Vol 465|6 May 2010|doi:10.1038/nature08960

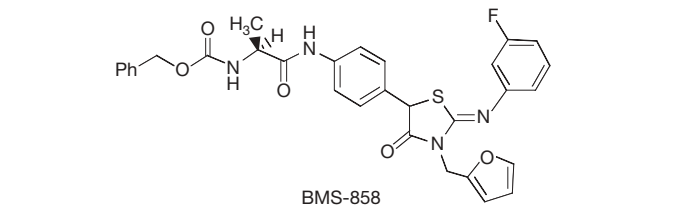
LETTERS

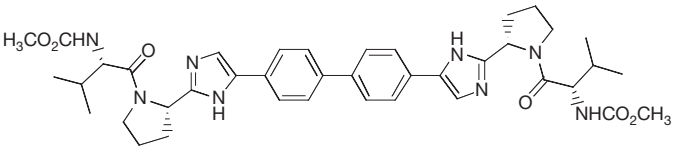
Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect

Min Gao[1], Richard E. Nettles[2], Makonen Belema[3], Lawrence B. Snyder[3], Van N. Nguyen[3], Robert A. Fridell[1], Michael H. Serrano-Wu[3], David R. Langley[4], Jin-Hua Sun[1], Donald R. O'Boyle II[1], Julie A. Lemm[1], Chunfu Wang[1], Jay O. Knipe[5], Caly Chien[2], Richard J. Colonno[1], Dennis M. Grasela[2], Nicholas A. Meanwell[3] & Lawrence G. Hamann[3]

The worldwide prevalence of chronic hepatitis C virus (HCV) infection is estimated to be approaching 200 million people[1]. Current therapy relies upon a combination of pegylated interferon-α and ribavirin, a poorly tolerated regimen typically associated with less than 50% sustained virological response rate in those infected with genotype 1 virus[2,3]. The development of direct-acting antiviral agents to treat HCV has focused predominantly on inhibitors of the viral enzymes NS3 protease and the RNA-dependent RNA polymerase NS5B[4]. Here we describe the profile of BMS-790052, a small molecule inhibitor of the HCV NS5A protein that exhibits picomolar half-maximum effective concentrations (EC_{50}) towards replicons expressing a broad range of HCV genotypes and the JFH-1 genotype 2a infectious virus in cell culture. In a phase I clinical trial in patients chronically infected with HCV, administration of a single 100-mg dose of BMS-790052 was associated with a 3.3 log_{10} reduction in mean viral load measured 24 h post-dose that was sustained for an additional 120 h in two patients infected with genotype 1b virus. Genotypic analysis of samples taken at baseline, 24 and 144 h post-dose revealed that the major HCV variants observed had substitutions at amino-acid positions identified using the *in vitro* replicon system. These results provide the first clinical validation of an inhibitor of HCV NS5A, a protein with no known enzymatic function, as an approach to the suppression of virus replication that offers potential as part of a therapeutic regimen based on combinations of HCV inhibitors.

We designed a mechanistically unbiased approach based on chemical genetics to identify chemical starting points for interfering with HCV replication. Our differentiating strategy centred on the identification of compounds functionally distinct from those acting on the traditional targets of antiviral research in this field, the NS3 protease and the NS5B RNA-dependent RNA polymerase[5–7]. Using a Con-1 genotype 1b replicon replicating in Huh-7 liver cells[8], we screened over one million compounds from the Bristol-Myers Squibb proprietary collection in high throughput mode. We used a dual assay format that simultaneously evaluated replication of the related flavivirus bovine viral diarrhoea virus (BVDV) and host cell cytotoxicity as an efficient means of preliminarily eliminating compounds without specificity for HCV[9]. Active inhibitors were further triaged by evaluation in biochemical assays for NS3 protease, NS3 helicase and NS5B polymerase in addition to screening against a panel of unrelated viruses. The iminothiazolidinone BMS-858 (Fig. 1) emerged as a weak but, more importantly, specific inhibitor of HCV RNA replication ($EC_{50} = 0.57\ \mu M$, half-maximum cytotoxic concentration (CC_{50}) = >50 μM) for which resistance was mapped to a tyrosine to histidine substitution at residue 93 in the NS5A protein (Y93H or Y2065H in the HCV polyprotein)[10]. BMS-858 formed the basis of an extensive series of chemical refinements that focused on improving antiviral potency, broadening inhibitory activity to encompass the HCV 1a genotype, and optimizing for oral bioavailability and sustained pharmacokinetic properties. After defining symmetry as an important contributor to antiviral activity[10], a discovery that preceded the disclosure of structural information (see below), we subsequently identified BMS-790052 (Fig. 1) as a development candidate for advancement into clinical trials[11]. (The studies leading to the identification of BMS-790052 and its preclinical profiling will be the subject of a series of separate publications.) This compound is the most potent HCV replication inhibitor reported so far, with mean EC_{50} values of 50 and 9 pM against genotype 1a and 1b replicons, respectively (data summarized in Table 1). BMS-790052 displays a therapeutic index (CC_{50}/EC_{50}) of at least 100,000 *in vitro* and is inactive towards a panel of 10 RNA and DNA viruses, with EC_{50} values greater than 10 μM, which confirms specificity for HCV (Supplementary Tables 1 and 2).





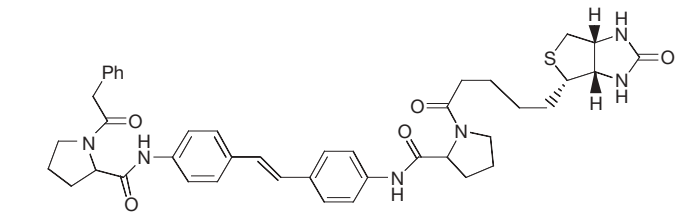


Figure 1 | Structures of the iminothiazolidinone BMS-858, BMS-790052, the biotin-tagged HCV NS5A inhibitor 1 and its inactive stereoisomer 2.

[1]Department of Virology, Bristol-Myers Squibb Research and Development, 5 Research Parkway, Wallingford, Connecticut 06492, USA. [2]Department of Discovery Medicine and Clinical Pharmacology, Bristol-Myers Squibb Research and Development, Princeton, New Jersey 08543, USA. [3]Department of Discovery Chemistry, [4]Department of Computer-Aided Drug Design, [5]Department of Metabolism and Pharmacokinetics, Bristol-Myers Squibb Research and Development, 5 Research Parkway, Wallingford, Connecticut 06492, USA.

Table 1 | *In vitro* antiviral profile of BMS-790052

Assay	EC_{50}
HCV replicon genotype 1a, H77	50 ± 13 pM
HCV replicon genotype 1b, Con1	9 ± 4 pM
HCV replicon genotype 2a, JFH	71 ± 17 pM
HCV replicon genotype 2a, JFH*	103 ± 36 pM
HCV replicon genotype 3a*	146 ± 34 pM
HCV replicon genotype 4a*	12 ± 4 pM
HCV replicon genotype 5a*	33 ± 10 pM
Infectious HCV, genotype 2a, JFH	28 ± 24 pM
BVDV replicon	9 μM
Infectious BVDV	12 μM
Cytotoxicity Huh-7 cells	17 ± 1 μM

* Data derived from hybrid replicons.

Upon further analysis, we determined that the inhibitory activity of BMS-790052 maps to the first 100 amino acids of HCV NS5A (see below). The antiviral activity of the compound towards additional genotypes was assessed by using replication-competent 1a[12] or 1b replicons to construct hybrids in which the entire NS5A coding region or the first 100 amino acids of NS5A from genotypes 2a, 3a, 4a and 5a replaced the corresponding sequence of the parent replicon[13]. This approach has been validated using genotype-specific inhibitors of HCV NS5A[10]. As summarized in Table 1, BMS-790052 demonstrates potent inhibitory activity towards all genotypes tested, with EC_{50} values ranging from 9 to 146 pM. In combination studies, BMS-790052 displayed additive-to-synergistic effects with interferon-α/ribavirin, an inhibitor of NS3 protease (ITMN-191), and both nucleoside and allosteric inhibitors of NS5B polymerase, which is indicative of the potential of this molecule as a candidate for combination therapy with other HCV therapeutic agents (data summarized in Table 2) (Supplementary Tables 3–7). More significantly, BMS-790052 is a potent inhibitor of the JFH-1 genotype 2a infectious virus that replicates in cell culture (EC_{50} = 28 pM), an assay considered to be a more biologically relevant *in vitro* cell culture system[14]. In addition, BMS-790052 displayed similar potency in Huh-7, HeLa and HEK293T cells (Supplementary Table 8), demonstrating that the function(s) of NS5A inhibited by BMS-790052 is (are) highly conserved in different cellular environments.

To characterize the resistance profile of BMS-790052, genotype 1a and 1b replicon cells were maintained in the presence of the drug at concentrations 5–20 times above the respective EC_{50} values. Direct sequencing of individual clones from both 1a- and 1b-resistant cells revealed multiple changes in the first 100 amino acids of NS5A that, when individually introduced into a wild-type replicon background, displayed a resistant phenotype to BMS-790052. Table 3 shows the results of the contribution of these substitutions to resistance in transient replication assays, which were used to ensure that the observed resistant phenotype was caused by inhibitor-induced substitution(s). For genotype 1b, L31V and Y93H were identified as the major resistant variants whereas changes were observed at residues M28 and Q30 of genotype 1a in addition to substitutions at residues L31 and Y93. In general, substitutions in genotype 1b NS5A confer only modest effects on the potency of BMS-790052 whereas substitutions in genotype 1a NS5A are associated with much higher levels of resistance in the replicon system. The most resistant 1a variant, L31V, is inhibited *in vitro* with an EC_{50} of approximately 20 nM, a plasma concentration that we have shown is readily achievable in humans after oral dosing (see below), which indicates that single-point substitutions should be suppressed *in vivo*. Notably, many of these resistant variants display reduced fitness *in vitro* and none are recorded as major species in the HCV sequence database, which suggests that natural selection prevents them from having a dominant advantage *in vivo*. Cyclosporin A and structurally related compounds can inhibit HCV replication *in vitro* and *in vivo*, and resistance has been mapped, in part, to domains II and III of NS5A[15]. To assess the potential for cross-resistance, the sensitivity of inhibition by cyclosporin A of genotype 1a replicons expressing a range of substitutions conferring resistance to BMS-790052 was examined. Cyclosporin A inhibition was not significantly affected by any of the substitutions studied, with EC_{50} values comparable to that observed with a wild-type genotype 1a replicon (Supplementary Table 9).

Table 2 | HCV replicon combination studies with BMS-790052

Interferon-α2β (intron A)	NS3 protease inhibitor	Nucleoside NS5B polymerase inhibitor NM-107	Non-nucleoside NS5B polymerase inhibitor
Synergistic	Additive/synergistic	Additive/synergistic	Additive/synergistic

To confirm that NS5A inhibitors bind to the viral protein, we prepared the biotin-tagged derivative **1**, with the natural (*S*)-configuration at both proline stereocentres, and its diastereomer **2** (Fig. 1). Compound **1** inhibited subgenomic viral RNA replication in the Con-1 genotype 1b replicon with an EC_{50} of 33 nM but was inactive towards a Y93H replicon ($EC_{50} > 10$ μM) whereas the diastereomer **2**, used as a control, was inactive *in vitro* ($EC_{50} > 10$ μM). After exposure to active inhibitor **1**, the replicon lysate was passed over streptavidin agarose beads, separated by SDS–polyacrylamide gel electrophoresis and probed with an antibody specific for HCV NS5A, with a parallel experiment using the inactive diastereomer **2** serving as a control. NS5A could only be pulled down efficiently with active inhibitor **1** but not by the inactive compound **2** (Fig. 2); NS3 and NS5B were not detected in pull-down experiments with **1**, suggesting selective binding to NS5A. In a similar experiment conducted with replicons expressing BVDV RNA, compound **1** failed to bind to BVDV NS5A.

HCV NS5A is a 447 amino-acid, zinc-binding phosphoprotein that plays a critically important but enigmatic role in the virus life cycle[16]. The substitutions conferring resistance to BMS-790052 map to domain I of the protein, which incorporates an amphipathic amino (N)-terminal α-helix (residues 5–25) thought to anchor the protein to the membrane[16]. The solid-state structure of fragments of domain I (residues 36–198 and 33–191) of HCV NS5A reveal dimeric complexes with patterns of interfacial recognition that anticipate an oligomeric form of the protein[17,18], postulated to sequester RNA within the replication complex[19]. The unique palindromic topology of BMS-790052 complements the dimeric structure of the NS5A protein. The location of the resistant substitutions suggests that the compound binds across the dimer interface, proximal to the N terminus of domain I between the protein and the membrane, and on the face opposite that of the putative RNA binding domain. We speculate that BMS-790052 may interfere with the precision of dimer association, effecting subtle structural distortions that directly or allosterically interfere with protein function. Under these circumstances, the exquisite inhibitory potency exhibited by BMS-790052 *in vitro* may be related to the disruption of the function of a limited number of NS5A dimers that compromise the activity of an oligomeric complex. However, although this hypothesis allows for an amplification of inhibitory effect, an alternative explanation relies on the inhibition of two or more activities of NS5A that interact synergistically. Further studies with BMS-790052 will be required to define its exact mode of action more fully and, conversely, the compound is a useful tool with which to study aspects of HCV NS5A function in viral replication. These investigations may be aided by compounds with an *in vitro* profile similar to **1**, which is restricted to genotype 1b inhibition[10].

BMS-790052 exhibited no significant effects in an extensive battery of *in vitro* receptor binding and enzymatic assays designed to assess promiscuity. Despite a molecular mass of over 700 daltons, the compound is orally bioavailable in four preclinical species, with plasma levels readily achieved that surpass the *in vitro* EC_{50}, and it distributes effectively to the liver.

In a randomized, double-blind, placebo-controlled, single ascending-dose study, BMS-790052 was administered at six dose levels to healthy, non-HCV-infected subjects over a range of 1 to 200 mg as an oral solution. The compound was safe and well tolerated up to 200 mg with

Table 3 | Resistance profile of genotype 1a and 1b replicons exposed to BMS-790052

1b replicon	Wild type	L31V	Y93H
Replication level (%)	100	144 ± 47	20 ± 6.8
BMS-790052 EC_{50} (pM)	2.6 ± 0.33	61 ± 15	49 ± 13
Fold resistance	1	24	19

1a replicon	Wild type	M28T	Q30H	Q30R	L31M	L31V	Y93C
Replication level (%)	100	31 ± 23	75 ± 31	41 ± 16	55 ± 15	117 ± 29	11 ± 7
BMS-790052 EC_{50} (pM)	5.9 ± 3.7	4,100 ± 360	8,700 ± 1,900	7,300 ± 1,100	2,100 ± 610	20,000 ± 6,000	11,000 ± 4,000
Fold resistance	1	695	1,475	1,237	356	3,390	1,864

no clinically relevant adverse effects. After oral administration, BMS-790052 was readily absorbed, with dose-proportional exposures over the studied dose range, and all subjects had drug concentrations greater than the protein-binding-adjusted EC_{90} for genotypes 1a and 1b, as measured in the replicon assay, at and beyond 24 h post-dose (Fig. 3). (The protein binding-adjusted EC_{90} figures were derived from an analysis of the effect of the addition of human serum on antiviral activity in replicons. In the presence of 40% human serum, the EC_{90} for BMS-790052 is 383 pM (0.28 ng ml^{-1}) for the genotype 1a replicon and 49 pM (0.04 ng ml^{-1}) for the genotyope 1b replicon.)

In a randomized, double-blind, placebo-controlled, single ascending-dose study, BMS-790052 was administered to subjects with genotype 1 chronic HCV at doses of 1, 10 and 100 mg as an oral solution. All subjects were infected with HCV genotype 1a except for two subjects at 10 mg and three subjects at 100 mg who were infected with genotype 1b. BMS-790052 was safe and well tolerated in HCV-infected subjects after single oral doses up to 100 mg. Specifically, there were no deaths, serious adverse events, discontinuations due to adverse events or clinically relevant adverse effects. Headache was the most frequent adverse event, reported by four subjects after administration of BMS-790052. In HCV-infected subjects, BMS-790052 had a mean plasma elimination half-life ranging from 10 to 14 h, and plasma drug levels were similar to those in non-HCV-infected subjects. After single oral doses of 10–100 mg BMS-790052, all subjects had 24-h plasma concentrations above the tenfold protein binding-adjusted EC_{90} for HCV genotypes 1a and 1b, suggesting the possibility for once daily administration. The plasma HCV RNA levels were measured for up to 6 days after administration; the mean decline from the time of administration to 144 h post-dose is shown in Fig. 4. A single milligram dose of BMS-790052 produced a mean 1.8 $\log_{10}$ reduction (range 0.2–3.0 $\log_{10}$) in HCV viral load measured 24 h after drug administration. Both the 10 and 100 mg doses produced a greater antiviral effect, with mean plasma viral RNA falling by 3.2 $\log_{10}$ (range 2.9–4.0 $\log_{10}$) and 3.3 $\log_{10}$ (range 2.7–3.6 $\log_{10}$), respectively, at 24 h post-dose. Moreover, the 100 mg dose resulted in a mean maximal HCV RNA decline of 3.6 $\log_{10}$ (range 3.0–4.1 $\log_{10}$) and a prolonged antiviral response was observed in two subjects infected with genotype 1b virus, with an HCV RNA measurement that reached the lower limit of quantification (less than 25 IU ml^{-1}) in one subject and 35 IU ml^{-1} in the other measured at hour 144. Genotypic analysis of samples taken at baseline (T0), 24 (T24) and 144 (T144) hours post-dose revealed that, in general, a marked reduction in viral load was required to detect major HCV variants. Substitutions were observed at amino-acid positions identified using the *in vitro* replicon system (Supplementary Tables 12–14): M28T, Q30H/R and L31M for genotype 1a, and Y93H for genotype 1b, results that suggest the usefulness of the replicon system for assessing resistance in response to inhibitor pressure *in vivo*. Follow-up samples were available for only one of these subjects, which revealed that HCV RNA had returned to near baseline; however, genotype analysis was not performed on this sample. As would be anticipated based on the *in vitro* replicon potency of BMS-790052, a greater and more sustained decline in HCV RNA was observed for subjects infected with genotype 1b (mean 3.6 $\log_{10}$ reduction (range 3.1–4.0 $\log_{10}$) and mean 3.1 $\log_{10}$ reduction (range 2.7–3.4 $\log_{10}$) in HCV viral load measured 24 h after a 10 and 100 mg dose, respectively) than for subjects infected with genotype 1a (mean 1.8 $\log_{10}$ reduction (range 0.2–3.0 $\log_{10}$), mean 2.9 $\log_{10}$ reduction (range 2.9–3.0 $\log_{10}$) and mean 3.6 $\log_{10}$ reduction (range 3.5–3.6 $\log_{10}$) in HCV viral load measured 24 h after a 1, 10 and 100 mg dose, respectively). The mean rates of decline for subjects who received 10 and 100 mg doses of BMS-790052 were similar up to 36 h after dosing, after which the mean decline was greater and more sustained in the subjects who received 100 mg. Subjects who received 1 mg of BMS-790052 had a lower mean decline in HCV RNA than subjects treated with 10 and 100 mg (Fig. 4). However, multiple-dose studies are needed to define the optimal dose range for maximal antiviral effect beyond the first phase of viral decline. The relationships between maximum decline from baseline in HCV RNA and drug pharmacokinetics exposures were explored using Pearson's correlation coefficients. All estimated Pearson's correlation coefficients were above 0.65, suggesting that the maximum declines in $\log_{10}$ HCV RNA and log pharmacokinetics exposures (BMS-790052 C_{max}, AUC(0-T), AUC(INF), C12 and C24) were positively correlated; that is, that the maximum declines in $\log_{10}$ HCV RNA increase with the exposure to BMS-790052.

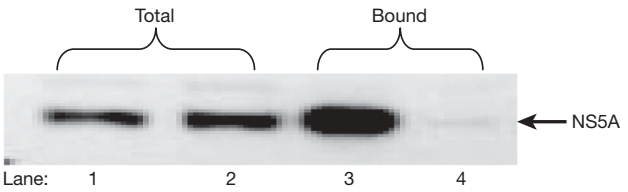


Figure 2 | The active inhibitor 1 binds to genotype 1b HCV NS5A whereas the inactive stereoisomer 2 does not. Genotype 1b replicon cells were exposed to either **1** or **2** for approximately 18 h before the cells were lysed. A portion of the supernatant was set aside to serve as input control for immunoblot analysis (lanes 1 and 2) while the remainder was mixed with streptavidin–agarose beads and incubated for several hours. Bound proteins were detected by immunoblotting with primary antibodies directed to HCV NS5A. Lanes 1 and 3 depict the results of experiments with the active inhibitor compound **1** whereas lanes 2 and 4 depict the results with the inactive stereoisomer **2**.

Individuals infected with HCV are at considerable risk of developing liver cirrhosis, end-stage liver disease and hepatocellular carcinoma[20]. The current standard of care for the treatment of HCV infection is a combination of weekly subcutaneous injections of pegylated interferon-α in conjunction with ribavirin, administered orally twice a day, for periods ranging from 24 to 72 weeks, depending on genotype[2,3]. However, the side effects associated with this therapeutic regimen impose a significant physiological and psychological burden on the patient. Moreover, sustained virological response rates in those infected with genotype 1 are typically less than 50% (refs 2, 3). The direct-acting HCV antiviral drug candidates currently reported to be in late-stage clinical development are inhibitors of NS3 protease and NS5B polymerase, more traditional antiviral targets. Although these compounds are currently being developed as an add-on therapy to the standard of care, there is considerable anticipation for combinations of direct-acting agents, which will require at least two mechanistically distinct inhibitors to suppress the emergence of resistant virus[4,21]. The preliminary *in vitro* and clinical profile of NS5A inhibitors described in this paper makes them a potentially valuable component of any interferon-free treatment regimen.

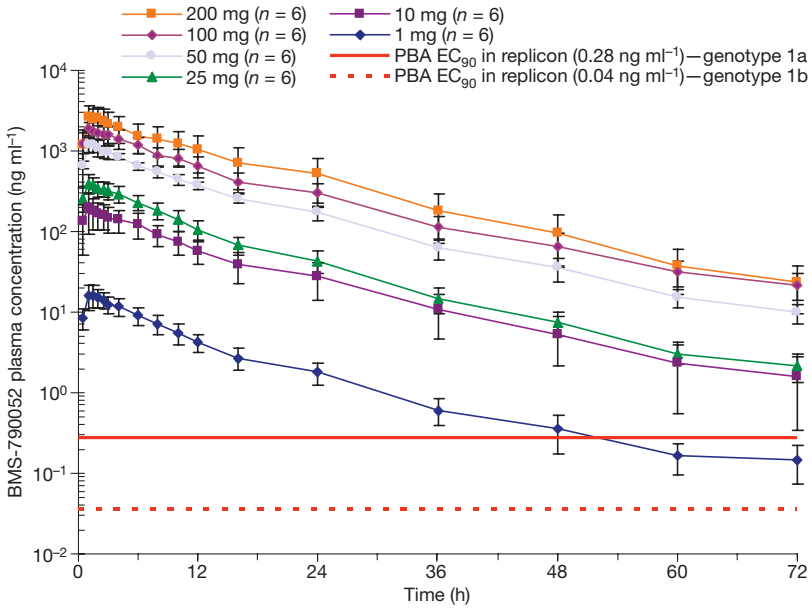


Figure 3 | Mean plasma concentration–time profile (time 0–72 h) of BMS-790052 after single oral administration of 1–200 mg of drug to healthy subjects. In a double-blind, placebo-controlled, sequential, single ascending-dose study, eight male or female subjects were randomized within each dose panel (1, 10, 25, 50, 100 and 200 mg) to drug or placebo in a ratio of 3:1. BMS-790052 or placebo was administered in the fasted state. The plasma samples obtained at various times were analysed for BMS-790052 by a validated liquid chromatography tandem mass spectrometry assay. Pharmacokinetic parameter values for individual subjects were derived by non-compartmental methods by a validated pharmacokinetic analysis programme. PBA EC_{90}, protein-binding-adjusted EC_{90} for the individual genotype in a replicon assay. Error bars, standard deviation.

The results with BMS-790052 provide clinical validation for the first in a new class of HCV inhibitors that target a viral protein with no known enzymatic function and an as yet poorly understood role in viral replication[16,22]. The strategy used to identify a lead HCV NS5A inhibitor and to optimize this molecule into a clinical candidate offers a contemporary demonstration of the effectiveness of an approach to drug discovery based on chemical genetics[6]. Indeed, this methodology is uniquely applicable to targets similar to HCV NS5A for which precise function is enigmatic and the development of biochemical assays could neither be anticipated nor are feasible. As a specific inhibitor of HCV, BMS-790052 exhibits a spectrum of genotype inhibition, *in vitro* potency, and efficacy and potency after single oral doses to chronically HCV-infected subjects that is collectively and individually unprecedented. Achieving high potency and selectivity against a non-mammalian target, the traditional goal of antiviral medicinal chemistry, has in the past translated into a wider therapeutic index in the clinic. Although preliminary, these data indicate that inhibitors of HCV NS5A offer considerable promise for the treatment of HCV infection. The *in vitro* data demonstrating additive to synergistic interactions with known HCV inhibitors

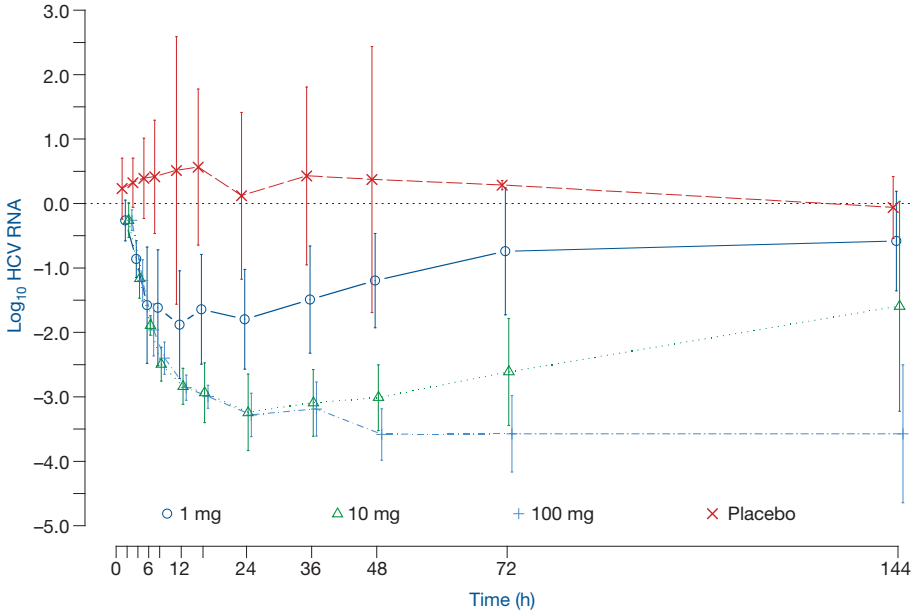


Figure 4 | Mean change in log_{10} HCV RNA with 90% confidence intervals after administration of single oral doses of BMS-790052 to HCV-infected patients. In a double-blind, placebo-controlled, sequential, single ascending-dose study, six subjects were randomized within each dose panel (1, 10, 100 mg) to drug or placebo in a ratio of 5:1. BMS-790052 or placebo was administered in the fasted state. Owing to a dosing error, all six subjects received BMS-790052 in the 1 mg panel. One subject in the 10 mg panel withdrew from the study 8 h after administration of the study drug for non-drug-related reasons; HCV RNA data from the subject are included up until the subject withdrew.

suggest that combinations of BMS-790052, with either current standard of care or, ultimately, emerging inhibitors of HCV NS3 and NS5B as part of a cocktail of direct-acting antiviral agents, may lead to therapeutic regimens with better tolerability and improved clinical outcomes[21].

METHODS SUMMARY

***In vitro* HCV replicon systems.** Construction and isolation of HCV replicons, including hybrid replicons for genotype coverage and replicon assays, have been described previously and are elaborated in the Supplementary Information[9,10]. For the genotype 1a replicon, the P1496L substitution in the *NS3* gene and the S2204I substitution in the *NS5A* gene were used as the adaptive substitutions for efficient replicon replication. For the genotype 1b replicon, a S2204I substitution in the *NS5A* gene was used as the adaptive mutation.

HCV-infected clinical study population. In total, 16 subjects received treatment with BMS-790052 and two subjects received placebo. Subjects selected for this study included men and women aged 18–49 years, inclusive, who were chronically infected with HCV genotype 1 and were treatment naive or treatment non-responders, defined as subjects who received the current standard of care (interferon and/or ribavirin) and who continued to have a detectable HCV RNA level (including relapsers) or subjects who did not attain a 2 $\log_{10}$ decline in HCV RNA levels at 12 weeks and stopped treatment; or treatment-intolerant subjects, defined as subjects who were unable to tolerate the toxicities associated with interferon and/or ribavirin; and who had not received another NS5A replication co-factor inhibitor; and who were not co-infected with human immunodeficiency virus, hepatitis B virus or HCV other than genotype 1.

Eligible subjects also had an HCV RNA viral load of at least 10^5 IU ml^{-1}, a documented FibroSure score of not more than 0.59, an aspartate aminotransferase to platelet ratio index of not more than 2 and a body mass index of 18–35 kg m^{-2}, inclusive. All subjects signed the informed consent form, and were screened and determined to be eligible based on medical history, physical examination and measurements, vital signs, a 12-lead electrocardiogram, and clinical laboratory measurements including serology.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 29 October 2009; accepted 26 February 2010.
Published online 21 April 2010.

1. Kim, W. R. Global epidemiology and burden of hepatitis C. *Microbes Infect.* **4,** 1219–1225 (2002).
2. Hoofnagle, J. H. & Seeff, L. B. Peginterferon and ribavirin for chronic hepatitis C. *N. Engl. J. Med.* **355,** 2444–2451 (2006).
3. Pearlman, B. L. Extended-therapy duration for chronic hepatitis C, genotype 1: the long and the short of it. *World J. Gastroenterol.* **14,** 3621–3627 (2008).
4. De Francesco, R. & Migliaccio, G. Challenges and successes in developing new therapies for hepatitis C. *Nature* **436,** 953–960 (2005).
5. Bartenschlager, R. Hepatitis C virus replicons: potential role for drug development. *Nature Rev. Drug Discov.* **1,** 911–916 (2002).
6. Stockwell, B. R. Exploring biology with small organic molecules. *Nature* **432,** 846–854 (2004).
7. Green, N., Ott, R. D., Isaacs, R. J. & Fang, H. Cell-based assays to identify inhibitors of viral disease. *Exp. Opin. Drug Discov.* **3,** 671–676 (2008).
8. Lohmann, V. *et al.* Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285,** 110–113 (1999).
9. O'Boyle, D. R. II *et al.* Development of a cell-based high-throughput specificity screen using a hepatitis C virus-bovine viral diarrhea virus dual replicon assay. *Antimicrob. Agents Chemother.* **49,** 1346–1353 (2005).
10. Lemm, J. A. *et al.* Identification of hepatitis C virus NS5A inhibitors. *J. Virol.* **84,** 482–491 (2010).
11. Bachand, C. *et al.* Hepatitis C virus NS5A inhibitors. World patent WO-2008021927 (2008).
12. Yi, M.-K. & Lemon, S. M. Adaptive mutations producing efficient replication of genotype 1a hepatitis C virus RNA in normal Huh7 cells. *J. Virol.* **78,** 7904–7915 (2004).
13. Pietschmann, T. *et al.* Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc. Natl Acad. Sci. USA* **103,** 7408–7413 (2006).
14. Bartenschlager, R. & Pietschmann, T. Efficient hepatitis C virus cell culture system: what a difference the host cell makes. *Proc. Natl Acad. Sci. USA* **102,** 9739–9740 (2005).
15. Fernandes, F. *et al.* Sensitivity of hepatitis C virus to cyclosporin A depends on nonstructural proteins NS5A and NS5B. *Hepatology* **46,** 1026–1033 (2007).
16. Schmitz, U. & Tan, S.-L. NS5A – from obscurity to new target for HCV therapy. *Recent Patents Anti-infect. Drug Discov.* **3,** 77–92 (2008).
17. Tellinghuisen, T. L., Marcotrigiano, J. & Rice, C. M. Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. *Nature* **435,** 374–379 (2005).
18. Love, R. A., Brodsky, O., Hickey, M. J., Wells, P. A. & Cronin, C. N. Crystal structure of a novel dimeric form of NS5A domain I protein from hepatitis C virus. *J. Virol.* **83,** 4395–4403 (2009).
19. Appel, N., Schaller, T., Penin, F. & Bartenschlager, R. From structure to function: new insights into hepatitis C virus RNA replication. *J. Biol. Chem.* **281,** 9833–9836 (2006).
20. Lauer, G. M. & Walker, B. D. Hepatitis C virus infection. *N. Engl. J. Med.* **345,** 41–52 (2001).
21. Manns, M. P. *et al.* The way forward in HCV treatment – finding the right path. *Nature Rev. Drug Discov.* **6,** 991–1000 (2007).
22. Holler, T. P., Parkinson, T. & Pryde, D. C. Targeting the non-structural proteins of hepatitis C virus: beyond hepatitis C virus protease and polymerase. *Exp. Opin. Drug Discov.* **4,** 293–314 (2009).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We acknowledge the following colleagues who are or who have been part of the broader HCV NS5A inhibitor drug discovery effort: drug discovery—J. L. Romine, J. E. Leet, D. R. St Laurent, F. Yang, O. D. Lopez, R. Lavoie, J. Goodrich, M. Deshpande, C. Bachand, E. H. Ruediger, C. A. James, D. H. Deon, A. Martel, A. C. Good, K. Mosure, D. Qiu, P. T. Nower, L. L. Valera, S. A. Voss, M. Liu and S. B. Roberts; clinical development—J.-C. Lopez-Talavera, E. Chung, A. H. Persson, J. Coumbis, M. Bifano, J. Dudley, M. DeMicco, T. Marbury, R. Goldwater, P. Northup, W. K. Kraft and M. Charlton. We also thank our colleagues M. I. Cockett, F. M. Cuss, C. P. Decicco, J. G. Houston and E. Sigal for their support and counsel.

Author Contributions R.A.F., J.A.L., D.R.O., J.-H.S., C.W. and M.G. designed and performed the replicon screen, constructed replicons and hybrid replicons, conducted genotype coverage and inhibitor-binding experiments, isolated and mapped resistant variants, designed *in vitro* combination studies and performed genotypic and phenotypic analysis of clinical samples. M.G., R.A.F., J.A.L. and R.J.C. designed the overall virology studies and provided input to the overall research direction. J.O.K. designed and interpreted *in vivo* pharmacokinetic studies and *in vitro* compound profiling. M.B., V.N.N., L.B.S. and M.H.S.-W. designed and synthesized the compounds, L.B.S., L.G.H. and N.A.M. provided direction to the chemistry research and contributed to the design of compounds. D.R.L. constructed models of HCV NS5A and contributed to the development of mechanistic hypotheses. R.E.N., C.C. and D.M.G. designed and interpreted human trials, and D.M.G. provided input to the overall research direction. M.G., R.E.N. and N.A.M. co-authored the manuscript with input from all co-authors, and N.A.M. compiled and edited the article.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the full-text version of the paper at www.nature.com/nature. Correspondence and requests for materials should be addressed to N.A.M. (Nicholas.Meanwell@bms.com).

METHODS

Replicon assays. Determination of the EC_{50} values of HCV NS5A inhibitors using the FRET assay was described previously[9]. For the luciferase assay, the replicon cells were maintained in culture at 37 °C, 5% CO_2 in DMEM with 10% FBS. Inhibitors were serially diluted in 100% dimethylsulphoxide (DMSO) and added to 96-well tissue culture plates (Costar catalogue number 3614) containing HCV replicon cells seeded approximately 12 h previously in 200-µl tissue culture media. The final concentration of DMSO was 0.5%, a concentration shown previously to be non-toxic to replicon cells (data not shown). After incubation for 72 h, cell plates were tested for replication activity and cytotoxicity. Cells plates were incubated for 5 h with CellTiter-Blue (10% final concentration; Promega number G8081) to determine cytotoxicity. After 5 h, the plates were equilibrated to ambient temperature with gentle shaking and read by Cytofluor (series 4000, PerSeptive Biosystems). The endpoint reading was performed at an excitation of 530 nm and emission of 580 nm with a gain of 40. Control wells containing DMSO only were set at 100% viability and the percentage cytotoxicity was determined for each concentration of the inhibitor by dividing the average value for wells containing compound by the average value for wells containing DMSO. After reading the CellTiter-Blue, the media and dye were removed from the cells, the plates were inverted and the remaining liquid was blotted with paper towels. Replication activity of the HCV genotype 1a cell lines was quantified using *Renilla* luciferase. Thirty microlitres of 1× *Renilla* luciferase lysis buffer (Promega number E291A) was added to each well and plates were incubated with gentle shaking at ambient temperature for 15 min. Forty microlitres of *Renilla* luciferase substrate (Promega number E290B) was added to each well, and signal was detected on a Top Count luminometer for light emission quantification. One hundred per cent activity was calculated for each cell line from the DMSO only wells; percentage activity was calculated for each concentration of the inhibitor by dividing the average value for wells containing compound by the average value for wells containing DMSO.

Specificity and cytotoxicity assays. Human immunodeficiency virus and canine parainfluenza virus. Susceptibility of human immunodeficiency virus and canine parainfluenza virus to compounds was determined by incubation in the presence of serial dilutions of the compound. For recombinant human immunodeficiency viruses expressing *Renilla* luciferase, antiviral activity was evaluated by measuring the production of luciferase in infected cells 5 days post-infection using the Dual Luciferase kit (Promega). For the canine parainfluenza virus assay, viral neuraminidase activity was used as a measure of viral production. Fluorescence was measured using an LJL Analyst plate reader at 360 nm excitation/485 nm emission.

Bovine viral diarrhoea virus (BVDV). The cell protection assay used to assess specificity of BMS-790052 was described previously[23]. Briefly, BVDV was placed onto cells in 96-well plates at different multiplicities of infection for 3 days in the presence and absence of compounds. Infected cells protected by compound-1453 were used as a positive control for protection from the cytopathic effect of BVDV.

BVDV replicon. A cell-based, high-throughput specificity screen using an HCV–BVDV dual replicon assay was described previously[9]. Briefly, BVDV replicon cells and HCV replicon cells were seeded in 96-well plates at a cell density of 10^4 per well (5,000 each cell line) in 80 µl of DMEM media containing 10% FBS. After incubation overnight (about 18 h), compounds were serially diluted in DMSO and media was added to individual wells. The cell plates were incubated for 3 days before assaying for cytotoxicity, HCV inhibition and BVDV inhibition using three separate but compatible assay methods.

Herpes simplex virus (HSV). Susceptibility of HSV viruses to compounds was determined by using a multi-cycle HSV growth assay. Fifty plaque forming units of HSV-1 (strain F) or HSV-2 (strain 186) suspended in 100 µl of MEM were added to Vero cells (10^4). After absorption for 1 h, 100 µl of MEM containing 2% FBS and 1 µl of threefold serial dilutions of compounds in DMSO were added to the cells. Acyclovir, an HSV inhibitor, was included as a control. After 4 days of incubation at 37 °C, Alamar blue solution was added and fluorescence was read at 530 nm excitation/580 nm emission for calculations of EC_{50} and CC_{50}.

Influenza. Inhibition of influenza A/WSN/33 (American Type Culture Collection number VR-825) replication was examined in MDBK cells (American Type Culture Collection number CCL-22). Viral neuraminidase activity was assayed as a measurement of influenza virus replication. Alamar blue metabolism was used for measurement of cytotoxicity. MDBK cells were resuspended in 2% FBS in MEM for the neuraminidase assay for virus production or in 10% FBS MEM for Alamar blue-based cytotoxicity assay. Media with and without virus was then added to the cell plates followed by test compounds and control compounds. Plates containing virus were incubated for 24 h in a 37 °C incubator at which time a neuraminidase substrate was added. Fluorescence generated by viral neuraminidase activity was read at 360 nm excitation/460 nm emission. Non-infected cell assay plates containing compound dilutions were allowed to incubate for an additional 72 h. Alamar blue solution was added and the plates were incubated for another 4 h. Plates were read at 530 nm excitation/580 nm emission. Percentage cytotoxicity was calculated by comparison with control wells. All CC_{50} values were calculated using the median effect equation.

Other positive-stranded viruses. Human coronavirus, poliovirus, Coxsackie virus A21 and human rhinovirus 2 were obtained from the American Type Culture Collection. MRC-5 cells were seeded in a 96-well plate and incubated at 37 °C. The next day, media were removed and 135 µl of DMEM with 2% FBS was placed on cells containing either the counterscreen virus at a multiplicity of infection of 0.1 or media alone. Dilutions of compounds in 100% DMSO were prepared and 10 µl of the dilution placed into 190 µl of DMEM with 2% FBS; 15 µl of the mix of compound/media was then added to the 135 µl of media/virus in the 96-well plate. The plates were further incubated for 5 days, after which a solution of Alamar blue was added to quantify protection and cytotoxicity.

Cytotoxicity. Cells were incubated in 96-well assay plates in the presence of serially diluted compounds for 5 days (MT-2, MRC-5 cells) or 3–4 days (all other cell types). Cell viability was quantified by using either an MTS assay for MT-2, or an Alamar blue assay for HEK-293 cells, HuH-7, Vero, MRC-5 or HeLa cells. Microscopic visual inspection of cell monolayers was also conducted periodically to confirm cytotoxicity measurements. All CC_{50} values were calculated using the median effect equation.

Isolation of hybrid replicons for assessing genotype coverage. The National Institutes of Health genotype 2a HCV full-length clone (pJ6CF) was obtained from J. Bukh. Genotypes 2b, 3a, 4a and 5a NS5A fragments were obtained from a panel of HCV-positive sera (Cliniqa Corporation). RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. First-strand complementary DNA synthesis with random primers (Superscript II Reverse Transcriptase and Platinum Taq DNA Polymerase High Fidelity, Invitrogen) and PCR with gene specific primers (Topo TA cloning kit, Invitrogen) were performed following the manufacturer's protocol. Hybrid replicons with the region encoding NS5A or the first 100 amino acids of NS5A from a different genotype were constructed by using recombinant overlapping PCR in Con-1 background. Clones were verified by DNA sequence analysis.

BMS-790052 resistance analysis. Methods for selecting, isolating and mapping resistant substitutions for BMS-790052 were described previously[10]. Briefly, HCV genotype 1a and 1b replicon cells were maintained in media containing BMS-790052 at a concentration of 5- to 20-fold above EC_{50} and 0.5 mg ml^{-1} G418. Replicon cells similarly treated with DMSO were maintained as controls. After approximately 4–5 weeks when cell growth was similar to DMSO-treated control cells, selected cells were expanded for resistance testing and analysis by PCR with reverse transcription.

The resistance profile and replication level (fitness) of variants were determined *in vitro* in transient replication assays after introducing specific substitutions into wild-type replicons.

NS5A inhibitor binding assay. Compound **1** and its inactive diastereomer **2** are biotin-containing analogues of BMS-790052 that were used as affinity probes for NS5A binding (Fig. 1). They differ only in the stereochemistry of the proline moieties of the compounds. Biotin-containing compounds (1 µM) were added to separate T-175 flasks of HCV 1b replicon cells (approximately 80% confluent) and cells were incubated under normal growth conditions for approximately 18 h. Cells were removed with EDTA-free Cell Dissociation Buffer (Invitrogen), centrifuged (4,000*g*, room temperature) and resuspended in ice cold NP-40 lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 1× Complete Protease Inhibitor Cocktail (Roche Applied Science)). After 15 min at 4 °C, nuclei were removed by centrifugation (1,500*g*, 10 min, 4 °C). A portion of the supernatant was saved as an input control for immunoblot analysis (see below) and the remainder was mixed with 30-µl streptavidin–agarose beads (Sigma) and incubated for several hours with gentle rocking at 4 °C. Beads were pelleted by brief centrifugation, washed several times (500 µl NP-40 lysis buffer), resuspended in SDS gel loading buffer and heated for 3 min at 100 °C. After a brief spin (14,000*g*, 5 min), supernatants were electrophoresed on 7.5% Criterion gels (Bio-Rad Laboratories) following the manufacturer's recommended procedure. Proteins were transferred to nitrocellulose and analysed by immunoblotting with primary antibodies directed at HCV NS5A, NS5B or NS3 and HRP-conjugated secondary antibodies (Sigma). Antibody complexes were detected by chemiluminescence with a Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer) following the manufacturer's instructions.

Clinical trials: ethical conduct and consent. The clinical trials were conducted in accordance with Good Clinical Practice, as defined by the International Conference on Harmonisation and in accordance with the ethical principles underlying European Union Directive 2001/20/EC and the United States Code of Federal Regulations, Title 21, Part 50 (21CFR50). The protocol and the subject informed consent received institutional review board/independent ethics committee approval/favourable opinion before initiation of the study. Freely given

written informed consent was obtained from every subject before participation in the clinical study.

Clinical trials: single ascending-dose study in HCV-infected subjects. Subjects could be treatment-naive or -experienced men or women, 18–49 years of age with HCV RNA $\geq 10^5$ IU ml^{-1} with non-cirrhotic compensated liver disease. The plasma samples obtained at various times were analysed for BMS-790052 by a validated liquid chromatography tandem mass spectrometry assay. Individual subject pharmacokinetic parameter values were derived by non-compartmental methods by a validated pharmacokinetic analysis programme. Plasma HCV RNA levels were determined using the Roche TaqMan HCV (qPCR) Quantitative Assay, which has a limit of detection of 10 IU ml^{-1} and range for quantification of 25–3.91 $\times$ 10^8 IU ml^{-1}. All samples were analysed by a central laboratory.

23. Sun, J.-H. *et al.* Specific inhibition of bovine viral diarrhea virus replicase. *J. Virol.* **77**, 6753–6760 (2003).