In Vitro Cross-Resistance Profile of Nucleoside Reverse Transcriptase Inhibitor (NRTI) BMS-986001 against Known NRTI Resistance Mutations

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BMS-986001 is a novel HIV nucleoside reverse transcriptase inhibitor (NRTI). To date, little is known about its resistance profile. In order to examine the cross-resistance profile of BMS-986001 to NRTI mutations, a replicating virus system was used to examine specific amino acid mutations known to confer resistance to various NRTIs. In addition, reverse transcriptases from 19 clinical isolates with various NRTI mutations were examined in the Monogram PhenoSense HIV assay. In the site-directed mutagenesis studies, a virus containing a K65R substitution exhibited a 0.4-fold change in 50% effective concentration (EC₅₀) versus the wild type, while the majority of viruses with the Q151M constellation (without M184V) exhibited changes in EC₅₀ versus wild type of 0.23- to 0.48-fold. Susceptibility to BMS-986001 was also maintained in an L74V-containing virus (0.7-fold change), while an M184V-only-containing virus induced a 2- to 3-fold decrease in susceptibility. Increasing numbers of thymidine analog mutation pattern 1 (TAM-1) pathway mutations correlated with decreases in susceptibility to BMS-986001, while viruses with TAM-2 pathway mutations exhibited a 5- to 8-fold decrease in susceptibility, regardless of the number of TAMs. A 22-fold decrease in susceptibility to BMS-986001 was observed in a site-directed mutant containing the T69 insertion complex. Common non-NRTI (NNRTI) mutations had little impact on susceptibility to BMS-986001. The results from the site-directed mutants correlated well with the more complicated genotypes found in NRTI-resistant clinical isolates. Data from clinical studies are needed to determine the clinically relevant resistance cutoff values for BMS-986001.

Nucleoside reverse transcriptase inhibitors (NRTIs) were the first antiretroviral agents approved for clinical use in HIV-infected patients. The genetic correlates for resistance to approved NRTIs are well known and include amino acid substitutions that result in resistance to a specific NRTI or mutations that confer resistance to multiple agents (1).

BMS-986001 is a novel thymidine analog NRTI (Fig. 1), currently in advanced clinical development (NCT01489046). It is significantly more potent against HIV-1 than stavudine (d4T) *in vitro* and has a favorable preclinical toxicity profile (2, 3), making it an appealing candidate for further development as part of combination therapy for HIV. BMS-986001 administered as monotherapy for 10 days in treatment-experienced (not exposed to any antiretroviral therapy in the previous 3 months) HIV-1-infected subjects resulted in a decrease in HIV-1 RNA of 0.97 to 1.28 log₁₀ copies/ml, comparing favorably with other NRTIs (4). BMS-986001 has also demonstrated reduced inhibition of host DNA polymerases and subsequently a favorable mitochondrial toxicity profile *in vitro* (2, 3). In addition, BMS-986001 did not significantly reduce mitochondrial DNA content in cultures of human proximal tubule epithelium, skeletal muscle, or subcutaneous adipocytes (5). BMS-986001 also showed no evidence of renal or bone toxicity in rats and cynomolgus monkeys receiving chronic high doses of BMS-986001 (6). Preliminary *in vitro* studies have demonstrated BMS-986001 activity against some NRTI-resistant HIV-1 mutants and a number of HIV-1 subtypes (3, 7–9; unpublished data). However, a systematic study of known mutations has not been carried out.

The reverse transcriptase (RT) substitution M184V is a common NRTI mutation selected by lamivudine (3TC) and emtricit-

abine (FTC), resulting in a >100-fold increase in half-maximal inhibitory concentration (IC₅₀) to these agents (10–14). The M184V substitution is also known to reduce susceptibility to abacavir (ABC) and didanosine (ddI). However, since the clinical cutoff for ABC is above the fold change in susceptibility associated with M184V, ABC is successfully used in combination with 3TC in the clinic (15–17). Similarly, M184V has been reported to be selected by BMS-986001 *in vitro*, resulting in reduced susceptibility to the agent (8, 9). Conflicting reports of additional amino acid changes that may further decrease susceptibility to BMS-986001 have been published (8, 9). Following 26 days *in vitro*, BMS-986001 selected for M184V (8). Continued selection of virus in cell culture, to a total of 81 days, generated a virus with a substantial decrease in susceptibility to BMS-986001 (130-fold change). This virus contained P119S/T165A substitutions in addition to M184V (8). Subsequent reports used recombinant virus and showed that viruses with the M184V and P119S/T165A/M184V genotypes exhibited a much lower level of resistance to BMS-

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FIG 1 Chemical structure of BMS-986001.

986001 (~2- to 3-fold and 5- to 10-fold decreased susceptibility versus the wild type, respectively) (7, 9).

In this study, the activity profile of BMS-986001 against viruses containing the M184V substitution and other known NRTI and non-NRTI (NNRTI) mutations was investigated using systematic *in vitro* analyses. Mutations were created in a control virus through site-directed mutagenesis (SDM), and mutant viruses were examined for susceptibility to BMS-986001 and five other NRTIs. Susceptibility to a series of clinical isolates with complex NRTI and NNRTI mutations was also examined in the Monogram PhenoSense assay and compared with the fold changes observed in the site-directed mutant experiments.

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MATERIALS AND METHODS

Recombinant virus, cells, and compounds. MT-2 cells, HEK 293T cells, and the proviral DNA clone of NL₄₋₃ were obtained from the NIH AIDS Research and Reference Reagent Program. MT-2 cells were propagated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 mM HEPES buffer (pH 7.55), and 2 mM L-glutamine. HEK 293T cells were propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated FBS, 10 mM HEPES buffer (pH 7.55), and 2 mM L-glutamine. Recombinant NL-Rluc virus, in which a section of the *nef* gene from the proviral clone of NL₄₋₃ was replaced with the *Renilla* luciferase gene, was constructed at Bristol-Myers Squibb. The replication-competent virus and defined mutants were harvested 3 days after transfection of HEK 293T cells with the modified proviral clone. Transfections were performed using Lipofectamine Plus (Invitrogen, Carlsbad, CA), according to the manufacturer's instruction. The titers of the recombinant viruses were determined in MT-2 cells using luciferase enzyme activity as a marker. Recombinant viruses were assayed against the NRTIs BMS-986001, ABC, tenofovir (TFV), d4T, FTC, zidovudine (ZDV), the protease inhibitor nelfinavir (NFV), or the NNRTI efavirenz (EFV). Compounds were either synthesized at Bristol-Myers Squibb (BMS-986001) or obtained from commercial sources.

Cell culture assays. Recombinant viruses containing the luciferase reporter gene were used to infect MT-2 cells in the presence of compound. Cells were infected for 1 h before being added to compounds in 96-well plates. The amount of virus was chosen to give approximately 2×10^6 luminescence units per 10^4 cells on day 5 postinfection; with the wild-type virus, the multiplicity of infection was 0.01. Compounds were serially diluted 3-fold, and 11 concentrations were plated in triplicate. After 5 days of incubation, cells were processed and quantitated for virus growth by the

amount of expressed luciferase. Maximum concentrations for each agent were 100 μ M for BMS-986001 and ABC, 50 μ M for d4T and FTC, and 3 μ M for ZDV, TFV, and NFV. Luciferase was quantitated using the Dual Luciferase kit from Promega (Madison, WI), with modifications to the manufacturer's protocol. The diluted Passive Lysis solution was premixed with the resuspended Luciferase Assay Reagent and then resuspended in Stop & Glo Substrate (2:1:1 ratio). A total of 50 μ l of the mixture was added to each aspirated well on assay plates, and luciferase activity was measured immediately on a Wallac TriLux (Perkin-Elmer, Waltham, MA). Luminescence output was imported into Microsoft Excel, and curves were calculated with XLfit using the Dose Response One Site equation: % inhibition = $100/[1 + (EC_{50}/\text{cmpd})^m]$, where EC_{50} is the 50% effective concentration, cmpd represents BMS-986001, and m represents the slope of the concentration response curve. Each virus was assayed in at least 3 to 5 separate experiments, and the fold change versus wild-type virus for each experiment was calculated and averaged. The protease inhibitor NFV was used in every experiment as a control, since NFV antiviral activity should be invariant with NRTI- and NNRTI-resistant viruses. EFV was included in the NNRTI resistance-associated mutation experiment as a positive control. Grubbs' test for outliers (GraphPad Software) was used to identify outliers at a P value of 0.05. A total of 12 values were identified as outliers, out of 882 values; only one value for BMS-986001 was an outlier, and it was discarded. The Student t test was used to determine whether fold changes were significantly different ($P \leq 0.05$) from wild-type virus. In the event that a half-maximal effective concentration (EC_{50}) could not be reached due to the upper limit of the test compound being investigated, the highest concentration of the test compound was used for calculation of the fold change in EC_{50} ($FC-EC_{50}$) and subsequent comparison versus wild-type virus values in the Student t test. Experiments with clinical isolates were run by Monogram Biosciences using their PhenoSense assay, and fold changes were calculated using their own control virus.

DNA mutagenesis. Modification of the NL₄₋₃ Rep-Rluc proviral clone was carried out via the addition of a unique (but translationally silent) SacII restriction site at position 4206, at the carboxy terminus of the *RT* gene. This allows for the replacement of the wild-type *RT* gene with mutagenized *RT* genes through the use of ApaI/SacII sites surrounding the *RT* gene in the clone. The ApaI site at position 2011 is unique in the proviral clone of NL₄₋₃. Mutations in *RT* were created by SDM using the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA). A subclone containing only the NL₄₋₃ *RT* gene was used for the mutagenesis. After the mutations were confirmed by sequencing, the *RT* gene was excised from the vector and recloned into the full-length Rep-Rluc proviral clone using the unique ApaI/SacII sites.

Clinical RT genes. A series of clinical isolates containing various NRTI mutations were sourced from Monogram Biosciences (South San Francisco, CA) and examined in the PhenoSense HIV assay at Monogram Biosciences. Only sequences at sites that encode established antiretroviral resistance within *RT* genes are known. Thus, background sequences in *RT* can vary in these clinical isolates.

RESULTS

Potency of BMS-986001 and other antiretrovirals against replicating virus. The EC_{50} s of BMS-986001 and other antiretroviral agents against NL₄₋₃ Rep-Rluc virus were evaluated (Table 1). In 43 separate experiments, the average EC_{50} of BMS-986001 was 0.323 μ M. EC_{50} s for the other agents were generally in line with

TABLE 1 Potency of BMS-986001 and other antiretroviral drugs^a against wild-type NL₄₋₃ Rep-Rluc HIV-1 virus

Mean $EC_{50} \pm$ SD (μ M)						
BMS-986001	ABC	d4T	FTC	TFV	ZDV	NFV
0.323 $\pm$ 0.119	0.088 $\pm$ 0.045	0.480 $\pm$ 0.242	0.129 $\pm$ 0.058	0.004 $\pm$ 0.003	0.011 $\pm$ 0.005	0.003 $\pm$ 0.001

^a ABC, abacavir; d4T, stavudine; FTC, emtricitabine; TFV, tenofovir; ZDV, zidovudine; NFV, nelfinavir.

TABLE 2 Fold changes in drug EC₅₀ of virus with M184V compared with wild-type NL₄₋₃ Rep-Rluc HIV-1^a

Virus and mutation(s)	Fold change in EC ₅₀ versus wild-type virus (± SD)						
	BMS-986001	ABC	d4T	FTC	TFV	ZDV	NFV
SDM viruses							
M184V	2.30 (± 0.73)*	4.55 (± 3.11)*	0.79 (± 0.86)	>354*	0.27 (± 0.23)*	0.70 (± 0.38)	1.06 (± 0.54)
P119S/T165A/M184V	4.83 (± 2.37)	4.54 (± 0.71)	0.85 (± 0.43)	408.67 (± 132.31)*	0.71 (± 0.35)	0.62 (± 0.21)	0.78 (± 0.26)
Clinical isolate ^b							
M184V	2.3	3.5	0.8	>103	0.6	0.4	

^a Mean EC₅₀s against wild-type NL₄₋₃ Rep-Rluc HIV-1 virus are listed in Table 1. *, *P* ≤ 0.05.^b *n* = 1 for all.

expected values, except that the EC₅₀ of TFV was also lower than previously described (18). Subsequent results are shown as fold change in EC₅₀ (FC-EC₅₀) compared with the EC₅₀ against the wild-type virus tested in the same experiment, with standard deviation included. Each comparison between site-directed mutants and wild-type virus was performed at least three times, with the exception of experiments with the SDM virus containing the P119S/T165/M184V substitutions, which were performed 2 or 3 times.

Activity against M184V-containing RT mutants. The substitutions M184V or P119S/T165A/M184V were introduced into RT by SDM prior to introduction into the recombinant NL₄₋₃ Rep-Rluc proviral clone. The resultant viruses were examined in a multicycle cell culture inhibition assay for susceptibility to BMS-986001. In addition to the recombinant NL₄₋₃ virus, the RT gene from a clinical isolate with an M184V substitution was analyzed in the PhenoSense HIV assay. Both the NL₄₋₃ recombinant virus and the clinical isolate with M184V exhibited a 2.3-fold decrease in susceptibility to BMS-986001 (Table 2). The NL₄₋₃ virus with the P119S/T165A/M184V substitutions exhibited a slightly greater decrease in susceptibility (FC-EC₅₀ = 4.8) than that with M184V alone. All three viruses exhibited similar decreases in susceptibility to ABC (3.5- to 4.6-fold). As expected, all viruses exhibited significantly decreased susceptibility to FTC (*P* ≤ 0.05 for NL₄₋₃ viruses). The FC-EC₅₀s of the three viruses to the other NRTIs tested (TFV, d4T, and ZDV) were all <1, suggesting enhanced susceptibility to these agents when M184V is present.

Activity against K65R-containing RT mutants. The K65R substitution is the signature clinical resistance mutation for TFV, decreasing susceptibility to the agent in enzymatic studies and in cell culture (19–22). K65R can also be selected by ABC, d4T, and ddI and impacts susceptibility to these agents, as well as to 3TC and FTC (12, 23–25). K65R or the double substitution K65R/

M184V was introduced into the NL₄₋₃ RepRluc backbone by SDM, and the resultant virus was tested for susceptibility to the various NRTIs. In addition, two clinical isolates, one with K65R and two NNRTI substitutions (Y181C/G190S) and one with K65R/M184V substitutions, were analyzed in the PhenoSense HIV assay. The FC-EC₅₀s observed for the recombinant K65R virus and the K65R/Y181C/G190S clinical isolate were 0.4-fold (*P* ≤ 0.05) and 0.2-fold, respectively (Table 3). These data support a synergistic relationship of BMS-986001 with K65R, which has also been described in previous studies (8; unpublished data). However, when M184V was added to K65R, both in the recombinant virus and in the clinical isolate, a 2- to 3-fold decrease in susceptibility to BMS-986001 was observed, similar to that seen with M184V alone.

Results with the other five NRTIs were as expected, with small decreases in susceptibility to ABC, d4T, and TFV observed for the K65R viruses and little change for ZDV. Susceptibility to FTC was decreased by K65R; however, the FC-EC₅₀ was relatively low (14.2- or 13.0-fold) compared with that observed when K65R/M184V was analyzed (>384-fold; *P* ≤ 0.05). The addition of M184V to K65R further decreased susceptibility to ABC (FC-EC₅₀ = 9.9 in the clinical isolate and 23.5 in the SDM virus) compared with K65R alone, as has been noted in clinical samples (26). Addition of M184V to K65R had little effect on the susceptibility of clinical isolates to TFV and d4T; however, in recombinant viruses, addition of M184V to K65R appeared to increase susceptibility to the drugs compared with K65R alone.

Activity against L74V-containing RT mutants. The L74V substitution is considered a major mutation resulting in reduced susceptibility to ABC and ddI (14, 15, 24, 27, 28). In order to determine the susceptibility of an RT gene with L74V to BMS-986001, a recombinant NL₄₋₃ Rep-Rluc virus with this substitution was generated, together with an L74V/M184V recombinant

TABLE 3 Fold changes in drug EC₅₀ of virus with K65R compared with wild-type NL₄₋₃ Rep-Rluc HIV-1^a

Virus and mutation(s)	Fold change in EC ₅₀ versus wild-type virus (± SD)						
	BMS-986001	ABC	d4T	FTC	TFV	ZDV	NFV
SDM viruses							
K65R	0.40 (± 0.16)*	3.03 (± 1.49)	2.03 (± 0.75)*	14.21 (± 11.80)	4.09 (± 2.63)	0.90 (± 0.61)	0.90 (± 0.50)
K65R/M184V	2.65 (± 0.85)*	23.54 (± 15.96)	1.15 (± 0.83)	>384*	2.44 (± 0.80)*	0.64 (± 0.44)	0.93 (± 0.36)
Clinical isolate ^b							
K65R/Y181C/G190S	0.2	2.7	1.4	13.0	1.5	0.5	
K65R/M184V	1.9	9.9	1.2	>103	1.5	0.4	

^a Mean EC₅₀s against wild-type NL₄₋₃ Rep-Rluc HIV-1 virus are listed in Table 1. *, *P* ≤ 0.05.^b *n* = 1 for all.

TABLE 4 Fold changes in drug EC₅₀ of virus with L74V compared with wild-type NL₄₋₃ Rep-*Rluc* HIV-1^a

SDM virus mutation(s)	Fold change in EC ₅₀ versus wild-type virus (± SD)						
	BMS-986001	ABC	d4T	FTC	TFV	ZDV	NFV
L74V	0.72 (± 0.19)*	2.99 (± 0.91)*	1.04 (± 0.32)	1.50 (± 0.89)	0.84 (± 0.24)	1.09 (± 0.74)	0.81 (± 0.28)
L74V/M184V	2.48 (± 0.98)*	5.98 (± 2.62)*	0.75 (± 0.19)*	>384*	1.06 (± 0.42)	0.68 (± 0.38)	1.13 (± 0.36)

^a Mean EC₅₀s against wild-type NL₄₋₃ Rep-*Rluc* HIV-1 virus are listed in Table 1. *, $P \leq 0.05$.

virus. The L74V virus remained susceptible to BMS-986001; it exhibited an FC-EC₅₀ of 0.7 (Table 4). When M184V was added, a decrease in susceptibility to BMS-986001 was recorded (FC-EC₅₀ = 2.5), similar to that observed with M184V alone (Table 2).

Virus with L74V demonstrated a 3-fold decrease in susceptibility to ABC compared with the wild type and remained susceptible to the other NRTIs tested. For the L74V/M184V-recombinant virus, a significant decrease in susceptibility to ABC (FC-EC₅₀ = 6.0) and FTC (FC-EC₅₀ > 384) was observed, as expected ($P \leq 0.05$).

Activity against TAM pathway-containing RT mutants. Thymidine analog mutations (TAMs), which are selected by thymidine analogs (ZDV and d4T) and confer resistance to all NRTIs, have been shown to develop by one of two pathways: TAM 1 (T215Y linked) and TAM 2 (T215F linked). M184V has been shown to be associated with both pathways (29).

TAM 1 pathway. A series of seven recombinant viruses were constructed based upon the stepwise progression of TAMs usually observed (30, 31) and were tested along with five RT genes from clinical isolates containing TAM 1 pathway mutations. The accumulation of TAM 1 pathway mutations in the NL₄₋₃ background decreased the susceptibility of the virus to BMS-986001 incrementally (Table 5). The same was true for the RT genes from clinical isolates when analyzed with the PhenoSense HIV assay. The addition of M184V to the recombinant viruses did not substantially affect susceptibility to BMS-986001. As expected, the TAM 1 pathway mutations generally decreased susceptibility to the other NRTIs.

TAM 2 pathway. A series of seven recombinant viruses with substitutions representing the TAM 2 pathway were generated. In addition, three clinical isolate RT genes containing multiple TAM 2 pathway mutations were analyzed in the PhenoSense HIV assay. Susceptibility of viruses with TAM 2 pathway mutations to BMS-986001 appeared to be predicted by the presence of T215F (Table 6). In the recombinant virus, the presence of T215F led to an 8.2-fold decrease in susceptibility to BMS-986001 ($P \leq 0.05$), which did not change substantially when additional pathway mutations were added. Two of the three clinical samples exhibited greater decreases in susceptibility to BMS-986001 than that observed with recombinant viruses, but they also contained TAM 1 and NNRTI mutations.

The presence of cumulative mutations in the recombinant and clinical viruses tended to result in decreased susceptibility not only to the earlier thymidine analogs, ZDV and d4T, but to all of the NRTIs tested.

Activity against Q151M-containing RT mutants. The Q151M complex (A62V, V75I, F77L, F116Y, and Q151M) is a rare pathway that generates broad cross-resistance to most NRTIs, perhaps with the exception of TFV, through discrimination of the NRTI (32–34). A series of five recombinant viruses containing the mutations from the complex observed in clinical isolates was exam-

ined, along with two RT genes from clinical isolates. Compared with the other NRTIs tested, viruses with the Q151M complex exhibited unique susceptibility to BMS-986001. The core A62V/V75I/Q151M recombinant virus showed a significant increase in susceptibility to BMS-986001, with an FC-EC₅₀ of 0.2 ($P \leq 0.05$). The addition of F116Y slightly increased the FC-EC₅₀ to 0.5, which then increased to 1.1 when F77L was also present (Table 7). Interestingly, when M184V was added onto the entire Q151M complex, there was a large decrease in susceptibility to BMS-986001, with the FC-EC₅₀ increasing from 1.1 to >38. This phenomenon was also observed with the two clinical isolates tested. The isolate without M184V demonstrated an FC-EC₅₀ of 0.3, while the isolate that contained the Q151M complex with M184V exhibited a 25-fold decrease in susceptibility to BMS-986001 compared with the wild type. This is a unique phenotype for BMS-986001, as the addition of M184V to K65R or L74V did not increase the susceptibility of the recombinant viruses above that observed with M184V alone, and adding M184V to viruses with TAM 1 or 2 pathway substitutions did not appear to have a large effect.

The other NRTIs tested, with the exception of TFV, exhibited decreased activity of various magnitudes against all of the recombinant viruses with the Q151M complex. Similar to BMS-986001, large decreases in susceptibility to ABC, ZDV, and FTC were recorded when M184V was present in addition to the Q151M complex.

Activity against T69ins-containing RT mutants. The T69 insertion pathway (T69ins) is another relatively rare multinucleoside resistance pathway (35). The substitution T69S, with the additional insertion of two or more amino acids at this position, is typically noted on a background of several TAMs and results in resistance to most NRTIs (36, 37). The mechanism of resistance associated with the T69ins pathway is increased excision activity, similar to that observed with TAMs (38).

In this study, two recombinant viruses were generated. One contained only the T69SSS insertion in NL₄₋₃, while the other contained the insertion along with mutations that should encode broad NRTI resistance (36). In addition, two clinical isolate genes were examined in the PhenoSense HIV assay, both containing the T69ins with additional mutations. As expected, the recombinant virus with only the T69SSS insertion was susceptible to all NRTIs, with the greatest decrease in susceptibility observed with FTC (FC-EC₅₀ = 4.1). Addition of T210W/T215Y to the recombinant virus resulted in ≥ 14 -fold decreases in susceptibility to the NRTIs tested, with the exception of TFV, which exhibited a 4.6-fold decrease in activity (Table 8). The susceptibility of the two clinical isolates containing RT genes with the T69ins to all NRTIs was reduced relative to the wild type.

Activity against NNRTI resistance mutation-containing RT mutants. The effect of NNRTI resistance-associated mutations on the activity of BMS-986001 and other NRTIs was evaluated by testing the activity of the agents against recombinant viruses con-

TABLE 5 Fold changes in drug EC₅₀ of virus with TAM 1 pathway mutations compared with wild-type NL₄₋₃ Rep-Rluc HIV-1^a

Fold change in EC ₅₀ versus wild-type virus (± SD)							
Virus and mutation(s)	BMS-986001	ABC	d4T	FTC	TFV	ZDV	NFV
SDM viruses							
M41L	1.55 (± 0.57)	0.79 (± 0.29)	1.57 (± 0.52)	0.65 (± 0.31)	0.58 (± 0.13)*	1.15 (± 0.69)	0.68 (± 0.21)
T215Y	5.91 (± 3.87)*	2.96 (± 1.53)*	2.23 (± 0.59)*	3.18 (± 1.62)*	2.30 (± 0.81)*	10.03 (± 6.36)*	1.39 (± 0.98)
M41L/T215Y	10.86 (± 7.98)*	3.31 (± 1.90)	4.48 (± 2.89)	2.85 (± 1.88)	2.32 (± 2.26)	21.54 (± 24.12)	1.07 (± 0.41)
M41L/L210W/T215Y	17.37 (± 9.92)*	2.33 (± 1.24)	2.60 (± 0.76)	3.64 (± 1.44)	2.41 (± 1.73)	31.75 (± 22.50)	0.85 (± 0.32)
M41L/D67N/L210W/T215Y	26.72 (± 18.46)*	3.24 (± 1.18)*	2.20 (± 0.15)*	6.93 (± 4.23)*	3.26 (± 3.24)	>122*	0.92 (± 0.20)
M41L/D67N/M184V/L210W/T215Y	16.95 (± 12.27)*	7.36 (± 4.65)*	6.60 (± 3.32)*	>264*	1.57 (± 0.86)	>24*	0.83 (± 0.32)
M41L/D67N/K70R/L210W/T215Y/K219Q	15.30 (± 7.71)	3.08 (± 1.16)	4.30 (± 0.50)*	24.41 (± 10.69)	1.23 (± 0.64)	98.17 (± 21.92)*	0.96 (± 0.33)
Clinical isolate ^b							
M41L/M184V/L210W/T215Y	4.4	6.2	1.2	>103	0.9	4.2	
M41L/A98G/K103N/M184V/L210W/T215Y	3.3	9.6	3.3	>103	2.8	261	
M41L/D67N/K70R/M184V/L210W/T215Y/K219E	19	6.9	1.9	>103	2.3	66.0	
M41L/D67N/K70R/Y181C/G190A/L210W/T215Y/K219E	14	5.6	4.7	6.4	3.7	638	
M41L/D67N/K70R/A98G/K101E/M184V/G190A/L210W/T215Y/K219E	>143	11.0	2.7	>103	6.0	726	

^a Mean EC₅₀s against wild-type NL₄₋₃ Rep-Rluc HIV-1 virus are listed in Table 1. *, P ≤ 0.05.
^b n = 1 for all.

TABLE 6 Fold changes in drug EC₅₀ of virus with TAM 2 pathway mutations compared with wild-type NL₄₋₃ Rep-Rluc HIV-1^a

Fold change in EC ₅₀ versus wild-type virus							
Virus and mutation(s)	BMS-986001	ABC	d4T	FTC	TFV	ZDV	NFV
SDM viruses							
T215F	8.21 (± 4.09)*	1.53 (± 1.18)	1.16 (± 0.63)	5.26 (± 2.56)*	1.19 (± 0.68)	8.59 (± 2.91)*	1.24 (± 0.32)
K219Q	1.20 (± 0.61)	0.92 (± 0.48)	1.53 (± 0.49)	1.51 (± 0.88)	0.80 (± 0.75)	1.61 (± 0.59)	1.34 (± 0.49)
T215F/K219Q	5.97 (± 3.22)*	3.75 (± 2.42)*	4.17 (± 1.87)*	8.37 (± 3.58)*	3.35 (± 2.07)*	8.70 (± 4.96)*	2.12 (± 0.63)*
D67N/T215F/K219Q	5.37 (± 3.07)*	2.11 (± 1.01)	4.58 (± 2.45)	5.16 (± 2.62)	1.02 (± 0.28)	11.75 (± 8.53)	1.16 (± 0.48)
D67N/K70R/T215F/K219Q	5.70 (± 1.07)*	2.90 (± 0.69)*	3.41 (± 1.17)*	4.57 (± 1.65)*	1.67 (± 0.27)*	17.35 (± 16.66)	1.29 (± 0.38)
D67N/K70R/K219Q	6.19 (± 2.27)*	1.46 (± 1.12)	0.96 (± 0.42)	1.55 (± 2.24)	2.14 (± 1.12)	4.35 (± 4.83)	1.19 (± 0.31)
D67N/K70R/M184V/T215F/K219Q	8.24 (± 2.59)*	4.21 (± 3.26)	1.50 (± 0.61)	>383	0.92 (± 0.73)	5.50 (± 1.62)*	1.12 (± 0.48)
Clinical isolate ^b							
M41L/D67N/K70R/K103N/L210W/T215F/K219Q	15.0	4.7	3.4	4.0	4.0	504	
D67N/T69N/K70R/A98G/M184V/G190S/T215F/K219Q	13.0	8.5	2.8	>103	1.4	18.0	
D67N/T69N/K70R/K103N/M184V/T215F/K219Q	4.8	5.1	1.3	>103	1.0	8.6	

^a Mean EC₅₀s against wild-type NL₄₋₃ Rep-Rluc HIV-1 virus are listed in Table 1. *, P ≤ 0.05.
^b n = 1 for all.

TABLE 7 Fold changes in drug EC₅₀ of virus with the Q151 M complex compared with wild-type NL₄₋₃ Rep-*Rluc* HIV-1^a

Virus and mutations	Fold change in EC ₅₀ versus wild-type virus						
	BMS-986001	ABC	d4T	FTC	TFV	ZDV	NFV
SDM viruses							
A62V/V75I/Q151 M	0.23 (± 0.06)*	7.98 (± 4.99)*	6.54 (± 4.41)*	8.17 (± 12.09)	1.17 (± 0.61)	2.02 (± 1.26)	1.08 (± 0.41)
A62V/V75I/F116Y/Q151 M	0.48 (± 0.07)*	8.56 (± 3.72)*	27.81 (± 31.62)	4.92 (± 3.85)	1.38 (± 1.09)	11.15 (± 8.83)*	1.98 (± 0.21)*
F77L/F116Y/Q151 M	0.67 (± 0.36)*	5.74 (± 2.42)*	25.87 (± 14.61)*	4.15 (± 2.98)*	1.03 (± 0.78)	10.69 (± 5.52)*	1.04 (± 0.52)
A62V/V75I/F77L/F116Y/Q151 M	1.08 (± 0.70)	5.42 (± 3.22)	52.95 (± 43.15)	6.76 (± 3.77)	1.73 (± 1.92)	67.46 (± 39.50)	1.16 (± 0.19)
A62V/V75I/F77L/F116Y/Q151 M/M184V	>38 *	119.65 (± 139.50)	>52*	>459*	2.39 (± 1.94)	>236	1.39 (± 0.51)
Clinical isolate^b							
A62V (mixture)/L74V/V75I (mixture)/K103N/Y115F/F116Y/Q151 M	0.3	35.0	15.0	28.0	1.3	25.0	
A62V/V75I/F77L/F116L/Q151 M/M184V	25.0	17.0	7.3	>103	0.9	76.0	

^a Mean EC₅₀s against wild-type NL₄₋₃ Rep-*Rluc* HIV-1 virus are listed in Table 1. *, *P* ≤ 0.05.^b *n* = 1 for all.

taining known mutations (Table 9). None of these common NNRTI resistance mutations affected susceptibility to BMS-986001, with FC-EC₅₀ ranging between 0.7 for Y181C and 1.5 for K103N. As would be expected, the presence of certain NNRTI resistance-associated mutations resulted in significant decreases in susceptibility to EFV and had relatively little effect on the activity of the other NRTIs tested. The addition of M184V to an NNRTI mutation resulted in a decrease in susceptibility to BMS-986001 similar to that obtained with M184V alone. In the PhenoSense assay, a clinical isolate containing the K103N/Y188L substitutions produced an FC-EC₅₀ for BMS-986001 of 0.5, while addition of M184V to K103N increased the FC-EC₅₀ to a value close to that observed with M184V alone (FC-EC₅₀ = 2.7).

DISCUSSION

As a new NRTI, BMS-986001 has limited clinical experience thus far, and it is not yet known which RT mutations will be selected by the drug during treatment, nor which ones will impact its clinical antiviral activity. However, it is important to understand the effect that known NRTI resistance mutations have on susceptibility to BMS-986001. *In vitro*, the only single substitution that has been selected for by BMS-986001 is M184V (8), which in this study has been shown to induce a 2.3-fold decrease in susceptibility to BMS-986001 (Table 2). In a 10-day monotherapy clinical study with BMS-986001 in antiretroviral treatment-experienced HIV-1-infected subjects, no selection of specific amino acid changes was observed (4), although only population sequencing was performed on these samples, and therefore, selection of minor resistance variants cannot be excluded. One subject receiving BMS-986001 had T215T/S at baseline, which was not present on day 11 or day 17 of the study. Two other subjects who received BMS-

986001 had K70K/R and K219K/R, respectively, detected on day 17, which were not present on population sequencing at baseline or day 11 of the study. All three subjects exhibited a good antiviral response to BMS-986001, with a range of plasma HIV-1 RNA decline from baseline to day 11 of 1.42 to 1.91 log₁₀ copies/ml. Notably, despite a history of previous treatment and failure of 3TC and/or FTC in several subjects, the M184V substitution was not detected at baseline, day 11, or day 17 of the study in any subject.

Here, we describe a systematic approach that confirms and expands on existing data on the cross-resistance profile of BMS-986001 against mutations known to encode resistance to currently approved NRTIs. In the current study, a replication-competent NL₄₋₃ virus was used as a backbone, and specific amino acid changes known to encode resistance to common NRTIs were made in the *RT* gene. The absolute EC₅₀ for BMS-986001 (0.323 μM; Table 1) against this virus is higher than what has been noted in most published reports. This may be due in part to the virus/cells used and other experimental conditions. In previous studies, using different viruses or cell lines, the EC₅₀ for BMS-986001 ranged from 0.0019 μM (HIV-III_B virus in peripheral blood mononuclear cells [PBMCs]) (39) to 0.49 μM (A012B virus in MAGI-CCR5 cells) (8). For HIV-III_B virus, EC₅₀s of 0.0019 (in PBMCs), 0.07 (MT-4 cells) (39), 0.1 (MT-4 cells) (8), and 0.25 (MT-2 cells) (39) were reported, while the EC₅₀ determined in the PhenoSense assay in this study was 0.070 μM. Interestingly, the only published report using the NL₄₋₃ virus described the EC₅₀ in TZM-bl cells as 0.37 μM, similar to the value described here (9). It should be noted that the mean concentration of BMS-986001 triphosphate in PBMCs from healthy donors treated with BMS-986001 was 0.71 pmol/10⁶ cells (0.71 × 10⁻¹⁸ mol/cell) (range,

TABLE 8 Fold changes in drug EC₅₀ of virus with the T69ins complex compared with wild-type NL₄₋₃ Rep-*Rluc* HIV-1^a

Virus and mutations	BMS-986001	ABC	d4T	FTC	TFV	ZDV	NFV
SDM viruses							
T69SSS	1.72 (± 0.73)	3.16 (± 1.70)*	1.33 (± 1.06)	4.06 (± 2.13)	2.31 (± 2.20)	1.83 (± 1.15)	1.29 (± 0.64)
T69SSS/T210W/T215Y	22.28 (± 9.51)*	13.60 (± 6.82)*	>35*	48.42 (± 54.15)	4.57 (± 4.66)	>103*	0.93 (± 0.40)
Clinical isolate^b							
A62V/T69ins/V75I/A98G/K103S/G190A	>143	17.0	13.0	21.0	22.0	>1067	
T69ins/K70R/M184V/K219Q	>143	14.0	7.1	>103	4.3	254	

^a Mean EC₅₀s against wild-type NL₄₋₃ Rep-*Rluc* HIV-1 virus are listed in Table 1. *, *P* ≤ 0.05.^b *n* = 1 for all.

TABLE 9 Fold changes in drug EC₅₀ of virus with NNRTI resistance-associated mutations compared with wild-type NL₄₋₃ Rep-Rluc HIV-1^a

Virus and mutation(s)	Fold change in EC ₅₀ versus wild-type virus (± SD)							
	BMS-986001	ABC	d4T	FTC	TFV	ZDV	NFV	EFV
SDM viruses								
K103N	1.49 (± 0.65)	1.11 (± 0.61)	1.03 (± 0.68)	0.98 (± 0.56)	1.00 (± 0.60)	1.58 (± 0.960)	1.05 (± 0.19)	58.70 (± 27.13)*
Y181C	0.68 (± 0.38)	1.05 (± 0.61)	0.51 (± 0.56)	0.63 (± 0.42)	1.10 (± 0.57)	0.43 (± 0.16)*	0.69 (± 0.28)	1.53 (± 0.39)*
G190A	1.08 (± 0.48)	1.04 (± 0.38)	1.53 (± 1.26)	1.00 (± 1.05)	0.82 (± 0.54)	1.12 (± 0.45)	0.68 (± 0.28)	5.73 (± 3.32)*
G190S	1.41 (± 0.73)	1.03 (± 0.39)	1.12 (± 0.53)	0.93 (± 0.50)	0.84 (± 0.52)	1.62 (± 0.57)	0.87 (± 0.37)	102.92 (± 36.72)*
V106A	1.15 (± 0.57)	1.05 (± 0.50)	1.27 (± 0.68)	0.71 (0.32)	0.94 (± 0.52)	1.54 (± 0.61)	0.78 (± 0.25)	2.21 (± 0.45)*
V106 M	1.32 (± 0.73)	1.81 (± 1.23)	1.03 (± 0.61)	0.69 (± 0.38)	0.96 (± 0.53)	0.89 (± 0.45)	0.87 (± 0.21)	13.21 (± 6.09)*
L100I	0.71 (± 0.34)	0.63 (± 0.26)*	0.68 (± 0.56)	0.51 (± 0.38)*	1.01 (± 0.58)	0.50 (± 0.20)*	0.68 (± 0.18)*	17.48 (± 4.90)*
Y181C/K103N	1.11 (± 0.37)	1.40 (± 0.82)	1.22 (± 0.84)	1.01 (± 0.77)	1.42 (± 1.18)	1.07 (± 0.21)	1.31 (± 0.24)*	56.64 (± 16.92)*
K103N/M184V	2.57 (± 1.03)*	3.36 (± 1.67)*	2.03 (± 2.24)	>1004*	0.96 (± 0.62)	0.73 (± 0.19)*	0.92 (± 0.64)	31.75 (± 16.69)*
Y181C/M184V	1.78 (± 0.79)	4.12 (± 2.69)	1.07 (± 0.50)	>1004*	0.93 (± 0.36)	0.57 (± 0.24)*	0.83 (± 0.21)	1.18 (± 0.29)
Clinical isolate ^b								
K103N/Y188L	0.5	1.2	0.8	1.4	0.7	0.6		
K103N/M184V	2.7	3.0	0.7	>103	0.6	0.4		

^a Mean EC₅₀s against wild-type NL₄₋₃ Rep-Rluc HIV-1 virus are listed in Table 1. *, *P* ≤ 0.05.
^b *n* = 1 for all.

0.10 to 3.00 pmol/10⁶ cells) (40). Assuming the volume of a resting PBMC is 200 femtoliters (2 × 10⁻¹³ liters/cell), this translates into a mean BMS-986001 triphosphate concentration of 3.6 μM, which is greater than any of the measured EC₅₀s. The clinical efficacy of BMS-986001 will depend upon a number of factors, which include the innate susceptibility of the virus for the drug and the intracellular triphosphate level (and the ratio of the two) that is required for good efficacy.

Substitutions within the RT gene were constructed based upon known resistance correlates for NRTIs. These substitutions included M184V, K65R, L74V, the two TAM pathways, and the Q151M and T69ins complexes. Combinations of substitutions were also made based upon changes typically observed in clinical studies. In addition, the data generated with SDM viruses using our assay were compared with results obtained from clinical isolates in the PhenoSense HIV assay. The experiments compared the changes in susceptibility to BMS-986001 with five other NRTIs (ABC, TFV, d4T, FTC, and ZDV). These comparator agents were chosen either because they are the most commonly used NRTIs in clinical practice today or because of structural similarity to BMS-986001. The data reported here for the comparator agents are in line with their known activity profile (12, 14, 15, 20, 22, 23, 25, 26, 28, 29, 33, 35–38, 41–43). The HIV protease inhibitor NFV was included as a control in all experiments and exhibited little variation throughout.

Although one cannot definitively predict the clinical resistance profile of BMS-986001 from these studies, several observations can be made. First, the M184V substitution confers a decrease in susceptibility to BMS-986001, similar to that observed for ABC. When this substitution is present with K65R or L74V, the decrease in susceptibility to BMS-986001 is similar to that observed for M184V alone. Adding M184V to either TAM pathway (when multiple TAMs were present) also did not appear to have a large effect on susceptibility to BMS-986001. Second, virus containing K65R, which confers resistance to several NRTIs including ABC, TDF, d4T, and ddI, was significantly more susceptible to BMS-986001, similar to observations with ZDV. Third, virus containing L74V, which confers resistance to ABC and ddI, remains suscep-

tible to BMS-986001. Fourth, viruses with increasing numbers of TAM 1 mutations generally display decreasing susceptibility to BMS-986001, reaching fold change levels of >10 once two TAMs are present. Viruses with increasing numbers of TAM 2 mutations exhibit relatively flat 4- to 8-fold increases in EC₅₀ against BMS-986001, due mainly to the T215F substitution, which exhibits an 8.2-fold increase by itself. Fifth, BMS-986001 displayed a unique profile against the Q151M complex, with the virus containing the A62V/V75I/Q151M genotype showing significantly increased susceptibility to BMS-986001 and the virus containing the entire complex of A62V/V75I/F77L/F116Y/Q151M maintaining susceptibility to BMS-986001 (FC-EC₅₀ = 0.2 and 1.1, respectively). However, addition of M184V to this genotype significantly decreased susceptibility to BMS-986001 (>38-fold). This observation was confirmed in the clinical isolates tested. Finally, recombinant virus with the T69ins complex (including T210W/T215Y) exhibited a relatively large decrease in susceptibility to BMS-986001, which was similar to observations with all other NRTIs. Clinical isolates that contained a T69 insertion with additional NRTI and NNRTI mutations generally exhibited large decreases in susceptibility to BMS-986001 and the other NRTIs. Based on these observations, BMS-986001 appears to have a predictable resistance profile which overlaps those of some NRTIs. BMS-986001 retains activity against viruses with K65R (a signature mutation of the commonly used drug TDF) and L74V (a major mutation resulting in reduced susceptibility to ABC). Finally, as with most other NRTIs, there seems to be little impact of common NNRTI mutations on susceptibility to BMS-986001. Previous reports describe similar results with a subset of the mutations assessed in our study (7, 8). Although Nitanda et al. reported that a virus with an M184V mutation exhibited an 11-fold reduction in susceptibility to BMS-986001 (8), this was not consistent with our study and other reports (3, 9). In addition, minor differences were observed in viruses containing the K103N substitution. In these reports, a virus containing K103N in a MAGI-CCR5 reporting system demonstrated an enhanced fold change in the BMS-986001 EC₅₀ of 0.59 relative to the control virus, whereas in our system, a 1.49-fold change in BMS-986001 EC₅₀ was seen in a

K103N-containing SDM. In addition, a K65R-containing HXB2 virus in the same reporting system produced a 0.87-fold change, which is higher than the 0.4-fold change in our system (Table 3). These minor differences could be related to the virus strain, cell lines, or reporter systems used.

The clinical relevance of these *in vitro* data remains to be determined. It is not yet known whether clinical administration of BMS-986001 will select for any of these mutations or what the signature mutation(s) for BMS-986001 will be. Furthermore, the fold increase in EC₅₀ required to diminish the antiviral effect of BMS-986001 *in vivo* remains to be determined. The clinical efficacy of BMS-986001 will depend upon a number of factors, including the innate susceptibility of the virus to the drug, the levels of the biologically active intracellular triphosphate achieved with clinical administration, and the ratio of the two (inhibitory quotient) that is required for good efficacy. Data from clinical trials of subjects with NRTI resistance mutations will be needed to determine the clinically relevant phenotypic and genotypic resistance thresholds for BMS-986001.

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