Genotypic and Phenotypic Analysis of Variants Resistant to Hepatitis C Virus Nonstructural Protein 5A Replication Complex Inhibitor BMS-790052 in Humans: *In Vitro* and *In Vivo* Correlations

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The NS5A replication complex inhibitor, BMS-790052, inhibits hepatitis C virus (HCV) replication with picomolar potency in preclinical assays. This potency translated *in vivo* to a substantial antiviral effect in a single-ascending dose study and a 14-day multiple-ascending dose (MAD) monotherapy study. However, HCV RNA remained detectable in genotype 1a–infected patients at the end of the MAD study. In contrast, viral breakthrough was observed less often in patients infected with genotype 1b, and, in several patients, HCV RNA declined and remained below the level of quantitation (<25 IU/mL) through the duration of treatment. Here, we report on the results of the genotypic and phenotypic analyses of resistant variants in 24 genotype 1–infected patients who received BMS-790052 (1, 10, 30, 60, and 100 mg, once-daily or 30 mg twice-daily) in the 14-day MAD study. Sequence analysis was performed on viral complementary DNA isolated from serum specimens collected at baseline and days 1 (4, 8, and 12 hours), 2, 4, 7, and 14 postdosing. Analyses of the sequence variants (1) established a correlation between resistant variants emerging *in vivo* with BMS-790052 treatment and those observed in the *in vitro* replicon system (major substitutions at residues 28, 30, 31, and 93 for genotype 1a and residues 31 and 93 for genotype 1b); (2) determined the prevalence of variants at baseline and the emergence of resistance at different times during dosing; and (3) revealed the resistance profile and replicative ability (i.e., fitness) of the variants. *Conclusion:* Although resistance emerged during monotherapy with BMS-790052, the substantial anti-HCV effect of this compound makes it an excellent candidate for effective combination therapy. (HEPATOLOGY 2011;54:1924-1935)

The hepatitis C virus (HCV) nonstructural protein 5A (NS5A) is a multifunctional protein with key roles in HCV replication. NS5A has also been implicated in the modulation of cellular signaling pathways.[1,2] Because it is required *in vivo* and *in vitro* for viral replication and has no known human homologs, NS5A provides an attractive target for therapeutic intervention.[3]

BMS-790052 is a potent HCV NS5A replication complex inhibitor, with 50% effective concentration (EC_{50}) values of 9 and 50 pM against genotype 1b and 1a replicons, respectively.[4,5] It is also potent against live virus (genotype 2a, JFH-1), with an EC_{50} of ~28 pM.[4] BMS-790052 has broad genotype coverage, with EC_{50} values ranging from pM to low nM for replicons with NS5A sequences derived from genotype 2a, 3a, 4a, and 5a.[4]

Proof of concept for BMS-790052 has been achieved clinically, where its exceptional *in vitro* potency translated to an *in vivo* effect in a single-ascending dose study.[4] In this study, marked HCV RNA decline (~2.9 $\log_{10}$) was needed for detection of resistant

Abbreviations: DAA, direct-acting antiviral; EC_{50}, 50% effective concentration; HCV, hepatitis C virus; MAD, multiple-ascending dose; NS5A, nonstructural protein 5A.

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variants, suggesting that the variants were likely present as preexisting minor quasi species and were uncovered by the efficient suppression of wild-type virus.[4] However, the ability of BMS-790052 to further suppress viral replication with continuous dosing could not be assessed in the single-ascending dose study. In addition, analysis of specimens from the single-ascending dose study did not reveal whether the resistance detected clinically correlates with resistance observed in the *in vitro* replicon system. In the multiple-ascending dose (MAD) study reported here, a total of 24 chronically infected patients (4 active patients per cohort) were treated with BMS-790052 at 1, 10, 30, 60, and 100 mg once-daily or 30 mg twice-daily for 14 days. The treated patients generally experienced rapid, marked viral load declines. However, HCV RNA remained detectable in all genotype 1a–infected patients, and viral breakthrough was observed during the course of treatment in the majority of these patients. In contrast, viral breakthrough was observed less often in patients infected with HCV genotype 1b, and, in several patients, HCV RNA dropped below the level of quantitation (<25 IU/mL).[6]

The primary aims of the genotypic and phenotypic analysis of specimens from the MAD study were to (1) determine whether clinically relevant resistant variants identified during 14 days of monotherapy with BMS-790052 would correlate with resistance selected *in vitro*; (2) assess the prevalence of variants at baseline and the emergence of resistance at different times during dosing; and (3) examine the resistance profile and replicative ability (i.e., fitness) of variants. This article describes the viral variants identified by population sequencing and the phenotypic analysis of these variants using transient replicon replication assays.

Because there is a large population of viral quasi species preexisting in infected individuals, variants that confer resistance to antiviral agents can be rapidly enriched and selected during antiviral treatment. BMS-790052, like other direct-acting antiviral (DAA) agents,[7-10] is, therefore, unlikely to be used as monotherapy. Variants with high levels of resistance to BMS-790052, which were identified in this MAD study, were evaluated for susceptibility to other DAA agents. Because no cross-resistance has been observed with other DAAs, our results suggest that BMS-790052 is an excellent candidate for combination therapy.

Patients and Methods

Compounds. The structure of the HCV NS5A inhibitor, BMS-790052, has been described previously.[4,5]

Genotypic Analysis of Clinical Specimens. Genotypic analysis of clinical specimens has been previously described.[4] To determine the relative sensitivity of detecting sequence variants, reconstitution experiments were performed with DNA mixtures containing both wild-type and resistant (Y93H) variants. Mixtures of wild-type and Y93H DNA, at ratios of 100:0, 95:5, 90:10, 80:20, and 60:40, were sequenced. The experiment revealed that the variant could be readily detected at 20% of the wild-type population (results not shown).

In Vitro Analysis of Replicon Variants. Substitutions were introduced into HCV reference replicons (genotype 1b, con1; genotype 1a, H77c), as previously described.[4,5] Inhibitor sensitivities and replicative ability (i.e., fitness) were assessed in transient replication assays, as previously described.[4]

Study Design and Clinical Specimens. To examine safety, pharmacokinetics, and antiviral effect across the potential clinical dose range, BMS-790052 was dosed as monotherapy in a double-blind, placebo-controlled, sequential panel, MAD study in patients chronically infected with HCV genotype 1. Six dose regimens were evaluated (1 mg once-daily, 10 mg once-daily, 30 mg once- or twice-daily, 60 mg once-daily, and 100 mg once-daily). Five patients in each panel were randomized to receive a 14-day course of orally administered BMS-790052 or placebo in a ratio of 4:1; thus, a total of 24 patients received BMS-790052.[6] The study was approved by the institutional review boards in all study centers and conducted in accord with good clinical practice and the ethical principles that have their origin in the Declaration of Helsinki.[6] Informed written consent was obtained from all patients. HCV RNA levels were determined using the Roche Cobas TaqMan HCV Test, v2.0 (lower limit of quantification, 25 IU/mL; lower limit of detection, 10 IU/mL; Roche, Pleasanton, CA) at baseline and days 1 (2, 4, 6, 8, 12, 16, and 20 hours post–first dose), 2, 3, 4, 5, 7, 9, 11, 14, 15, 16, 17, 21, and 28. Viral breakthrough was defined as an HCV RNA increase by at least 0.5 $\log_{10}$ after HCV RNA nadir while receiving BMS-790052. Serum specimens were collected for potential genotypic analysis at baseline and days 1 (4, 8, and 12 hours post–first dose), 2, 4, 7, and 14. After amplification of the NS5A coding region, a genotypic analysis was performed by population sequencing to determine the emergence of viral variants after the administration of multiple doses of BMS-790052. The complete study design and resistance analysis methodology have been described elsewhere.[5,6]

Results

Genotypic analysis of HCV NS5A complementary DNA was performed at baseline and seven time points

Table 1. Resistance Profile of BMS-790052 in the *In Vitro* 1b Replicon System

Replicon*	Replication Level (%) Average (SD)	EC_{50} (ng/mL) Average (SD)†	Fold Resistance
WT	100	0.0019 (0.0007)	1
L31M	99 (23)	0.0062 (0.0014)	3
L31V	158 (54)	0.053 (0.015)	28
Q54H	83 (18)	0.0024 (0.0003)	1
Q54N	83 (29)	0.0027 (0.0006)	1
Y93H	27 (16)	0.046 (0.018)	24
L31M-Y93H	70 (68)	13.5 (12.2)	7,105
L31V-Y93H	50 (38)	28.1 (24.7)	14,789
Q54H-Y93H	22 (7)	0.018 (0.005)	9
L31V-Q54H-Y93H	189 (25)	36.1 (7.7)	19,000

*Genotype 1b replicon: Con1 with cell culture replication enhancing mutation S2204I.

†The conversion factor for BMS-790052 is ~1.35. For example, 1 ng/mL = 1.35 nM.

Abbreviations: EC_{50}, 50% effective concentration; SD, standard deviation; WT, wild type.

(days 1 [4, 8, and 12 hours post–first dose], 2, 4, 7, and 14) for all patients receiving BMS-790052 when HCV RNA levels were >1,000 IU/mL and, in some instances, when HCV RNA levels were <1,000 IU/mL. Variants identified within the N-terminal region of NS5A by population sequencing are shown in Tables 1 and 2. Transient replication assays were used to assess the contribution of amino acid substitutions to BMS-790052 resistance and to estimate the relative replicative ability (i.e., fitness) of the variants. Many of these substitutions were previously identified during *in vitro* replicon studies, and others are novel substitutions.[4,5,11] Values for previously described substitutions (Tables 1 and 2) have been updated to reflect additional test occasions.

HCV RNA levels observed during the 14-day monotherapy study are summarized for each dosing cohort in Fig. 1A-F. NS5A variants identified from individual patients treated with BMS-790052 are summarized in Table 3A-F. The percent values shown in the tables are estimates based on population sequencing chromatograms. Based on the results of reconstitution experiments, variants present at ≥20% are readily detectable from the chromatograms (see Materials and Methods). We were also able to estimate variants that were present at less than 20% when they were detected at previously characterized NS5A resistance sites (residues 28, 30, 31, and 93 of genotype 1a and 31 and 93 for genotype 1b). Results from each dosing cohort are reported on below.

The 1-mg Once-Daily Cohort. Figure 1A shows HCV RNA levels, and Table 3A shows resistant substitutions identified in the specimens derived from patients treated with 1 mg of BMS-790052. Known resistant variants were not detected in baseline specimens from any of the patients in this cohort.

Patients A and B (genotype 1a) experienced maximal HCV RNA declines of ≥2.0 $\log_{10}$ (Fig. 1A). An H58P substitution (≤10%; data not shown) was detected in the baseline specimen derived from patient B; however, phenotypic analysis indicates that this variant does not confer resistance to BMS-790052 (Table 2). At day 14, resistant variants were detected at multiple residues, including M28, Q30, L31, and Y93 (Table 3A).

Patients C and D (genotype 1b) had relatively low viral loads at baseline; HCV RNA declined to <25 IU/mL at day 3 and 12 hours post–first dose, respectively (Fig. 1A). Although viral sequences were determined from some specimens with HCV RNA <1,000 IU/mL, no variants were detected at any of the time points analyzed.

The 10-mg Once-Daily Cohort. A more substantial HCV RNA decline was observed in the 10-mg cohort, compared to the 1-mg cohort (compare Fig. 1A and B). No known resistant variants were detected from the baseline or the 4-hours post–first-dose specimens.

Patients E and F (genotype 1a) experienced maximal HCV RNA declines (3.2 $\log_{10}$ and ~2.8 $\log_{10}$) at days 7 and 2, respectively (Fig. 1B). Population sequencing revealed ~100% substitution at residue 93 for patient E at day 14 (Y93H; Table 3B). The

Table 2. Resistance Profile of BMS-790052 in the *In Vitro* 1a Replicon System

Replicon*	Replication level (%) Average (SD)	EC_{50} (ng/mL) Average (SD)†	Fold Resistance
WT	100	0.0044 (0.0028)	1
M28A	27 (25)	20.2 (13.3)	4,591
M28T	31 (23)	3.0 (0.3)	682
M28V	16 (11)	0.0055 (0.0019)	1.3
Q30E	130 (56)	110.9 (66.0)	25,205
Q30H	75 (31)	6.5 (1.4)	1,477
Q30R	41 (16)	5.4 (0.8)	1,227
Q30K	19 (9)	108 (52)	24,545
L31M	55 (15)	1.5 (0.5)	341
L31V	117 (29)	14.9 (4.4)	3,386
H58D	92 (9)	2.2 (0.3)	500
H58P	266 (261)	0.0053 (0.0006)	1.2
Y93C	11 (7)	8.2 (3.0)	1,864
Y93H	18 (11)	23.9 (7.0)	5,432
Y93N	13 (8)	208.9 (47.9)	47,477
M28V-Q30R	147 (55)	1.4 (0.013)	350
Q30H-Y93H	20 (6)	409.8 (153.6)	93,136
Q30R-H58D	60 (12)	1,867 (46)	424,318

*Genotype 1a replicon: H77C with cell culture replication enhancing mutations P1496L and S2204I.

†The conversion factor for BMS-790052 is ~1.35. For example, 1 ng/mL = 1.35 nM.

Abbreviations: EC_{50}, 50% effective concentration; SD, standard deviation; WT, wild type.

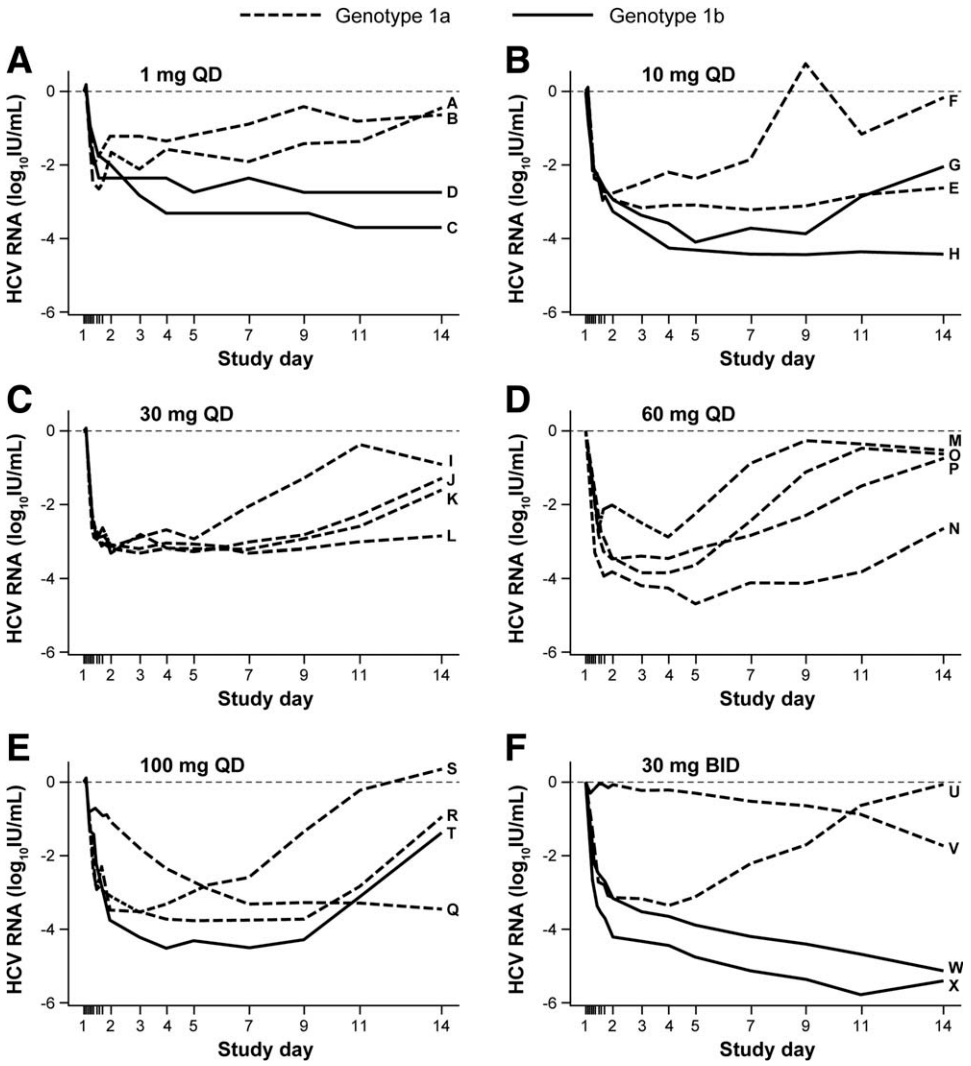

Fig. 1. Individual change from baseline in $\log_{10}$ HCV RNA by dose and HCV genotype. HCV RNA was set to 25 or 10 IU/mL, if observed value was less than lower limit of quantification (25 IU/mL) or lower limit of detection (10 IU/mL) respectively, when deriving changes from baseline. Dashed and solid lines indicate patients with HCV genotype 1a and 1b, respectively. A to X, patient designations; BID, twice-daily; HCV, hepatitis C virus; QD, once-daily.

genotype 1a Y93H variant replicated poorly in the transient replication assay, but displayed a moderate level of resistance, with an EC_{50} value of 23.9 ng/mL or 32.3 nM (Table 2). At day 14, multiple variants, including Q30E/H/R, L31M/V, and Y93H, were detected in specimens from patient F (Table 3B). Q30E conferred a relatively high level of resistance to BMS-790052, with an EC_{50} value of 110.9 ng/mL or 150 nM (Table 2).

Patients G and H (genotype 1b) experienced HCV RNA declines ($\sim$3.7 $\log_{10}$ and $>$4.4 $\log_{10}$) at day 7 (Fig. 1B). Population sequence traces from patient G on day 14 revealed complete replacement of the wild-type amino acids at residues 31 ($\sim$50% each of L31V and L31M) and 93 (100% Y93H), indicating linkage of these resistant substitutions. In the replicon system, genotype 1b variants with L31 or Y93 single amino acid substitution conferred minimal resistance, whereas double amino acid substitution variants (L31V-Y93H and L31M-Y93H) conferred much higher levels of resistance (Table 1). In patient H, HCV RNA levels decreased to $<$1,000 IU/mL at day 2 and $<$25 IU/mL at days 7 and 14 (Fig. 1B). Sequence traces from baseline revealed Q54H/N ($\sim$50% each) substitutions in NS5A. Not surprisingly, the Q54H/N variants did not confer appreciable resistance to BMS-790052 in the replicon assay (Table 1). No resistant variants were detected in day 1 specimens (4, 8, and 12 hours) from this patient.

The 30-mg Once-Daily Cohort. All patients (I, J, K, and L) were infected with HCV subtype 1a. Known resistant variants were not detected in the baseline specimens.

All patients experienced maximal declines in HCV RNA of $\geq$3.2 $\log_{10}$, followed by varying levels of breakthrough with the appearance of known resistant substitutions (Fig. 1C). In general, M28T, Q30R, and Y93C were the earliest variants detected (as early as 8 hours post–first dose in patient I), but an assortment of other variants emerged at later time points in all patients (Table 3C). In 3 patients, Q30E emerged as a

Table 3A. Resistance Profile of HCV Patients Treated Once-Daily With 1 mg of BMS-790052

Patient	GT	Time (HCV RNA, IU/mL)	M28 (1a)*	Q30 (1a)*	L31*	Y93*
Patient A	1a	Baseline (1.06×10^6)	WT	WT	WT	WT
		4 hours	WT	WT	WT	WT
		8 hours	T (∼10%)	WT	WT	WT
		12 hours	WT	WT	WT	WT
		Day 2	WT	H (∼10%)	WT	WT
		Day 4†	WT	WT	WT	WT
		Day 7	T (∼30%)	R (∼30%)	M (∼15%)	C (∼15%)
		Day 14	T (∼45%)	R (∼20%) H (∼10%)	M (∼15%)	C (∼10%)
Patient B	1a	Baseline (3.23×10^5)	WT	WT	WT	WT
		4 hours	WT	WT	WT	WT
		8 hours	WT	WT	WT	WT
		12 hours	WT	WT	WT	WT
		Day 2	WT	WT	WT	WT
		Day 4	WT	WT	WT	WT
		Day 6‡	T (∼10%)	R (∼5%)	M (∼10%)	WT
		Day 14	T (∼10%)	R (∼5%)	M (∼50%)	WT
			L28 (1b)	**R30 (1b)**	**L31**	**Y93**
Patient C	1b	Baseline (5.45×10^4)	WT	WT	WT	WT
		4 hours	WT	WT	WT	WT
		8 hours	WT	WT	WT	WT
		12 hours	WT	WT	WT	WT
		Day 2	WT	WT	WT	WT
		Day 4	ND	ND	ND	ND
		Day 7	ND	ND	ND	ND
		Day 14	ND	ND	ND	ND
Patient D	1b	Baseline (5.77×10^3)	WT	WT	WT	WT
		4 hours	ND	ND	ND	ND
		8 hours	ND	ND	ND	ND
		12 hours	ND	ND	ND	ND
		Day 2	ND	ND	ND	ND
		Day 4	ND	ND	ND	ND
		Day 7	ND	ND	ND	ND
		Day 14	ND	ND	ND	ND

*(%) Quantitative estimates of variants based on chromatograms from population sequencing (estimated from the average of at least two polymerase chain reaction [PCR] products, unless otherwise indicated).

†Resistance data were derived from one PCR product.

‡A sample from day 6, instead of day 7, was collected and analyzed.

Abbreviations: GT, genotype; ND, not determined (HCV RNA <1,000 IU/mL); WT, wild-type consensus.

dominant variant; Q30E is associated with a relatively high level of resistance (EC_{50} value: 110.9 ng/mL or 150 nM; Table 2).

The 60-mg Once-Daily Cohort. All patients (M, N, O, and P) were infected with HCV genotype 1a and experienced maximal HCV RNA declines of $\geq$2.9 $\log_{10}$. HCV RNA remained detectable in all of the patients, and viral breakthrough was observed at the end of treatment (Fig. 1D).

The preexisting resistance variant, Q30R, was detected (∼10%) at baseline in patient M (Table 3D). However, continuous HCV RNA decline suggests that this variant was, at least initially, suppressed by the 60-mg dose of BMS-790052 (Fig. 1D). At day 14, a variant with Q30H and Y93H linkage was detected in this patient (Table 3D). The level of resistance of genotype 1a variant Q30H-Y93H was high, with an EC_{50} value of 409.8 ng/mL or ∼553 nM (Table 2). For patients N and O, Q30E and Y93N were observed at day 14. These variants conferred substantial resistance to BMS-790052 (EC_{50} values: 110.9 or 150 nM and 208.9 ng/mL or 282 nM, respectively; Table 2). For patient P, a Q30R variant was first detected 12 hours post–first dose and became the only variant detected at day 14 (Table 3D). Because the plasma exposure of BMS-790052 at day 14 in this patient was 86.8 ng/mL or ∼117 nM (data not shown), and the EC_{50} value for a genotype 1a replicon containing the Q30R substitution in NS5A is ∼7 nM or 5.4 ng/mL (Table 2), a rigorous investigation was triggered to understand the basis of the resistance observed in patient P. A detailed analysis of this resistant variant will be presented elsewhere.

Table 3B. Resistance Profile of HCV Patients Treated Once-Daily With 10 mg of BMS-790052

Patient	GT	Time (HCV RNA, IU/mL)	Q30 (1a)*	L31*	Q54 (1b)*	Y93*
Patient E	1a	Baseline (4.24×10^5)		Failed to amplify PCR products		
		4 hours	WT	WT	WT	WT
		8 hours†	WT	WT	WT	WT
		12 hours		Failed to amplify PCR products		
		Day 2	ND	ND	ND	ND
		Day 4	ND	ND	ND	ND
		Day 7	ND	ND	ND	ND
		Day 14†	WT	WT	WT	H (∼100%)
Patient F	1a	Baseline (1.96×10^6)	WT	WT	WT	WT
		4 hours	WT	WT	WT	WT
		8 hours	R (∼10%) H (<5%)	WT	WT	WT
		12 hours	R (∼40%) H (∼20%)	(V<5%)	WT	H (∼10%)
		Day 2	R (∼40%) H (∼30%)	WT	WT	H (∼10%)
		Day 4	R (∼40%) H (∼30%)	V (∼10%)	WT	H (∼20%)
		Day 7	R (∼30%) H (∼40%)	V (∼15%)	WT	H (∼10%)
		Day 14	R (∼20%) H (∼15%) E (∼5%)	V (∼50%) M (∼5%)	WT	H (∼10%)
Patient G	1b	Baseline (3.22×10^5)	WT	WT	WT	WT
		4 hours	WT	WT	WT	WT
		8 hours	WT	WT	WT	WT
		12 hours	WT	WT	WT	WT
		Day 2	ND	ND	ND	ND
		Day 4	ND	ND	ND	ND
		Day 7	ND	ND	ND	ND
		Day 14	WT	M (∼50%) V (∼50%)	WT	H (∼100%)
Patient H	1b	Baseline (6.82×10^5)	WT	WT	H (∼50%) N (∼50%)	WT
		4 hours	WT	WT	N (∼100%)	WT
		8 hours	WT	WT	N (∼100%)	WT
		12 hours	WT	WT	N (∼100%)	WT
		Day 2	ND	ND	ND	ND
		Day 4	ND	ND	ND	ND
		Day 7	ND	ND	ND	ND
		Day 14	ND	ND	ND	ND

*(%) Quantitative estimates of variants based on chromatograms from population sequencing (estimated from the average of at least two polymerase chain reaction [PCR] products, unless otherwise indicated).

†Resistance data were derived from one PCR product.

Abbreviations: GT, genotype; ND, not determined (HCV RNA <1,000 IU/mL); WT, wild-type consensus.

The 100-mg Once-Daily Cohort. All patients (3 infected with genotype 1a [patients Q, R, and S] and 1 with genotype 1b [patient T]) experienced HCV RNA declines $\geq$3.5 $\log_{10}$ (Fig. 1E).

Preexisting resistance variants were not detected in the 3 patients (Q, R, and S; Table 3E) infected with the genotype 1a virus. Variants with substitutions that yield low or moderate levels of resistance, such as M28T/V, Q30H, and H58D, were detected at early time points (Table 3E); variants with substitutions yielding higher levels of resistance, such as Q30E and Q30R-H58D (EC_{50} value: 1,867 ng/mL or ∼2,521 nM), became apparent at later time points (Tables 1 and 3E). For the genotype 1b patient T, population sequencing revealed that Q54H and Y93H substitutions were present at ∼100% in all time points analyzed. This variant was clearly suppressed by BMS-790052 at early time points (Fig. 1E). Q54H did not confer resistance, whereas Q54H-Y93H displayed a resistance profile similar to the Y93H variant (Table 1). At day 14, L31V, Q54H, and Y93H were all present at ∼100%, indicating linkage of these resistant variants in the rebounding virus (Table 3E). The genotype 1b L31V-Q54H-Y93H variant conferred a moderate level of resistance, with an EC_{50} value of 36.1 ng/mL or 48.7 nM (Table 1).

Table 3C. Resistance Profile of HCV Patients Treated Once-Daily With 30 mg of BMS-790052

Patient	GT	Time (HCV RNA, IU/mL)	M28 (1a)*	Q30 (1a)*	L31*	Y93*
Patient I	1a	Baseline (1.40×10^7)	WT	WT	WT	WT
		4 hours	WT	WT	WT	WT
		8 hours†	T (∼75%)	R (∼25%)	WT	WT
		12 hours	T (∼70%)	WT	M (∼15%)	WT
		Day 2	T (∼65%)	R (∼15%)	WT	WT
				E (∼10%)		
				K (∼10%)		
		Day 4	T (∼55%)	R (∼5%)	M (∼5%)	C (∼25%)
		Day 7	T (∼40%)	E (∼17%)	M (<5%)	C (∼10%)
				H (∼5%)	V (∼10%)	H (<5%)
				R (∼10%)		N (<5%)
		Day 14	T (∼5%)	E (∼70%)	V (∼15%)	H (<5%)
				R (<5%)		N (∼5%)
Patient J	1a	Baseline (1.26×10^6)	WT	WT	WT	WT
		4 hours	WT	WT	WT	WT
		8 hours	WT	WT	WT	WT
		12 hours	T (∼5%)	WT	WT	WT
		Day 2	ND	ND	ND	ND
		Day 4	ND	ND	ND	ND
		Day 7	WT	R (∼35%)	V (∼15%)	H (∼50%)
		Day 14	T (∼ 25%)	E (∼65%)	V (∼5%)	WT
				K (∼5%)		
Patient K	1a	Baseline (4.77×10^6)	WT	WT	WT	WT
		4 hours	WT	WT	WT	WT
		8 hours	WT	WT	WT	WT
		12 hours	T (∼10%)	R (∼10%)	WT	WT
		Day 2	WT	R (∼25%)	M (∼20%)	WT
				H (∼20%)		
		Day 4	T (∼20%)	H (∼25%)	WT	C (∼5%)
				R (∼10%)		H (∼5%)
				E (∼5%)		
		Day 7	T (∼10%)	R (∼25%)	M (∼5%)	C (∼10%)
				H (∼20%)		H (∼5%)
				E (∼5%)		N (∼5%)
		Day 14	WT	E (∼50%)	WT	WT
Patient L	1a	Baseline (5.60×10^6)	WT	WT	WT	WT
		4 hours	WT	WT	WT	WT
		8 hours		Failed to amplify PCR products		
		12 hours		Failed to amplify PCR products		
		Day 2†	T (∼50%)	WT	WT	C (<5%)
		Day 4		Failed to amplify PCR products		
		Day 7	T (∼30%)	E (∼15%)	V (∼5%)	C (∼5%)
				H (∼5%)		
				R (∼15%)		
		Day 14		Failed to amplify PCR products		

*(%) Quantitative estimates of variants based on chromatograms from population sequencing (estimated from the average of at least two polymerase chain reaction [PCR] products, unless otherwise indicated).

†Resistance data were derived from one PCR product.

Abbreviations: GT, genotype; ND, not determined (HCV RNA <1,000 IU/mL); WT, wild-type consensus.

The 30-mg twice-daily cohort. HCV RNA remained detectable in 2 patients infected with genotype 1a virus (patients U and V; Fig. 1F). In contrast, HCV RNA became virtually undetectable at the end of treatment in 2 genotype 1b patients (W and X; >5 $\log_{10}$ HCV RNA decline; Fig. 1F).

For patient U, multiple variants of low abundance were observed 12 hours post–first dose. A variant with the highly resistant Q30E substitution emerged as a predominant species at day 14 (∼70%; Table 3F). For patient V, a consensus Q30H substitution was detected at all time points, including baseline. The Y93H substitution was also detected at levels of 45%-60% during the course of treatment, suggesting a linkage of two resistant substitutions at baseline. Genotype 1a Q30H-Y93H conferred a high level of resistance to BMS-790052 (EC_{50} value: 409.8 ng/mL or 553 nM; Table 2).

Table 3D. Resistance Profile of HCV Patients Treated Once-Daily With 60 mg of BMS-790052

Patient	GT	Time (HCV RNA, IU/mL)	M28*	Q30*	L31*	Y93*
Patient M	1a	Baseline (1.66×10^7)	V (∼40%)	R (∼10%)	WT	WT
		4 hours	V (∼45%)	R (∼20%)	WT	WT
		8 hours	V (∼30%)	R (∼20%)	WT	WT
		12 hours	A (∼5%)	R (∼60%)	WT	C (∼5%)
			T (∼5%)			
			V (∼55%)			
		Day 2	V (∼75%)	R (∼75%)	WT	C (∼5%)
		Day 4	A (∼15%)	H (∼20%)	WT	H (∼7.5%)
			T (∼10%)	R (∼60%)		
			V (∼50%)			
		Day 7	A (∼30%)	H (∼70%)	WT	H (∼70%)
			T (∼30%)	R (∼30%)		
			V (∼30%)			
		Day 14	A (∼5%)	H (∼95%)	WT	H (∼95%)
			T (∼5%)	R (∼5%)		
			V (∼5%)			
Patient N	1a	Baseline (1.84×10^6)	V (∼5%)	WT	WT	WT
		4 hours	V (<5%)	WT	WT	WT
		8 hours	V (<5%)	WT	WT	H (<5%)
		12 hours	ND	ND	ND	ND
		Day 2	ND	ND	ND	ND
		Day 4	ND	ND	ND	ND
		Day 7	ND	ND	ND	ND
		Day 14	A (∼7.5%)	E (∼45%)	WT	N (∼10%)
			T (∼42.5%)	K (∼25%)		
			V (∼10%)	R (∼15%)		
Patient O	1a	Baseline (8.90×10^6)	WT	WT	WT	WT
		4 hours	WT	WT	WT	WT
		8 hours	WT	WT	WT	WT
		12 hours	WT	WT	WT	WT
		Day 2	WT	H (∼33%)	WT	WT
				E (∼20%)		
		Day 4	WT	E (∼10%)	WT	N (∼75%)
		Day 7	T (∼5%)	E (∼50%)	V (∼5%)	H, N, C (≤5% each)
				K, R (∼5% each)		
		Day 14	T (∼10%)	E (∼70%)	WT	N (∼10%)
				R (∼10%)		
Patient P	1a	Baseline (2.44×10^7)	WT	WT	WT	WT
		4 hours	WT	WT	WT	WT
		8 hours	WT	WT	WT	WT
		12 hours	WT	R (∼50%)	WT	WT
		Day 2	T (∼5%)	R (∼35%)	WT	WT
				H (<5%)		
		Day 4	WT	R (∼95%)	WT	WT
		Day 7	WT	R (∼100%)	WT	WT
		Day 14	WT	R (∼100%)	WT	WT

*(%) Quantitative estimates of variants based on chromatograms from population sequencing (estimated from the average of at least two polymerase chain reaction [PCR] products, unless otherwise indicated).

Abbreviations: GT, genotype; ND, not determined (HCV RNA <1,000 IU/mL); WT, wild-type consensus.

Patients W and X (genotype 1b) experienced continuous HCV RNA decline during the course of treatment, and HCV RNA became virtually undetectable at day 14, with >5 $\log_{10}$ drop from baseline (Fig. 1F). Known resistant variants were not detected at any time during treatment, suggesting that any preexisting genotype 1b–resistant variants in these 2 patients were either at extremely low levels or fully suppressed by 30-mg twice-daily dosing.

During the phenotypic analysis of NS5A variants, DAA agents, such as an NS3 protease inhibitor and/or an NS5B polymerase inhibitor, were used as controls for all analyses. Consistent with our previous report,[5] NS5A variants are susceptible to these agents in the replicon system (Table 4), suggesting that these variants would likely be suppressed by combination therapy.[5]

Discussion

BMS-790052 is a novel HCV replication complex inhibitor, and the 14-day monotherapy study provided

Table 3E. Resistance Profile of HCV Patients Treated Once-Daily With 100 mg of BMS-790052

Patient	GT	Time (HCV RNA, IU/mL)	M28 (1a)*	Q30 (1a)*	L31*	H58 (1a)†	Y93*
Patient Q	1a	Baseline (1.05×10^6)	WT	WT	WT	WT	WT
		4 hours	WT	WT	WT	WT	WT
		8 hours	WT	WT	WT	WT	WT
		12 hours	WT	WT	WT	WT	WT
		Day 2	WT	WT	WT	WT	WT
		Day 4	T (∼65%)	H (∼15%)	WT	D (∼20%)	WT
		Day 7	T (∼35%)	WT	WT	WT	H (∼65%)
		Day 14	T (∼50%)	E (∼50%)	WT	WT	C (∼35%)
				H (∼20%)			
Patient R	1a	Baseline (3.54×10^5)	WT	WT	WT	WT	WT
		4 hours	WT	H (∼20%)	WT	WT	WT
		8 hours	T (<5%)	H (∼45%)	WT	WT	WT
		12 hours	WT	H (∼40%)	WT	WT	H (∼20%)
		Day 2	ND	ND	ND	ND	ND
		Day 4	ND	ND	ND	ND	ND
		Day 7	ND	ND	ND	ND	ND
		Day 14	T (∼50%)	E (∼10%)	WT	WT	WT
				H (∼40%)			
Patient S	1a	Baseline (1.04×10^7)	WT	WT	WT	WT	WT
		4 hours	WT	WT	WT	WT	WT
		8 hours	V (∼10%)	WT	WT	WT	WT
		12 hours	Failed to amplify PCR products				
		Day 2	WT	H (∼5%)	WT	WT	H (∼80%)
		Day 4	WT	E (∼10%)	M (∼5%)	D (<5%)	C (∼5%)
				R (<5%)			H (∼45%)
							N (∼15%)
		Day 7	WT	E (<5%)	WT	D (∼45%)	H (∼15%)
				R (∼45%)			N (∼15%)
		Day 14†	WT	R (∼100%)	WT	D (∼100%)	WT
			L31	**Q54**			**Y93**
Patient T	1b	Baseline (1.99×10^7)	WT	H (∼100%)			H (∼100%)
		4 hours	WT	H (∼100%)			H (∼100%)
		8 hours	WT	H (∼100%)			H (∼100%)
		12 hours	Failed to amplify PCR products				
		Day 2	Failed to amplify PCR products				
		Day 4	ND	ND			ND
		Day 7	ND	ND			ND
		Day 14	V (∼100%)	H (∼100%)			H (∼100%)

*(%) Quantitative estimates of variants based on chromatograms from population sequencing (estimated from the average of at least two polymerase chain reaction [PCR] products, unless otherwise indicated).

†Resistance data were derived from one PCR product.

Abbreviations: GT, genotype; ND, not determined (HCV RNA <1,000 IU/mL); WT, wild-type consensus.

a unique opportunity not only to study the anti-HCV effect, but also to investigate the emergence of resistance. Studies of *in vitro* resistance to BMS-790052 using different levels of selective pressure and different replicon cell lines have been described.[5] All amino acid substitutions associated with resistance to this class of inhibitors have mapped to the N-terminal region of NS5A.[4,5,11] Patients receiving BMS-790052 monotherapy in the MAD study generally experienced rapid, marked viral load declines at early time points. However, viral breakthrough, especially in patients infected with genotype 1a virus, was observed at later time points and was associated with the emergence of resistance. The majority of resistant substitutions observed *in vivo* were at residues 28, 30, 31, and 93 for genotype 1a and at residues 31 and 93 for genotype 1b. This is consistent with the resistance identified *in vitro*, confirming the utility of the replicon system for assessing levels of resistance in response to BMS-790052 exposure.

In general, the single amino acid substitutions and some double amino acid substitutions (Q54H-Y93H) that were observed in genotype 1b conferred minimal resistance (1- to 28-fold; Table 1); however, some double amino acid substitutions in genotype 1b, such as L31V-Y93H, conferred high levels of resistance (14,789-fold; Table 1). For genotype 1a, the major resistance substitutions observed in the study delivered

Table 3F. Resistance Profile of HCV Patients Treated Twice-Daily With 30 mg of BMS-790052

Patient	GT	Time (HCV RNA, IU/mL)	M28 (1a)*	Q30 (1a)*	L31*	H58 (1a)*	Y93*
Patient U	1a	Baseline (1.95×10^7)	WT	WT	WT	WT	WT
		4 hours	WT	WT	WT	WT	WT
		8 hours	WT	WT	WT	WT	WT
		12 hours	T (~30%)	R (~10%) H (~10%)	M (~5%)	D (~5%)	H (~7.5%)
		Day 2†	WT	WT	V (~10%)	WT	WT
		Day 4	WT	WT	WT	WT	WT
		Day 7	T (~25%)	R (~5%) E (~60%)	WT	D (<5%)	H (<5%)
		Day 14	T (~8%)	R (~10%) E (~70%)	WT	D (~8%)	WT
Patient V	1a	Baseline (4.35×10^6)	WT	H (~100%)	WT	WT	H (~45%)
		4 hours	WT	H (~100%)	WT	WT	H (~55%)
		8 hours	WT	H (~100%)	WT	WT	H (~55%)
		12 hours	WT	H (~100%)	WT	WT	H (~55%)
		Day 2	WT	H (~100%)	WT	WT	H (~55%)
		Day 4	WT	H (~100%)	WT	WT	H (~55%)
		Day 7	WT	H (~100%)	WT	WT	H (~55%)
		Day 14	WT	H (~100%)	WT	WT	H (~60%)
Patient W	1b	Baseline (7.50×10^6)	WT	WT	WT	WT	WT
		4 hours	Failed to amplify PCR products				
		8 hours	WT	WT	WT	WT	WT
		12 hours	Failed to amplify PCR products				
		Day 2	WT	WT	WT	WT	WT
		Day 4	WT	WT	WT	WT	WT
		Day 7	ND	ND	ND	ND	ND
		Day 14	ND	ND	ND	ND	ND
Patient X	1b	Baseline (6.63×10^6)	WT	WT	WT	WT	WT
		4 hours	WT	WT	WT	WT	WT
		8 hours	WT	WT	WT	WT	WT
		12 hours	WT	WT	WT	WT	WT
		Day 2	ND	ND	ND	ND	ND
		Day 4	ND	ND	ND	ND	ND
		Day 7	ND	ND	ND	ND	ND
		Day 14	ND	ND	ND	ND	ND

*(%) Quantitative estimates of variants based on chromatograms from population sequencing (estimated from the average of at least two polymerase chain reaction [PCR] products, unless otherwise indicated).

†Resistance data were derived from one PCR product.

Abbreviations: GT, genotype; ND, not determined (HCV RNA <1,000 IU/mL); WT, wild-type consensus.

greater resistance than single substitutions in genotype 1b (Table 2). Some of them displayed poor replicative ability, especially variants with substitutions at residue 93. However, other variants, such as Q30E and L31V, replicated as well as the wild-type replicon. Some variants with double amino acid substitutions (such as Q30R-H58D) also conferred high levels of resistance in the transient replication assays (Table 2). This can, at least partially, explain why viral breakthrough was more commonly observed in patients infected with genotype 1a than those infected with genotype 1b.

The frequencies of substitutions conferring resistance to BMS-790052 in the NS5A region (residues 28-32 and 93) were examined in sequences deposited in the European HCV database (http://euhcvdb.ibcp.fr/euHCVdb/, accessed on March 23, 2010).[5] In this study, the variant with Q30R-H58D substitutions displayed the highest level of resistance (>400,000-fold) (Table 2). Though Q30R is a common genotype 1a BMS-790052-resistant mutation,[5,6] the H58D substitution was not detected in the European HCV database. However, H58D was also identified in patient Q who, like patient S, was a member of the 100-mg cohort. H58D was detected at day 4 in patient Q, but not at any other time points (Table 3E), suggesting that resistant variants can emerge from different paths under similar selective pressures.

Previously characterized resistant variants were detected in only 3 of 24 patients' (M, T, and V) baseline specimens by population sequencing. Patient M (60-mg cohort) was infected with genotype 1a virus. A Q30R substitution was detected at a level of ~10% at baseline, but reached ~60% at day 4. This profile suggests that the Q30R variant was suppressed by the 60-mg dose of BMS-790052, with a slower rate of

Table 4. Wild-Type and BMS-790052-Resistant Replicons Are Equally Sensitive to NS3 Protease and NS5B Polymerase Inhibitors

	EC_{50} (nM)			
Inhibitor*	Parent (1a)	Q30K	Y93H	Q30R-H58D
BMS-790052	0.021 ± 0.002	82 ± 9.7	52 ± 20	>2,000
NS3 protease inhibitor (BMS-650032)	6.6 ± 1.9	3.4 ± 0.8	2.3 ± 0.5	2.9 ± 1.0
NS5B polymerase inhibitor (BMS-791325)	7.5 ± 0.3	2.6 ± 0.4	4.1 ± 0.5	5.4 ± 0.8

*Genotype 1a replicon cell lines with indicated substitutions in NS5A were assayed for sensitivity to hepatitis C virus inhibitors.
Abbreviations: NS, nonstructural protein; EC_{50}; 50% effective concentration.

decline than wild-type virus at day 1. Replacement of the Q30R variant with a Q30H-Y93H variant at day 14 suggests that this double amino acid substitution variant with a high level of resistance (EC_{50} value: 409.8 ng/mL or 553 nM; Table 2) was selected. Selection of this highly resistant linked variant could explain the viral breakthrough observed in patient M during treatment with 60 mg of BMS-790052. Patient T in the 100-mg cohort was infected with genotype 1b virus with baseline resistance (Q54H-Y93H). Consistent with the *in vitro* resistance profile of this variant (Table 1), patient T experienced a maximal HCV RNA decline of ~4.5 log_{10} at day 7, suggesting that the variant with a Q54H-Y93H substitution (100%) was initially suppressed by BMS-790052. The EC_{50} value of L31V-Q54H-Y93H, the dominant variant at day 14, was 36.1 ng/mL or ~49 nM. Trough concentrations of BMS-790052 in patient T were 153-546 ng/mL or 207-737 nM (data not shown), much higher than the EC_{50} value for L31V-Q54H-Y93H. Experiments to determine why the virus was not suppressed in patient T are ongoing. Patient V in the 30-mg twice-daily cohort was also infected with genotype 1a virus. A Q30H-Y93H variant, with a high level of resistance to BMS-790052, was detected at baseline (~100% of Q30H and ~45% of Y93H; Table 3F). It is likely that the presence of this variant at baseline accounts for the lack of viral suppression in patient V.

As we observed in the single-ascending dose study, significant HCV RNA decline was required to detect resistance variants by population sequencing.[4] This observation suggests that these variants were either present at very low levels at baseline or were initially inhibited by BMS-790052. Because variants, such as genotype 1a Q30H in patient R (100-mg cohort), were detected at 4 hours (the first time point) postdosing, it is likely that the Q30H variant preexisted at baseline. Clonal analysis of the baseline specimens could address this possibility.

From a virology point of view, the antiviral effect of a specific DAA is mainly determined by two factors: intrinsic potency and resistance barrier. Because of the exceptional potency of BMS-790052, patients generally experienced an initial sharp HCV RNA decline, indicative of the inhibition of wild-type virus. A slow second phase of viral decline or a slight viral rebound was observed at later time points, consistent with an accumulation of resistant variants and suggesting the adaptation or selection of resistant variants with enhanced fitness. The emergence of resistance suggests that BMS-790052, like NS3 protease inhibitors[12] and NS5B polymerase allosteric inhibitors,[13] may have a low genetic barrier for resistance. Only a single-nucleotide change (UAU or UAC to AAU or AAC) at residue 93 (Tyr to Asn) of genotype 1a NS5A is required for HCV to acquire clinical resistance to BMS-790052 (Table 2). Furthermore, through either accumulation or novel mutation, linked substitutions emerged, such as Q30R-H58D (patient S, 100-mg cohort; Table 3E), which conferred a high level of resistance.

Questions not addressed in the current study remain. For example, how common is the linkage of resistance substitutions? The possible linkage of two or more substitutions may only be recognized by population sequencing when the substitution for each residue is >50%. Clonal analysis will reveal the frequency of linkage, and phenotypic analysis of variants with linked substitutions will provide useful information about the level of resistance contributed by linked variants. In addition, the rate of decay of resistant variants after cessation of dosing is currently unknown; however, studies to address this are ongoing.

To maximize the anti-HCV response and minimize resistance, combination therapy, similar to current HIV treatment, could be used to enhance the resistance barrier. During combination therapy, variants with multiple substitutions, generally accompanied by reduced fitness, are required to generate clinical resistance. Our *in vitro* data show that BMS-790052-resistant variants remain fully sensitive to inhibitors of the NS3 protease and the NS5B polymerase,[5] demonstrating the potential for effective combination treatment with DAAs that have different mechanisms of actions and targets.

In conclusion, plasma trough concentrations of BMS-790052 monotherapy at the dose range used for the MAD study were not sufficient to prevent all viral breakthroughs because of the emergence of resistant variants. Because BMS-790052 is a novel class of HCV inhibitor with a demonstrated antiviral response in genotype 1–infected patients, it is anticipated that BMS-790052 will be an excellent candidate for combination therapy with interferon plus ribavirin and/or other small-molecule HCV inhibitors. It is also anticipated that combination therapy will suppress the selection of resistant variants.

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