Preclinical Profile and Characterization of the Hepatitis C Virus NS3 Protease Inhibitor Asunaprevir (BMS-650032)

Fiona McPhee,1# Amy K Sheaffer,1 Jacques Friborg,1 Dennis Hernandez,1 Paul Falk,1 Guangzhi Zhai,1 Steven Levine,1 Susan Chaniewski,1 Fei Yu,1 Diana Barry,1 Chaoqun Chen,1 Min S. Lee,1 Kathy Mosure,2 Li-Qiang Sun,3 Michael Sinz,2 Nicholas A. Meanwell,3 Richard J. Colonno,1* Jay Knipe,2 and Paul Scola3

Affiliation(s): Discovery Virology,1 MAP Discovery Support,2 and Department of Medicinal Chemistry,3 Bristol-Myers Squibb, Research and Development, Wallingford, Connecticut, USA

*Currently at Presidio Pharmaceuticals Inc., San Francisco, CA

#Corresponding author: Fiona McPhee, Bristol-Myers Squibb Research and Development, 5 Research Parkway, Wallingford, CT 06492. Tel: 203-677-7573. Fax: 203-677-0688. fiona.mcphee@bms.com.

Running title: Preclinical Characterization of Asunaprevir
Asunaprevir (ASV; BMS-650032) is a hepatitis C virus (HCV) NS3 protease inhibitor that has demonstrated efficacy in patients chronically infected with HCV genotype 1 when combined with interferon alfa and/or the NS5A replication complex inhibitor daclatasvir. ASV competitively binds to the NS3/4A protease complex, with $K_i$ values of 0.4 and 0.24 nM against recombinant enzymes representing genotypes 1a (H77) and 1b (J4L6S), respectively. Selectivity was demonstrated by the absence of any significant activity against the closely related GB virus-B NS3 protease and a panel of human serine or cysteine proteases. In cell culture, ASV inhibited replication of HCV replicons representing genotypes 1 and 4 with 50% effective concentration ($EC_{50}$) values ranging from 1 to 4 nM, with weaker activity against genotypes 2 and 3 ($EC_{50}$ 67 to 1,162 nM). Selectivity was again demonstrated by the absence of activity ($EC_{50} >12 \mu M$) against a panel of other RNA viruses. ASV exhibited additive to synergistic activity in combination studies with interferon alfa, ribavirin, and/or inhibitors specifically targeting NS5A or NS5B.

Plasma and tissue exposures in vivo in several animal species indicated that ASV displayed a hepatotrophic disposition (liver-to-plasma ratios ranging from 40- to 359-fold across species). Twenty-four hours post-dose, liver exposures across all species tested were >80-fold above the inhibitor $EC_{50}$ values observed using HCV genotype-1 replicons. Based on these virologic and exposure properties, ASV holds promise for future utility in a combination with other anti-HCV agents in the treatment of HCV-infected patients.
Hepatitis C virus (HCV) infects an estimated 170 million individuals worldwide (52). Primary infection is asymptomatic; however, most HCV infections progress to a chronic state that can gradually evolve into cirrhosis, liver failure, and/or hepatocellular carcinoma. In the United States, these clinical manifestations are the leading indication for liver transplantation (20).

Seven major genotypes and several subtypes exist based on the sequence heterogeneity of the HCV genome; their distribution, transmission, and disease progression differ substantially (25). While the distribution of genotypes 1 and 2 is global, genotype 3 predominates in Southeast Asia, genotype 4 in Africa and Egypt, genotype 5 in South Africa, and genotype 6 primarily in Hong Kong and Vietnam. In the United States, genotype 1 accounts for greater than 70% of all HCV infections (24). Although genotype does not predict the outcome of infection, different genotypes are associated with variable responses to interferon-based treatment options (17, 20, 29). Recently, the direct-acting antivirals (DAAs) boceprevir and telaprevir were approved for use in combination regimens with pegylated interferon alfa and ribavirin, resulting in enhanced sustained virologic response (SVR) rates (66 to 75%) in genotype 1 treatment-naive patients (22, 39). Although these regimens are more efficacious in patients infected with genotype 1, complicated three-times-daily dosing schedules and significant side effects, such as anemia, add further limitations to the well-documented tolerability issues associated with interferon-based regimens, and can lead to higher discontinuation rates or dose reductions (20, 32, 50). This highlights the need for more effective, better-tolerated therapeutic agents for HCV infection.
HCV is a Hepacivirus, and member of the Flaviviridae family (43) that comprise small, enveloped, single-stranded, positive sense RNA viruses (33). The 9.6-kb HCV RNA genome consists of one large open reading frame flanked on the 5’ and 3’ ends by untranslated regions important for efficient replication and translation of the polyprotein. The HCV polyprotein is processed into proteins that are essential for the virus replication cycle (2). The bi-functional NS3 protein consists of an N-terminal catalytic subunit that, when complexed with its activating cofactor NS4A, is required for cleavage of the polyprotein and subsequent viral replication (3, 41). Inhibition of proteolytic cleavage has been an effective approach for anti-HCV therapy since its identification (2). Efforts were aided early on by the availability of a crystal structure of the enzyme’s NS3/4A protease active site (1). Using the crystal structure in conjunction with computer-aided structure design, peptido-mimetic compounds were designed to occupy the enzyme active site, resulting in highly active, selective inhibitors of the HCV NS3/4A serine protease complex (7, 9, 27).

A structure-based drug discovery approach used computer-generated models of the tripeptide core of BILN-2061 (ciluprevir) (26) bound to the HCV NS3/4A protease complex (31). When the carboxylate moiety of a ciluprevir derivative was replaced with a proprietary cyclopropyl acylsulfonamide moiety, a significant enhancement in both intrinsic and cell activity was observed when compared with the equivalent carboxylate derivative (31). Lead optimization subsequently led to the identification of the selective NS3 protease inhibitor (PI) asunaprevir (ASV; BMS-650032) (Fig. 1) (45). Here we report the preclinical profile that supported the selection of ASV for subsequent clinical development.
MATERIALS AND METHODS

Compounds, cells, and viruses. ASV (N-(tert-butoxycarbonyl)-3-methyl-L-valyl-(4R)-N-((1R,2S)-
1{[(cyclopropylsulfanyl)amino]carbonyl}-2-vinylcyclopropyl)-4-{(4-methoxy-7-chloroisouquinolin-
1-yl)oxy]-L-prolinamide), daclatasvir, and the HCV NS5B polymerase inhibitor BMS-791325 were
synthesized by Bristol-Myers Squibb (BMS) (19, 23, 45). The NS3 PI telaprevir (VX-950 is used to
denote compound synthesized at BMS), the pyrimidine analog 2′-C-methylcytidine, the non-
nucleoside inhibitor HCV-796, and NM-107, the active component of the prodrug valopicitabine
(12), were synthesized at BMS (6). Recombinant human interferon α-2b (rIFNα, Intron A) was
from Myoderm Medical Supply (Norristown, PA). Ribavirin was from Sigma-Aldrich Co. (St.
Louis, MO). HCV serum samples of strains (H77c, J4L6S, HC-J6, HC-J8, SAS2, ED43, SA13, and
HK-6A) representing six major HCV genotypes and GB virus-B (GBV-B) were a gift from Jens
Bukh. Cell lines included human hepatoma cells, HuH-7 (Ralf Bartenschlager, University of
Heidelberg, Germany), HuH-7.5 (APATH, Brooklyn, NY), MT2 (NIH AIDS Research and Reference
Reagent Program), HepG2, HeLa, HEK293, and MRC5 (American Type Culture Collection; ATCC).
The attached cell lines HuH-7, HeLa, HEK293, and MRC5 were propagated in Dulbecco’s
modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS)
and 2 mM L-glutamine. Huh-7 cells that stably maintain HCV replicons were propagated as
subconfluent monolayers in DMEM containing 10% FBS, 2 mM L-glutamine, and 0.5 mg/ml
Geneticin (G418; Invitrogen Corp., Carlsbad, CA) (13, 45). MT-2 and HepG2 cells were
propagated in RPMI-1640 containing 10% heat-inactivated FBS and 2 mM L-glutamine. Human
rhinovirus serotype 2 (HRV2) and human coronavirus strain OC43 (HCoV OC43) were from
ATCC. HRV2 was propagated in HeLa cells; HCoV OC43 was propagated in MRC5 cells. The
proviral DNA clone of HIV NL4-3 was obtained from the NIH AIDS Research and Reference
Reagent Program.

**HCV NS3/4A protease genotype assays.** Published methods for construction, expression, and purification of recombinant full-length NS3/4A complexes representing HCV genotypes 1a (H77c) and 1b (J4L6S) (46) were applied to generate homogeneous full-length NS3/4A protease complexes representing the six major HCV genotypes (HC-J6, HC-J8, SS2, ED43, SA13, and HK-6A). The susceptibility of purified recombinant NS3/4A protease complexes was assessed using fluorescence resonance energy transfer (FRET) assays (47). The 50% inhibitory concentration (IC50) was generated as described previously (14).

**Enzyme-based selectivity assays.** To assess *in vitro* selectivity, compounds were counter-screened against GBV-B NS3/4A protease, human neutrophil elastase (Sigma), porcine pancreatic elastase (Sigma), human \( \alpha \)-chymotrypsin (Sigma), human cathepsin A (R&D Systems, Minneapolis, MN), and human liver cathepsin B (Calbiochem, San Diego, CA). Compounds were diluted in assay buffer in 10% dimethyl sulfoxide (DMSO). GBV-B NS3/4A protease (0.3 nM) replaced HCV-NS3/4A protease in the FRET-based assay, described above. All other assays were performed in 96-well plates with 1% DMSO and measured continuous substrate hydrolysis at 405 nm on a Spectramax (Molecular Devices, Sunnyvale, CA) plate spectrophotometer.

Human neutrophil elastase, porcine pancreatic elastase, and human pancreatic \( \alpha \)-chymotrypsin reactions contained 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 0.05% Tween-20, and 20 nM human, 25 nM porcine elastase, or 1 nM chymotrypsin. Reactions were initiated by addition of substrate (elastases, 5 \( \mu \)M succ-AAPV-AMC [Sigma]; chymotrypsin, 5 \( \mu \)M LLVY-AMC.
Cathepsin A assays were performed per manufacturer’s instructions with Mca-RPPGFSAFK(Dnp)-OH (R&D Systems) as substrate; reactions were monitored at Ex/Em 565/580 nm. Cathepsin B assays contained 50 mM NaOAC pH 5.5, 1 mM TCEP, 5 nM cathepsin B, and 2 μM Z-FR-AMC (Calbiochem) as substrate, and monitored at Ex/Em 360/460 nM. Factor XA assays contained 100 mM NaPO₄ pH 7.5, 0.5% PEG-8000, 200 mM NaCl, 1 nM human Factor XA (Enzyme Research Laboratories, South Bend, IN), and 400 μM S-2222 (Diapharma, West Chester, OH) as substrate. Factor XIA assays contained 50 mM HEPES pH 7.5, 0.1% PEG-8000, 5 mM KCl, 145 mM NaCl, 0.14 nM human Factor XIA (Haematologic Technologies, Essex Junction, VT), and 250 μM S-2366 (Diapharma) as substrate. Factor VIIA assays contained 50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM CaCl₂, 0.1% PEG-8000, 1.8 nM FVIIA (Novo Screen), and 1 mM S-2288 (Diapharma) as substrate. Kallikrein assays contained 50 mM Tris pH 7.5, 100 mM NaCl, 0.5% PEG-8000, 0.2 nM kallikrein (Enzyme Research Laboratories), and 400 μM S-2302 (Diapharma) as substrate. Thrombin assays contained 100 mM sodium phosphate pH 7.5, 200 mM NaCl, 0.5% PEG-8000, 0.2 nM thrombin (Enzyme Research Laboratories), and 200 μM S-2366 (Diapharma) as substrate. Trypsin assays contained 100 mM NaPO₄ pH 7.5, 200 mM NaCl, 0.5% PEG-8000, 0.332 nM trypsin (Sigma), and 200 μM S-2222 (Diapharma) as substrate.

Cell lines and viral constructs. The genotype 1b (Con1) replicon cell line was constructed as described previously (16). A replicon cell line representing genotype 1a (H77) was generated as described (14), except that mutations were introduced into the genes encoding the NS3 helicase domain (proline to leucine at position 1496) and NS5A (serine to isoleucine at position 2204) to improve replication in cell culture (21). A genotype 2a (JFH-1) cell line was generated.
(15) by introducing the JFH-1 sequence from NS3 to NS5B (51) into the genotype 1b (Con1) backbone. Recombinant HCV hybrid replicon clones, in which the NS3 protease domain (amino acids 1–181) from Con1 or JFH-1 replicons was replaced with NS3 protease sequences from HCV genotypes 2b (HC-J8) and 3a (S52), were generated as described (11). PCR primers for amplifying and inserting the 2b (HC-J8) NS3 protease sequence contained anchoring sequences for PCR-mediated restrictionless cloning into the 2a (JFH-1) replicon (forward oligonucleotide 5’ GCT CCC ATT ACT GCT TAC ACT CAG CAA AC 3’, reverse oligonucleotide 5’ CAC AGC TGG CGG CGT ACT GTT G 3’). Primers for inserting 3a (S52) NS3 sequences into a 1b (Con1) carrying two cell culture adaptive mutations (A1289G and S1560G) in the NS3 helicase were designed similarly (forward oligonucleotide 5’ CCT TTG AAA AAC ACG ATA ATA CCA TGG CGC CGA TCA CAG CAT ACG CCC AGC 3’, reverse oligonucleotide 5’ GGG ACG AGT TGT CCG TGA AGA CCG GAG ACC TAG CCT GTG TAA GGG 3’). To construct plasmids harboring consensus 2b (HC-J8) and 3a (S52) NS3/4A regions, first-strand cDNA was synthesized using random hexamers and a Superscript II First-Strand Synthesis System for RT-PCR (Life Technologies, Grand Island, NY).

The HCV genotype 2b NS3/4A gene was PCR-amplified using primers 5’-GTG AAG CTT ATG GCT CCC ATT ACT GCT TAC ACT CAG-3’ and 5’-GAT CCT GCA GTT AGC ATT CTT CCA TCT CAT CAA AG-3’, cloned into pCR2.1 (Life Technologies), and sequenced. A consensus NS3/4A sequence was assembled, and a HindIII-EcoRI fragment containing the NS3/4A sequence was cloned into pcDNA3.1P-Zeo (Life Technologies) creating construct p50A-1. The HCV genotype 3a NS3/4A gene was PCR-amplified using primers 5’-GCT CTA GAA GCT TAT GGC CCC GAT CAC AGC ATA CGC CCA-3’ and 5’-GAT CCT GCA GTT AGC ACT CCT CCA TCT CAT CAA AG-3’, cloned into pCR2.1 (Life Technologies), and sequenced. A consensus HCV genotype 3a NS3/4A clone was assembled creating
p50A-1 and p51-1 were used as templates for “touchdown” PCR amplification (Life Technologies) using Platinum Taq High Fidelity DNA polymerase. PCR products were purified using the DNA Clean and Concentrate kit (Zymo Research, Irvine, CA). Purified amplicon (2 µg) was used as a “mega” primer in a QuikChange II XL mutagenesis reaction (Agilent Technologies, Santa Clara, CA) using recombinant plasmids containing 2a (JFH-1) or 1b (Con1) replicon sequences as template. All recombinant NS3 hybrid replicon clones were confirmed by DNA sequencing.

HCV genotype 4a NS3 protease sequences (amino acids 1–181) from strain ED43 were PCR-amplified and cloned into the 1b (Con1) replicon shuttle vector (46), which includes a renilla luciferase (RLuc) reporter for quantifying HCV replication. Constructs were verified by sequence analysis and alignments were performed using Sequencher software (Gene Codes, Ann Arbor, MI). The genotype 4a NS3 protease sequence was amplified, cloned into the shuttle vector, and transformed into STBL-2 cells. DNA template was prepared and replicon RNA was transcribed from the T7 promoter for subsequent phenotyping in a transient HCV replicon assay (46).

A recombinant HIV NL-RLuc virus, in RLuc replaced a section of nef from NL4-3, was constructed by co-transfection of two plasmids: pNLRLuc and pVSVenv (4). The pNLRLuc contains the NL-RLuc DNA cloned into pUC18 at the PvuII site, while pVSVenv contains the gene for VSV G protein linked to an LTR promoter. Transfections were performed at a 1:3 ratio of pNLRLuc to pVSVenv on 293T cells using LipofectAMINE PLUS (Invitrogen), and the resulting pseudotype virus was titered in MT-2 cells. The bovine viral diarrhea virus (BVDV) luciferase replicon genome carrying an in-frame firefly luciferase reporter gene was described previously (36).
**Cell culture virology assays.** For HCV replicons containing an RLuc reporter, compound antiviral activities (50% effective concentration; EC₅₀), were determined as described (46). For replicons lacking a reporter gene, activity was measured using a FRET-labeled peptide substrate (10 μM), as described (46). BVDV assays were performed as described (36), except that incubations were maintained for 4 days.

Antiviral susceptibility of HIV, HRV2, and HCoV was determined by incubation with serial dilutions of compound. For recombinant HIV viruses expressing RLuc, antiviral activity was evaluated by luciferase assay 5 days postinfection using the Dual Luciferase kit (Promega). The diluted passive lysis solution was premixed with both the luciferase assay substrate and the Stop & Glo substrate (2:1:1 ratio). To each aspirated sample well, 40 μl of the mixture was added and luciferase activity was measured immediately on a Wallac TriLux (PerkinElmer, Waltham, MA). For determination of activities against HRV2 and HCoV OC43, compound and virus (multiplicity of infection: 0.1) were incubated with cells for 96 h, and cell protection was used as a measure of virus production. For the cell protection assay, 17 μl of Cell-Titer Blue reagent (Promega) was added to each well. Plates were then incubated for 2 h at room temperature before fluorescence was measured using a SpectraMAX Gemini EM instrument (Molecular Devices) set to Ex/Em 530/580 nm.

Cytotoxicity was determined by incubating cells (3,000–10,000 cells/well) with serially diluted test compounds or DMSO for 5 days (MT-2 cells) or 4 days (all other cell types). Cell viability was quantitated using an MTS assay for MT-2 or a Cell-Titer Blue reagent (Promega, Madison, WI) assay for HEK-293, HuH-7, HepG2, or MRC5 cells, and 50% cytotoxic concentration (CC₅₀)
values were calculated. For the HCV and BVDV replicon assays, CC50 values were determined from the same wells that were later used to determine EC50 values.

**Inhibitor combination studies.** For combination studies, inhibitors (rIFNα and inhibitors of NS3, NS5A, and NS5B) were each tested at 11 concentrations. Stock solutions, 200-times final assay concentrations, were prepared by 2- or 3-fold dilution in DMSO prior to addition to cells/media. Compounds were tested alone and in two- or three-drug combinations with ASV. HCV genotype 1b (Con1) replicon cells were exposed to compounds for 4 days and inhibition was determined using the FRET-labeled peptide assay (46). Cytotoxicity of combined agents was monitored in parallel by Cell-Titer Blue staining. Concentration–response curves were used to assess the degree of antagonism, additivity, or synergy at the 50, 75, or 90% effective level. Combination effects were analyzed using in-house software to determine combination indices and 95% confidence intervals, applying the definition of Loewe’s synergy cited by Chou (10). The analysis starts by fitting concentration–response curves to normalized responses from each therapy. These curves are fit as a four-parameter logistic model with a common minimum and maximum across all therapies. Conceptually, this equation can be written as:

\[
\text{response}_j = A + \frac{D - A}{1 + \left(\frac{c_1^j c_2^j c_3^j c_4^j c_5^j}{\text{concentration}_j}\right)^{x_3}_2^{x_2}_1^{x_1} g_3 g_2 g_1},
\]

where \( I_j = \begin{cases} 1 & \text{if therapy} = j \\ 0 & \text{otherwise} \end{cases} \)

for \( j = 1, 2, 3, 4, \) or 5.

In the above equation, response represents the normalized data. For this assay, \( A \) is the bottom plateau common to all curves, \( D \) is the common top plateau, \( B^1 \) is the “slope” parameter for the
jth therapy, and $C_j$ is the concentration that produces an effect equal to the average of $A$ and $D$ for the jth therapy. The concentration response curves are then inverted, and used to compute the combination index:

\[ CI_{\alpha} = \frac{\alpha[A]_B}{\alpha[A]} + \frac{\alpha[B]_A}{\alpha[B]} \]

In the above equation, for a given effective level, $\alpha$, $\alpha[A]$ and $\alpha[B]$ are the concentrations of drugs that produce that specific level of effect individually; and $\alpha[A]_B$ and $\alpha[B]_A$ represent the concentrations of drugs in combination that would produce the same level of effect.

Combination studies with ribavirin were carried out essentially as described previously, using MacSynergy II software developed by Prichard and Shipman (12). For each combination, six to eight replicate plates were analyzed per experiment. Antiviral activity was measured after 4 days in culture using an RLuc reporter expressed by the HCV genotype 1b replicon (16). A representative plate from each experiment was stained in parallel with Cell-Titer Blue reagents to assess cytotoxicity. Ribavirin concentrations that exhibited cytotoxic effects >15% (typically, concentrations of 45 $\mu$M and 135 $\mu$M) were excluded from antiviral analysis. As recommended by guidelines in the MacSynergy II user manual, values of synergy or antagonism under 25 $\mu$M$^2$/% at 95% confidence were considered insignificant, values over 100 indicated strong synergy, and values less than 100 indicated strong antagonism. In three-dimensional plots, an overall response below the 0% plane was considered to indicate antagonism, a response above indicated synergy, and a flat surface indicated additivity.
In vitro protocols for predicting permeability, metabolic stability, and protein binding.

Compound permeability was assessed by the non-cell-based Parallel Artificial Membrane Permeability Assay (PAMPA), as well as the cell-based Caco-2 cell model. PAMPA permeability assays were conducted in triplicate with 100 μM compound at room temperature for 4 h using pION® lipid solution, at pH 7.4 and 5.5. Caco-2 cells (ATCC) were seeded at 80,000 cells/cm² on a 24-well polycarbonate filter membrane and incubated at 37°C with 5% CO₂ and 95% relative humidity for approximately 21 days, with medium changes every 2 days. Culture medium consisted of DMEM with 10% FBS, 1% nonessential amino acids, 1% L-glutamine, 100 U/ml penicillin-G, and 100 µg/ml streptomycin. The assay was performed at pH 7.4 using Hank’s balanced salt solution containing 10 mM HEPES as transport medium. Studies were initiated by adding a buffer-containing compound to either the apical (A-to-B transport) or basolateral (B-to-A transport) side of the monolayer, and incubating for 2 h at 37°C. Compound transport was determined by liquid scintillation counting. Permeability coefficients (Pc) were determined using the equation: \( Pc = \frac{dA}{dt \cdot S \cdot C_0} \), where \( dA/dt \) is the flux of test compound across the monolayer (nmol/sec), \( S \) is the surface area of the cell monolayer (0.33 cm²), and \( C_0 \) is the initial concentration (μM) in the donor compartment.

In vitro metabolic stability/clearance was determined from duplicate 96-well reactions containing liver microsomes isolated from rat (Sprague–Dawley; BD Biosciences, Woburn, MA), dog (beagle; BD Biosciences), human (pooled samples; BD Biosciences), and monkey (cynomolgus; BD Biosciences). Assays contained 0.5 μM compound, 1% acetonitrile, 1 mg/ml protein, 100 mM potassium phosphate buffer pH7.4, and 6.7 mM magnesium chloride. Reactions were initiated by addition of 1 mM NADPH, incubated at 37°C for 0, 10, 20, 30, 40, or
60 min, and quenched by addition of two volumes of acetonitrile. Mixtures were centrifuged at
3000 × g in an Allegra X-12 centrifuge with an SX4750 rotor (Beckman Coulter Inc., Fullerton,
CA) at 10°C for 10 min; supernatants were analyzed using liquid chromatography (LC)/mass
spectrometry (MS)/MS. Several compounds were run in parallel as quality controls for low,
medium, and high clearances. Compound disappearance rates were determined by regression
analysis of concentration time profiles.

Protein binding was determined in fresh rat, dog, cynomolgus monkey, and human sera (pooled
samples from Bioreclamation Inc., Hicksville, NY). Protein binding was also determined in the
replicon assay medium (DMEM containing L-glutamine and 4% FBS), with and without 40%
human serum, using equilibrium dialysis (Dianorm Equilibrium Dialyser, Munich, Germany) in
0.1 M sodium chloride, 4 mM potassium chloride, 1 mM magnesium sulfate, and 30 mM
sodium phosphate, pH 7.4 and dialysis membranes (HTDialysis, LLC, Gales Ferry, CT) with a
12,000- to 14,000-Dalton MW cut-off. Dialysis cells (HTDialysis, LLC, Gales Ferry, CT) were
rotated at 20 rpm on an orbital shaker at 37°C with 10% CO₂ and 85% humidity. Protein binding
was determined at a nominal compound concentration of 10 µM (N=3). At 7 hours, buffer
samples (100 µl) were collected in a 96-well plate that contained 100 µl of species-specific
serum. Serum samples (100 µl) were collected in a 96-well plate that contained protein binding
buffer. A mixed-matrix solution contained 50% protein binding buffer and 50% blank serum of
the species under investigation and was used to prepare standard curves. Samples were
analyzed using LC/MS/MS and percent protein binding was calculated from the concentration
in buffer to that in serum.
In vivo exposure studies. The amorphous free acid form of ASV was used in all studies. Plasma and tissue exposures were characterized in male FVB mice (20–25 g; Harlan Breeding Laboratories, Indianapolis, IN), male Sprague–Dawley rats (300–350 g; Hilltop Lab Animals, Scottsdale, PA) with indwelling cannulae implanted in the jugular vein or common bile duct, male beagle dogs (ca 9–12 kg; Marshall Farms, North Rose, NY) bearing vascular access ports, and male cynomolgus monkeys bearing vascular access ports (3.1–5.7 kg; Charles River Biomedical Research Foundation, Houston, TX). All animal procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of the test facility. In all studies, coagulated blood samples taken after dosing were centrifuged at 4°C (1500–2000 \( \times \) g); plasma samples were stored at −20°C until analyzed. Tissue samples were rinsed, blotted dry, weighed, and stored frozen. ASV in plasma and tissue samples was analyzed by LC/MS/MS. The lower limit of quantification (LLOQ) of ASV was 1 nM in plasma samples of all species, 5 nM in mouse and rat tissues, and 50 nM in dog and monkey tissues.

Mice (N=9 per group, fasted overnight) received ASV by oral gavage (5 mg/kg; vehicle of PEG-400/ethanol, 9:1). Blood samples (~0.2 ml) were obtained by retro-orbital bleeding at 0.25, 0.5, 1, 3, 6, 8, and 24 h after dosing. Within each group, three animals were bled at 0.25, 3, and 24 h, three at 0.5 and 6 h, and three at 1 and 8 h, resulting in a composite pharmacokinetic profile. Livers and brains were also removed from mice at the terminal sampling points.

Rats (N=3 per group, fasted overnight) received ASV (amorphous free acid) by oral gavage (3, 5, 10, and 15 mg/kg) in PEG-400/ethanol (9:1). Serial blood samples (~0.3 ml) were obtained from the jugular vein at predose (0 h) and 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 24, and 48 h postdose.
assess tissue exposure, rats were orally administered ASV (5 or 15 mg/kg, same vehicle as above), and blood, liver, and heart samples from two rats/group were obtained at 0.17, 0.5, 1, 2, 4, 6, 8, 24, 48, and 72 h after dosing.

Dogs (N=3, fasted overnight) were administered ASV by oral gavage at 3 or 6 mg/kg (3 mg/ml in 85% PEG-400/15% water). Serial blood samples were collected from vascular access ports at 0.08, 0.167, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 24, and 48 h postdose. To assess tissue exposure, six male dogs (8.4–12.5 kg) were orally administered ASV (6 mg/kg) and blood, liver, and spleen samples were obtained from one dog each at 1, 3, 7, 24, 48, and 72 h postdose.

Monkeys (N=3 males, fasted overnight) were administered ASV by oral gavage at 3 mg/kg (3 mg/ml in 85% PEG-400/15% water). Blood samples were collected at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, and 24 h postdose. To assess tissue exposure, three male and six female cynomolgus monkeys (2–5 kg) were orally administered ASV (10 mg/kg), and blood, liver, and spleen samples were obtained from 1 male each at 2, 8, and 24 h postdosing and from 1 female each at 0.5, 2, 4, 8, 24, and 30 h postdosing.
RESULTS

Proteolytic enzyme activity. To assess the potential genotype spectrum coverage by ASV, enzyme IC$_{50}$ values against representatives of six major genotypes of HCV NS3/4A protease were determined (Table 1). These *in vitro* potencies were benchmarked against the NS3 PI VX-950 (Table 1), recently approved for use in a combination regimen with pegylated interferon alfa/ribavirin in patients infected with HCV genotype 1. ASV inhibited the NS3 proteolytic activity of genotype 1a (H77 strain) and genotype 1b (J4L6S strain), with IC$_{50}$ values of 0.7 and 0.3 nM, respectively. Against NS3/4A protease complexes representing genotypes 2 through 6, IC$_{50}$ values ranged from 0.9 to 320 nM. The *in vitro* potencies for VX-950 ranged from 12 to 537 nM against enzymes representing genotypes 1 through 6.

The mechanism of inhibition was established for ASV using purified recombinant full-length HCV NS3/4A protease complexes. The kinetic constants of the HCV NS3/4A proteases representing genotype subtypes 1a and 1b, measured against the FRET-labeled peptide substrate loosely representing the HCV NS4A/NS4B cleavage site (47), were as follows: $k_{\text{cat}}/K_m$ (rate of catalysis divided by Michaelis–Menten constant) = 0.9 ± 0.0 and 1.3 ± 0.5 μM$^{-1}$ s$^{-1}$, respectively. In order to determine the inhibition constant, $K_i$, apparent $K_m$ determinations for the FRET-labeled peptide substrate were calculated in the presence of increasing concentrations of ASV. Analysis of the data revealed that as concentrations increased, the apparent $K_m$ for substrate increased while the maximal enzyme turnover rate ($V_{\text{max}}$) remained constant, typical of a first-order competitive inhibitor. Slope replots from each of the resulting Lineweaver–Burk graphs were plotted against the compound concentration. The graph in Fig. 2 represents the data from a typical experiment. The average $K_i$ values for ASV were
approximately 0.4 nM and 0.2 nM for the HCV NS3/4A protease complexes representing genotype 1a and genotype 1b, respectively. This mechanism of inhibition differs from that reported for telaprevir. The acylsulfonamide of ASV interacts with the NS3 protease catalytic site in a non-covalent manner, whereas the α-ketoamide moiety of telaprevir covalently binds to the catalytic serine of NS3/4A protease and slowly reverses over time (38).

The substrate specificity determinant of proteases is generally defined by the side chains flanking the cleavage site (35). The naturally occurring trans-cleavage substrates for HCV NS3/4A protease exhibit a strict requirement for a cysteine residue in the P1 position of the scissile bond. Owing to the large number of proteases present in the body, selectivity is an important feature for PIs designed as therapeutic agents. ASV selectivity was subsequently determined by evaluating its inhibitory effect on a panel of purified recombinant proteases (Table 2). Reference enzymes that are structurally and/or mechanistically similar to the target enzyme or of clinical significance were chosen for the in vitro selectivity studies. ASV exhibited an excellent selectivity index (>40,000) against all serine/cysteine proteases evaluated, which included human leukocyte elastase (human “sputum” elastase), porcine pancreatic elastase, and three members of the chymotrypsin family of serine proteases, when compared with the IC₅₀ value against genotype 1a (H77c) NS3/4A protease. GBV-B belongs to the Flaviviridae family and is closely related to the human HCV. Although GBV-B and HCV only share approximately 30% amino acid homology within the NS3 protease domain, the catalytic triad and the S1 specificity determinant residue, a phenylalanine at residue 154, are conserved. In addition, GBV-B and HCV NS3/4A proteases have overlapping substrate specificities. Against purified recombinant GBV-B NS3/4A protease, ASV had a selectivity index of approximately 30,300 for
the HCV NS3/4A protease representing the genotype 1a H77c strain. These ASV selectivity indices compared favorably with those calculated for VX-950 against closely related human serine proteases and GBV-B versus its activity against the HCV genotype 1a NS3/4A protease (VX-950 selectivity index ≥140; Table 2).

**Antiviral activity in cell culture.** The activity of ASV against HCV RNA replication was measured using replicons representing the most clinically pertinent genotypes, 1a and 1b, as well as the genotype 2a JFH-1 strain (51). In addition, its effects against hybrid replicons encoding the NS3 protease domain representing genotypes 2b, 3a, or 4a were also monitored. Inhibition of HCV RNA replication by ASV was monitored using a luciferase reporter assay; however, comparable inhibitory effects could be determined by directly measuring HCV RNA or protein expression (data not shown) (36). A summary of ASV EC50 values generated using these assays is shown in Table 3. Against replicons encoding the NS3 protease domains representing of genotypes 1a, 1b, and 4a, EC50 values ranged from 1.2 to 4.0 nM. These compared favorably with the 427 to 1,137 nM values determined for VX-950. Against replicons encoding the NS3 protease domains of genotypes 2a and 3a, ASV exhibited significantly reduced activity (EC50 values ranging from 67 to 1,162 nM), similar to results obtained for VX-950 (EC50 values ranging from 108 to 2,731 nM) (Table 3).

ASV specificity was examined in different cell culture systems. ASV demonstrated no significant activity against BVDV, a pestivirus that is closely related to flaviviruses (48), and other RNA viruses at concentration exceeding 10 µM (Table 4). Against a panel of cell lines representing liver, T-lymphocytes, lung, cervix, and embryonic kidney, CC50 values ranged from 11 to 38 µM.
Inhibitor combination studies in the HCV replicon. The effects of combination therapy have been explored using HCV genotypes 1a and 1b replicon systems to determine whether drug combinations result in additive, antagonistic, or synergistic interactions. Seven HCV antivirals, including rIFNα, ribavirin, daclatasvir (HCV NS5A replication complex inhibitor), and NS5B inhibitors targeting the nucleoside-binding site (NM-107), and allosteric inhibitors targeting site I (BMS-791325) or site II (HCV-796) were tested in two-drug combinations with ASV. The NS3 PI and rIFNα were combined at three different ratios. Concentration response curves were fit to assess the antiviral effects of the drug treatment combinations and the degree of antagonism, additivity, or synergy was determined at the 50, 75, and 90% effective levels. The combination indices were calculated using the definition of Loewe’s synergy cited in Chou (10). The results showed a mixture of additive to synergistic effects for rIFNα, as determined from the combination indices (Table 5). Additive to synergistic effects were also observed for daclatasvir (synergistic/additive) and NS5B inhibitors (additive or synergistic additive) in two-drug combination studies (data shown in Supplementary Tables 1–3). Importantly, no drug antagonism was observed in any of the combinations evaluated. Effects of combining ASV in three-drug combinations were also explored. Combinations of the NS3 PI with rIFNα and daclatasvir or rIFNα and BMS-791325 yielded mixed synergy/additivity or additivity, respectively. As with the two-drug combinations, no antagonism was observed at any of the effective doses (Supplementary Tables 4 and 5). For combination studies between ASV and ribavirin, a technique similar to that used by Coelmont et al. (12) was used rather than the
The method described by Chou, which relies on obtaining a full EC_{50} curve in the absence of any overlapping cytotoxicity. In the genotype 1b system, ribavirin concentration–response curves resulted in EC_{50} and CC_{50} values that were not well separated enough for analysis by the method of Chou (20 ± 5 µM and 68 ± 23 µM, respectively). Combination response data were analyzed using the MacSynergy II software developed by Prichard and Shipman (University of Michigan, 1993), with ASV demonstrating overall additivity (Supplementary Fig. 1).

**In vitro permeability, metabolic stability, and serum protein binding.** The permeability of ASV was examined using a PAMPA permeability model and the cell-based Caco-2 bi-directional permeability assay. The Pc values for the NS3 PI in the PAMPA permeability model were >473 nm/sec (pH 5.5) and >492 nm/sec (pH 7.4), similar to those of compounds that exhibit good (>80%) absorption in humans. In the Caco-2 cell bi-directional permeability assay, ASV permeability was dependent on the initial drug concentration; (B-A)/(A-B) efflux ratios greater than unity (~31-fold at 5 µM and ~3-fold at 25 µM) were calculated, suggesting that it is a substrate of efflux transporters such as P-glycoprotein (Table 6).

The rate of metabolism of ASV was evaluated in NADPH-fortified liver microsomes of various species. Using the rates of loss of parent compound at 0.5 µM, the predicted hepatic intrinsic clearance (CL_{h,intr}) values were 13.3, 13.3, 31.7, and 8.5 ml/min/kg for rat, dog, monkey, and human, respectively. These values represent approximately 24, 43, 69, and 41% of hepatic blood flow in rat, dog, monkey, and human and indicate that the NS3 PI may exhibit low to intermediate metabolic clearance. These results indicate that, as a fraction of hepatic blood...
flow, the predicted human CL_{h,int} of ASV (41%) is more similar to that predicted for dog (43%) than for rat (24%) or monkey (69%).

Serum protein binding can often attenuate the clinical efficacy of antiviral agents. The extent of ASV serum protein binding was assessed across species by equilibrium dialysis, while the direct effect of human serum binding on its antiviral activity was also examined in a replicon cell-based assay. In protein binding assays, ASV (10 µM) was 98.8 ± 0.5% bound to human serum protein and 97.2–98.8% bound to serum proteins in the animal species studied (rat, dog, and cynomolgus monkey). Additionally, protein binding of the compound (0.5, 3, and 10 µM) was determined in the replicon assay medium (containing 4% FBS) and found to be 77.6 ± 1.5, 76.0 ± 4.8, and 68.1 ± 1.1% bound at each of the concentrations evaluated, respectively. When the replicon buffer contained 40% human serum, ASV protein binding was increased to 97.4 ± 0.66, 97.7 ± 0.31, and 97.6 ± 0.54% bound at 0.5, 3, and 10 µM, respectively. Supplementation of 40% human serum to an HCV genotype 1b replicon assay resulted in a loss in ASV potency of approximately 6.5-fold. This loss in EC_{50} value compared with a 6.8-fold loss observed for VX-950 (data not shown).

**In vivo plasma and tissue exposures across species.** Plasma and liver concentration, as well as that in other organs, were assessed in mouse, rat, dog and monkey, with results summarized in Table 7. FVB mice were dosed orally (PO) with ASV, and drug exposure was monitored over 24 h. Liver concentrations in all animals were highly elevated over those in plasma (Table 7) with liver-to-plasma ratios of ≥80. ASV was not detected in brain, indicating minimal penetration into that tissue. Similar studies were carried out in rats; the time courses of tissue (liver and
heart) and plasma concentrations of ASV were monitored through 48 or 72 h after PO dosing. Analysis of samples revealed that liver contained the highest concentrations of ASV, with the elevated liver-to-plasma area under the curve (AUC) ratios (Table 7) similar to those observed in mice using a more limited data set. Compound exposure in the hearts of these rats was minimal when detected. In dogs, a time course of tissue (liver and spleen) and plasma concentrations were determined through 72 h after PO dosing. Liver contained the highest concentrations of compound, and the liver-to-plasma ratios at each of the sampling times were variable, ranging from 10 to approximately 410; with a ratio of 40 obtained using AUC values. The other tissues (spleen) contained lower levels of ASV than liver, with tissue-to-plasma ratios at the various sampling times ranging from 0.5 to 1. In monkeys, the time course of tissue (liver and spleen) and plasma concentrations of ASV were determined through 72 h after PO dosing. Over the 72-h study duration, liver tissue again contained the highest concentration of compound, while spleen contained considerably less, with concentrations in most samples were below the LLOQ by LC/MS/MS. While the liver-to-plasma ratios of ASV at each of the sampling times were variable, ranging from 39 to approximately 320, the estimate based on the AUC values was 151. The observed liver-to-plasma ratio after oral dosing to monkeys (~150) was comparable with the ratios observed in mice or rats (approximately >200) and higher than that for dogs (~40). When considering dose prediction analyses (unpublished results), it was assumed that the high liver levels observed across preclinical species would also translate to humans.
DISCUSSION

ASV is a linear peptide mimetic HCV NS3 PI, discovered using a structure-based drug design approach to replace the carboxylate moiety of ciluprevir derivatives, with a proprietary P1' acylsulfonamide cyclopropyl moiety with the aim of significantly enhancing potency (Fig. 1) (7).

Comprehensive profiling has demonstrated that ASV is a highly selective inhibitor of HCV NS3/4A protease complex enzymatic activity, effectively inhibiting four major HCV genotype classes (Table 1). It is most active against proteases representing genotype 1, the genotype with the highest unmet medical need, while isolates representing genotypes 2 and 3 were the least susceptible to inhibition. The reduced activity against genotype 3 was anticipated, since the reported salt bridge that forms between the charged NS3 amino acid residues R155 and D168 in genotypes 1, 4, 5, and 6 (49), stabilizing the interaction of the bulky P2 moiety of ASV with the enzyme, would not form. This is a consequence of the NS3-168 amino acid being a glutamine in genotype 3a. Replacement of the charged D168 for neutral residues, predicted to weaken or destroy the 155:168 salt bridge, results in a reduced susceptibility to ASV (30). For the genotype 2 subtype, the residue at NS3-122 is either a lysine (genotype 2a) or arginine (genotype 2b). This residue is close to charged residues D168 and R123, and replacement of the neutral serine by arginine could lead to conformational changes affecting the binding of ASV and NS3 PIs with similar bulky P2 moieties (49).

Kinetic characterization of ASV against HCV genotype 1a and 1b NS3/4A protease complexes in homogeneous in vitro assays indicated that it is a classic competitive inhibitor with \( K_i \) values in the picomolar range. The classic competitive inhibition curve, as calculated from a Lineweaver–Burk plot (Fig. 2), confirmed that ASV is a non-covalent, single-step competitive inhibitor rather
than a covalent (telaprevir and boceprevir) or non-covalent (danoprevir), two-step slow reversible inhibitor (40). In theory, NS3 PIs exhibiting slower off-rates from the enzyme could confer greater efficacy in patients and result in lower doses and less frequent dosing. However, the off-rate would need to be longer than the drug exposure half-life to have impact in patients. Since telaprevir and boceprevir are administered 3 times daily at high doses and danoprevir appears likely to require boosting with ritonavir to achieve twice-daily administration (18), it is unclear whether the observed *in vitro* slow off-rates confer any advantage over ASV. However, single- and multiple-ascending dose monotherapy clinical studies with ASV indicated rapid robust responses at lower doses than reported for telaprevir, boceprevir, or danoprevir (37).

ASV was shown to be highly specific for the HCV NS3 serine protease, demonstrating micromolar activity against the closely related GBV-B NS3 protease (Table 2). Furthermore, it exhibited little or no activity against a panel of human serine or cysteine proteases including elastase, the most homologous of these proteases. The selectivity index calculated for ASV was significantly greater than calculated for VX-950 (telaprevir), which demonstrated submicromolar to low micromolar activities against a number of human serine and cysteine proteases. The reported exposure of telaprevir administered to HCV-infected patients (*C*\text{max}, 3.5 µg/ml; *C*\text{min}, 2 µg/ml) is higher than the observed intrinsic potencies against some of these human proteases. This may have potential implications with respect to off-target side effects such as the skin orders (rash and pruritus) reported with telaprevir (50). The reduced selectivity of VX-950 (cathepsin A IC\text{50} ~0.3 µM) may also limit the potential of combining this drug with HCV nucleos(t)ide prodrugs. Cathepsin A is believed to be involved in the initial activation of the
prodrug moiety of the NS5B polymerase nucleotide PSI-7977 via cleavage of the amino acid ester moiety (34). Taken together, these results suggest that the selectivity profile of ASV has the potential to result in improved tolerability over telaprevir in vivo.

HCV genotypes 1a, 1b, and 4 account for the majority of HCV infections. These patients represent some of the more difficult-to-treat populations using the currently available interferon-alfa-based therapies; therefore, a requirement for DAAs with promising activity against these genotypes still exists. HCV replicon cell systems representing NS3 protease genotype subtypes 1a and 1b and 4a were susceptible to inhibition by ASV, as demonstrated by EC_{50} values in the low nanomolar range (Table 3). This in vitro antiviral activity correlates with the high rates of early viral suppression observed among genotype 1 and 4 patients treated with ASV in combination with pegylated interferon alfa/RBV in Phase 2 studies (5). Cellular proteases are unlikely to be affected given the calculated in vitro therapeutic index of ≥2,750 for ASV. ASV was also shown to exert minimal or no antiviral activity against other RNA viruses, including BVDV, HRV, HCoV, and HIV, further confirming its specificity for HCV. BVDV is the only virus encoding a serine protease, albeit of low sequence homology (52%) (33), and these selectivity results hold potential for ASV exerting minimal off-target activities. In fact, in early clinical studies (37), rash and neutropenia did not appear to be associated with the administration of ASV.

Resistance to direct-acting NS3 PIs has been shown to rapidly emerge during monotherapy (44). To increase the barrier to resistance, clinical studies have shown that combination therapies can achieve improved sustained viral responses (22, 39). For an investigational agent to be
combined with another HCV agent in patients, it is important to first demonstrate that no antagonism is observed in \textit{in vitro} combination studies (34). Under the experimental conditions outlined here, additive to synergistic effects were obtained when ASV was examined in combination with HCV inhibitors, representing distinct mechanisms of action. Importantly, no enhanced cytotoxicity was observed with any of the combinations tested.

Assessment of the \textit{in vitro} permeability properties of ASV indicated that the compound should be readily absorbed. However, this absorption may be influenced by efflux transporters such as P-glycoprotein (Table 6) and lead to variable drug exposure. ASV was predicted to exhibit low to intermediate metabolic clearance, with calculated human hepatic clearance being more similar to that predicted for dogs than for rats or monkeys. While ASV was shown to be highly bound to serum proteins, its intrinsic potency was not attenuated in an NS3/4A protease enzyme assay supplemented with albumin to a concentration equivalent to that in human serum (data not shown). However, modest attenuation of anti-NS3 protease activity was observed in an HCV genotype 1b replicon assay supplemented with 40% human serum. This fold-loss in potency is comparable with values obtained for other NS3 PIs.

The exposure profile of ASV across four different animal species (mouse, rat, dog, and monkey) revealed its hepatotropic disposition in all tested species, as indicated by a high exposure in liver versus plasma (Table 7). Compound exposure in other tissues examined, such as brain or spleen, was comparable to or less than that observed in plasma. Subsequent studies indicated that there was active uptake of ASV into the hepatocyte and elimination via the bile across species (unpublished results), suggesting that the compound was enriched in hepatocytes. This
is considered a desirable property given that HCV is a disease of the liver, with viral replication occurring in the hepatocyte. Moreover, limited systemic exposure of the compound would reduce the potential for non-specific side effects. Using the animal data to provide insight into potential doses and exposures in humans suggests that clearance in humans was closer to that of dogs. After dogs were administered an oral dose of 6 mg/kg of ASV, the $C_{\text{min}}$ value in the liver was ~100-fold the EC$_{50}$ value against genotype 1a replicons supplemented with 40% human serum. Using a simple body surface area conversion (42), a dose of approximately 200 mg was predicted to provide sufficient efficacy in humans (data not shown). This ASV dose administered twice daily provided rapid and robust antiviral activity in monotherapy studies and when combined with pegylated interferon and ribavirin (5, 37), and was subsequently chosen for Phase 2b studies.

Based on its overall preclinical profile, ASV was considered a suitable investigational agent for evaluation in clinical trials examining dual, triple, and quadruple combination approaches for the treatment of patients chronically infected with HCV genotypes 1 and 4 (5, 8, 28). The efficacy and safety of ASV were recently explored in a dual-DAA regimen of ASV combined with daclatasvir in difficult-to-treat genotype 1 null responders in Phase 2a studies (8, 28). In one study, 36% of patients achieved SVR following 24 weeks of treatment, providing proof-of-concept that an interferon-sparing regimen can cure HCV infection. This SVR rate is similar to the ~33% rate reported for a 48-week triple regimen of telaprevir combined with pegylated interferon alfa and ribavirin in genotype 1 null responders (50). Analyses of virologic failures in the dual-DAA regimen demonstrated that all the patients were infected with HCV genotype 1a and that the emergent NS3 protease-resistant variants detected were similar to those reported
in vitro (30). However, in a study in which all patients were infected with genotype 1b, all patients receiving ASV and daclatasvir for 24 weeks achieved SVR, suggesting that genotype 1 subtype can strongly influence virologic outcome (8).

In conclusion, the preclinical profile and subsequent data from early clinical trials support further research to assess the benefits of ASV-containing combination regimens in larger clinical studies.
Acknowledgements

The authors would like to thank David Stock for his development of the in-house combination study software, Burt Rose for constructing plasmids p50A-1 and p51-1, and Mark Cockett for his continued support of the program. Editorial assistance with the preparation of this manuscript was provided by Andrew Street, Ph.D. of Articulate Science and was funded by Bristol-Myers Squibb.
REFERENCES


Merck & Co. 2011. VICTRELIS™ (boceprevir) [package insert].


### TABLE 1. Potency of ASV and VX-950 against recombinant full-length HCV NS3/4A protease complexes representing different HCV genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>ASV ( IC_{50} ) (nM)(^b)</th>
<th>VX-950(^a) ( IC_{50} ) (nM)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a (H77)</td>
<td>0.70 ± 0.06</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>1b (J4L6S)</td>
<td>0.30 ± 0.02</td>
<td>22 ± 6</td>
</tr>
<tr>
<td>2a (HC-J6)</td>
<td>15 ± 1</td>
<td>95 ± 17</td>
</tr>
<tr>
<td>2b (HC-J8)</td>
<td>78 ± 2</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>3a (SS2)</td>
<td>320 ± 13</td>
<td>537 ± 4</td>
</tr>
<tr>
<td>4a (ED43)</td>
<td>1.6 ± 0.1</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>5a (SA13)</td>
<td>1.7 ± 0.2</td>
<td>38 ± 3</td>
</tr>
<tr>
<td>6a (HK-6A)</td>
<td>0.9 ± 0.1</td>
<td>86 ± 2</td>
</tr>
</tbody>
</table>

\(^a\) VX-950 was pre-incubated with each of the respective NS3/4A protease complexes for 30 min before adding substrate to obtain optimum potency. Pre-incubation of ASV and enzyme was not required to enhance potency.

\(^b\) Data indicate means ± SD from at least three independent experiments.
**TABLE 2.** Potency of ASV and VX-950 against a panel of serine and cysteine proteases

<table>
<thead>
<tr>
<th>Genotype</th>
<th>ASV $IC_{50}$ (μM)$^a$</th>
<th>VX-950 $IC_{50}$ (μM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV NS3/4A (1a[H77])</td>
<td>0.0007 ± 0.0001</td>
<td>0.012 ± 0.002</td>
</tr>
<tr>
<td>GBV-B NS3/4A</td>
<td>21 ± 6</td>
<td>7.7 ± 0.0</td>
</tr>
<tr>
<td>Human leukocyte elastase</td>
<td>&gt;50</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>PPE</td>
<td>&gt;50</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td>Human chymotrypsin</td>
<td>&gt;50</td>
<td>45 ± 8</td>
</tr>
<tr>
<td>Human cathepsin A</td>
<td>&gt;5$^b$</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td>Human cathepsin B</td>
<td>&gt;50</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Trypsin</td>
<td>&gt;30</td>
<td>ND$^c$</td>
</tr>
<tr>
<td>Thrombin</td>
<td>&gt;30</td>
<td>ND</td>
</tr>
<tr>
<td>Factor Vlla</td>
<td>&gt;30</td>
<td>ND</td>
</tr>
<tr>
<td>Factor Xa</td>
<td>&gt;30</td>
<td>ND</td>
</tr>
<tr>
<td>Factor Xla</td>
<td>&gt;30</td>
<td>ND</td>
</tr>
<tr>
<td>Kallikrein</td>
<td>&gt;30</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$ Data indicate means ± SD from at least three independent experiments.

$^b$ Assay interference observed at 20 μM.

$^c$ ND, not determined.
**TABLE 3.** Cell culture potency of ASV and VX-950 in HCV replicons

<table>
<thead>
<tr>
<th>Genotypea</th>
<th>Stable cell line or transient assayb</th>
<th>ASV EC50 (nM)c</th>
<th>VX-950 EC50 (nM)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a (H77c)</td>
<td>Stable cell line</td>
<td>4.0 ± 0.3</td>
<td>995 ± 11</td>
</tr>
<tr>
<td>1b (Con1)</td>
<td>Stable cell line</td>
<td>1.2 ± 0.3</td>
<td>427 ± 3</td>
</tr>
<tr>
<td>2a (JFH-1)</td>
<td>Stable cell line</td>
<td>230 ± 74</td>
<td>229 ± 32</td>
</tr>
<tr>
<td>3a (SS2)</td>
<td>Stable cell line</td>
<td>1162 ± 274</td>
<td>2731 ± 188</td>
</tr>
<tr>
<td>2a (JFH-1)</td>
<td>Transient</td>
<td>67 ± 23</td>
<td>108 ± 12</td>
</tr>
<tr>
<td>2b (HC-J8)</td>
<td>Transient</td>
<td>480 ± 104</td>
<td>1236 ± 96</td>
</tr>
<tr>
<td>1b (Con1)</td>
<td>Transient</td>
<td>1.3 ± 0.1</td>
<td>266 ± 77</td>
</tr>
<tr>
<td>4a (ED43)</td>
<td>Transient</td>
<td>1.8 ± 0.2</td>
<td>1137 ± 223</td>
</tr>
</tbody>
</table>

a Genotypes 2b, 3a, and 4a are NS3 protease hybrid-derived replicons.
b Luciferase reporter used as readout.
c Data indicate means ± SD from at least three independent experiments.
### TABLE 4. Selectivity of ASV 799

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cell type</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (µM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; (µM)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV-1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>HuH-7</td>
<td>0.004 ± 0.0003</td>
<td>25 ± 5</td>
</tr>
<tr>
<td>BVDV&lt;sup&gt;c&lt;/sup&gt;</td>
<td>HuH-7</td>
<td>12 ± 2</td>
<td>32 ± 4</td>
</tr>
<tr>
<td>HCoVOC43</td>
<td>MRC5</td>
<td>&gt;32</td>
<td>32 ± 5</td>
</tr>
<tr>
<td>HRV2</td>
<td>MRC5</td>
<td>&gt;32</td>
<td>32 ± 5</td>
</tr>
<tr>
<td>HIV</td>
<td>MT-2</td>
<td>13 ± 2</td>
<td>34 ± 3</td>
</tr>
<tr>
<td>NA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>HepG2</td>
<td>NA</td>
<td>38 ± 5</td>
</tr>
<tr>
<td>NA</td>
<td>HeLa</td>
<td>NA</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>NA</td>
<td>HEK293</td>
<td>NA</td>
<td>11 ± 2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data indicate means ± SD from at least three independent experiments.

<sup>b</sup> Subgenomic replicon.

<sup>c</sup> Full-length replicon.

<sup>d</sup> NA, not applicable.
### TABLE 5. Activity of ASV in combination with rIFNα

<table>
<thead>
<tr>
<th>Expt</th>
<th>ASV EC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>rIFNα EC&lt;sub&gt;50&lt;/sub&gt; (units/ml)</th>
<th>Ratio of rIFNα to ASV</th>
<th>Combination indices (confidence interval)</th>
<th>Overall result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.7</td>
<td>12.8</td>
<td>25:1</td>
<td>0.97 (0.89, 1.06)</td>
<td>Additivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10:1</td>
<td>0.77 (0.70, 0.84)</td>
<td>Synergy/additivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4:1</td>
<td>0.74 (0.67, 0.80)</td>
<td>Synergy/additivity</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>24.5</td>
<td>25:1</td>
<td>0.71 (0.66, 0.77)</td>
<td>Synergy</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10:1</td>
<td>0.95 (0.88, 1.01)</td>
<td>Synergy/additivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4:1</td>
<td>0.71 (0.65, 0.77)</td>
<td>Synergy</td>
</tr>
</tbody>
</table>
### TABLE 6. Bidirectional permeability of ASV in Caco-2 cells as a function of concentration

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (µM)</th>
<th>Pc (nm/sec)</th>
<th>Efflux ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A-B</td>
<td>B-A</td>
</tr>
<tr>
<td>ASV</td>
<td>5</td>
<td>11 ± 2</td>
<td>341 ± 20</td>
</tr>
<tr>
<td>ASV</td>
<td>25</td>
<td>54 ± 3</td>
<td>174 ± 22</td>
</tr>
<tr>
<td>Control(^b)</td>
<td>5</td>
<td>9 ± 1</td>
<td>167 ± 25</td>
</tr>
</tbody>
</table>

\(^a\) Pc, permeability coefficient; data indicate means ± SD from three independent experiments.

\(^b\) Control compound was the P-glycoprotein substrate digoxin.
TABLE 7. Tissue and plasma concentrations of ASV after PO dosing across speciesa

<table>
<thead>
<tr>
<th>Speciesb (PO dose, mg/kg)</th>
<th>Sample analyzed</th>
<th>C24 (nM)</th>
<th>T/P AUC ratio</th>
<th>Fold GT1a EC50 valuec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse (5)</td>
<td>Serum</td>
<td>&lt;LLOQ</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>714 ± 330d</td>
<td>&gt;80e</td>
<td>179 (28)</td>
</tr>
<tr>
<td>Rat (15)</td>
<td>Plasma</td>
<td>38</td>
<td>-</td>
<td>9.5 (1.5)</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>11986</td>
<td>359</td>
<td>2997 (461)</td>
</tr>
<tr>
<td>Dog (6)</td>
<td>Plasma</td>
<td>6</td>
<td>-</td>
<td>1.5 (0.2)</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>2541</td>
<td>40</td>
<td>635 (98)</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>&lt;LLOQ</td>
<td>0.5</td>
<td>ND</td>
</tr>
<tr>
<td>Monkey (10)</td>
<td>Plasma</td>
<td>&lt;LLOQ</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>441</td>
<td>151</td>
<td>110 (17)</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>&lt;LLOQ</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

a C24, concentration at 24 h postdose; GT, genotype; L, liver; LLOQ, lower limit of quantification;

b Mice (n = 3 per time point) were dosed at 5 mg/kg and followed for 24 h, rats (n = 2 per time point) were dosed at 15 mg/kg PO and followed for up to 72 h postdose, dogs (n = 1 per time point) were dosed at 6 mg/kg PO and followed for up to 72 h, and monkeys (n = 1 or 2 per time point) were dosed at 10 mg/kg PO and followed for up to 30 h postdose. The average values are shown in the table for rat and monkey, whereas only single values were obtained for dog.
Fold EC$_{50}$ multiples of ASV were calculated by dividing the C$_{24}$ value by the EC$_{50}$ value for ASV in HCV genotype 1a replicons (EC$_{50}$ ≈4 nM). Values in parentheses represent a similar calculation using the ASV EC$_{50}$ in 40% human serum (EC$_{50}$ ≈26 nM).

Average value ± standard deviation is provided. Liver-to-serum ratio determined.
**Figures Legends**

**FIG 1.** Chemical structure of ASV (BMS-650032): \(N\)-(tert-butoxycarbonyl)-3-methyl-L-valyl-(4R)-
\(N\)-((1R,2S)-1[((cyclopropylsulfonyl)amino)carbonyl]-2-vinylcyclopropyl) -4-[[4-methoxy-7-
chloroisquinolin-1-yl]oxy]-L-prolinamide (45). P and P’ labeling indicates the ASV moieties that
correspond to the natural HCV substrate positions occupying the NS3 protease active site.

**FIG 2.** Lineweaver–Burk plot and \(K_i\) determination for asunaprevir (ASV). (A) Lineweaver–Burk
plot of 1/V (reciprocal of rate of change of fluorescence) versus 1/S (reciprocal of the substrate
concentration). (B) \(K_i\) determination plot of the apparent \(K_m\) value (slope obtained from the
Lineweaver–Burke plot) versus ASV concentration.