Preclinical Characterization of BMS-791325, an Allosteric Inhibitor of Hepatitis C Virus NS5B Polymerase

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BMS-791325 is an allosteric inhibitor that binds to thumb site 1 of the hepatitis C virus (HCV) NS5B RNA-dependent RNA polymerase. BMS-791325 inhibits recombinant NS5B proteins from HCV genotypes 1, 3, 4, and 5 at 50% inhibitory concentrations (IC$_{50}$) below 28 nM. In cell culture, BMS-791325 inhibited replication of HCV subgenomic replicons representing genotypes 1a and 1b at 50% effective concentrations (EC$_{50}$s) of 3 nM and 6 nM, respectively, with similar (3 to 18 nM) values for genotypes 3a, 4a, and 5a. Potency against genotype 6a showed more variability (9 to 125 nM), and activity was weaker against genotype 2 (EC$_{50}$, 87 to 925 nM). Specificity was demonstrated by the absence of activity (EC$_{50}$s of >4 µM) against a panel of mammalian viruses, and cytotoxic concentrations (50%) were >3,000-fold above the HCV EC$_{50}$. Resistance substitutions selected by BMS-791325 in genotype 1 replicons mostly mapped to a single site, NS5B amino acid 495 (P495A/S/L/T). Additive or synergistic activity was observed in combination studies using BMS-791325 with alfa interferon plus ribavirin, inhibitors of NS3 protease or NS5A, and other classes of NS5B inhibitor (palm site 2-binding or nucleoside analogs). Plasma and liver exposures *in vivo* in several animal species indicated that BMS-791325 has a hepatotropic disposition (liver-to-plasma ratios ranging from 1.6- to 60-fold across species). Twenty-four hours postdose, liver exposures across all species tested were ≥10-fold above the inhibitor EC$_{50}$s observed with HCV genotype 1 replicons. These findings support the evaluation of BMS-791325 in combination regimens for the treatment of HCV. Phase 3 studies are ongoing.

C hronic infection with hepatitis C virus (HCV) is estimated to affect 130 to 170 million people worldwide, and its long-term sequelae represent a major and increasing public health concern (1). The virus—a member of the *Hepacivirus* genus of the *Flaviviridae*— has six major genotypes and is highly mutable (2). Acute HCV infection is often asymptomatic, and approximately 80% of acute infections progress to chronic disease. Of these, some 10 to 20% will ultimately develop cirrhosis and/or complications of chronic liver disease within 20 to 30 years, and 1 to 5% will develop hepatocellular carcinoma (3–7). The World Health Organization estimates that, globally, 350,000 people die of HCV-related complications each year (8).

Chronic HCV infection is curable in many patients, but the existing standard of care has a number of deficiencies. Treatment is largely—though no longer exclusively—based on parenteral administration of pegylated alfa interferon (IFN-α) together with the oral broad-spectrum antiviral ribavirin (RBV), for a period of up to 48 weeks depending on HCV genotype (9, 10). However, IFN-α/RBV efficacy is not equal across HCV genotypes. Genotype (GT) 1, which predominates in Europe, Japan, and the United States, is particularly difficult to treat, and rates of sustained virologic response (SVR) posttreatment seldom exceed 50% for GT 1 with IFN-α/RBV alone (9, 10). Two direct-acting antiviral (DAA) inhibitors of HCV NS3 protease, boceprevir and telaprevir, were approved in 2011 for use in combination with IFN-α/RBV for GT 1 treatment. The use of either of these inhibitors with IFN-α/RBV elevates SVR rates to approximately 70% in treatment-naive patients (11, 12). Two further DAA inhibitors were approved by the U.S. Food and Drug Administration in late 2013. Sofosbuvir, a nucleoside analog inhibitor of the HCV NS5B polymerase (discussed below), is indicated for use with IFN-α/RBV for treatment of GT 1 and GT 4 and with RBV alone for GT 2 and GT 3. Simepre-

vir, another NS3 protease inhibitor, is indicated for use with IFN-α/RBV for GT 1 treatment. Both these newer agents in combination with IFN-α/RBV have further improved GT 1 treatment responses, with treatment-naive SVR rates of approximately 80% for simeprevir (13, 14) and approximately 90% for sofosbuvir (15). However, both IFN-α and RBV have severe and treatment-limiting side effects that result in discontinuations and contraindications for a significant minority of patients (9, 10, 16, 17), and this adverse-event burden may be further elevated by the addition of a DAA—for example, telaprevir and boceprevir are associated with a number of toxicities such as rash, pruritus (telaprevir), and the exacerbation of treatment-related anemia (12). Thus, although the combination of IFN-α/RBV with a DAA represents an improvement in patient therapy for the more refractory HCV genotypes, there is still an unmet medical need for new agents and treatment regimens with improved efficacy and tolerability profiles.

The HCV life cycle presents a number of opportunities for chemotherapeutic intervention. Three HCV proteins have been
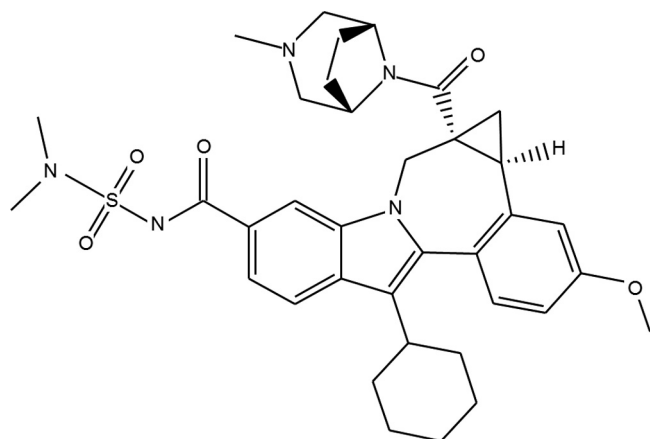
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FIG 1 Structure of BMS-791325.

the main focus for development of direct-acting small-molecule HCV antivirals: the NS3 protease, the multifunctional NS5A protein, and the NS5B RNA-dependent RNA polymerase. In addition to the four currently approved for treatment, a number of other DAAs are in clinical development, and their approval is anticipated to establish a new standard of care based on all-oral regimens—including ribavirin-free oral regimens—that offer shorter treatment durations with significantly improved convenience, tolerability, and efficacy across HCV genotypes.

The HCV NS5B polymerase is a particularly attractive target for intervention. As the catalytic core of the replicase complex, NS5B polymerase is critical for viral proliferation (18–20). It has the right-handed finger-palm-thumb domain structure typical of polymerases (21) with four individually targetable binding sites for allosteric small-molecule inhibitors: two sites in the thumb region (thumb sites 1 and 2) that are separate and two in the palm (palm sites 1 and 2) that partially overlap (22, 23). In addition, competitive inhibition by nucleoside analogs is also possible (24). One NS5B inhibitor—the nucleoside analog sofosbuvir (Sovaldi; GS-7977) (25)—is already approved for the treatment of HCV GTs 1 to 4 and a second, dasabuvir (ABT-333; a palm site 1 inhibitor) (26), is currently in phase 3 development. A third inhibitor, deleobuvir (BI 207127; thumb site 1) (27), was recently withdrawn from development. Each of these NS5B inhibitors has been combined with DAAs targeting other HCV proteins and/or combined with RBV to deliver high rates of SVR, a milestone closely associated with cure.

BMS-791325 (Fig. 1) is currently in phase 3 clinical development. It binds to thumb site 1 of the NS5B polymerase and is one of the most potent NS5B inhibitors in clinical development. As with other thumb site 1 inhibitors, binding of BMS-791325 forces the polymerase to adopt a catalytically inactive "open" conformation by displacing the Δ1 loop, part of the fingers domain, from a lipophilic pocket in the thumb (28, 29). We report here the preclinical characterization of BMS-791325 that supported its selection for clinical development, including its selectivity for HCV NS5B polymerase, its *in vitro* resistance profile, and its antiviral activity, both alone and in combination with other HCV antivirals.

MATERIALS AND METHODS

Cell lines, viruses, and HCV inhibitors. Huh-7 cells were obtained from Ralf Bartenschlager of the University of Heidelberg, Germany. MT-2 cells

were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. Vero, HeLa, MDBK, MRC5, and HEK293 cells were obtained from the American Type Culture Collection (ATCC). Huh-7 and MRC5 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Vero and MDBK cells were propagated in minimum essential medium (MEM), and MT-2 cells were propagated in RPMI 1640, supplemented as described above. Bovine viral diarrhea virus (BVDV) and GT 1a and 1b HCV replicon cell lines have been described previously (30, 31) and were propagated in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, with or without 0.3 to 0.5 mg/ml Geneticin (G418). To construct a subgenomic 2a replicon clone, recombinant PCR was used to place the NS3 to 3′ untranslated region (UTR) sequences of the JFH-1 2a infectious clone (32) into the GT 1b replicon backbone described above. Human influenza virus (A/WSN/33), human rhinovirus 2, human coronavirus, poliovirus, and coxsackie virus A21 were obtained from the ATCC.

BMS-791325, daclatasvir (DCV; an investigational NS5A replication complex inhibitor) (33), and asunaprevir (ASV; an investigational NS3 protease inhibitor) (34) were synthesized by Bristol-Myers Squibb, as were HCV reference inhibitors HCV-796, a palm site 2 nonnucleoside inhibitor of NS5B (35), and NM-283, a prodrug of the anti-NS5B ribonucleoside analog 2′-C-methylcytidine (36). Recombinant alfa-2a interferon (Intron A) was obtained from Myoderm Medical Supply (Norristown, PA, USA).

Construction of hybrid HCV replicons. Recombinant HCV hybrid replicon clones were constructed, which replaced Con1 or H77c NS5B with clinical isolate sequences from HCV GTs 1 to 6. A GT 1b shuttle replicon was prepared by introducing unique SpeI and SnaBI restriction sites using the QuikChange mutagenesis protocol (Stratagene, La Jolla, CA, USA). The SpeI site was located at the end of *NS5A*, approximately 20 nucleotides upstream of the start of *NS5B*, resulting in an alanine-to-threonine alteration, which has no effect on replicon replication efficiency or compound susceptibility. The SnaBI site was created 6 bp downstream of the *NS5B* stop codon. For GT 1a, a 1a shuttle replicon with unique restriction sites SpeI and ClaI was generated.

Patient sera were obtained from Cliniqa Corporation (Fallbrook, CA, USA) for GTs 1a and 1b and from Boca Biolistics (Coconut Creek, FL, USA) for GTs 2 to 5. GT 6 patient sera were obtained from SeraCare Life Sciences (Milford, MA, USA) or provided by Huy Trinh. Viral RNA was isolated using a QIAamp MinElute virus vacuum kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. First-strand cDNA synthesis was performed using random primers and the Superscript III reverse transcriptase (RT) kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. *NS5B* regions were amplified using degenerate primers designed by examination of published HCV *NS5B* sequences. Patient PCR products were sequenced and used to replace the *NS5B* gene of the GT 1a shuttle replicon for GT 3a and the GT 1b shuttle replicon for GTs 2b to 6 using standard cloning techniques. In addition, for HCV GTs 2b, 4a, and 5a, patient-derived *NS5B* sequences known to generate viable chimeric replicons (37) were synthesized by DNA 2.0 and cloned into the GT 1b shuttle replicon.

To generate stable cell lines, replicon clones were linearized with restriction enzymes and transcribed *in vitro* using the Promega T7 RiboMax RNA production kit (Madison, WI, USA) according to the manufacturer's directions. Transcribed RNA (5 μg) was electroporated into $5 × 10^6$ Huh-7 cells, and after 24 h, selective medium containing 0.25 mg/ml G418 was added. Medium was changed every 3 to 5 days. After approximately 3 weeks of selection, individual colonies were expanded for further analysis.

Cell culture assays. To evaluate antiviral activity, HCV replicon cells were incubated in 96-well plates in the presence of compound for 3 days. For replicons containing a luciferase reporter gene, *Renilla* luciferase activity was then assayed using a *Renilla* luciferase assay system or a Dual-Glo luciferase assay system (Promega Corporation, Madison, WI, USA), according to the manufacturer's directions. Plates were read on a Top-

Count NXT microplate scintillation and luminescence counter (Packard Instrument Company, Meriden, CT, USA). The antiviral activity of test compounds, expressed as the 50% effective concentration (EC_{50}), was determined as described previously ([38]). To study the effect of human serum (HS) on compound efficacy, the standard 10% fetal bovine serum in cell culture experiments was supplemented with 40% human serum.

To evaluate selectivity and cytotoxicity, BVDV assays were performed as described previously ([31]). Susceptibility of HIV, herpes simplex virus (HSV), influenza virus, and canine parainfluenza virus (CPIV) was determined by incubation with serial dilutions of compound. For recombinant HIVs expressing *Renilla* luciferase, antiviral activity was evaluated by measuring the production of luciferase in infected cells 5 days postinfection. For the CPIV and influenza virus assays, viral neuraminidase activity was used as a measure of viral production. Susceptibility of HSVs to compounds was determined using a multicycle HSV growth assay. For human coronavirus, poliovirus, coxsackie virus, and rhinovirus, MRC5 cells were infected at a multiplicity of infection of 0.1, incubated for 5 days with or without dilutions of compound, and then treated with alamarBlue to quantitate protection and cytotoxicity. To evaluate cytotoxicity for all other cell types, cells were incubated in the presence of serially diluted compounds for 3 to 4 days and cell viability was quantitated using either an XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide] assay for MT-2 cells or an alamarBlue assay for Huh-7, MDBK, and Vero cells. All 50% cytotoxic concentration (CC_{50}) values were calculated using the median effect equation.

Transient replication assays. Replicon clones were linearized with ScaI and transcribed *in vitro* using the Ambion T7 MegaScript kit (Ambion, Austin, TX, USA) according to the manufacturer's directions. Transcribed RNA (3 to 5 μg) was transfected into cured Huh-7 cells ($\sim 2 \times 10^6$ cells in 60-mm dishes) with DMRIE-C reagent (Invitrogen) according to the manufacturer's protocol. After 4 to 6 h, transfected cells were transferred to 96-well assay plates (10^4 cells/well) and incubated in the presence of inhibitors for 72 h. *Renilla* luciferase assays were performed as described above.

Resistance selection and analysis. For selection of BMS-791325-resistant replicons, HCV GT 1a and 1b replicon cells were passaged in medium containing 0.5 mg/ml G418 and BMS-791325 at a concentration of 5 to 20 EC_{50}s (up to 0.2 μM for GT 1b and 0.1 μM for GT 1a). Cells were selected with dimethyl sulfoxide (DMSO) in parallel as a control. Cells were split or fed with fresh medium containing DMSO or BMS-791325 twice weekly to maintain a subconfluent monolayer. After approximately 5 weeks, selected cells were expanded for resistance testing and reverse transcription-PCR (RT-PCR) analysis. RNA was isolated from populations of resistant cells using TRIzol (Invitrogen Corp., Carlsbad, CA, USA) in accordance with the manufacturer's directions. First-strand cDNA synthesis was performed on 1 to 3 μg of total RNA using Superscript III reverse transcriptase (Invitrogen) primed with random hexamers. PCR was performed on the cDNA using pairs of primers flanking the *NS5B* gene. PCR products were sequenced, and mutations were identified relative to DMSO-treated control populations. For clonal analysis, PCR products were purified and cloned using TOPO PCR cloning methods (Invitrogen).

To generate drug-resistant replicons for analysis, point mutations were introduced with the QuikChange II XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions and confirmed by sequencing.

Cell-based inhibitor combination assays. For combination studies, inhibitors were each tested at 11 concentrations. The compounds were tested as monotherapies and in combinations at various concentration ratios. A defined set of drug concentrations and ratios was achieved in a final concentration of 0.5% DMSO. The final drug concentration range for each compound as monotherapy was as follows: 1.7×10^{-5} to 1 μM for BMS-791325, 1.7×10^{-5} to 1 nM for DCV, 4×10^{-6} to 0.25 μM for ASV, 4.2×10^{-5} to 2.5 μM for HCV-796, 0.017 to 1,000 U/ml for IFN-α, 8×10^{-3} to 500 μM for ribavirin, and 8×10^{-4} to 50 μM for BMS-

790453. For individual drug studies (with the exception of ribavirin), the EC_{50} was selected as the midpoint for the concentration range tested. When the compound was used in double combinations at a ratio of 2.5 relative to the second inhibitor, the concentration range of the compound was increased by 2.5-fold. Cells were exposed to compounds for 3 days, and the amount of HCV inhibition was then determined using the Dual-Glo luciferase assay system as described above. The potential cytotoxicities of these combined agents were also analyzed in parallel by alamarBlue staining. The degree of antagonism, additivity, or synergy was determined from combination dose-response curves, which were fitted to assess the antiviral effects of the drug treatment combinations. The concentration ratios were analyzed using the method of Chou ([39]). All estimates were computed using SAS Proc NLIN biostatistical software and a four-parameter logistic. Combination indices (50%, 75%, and 90% effective) were tested for departure from additivity using isobologram methods. Asymptotic confidence intervals were also calculated for each of the combination indices. These intervals are used to test for departure from additivity by comparing the bounds to 1: a lower bound of the interval greater than 1 indicates antagonism, an upper bound of less than 1 indicates synergism, and a value of 1 contained in the interval indicates additivity. Mixed results, where synergy is established for some combination indices and/or inhibitor ratios and additivity is established for others, are reported as "synergy/additivity."

HCV NS5B polymerase cloning, expression, and purification. The cDNAs encoding HCV NS5B proteins of GTs 1a (H77c), 1b (Con1), 2a (JFH-1), 2a (HC-J6), 2b (database consensus), 3a (database consensus), 4a (database consensus and two patient sequences), 5a (database consensus), and 6a (database consensus) were cloned into the pET21a expression vector (database consensus sequences are provided in the supplemental material). Each untagged protein was expressed with an 18-amino-acid C-terminal truncation that enhances polymerase solubility. The *Escherichia coli* competent cell line BL21(DE3) was used for expression of the protein. Proteins were purified using heparin-Sepharose and poly(U)-Sepharose chromatography as described elsewhere ([40]).

HCV NS5B polymerase assays. RNA synthesis was measured by detecting the incorporation of radiolabeled nucleotides. Regardless of the assay format used to measure enzyme activity, inhibition by BMS-791325 was detected as a decrease in the incorporation of radiolabeled nucleotides compared with an untreated control. One format of the polymerase reaction mixture contained pGpG primer (8.6 μM), homopolymeric C template (0.35 nM), NS5B enzyme (2.8 nM), and 1 to 5 μM GTP (depending on the genotype of the enzyme) plus [^{33}P]GTP substrate (1 μCi, 3,000 Ci/mmol; Amersham, GE Healthcare, Piscataway, NJ). After a $\geq$1-h preincubation of NS5B polymerase, template, and compound (serially diluted 1:3 in DMSO and transferred to 96-well assay plates [Corning] for a final DMSO concentration of 2%), RNA synthesis was initiated by the addition of primer and GTP. Reaction mixtures (total volume of 0.06 ml) were incubated at 30°C for 15 to 50 min, depending on the genotype of the NS5B polymerase tested. The newly synthesized RNA product was precipitated with 10% trichloroacetic acid (TCA), harvested onto 96-well GF/B filter plates (PerkinElmer, Shelton, CT, USA), and quantified on a Packard TopCount NXT gamma counter. Another format of the polymerase reaction has been described previously in detail ([40]). Briefly, the reaction mixture contained biotinylated oligo(dT)$_{12}$ primer precaptured on streptavidin-coupled polystyrene imaging beads (GE Healthcare) by incubating primer and beads in assay buffer (20 mM HEPES, pH 7.5, 2.5 mM KCl, 2.5 mM MgCl$_2$, 1 mM dithiothreitol [DTT], 1.6 U RNase inhibitor [Promega], 0.1 mg/ml bovine serum albumin [BSA] [Promega], 2% glycerol) at room temperature for 1 h. Unbound primer was removed with the supernatant after centrifugation. The primer-bound beads were suspended in 20 mM HEPES buffer, pH 7.5, and used in the assay at final concentrations of 15 nM primer and 0.33 mg/ml beads. Compounds were serially diluted 1:3 in DMSO and transferred to 384-well assay plates (Corning) to give a final DMSO concentration of 2%. The order of addition was 0.01 ml water, 0.01 ml enzyme (7 nM) diluted in assay buffer,

0.01 ml template (0.2 nM), and [³H]UTP (0.3 mCi, 0.29 mM); concentrations are final. After a preincubation period of 24 h, the polymerase reaction was initiated by the addition of primer-bound beads. Reactions were allowed to proceed overnight at 30°C and terminated by the addition of 0.01 ml EDTA (50 mM). After ≥15 min, plates were read on a LEADseeker multimodality imaging system (Amersham).

The 50% inhibition value (IC_{50}) was calculated using a four-parameter logistic equation:

$$Y = A + \frac{B - A}{1 + \left(\frac{C}{x}\right)^D}$$

where A and B denote minimal and maximal % inhibition, respectively; C is the IC_{50}; D is the Hill slope; x represents compound concentration; and Y is the signal observed.

Polymerase selectivity assays. The selectivity of BMS-791325 was evaluated with a panel of enzymes unrelated to HCV NS5B, including bovine polymerase α, human polymerase β and γ, BVDV polymerase, Klenow polymerase, HCV protease, HIV reverse transcriptase, and HIV integrase. Compounds were also tested for nonspecific DNA and RNA binding.

Bovine polymerase α (Chimerx, Milwaukee, WI, USA), human polymerase β (Chimerx), Klenow polymerase (New England BioLabs, Ipswich, MA, USA), and HIV-1 reverse transcriptase (Amersham Biosciences) were assayed by scintillation counting of ³³P-labeled, TCA-precipitated primer extension products essentially as described by the manufacturers.

Human polymerase γ was obtained from W. C. Copeland at the National Institute of Environmental Health Sciences (Research Triangle Park, NC, USA) and assayed essentially as described previously (41). Due to enzyme instability, no preincubation of enzyme and inhibitor was performed. The assay measures the incorporation of radiolabeled [³H]TTP. The reaction mixture contains 0.25 μg poly(A) template, biotinylated dT primer bound to streptavidin scintillation proximity assay (SPA) beads, [³H]dTTP, and 10 ng human polymerase γ in a reaction volume of 35 μl.

BVDV polymerase was isolated and assayed as described for HCV NS5B, except that the assay concentration of unlabeled GTP was increased to a final concentration of 20 μM.

HIV integrase was assayed by strand transfer activity of purified integrase enzyme as described previously (42).

DNA binding was tested in a competition assay using the fluorescent ligand Picogreen (Molecular Probes, Invitrogen). Compounds titrated in DMSO were incubated in 50 mM Tris buffer (pH 7.4) in the presence and absence of 10 μg/ml DNA (1-kb ladder from Invitrogen) for 30 min at room temperature. The final concentration of DMSO was 2%. Picogreen commercial stock was diluted 1 to 200, and 50 μl was added to each well. Plates were read with a Gemini fluorescence plate reader (485-nm excitation and 530-nm emission).

RNA binding was tested according to the protocol described for a 96-well format in a Ribogreen kit (Molecular Probes, Invitrogen).

In vivo exposure studies. Plasma and liver tissue exposure to BMS-791325 was assessed in rats, dogs, and monkeys. Male Sprague-Dawley rats (280 to 330 g; $n = 18$; Hilltop Lab Animals, Inc., Scottdale, PA, USA) were administered BMS-791325 (10 mg/kg of body weight) in polyethylene glycol 400 (PEG-400) by oral gavage, and blood and liver samples were obtained at 10 min, 30 min, and 1, 2, 4, 6, 8, 24, and 48 h postdose (two rats per time point). Male beagle dogs (≈9 to 12 kg; $n = 6$; Marshall Farms USA Inc., North Rose, NY, USA) were orally dosed with BMS-791325 (3 mg/kg) in a mixture of PEG-400 (95%, vol/vol), povidone K-30 (2%), *d*-α-tocopherol polyethylene glycol succinate (TPGS; 2%), and Tween 80 (1%), and blood and liver samples were obtained at 1, 4, 6, 24, 48, and 72 h postdose (one dog per time point). Male cynomolgus monkeys (2.5 to 4 kg; $n = 4$; Charles River Biomedical Research Foundation, Houston, TX, USA) were orally administered BMS-791325 (3 mg/kg) in a mixture of sodium phosphate buffer (0.1 M; 93%, wt/wt), hydroxypropylcellu-

lose-SL (5%), and TPGS (2%), and blood and tissue samples were taken at 1, 4, 8, and 24 h postdose (one monkey per time point). Vehicles were optimized for each species based on both specific dosing volume and tolerability requirements and optimization of formulation properties as the compound proceeded through pharmacokinetic testing. All animal procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of the test facility. Plasma samples were stored at −20°C until analyzed. Liver samples (approximately 2 to 3 g per animal) were rinsed, blotted dry, weighed, and stored frozen. BMS-791325 was assayed in plasma and tissue using a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) methodology using standard curves fitted by weighted (1/concentration²) linear regression over the range of 2 to 10,000 nM. Liquid chromatography-tandem mass spectrometry conditions were as follows. We used a Cohesive Aria high-pressure liquid chromatography (HPLC) system consisting of 8 Shimadzu LC-10ADvp pumps with 2 SCL-10Avp system controllers (Columbia, MD) and a dual-arm CTC Analytics HTS PAL autosampler (Switzerland) equipped with a cooling stack that maintained samples at 10°C during analysis. The online extraction column used was a Cyclone-P mixed polymer column (0.5 mm by 50 mm, 60 μM particles; Cohesive Technologies, Franklin, MA) at room temperature. The analytical column used was a Supelguard Ascentis C_{18} column (3.0 mm by 20 mm, 5 μM particles; Supelco, Bellefonte, PA) at room temperature. The mobile phase for the online extraction consisted of 10 mM ammonium acetate in water (A) and 40/40/20 acetonitrile/isopropanol/acetone (B) and was delivered as a gradient at a flow rate of 2.0 ml/min to the turbulent-flow chromatography (TFC) extraction column. The mobile phases for the analytical column consisted of 0.1% formic acid in water (A) and 0.1% formic acid acetonitrile (B) and were delivered as a gradient at a flow rate of 1.5 ml/min. The TFC column was interfaced with an API4000 LC-MS/MS system (AB Sciex Instruments, Toronto, Canada) equipped with an Ionspray ionization interface operating in the positive ionization mode. The source temperature was 600°C. Detection of the analyte was achieved through selected reaction monitoring. Standards were analyzed in duplicate. Quality control (QC) samples in a blank biological matrix at concentrations within the standard curve were analyzed as replicates within each analytical set. The predicted concentrations of more than 83.7% of the QCs from various different matrices were within 20% of nominal values, indicating acceptable assay performance. Standard pharmacokinetic parameters (24-h concentration [C_{24}] and area under the concentration-time curve at 24 h [AUC_{24}] or extrapolated to infinity [AUC_{inf}]) were derived from concentration-time data using noncompartmental methods in KINETICA version 4.2 (InnaPhase Corporation, Philadelphia, PA, USA).

RESULTS

BMS-791325 antiviral activity, cytotoxicity, and specificity for HCV NS5B. The *in vitro* potency, specificity, and therapeutic index of BMS-791325 were evaluated in a series of experiments testing antiviral activity against purified enzymes, replicons, and a panel of RNA and DNA viruses in cell culture. In addition, a number of cell lines derived from various tissue origins were used to assess its cytotoxicity.

BMS-791325 demonstrated potent and selective inhibition of HCV GT 1a and 1b replicons, with EC_{50}s of 3 nM and 6 nM, respectively (Table 1). It did not significantly affect the replication of a panel of other RNA and DNA viruses, including the closely related BVDV, with EC_{50}s of >4 μM, whereas control inhibitors for each virus gave the expected EC_{50}s (Table 1). BMS-791325 cytotoxicity (CC_{50}) was ≥14 μM in a panel of cell lines representing liver, T lymphocytes, lung, and kidney following incubation for 3 to 4 days (Table 2). Its antiviral activity against HCV (GT 1b; EC_{50}, 6 nM) in Huh-7 replicon cells was more than 3,000-fold below cytotoxic concentrations.

The effect of human serum binding on antiviral activity was

TABLE 1 Antiviral activity and selectivity of BMS-791325 in cell culture assays

Replicon/virus[a]	Cell line	EC$_{50}$ (μM)	Selectivity index[b]
HCV replicon 1a (H77c)	Huh-7	0.003 ± 0.001[c]	
HCV replicon 1b (Con1)	Huh-7	0.006 ± 0.002[c]	
BVDV replicon	Huh-7	>14	>2,333
BVDV	MDBK	11	1,833
HIV	MT-2	>14	>2,333
HSV-1 and -2	Vero	>4	>667
Influenza virus	MDBK	>29	>4,833
CPIV	Vero	>32	>5,333
Human rhinovirus	MRC5	>16	>2,667
Coxsackie virus	MRC5	>16	>2,667
Poliovirus	MRC5	>16	>2,667
Human coronavirus	MRC5	>16	>2,667

[a] Positive controls run in the experiments: BVDV (EC$_{50}$ of UPS-1453, 0.6 μM), HIV (EC$_{50}$ of BMS-035, 1.5 nM), HSV-1 and -2 (EC$_{50}$s of acyclovir, 0.08 and 0.31 μM, respectively), influenza virus (EC$_{50}$ of BMS-659, 9 μM), CPIV (EC$_{50}$ of BMS-011, 0.1 μM), human rhinovirus (EC$_{50}$ of rupintrivir, 9 nM), coxsackie virus (EC$_{50}$ of rupintrivir, 135 nM), and poliovirus (EC$_{50}$ of rupintrivir, 82 nM). Compounds BMS-035, BMS-659, and BMS-011 are Bristol-Myers Squibb proprietary compounds that inhibit HIV, influenza virus, and CPIV, respectively.
[b] EC$_{50}$ for virus or replicon/EC$_{50}$ for HCV replicon 1b.
[c] Means ± standard deviations from two or more independent experiments.

evaluated. Results showed a modest 4.4-fold decrease in susceptibility in GT 1b replicons supplemented with 40% human serum during the assay period relative to unsupplemented controls.

BMS-791325 inhibitory activity was also highly selective for the HCV NS5B polymerase when tested against a panel of unrelated microbial and mammalian enzymes (Table 3). The IC$_{50}$ against isolated HCV NS5B (GT 1b) was 3 nM, more than 8,000-fold lower than that for any other polymerase tested or for HIV-1 integrase. In contrast, control inhibitors used for each assay inhibited the enzymes with the expected IC$_{50}$s (Table 3). In addition, BMS-791325 did not display any nonspecific DNA or RNA binding (IC$_{50}$, >20 μM) in competition assays against sensitive nucleic acid-binding probes (Table 3).

Genotype-specific activity of BMS-791325. The sequence divergence among the major HCV genotypes is significant (>30%) (2) and may result in substantial intergenotype variability in the activity of an inhibitor. Similarly, sequence diversity and the adaptive potential of the virus under *in vivo* selective pressures may result in intragenotype variability between clinical isolates. Since replicon culture systems are not available for all six of the major HCV genotypes, the cross-genotype activity of BMS-791325 was investigated by evaluating antiviral activity against hybrid replicons bearing clinical NS5B sequences and enzymatic inhibition of purified NS5Bs from GTs 1 to 6.

The results of the two sets of experiments show similar profiles

TABLE 2 Cytotoxicity of BMS-791325 in cell culture assays

Cell line	Tissue	CC$_{50}$ (μM)[a]
Huh-7	Liver	20, 26
Vero	Kidney	29, 32
MDBK	Kidney	30, 43
MRC5	Lung fibroblasts	16, 18
MT-2	T lymphocytes	14, 14

[a] Results from two independent experiments.

TABLE 3 *In vitro* enzyme inhibition and nucleic acid binding by BMS-791325

Enzyme or assay type[a]	BMS-791325 IC$_{50}$ (μM)[b]
HCV NS5B 1b (Con1)	0.003
Bovine polymerase α	>25
Human polymerase β	>25
Human polymerase γ	>25
BVDV polymerase	>25
HIV reverse transcriptase	>25
Klenow polymerase	>25
HIV integrase	>40
DNA binding	>25
RNA binding	>20

[a] Positive controls were run in each experiment: mammalian DNA polymerases (IC$_{50}$ of ddTTP, 5 to 20 mM), BVDV polymerase (IC$_{50}$ of BMS-514, 6.3 ± 2.6 mM), HIV RT (IC$_{50}$ of Sustiva, 0.017 ± 0.017 mM), Klenow polymerase and nucleoside triphosphate binding (IC$_{50}$ of actinomycin D, 1.9 ± 0.4 mM), and HIV integrase (IC$_{50}$ of BMS-035, 0.004 ± 0.001 mM). Compounds BMS-514 and BMS-035 are Bristol-Myers Squibb proprietary compounds that inhibit BVDV polymerase and HIV integrase, respectively.
[b] From two or more independent experiments.

of genotypic coverage by BMS-791325 (Table 4). For clinical NS5B sequences in hybrid replicons, EC$_{50}$s ranged from 1.6 to 5.3 nM for GT 1a and from 3.5 to 9.5 nM for GT 1b, similar to values obtained from the H77c and Con1 lab strains (3.2 nM and 6.0 nM, respectively). The GT 1a patient-derived NS5Bs were 97 to 98% identical to H77c NS5B at the amino acid level, whereas the GT 1b NS5Bs were 96 to 97% identical to Con1 NS5B. This level of conservation is similar to that observed among other HCV GT 1a and 1b NS5B database sequences. The H77c and Con1 NS5Bs are only 88% identical, yet BMS-791325 shows similar potencies on GT 1a and 1b, suggesting that BMS-791325 should exhibit good activity against HCV GT 1-infected patients. BMS-791325 also displayed potency similar to that for GT 1 against hybrid replicons containing clinical NS5B sequences from GTs 3a, 4a, and 5a (EC$_{50}$s, 0.8 to 18.0 nM). GT 2 replicons, however, were approximately 1 to 2 orders of magnitude less susceptible to BMS-791325 than were the other genotypes evaluated. BMS-791325 exhibited high potency against GT 6a hybrid replicons generated from two patients (pt-752 and pt-tt003), with EC$_{50}$s (8.6 nM and 9.7 nM) similar to those observed for GT 1. However, a GT 6a replicon generated from a third patient (pt-hn001) was approximately 9-fold less susceptible to BMS-791325.

Consistent with the replicon results, *in vitro* polymerase assays gave BMS-791325 IC$_{50}$s that ranged from 1.8 nM to 27.1 nM for GT 1, 3, 4, and 5 NS5B and were approximately 60 nM for GT 6 and 164 to 228 nM for GT 2a and GT 2b. The good concordance observed between BMS-791325 potency in the *in vitro* polymerase and cell-based replicon assays confirms that inhibition of viral RNA replication is indeed due to inhibition of NS5B polymerase activity. Results from both the enzyme and cell-based assays also indicate that BMS-791325 is a potent inhibitor of GTs 1a, 1b, 3a, 4a, and 5a with variability observed in potency against GT 6 sequences.

Selection of resistance *in vitro*. To gain insight into the mechanism of BMS-791325 resistance, the HCV replicon system was used to select resistance-associated substitutions in NS5B that may emerge during treatment. Passage of replicon cells in the presence of BMS-791325 resulted in significant decreases in susceptibility to BMS-791325 versus cocultured controls treated with

TABLE 4 BMS-791325 activity against major HCV genotypes in patient-derived chimeric replicons and in *in vitro* NS5B polymerase assays

NS5B genotype	Isolate	Chimeric replicon EC$_{50}$ (nM)^{a,b}	Enzyme IC$_{50}$ (nM)^a
1a	H77c	3.2 ± 2.4	3.3 ± 1.1
	pt-5-22	1.6 ± 0.3	ND^h
	pt-6-14	5.3 ± 2.3	ND
	pt-8-3	4.3 ± 1.5	ND
	pt-2-1	4.5 ± 2.1	ND
1b	Con1	6 ± 1.5	4.2 ± 2.1
	pt-7-4	3.5 ± 0.7	ND
	pt-10-5	9.5 ± 0.7	ND
2a	JFH-1	87 ± 2.8	165 ± 133
	J6	498 ± 229	228 ± 72
2b	Patient consensus^c	ND	164 ± 39
	pt-H6	480 ± 327	ND
	pt-308	>1,000	ND
3a	Patient consensus^d	ND	1.8 ± 0.1
	pt-002	4.7 ± 1.5	ND
	pt-007	3.5 ± 2.1	ND
	pt-341	9.5 ± 3.5	ND
	pt-342	7.5 ± 0.7	ND
4a	Database consensus^e	ND	19.9 ± 9.6
	pt-H2	6 ± 2.9	ND
	pt-A	3 ± 0.6	7.6 ± 1.9
	pt-B	18 ± 2.5	27.1 ± 13.1
5a	Database consensus^f	ND	4.8 ± 2.4
	pt-h3	4.3 ± 0.6	ND
	pt-010	0.8 ± 0.3	ND
	pt-011	1.2 ± 0.7	ND
	pt-019	2.8 ± 1.7	ND
6a	Patient consensus^g	ND	61.6 ± 30.3
	pt-752	8.6 ± 2.5	ND
	pt-tt003	9.7 ± 3.5	ND
	pt-hn001	79.5 ± 24.5	ND

^{a} Mean (± standard deviation) from three or more independent experiments.
^{b} Antiviral activity against HCV GTs 1, 3, 4, 5, and 6 was 250- to 25,000-fold below cytotoxic concentrations, while activity against GT 2 was <20- to 230-fold below cytotoxic concentrations.
^{c} Consensus from chimp infected with patient serum HC-J8 (97.4% identical to European HCV database GT 2b consensus).
^{d} Consensus from chimp infected with patient serum S52 (97.8% identical to European HCV database GT 3a consensus).
^{e} Synthesis based on previous GenBank consensus from 11 sequences (99.8% identical to European HCV database GT 4a consensus).
^{f} Synthesis based on previous GenBank consensus from 5 sequences (98.3% identical to European HCV database GT 5a consensus).
^{g} Consensus from patient serum (97.2% identical to the current European HCV database GT 6a consensus).
^{h} ND, not determined.

DMSO. Selection at 10× wild-type EC$_{50}$ (40 nM and 70 nM for GTs 1a and 1b, respectively) generated GT 1a replicon cells with EC$_{50}$s 34- to 46-fold greater (136 to 184 nM) than that of control and GT 1b cells with EC$_{50}$s 25- to 28-fold greater (175 to 196 nM). After selection at 20× wild-type EC$_{50}$ (80 nM and 140 nM for GTs 1a and 1b, respectively), GT 1a elevations (34- to 35-fold; 136 to 140 nM) were similar to those observed at 10×, whereas GT 1b

TABLE 5 Transient replication assays of BMS-791325 resistance-associated NS5B variants selected *in vitro*

Variant	Genotype 1a			Genotype 1b		
	EC$_{50}$ (nM)^a	Fold change^b	Replication efficiency^b	EC$_{50}$ (nM)^a	Fold change^b	Replication efficiency^b
Wild type	5.0 ± 0.6		1.0	8 ± 0.5		1.0
P495A	59 ± 5	12	0.7	115 ± 29	14	0.4
P495S	336 ± 36	67	0.2	350 ± 57	44	0.1
P495L	154 ± 25	31	0.3	290 ± 111	36	0.1
P495T	293 ± 124	59	0.1			
L392I^c		5–7	0.1–0.4			

^{a} Mean ± standard deviation from two or more independent experiments.
^{b} Versus wild type.
^{c} Data from work of Pelosi et al. (38).

elevations were somewhat higher (34- to 40-fold; 238 to 280 nM) than that at 10×.

In this study, genotypic analysis of the selected cells identified substitutions conferring resistance to BMS-791325 only at a single residue of NS5B (P495) for both GT 1a and GT 1b. The substitutions P495A, P495S, and P495L were common to the two genotypes, with an additional P495T substitution observed in GT 1a only. Each change, introduced to a wild-type replicon backbone by site-directed mutagenesis, resulted in various degrees of BMS-791325 resistance in transient replication assays (Table 5). All substitutions conferred in excess of a 10-fold loss of susceptibility versus the wild type. In general, the relative replication efficiency of the substituted replicons compared with the wild type was inversely correlated with the degree of resistance (Table 5). No change in susceptibility to asunaprevir, daclatasvir, HCV-796, or NM-283 was observed for any P495 substitution in transient assays of the GT 1a replicon (data not shown).

Naturally occurring variations at three positions in NS5B that are potentially associated with BMS-791325 resistance were examined in HCV GTs 1 to 6 in sequences from the European HCV database (http://euhcvdb.ibcp.fr/euHCVdb/). The three loci were as follows: P495 substitutions, as observed in these experiments; L392I, observed previously in GT 1a under *in vitro* selection and conferring low-level (5- to 7-fold) loss of susceptibility (38); and V494A, also observed in currently unpublished data to be associated with low-level (2- to 3-fold)-reduced GT 1a susceptibility to BMS-791325.

As shown in Table 6, the predominant amino acid associated with BMS-791325 resistance, residue P495, was conserved in all sequences for GTs 1 to 5 and in 95% of GT 6a sequences. Likewise, L392 was highly conserved (>92%) across all genotypes except GT 2, where isoleucine was present at this position in 87% and 96% of GT 2a and GT 2b sequences, respectively. An L392I substitution in GT 1a confers approximately 6-fold resistance to BMS-791325, suggesting that the naturally occurring I392 residue in GT 2 may contribute to the reduced BMS-791325 susceptibility observed. Another contributor is likely to be residue 494; V494 was 100% conserved across GT 1, GT 4, and GT 5 sequences, but more than 95% of GT 2 sequences in the database carried A494. Interestingly, although V494 was not present in GT 3a sequences at all, the C494 variant in this genotype did not affect BMS-791325 susceptibility (EC$_{50}$s, 4 to 10 nM across four GT 3a patient isolates). In GT 6a sequences, the A494 variant was present in a significant minority of database sequences (21%) with V494 con-

TABLE 6 Prevalence in the European HCV database (http://euhcvdb .ibcp.fr/euHCVdb/) of polymorphisms at BMS-791325 resistance-associated loci

Genotype (n)	Residue/polymorphism at NS5B position (% of sequences):		
	392	494	495
1a (215)	L (97)	V (100)	P (100)
	F (2.5)		
	P (0.5)		
1b (368)	L (92)	V (100)	P (100)
	I (6)		
	F (2)		
2a (23)	I (87)	A (100)	P (100)
	L (9)		
	X (4)		
2b (27)	I (96)	A (96)	P (100)
	L (4)	V (4)	
3a (25)	L (100)	C (100)	P (100)
4a (15)	L (100)	V (100)	P (100)
5a (4)	L (100)	V (100)	P (100)
6a (19)	L (100)	V (79)	P (95)
		A (21)	L (5)

served in the remaining 79%. It is of note, therefore, that A494 was present in the NS5B sequence from the GT 6a patient isolate pt-hn001, discussed above, which demonstrated lower susceptibility to BMS-791325 in a hybrid replicon culture than did the other two GT 6a patient sequences evaluated.

BMS-791325 cross-resistance with other HCV inhibitor classes. BMS-791325 retained full activity against mutations conferring resistance to other HCV inhibitors (Table 7). BMS-791325 was as active against HCV GT 1b replicons carrying mutations known to convey drug resistance to active-site (nucleoside) NS5B inhibitors (S282T) (43), NS3 protease inhibitors (R155Q, A156V, and D168V) (44), or NS5A replication complex inhibitors

TABLE 7 BMS-791325 activity against drug-resistant genotype 1b replicons

Protein	Mutation	BMS-791325 EC$_{50}$ (nM)^a	Fold change (vs wild-type Con1)
NS5B polymerase	Wild-type Con1	5.0 ± 1.0	
	S282T	4.0 ± 1.4	0.8
	L392I	35 ± 12	7
	P495S	192 ± 21	38
	M423T	5.0 ± 1.0	1
	M414T	7.0 ± 3.0	1.4
	C316Y	2.0 ± 1.0	0.4
NS3 protease	R155Q	5.3 ± 2.2	1
	A156V	2.4 ± 0.4	0.5
	D168V	3.0 ± 0.2	0.6
NS5A	Y93H	3.8 ± 0.9	0.8
	L31V + Y93H	2.9 ± 0.6	0.6

^{a} Mean (± standard deviation) from two or more independent experiments.

TABLE 8 Summary of BMS-791325 activity in drug combination studies

Drug(s) in combination	Activity
IFN-α + RBV	Additive/synergistic
ASV	Additive
DCV	Synergistic/additive
HCV-796	Synergistic
NM-283	Synergistic/additive
DCV + ASV	Additive

(Y93H ± L31V) (30, 45) as it was against the wild-type replicon. BMS-791325 activity was also unaffected by resistance mutations to nonnucleoside NS5B inhibitors targeting thumb site 2 (M423T) (29), palm site 1 (M414T) (46), or palm site 2 (C316Y) (47) but was reduced by known mutations to other thumb site 1 inhibitors (L392I and P495S) (38, 48, 49). The lack of cross-resistance to inhibitors targeting other HCV proteins, or different sites on NS5B, supports the use of BMS-791325 in DAA combination regimens, including those using multiple polymerase inhibitors.

***In vitro* drug combination studies.** Monotherapy treatment of a highly mutable, rapidly replicating pathogen can result in the rapid emergence of drug resistance (50), which has led to the development of combination regimens as a successful strategy to prevent resistance-associated therapeutic failure. Since HCV therapy with the newer agents under development is likely to benefit from the combination approach, the antiviral activity of BMS-791325 in combination with representative agents against the major HCV drug targets was examined in GT 1a replicon cultures. BMS-791325 was evaluated in dual- and triple-drug combinations with ASV (NS3 protease inhibitor), DCV (NS5A replication complex inhibitor), HCV-796 (a palm site 2 nonnucleoside NS5B inhibitor), NM-283 (a nucleoside analog NS5B inhibitor), and (unpegylated) alfa-2a interferon plus RBV (Table 8; see also Tables S1 to S6 in the supplemental material). In two-drug combinations, the compound displayed at least additive interactions with all drugs and combinations tested and was synergistic with HCV-796 across all drug ratios. Three-drug combinations of BMS-791325 with DCV and ASV, or alfa-2a interferon and RBV, also yielded additive to synergistic interactions. No antagonism was observed in any of the combinations evaluated.

***In vivo* plasma and tissue exposures across species.** Plasma and liver concentrations of BMS-791325 were assessed in rats, dogs, and monkeys following oral dosing (Table 9). Rats dosed with BMS-791325 were monitored over 48 h, dogs were monitored over 72 h, and monkeys were monitored over 24 h. Liver contained the highest concentrations of compound across all species tested, although the liver-to-plasma ratios in dog, as determined either by concentration at the 24-h time point or using 24-h AUC values, ranged from 1.6- to 2.0-fold. Over the study duration, monkeys showed the highest liver-to-plasma ratios across the species, measured both by 24-h concentration (60:1) and by AUC (24:1). In all species, liver BMS-791325 concentration at 24 h postdose was more than 10-fold above GT 1, 3, and 5 replicon EC$_{50}$s; between 6.7- and 40-fold above GT 4 replicon EC$_{50}$s; and 1.5- to 14-fold above GT 6 replicon EC$_{50}$s.

DISCUSSION

BMS-791325 showed robust antiviral activity in an early single-ascending-dose study, with mean HCV RNA declines of approx-

TABLE 9 Liver and plasma 24-h concentrations and AUC values of BMS-791325 in nonclinical animal models[a]

Animal (dose) and pharmacokinetic parameter	Liver	Plasma	Liver/plasma ratio
Rat (10 mg/kg)			
C_{24} (μM)	0.63	0.05	12.2
AUC_{inf} (μM · h)	645.82	42.99	15
Dog (3 mg/kg)			
C_{24} (μM)	2.13	1.33	1.6
AUC_{inf} (μM · h)	152.92	78.16	2
Monkey (3 mg/kg)			
C_{24} (μM)	0.12	0.002	60
AUC_{24} (μM · h)	51.32	2.13	24.1

[a] Values were derived from one animal (dog and monkey) or two animals (rat) per time point.

imately 1.5 and 2.5 $\log_{10}$ IU/ml at 24 h after administration of a single 100-mg or 300-mg dose, respectively, as described in the accompanying paper (51). Here, we report the preclinical profile of BMS-791325 that supported its selection for clinical development and which demonstrates the correlation between *in vitro* and *in vivo* potency and resistance.

In these studies, BMS-791325 displayed highly selective, nanomolar inhibition of recombinant HCV NS5B polymerase *in vitro* for all major HCV genotypes, with much weaker activity observed against GT 2. This inhibition resulted in potent antiviral activity in cell-based subgenomic replicons of HCV expressing consensus and/or clinical NS5B sequences for GTs 1a, 1b, 3a, 4a, 5a, and 6a. The antiviral activity observed against multiple clones for GTs 1, 3, 4, and 5 suggests that baseline heterogeneity within these genotypes is likely to have minimal impact on response to BMS-791325. BMS-791325 genotype specificity appears to be typical for NS5B thumb site 1 inhibitors, as reduced GT 2 activity has also been observed for other compounds active at this site (37, 49, 52, 53). This loss of activity in GT 2 apparently results from specific differences in amino acid sequence (L392I, V494A, and V499A) and protein conformation within the binding pocket (49, 53). The distribution of GT 2 is global, accounting for 10 to 20% of HCV infections overall and 15% of infections in the United States, while GT 1, which is susceptible to inhibition by BMS-791325, accounts for more than 80% of all HCV infections.

For GT 6a, although the three patient-derived NS5B sequences were 97 to 98% identical at the amino acid level, two showed susceptibilities to BMS-791325 in hybrid replicons (9 to 10 nM) similar to those of other non-GT 2 sequences, while the third was approximately 9-fold less susceptible (80 nM). Reduced susceptibility was most likely associated with an NS5B-V494A polymorphism present in this patient's virus and in approximately one-fifth of GT 6a sequences in the European HCV database. Based on mean plasma trough concentrations of approximately 380 to 400 nM following 14 days or 12 weeks of treatment with 75 mg BMS-791325 twice daily in phase 2 studies (54)—which are above the GT 6a V494A replicon EC_{90} values—HCV GT 6a variants with baseline V494A would likely be suppressed at this dose.

In vitro selection of BMS-791325 resistance variants at NS5B amino acid 495 (proline) is also typical of thumb site 1 inhibitors (27, 48, 54–56). The substitutions observed (P495A/S/L/T) con-

ferred variable levels of resistance in GT 1a and 1b replicons, with the lowest (12- and 14-fold, respectively) observed for P495A and the highest (67- and 44-fold, respectively) observed for P495S. In previously described replicon experiments by Pelosi et al. (38), an L392I substitution in NS5B—conferring 6- to 16-fold elevations in BMS-791325 EC_{50}s—was observed in GT 1a under coselection with BMS-791325 and DCV. This substitution occurred in combination with DCV-resistance-associated substitutions in NS5A and was not seen under joint selection by BMS-791325 and the NS3 protease inhibitor ASV (38) or under selection by BMS-791325 alone either by Pelosi et al. or in these experiments. Substitutions at NS5B codon 495 were also seen by Pelosi et al. (38) in combination with the same NS5A resistance substitutions but not in combination with L392I. The L392I substitution may therefore represent an alternative pathway to thumb site 1 inhibitor resistance that is more readily selected under joint selective pressure with other DAAs such as an NS5A inhibitor.

The observation that the EC_{50} fold change and relative replication capacity associated with proline 495 variants were inversely correlated suggests that the genetic barrier associated with the development of higher levels of drug resistance at this key position may support reversion to the wild type when inhibitor selection is removed. Although amino acid 495 was the sole locus of NS5B resistance identified in these replicon studies, in which selection was examined at 10- to 20-fold EC_{50} levels, *in vivo* selection in virologic breakthroughs from an ongoing phase 2a study of BMS-791325 with pegylated alfa-2a interferon and RBV has shown treatment-emergent linked substitutions at positions 495 and 421 (A421V) (57). The A421V substitution elevated P495-associated phenotypic resistance in GT 1a (H77c) replicons by a factor of 3 without further reducing the replication capacity but conferred only minimal ($\approx$3-fold) levels of resistance itself (57). Although BMS-791325 did not select for A421V in these studies, this substitution was seen by Pelosi et al. in GT 1a replicons under selection with BMS-791325 either alone or in combination with DCV, but it was not linked with P495 variants (38). Further data will be needed to establish the significance of A421V as a possible compensatory mutation for P495 resistance and whether selection of the linked variants *in vivo* but not *in vitro* represents an effect of combination treatment or of *in vivo* selective pressure on whole-virion replication.

In vitro combination experiments showed that BMS-791325 has at least additive antiviral activity with representative agents from the major classes of oral direct-acting HCV antivirals, as well as with alfa-2a interferon and RBV. No antagonism was seen with any agent at any concentration or drug/drug ratio. These data are supportive of BMS-791325 in all-oral DAA regimens for GT 1 and also as the third agent in a mixed oral/parenteral combination with IFN-α/RBV. The synergistic antiviral activity seen with combinations of BMS-791325 and the palm site 2 NS5B inhibitor, HCV-796, demonstrates the potential value of approaching a single target with two inhibitors that bind to different sites and share no cross-resistance. The presence of three nonoverlapping allosteric inhibitor-binding sites on NS5B offers an opportunity for the sequential or combined use of nonnucleoside inhibitors in HCV infection, contrasting with the experience for HIV-1. Although nonnucleoside HIV-1 reverse transcriptase inhibitors have been important components of antiretroviral combination regimens since the introduction of nevirapine—the first inhibitor—in 1996, the presence of only a single allosteric site on the polymerase

results in significant cross-resistance between individual agents that severely limits their sequential use after virologic failure (58). The potential for synergistic HCV suppression with combinations of nonnucleoside NS5B inhibitors further emphasizes the flexibility of this class of agent, although such combinations have not been clinically studied and it remains unknown how such a convergent approach would impact the emergence of multidrug NS5B resistance.

A hepatotropic disposition *in vivo* is a potentially beneficial property for drugs active in liver-specific disease, simultaneously maximizing target organ levels and reducing peripheral exposure and the associated potential for off-target adverse events. The liver-to-plasma ratios observed for BMS-791325 in rats, dogs, and monkeys were similar, although lower in magnitude, to the highly hepatotropic ratios seen for the NS3 protease inhibitor ASV in these species (34). In the case of ASV, accumulation occurs chiefly through active transport by liver organic anion-transporting polypeptide (OATP) 1B1 and OATP 2B1 at physiologically relevant concentrations (59). For BMS-791325, the mechanism of liver sequestration and the relative contributions of active and passive uptake remain to be elucidated. Hepatic exposures in all three animal species at 24 h postdose were greater than the GT 1 replicon protein-adjusted EC_{90} values, indicating that effective oral dosing would be achievable in humans.

In conclusion, these preclinical data show strong and selective nanomolar inhibition of HCV GTs 1, 3, 4, 5, and 6 by BMS-791325, with a large therapeutic window between antiviral and cytotoxic concentrations, and a very low potential for interaction with human polymerases at therapeutic levels. The antiviral properties of this compound indicate its suitability for use with a variety of other HCV therapeutics in combination regimens. Phase 2 studies of BMS-791325 included its use with DCV and ASV in an all-oral regimen in GT 1 and GT 4 treatment-naive patients and GT 1 patients with prior null response to IFN-α/RBV (study AI443-014; clinicaltrials.gov ID NCT01455090) (60), and phase 3 studies of this 3-DAA regimen are ongoing.

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