Resistance Analysis and Characterization of a Thiazole Analogue, BP008, as a Potent Hepatitis C Virus NS5A Inhibitor

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Hepatitis C virus (HCV) is a global health problem, affecting approximately 3% of the world's population. The standard treatment for HCV infection is often poorly tolerated and ineffective. Therefore, the development of novel or more effective treatment strategies to treat chronic HCV infection is urgently needed. In this report, BP008, a potent small-molecule inhibitor of HCV replication, was developed from a class of compounds with thiazol core structures by means of utilizing a cell-based HCV replicon system. The compound reduced the reporter expression of the HCV1b replicon with a 50% effective concentration (EC₅₀) and selective index value of 4.1 ± 0.7 nM and $>12,195$, respectively. Sequencing analyses of several individual clones derived from BP008-resistant RNAs purified from cells harboring HCV1b replicon revealed that amino acid substitutions mainly within the N-terminal region (domain I) of NS5A were associated with decreased inhibitor susceptibility. Q24L, P58S, and Y93H are the key substitutions for resistance selection; F149L and V153M play the compensatory role in the replication and drug resistance processes. Moreover, BP008 displayed synergistic effects with alpha interferon (IFN- α), NS3 protease inhibitor, and NS5B polymerase inhibitor, as well as good oral bioavailability in SD rats and favorable exposure in rat liver. In summary, our results pointed to an effective small-molecule inhibitor, BP008, that potentially targets HCV NS5A. BP008 can be considered a part of a more effective therapeutic strategy for HCV in the future.

Hepatitis C virus (HCV) is the leading cause of hepatitis C and liver disease, which affect nearly 160 million individuals worldwide (28). HCV can establish a persistent chronic infection that often increases the risk of developing liver fibrosis, steatosis, cirrhosis, and in some cases, hepatocellular carcinoma (19). The current standard of care for the treatment of HCV infection relies on the combination of alpha interferon (IFN- α) and the nucleoside analog ribavirin, which is poorly tolerated and may eventually lead to a suboptimal response rate. Furthermore, the treatment is associated with a high incidence of adverse effects, including flu-like symptoms, depression, and anemia (14, 41). Therefore, the development of specific antiviral therapies for hepatitis C with improved efficacy and better tolerance is a major public health objective and is urgently important.

HCV is a positive-strand RNA virus that has been classified as the sole member of the genus *Hepacivirus* within the *Flaviviridae* family. The HCV genome consists of a single strand of RNA of about 9,600 nucleosides with a large open reading frame encoding a polypeptide precursor of about 3,010 amino acids. The polyprotein is cleaved cotranslationally and posttranslationally by both cellular and viral proteases to yield structural proteins C, E1, E2, and p7, which are required for viral assembly, along with non-structural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B, which are involved in membrane-associated RNA replication, viral assembly, and release (1, 21, 22, 40). HCV NS3 is a bifunctional protein with an amino-terminal domain that has serine protease activity and a carboxy-terminal domain that shows helicase/NT-Pase activity (2, 24, 27). The small hydrophobic protein NS4A serves as a cofactor for NS3 protease and helicase activities. The association of NS4A with the NS3 protease domain is essential for enzymatic function, stability, and anchoring to the cellular membranes (46, 48). NS4B is an integral membrane protein that plays a direct role in the remodeling of host cell membranes for the

formation of the membranous web, which presumably is responsible for HCV replication complex assembly (10, 12). NS5A is a large hydrophobic and membrane-associated phosphoprotein, containing three domains and an amphipathic α -helix at its amino terminus that promotes membrane association (13, 18, 20, 54, 56). The amino terminus of NS5A (domain I, residues 1 to 213) contains a zinc and RNA binding motif (38, 56). Mutations disrupting either the zinc binding or membrane anchor of NS5A result in the complete inhibition of RNA replication (11, 47, 55). NS5B, the C-terminal cleavage product of the polyprotein, functions as the viral RNA-dependent RNA polymerase (23, 44). Previous studies have indicated that the NS3-NS5B proteins formed the HCV replicase complex and that all members are important for HCV replication (3, 36, 37).

To date, there is no vaccine to prevent or cure HCV infection. Therefore, the development of new direct-acting antiviral agents (DAA) to treat HCV infection is a major focus of drug discovery efforts. In the past, viral enzymes were the most advanced targets for drug development. NS3-4A protease inhibitors and NS5B polymerase inhibitors have garnered the most attention as drug targets, with several candidates recently showing great promise in

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clinical trials (26, 31, 45). However, the promising development of nonenzymatic inhibitors of HCV NS5A showed that non-enzyme HCV viral proteins also can be good DAA targets (17). Recently, two drugs, telaprevir and boceprevir, targeting HCV NS3-4A protease, were approved to treat HCV-infected patients in conjunction with IFN- α and ribavirin (25, 33). Antiviral drugs often have been shown to cause the emergence of drug-resistant mutations that lead to patients not responding to drug treatment. Therefore, exploring new mechanisms for anti-HCV drug design is highly desirable.

In this report, we used a cell-based replicon assay to develop a potent compound that inhibits HCV1b replication. Overall, our data suggested that this small-molecule inhibitor targets the domain I region of the HCV1b NS5A protein and confirmed that the function of NS5A in replication can be regulated by small molecules. In addition, the combination study and pharmacokinetic analysis of the inhibitor supported the use of this inhibitor as part of a new therapeutic strategy in the treatment of HCV.

MATERIALS AND METHODS

***Escherichia coli* and yeast strains.** Frozen, competent *E. coli* strain C41, a derivative of BL21(DE3) (43), was purchased from OverExpress Inc. Standard yeast medium and transformation methods were used (6). *Saccharomyces cerevisiae* YPH857 was purchased from the ATCC. Genotype YPH857 is *MAT α ade2-101 lys2-801 ura3-52 trp1- Δ 63 HIS5 CAN1 his3- Δ 200 leu2- Δ 1 cyh2*. Competent yeast cells were prepared using the lithium acetate procedure (6).

Construction of HCV1b and HCV2a reporter replicons and HCV2a infectious cDNA clones. The cloning of the constructs was based entirely on homologous recombination in yeast cells (6), and plasmid amplification was in C41 cells; recombinant DNA *in vitro* ligation was not used. Huh7.5-HCV1b replicon (a HCV genotype 1b [con1-based] subgenomic replicon clone with cell culture-adaptive mutation S2204I in NS5A) was obtained from Apath LLC (Brooklyn, NY) and modified by inserting the humanized *Renilla* luciferase (hRluc) gene accompanied with the gene encoding the 2A protease of foot-and-mouth disease virus (FMDV 2A) flanked at the 5' end of the neomycin resistance gene (hRluc-2A-neo), subcloned into the pRS423 shuttle vector (50), and named pRS-Luc-HCV1bRep replicon plasmid. The HCV genotype 2a replicon construct, pSGR-Luc-JFH1 (53), was kindly provided by Paul Targett-Adams and modified by inserting a yeast replication origin 2 μ m and yeast selection marker (*His*) derived from pRS423 and replacing the firefly luciferase gene with the hRluc-2a-neo gene as a reporter and selection marker in cell culture, and it was named the pRlu-JFH1Rep replicon plasmid. A 5'-flanking T7 RNA polymerase promoter allowed for the production of runoff RNA transcripts with authentic 5'- and 3'-terminal sequences. A full-length HCV2a infectious cDNA clone, pJFH-1 (58), that replicates and produces virus particles that are infectious in cell culture (HCVcc) was kindly provided by Takaji Wakita. A full-length HCV2a infectious cDNA clone harboring the reporter luciferase gene, pJFH-1-hRluc, was constructed by inserting the hRluc-2A DNA fragment between the HCV 5' N-terminal region and core gene. To construct pJFH-1-hRluc, a DNA fragment comprised of FMDV-2A and JFH-1 structural genes, C-E1-E2-p7-NS2, was synthesized by PCR using pJFH1 as a template. pJFH-1-hRluc plasmid was created by the digestion of pRlu-JFH1Rep plasmid with ClaI and NruI restriction enzymes to remove the FMDV-2A-EMCV-IRES DNA fragment, which was replaced with the DNA fragment FMDV-2A-C-E1-E2-p7-NS2 by homologous recombination in yeast cells. All constructed plasmids were amplified in the C41 bacteria strain and verified by DNA sequencing.

Cell culture and HCV inhibitors. Huh7.5 cells and their derivative HCV replicon cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco/BRL) that was supplemented with 100 U/ml penicillin-streptomycin (Gibco/BRL), 0.1 mM nonessential amino acid

FIG 1 Chemical structure of BP008.

(NEAA; Gibco/BRL), and 10% heat inactive fetal bovine serum (FBS) at 37°C in an atmosphere of 5% CO₂. The HCV replicon cell lines were isolated from colonies as described by Lohman et al. (37). The culture medium of replicon cells was additionally supplemented with 0.25 to 0.5 mg/ml G418 unless specified otherwise. Compound BP008 was synthesized at the Institute of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Taiwan. The structure of the NS5A inhibitor, BP008, is shown in Fig. 1. 2'-C-methyladenosine (2'CMA) (42) and VX-950 (35) were purchased from Carbosynth (Berkshire, United Kingdom) and Acme Biosciences (Belmont, CA), respectively. The compounds were stored at -20°C as 10 to 500 mM dimethyl sulfoxide (DMSO) stock solutions until being used in the assay. IFN- α was purchased from Calbiochem (La Jolla, CA) and stored at -80°C.

The inhibitory assay for HCV replicon. Totals of 1×10^4 (high-throughput screening assay) or 1×10^5 (regular assay) cells/well were seeded into a 96- or 12-well plate, respectively, and incubated for 4 h. Medium then was aspirated and replaced with 0.1 (96-well plate) or 1 ml (12-well plate) of complete medium containing a single compound or combinations at the serial concentration(s). Plates with compounds were incubated for 72 h and then assayed for luciferase expression (Promega). The 50% effective concentration (EC₅₀) of each compound was determined independently and used to determine the range of concentrations used for the combination experiments. All data are presented as the means $\pm$ standard deviations (SD) from more than three independent experiments. The selectivity index (SI) was calculated as the ratio of the CC₅₀ (drug concentration required to reduce cell growth by 50%) to the EC₅₀.

High-throughput screening. The high-throughput screening was done at the Division of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Taiwan. The compound libraries used in this primary screening were purchased from Chemical Diversity Laboratory (San Diego, CA) and had >95% purity. Compounds from a compound library with diverse structures were provided as DMSO stock solutions at 10 mM. Luciferase assay was applied to measure the luciferase activity of stable HCV1b reporter replicon cells by incubating with various compounds from compound libraries at a concentration of 10 μ M.

Cytotoxicity assay. The sensitivity of cell lines to inhibitors was examined using a [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, Huh7.5 cells were plated at a density of 1×10^5 cells per well in 12-well plates containing 1 ml of culture medium for 4 h. Serially diluted compounds or DMSO (positive control) was added, and the plates were incubated for an additional 72 h. The MTT reagent then was added into each well, and the plates were incubated for 3 h at 37°C in a humidified 5% CO₂ atmosphere before reading at a wavelength of 563 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader. All data are presented as the means $\pm$ SD from four independent experiments.

Inhibition of HCV particle formation by small molecules. To quantify the inhibitory effect of BP008 on HCV particle formation, the HCV replication of BP008-treated and untreated cells was quantitated by luciferase activity assay, as described previously (58, 60). *In vitro*-transcribed RNA derived from full-length HCV2a infectious cDNA clone with the reporter luciferase gene pJFH-1-hRluc was delivered to Huh7.5 cells by

electroporation. A total of 1×10^5 cells per well were seeded into 12-well plates and incubated for 4 h. Medium then was aspirated and replaced with 1 ml of complete medium containing BP008 at the serial concentration. Plates with compounds were incubated for 72 h, and then the medium was used to infect Huh7.5 cells. Huh7.5 cells were seeded in 12-well plates (1×10^5 cells/well) in DMEM with 10% FBS 24 h before infection. HCVcc-containing supernatant was added to Huh7.5 cells. After 72 h of incubation at 37°C, total cell lysate was assayed for luciferase expression (Promega).

Isolation of resistant replicons. The selection of resistant replicon cells was performed by growing HCV genotype 1b replicon cells in a medium containing a 5 μ M concentration of BP008. Medium containing the compound was added to monolayers of HCV1b-neo replicon cells at ~25% confluence in the presence of 0.2 to 0.4 mg/ml G418. Replicon cells maintained in the presence of dimethyl sulfoxide (DMSO) were used as a control. After 40 days, total RNA was isolated from both control replicon cells and homogeneous cell lines containing compound using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The RNAs were amplified by reverse transcription-PCR (RT-PCR). The PCR products of NS3-NS5B were gel purified and subcloned to the vector pRS-Luc-HCV1bRep to replace parental NS3-NS5B by homologous recombination in yeast. Thirty-six colonies of plasmids were purified from yeast cells and reamplified in the *E. coli* strain C41 for DNA sequencing.

Construction of molecular clones containing specific mutations. To create point mutations derived from resistant clones, the amino acid substitutions Q24L, P58S, P58T, P58L, Y93H, F149L, and V153M were introduced into the pRlu-HCV1b plasmid either individually or in combination with primers, as shown in the table in the supplemental material. The PCR products were gel purified and joined by overlapping PCR to form the fragments containing the following single, double, or triple mutations for homologous recombination with linearized pRlu-HCV1b plasmids (digested with HpaI): P58S+Y93H, P58T+Y93H, P58L+Y93H, Y93H+F149L, Y93H+V153M, Q24L+Y93H, Q24L+Y93H+F149L, and Q24L+Y93H+V153M. The mutant replicon plasmids were purified from yeast cells and then reamplified and maintained in *E. coli* strain C41. All constructs were sequenced to confirm the presence of the desired mutation and to ensure that there were no additional changes.

RNA transcription and transient replicon assay. RNA transcripts were synthesized *in vitro* by using ScaI-digested DNAs and the T7 MegaScript transcription kit (Ambion) according to the manufacturer's directions. The transient replicon assay was performed for the quantification of the compound-mediated inhibition of viral translation (9). The RNA transcripts were transfected into Huh7.5 cells by electroporation as described previously (4). Various concentrations of BP008 or the control medium were added to each well and assayed to determine the luciferase activities at 4 and 72 h posttransfection. The cells were lysed for luminometry, and the luciferase assay was performed by mixing 5 μ l of lysate with 25 μ l of the *Renilla* luciferase assay reagent (Promega). For the quantification of the compound-mediated inhibition, the relative luciferase activity derived from the mock-treated cells was set to 100% (61).

Serum shift assay. In the serum shift assay, the inhibitory activity of BP008 was determined using replicon 1b in the presence of 10, 20, 30, 40, and 50% extracellular fetal bovine serum or 10 and 40% extracellular normal human serum (NHS). In the absence or presence of serially diluted BP008, the percentage of inhibition was determined by a 50% reduction in *Renilla* luciferase activity (EC_{50}) or a reduction of 90% (EC_{90}) compared to levels for the control after 72 h of incubation.

Inhibitor combination study. Luciferase reporter-linked HCV replication assays were used to evaluate the potential use of BP008 in combination therapy with IFN- α , an NS3 protease inhibitor (VX-950), and a nucleoside inhibitor of NS5B (2'CMA), respectively. For the combination index model, the cells were incubated for 72 h with serial dilutions of either IFN- α , VX-950, or 2'CMA with BP008 and a fixed-ratio combination of inhibitors below their cytotoxic concentrations. CalcuSyn (BioSoft) was used to analyze the data obtained from the 72-h luciferase-based

TABLE 1 Potency of BP008 on HCV replicon cell lines and virus particle formation

HCV genotype	EC_{50}^a (nM)	EC_{90}^a (nM)	CC_{50}^a (μ M)	Selective index
1b, Con1	4.1 ± 0.7	56.4 ± 14.8	>50	>12,195
2a, JFH1	807.6 ± 274.7	$1,174.6 \pm 404.6$	>50	>62
2a, infectious HCV, JFH1	175.6 ± 21.0	573.8 ± 113.6	>50	>284

^a Means $\pm$ standard deviations determined from the parental cell line ($n \geq 3$).

HCV replicon assay and quantify differences between observed effects and predicted ones. Compound interactions and concentration ratios were quantified by the approach described by Chou and Talalay (7). The degrees of synergistic and additive effects were evaluated by the median-effect principle with the combination index (CI) calculation (7). CIs at the EC_{50} , EC_{70} , and EC_{90} levels also were determined (51). In total, six combinations were evaluated with three to eight experiment replicates per condition. By convention, a CI of 0.9 was considered synergistic, a CI of >0.9 or <1.1 was considered additive, and a CI of >1.1 was deemed antagonistic.

Pharmacokinetics. A solution of BP008 (1 mg/ml) was prepared by dissolving the appropriate amount of compound in a solution of polyethylene glycol 400 (PEG 400) in water (20/80, vol/vol). Male Sprague-Dawley (SD) rats, weighting 250 to 350 g each and at an age of 8 to 10 weeks, were obtained from BioLASCO, Ilan, Taiwan. Single 1 mg/kg of body weight intravenous and oral doses of BP008 were administered separately to a group of three male rats during each dosing regimen. At 0 (prior to dosing), 2, 5, 15, and 30 min and 1, 2, 4, 6, 8 and 24 h after intravenous dosing and at 0, 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h after oral dosing, a blood sample (150 μ l) was collected from each animal via the jugular vein cannula and stored in ice. Plasma was separated from the blood by centrifugation (7,500 rpm for 15 min) and stored in a freezer (−30°C). All samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Forty microliters of rat plasma was mixed with 75 μ l of an acetonitrile solution containing 250 ng/ml of an internal standard, and then the mixture was vortexed for 30 s and centrifuged at $15,000 \times g$ for 20 min at room temperature. The supernatant was transferred to a clean tube, and then a volume of 15 μ l of the supernatant was injected onto the LC-MS/MS. The animals used in the pharmacokinetic study were housed and the experiments were carried out at an International Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facility at the National Health Research Institutes, Zhunan, Taiwan. The Institutional Animal Care and Use Committees of the National Health Research Institutes approved uses of animals in this study (approval number NHRI-IACUC-097047-A).

Liver/plasma ratio determination. BP008 was administered orally to male Sprague-Dawley rats at doses of 2, 10, and 50 mg/kg. Twenty-four hours after drug administration, the plasma was obtained and the livers were collected. The plasma and liver samples were frozen and stored at −80°C until analysis. For the plasma samples, the sample treatment was the same as that for the pharmacokinetic study samples described above. For the liver samples, the removed liver tissues were weighed and homogenized with two times the volumes (vol/wt) of acetonitrile from an ice bath and vortexed for 30 s. After centrifugation at $14,000 \times g$ for 20 min at room temperature, the supernatant was transferred to a clean tube for analysis.

LC-MS/MS analysis. The chromatographic system consisted of an Agilent 1200 series LC system and an Agilent ZORBAX Eclipse XDB-C8 column (5 μ m; 3.0 by 150 mm) interfaced to an MDS Sciex API4000 Q TRAP tandem mass spectrometer in the positive scanning mode at 600°C. Data were acquired via multiple reaction monitoring (MRM). A gradient high-performance liquid chromatography (HPLC) method was employed for separation. Mobile phase A consisted of water containing 0.1% formic acid, and mobile phase B consisted of acetonitrile. The gradient

FIG 2 Inhibition of HCV1b RNA replication by BP008. BP008 inhibits the HCV replication stages (24 to 72 h) rather than the viral translation stages (4 to 8 h). The HCV1b replicon was electroporated into Huh7.5 cells, which then were maintained in the absence (DMSO) or presence of 500 nM BP008. *Renilla* luciferase (Rluc) activity was monitored at the indicated time points posttransfection. The numbers above the BP008-treated time points represent the percentages of luciferase signals relative to the DMSO-treated controls (100%), and the data are presented as means $\pm$ standard errors of the means from four independent experiments. ***, $P < 0.001$ compared to the DMSO group.

profiles were (in min/%B [% of mobile phase]) 0.0 to 1.1/5, 1.2 to 3.9/95, and 4.0 to 5.0/5. The flow rate was 0.5 ml/min.

RESULTS

Identification of HCV inhibitors. The availability of a cell-based high-throughput HCV replicon provides an ideal system to study viral replication and to screen for new antiviral compounds. In this study, we utilized a Con-1 genotype 1b replicon (with reporter luciferase) replicating in Huh7.5 liver cells for high-throughput screening. We started with a compound library containing 35,000 diverse compounds, from which 25 candidates were discovered to effectively suppress HCV1b replicon activity with calculated EC_{50} s of less than 1.5 μ M and lower intrinsic cytotoxicity. Interestingly, we discovered that 6 of the 25 lead compounds have a common thiazole core structure. After further systematic optimization (data not shown), BP008 emerged as a specific inhibitor of HCV

replication and displayed improved potency against genotype 1b and 2a replicons, as well as against 2a infectious virus, all with calculated CC_{50} values of more than 50 μ M and EC_{50} s of 4.1, 807.6, and 175.6 nM, respectively (Table 1). BP008 displays a selective index (CC_{50}/EC_{50}) of more than 12,195 for the HCV genotype 1b replicon and more than 284 for genotype 2a infectious virus *in vitro*. Moreover, the susceptibility of genotype 1b to BP008 was 196-fold greater than that of genotype 2a replicon cells (Table 1).

BP008 reduces the level of viral RNAs but not translational efficiency of viral RNAs in cells transfected with HCV1b replicon RNAs. To distinguish the inhibition of viral translation from the inhibition of RNA synthesis, we monitored the reduction rate of reporter gene expression levels as an indicator of the inhibition of BP008. The HCV1b reporter replicon construct, pRS-Luc-HCV1bRep, was transcribed *in vitro* and transfected into Huh7.5 cells. Luciferase activity was monitored several times during a period of 72 h posttransfection (Fig. 2). The luciferase activity reached a peak level at around 72 h posttransfection, and the level of luciferase activity was sustained until 72 h posttransfection in the absence of BP008 (data not shown). The luciferase activity peaked within the first 8 h posttransfection and also after 72 h posttransfection, representing viral translation and RNA replication, respectively (49). We measured the luciferase activity at 4, 8, 24, 48, and 72 h posttransfection, where BP008 had minimal effect on Rluc signals at 4 and 8 h posttransfection, but the signals were significantly reduced by 26, 93, and 99% at 24, 48, and 72 h posttransfection, respectively (Fig. 2). In summary, our data demonstrated a significant suppression of viral RNA synthesis by BP008.

Isolation and characterization of genotype 1b replicons resistant to BP008. The potential for viral resistance is a key point in managing HCV infection through DAA strategies (29, 57). Therefore, we further studied and characterized the resistance profile of BP008. The cell clones resistant to BP008 were obtained by culturing HCV genotype 1b replicon cells in the presence of G418 and increasing concentrations of BP008, ranging from 50- to 500-fold

amino acid	BP008 resistant individual clone																																				
pB77	p20	p47	p26	p36	p42	p44	p51	p22	p28	p53	p13	p4	p35	p7	p9	p5	p19	p10	p12	p16	p29	p32	p17	p6	p27	p39	p2	p11	p40	p50	p45	p31	p37	p54	p38	p46	
21 T													I	I	I																						
24 Q				H		R																			L	L	L	L	L	L	L	L	H				L
28 L																	P		M																		
31 L																																		V			
37 F					S																																
44 K								R																			E										
58 P	L	L	S	S	S	S	S	S	S	S	S	L	T	T	T																						
68 K							R												R																	R	
85 H																																				Y	
93 Y	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	
97 P															S	S																					
132 G																							D														
133 M	V																									T											
149 F																													L	L	L	L	L	L	L	L	
153 V																M	M	M	M	M	M	M	M	M	M	M	M										

FIG 3 Amino acid changes in genotype 1b HCV NS5A derived from cells resistant to 5 μ M BP008.

TABLE 2 Effects of genotype 1b HCV NS5A amino acid substitutions on BP008 potency

Amino acid substitution(s)	Frequency (%)	EC ₅₀ ^a (nM)	Fold resistance	EC ₉₀ ^a (nM)	Fold resistance	Replication level ^a	Fold replication
Parental		1.0 ± 0.3	1	4.0 ± 1.0	1	100	1
Q24L	25	8.9 ± 1.5	9	45.2 ± 5.4	11	78.5 ± 17.9	0.79
P58S	25	8.9 ± 4.1	9	66.4 ± 8.3	17	57.1 ± 10.5	0.57
P58T	8	5.4 ± 3.6	5	39.7 ± 12.3	10	37.2 ± 3.5	0.37
P58L	8	8.7 ± 4.1	9	49.9 ± 15.6	13	21.4 ± 6.5	0.21
Y93H	100	177.2 ± 42.1	174	833.9 ± 33.6	211	6.5 ± 2.2	0.07
F149L	22	1.0 ± 0.3	1	5.2 ± 0.2	1	90.7 ± 21	0.91
V153M	31	1.3 ± 0.5	1	8.1 ± 1.0	2	96.1 ± 27.7	0.96
P58S+Y93H	25	1,420.9 ± 660.7	1,393	>5,000	1,250	2.5 ± 1.2	0.03
P58T+Y93H	8	986.3 ± 176.0	967	>5,000	1,250	1.7 ± 0.8	0.02
P58L+Y93H	8	967.5 ± 213.9	949	>5,000	1,250	1.3 ± 0.9	0.01
Y93H+F149L	22	186.9 ± 19.1	183	852.0 ± 87.6	216	8.6 ± 5.5	0.09
Y93H+V153M	31	305.2 ± 42.1	299	1,206.5 ± 47.4	305	24.0 ± 4.1	0.24
Q24L+Y93H	25	330.0 ± 42.8	324	3,652 ± 462.4	925	33.4 ± 2.4	0.33
Q24L+Y93H+F149L	11	1,862.3 ± 427.9	1,826	4,405.7 ± 1,570.1	1,115	13.1 ± 3.9	0.13
Q24L+Y93H+V153M	8	2,135.4 ± 599.8	2,094	6,697.0 ± 1,510.4	1,695	21.1 ± 4.0	0.21
HCVrep2a		1,033.8 ± 36.5	1,014	1,299.3 ± 149.6	329	4,218.7 ± 809.8	42.19

^a Means ± standard deviations determined from transient assays ($n \geq 3$).

its EC₅₀. Selection experiments revealed that the replication of the cognate replicons was resistant to inhibition by BP008, and that they displayed a loss of potency compared to that of the parental cell lines. Compared to the parental cells, the selected cells (showing BP008 resistance [BP008R]) were determined to be greater than 1,220-fold more resistant, going from an EC₅₀ of 4.1 nM for parental cells to an EC₅₀ of more than 5,000 nM for BP008R cells.

Direct DNA sequencing of individual clones containing NS3-NS5B from genotype 1b-resistant cells revealed predominant multiple changes in the N terminus of NS5A (summarized in Fig. 3 and Table 2). Q24L (20%), P58S (24%), Y93H (100%), F149L (22%), and V153M (24%) were the predominant mutations observed in resistant clone selections (Fig. 3 and Table 2). In contrast, P58T and P58L were observed less frequently. In total, 100% of the cDNA clones isolated from cells treated with BP008 contained a mutation at Y93 (Fig. 3 and Table 2). Amino acid substitutions at Q24, P58, Y93, F149, and V153 were not detected in NS5A cDNA clones isolated from DMSO-treated control cells (data not shown). Substitutions at Q24, P58, and Y93 of NS5A are common mutations in HCV drug resistance studies (16, 17, 32), signifying that these residues play an important role in the drug-resistant functions of HCV.

Validation of mutations responsible for the resistant phenotype. To determine the effect of the mutations, the resistant phenotypes were further validated by engineering one or more mutations into an HCV genotype 1b replicon that contained a luciferase reporter gene, which can be used to monitor replication in a transient reporter assay. The replication of the parental and mutant clone replicons was monitored over time in the presence or absence of BP008. The time of maximum replication efficiency for both the parental and mutant RNAs was determined to be 72 h posttransfection (data not shown). As shown in Table 2, the replication efficiencies of the Q24L, P58S, P58T, P58L, and Y93H replicons were 78.5% ± 17.9%, 57.1% ± 10.5%, 37.2% ± 3.5%, 21.4% ± 6.5%, and 6.5% ± 2.2% of the level of the parental replicon at 72 h, respectively. This result indicates that these resistant mutants had reduced fitness, with the amino acid substitution Y93H revealing the lowest replication capacity. In the previous

study by Gao et al., substitutions at residue 93 also had a great effect on replication fitness (16). The replication efficiencies of F149L and V153M were 90.7% ± 21% and 96.1% ± 27.7% of the level of the parental replicon, respectively, indicating that F149L and V153M mutants did not affect fitness. Our data revealed that 100% of the BP008-resistant clones contained a combination of two or three amino acid substitutions at residue 24, 58, 93, 149, or 153 (Fig. 3). The complexity of the resistance pattern was verified by the analysis of individual cDNA clones (Fig. 3). While many different combinations were observed, the combination of P58S+Y93H or Q24L+Y93H was the most prevalent pattern, being seen in 25% of the cases (Fig. 3). Furthermore, to determine the phenotypes of variants with linked mutations, replicons with the representative combinations P58S+Y93H, P58T+Y93H, P58L+Y93H, Q24L+Y93H, Y93H+F149L, Y93H+V153M, Q24L+Y93H+F149L, and Q24L+Y93H+V153M were tested in transient replication assays. These variants exhibited an impaired replication capacity of 1.3 to 33.4% relative to that of the parental clone (Table 2).

Individual amino acid substitutions Q24L, P58S/T/L, and Y93H exhibited different levels of resistance to BP008 with increasing EC₅₀s ranging from 5- to 174-fold above the parental

TABLE 3 Effects of serum on antiviral activity of BP008 to HCV1b replicon cell lines

Serum (%)	HCV1b replicon results			
	EC ₅₀ ^a (nM)	Shift fold	EC ₉₀ ^a (nM)	Shift fold
FBS				
10	4.1 ± 0.7	1.0	56.4 ± 14.8	1.0
20	11.5 ± 2.0	2.8	96.8 ± 18.8	1.7
30	21.1 ± 1.2	5.1	153.8 ± 18.2	2.7
40	32.9 ± 6.6	8.0	204.0 ± 44.2	3.6
50	47.7 ± 7.2	11.6	247.8 ± 77.8	4.4
NHS				
10	38.0 ± 7.8	1.0	177.7 ± 32.9	1.0
40	125.3 ± 37.1	3.3	522.1 ± 15.6	2.9

^a Mean ± standard deviation determined from the HCV1b replicon cell line ($n \geq 3$).

TABLE 4 Potencies of BP008, IFN- α , VX-950, and 2'CMA on HCV1b replicon cell lines

Compound	EC ₅₀ ^a (nM)	EC ₉₀ ^a (nM)	CC ₅₀ (μ M)
BP008 (nM)	7.0 $\pm$ 1.1	51.0 $\pm$ 8.9	>50,000
IFN- α (IU/ml)	55.2 $\pm$ 51.0	204.8 $\pm$ 106.3	>2,000
VX-950 (nM)	280.2 $\pm$ 89.8	631.2 $\pm$ 55.3	>5,000
2'CMA (nM)	219.7 $\pm$ 32.1	644.9 $\pm$ 87.3	>25,000

^a Means $\pm$ standard deviations determined from the HCV1b replicon cells ($n \geq 5$).

control (Table 2). When P58S was combined with Y93H on the same replicon, the effects on the inhibitor increased dramatically to give a 1,393-fold boost in resistance. Likewise, Q24L, P58T, or P58L alone produced a merely 5- to 9-fold increase in resistance, but much greater resistance of 324-, 967-, and 949-fold was produced when combined with Y93H. On the other hand, F149L and V153M, identified in a single NS5A cDNA clone, did not affect BP008 potency as a single mutation (Fig. 3), but the combination of Y93H and F149L or Y93H and V153M produced a 183- or 299-fold resistance, respectively. However, compared to Y93H alone, the substitution of V153 increased the drug resistance to 1.7-fold and improved the replication capacity to 3.4-fold. A substitution of F149 did not reveal this phenomenon.

In addition, compared with the parental replicon, when Q24L was combined with Y93H and either F149L or V153M (Q24L+Y93H+F149L or Q24L+Y93H+V153M) on the same replicon, the effects on the inhibitor increased dramatically by 1,826- and 2,094-fold. These multiple variants displayed very high levels of resistance to BP008 (EC₅₀, 183- to 2,094-fold; EC₉₀, 216- to 1,695-fold) relative to the parental clone (Table 2). This suggests that the primary conformation of NS5A, or of NS5A in the replication complex, is the predominant determinant for inhibitor sensitivity, while residues 24, 58, 93, 149, and 153 are the determinants for resistance selection.

Protein binding activity of BP008. To evaluate the effect of serum protein binding on BP008 activity, the percentage of fetal bovine serum (FBS) and normal human serum (NHS) was added to examine the binding of BP008. Our results revealed that the EC₅₀s in the presence of 10, 20, 30, 40, and 50% FBS were 4.1 $\pm$ 0.7, 11.5 $\pm$ 2.0, 21.1 $\pm$ 1.2, 32.9 $\pm$ 6.6, and 47.7 $\pm$ 7.2 nM, and the EC₉₀s were 56.4 $\pm$ 14.8, 96.8 $\pm$ 18.8, 153.8 $\pm$ 18.2, 204.0 $\pm$ 44.2, and 247.8 $\pm$ 77.8 nM, respectively. The EC₅₀s in the presence of 10 and 40% NHS were 38.0 $\pm$ 7.8 and 125.3 $\pm$ 37.1 nM, respectively, and the EC₉₀s were 177.7 $\pm$ 32.9 and 522.1 $\pm$ 15.6 nM, respectively (Table 3). While the activity of BP008 in higher serum con-

centrations was more favorable than that in regular concentrations of serum (10%), we observed that the EC₅₀s and EC₉₀s were shifted 2.8- to 11.6-fold and 1.7- to 4.4-fold, respectively (Table 3). These results indicate that there is an apparent minor shift in the potency of BP008 in the presence of higher serum concentrations.

In vitro combination studies with IFN- α , VX-950, and 2'CMA. Single-agent or standard-of-care therapies of viral infections often lead to clinical drug resistance. Therefore, more effective and better-tolerated combination therapies are urgently needed. To evaluate the effect of BP008 used in combination with other HCV inhibitors, the inhibitory activity of pairwise combinations of IFN- α , VX-950, or 2'CMA with BP008 were analyzed using a genotype 1b replicon encoding a luciferase reporter gene. In this system, BP008 has a calculated EC₅₀ of 7.0 $\pm$ 1.1 nM, whereas IFN- α , VX-950, and 2'CMA have an EC₅₀s of 55.2 $\pm$ 51 IU/ml, 280.2 $\pm$ 89.8 nM, and 219.7 $\pm$ 32.1 nM, respectively (Table 4).

BP008 was mixed with different ratios of IFN- α , VX-950, or 2'CMA, and serial dilutions of each mixture were determined thereafter. The degree of inhibition for each drug combination was analyzed according to the median-effect principle using the combination index calculation at 50, 75, and 90% (7). In three independent experiments, the combination of BP008 with IFN- α , VX-950, or 2'CMA produced synergistic effects at the 50, 75, and 90% effective doses (Table 5). No cytotoxicity was observed for BP008, IFN- α , VX-950, and 2'CMA at the concentrations used in these experiments.

Pharmacokinetic properties of BP008. To evaluate the pharmacokinetic behavior *in vivo*, blood samples were collected at intervals of up to 24 h after BP008 was administered to rats by oral gavage or intravenous injection. There were no treatment-related effects on body weight or clinical observation, and there were no deaths. LC-MS/MS was used to analyze the plasma concentration of BP008, and the calculated pharmacokinetic parameters of BP008 after dosing at 1 mg/kg intravenously and 2 mg/kg orally are summarized in Table 6 and Fig. 4. At an intravenous bolus of 1 mg/kg, BP008 was cleared at a plasma clearance (CL) of 2.0 $\pm$ 4.1 ml/min/kg; the volume of distribution at steady state (V_{ss}) was 1.5 $\pm$ 0.5 liters/kg, and the half-life ($t_{1/2}$) was 2.1 $\pm$ 0.3 h. BP008 was rapidly absorbed after an oral dose of 2 mg/kg to rats, with a time to maximum concentration of orally administered drug in plasma (T_{max}) of 1.2 $\pm$ 0.8 h. The plasma $t_{1/2}$ was 4.4 $\pm$ 3.8 h with an acceptable percent oral bioavailability (F) (31.6%). The concentrations of BP008 in plasma and liver at different doses re-

TABLE 5 Mutual influences between BP008 and IFN- α or VX-950 or 2'CMA as analyzed by the Loewe additivity model

Combination compound	Ratio of BP008 to other compound	CI value for ^a :			Influence
		ED ₅₀	ED ₇₅	ED ₉₀	
IFN- α	1:1	0.48 $\pm$ 0.18	0.40 $\pm$ 0.10	0.34 $\pm$ 0.05	Synergistic
	2.5:1	0.52 $\pm$ 0.25	0.45 $\pm$ 0.17	0.40 $\pm$ 0.10	Synergistic
	1:2.5	0.26 $\pm$ 0.09	0.23 $\pm$ 0.07	0.20 $\pm$ 0.05	Synergistic
VX-950	1:1	0.44 $\pm$ 0.26	0.37 $\pm$ 0.21	0.32 $\pm$ 0.17	Synergistic
	2.5:1	0.31 $\pm$ 0.12	0.26 $\pm$ 0.07	0.22 $\pm$ 0.04	Synergistic
	1:2.5	0.32 $\pm$ 0.10	0.24 $\pm$ 0.05	0.19 $\pm$ 0.07	Synergistic
2'CMA	1:1	0.27 $\pm$ 0.04	0.23 $\pm$ 0.03	0.19 $\pm$ 0.03	Synergistic
	2.5:1	0.67 $\pm$ 0.15	0.51 $\pm$ 0.08	0.40 $\pm$ 0.03	Synergistic
	1:2.5	0.19 $\pm$ 0.03	0.15 $\pm$ 0.03	0.12 $\pm$ 0.03	Synergistic

^a Means $\pm$ standard deviations determined from the HCV1b replicon cells ($n \geq 3$).

TABLE 6 Pharmacokinetic parameters of BP008 in Sprague-Dawley rats dosed orally at 2 mg/kg and intravenously at 1 mg/kg^a

Route	Dose (mg/kg)	$t_{1/2}$ (h)	CL (ml/min/kg)	V_{ss} (liter/kg)	C_{max} (ng/ml)	T_{max} (h)	F (%)
Intravenous	1	2.1 ± 0.3	20 ± 4.1	1.5 ± 0.5			
Oral	2	4.4 ± 3.8			115 ± 35.4	1.2 ± 0.8	31.6

^a $t_{1/2}$, elimination half-life; CL, plasma clearance; V_{ss} , volume of distribution at steady state; C_{max} , maximum concentration of orally administered drug in plasma; T_{max} , time to maximum concentration of orally administered drug in plasma; F, percent oral bioavailability. The values represent averages from three independent experiments.

vealed that BP008 levels increased in a dose-dependent manner. The liver/plasma ratios at three different doses ranged from 1.8 to 2.6 (Table 7).

DISCUSSION

In the present study, we used a high-throughput screening method to analyze BP008, an HCV replication inhibitor with a selective antiviral spectrum and potent inhibitory activity *in vitro*. We found that BP008 is an effective inhibitor of genotype 1b HCV replicon replication, while it modestly affected genotype 2a replicon (Table 1). The resistance selection and validation of responsible mutations revealed that the HCV1b NS5A residues Q24L, P58S/T/L, and Y93H were identified as key substitutions, while F149L and V153M played a compensatory role in the replication and drug resistance processes (Table 2). In addition, a pharmacokinetic behavior study demonstrated that BP008 has good oral bioavailability and exposure in SD rats, making it a promising development candidate for HCV treatment. More importantly, our data suggested that BP008 is a small-molecule inhibitor of HCV1b NS5A, a protein without any known enzymatic activity (see the discussion in references 16, 17, and 32). This finding of inhibiting hepatitis C virus replication through NS5A could be valuable as a potential therapeutic strategy to treat HCV.

Resistance studies of BP008 revealed that amino acid residues Q24L, P58S/L/T, Y93H, F149L, and V153M within HCV1b NS5A were the predominant mutations associated with BP008 resistance (Fig. 3). In transient replicon assays, replicons containing these

mutations showed substantially reduced susceptibility to BP008 (Table 2). In particular, individual changes at Y93 had the strongest effect on the susceptibility of HCV1b replicons to BP008. Our study revealed that the Y93H mutant replicated only at 6.5% of the level of the parental type, indicating that its fitness is greatly reduced, although it affected BP008 potency significantly. Also, the same Y93H mutation was found to be the key drug resistance mutation within HCV1b replicon variants selected from other NS5A inhibitors, such as BMS-824, BMS-858 (32), and BMS-790052 (16). In addition to the Y93H alteration, although they were identified at lower frequencies in BP008-resistant HCV1b replicon cells, substitutions at residues 24 and 58 also were capable of acting as primary resistance mutations, as they moderately reduced BP008 potency in transient assays. The prevalence of mutations at residues Y93, Q24, and P58 was determined to be 8, 24, and 18%, respectively, from HCV1b patient sequences deposited in the European HCV database (8). The lower prevalence of mutation at residue Y93 within HCV1b patient sequences may be related to the greatly reduced fitness by the mutation at Y93, e.g., Y93H. Interestingly, amino acid residues Y93 and P58, but not Q24, are conserved between genotype 1b and 2a HCV genomes, implying that residue T24 within the HCV2a genome is responsible for the higher EC_{50} of BP008 for genotype 2a (807.6 nM) than 1b (4.1 nM) replicons (Table 1). Unlike Q24 and P58 residues found in primary drug resistance sites of BP008-resistant HCV1b replicon variants, L31V and Q54L are the primary drug resistance mutations derived from BMS-824- and BMS-858-resistant HCV1b replicon cells (32), while L31F/V and P32L were identified as primary resistance mutations in BMS-790052-resistant HCV1b replicon cells (16). These results indicated that BP008 has a mode of action that is similar to that of other NS5A inhibitors (BMS-824, BMS-858, and BMS-790052) due to similar drug resistance profiles.

The BP008 resistance profile suggested that the mutations (Q24L, P58S/T/L, Y93H, F149L, and V153M) in HCV NS5A are the important determinants for mediating BP008 sensitivity. Those drug resistance mutations were located mainly at the N-terminal domain I of NS5A protein. The structure of domain I was determined and shown to form a dimer via contacts near the

FIG 4 Pharmacokinetics of BP008 after intravenous (IV) or oral (per os [PO]) administration to SD rats. SD rats were given an intravenous or oral dose of 1 or 2 mg/kg BP008 in a solution of PEG 400 and water (20/80, vol/vol). At 0 (prior to dosing), 2, 5, 15, and 30 min and 1, 2, 4, 6, and 8 h after intravenous dosing and at 0.25, 0.5, 1, 2, 4, 6 and 8 h after oral dosing, a blood sample (150 μ l) was collected from each animal via the jugular vein cannula. Plasma was separated from the blood by centrifugation (7,500 rpm for 15 min). All samples were analyzed by LC-MS/MS. The data were presented as means $\pm$ SD ($n = 3$).

TABLE 7 Liver and plasma concentrations of BP008 in male Sprague-Dawley rats dosed orally at 2, 10, and 50 mg/kg

Dose (mg/kg)	Time (h)	Concn in liver ^a (ng/g)	Concn in plasma ^a (ng/ml)	Liver/plasma ratio ^b
2	24	1.1 ± 0.9	0.6 ± 0.3 ^c	1.8
10	24	12.4 ± 8.6	5.7 ± 2.5	2.2
50	24	226.9 ± 179.2	89.1 ± 28.3	2.6

^a Means $\pm$ standard deviations determined from three independent experiments. The represented plasma concentrations are estimated values.

^b Ratio of concentration of BP008 in liver to concentration in plasma.

^c This value was below the limit of quantitation (1 ng/ml).

N-terminal ends of the molecules, which can adopt different conformations (38, 56). Tyrosine-93, the major residue involved in BP008 resistance, is located near the dimer interface between two NS5A molecules in the structure proposed by Tellinghuisen et al. (38, 56). The identification of Y93 as an important site of BP008 resistance suggests that the dimer formation of NS5A could be affected by the binding of an inhibitor. If transitioning between alternate dimer configurations is important for HCV replication, one can speculate that BP008 disrupts this transition by binding across the dimer interface and stabilizing one conformation over the other. However, there is no experimental evidence to support such a model at this time. Further work, including cocrystallization studies, will be essential to better understand the interaction and molecular mechanism of the inhibition of BP008 and related inhibitors.

With regard to how BP008 affects HCV RNA replication, our transient replicon assay indicated that BP008 does not affect the translation efficiency of HCV1b replicon RNA but significantly reduces the synthesis of HCV viral RNA (Fig. 2). Moreover, results from the BP008 resistance and potency mapping experiments demonstrated that the N terminus of NS5A is the region responsible for the BP008-mediated inhibition of HCV1b replicon activity (Fig. 3 and Table 2). The *in vitro* binding experiment of the BMS-790052 analogue suggested that BMS-790052 inhibits HCV replication by directly binding to HCV NS5A (17). Since BMS-790052 and BP008 resistance data share high degrees of similarity in the drug resistance mutations within the N terminus of NS5A, BP008 is likely to interfere with the roles of NS5A during HCV replication cycle, possibly via binding to NS5A. Interestingly, due to recent reports showing that BMS-790052 and other NS5A inhibitors can cause changes in the subcellular redistribution of HCV NS5A protein (30, 52), it was hypothesized that NS5A inhibitors distort the conformation of NS5A dimer that is communicated allosterically to NS5A molecules in an oligomeric complex. Furthermore, BMS-790052 was shown to interrupt two distinct functions (*cis*- and *trans*-acting functions) of NS5A in HCV RNA replication, where *cis*-acting function is part of the replication complex and *trans*-acting function occurs outside the replication complex (15). In all, the participation of NS5A in many steps or aspects of HCV viral replication demonstrated that NS5A is a good target for future drug development. The detailed mechanism by which NS5A inhibitors affect HCV replication remains unclear. More studies are needed to investigate if BP008 inhibits HCV replication using a mechanism similar to that of other NS5A inhibitors.

One of the major problems in clinical chemotherapy of viral infections using a single agent is the rapid development of viral resistance, therefore the development of effective combination therapy is urgently needed. For instance, IFN- α , with known *in vitro* antiviral activities, is being used synergistically in combination therapy of chronic HCV infections (5). Our combination study using HCV1b replicon cells showed synergistic antiviral actions between BP008 and IFN- α , VX-950, or 2'CMA (Table 5). More importantly, no antagonistic antiviral effects or enhanced cytotoxicity was observed in any of these combinations. Although the EC₅₀ (55.2 IU/ml) of IFN- α for stable HCV1b replicon cells was higher than the values reported by other groups (Table 4) (34, 35, 39, 59), the EC₅₀ of IFN- α for our parental HCV1b replicon measured by transient HCV1b replicon assay was in the region of 3 IU/ml (data not shown), comparable to most of the values re-

ported by others. Moreover, several mutations within the C terminus of NS5A were identified by sequencing analysis of cDNAs derived from viral RNAs of our stable HCV replicon cells compared to the sequences of parental HCV1b replicon plasmid for transient replicon assay (data not shown). We suspect that the mutations identified in the NS5A region of our stable HCV replicon cells confer resistance to IFN- α . Considering that BP008 can yield high-level synergy with clinical standard-of-care inhibitors and inhibitors targeting the HCV NS3 protease or NS5B polymerase, incorporating BP008 in future anti-HCV cocktails can provide major advantages over single-drug therapy and represents an attractive paradigm to improve current virologic response rates. In addition to drug resistance, another problem with chemotherapy is the reduced therapeutic efficiency caused by the high proportion of drug binding to plasma or serum proteins. In our protein binding study, we revealed 2.8- (20% FBS) to 11.6-fold (50% FBS) increases compared to levels for the standard conditions (either 10% FBS or NHS), suggesting that drug-serum protein binding has no apparent effect on the therapeutic efficiency of BP008 (Table 3).

Furthermore, pharmacokinetic data on BP008 suggested favorable systemic and hepatic drug exposures which may be supportive of once-daily dosing with moderate $t_{1/2}$ and plasma clearance (Table 7). The oral dose-proportionality study also showed that at 24 h after dosing for three different doses tested (liver/plasma ratios ranged from 1.8 to 2.6), BP008 liver concentrations were achieved related to plasma concentrations, indicating that the liver concentrations of BP008 remained above or close the EC₅₀ for the three doses examined, even though the plasma drug exposure dropped lower than the EC₅₀ at 24 h postdosing. Dose selection and optimization therefore are essential in future drug development to ensure that certain liver drug levels are maintained for the treatment of liver diseases, especially in the case of HCV infection.

In summary, our study on the identification and characterization of BP008 represents the first step toward developing a new chemotype for anti-HCV therapy. Moreover, based on recent experimental, crystallographic, homology modeling, and mutagenesis studies of NS5A, we believe that BP008 has a different mode of action by interfering with RNA replication. At the same time, by the optimization of BP008, we obtained a series of interesting NS5A inhibitors (data not shown) which provide a useful means to study NS5A function and to develop more specific and potent anti-HCV agents. Further work with this inhibitor series should allow us to explore the molecular mechanism of action of BP008, advance our understanding of the NS5A protein, and eventually demonstrate that inhibitors targeting a nontraditional target like NS5A can have great antiviral effects.

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