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In Vitro and *In Vivo* Antiviral Activity and Resistance Profile of the Hepatitis C Virus NS3/4A Protease Inhibitor ABT-450

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ABSTRACT

The development of direct-acting antiviral agents is a promising therapeutic advance in the treatment of hepatitis C virus (HCV) infection. However, rapid emergence of drug resistance can limit efficacy and lead to cross-resistance among members of the same drug class. ABT-450 is an efficacious inhibitor of HCV NS3/4A protease, with 50% effective concentration values of 1.0, 0.21, 5.3, 19, 0.09, or 0.69 nM against stable HCV replicons with NS3 protease from genotypes 1a, 1b, 2a, 3a, 4a, or 6a, respectively. *In vitro*, the most common amino acid variants selected by ABT-450 in genotype 1 were located in NS3 at positions 155, 156, and 168, with the D168Y variant conferring the highest level of resistance to ABT-450 in both genotype 1a and 1b replicons (219- and 337-fold, respectively). In a three-day monotherapy study in HCV genotype 1-infected patients, ABT-450 was coadminstered with ritonavir, a cytochrome P450 3A4 inhibitor, shown previously to markedly increase peak, trough, and overall drug exposures of ABT-450. A mean maximum HCV RNA decline of 4.02 $\log_{10}$ was observed at the end of the 3-day dosing period across all doses. The most common variants selected in these patients were R155K and D168V in genotype 1a and D168V in genotype 1b. However, selection of resistant variants was significantly reduced at the highest ABT-450 dose compared to lower doses. These findings were informative for the subsequent evaluation of ABT-450 in combination with additional drug classes in clinical trials in HCV-infected patients.

42 **INTRODUCTION**

43 Hepatitis C virus (HCV) infection is a global health problem, with 160-180 million
44 individuals infected worldwide (1, 2). Chronic HCV infection can lead to serious liver
45 disease, including cirrhosis, liver failure, and hepatocellular carcinoma. There are 7
46 major HCV genotypes, which differ in their geographic distribution, disease progression,
47 and response to therapy (3). In the United States, Europe, and Japan, genotype 1 is the
48 most prevalent genotype, and globally it accounts for approximately 60% of HCV
49 infections (4).
50
51 Therapy for those infected with HCV genotype 1 improved with the approval of the
52 NS3/4A protease inhibitors (PIs) telaprevir, boceprevir, and more recently simeprevir (5-
53 10). Although the addition of a PI to pegylated interferon (pegIFN) and ribavirin (RBV)
54 therapy significantly improved sustained virologic response (SVR) rates compared to
55 pegIFN/RBV therapy alone, IFN-based therapies are associated with treatment-limiting
56 toxicities (11). In addition, there are many patients who are ineligible for IFN-based
57 treatment due to comorbidities such as depression (12). Early clinical trials with these
58 PIs also demonstrated that drug resistance developed within days after initiation of
59 treatment (13-15). The rapid selection of resistant variants is facilitated by a high rate of
60 virus production and the infidelity of the HCV RNA polymerase (16). Thus, there is a
61 need for effective treatments for HCV genotype 1 infection that eliminate the need for
62 IFN, while increasing SVR rates and reducing the development of resistance.
63

HCV is a positive-sense single-stranded RNA virus with a genome that consists of a single large open reading frame flanked by 5' and 3' untranslated regions. The large open reading frame is translated into a polyprotein, which is subsequently proteolytically processed into the proteins that are necessary for viral replication (17). The viral NS3/4A protease is essential for this process, and is a validated drug target as evidenced by the approval of the linear peptidomimetic covalent inhibitors telaprevir and boceprevir, and the macrocyclic noncovalent peptidomimetic inhibitor simeprevir. ABT-450, identified by AbbVie and Enanta as a lead compound, is a macrocyclic noncovalent peptidomimetic inhibitor of HCV NS3/4A protease that is in clinical development at AbbVie for use in combination with the NS5A inhibitor ombitasvir (formerly known as ABT-267) and the non-nucleoside NS5B polymerase inhibitor dasabuvir (formerly known as ABT-333), with or without RBV for the treatment of chronic HCV infection (18-22).

ABT-450 is metabolized primarily by cytochrome P450 (CYP) 3A4. In a prior study of healthy volunteers, co-administration of ABT-450 with a low dose of the CYP3A4 inhibitor ritonavir (combination denoted ABT-450/r) dramatically increased peak, trough, and overall ABT-450 plasma concentrations as well as half-life, resulting in sustained high plasma levels with once-daily dosing (23, 24). In this paper, we report the mean viral load declines and resistance-associated variants (RAVs) observed after 3 days of therapy in HCV genotype 1-infected patients treated with 1 of 3 doses of ABT-450 (50 mg, 100 mg, or 200 mg) combined with ritonavir 100 mg (50/100 mg, 100/100 mg, or 200/100 mg). We also report the *in vitro* inhibitory activity of ABT-450 against wild-type genotypes 1a, 1b, 2a, 3a, 4a, and 6a (genotype 5 has not been evaluated, as attempts

to generate a functional chimeric replicon containing NS3 protease from genotype 5 have been unsuccessful), as well as its *in vitro* resistance profile in genotype 1.

MATERIALS AND METHODS

Compound. ABT-450, (2R,6S,12Z,13aS,14aR,16aS)-N-(cyclopropylsulfonyl)-6-{[(5-methylpyrazin-2-yl)carbonyl]amino}-5,16-dioxo-2-(phenanthridin-6-yloxy)-1,2,3,6,7,8,9,10,11,13a,14,15,16,16a tetradecahydrocyclopropa[e]pyrrolo[1,2-a][1,4] diazacyclopentadecine-14a(5H)-carboxamidehydrate (Figure 1) was synthesized at AbbVie (North Chicago, IL) (25).

Replicon cell lines. The genotype 1a and 1b stable subgenomic replicon cell lines used for compound characterization in cell culture are derived from HCV strains 1a-H77 and 1b-Con1 (Genbank accession number NC_004102 and AJ238799, respectively) (Figure 2A). Both constructs are bicistronic subgenomic replicons similar in structure to those described by Lohmann et al (26). The genotype 1a replicon contains the 5' nontranslated region (NTR) from 1a-H77 followed by a firefly luciferase reporter gene and the neomycin phosphotransferase (Neo) gene, which together comprise the first cistron of the bicistronic replicon construct. This is followed by the EMCV IRES and the second cistron containing the 1a-H77 NS3-NS5B coding region with adaptive mutations encoding E1202G, K1691R, K2040R, and S2204I, and finally the 1a-H77 3' NTR. The 1b-Con1 replicon construct is similar in structure to the 1a-H77 replicon; however the 5' NTR, NS3-NS5B coding region, and 3' NTR are derived from 1b-Con1. The adaptive mutations in the 1b-Con1 replicon are those encoding K1609E, K1846T, and Y3005C,

and this replicon construct contains a poliovirus IRES between the HCV 5' NTR and the firefly luciferase gene (27).

In addition to the genotype 1a and 1b replicons, chimeric replicons in the 1b-Con1 background were generated by insertion of the region encoding the first 251 amino acids of NS3 from genotype 3a, 4a, or 6a in place of the corresponding region in a 1b-Con1 NS2-NS5B replicon that contained adaptive mutations encoding the same amino acid changes as found in the 1b-Con1 NS3-NS5B replicon described above (Figure 2A). In the genotype 3a replicon, the region encoding NS4A amino acids 21-32 (numbered relative to NS4A open reading frame) of the 1b-Con1 backbone was also replaced with the corresponding residues from genotype 3a. In the genotype 4a replicon, the region encoding the 20 amino acids at the C-terminus of NS2 from the 1b-Con1 backbone was replaced with the corresponding region from genotype 4a. In the genotype 6a replicon, the region encoding the entire 54 amino acids of NS4A was also replaced with the corresponding region from genotype 6a. The genotype 3a and 4a sequences were each derived from a population sequence from a single treatment-naïve HCV-infected patient. The NS3 and NS4A genes of the genotype 6a replicon were synthetically constructed based on generation of a consensus sequence derived from the alignment of fifteen sequences in GenBank. Stably replicating cell lines were generated by transfecting RNA transcribed *in vitro* from each of the replicon constructs into a Huh7-derived cell line and selecting for individual colonies using 400 µg/ml G418 (28). Activity of ABT-450 against genotype 2a JFH-1 (Genbank accession number AB047639) was determined at Southern Research Institute (Birmingham, AL) with a quantitative reverse-transcriptase

PCR (qRT-PCR) assay using a subgenomic replicon that did not contain a luciferase reporter (29, 30).

Antiviral activity in cell culture. Replicon cell lines were maintained in Dulbecco's modified Eagle medium (DMEM), supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, and 200 µg/mL G418, all of which were from Invitrogen (Carlsbad, CA), as well as 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA). The inhibitory effect of ABT-450 was evaluated by incubating replicon-containing cells in the presence of a series of ABT-450 dilutions for 3 days in the same medium containing 5% FBS, followed by measurement of firefly luciferase activity using the Luciferase Assay System (Promega, Madison, WI). In assays measuring inhibitory activity in the presence of human plasma, the medium contained 40% human plasma (Bioreclamation, Westbury, NY) and 5% FBS. The percent inhibition of HCV RNA replication was calculated for each compound concentration and the 50% effective concentration (EC_{50}) value was calculated using nonlinear regression sigmoidal dose-response variable slope curve fitting to the 4-parameter logistic equation (31) and GraphPad Prism 4 software. The cytotoxicity of ABT-450 was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma-Aldrich, St. Louis, MO) colorimetric assay (32). The 50% cytotoxicity concentration (CC_{50}) was calculated using nonlinear regression sigmoidal dose- response variable slope curve fitting as described above.

***In vitro* resistance selection.** The 1a-H77 and 1b-Con1 replicon cell lines (10^5 cells) were plated in 150 mm cell culture plates and grown in the presence of G418 (400 μg/ml) and ABT-450 at a concentration that was 10-, 100-, or 500-fold above the EC_{50} value for the respective cell line. After approximately three weeks of treatment, most cells were cleared of replicon RNA, and therefore were unable to survive in the G418-containing medium. The cells containing resistant replicon variants survived and formed colonies that were isolated and further expanded. In order to characterize resistant replicon variants, total RNA was extracted from the expanded colonies, the NS3 protease coding region was amplified by RT-PCR using gene-specific primers, and the nucleotide sequence of the amplified samples was determined.

Antiviral activity against a panel of resistant mutants. The 1a-H77 and 1b-Con1 subgenomic replicon shuttle vector constructs used for introduction of mutations of interest in the NS3 gene were similar to the replicon cell line constructs described above, but in both cases the Neo gene was not present, and the HCV NS2 gene was inserted between the EMCV IRES and the NS3 gene (Figure 2B). In addition, the 1a-H77 replicon construct had the adaptive mutation in NS3 protease encoding E1202G replaced with one encoding P1496L in NS3 helicase. An AscI restriction site was introduced into the NS2 gene 62 nucleotides upstream of the 5' end of the NS3 gene and a BstBI restriction site was introduced within the helicase domain of NS3 after the NS3 amino acid 251 codon. The introduction of these restriction sites did not result in an amino acid insertion or change in either the genotype 1a or 1b replicon. Mutations encoding resistance-associated variants were introduced by site-directed mutagenesis and

confirmed by sequence analysis. Subgenomic replicon RNA was generated by linearization of plasmid DNA followed by *in vitro* transcription. Replicon RNA was transfected into Huh7-derived cells and inhibition of replication of the HCV replicon by ABT-450 was measured using the luciferase assay as described above, except that cells were incubated for 4 days rather than 3 days prior to lysis. Replication efficiency was calculated as a percentage of wild-type replication using the following equation: 100x{(mutant four day luciferase activity/wild-type four day luciferase activity)/(mutant four hour luciferase activity/wild-type four hour luciferase activity)}.

Antiviral activity against a panel of genotype 1a and 1b isolates. The HCV 1a-H77 and 1b-Con1 replicon shuttle vectors described above were used to generate replicons containing NS3 genes from a panel of genotype 1a and 1b patient isolates, for assessing the activity of ABT-450. These replicon shuttle vectors allowed insertion of the region encoding the complete NS3 protease domain without adaptive mutations. The region encoding the C-terminal 20 amino acids of NS2 and amino acids 1-251 of NS3 was amplified by RT-PCR of viral RNA from genotype 1a and 1b isolates using primers incorporating AscI and BstBI restriction sites. The amplified products were inserted into the appropriate shuttle vector and the EC_{50} values of ABT-450 were evaluated in transient assays as described above.

Clinical study design. Study M11-602 was a randomized, multiple dose, placebo-controlled, blinded (active *versus* placebo), dose-ranging Phase 2a clinical trial to explore the safety, tolerability, pharmacokinetics, and antiviral activity of three direct acting

antiviral agents (DAAs), ABT-450/r and 2 non-nucleoside inhibitors of HCV NS5B polymerase, dasabuvir and ABT-072, in genotype 1 HCV-infected treatment-naïve patients in the United States and Puerto Rico (33). All of the patients provided written informed consesnt. The study was performed in accordance with Good Clinical Practice guideline and the principles of the Declaration of Helsinki, and the study protocol was approved by the the relevant institutional review boards and regulatory agencies. In this study, 75 patients meeting all eligibility criteria and none of the exclusion criteria were randomized to receive various doses of ABT-450/r, dasabuvir, or ABT-072. Only data from the 24 patients treated with ABT-450/r are discussed in this report. Eligibility criteria for Study M11-602 included: age 18 to 65 years, body mass index (BMI) ≥18 and <35 kg/m^2, chronic HCV genotype 1 infection for at least 6 months prior to study enrollment, plasma HCV RNA level ≥100,000 IU/mL at screening, liver biopsy within the past 3 years with histology consistent with HCV-induced liver damage, and no evidence of cirrhosis. Exclusion criteria included: liver biopsy with a METAVIR fibrosis score of 3 or 4, positive test result for hepatitis B surface antigen or anti-HIV antibodies, history of major depression within the 2 years prior to enrollment, history of disease precluding the use of pegIFN or RBV, and unresolved clinically significant diseases other than HCV.

Patients were randomized to receive 1 of 3 doses of ABT-450/r (50/100 mg, 100/100 mg, or 200/100 mg) or placebo once daily (QD). Following 3 days of monotherapy, pegIFN alfa-2a 180 µg/week and weight-based RBV 1000-1200 mg/day were added and the same dose of ABT-450/r or placebo was continued to complete a total of 12 weeks. At week

12, ABT-450/r or placebo was discontinued, and patients received pegIFN/RBV alone for up to 36 additional weeks.

Pharmacokinetic evaluations. ABT-450 and ritonavir concentrations were determined using a liquid chromatography tandem mass spectroscopic method (LC-MS/MS) with a lower limit of quantitation of 0.5 ng/mL (ABT-450) and 5 ng/mL (ritonavir). Pharmacokinetic analyses were conducted using WinNonlin Professional version 5.2 (Pharsight Corporation, CA).

Efficacy analysis. HCV RNA was measured using the COBAS TaqMan HCV Test v2.0 real-time reverse transcriptase-PCR (RT-PCR) assay (Roche, Pleasanton, CA), with a lower limit of quantitation = 25 IU/mL and a lower limit of detection = 10 IU/mL. Virologic response was assessed as HCV RNA decrease from baseline in $\log_{10}$ IU/mL.

Sequence analysis of patient samples. Viral RNA was isolated from the plasma of HCV-infected patients at baseline (prior to the first dose on Day 1) and after 3 days of ABT-450/r dosing (prior to the first dose on Day 4) from samples with viral load ≥500 IU/mL by either an automated method using the m2000 instrument (Abbott Molecular, Inc., Des Plaines, IL) or the QIAamp Viral RNA Mini Kit (QIAgen, Valencia, CA). RT-PCR was performed with the Superscript III One-Step RT-PCR System with Platinum *Taq* High Fidelity (Invitrogen, Carlsbad, CA) using sense and antisense primers located outside of NS3/4A. Nested PCR was performed with Platinum *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA) using sense and antisense primers specific for the NS3

protease domain. The NS3 protease domain PCR fragment was inserted into pJET1.2 vector (Fermentas, Glen Burnie, MD), plasmid DNA was isolated from individual colonies, and the plasmid DNA insert was sequenced. An average of 87 clones per sample were sequenced with a minimum of 75 clones sequenced from each sample.

Phenotypic analysis of patient samples. The nested PCR products from patient samples described above were digested and cloned into the appropriate genotype 1a or 1b replicon shuttle vector. Plasmid DNA and *in vitro* transcribed RNA were prepared and transfected into Huh7-derived cells and luciferase assays were performed with EC_{50} values calculated as described above.

RESULTS

***In vitro* activity.** ABT-450 inhibited genotype 1a-H77 and 1b-Con1 HCV subgenomic replicons in cell culture with EC_{50} values of 1.0 and 0.21 nM, respectively. In the presence of 40% human plasma the EC_{50} values increased by 17- to 24-fold (Table 1). The CC_{50} of ABT-450 was >37 μM, resulting in an *in vitro* selectivity index of ≥ 37,000-fold. ABT-450 demonstrated activity across multiple HCV genotypes, with an EC_{50} value of 5.3 nM against genotype 2a JFH-1 subgenomic replicon, and EC_{50} values of 19, 0.09, and 0.69 nM against replicons containing NS3 protease from genotypes 3a, 4a, and 6a, respectively. As has been observed for other NS3/4A protease inhibitors such as simeprevir and asunaprevir, ABT-450 has reduced potency against genotype 3a, likely caused by the presence of the NS3 polymorphism D168Q, commonly found in this genotype (34, 35).

Activity of ABT-450 against chimeric replicons containing NS3 protease from genotype 1 patients. To determine the breadth of coverage in genotype 1, the activity of ABT-450 was characterized against chimeric replicons containing sequences derived from 11 genotype 1a- and 9 genotype 1b-infected patients. EC_{50} values ranged from 0.43 to 1.87 nM against the genotype 1a isolates and 0.033 to 0.087 nM against the genotype 1b isolates (Table 2), indicating that ABT-450 can inhibit NS3 proteases across a broad range of genotype 1 isolates.

Characterization of resistance. HCV genotype 1a and 1b subgenomic replicon cell lines were passaged in the presence of ABT-450 at concentrations 10-, 100- or 500-fold over EC_{50}. No colonies survived selection at 100- or 500-fold over EC_{50} in genotype 1a, and no colonies survived at 500-fold over EC_{50} in genotype 1b. The following major variants in HCV NS3 were observed in selected colonies: R155K, D168E, and D168N in 1a-H77; and A156T, D168H, D168V, and D168Y in 1b-Con1 (Table 3). In order to achieve a more complete understanding of the ABT-450 resistance profile, its activity was assessed against HCV replicons containing NS3 variants that have been observed in HCV-infected patients treated with the HCV protease inhibitors telaprevir, boceprevir, simeprevir, asunaprevir, danoprevir, or faldaprevir (36-41). Most of the tested variants conferred less than 100-fold resistance to ABT-450. R155K in genotype 1a, which has been shown to confer resistance to virtually all HCV NS3/4A protease inhibitors, confers 37-fold reduced susceptibility to ABT-450. In both genotypes 1a and 1b, the D168V variant has been shown to confer resistance to the macrocyclic and acyclic non-covalent

293 protease inhibitors such as simeprevir, asunaprevir, danoprevir, or faldaprevir, but not to
294 the linear keto amide class of covalent inhibitors such as telaprevir and boceprevir. This
295 variant confers 96- or 159-fold reduced susceptibility to ABT-450 in genotype 1a or 1b,
296 respectively. The highest level of resistance to ABT-450 among the evaluated variants
297 was observed with the D168Y variant, which conferred 219- or 337-fold reduced
298 susceptibility in genotype 1a or 1b, respectively.
299
300 **Three day monotherapy pharmacokinetics and antiviral efficacy in study M11-602.**
301 Twenty-four treatment-naïve patients infected with HCV genotype 1 (8 per group) were
302 randomized in Study M11-602 and administered ABT-450/r doses of 50/100 mg, 100/100
303 mg, or 200/100 mg QD for 3 days. Demographic and baseline characteristics were
304 similar between groups. Mean baseline HCV RNA levels were 6.86 $\log_{10}$ IU/mL for
305 patients receiving placebo and 6.74 $\log_{10}$ IU/mL for patients receiving ABT-450/r (33).
306 Pharmacokinetic analysis of samples from the HCV-infected patients administered
307 different doses of ABT-450 showed a greater than dose-proportional increase in ABT-
308 450 exposure with increasing dose through 3 days of treatment with ABT-450/r (Figure
309 3) (24). The mean trough concentration increased 58-fold when the ABT-450 dose was
310 increased from 50 mg to 200 mg. HCV RNA levels were analyzed during the 3-day
311 monotherapy treatment. The mean maximum HCV RNA decrease from baseline was
312 4.02 ± 0.43 $\log_{10}$ IU/mL for all patients receiving ABT-450/r compared with 0.36 ± 0.13
313 $\log_{10}$ IU/mL for patients receiving placebo ($P<0.001$) (33). Similar HCV RNA decreases
314 were seen with all ABT-450/r doses at the end of the 3-day treatment (Figure 4).
315

316 **Phenotypic evaluation of *in vivo* resistance development.** Phenotypic analyses of
317 viral isolates from baseline (before the first dose was administered) and at the end of 3
318 days of ABT-450/r monotherapy were performed in order to characterize the selection of
319 resistant variants. The development of phenotypic resistance to ABT-450 during 3 days
320 of dosing was assessed by calculating the fold change in EC_{50} at the end of the 3-day
321 monotherapy compared to baseline (Table 4). Thirteen of the 24 patients (12 of 19
322 infected with genotype 1a and 1 of 5 infected with genotype 1b) had a viral load level
323 sufficient (≥ 500 IU/mL) to allow amplification of the target gene at the end of the 3 day
324 dosing period (5, 3, and 5 patients in the ABT-450/r 200/100, 100/100, and 50/100 mg
325 treatment groups, respectively).
326
327 Among the genotype 1a-infected patients with amplifiable samples, 4 of 5 (80%) from
328 the ABT-450/r 50/100 mg group, 2 of 2 (100%) from the ABT-450/r 100/100 mg group,
329 and 0 of 5 (0%) from the ABT-450/r 200/100 mg group showed a 3- to 12-fold loss of
330 susceptibility to ABT-450 relative to the baseline value for that patient. The remaining
331 samples had less than a 3-fold loss of susceptibility to ABT-450 relative to the baseline
332 value for that patient. The single genotype 1b-infected patient with amplifiable HCV
333 RNA, who received ABT-450/r 100/100 mg QD, showed a 386-fold loss of susceptibility
334 to ABT-450 relative to the baseline value for that patient.

335 Since many of the clinical samples at Day 4 contained a mixture of NS3 variants
336 (including wild-type), and each of these variants may have differing replication fitness
337 and variable susceptibility to ABT-450, the EC_{50} against clinical isolates is a reflection of
338 the multiple variants present in the quasispecies. Therefore the EC_{50} values from

reference replicons harboring single defined resistant variants differ from the clinical isolates.

Sequence analysis at baseline and on Day 4 (after 3 days of ABT-450/r monotherapy). In order to identify known RAVs at baseline and to monitor the emergence of variants at the end of ABT-450/r monotherapy, clonal nucleotide sequence analysis was performed on the NS3 protease gene from all baseline and Day 4 samples with HCV viral titer ≥500 IU/mL. The analysis of the baseline samples focused on changes at amino acid positions 155, 156, and 168 of NS3, since these are the positions at which resistant variants were identified during *in vitro* selection experiments with ABT-450, and where variants have most commonly emerged in patients treated with other NS3/4A protease inhibitors. The consensus amino acids at these positions, based on the prototypic standards, are arginine (R) at amino acid position 155 (R155), alanine (A) at 156 (A156), and aspartic acid (D) at 168 (D168). For all of the patients in this study, the HCV NS3 sequence at baseline was identical to the prototypic sequence at these 3 amino acid positions.

The analysis of the viral isolates obtained at the end of the 3-day monotherapy period also focused on changes at amino acids 155, 156, and 168, as no emergent variants were observed in samples from more than 1 patient at any other amino acid position in NS3/4A. In the ABT-450/r 200/100 mg dose group, clonal sequences from 4 of 5 samples did not contain any known RAVs at the end of 3 days of dosing, while clonal sequences from 1 sample contained D168V (Figure 5). In contrast, sequences from all

patients receiving lower doses of ABT-450 contained RAVs. Samples from the 3 patients from the ABT-450/r 100/100 mg dose group that could be amplified and sequenced had a high percentage of clones containing RAVs, most commonly R155K or D168V. Similarly, samples from the 5 patients in the ABT-450/r 50/100 mg dose group that were sequenced contained clones with known RAVs; R155K was the most common RAV identified. In most of the genotype 1a-infected patients who received the 50 or 100 mg dose of ABT-450, a number of other RAVs which confer modest levels of resistance to ABT-450, such as R155G, R155S, R155T, R155W, D168A and/or D168E, were present at lower prevalence.

DISCUSSION

ABT-450 is an inhibitor of HCV NS3/4A protease with nanomolar activity against genotypes 1, 2, 3, 4, and 6. We assessed the resistance-associated variants selected in vitro by ABT-450 since these variants may have clinical implications relative to other HCV protease inhibitors. Resistance selection experiments with ABT-450 in genotype 1a and 1b replicons identified RAVs at amino acid positions 155 and 168 in genotype 1a, and at positions 156 and 168 in genotype 1b. When ABT-450 was evaluated against a panel of NS3 variants that have been observed in patients treated with other HCV NS3/4A protease inhibitors, cross-resistance was noted with variants known to confer resistance to other protease inhibitors. The D168Y variant conferred the highest level of resistance to ABT-450 in both genotypes 1a and 1b.

In study M11-602, the mean maximum HCV RNA decrease from baseline was 4.02 ± 0.43 $\log_{10}$ IU/mL for all patients receiving ABT-450/r, and was similar across all 3 ABT-450/r doses during the 3-day treatment (Figure 4). While the decrease in viral RNA was independent of dose, resistance analysis from the ABT-450/r monotherapy portion of this study demonstrated a relationship between higher ABT-450 doses and a lower likelihood of emergence of RAVs. Among the 13 patients in whom HCV RNA could be amplified and sequenced after 3 days of monotherapy (12 with genotype 1a and 1 with genotype 1b), clonal sequencing of samples from 4 of the 5 patients who received the highest ABT-450 dose (200 mg QD) did not identify RAVs at positions 155, 156, or 168, while one sample contained D168V. In contrast, samples from all patients receiving lower doses of ABT-450 contained multiple RAVs, most commonly R155K or D168V. These findings suggest that, despite similar decreases in HCV RNA levels, ABT-450 exposures associated with the 50 mg dose were not sufficient to suppress replication of the R155K and D168V variants, which are associated with approximately 30 to 100-fold loss in ABT-450 susceptibility. In contrast, the higher exposures achieved with the 200 mg dose suppressed emergence of all detectable RAVs at Day 4 in a substantial proportion of patients. These findings suggest that the ABT-450 exposures achieved with all tested doses were adequate to suppress replication of wild-type HCV, but only those at the 200 mg dose consistently prevented emergence of virus harboring RAVs during the 3 days of treatment. These 3-day monotherapy findings are consistent with resistance analyses conducted in a dose-ranging phase 2 trial, AVIATOR (M11-652, NCT01464827), in which ABT-450/r was administered for up to 24 weeks. In this study, the most prevalent treatment-emergent variant in NS3 among patients who experienced virologic failure was

D168V; treatment-emergent R155K was seen infrequently in virologic failure patients who received an ABT-450 dose of ≥150 mg (42).

HCV exists as a quasispecies containing all possible pre-existing single RAVs even before the onset of drug pressure (16). Thus sufficient and ongoing drug exposure for the duration of the dosing interval is critical to maintain viral suppression. It has been observed previously that higher plasma concentrations of HCV NS3 protease inhibitors such as telaprevir result in both greater antiviral activity and lower selection of resistant HCV variants (40). Preclinical data suggested that the plasma levels of HCV protease inhibitors might be boosted with ritonavir coadministration (43). Based on these observations, we utilized ritonavir for pharmacokinetic enhancement of ABT-450. Our hypothesis was that a strategy based on achieving consistently high ABT-450 exposures throughout the 24-hour dosing period may reduce the risk of emergence of resistance, improve efficacy, and allow use of a lower dose of ABT-450 while permitting once-daily dosing.

The strategy of ritonavir pharmacokinetic enhancement to increase drug exposures has long been utilized in antiretroviral therapy for the treatment of human immunodeficiency virus (HIV) infection. Co-administration of HIV protease inhibitors with ritonavir improves efficacy by maintaining peak, trough and overall drug exposures of the protease inhibitor at levels necessary to inhibit replication of both wild-type virus and resistant variants (44). High rates of HIV suppression despite less than optimal medication adherence has been attributed to the “forgiveness of ritonavir” and the favorable PK

profile of boosted HIV protease inhibitors (45, 46), which have been first-line therapies for more than a decade. In addition, by prolonging the drug's half-life, ritonavir also permits less frequent dosing. These advantages have resulted in the widespread use of low-dose ritonavir to enhance the pharmacokinetics of HIV protease inhibitors (47, 48).

A comparison of pharmacokinetic parameters in healthy volunteers receiving ABT-450 alone or combined with ritonavir 100 mg demonstrated that ritonavir substantially increases ABT-450 plasma concentration and half-life, with trough concentrations (C_{24}) approximately 340-fold higher in the presence of ritonavir compared to ABT-450 alone (24, 49). Based on pharmacokinetic data in healthy patients, the trough ABT-450 concentrations achieved with a daily dose of 200 mg of ABT-450 and 100 mg of daily ritonavir are predicted to be greater than that seen with 1000 mg twice daily ABT-450 in the absence of ritonavir.

Thus, co-administration of ABT-450 with low-dose ritonavir enhances ABT-450 exposures considerably. While the relationship between plasma ABT-450 levels and those achieved in hepatocytes is unclear, rapid suppression of HCV RNA and sustained virologic response after the end of treatment in patients who received ABT-450 in Study M11-602 suggest that co-administering an adequate dose of ABT-450 with ritonavir achieves ABT-450 concentrations at the site of action well in excess of the EC_{50} of RAVs (50). As the emergence of RAVs may lead to treatment failure, inhibition of these variants, resulting in more complete HCV suppression, may account for the high response rates seen in large trials of DAA combination therapy that include ABT-450/r, where

ABT-450 was administered at a dosage of 150 mg QD with ritonavir 100 mg QD, in a coformulated tablet (18-22).

In conclusion, RAVs in NS3 result in decreased activity of ABT-450 *in vitro*; however, when ABT-450 exposures are optimized through use of ritonavir for pharmacokinetic enhancement, emergence of these variants is prevented or delayed *in vivo*. This finding may explain the low rates of virologic breakthrough seen in the phase 3 clinical trials of ABT-450/r, ombitasvir and dasabuvir with or without RBV, since similarly constructed drug regimens that did not use pharmacokinetic enhancement have been associated with high rates of virologic breakthrough (51, 52). In addition, the lower dosing of ABT-450, facilitated by the boosting effect of ritonavir, may correlate with the low rates of treatment discontinuation and good tolerability seen in these large clinical trials (18-22).

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476 **FIGURE LEGENDS**

477 **FIG 1** Chemical structure of ABT-450

478

479 **FIG 2** Schematic diagrams of HCV replicons used in this study. (A) Replicons for
480 stable cell lines. (B) Replicons for transient assays.
481 C, cysteine; E, glutamic acid; Delta ribozyme, ribozyme from hepatitis delta virus; E,
482 glutamic acid; EMCV, encephalomyocarditis virus; F-luc, firefly luciferase; G, glycine; I,
483 isoleucine; IRES, internal ribosome entry site; K, lysine; L, leucine; Neo, neomycin
484 phosphotransferase gene; NTR, non-translated region; P, proline; Polio, poliovirus; R,
485 arginine; S, serine; T, threonine, Y, tyrosine.

486

487 **FIG 3** Mean (± SD) ABT-450 plasma C_{trough} levels after 3-day monotherapy in HCV
488 genotype 1-infected patients following various ABT-450/r doses.

489

490 **FIG 4** Mean (± SE) HCV RNA change from baseline during 3-day treatment with ABT-
491 450/r in HCV genotype 1-infected patients.
492 BL, baseline; PBO, placebo; P/R, pegylated interferon plus ribavirin.

493

494 **FIG 5** Distribution of wild-type virus and resistant variants in patient samples with viral
495 load ≥500 IU/mL after 3 days of treatment with ABT-450/r.
496 A, alanine; D, aspartic acid; E, glutamic acid; G, glycine; K, lysine; R, arginine; S, serine;
497 T, threonine; V, valine; W, tryptophan; Y, tyrosine.

498

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Table 1: Activity of ABT-450 against replicon cell lines with NS3 from different genotypes in the presence or absence of 40% human plasma

Human Plasma	EC_{50} (nM)					
	1a	**1b**	**2a**	**3a**	**4a**	**6a**
0%	1.0 ± 0.33	0.21 ± 0.07	5.3 ± 1.2	19 ± 5.2	0.09 ± 0.03	0.69 ± 0.09
40%	17.3 ± 5.7	5.2 ± 2.0	ND	ND	ND	ND

ND: Not determined

Table 2: Antiviral activity of ABT-450 in transient HCV replicon assays using chimeric replicons containing NS3 protease genes from HCV genotype 1-infected patients

GT1a		GT1b	
Sample	**EC_{50}, nM**	**Sample**	**EC_{50}, nM**
1a-H77[a]	0.96	1b-Con1[a]	0.033
1	0.43	1	0.062
2	0.69	2	0.046
3	0.65	3	0.056
4	1.87	4	0.058
5	0.60	5	0.087
6	0.65	6	0.033
7	0.89	7	0.043
8	0.68	8	0.074
9	1.30	9	0.067
10	0.62		
11	1.03		
Mean	0.86	Mean	0.058

GT: Genotype

a. GenBank accession numbers for 1a-H77 and 1b-Con1 are AF011751 and AJ238799, respectively. Infected patient samples were obtained from ProMedDx (Norton, MA).

721 **Table 3: Loss in activity for ABT-450 against resistant variants selected by NS3/4A**
722 **protease inhibitors**

Replicon	NS3 Variant	Fold change in EC_{50} relative to wild-type	Replication capacity (% of wild-type)
1a-H77[a]	V36A	3	130
	V36L	2	82
	V36M	2	81
	F43L	20	17
	T54S	0.4	6
	V55I	1	81
	Q80K	3	91
	Q80L	2	38
	Q80R	2	44
	R155G	14	2
	R155K[b]	37	31
	R155S	7	2
	R155T	7	5
	R155W	11	5
	A156T	17	5
	D168A	50	35
	D168E[b]	14	34
	D168H	62	24
	D168N[b]	13	28
	D168V	96	2
	D168Y	219	4
	V36M + R155K	79	29
	Q80K + R155K	19	77
1b-Con1[a]	T54A	1	59
	V55A	1	14
	R155K	40	73
	R155Q[b]	-	<0.5%
	A156S	0.5	61
	A156T[b]	7	19
	D168A	27	69
	D168E	4	80
	D168H[b]	76	108
	D168T	49	129
	D168V[b]	159	157
	D168Y[b]	337	70
	V170A	1	64

723 a. GenBank accession numbers for 1a-H77 and 1b-Con1 are AF011751 and AJ238799,
724 respectively.
725 b. Selected by ABT-450 *in vitro*
726

727 **Table 4: Phenotypic resistance after 3 days of treatment with ABT-450/r in HCV**
728 **genotype 1-infected patients**

ABT-450/r Treatment	Patient	Genotype	EC_{50}, nM		
			Baseline	After 3 days of dosing	Fold Change
200/100 mg QD	1	1a	1.31	1.14	0.9
	2	1a	1.36	1.06	0.8
	3	1a	3.11	3.23	1.0
	4	1a	2.42	2.57	1.1
	5	1a	0.56	0.86	1.5
100/100 mg QD	6	1a	1.55	10.2	6.6
	7	1b	0.04	15.5	386
	8	1a	2.80	8.84	3.2
50/100 mg QD	9	1a	0.81	8.88	11
	10	1a	5.97	66.7	11
	11	1a	1.52	11.5	7.6
	12	1a	1.01	1.69	1.7
	13	1a	1.54	9.78	6.4

729 QD: Once daily