and TaqMan RT-PCR to be about 37 nM and 54.67±4.11 nM, respectively (Fig. 3B, C).

It has been demonstrated that certain antiviral compounds show markedly reduced activity in vivo or in cell-based assays because of their great propensity to bind serum proteins (45). Since R803 has a high rate (>99%) of protein binding (data not shown), it was important to address whether protein binding would weaken R803’s anti-HCV activity. Thus, EC$_{50}$ of R803 was measured in replicon cells cultured in media containing increasing concentrations of human serum (Irvine Scientific). Western blot analysis demonstrated that the elevation of human serum concentration, up to 25% (v/v) of the culture media, had only negligible effect on the potency of R803 (Fig. 3D). It is possible that the compound was already bound to the
proteins in the culture medium before the addition of human serum and the addition of human  
supernatant did not affect that binding. Further increase of serum concentration resulted in reduced 
cell viability (data not shown), prohibiting effective evaluation of R803 under these culture  
conditions.

To assess the general effect of R803 on cell proliferation, a panel of primary cells and  
transformed human cell lines were treated with increasing doses of R803 for 48 hrs, and the  
effect on cell proliferation was measured by MTS-based cell viability assay. CC_{50} of R803  
was found to range from 2 µM to > 10 µM, depending on cell types and proliferation status  
(Table 1). It is noteworthy that R803 had no detectable cytotoxic effects on cultured primary  
human hepatocytes at compound concentration of up to 10 µM, by either measuring cell  
viability using MTS-based analysis (Table 1) or quantifying enzymes released from damaged  
hepatocytes (data not shown). To calculate the therapeutic window (the ratio of CC_{50}/EC_{50}) in  
the cell culture model, the antiviral activity as well as the cytotoxicity of R803 was evaluated  
simultaneously in PLNCP54. With the antiviral activity at 28.77±7.44 nM and the cytotoxicity  
at 2.66±0.54 uM, the therapeutic window was calculated to be 92.

It is well documented that T cells play pivotal roles in HCV clearance in patients (7).  
Two counter assays were performed to examine whether R803 could inhibit T cell activation.  
In one assay, Jurkat T cells were stimulated with either C305 (a T cell receptor antibody) or  
phorbol 12-myristate 13-acetate (PMA) with or without the presence of R803, and T cell  
activation was quantified by cell surface CD69 expression. R803 did not show any inhibitory  
activity in the Jurkat activation test (data not shown). In a second assay, primary human T  
cells, with or without IL-2 stimulation, were treated with increasing doses of R803, and cell  
proliferation was quantified by measuring cell number increase 72 hrs after compound
incubation. The results demonstrated that, in contrast to R050 (a known inhibitor of T cell
signaling) (8), R803 did not affect primary T cell growth, with or without IL-2 stimulation
(data not shown).

**Antiviral specificity of R803.** To examine anti-viral specificity, R803 was tested
against heterologous, positive-strand RNA viruses, such as poliovirus (PV), yellow fever virus
(YFV), and bovine viral diarrhea virus (BVDV), in cell culture. Specifically, R803 was
assayed in a PV replicon system, in which the region of the PV genome encoding viral
structural proteins was replaced by the firefly luciferase coding sequence (33). Increasing
doses of R803 were added to Huh-7 cells transfected with the PV replicon RNA, and the level
of RNA replication was monitored by quantifying firefly luciferase activity. No difference was
noticed between mock (DMSO) and R803 treatment, suggesting the compound did not have
anti-PV activity. Similarly, R803 was inactive in antagonizing BVDV- or YFV-induced
cytopathic effect in cultured MDBK or Vero cells, respectively. PV is a prototype positive-
strand RNA virus, and BVDV and YFV are HCV-related *Flaviviridae* family members.

Despite the fact that HCV and these RNA viruses express homologous viral enzymes and share
common replication strategies, R803 was active only in HCV replicon assays (Table 2), an
observation strongly suggesting that R803 is an HCV-specific inhibitor.

HCV exists in six genotypes, and an ideal HCV antiviral should be active against most,
if not all of the genotypes. R803 was tested in replicon variants that were available to us,
including those derived from genotypes 1a (Rep1aNeo), 1b (con1), and 2a (JFH-1). As shown
in Fig. 4, R803 was substantially more potent against genotype 1a and 1b replicons (EC$_{50}$~ 30
nM) than the genotype 2a replicon (EC$_{50}$~ 1,000 nM). Analogous genotype-differential
response to R803 was also observed in HCV virus culture assays involving genotype 1a and 2a viruses (unpublished data).

**Testing R803 against individual HCV targets.** To investigate its anti-HCV mechanism, R803 was further tested against individual HCV targets both *in vitro* and in cell-based assays. A cell-free translation assay, programmed with a synthetic dicistronic mRNA (cap-*Renilla* luciferase-HCV IRES-firefly luciferase), was employed to test whether R803 could block HCV IRES-mediated protein translation. At concentrations up to 40 µM, R803 did not show any effect on the expression of the firefly luciferase in the cell-free translation system. A parallel test showed that R803 treatment did not affect a Huh-7 cell line expressing the dicistronic mRNA either, indicating the compound might not act as an HCV IRES inhibitor. Likewise, no activity was detected at R803 concentrations ≥ 40 µM in biochemical assays for the HCV NS2-3 cysteine protease, and ≥ 10 µM against the NS3-4a serine protease, the NS3 NTPase/helicase, and the NS5b RdRp (Table 3).

A transient transfection-infection (TTI) assay was developed to explore the possibility that R803 might only inhibit a natively expressed HCV IRES, NS2-3 cysteine protease, and the NS3-4a serine protease in cells. In the study, Huh-7 cells were transfected with replicon plasmid PLNCP or pHuh-mono (Fig. 1). The RNA transcriptions of both constructs were under the control of the T7 promoter. Four hours following transfection, cells were infected with VTF-T7, a recombinant vaccinia virus that expresses bacterial phage T7 polymerase (14). The cells were able to produce a significant amount of viral proteins under the control of the viral IRES, and supported efficient cleavage by the NS2-3 cysteine protease and the NS3-4a serine protease. R803, at concentrations ≥ 1 µM inhibited neither the IRES-controlled protein translation nor the HCV proteases in the treated cells. In contrast, BILN 2061, a published
inhibitor of the NS3-4a serine protease (28), was active in blocking NS3-4a-mediated protein
processing (Fig. 5).

The potency of R803 was compared in PLN5 and PLNCP54, a pair of identical HCV
replicons except for the NS2 coding region (Fig. 1). No significant differences were found in
their response to R803 treatment, suggesting NS2-3 was not the target of R803 (data not
shown). This result was in agreement with the observation that R803 was unable to block
NS2-3 processing in the \textit{in vitro} translation-based test or in the TTI assay (Table 3).

Taken together, the available data suggest that R803 did not show significant inhibitory
effect on the viral IRES, the NS2-3 cysteine protease, the NS3-4a serine proteases, the NS3
NTPase/helicase, or the NS5b RdRp. These data, however, did not exclude the possibility that
R803 might inhibit one of these viral functions presented only in the viral replication complex
inside replicon cells.

\textbf{The antiviral effect of R803 combined with IFN-\(\alpha\) or viral enzyme inhibitors in the
replicon.} The potential interaction between R803 and IFN-\(\alpha\)/ribavirin therapy was assessed in
the replicon model by a modified fixed ratio isobologram method (13). Stock solutions of
R803 and IFN-\(\alpha\) were mixed by a fixed ratio and serially diluted for the determination of
antiviral potency against replicon. The fractional IC\(_{50}\) (FIC\(_{50}\)) of R803 and IFN-\(\alpha\) combination
for this specific mixing ratio was calculated. More FIC\(_{50}\)s will be generated using several
different mixing ratios of R803 and IFN-\(\alpha\). All the FIC\(_{50}\)s will then be used to calculate a
meanFIC\(_{50}\) value to evaluate the interaction between R803 and IFN-\(\alpha\). A meanFIC\(_{50}\) score of
1 indicates additive effect, a meanFIC\(_{50}\) score of less than 1 indicates synergistic effect between
the two testing compounds, while a meanFIC\(_{50}\) score of more than 1 indicates antagonistic
effects between the two testing compounds (13). The ribavirin concentration was fixed at 20
µM because the commercial ribavirin displayed cytotoxic effect in the replicon at higher concentrations. This also helps to simplify the study. The combination of R803 and IFN-α/ribavirin scored a mean FIC<sub>50</sub> value of 0.90±0.05, indicating a weak synergy between the testing compounds. Moreover, the combination did not cause any notable cytotoxic effect using cellular tubulin as an indicator (data not shown). These results clearly demonstrated that R803 is not only compatible with, but could also enhance the anti-HCV activity of IFN-α/ribavirin in the replicon model.

Similarly, studies were designed to elucidate the combination of R803 and BILN 2061, an active site inhibitor of the HCV NS3-4a serine protease (28). Results show that the combination of R803 and BILN 2061 scored a mean FIC<sub>50</sub> value of 1.05±0.12 in the replicon model. As a result, no synergy was found between R803 and BILN 2061. Another experiment that studied the combination of R803 and an allosteric RdRp inhibitor (20) scored a mean FIC<sub>50</sub> of 1.09±0.05, also indicating an additive effect between R803 and this RdRp inhibitor. The number might indicate a very slight antagonistic effect, but it is just too close to differentiate.

**Comparison of R803 and IFN-α in the replicon model.** Further studies were performed to compare the efficacy of R803 and IFN-α in the replicon during extended treatment. Specifically, replicon cells were treated with either the DMSO control, 500 nM of R803, or 25 IU/ml of IFN-α (approx. 15X EC<sub>50</sub> of the respective agents based on Western blot assay). Cells were split at a dilution of 1:4 every 2-3 days, and RNA samples were taken at each split to measure HCV RNA levels by TaqMan RT-PCR. Media containing fresh DMSO, R803 or IFN-α were added at each cell split to maintain continuous treatment. During the 14-day treatment period, replicon cells that were exposed to 500 nM concentration of R803 showed a gradual decrease in viral RNA levels; on day 14 the HCV RNA titer was reduced by
more than 1000-fold. In contrast, parallel treatment with 25 IU/ml IFN-α yielded ~22-fold reduction in HCV RNA titer after 14 days of treatment (Fig. 6A). Moreover, no viral RNA rebound was observed after the cessation of R803 treatment (Fig. 6B, black bars), suggesting the compound had a potential to “cure” the replicon.

Significant amounts of HCV RNA persisted after the 14-day treatment with IFN-α (Fig. 6A, gray bars). To test whether these HCV replicon that survived IFN-α treatment would respond to R803 treatment, replicon cells treated with 25 IU/ml IFN-α for 14 days were switched to either DMSO or a 500 nM concentration of R803 for an additional 2 weeks. The HCV RNA titer dropped from 3.2x10^5 gc/ng GAPDH mRNA to a level around the detection limit of TaqMan RT-PCR (Fig. 6B, shaded bars), whereas that of the DMSO control group remained unchanged (Fig. 6B, striped bars). These results indicate that the IFN-α-refractory HCV RNA population was sensitive to R803 treatment.

DISCUSSION

The application of the replicon system in anti-HCV drug screening has several advantages. First, in the replicon system HCV proteins are presented in their functional form in a cell line derived from human liver tissue. The HCV RNA replication process entails complex interactions and temporal regulations between viral proteins, cellular factors, and the ER-derived membrane. Only a limited portion of these processes can be reconstituted biochemically. The replicon system allows screening of critical viral targets such as HCV NS4b, NS5a, and a network of protein-protein interactions for which meaningful biochemical assays are not available. Furthermore, inhibitors identified from replicon screens should have
acceptable cell permeability and intracellular stability, both critical characteristics for drug-like compounds. In this report we described the development of a high throughput screening assay using modified replicon cell lines, and the discovery of R706 from the screening of a 230,000-member chemical library. R706 and its analogue R803 inhibit HCV replication effectively as demonstrated by the dose-dependent reduction of HCV protein and RNA in the treated cells.

On the other hand, there are disadvantages for replicon screens. Considerable effort is required to dissect the molecular mechanism of replicon inhibition by small molecule inhibitors. Drug-resistance genotyping is a classic method to identify the target of a given compound. Indeed, the method has been successfully used to map drug-resistant alleles for inhibitors of the NS3-4a protease (34, 40) and the NS5b RdRp (43, 44). It is noteworthy that these inhibitors were all derived from biochemical screens or structure-based drug design with defined viral enzyme targets. There are no reports thus far of the successful identification of targets for any compounds derived from a replicon screen. The lack of target identification may reflect that the replicon-based screen is still at an early stage, or more likely that the lack of viral exit and entry in HCV replicon cells makes genetic selection of resistant variants more difficult, if not intractable. In the case of the R803 scaffold, the mechanism of action remains elusive despite extensive biochemical and cell-based analysis of potential on-target activity, and by genetic selection and analysis of R803-resistant replicons. In particular, the lengthy selection with R706 or R803 led to the selection of replicons with either reduced compound permeability or replicon that had accumulated multiple mutations, each of which contributes only weak resistance to R803 (data not shown). We conclude that R803 does not inhibit NS2-3 cysteine protease, the NS3-4a serine protease, the NS3 NTPase/helicase, the HCV IRES, or the NS5b RdRp activities under the described assay conditions. Current data can not
discriminate whether the actual target is of viral or cellular origin. Nonetheless, R803 is a specific inhibitor targeting a critical event in the HCV RNA replication cycle, it has no detectable inhibitory activity against PV, YFV, or BVDV, a HCV related positive-strand RNA virus. In a recent experiment, in chimpanzees chronically infected by HCV, administration of a derivative of R803 reduced the viral titer by 1 to 2 logs after 7 days of treatment (unpublished data). In the meantime, simultaneous monitoring of the elevated liver enzymes of the chimpanzees over the treatment period showed no obvious liver damage. This data also indicated that the compound probably have a specific antiviral mechanism rather than inhibiting the viral replication by a non-specific cytotoxic mechanism.

It is intriguing that genotypes 1 and 2a displayed differential response (>30-fold shift in EC₅₀) to R803 treatment, in both replicon and live virus assays. It indicates that R803 probably inhibits a viral target, or perhaps a cellular factor(s) that is involved in HCV replication in a genotype-specific manner. Ongoing analysis of R803 in 1b/2a inter-genotypic chimeras may provide additional hints regarding its mechanism of action. Due to the high degree sequence heterogeneity amongst HCV genotypes (e.g. genotype 1b and 2a differ by approximately 30% at the amino acid level), it is not surprising for an HCV inhibitor to show differential antiviral effects in different HCV genotypes, both in the HCV replicon model (41) and in human clinical studies (21, 48). It is clinically desirable to broaden the antiviral spectrum of R803-like HCV inhibitors by further structure-activity iterations, and the replicon model would be a crucial reagent for the selection of inhibitors with cross genotype activities.

The transient transfection VTF-T7 infection assay (TTI) has been widely used in functional analyses of viral and cellular proteins, such as the analysis of HCV polyprotein processing (16). It is also a useful cell-based system, complementary to the HCV replicon, for the study
of a compound's antiviral mechanism. Because TTI uncouples HCV RNA translation and
protein cleavage processes from the HCV RNA replication cycle, it allows the analysis of the
potential impact of antiviral compounds on the HCV IRES, the NS2-3 cysteine protease, and
the NS3-4a serine protease in a single cycle of protein translation and proteolytic processing.
For instance, TTI testing of BILN 2061, yielded the accumulation of unprocessed viral protein
precursors, a phenotype expected for an NS3-4a serine protease inhibitor. We also identified
replicon inhibitors capable of blocking viral IRES-mediated translation with the TTI assay
(unpublished data).

Significant progress has been made recently in the development of efficient HCV
replication systems, such as replicons expressing the green fluorescent protein (GFP) (46), as
well as the fully infectious genotype 2a virus culture (35, 51, 54, 9). It is plausible to use these
systems to isolate drug-resistant viral populations and perhaps elucidate a viral target for R803
or other inhibitors identified by high-throughput replicon screens.

R803 has a number of attractive properties: as a simple chemical entity, R803 can be made
by short convergent synthesis; it is readily scaleable from commercially available starting
materials; and contains sites suitable for chemical optimization (D. A. Goff et al. manuscript in
preparation). As an antiviral agent, R803 exhibits anti-HCV potency, selectivity, and has the
potential to be used in combination with IFN-α and/or other classes of HCV inhibitors. In the
HCV replicon model, it induces far fewer drug-resistant replicon clones than other known
inhibitors of the NS3-4a protease and the NS5b RdRp (data not shown). We conclude that
R803 and its derivatives are a class of novel HCV inhibitors that can be explored as a potential
HCV therapeutic agent.
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FIGURE LEGENDS

Figure 1. A schematic presentation of the gene organization of the replicons. The dark vertical bars adjacent to HCV 5’ NTR represent amino acids 1-16 of HCV core (39, 23). 5’ NTR, 5’ non-translated region; Neo’, neomycin resistant gene; EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site; Hygro’, hygromycin resistant gene; Ubi, ubiquitin; PV, poliovirus.

Figure 2. R706, a substituted diphenyl heterocyclic compound identified from a high throughput replicon screen. (A) Chemical structure of R706. Anti-replicon activity measured by (B) luciferase- and (C) TaqMan RT-PCR-based assays. HCV RNA ge, HCV RNA genome equivalent copies. The data shown are from a single representative experiment and were reproduced several times. The luciferase-based and TaqMan-based experiments were performed in triplicates and the error bars represent the standard deviations.

Figure 3. R803 is a lead compound derived from structure-activity iterations of the R706 scaffold. (A) Chemical structure of R803. (B) Dose-dependent inhibition of HCV RNA replication shown by TaqMan RT-PCR. Experiment was performed in triplicates and the error bars represent the standard deviations. The data shown are from a single representative experiment and were reproduced several times. (C) R803 vs. R706 in a Western blot-based replicon assay. (D) The anti-replicon activity of R803 under various human serum concentrations in the culture media.
Figure 4. R803 induced differential responses in HCV replicons derived from genotypes 1a, 1b, and 2a isolates. HCV replicons derived from genotype 1a (30), 1b (42), and 2a (25) were treated with varying concentrations of R803 for 48 hrs, and the cell lysates were analyzed by Western blot using a genotype 1b NS3-specific monoclonal antibody and a β-tubulin antibody (Panel A) or a genotype 2a NS3-specific monoclonal antibody and a β-actin antibody (Panel B, C). The data shown are from a single representative experiment and were reproduced several times.

Figure 5. Evaluation of R803 and BILN 2061 in a transient transfection, VTF-T7 infection (TTI) assay. (A) R803 or (B) BILN 2061 was tested in a transient transfection, VTF-T7 infection assay. Cells were lysed and subjected to Western blot analysis with an HCV NS5b-specific monoclonal antibody. The data shown are from a single representative experiment and were reproduced several times.

Figure 6. IFN-α vs. R803 in the replicon model. (A) 9-13 replicon cells were treated with either 25 IU/ml of IFN-α or 500 nM of R803 (approx. 16X of EC_{50} of the respective agent) for 14 days, and cell samples were collected at each time point of cell split. HCV RNA titer of each sample was measured by TaqMan RT-PCR, normalized to the endogenous GAPDH mRNA. (B) Replicon cells were treated with 500 nM R803 for 14 days, and were then switched to 1% DMSO alone for 12 days; cells were collected at each split as specified, and GAPDH mRNA normalized to HCV RNA titer was measured (black bars). Replicon cells
treated with 25 IU/ml IFN-a for 14 days were switched to either 1% DMSO (striped bars) or 500 nM R803 (shaded bars) for 12 days. Samples were withdrawn and analyzed as above. The detection limit for TaqMan RT-PCR in this study was approx. 50–100 HCV RNA ge/ng GAPDH mRNA. The data shown are from a single representative experiment and were reproduced several times. The experiments were performed in triplicates and the error bars represent the standard deviations.
TABLE 1. The effect of R803 on viability of cultured human cells

<table>
<thead>
<tr>
<th>Cell</th>
<th>Type</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocyte</td>
<td>primary</td>
<td>&gt;10</td>
</tr>
<tr>
<td>T cell&lt;sup&gt;b&lt;/sup&gt;</td>
<td>primary</td>
<td>&gt;10</td>
</tr>
<tr>
<td>HUVEC</td>
<td>primary</td>
<td>5.5</td>
</tr>
<tr>
<td>HMEC</td>
<td>primary</td>
<td>2.0</td>
</tr>
<tr>
<td>Huh-7</td>
<td>hepatoma</td>
<td>2.0</td>
</tr>
<tr>
<td>Replicon 9-13</td>
<td>hepatoma</td>
<td>4.0</td>
</tr>
<tr>
<td>A549</td>
<td>lung cancer</td>
<td>7.0</td>
</tr>
<tr>
<td>BJAB</td>
<td>B lymphoma</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Jurkat E6.1</td>
<td>T leukemia</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

<sup>a</sup>HUVEC, human umbilical vein endothelial cell; HMEC, human mammary epithelial cell; BJAB, Burkitt-like lymphoma cell line; Jurkat E6.1, human T cell leukemia Jurkat cell line E6-1. Cells were incubated with R803 for 48h (or 72h with primary T cells) and cell viability was measured by cell counts (for primary T cells) or using CellTiter 96® Reagent (for all the rest of the cell types).

<sup>b</sup>Primary human T cells were tested in stationary phase (without stimulation) or proliferative phase (stimulated with anti-CD3/anti-CD28 coated on culture plates in the presence of 40U/ml IL-2). Replicate assays were not performed, so the SD is not available.
TABLE 2. Antiviral specificity of R803

<table>
<thead>
<tr>
<th>Viral System&lt;sup&gt;b&lt;/sup&gt;</th>
<th>R803&lt;sup&gt;EC&lt;sub&gt;50&lt;/sub&gt;&lt;/sup&gt; Response (µM)</th>
<th>R803&lt;sup&gt;CC&lt;sub&gt;50&lt;/sub&gt;&lt;/sup&gt; (cell line)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV replicon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gt 1a</td>
<td>0.037</td>
<td>3.0 (Huh-7)</td>
</tr>
<tr>
<td>gt 1b</td>
<td>0.037</td>
<td>3.0 (Huh-7)</td>
</tr>
<tr>
<td>gt 2a</td>
<td>1.0</td>
<td>3.0 (Huh-7)</td>
</tr>
<tr>
<td>YFV</td>
<td>≥ 5.1</td>
<td>5.1 (Vero)</td>
</tr>
<tr>
<td>BVDV</td>
<td>≥ 2.1</td>
<td>2.1 (MDBK)</td>
</tr>
<tr>
<td>PV-Luc replicon</td>
<td>≥ 10</td>
<td>≥10 (Huh-7)</td>
</tr>
</tbody>
</table>

<sup>a</sup>gt, genotype; YFV, yellow fever virus; BVDV, bovine viral diarrhea virus; PV-Luc replicon, a poliovirus (PV) replicon in which the PV capsid region is replaced with a luciferase coding sequence; MDBK, Madin-Darby bovine kidney epithelial cell; Vero, African green monkey kidney cell.

<sup>b</sup>HCV replicon cells were incubated with R803 for 48h and assessed by Western blot. YFV and BVDV infection assay was based on the viral-induced cytopathic effect (CPE), and R803 was tested for its ability to block CPE. PV-Luc replicon RNA was transfected into Huh-7 cells, and R803 was added after the transfection. Luciferase activity, an indicator of PV replication, was measured 18h post R803 addition. At 18h time point, R803 did not cause significant cytotoxicity in Huh-7 cells. Replicate assays were not performed, so the SD is not available.
TABLE 3. Summary of individual target assays for R803$^a$

<table>
<thead>
<tr>
<th>HCV Target</th>
<th>Assay Type</th>
<th>Control Compound</th>
<th>R803 Activity (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRES</td>
<td>IVT; IRES cell line; TTI</td>
<td>None</td>
<td>≥ 10</td>
</tr>
<tr>
<td>NS2-3$^{pro}$</td>
<td>IVT; TTI; PLNCP54 replicon</td>
<td>Chloro-methylketones$^b$</td>
<td>≥ 40</td>
</tr>
<tr>
<td>NS3$^{helicase}$</td>
<td>Biochemical</td>
<td>ADP*BeF$_3$</td>
<td>≥ 10</td>
</tr>
<tr>
<td>NS3-4a$^{pro}$</td>
<td>Biochemical; TTI</td>
<td>BILN 2061$^c$</td>
<td>≥ 10</td>
</tr>
<tr>
<td>NS5a</td>
<td>Membrane association</td>
<td>None</td>
<td>≥ 10</td>
</tr>
<tr>
<td>NS5b$^{RdRp}$</td>
<td>Biochemical</td>
<td>Compound 54$^d$</td>
<td>≥ 10</td>
</tr>
</tbody>
</table>

$^a$Serially diluted R803 samples were tested in the assays at concentrations up to 40 µM. IVT, *in vitro* translation; TTI, transient transfection and VV-T7 infection assay.

$^b$Chloromethyl 2-hydroxy-5-methylcyclohexyl ketone was the most potent inhibitor in the IVT-based NS2-3 cysteine protease assay with an IC$_{50}$ at ~90 µM.

$^c$An active site inhibitor reported by Lamarre et al. (28).

$^d$An indole-N-acetamide derivative reported by Harper et al. (20).

The experiment has been repeated for several times. Standard deviations are not available since the activities all exceed the highest tested compound concentration.
Fig. 2

A

B

C

EC50 = 84 nM

Relative Luciferase Activity

Relative Luciferase Activity

HCV RNA ge/GAPDH mRNA Ratio

R706 Concentration (µM)
Fig. 3

A. Chemical structure of R803.

B. Graph showing HCV RNA levels (x10^6) in serum per ng GAPDH mRNA vs. R803 concentration (µM).

C. Western blot analysis showing NS3 and β-tubulin expression levels at different R803 concentrations (µM).

D. Western blot analysis showing NS3 and β-tubulin expression levels at different serum concentrations (%).
A. HCV genotype 1a (Rep1aNeo)

B. HCV genotype 1b (Con1)

C. HCV genotype 2a (JFH-1)
Fig. 5

A

R803 (nM)

0.0 12.3 33.3 111.1 333.3 1000.0

NS5b

B

BILN 2061 (nM)

0.0 1.2 3.7 11.1 33.3 100.1

NS5b-containing precursors

NS5b