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In Vitro Antiviral Characteristics of HIV-1 Attachment Inhibitor BMS-626529, the Active Component of the Prodrug BMS-663068

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BMS-663068 is the phosphonooxymethyl prodrug of BMS-626529, a novel small-molecule attachment inhibitor that targets HIV-1 gp120 and prevents its binding to CD4$^+$ T cells. The activity of BMS-626529 is virus dependent, due to heterogeneity within gp120. In order to better understand the anti-HIV-1 spectrum of BMS-626529 against HIV-1, *in vitro* activities against a wide variety of laboratory strains and clinical isolates were determined. BMS-626529 had half-maximal effective concentration (EC_{50}) values of <10 nM against the vast majority of viral isolates; however, susceptibility varied by >6 $\log_{10}$, with half-maximal effective concentration values in the low pM range against the most susceptible viruses. The *in vitro* antiviral activity of BMS-626529 was generally not associated with either tropism or subtype, with few exceptions. Measurement of the binding affinity of BMS-626529 for purified gp120 suggests that a contributory factor to its inhibitory potency may be a relatively long dissociative half-life. Finally, in two-drug combination studies, BMS-626529 demonstrated additive or synergistic interactions with antiretroviral drugs of different mechanistic classes. These results suggest that BMS-626529 should be active against the majority of HIV-1 viruses and support the continued clinical development of the compound.

Despite advances in HIV treatment, there is a continuing need for the development of new antiretroviral drugs and regimens because of safety and long-term tolerability concerns with existing treatment options and the emergence of resistance (10). The process of HIV entry depends on multiple sequential steps that are initiated by the binding of the viral gp120 envelope glycopeptide to the host cell CD4 receptor, followed by coreceptor binding and membrane fusion (21, 35). Antiretroviral drugs that target these steps include maraviroc, which prevents HIV-1 binding to the CCR5 (C-C chemokine receptor type 5) coreceptor, (12), and enfuvirtide, an injectable peptide that inhibits gp41-mediated fusion of the host and viral cell membranes (22). However, neither of these agents offers a complete solution to the inhibition of HIV entry, with the efficacy of maraviroc limited by issues that include the presence of CXCR4-using or dual/mixed virus and coreceptor switching, while the utility of enfuvirtide is restricted by requirements for twice-daily injections and local injection site adverse reactions. Currently there are no licensed agents that target the first step of HIV entry, the binding of gp120 to CD4.

Small-molecule inhibitors of gp120 attachment to CD4 have been described (3, 13, 17, 38), and proof of concept for this class was achieved in a phase IIa 8-day monotherapy study that examined the progenitor attachment inhibitor BMS-488043 (14). However, while BMS-488043 demonstrated potent antiviral activity in this study, significant variability in individual half-maximal effective concentration (EC_{50}) values was observed (14, 41).

The exact mechanism of action of this class of compounds remains under investigation. BMS-488043 has been shown to stabilize a conformation of gp120 that does not recognize CD4, thereby interfering with its initial association with CD4 (16). Additionally, this class of compounds may also form a ternary complex with gp120 and CD4 and interfere with gp41 unmasking (28). While the CD4 binding site of gp120 has shown little propensity for polymorphic substitution, heterogeneity in gp120 sequences and hence structure is believed to be the underlying reason for the broad range of EC_{50}s observed with BMS-488043 (41). In addition, BMS-488043 displayed limited oral bioavailability attributed to issues with dissolution and suboptimal pharmacokinetics, properties that ultimately resulted in discontinuation of its development. A major goal of our drug discovery program was to increase the inhibitory potency of the attachment inhibitors against specific HIV-1 isolates, with the belief that this would translate into increased inhibitory potency against a broader range of envelope sequences. This effort led to the discovery of BMS-626529 (Fig. 1), an attachment inhibitor predicted to be more efficacious than BMS-488043.

The generally low solubility and poor intrinsic dissolution properties associated with the previous small-molecule attachment inhibitors extended to BMS-626529. This deficiency was successfully addressed by development of a phosphonooxymethyl prodrug, BMS-663068 (Fig. 1). This prodrug moiety was designed to increase the solubility of the compound in the gut. The prodrug is thought to be cleaved by alkaline phosphatase, located on the luminal surface of the small intestine brush border membranes, releasing BMS-626529. Due to its good membrane permeability, BMS-626529 is then rapidly absorbed (20, 37). In healthy volunteers, BMS-663068 demonstrated good exposure following oral administration, reflecting effective conversion to BMS-626529 and subsequent rapid absorption (20). The pharmacokinetic pro-

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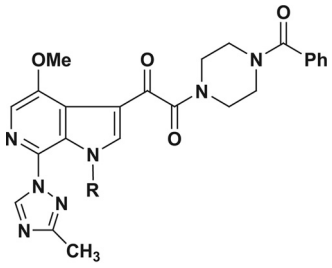

R = H: BMS-626529

R = $CH_2OP(O)(OH)_2$: BMS-663068

FIG 1 Structures of BMS-626529 and the prodrug, BMS-663068.

file of BMS-663068 was further optimized by the development of an extended-release formulation (31). BMS-626529, dosed as BMS-663068, demonstrated potent antiviral activity when administered once or twice daily, with and without ritonavir, in an 8-day monotherapy study of treatment-naïve and treatment-experienced HIV-1-infected subjects, all of whom were infected with subtype B virus (31a).

The present study investigated the *in vitro* antiviral characteristics of BMS-626529. Its activity was examined in peripheral blood mononuclear cells (PBMCs) against a large cohort of clinical isolates of various HIV-1 subtypes with either CCR5 and/or CXCR4 tropism. In addition, envelopes from clinical isolates of different subtypes with or without nonnucleoside reverse transcriptase inhibitor (NNRTI), nucleoside/nucleotide reverse transcriptase inhibitor (NRTI), or protease inhibitor (PI) resistance were assessed for BMS-626529 susceptibility by using the Monogram PhenoSense Entry assay. Also, the binding of BMS-626529 to purified gp120 was investigated, and the antiviral activities of BMS-626529 in combination with approved and preclinical antiretroviral compounds representing different classes were determined.

(Some of these data were presented previously at the 18th Annual Congress on Retroviruses and Opportunistic Infections, Boston, MA, 27 February to 2 March 2011.)

MATERIALS AND METHODS

Compounds. BMS-626529 and BMS-488043 were synthesized at Bristol-Myers Squibb. Efavirenz, atazanavir, BMS-538203 (a strand transfer integrase inhibitor [36]), maraviroc, emtricitabine, AMD-3100, and enfuvirtide (T-20) were purchased from external sources or synthesized using published or known reactions (4; M. A. Walker, J. Banville, R. Remillard, and S. Plamondon, 2003, PCT international application WO 2003049690; T. Wang et al., 2004, U.S. patent application 20,040,110,785). Abacavir, lamivudine, tenofovir, lopinavir, and ritonavir were extracted from commercial formulations of the prescribed drugs and purified using published or common techniques. Tenofovir was tested as tenofovir disoproxil fumarate. Purities of the anti-HIV agents were greater than 95% except for maraviroc and AMD-3100 (>90%). The compounds were dissolved in 100% dimethyl sulfoxide (DMSO; Merck KGaA, Darmstadt, Germany) and serially diluted to the desired concentration such that the final DMSO concentration in cell culture assays was 1%. Maraviroc, tested in the PhenoSense Entry assay, was sourced by Monogram Biosciences and used according to their protocol.

Viruses and cells. PBMCs were isolated by Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO) density gradient centrifugation of heparinized blood from HIV-1-negative subjects. PBMCs were cultured in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY) containing 20% fetal bovine serum (FBS; Gibco), 20 U/ml of penicillin (Gibco), 250 μg/ml of streptomycin (Gibco), and 1 ng/ml of recombinant human interleukin-2 (Invitrogen, Camarillo, CA) and stimulated with phytohemagglutinin (Sigma-Aldrich) at 2 to 4 μg/ml for 2 to 3 days prior to use. MT-2 and PM1 cells were cultured in RPMI 1640 medium (Gibco) containing 10% FBS, 2 mM L-glutamine, and 100 μg/ml penicillin-streptomycin.

HIV-1 laboratory strains and clinical isolates were obtained through the NIH AIDS Research and Reference Reagent Program, which also provided coreceptor tropism data for each of the clinical isolates. A total of 134 clinical isolates for use in the PhenoSense Entry assay (below) were obtained from Bristol-Myers Squibb-sponsored clinical trials (including the CASTLE study [29]). These isolates were chosen from available clinical samples based upon subtype and geographic location. In addition, 50 subtype B envelopes from isolates containing NRTI, NNRTI, and PI resistance mutations, along with 41 subtype A and 17 subtype C envelopes for use in the PhenoSense Entry assay, were obtained from the Monogram Biosciences collection (South San Francisco, CA). Informed consent was obtained for use of all clinical isolates.

Cytotoxicity assays. Cytotoxicity assays were performed in the presence of serially diluted BMS-626529 for up to 6 days, and cell viability was quantitated using an XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) assay (39). To determine CC_{50} values (concentration of drug required to kill 50% of cells), laboratory-adapted cells were initially plated at a density of 0.1×10^6 cells/ml. In the absence of compounds, the cell densities typically reached 1.0×10^6 to 1.2×10^6/ml after 6 days (18).

Drug susceptibility assays using laboratory virus strains. MT-2 (for CXCR4 or dual-tropic viruses) or PM1 (for CCR5-tropic viruses) cells were infected with virus at a multiplicity of infection of 0.005 and incubated in the presence of serial dilutions of drug at 37°C for 4 to 6 days. Virus yields were quantified by determination of reverse transcriptase (RT) activity (33) for CXCR4 viruses or by a p24 enzyme-linked immunosorbent assay (ELISA; PerkinElmer Life Sciences, Inc., Boston, MA) for CCR5 viruses.

Drug susceptibility assays using clinical isolates. Pellets of PBMCs were infected with clinical isolates at a multiplicity of infection of 0.005 and incubated in a 0.5-ml volume at 37°C for 3 h prior to resuspension in medium and addition to plates containing serial dilutions of drug. The final cell density was 1×10^6 cells/ml. Plates were incubated at 37°C, and virus yields were monitored from day 5 postinfection by using a p24 ELISA kit according to the manufacturer's instructions. The incubation was terminated when the control infection yielded a level of p24 in the supernatant within a dynamic range ($0.6 < A_{490} < 2.0$).

Drug susceptibility assays using envelopes derived from clinical isolates. Plasma samples obtained during Bristol-Myers Squibb-sponsored trials were tested by Monogram Biosciences, together with additional samples from the Monogram collection. Drug susceptibilities of the envelopes were determined using the PhenoSense Entry assay (9). Envelope sequences (gp160) were amplified by reverse transcriptase PCR (RT-PCR) and ligated into the pCXAS expression vector. Envelope expression vectors were prepared as large pools of sequences (>200) in order to ensure an accurate representation of the diversity of viral quasispecies present in each sample. Recombinant HIV-1 pseudovirus stocks were prepared by cotransfecting HEK293 cells with the envelope expression vectors and a replication-defective HIV-1 genomic vector containing luciferase within the deleted envelope region. Recombinant pseudovirus particles were used to infect U87 cell lines expressing CD4/CCR5/CXCR4. Drug susceptibility was measured by comparison of luciferase activities in the presence and absence of BMS-626529. Drug susceptibility data were provided as half-maximal inhibitory concentration (IC_{50}) values by Monogram Biosciences and are reported as such.

gp120 expression and purification. Soluble gp120 was expressed from plasmid pCDNA3.1/$gp120_{JRFL}$, encoding the gp120 glycoprotein domain of the HIV-1 JRFL envelope, as previously described (13, 26). gp120-containing supernatants from 293T cells transfected with plasmid

by using Lipofectamine Plus (Invitrogen Life Technologies, Rockville, MD) were concentrated ~30-fold by ultrafiltration (YM30; Millipore Corporation, Bedford, MA). The concentrate was dialyzed against 20 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and absorbed onto a 10-ml column of lectin-Sepharose 4B (AP Biotech, Piscataway, NJ), previously preequilibrated with 10 column volumes of buffer A (20 mM Tris-HCl [pH 8.0], 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$) at 4°C at 1 ml/min. Following a 20-column-volume wash with buffer A containing 1 M NaCl, gp120 was eluted with 10 column volumes of elution buffer (20 mM Tris-HCl [pH 8.0], 0.5 M α-methyl mannoside, 0.1 mM EDTA). The eluent was dialyzed against 25 mM Tris-HCl (pH 7.6) and purified on a 5-ml HiTrap Q column (AP Biotech), using a 0-to-1.0 M NaCl gradient buffered with 20 mM Tris-HCl (pH 8.0). The gp120-containing fractions were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stored at −70°C.

Affinity and off-rates of attachment inhibitors from gp120. Micro BioSpin 6 columns (Bio-Rad Laboratories, Hercules, CA) were used to measure the binding of [^{3}H]BMS-488043 or [^{3}H]BMS-626529 to gp120. Binding solutions (30 μl) containing 25 mM Tris-HCl (pH 7.5), 125 mM NaCl, 50 nM $gp120_{JRFL}$, and serial dilutions of [^{3}H]BMS-488043 or [^{3}H]BMS-626529 were allowed to equilibrate and then adsorbed to a MicroBioSpin 6 column (Bio-Rad Laboratories). The column was centrifuged (~14,000 rpm) for 5 min, the eluent was collected, and radioactivity was determined with a scintillation counter. To measure dissociative kinetics, 150 nM [^{3}H]BMS-626529 or 90 nM [^{3}H]BMS-488043 was incubated with 60 nM gp120 at ambient temperature for 1 h to achieve equilibrium binding, and then a large molar excess (14-fold) of soluble CD4 protein (Jackson Immunodiagnostics, Avondale, PA) was added to drive dissociation. Aliquots were taken at the indicated time intervals, adsorbed to a spin column, and centrifuged, and the radioactivity in the eluent was quantitated. Comparison of the tritium signal from parallel samples with and without the soluble CD4 challenge allowed for the determination of the percent compound bound.

Two-drug combination studies. All combinations, except for those with maraviroc, were examined in MT-2 cells infected with a CXCR4-tropic virus, HIV-1 LAI. The combinations with maraviroc were performed in PM1 cells infected with a CCR5-tropic virus, HIV-1 Bal. In both cases, the multiplicity of infection was 0.005, and cells were seeded at a density of 0.1×10^6 cells/ml in plates containing serial dilutions of the test compounds. The drug combinations were set up using molar ratios of the two drugs at 1:1, 1:2.5, and 2.5:1 times the known EC_{50} for each drug (determined in prior experiments). Each drug ratio consisted of an array of 3-fold serial dilutions, with the concentrations originally designed to start at 300 or 750 times the EC_{50} of the respective compound. Plates were incubated at 37°C, and on day 5 postinfection, a 20-μl sample from each well was harvested and quantitated in a reverse transcriptase assay (33). Combinations with efavirenz were evaluated in a dye reduction cytotoxicity (MTS) assay (6), whereas those with maraviroc were tested on day 6 postinfection in a p24 ELISA (PerkinElmer Life Sciences, Inc.).

Statistical analysis. For the cytotoxicity assays and drug susceptibility assays using laboratory strains and clinical isolates, CC_{50}s and EC_{50}s, respectively, were calculated using XLfit software (Microsoft, Redmond, WA). The results from at least two independent experiments were used. For the PhenoSense Entry assay, IC_{50}s were estimated from inhibition curves generated by bootstrap analysis of the luciferase activity versus drug concentration data. Percentile IC_{50}s were calculated using R version 2.14.0, using the quantile() function, with the "type =" option set to 8. For assessment of dissociation kinetics, data were fit to exponential decay equations using Graphpad Prism, version 6.0. For the combination studies, concentration-response curves were estimated for each individual drug and combination using the median effect equation fitted using a nonlinear regression routine (Proc Nlin) in PC SAS version 8.01 (SAS Institute Inc., Cary, NC). Curves with an R^2 value of less than 0.90 were deleted. To assess antiviral effects of different drug combination treatments, combination indices were calculated according to the method of Chou and Rideout (8).

TABLE 1 *In vitro* activity of BMS-626529 against laboratory strains of HIV-1

Coreceptor tropism	Virus	Host cell type	EC_{50} ± SD (nM) for:	
			BMS-488043	BMS-626529
CCR5	JRFL	PM1	3.0 ± 0.9	0.4 ± 0.1
	SF-162	PM1	4.7 ± 0.6	0.5 ± 0.2
	Bal	PM1	23.0 ± 0.6	1.7 ± 0.5
CXCR4	LAI	MT-2	4.1 ± 1.8	0.7 ± 0.4
	NL4-3	MT-2	17.4 ± 8.5	2.2 ± 0.6
	MN	MT-2	>1,000	14.8 ± 5.2
	IIIb	MT-2	160 ± 78	16.2 ± 1.7
	RF	MT-2	>2,000	>2,000
Dual	89.6	PM1	≥871	57.6 ± 11.4

RESULTS

BMS-626529 exhibits low cytotoxicity in cell culture. The cytotoxicity profile of BMS-626529 was examined in several cell types from different human tissues. CC_{50} values of >200 μM were observed in MT-2 (T lymphocytes), HEK293 (kidney), HEp-2 (larynx), HepG2 (liver), HeLa (cervix), HCT116 (colorectal), MCF-7 (breast), SK-N-MC (neuroepithelium), HOS (bone), H292 (lung), and MDBK (bovine kidney) cells measured after 3 or 6 days in culture. CC_{50} values of 105 and 192 μM were obtained in the T-cell line PM1 and in PBMCs, respectively, following 6 days in culture. These results showed that BMS-626529 exhibits low cytotoxicity in cell culture (data not shown).

BMS-626529 demonstrates activity against the majority of laboratory strains of HIV-1. The hypothesis underlying the optimization of HIV-1 attachment inhibitors was that, by enhancing the inhibitory potency against a specific target virus, the overall spectrum of activity would be improved. The target envelope chosen for optimization of the attachment inhibitor class was from the CXCR4-tropic LAI virus, and BMS-626529 was identified as a highly potent inhibitor of the cellular attachment of this virus (19). BMS-626529 exhibits an average EC_{50} against LAI of 0.7 ± 0.4 nM (mean ± standard deviation), which is approximately 6-fold more potent than BMS-488043 (EC_{50}, 4.1 ± 1.8 nM) (Table 1). Compared with the activity of BMS-488043 against a cohort of laboratory strains, BMS-626529 exhibited increased inhibitory potency against almost all of the viruses tested. Against the JRFL, SF-162, NL4-3, and IIIb viruses, BMS-626529 exhibited inhibitory potency increases of 7- to 10-fold compared with BMS-488043, while there was a greater increase in inhibitory potency against the Bal virus (14-fold), 89.6 viruses (15-fold), and MN virus (>67-fold). These data are consistent with the aforementioned guiding hypothesis; thus, BMS-626529 may exhibit a broader spectrum of activity than BMS-488043. As expected, there was a range of susceptibilities within the laboratory strains to BMS-626529, but most strains exhibited high susceptibility without regard to coreceptor tropism. Only one strain, RF, demonstrated resistance to both BMS-488043 and BMS-626529, with an EC_{50} of >2,000 nM for both agents.

BMS-626529 demonstrates activity against the majority of clinical isolates of HIV in PBMCs. In order to gain a broader

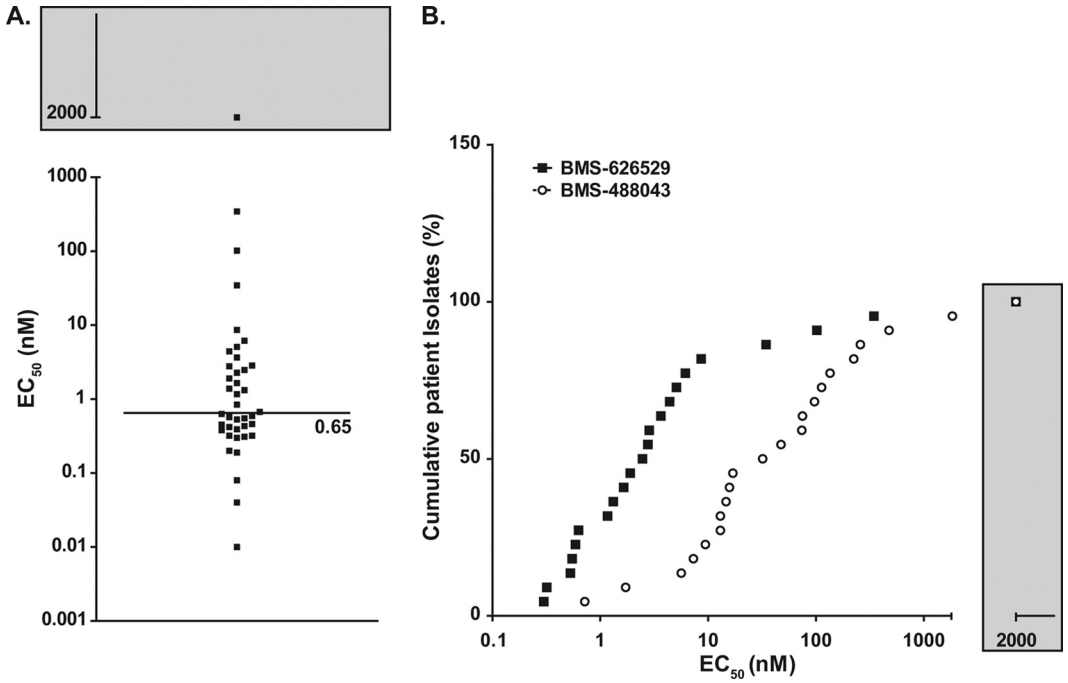

FIG 2 (A) Distribution of EC_{50}s for BMS-626529 against HIV-1 subtype B clinical isolates in the PBMC assay. The gray area represents concentrations above the highest concentration tested in the assay; thus, the determined values were greater than the indicated EC_{50}. The horizontal line represents the median EC_{50} for the cohort. (B) Comparison of EC_{50}s for BMS-626529 and BMS-488043 against identical HIV-1 subtype B clinical isolates in the PBMC assay. The EC_{50}s for the 22 viruses that were analyzed against each compound were plotted from lowest to highest EC_{50}. The gray area represents concentrations above the highest concentration tested in the assay; thus, the determined values were greater than the indicated EC_{50}.

understanding of the variability in susceptibilities of clinical isolates to the attachment inhibitor, samples from the NIH AIDS Repository were examined in antiviral assays performed in PBMCs. A total of 88 HIV-1 viruses were tested, of which 40 were subtype B. Table S1 in the supplemental material shows the EC_{50}s for BMS-626529 and BMS-488043 against these subtype B viruses. The values for BMS-626529 against these 40 subtype B viruses are shown graphically in Fig. 2A, while Table 2 shows the calculated percentile EC_{50}s against subtype A, B, and C viruses. As anticipated, significant variability in susceptibilities to BMS-626529 was observed across the subtype B viruses. BMS-626529 exhibited an EC_{50} of 0.01 nM against the most susceptible virus and an EC_{50} of >2,000 nM against the least susceptible virus. Using data from these 40 viruses, it is estimated that a BMS-626529 concentration of 0.65 nM would exceed the EC_{50} against 50% of subtype B viruses, while a concentration of 25 nM would be higher than the EC_{50} for 90% of subtype B viruses (Table 2). These data represent a significant improvement over BMS-488043 (although it should be noted that fewer viruses were analyzed), for which a concentration of 39.8 nM would be greater than the EC_{50} for 50% of the viruses, while a concentration of 1,040 nM was estimated to exceed the EC_{50} for 90% of the viruses (Table 2). Again, this suggests that BMS-626529 has improved activity against the spectrum of isolates examined. There were 22 subtype B viruses that were examined against both BMS-488043 and BMS-626529. Figure 2B shows a direct comparison of the EC_{50}s against these viruses. Interestingly, all viruses were more susceptible to BMS-626529 than to BMS-488043, and the majority were notably more susceptible (see Table S1 in the supplemental material).

TABLE 2 Calculated percentile EC_{50}s for BMS-626529 and BMS-488043 against HIV-1 subtype A, B, and C clinical isolates in the PBMC assay

Drug and virus subtype (*n*)	Percentile EC_{50} (nM)				
	50th	75th	80th	85th	90th
BMS-626529					
A (13)	10.0	1,000[a]	2,000[a]	2,000[a]	2,000[a]
B (40)	0.65	2.82	4.11	5.75	25.0
C (11)	9.32	74.6	90.3	102	315
BMS-488043					
A (13)	900	2,000[a]	2,000[a]	4,000[a]	5,000[a]
B (22)	39.8	142	231	327	1040
C (10)	934	3,052	4,150	5,121	5,656

[a] The exact EC_{50} was not observed, and only the highest observed concentration was recorded. In these cases, in order to perform calculations to determine percentiles, the observed highest concentration was treated as the EC_{50}. The number (*n*) of isolates used for each calculation is also shown.

In addition to the subtype B viruses, a total of 48 clinical isolates from HIV-1 subtypes A, C, B′, D, AE, F, BF, and G and group O were examined for susceptibility to BMS-626529. The comparative data are shown in Table S1 and Fig. 3. The data set includes 13 subtype A, 11 subtype C, 3 subtype B′, 5 subtype D, 8 subtype AE, 2 subtype F, 1 subtype BF, and 3 subtype G isolates and 2 viruses from group O. BMS-626529 displayed inhibitory potency against isolates from all subtypes, with the exception of subtype AE and group O viruses. Variability in susceptibility between viruses and among subtypes was observed (Fig. 3), but again, the non-subtype B clinical isolates showed increased susceptibility to BMS-626529 compared with BMS-488043 (see Table S1). None of

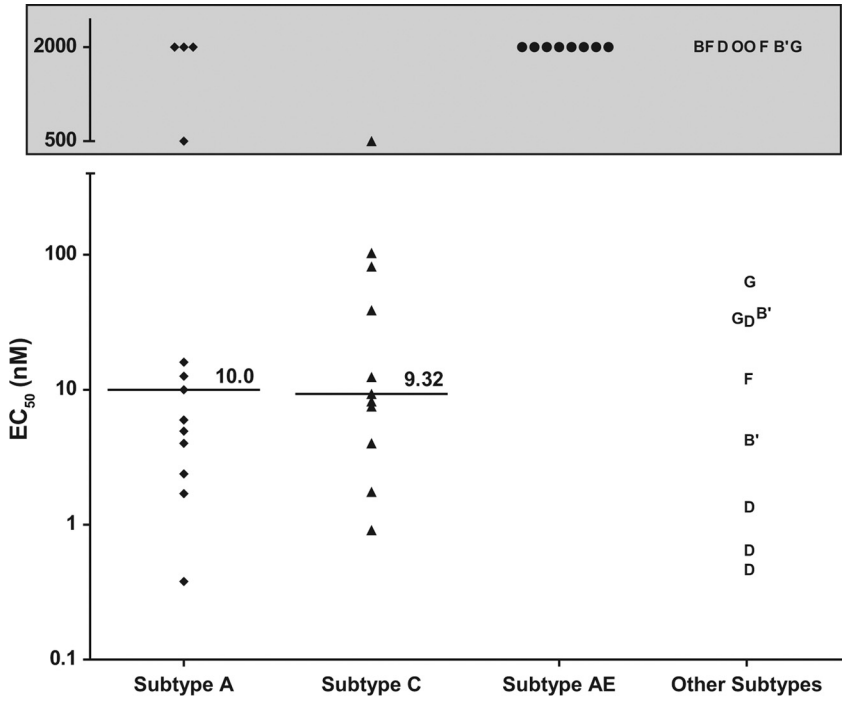

FIG 3 Distribution of EC_{50}s for BMS-626529 against HIV-1 non-subtype B clinical isolates in the PBMC assay. The gray area represents concentrations above the highest concentration tested in the assay; thus, the determined values were greater than the indicated EC_{50}. The horizontal lines represent the median EC_{50}s for subtypes A and C. Subtypes A, C, and AE are shown separately, while isolates from subtypes B′, D, F, BF, and AG and group O are combined.

the eight subtype AE isolates were susceptible to BMS-626529 or BMS-488043, suggesting that this subtype may be inherently resistant to the attachment inhibitors. Also, although there were only two group O viruses examined, neither of these exhibited susceptibility to BMS-626529, even at the highest concentrations examined (EC_{50}s of >2,000 nM). Comparison of the percentile EC_{50}s for subtype A, B, and C viruses suggested that subtype A viruses may be somewhat less susceptible to BMS-626529 than subtype B and C viruses (Table 2). However, the small numbers of subtype A (13) and subtype C (11) isolates tested did not allow for definitive conclusions to be drawn.

While the vast majority of isolates tested were HIV-1, the susceptibility of the HIV-2 strain 287 to BMS-626529 was also investigated. BMS-626529 exhibited no activity against HIV-2 strain 287 up to the highest concentration tested (EC_{50}, >2,000 nM). It was also inactive against three RNA viruses (EC_{50}, >100 μM against respiratory syncytial virus, influenza A virus, and canine parainfluenza virus) and refractory in an *in vitro* replicon system for hepatitis C virus (EC_{50}, >75 μM). In biochemical assays, BMS-626529 was inactive at a concentration of 30 μM against three HIV-1-encoded enzymes: integrase, protease, and reverse transcriptase. It was also inactive at a concentration of 25 μM against polymerases from two unrelated RNA viruses (hepatitis C virus and bovine viral diarrhea virus) (unpublished data).

The susceptibility of clinical isolates from all HIV-1 subtypes to BMS-626529 was also examined according to their coreceptor tropism. The tropism of the viruses was obtained from the description of the isolates from the NIH Repository. The majority of the clinical isolates tested were CCR5 tropic ($n = 73$), almost half of which were subtype B ($n = 35$), with the remainder from subtypes A, C, B′, D, AE, F, and G and group O. In addition, eight isolates were CXCR4 tropic, and seven were dual tropic. There was considerable variation in activity of BMS-626529 against the clinical isolates within each coreceptor usage group, with EC_{50}s ranging from picomolar levels to >2,000 nM (Fig. 4). The median EC_{50}s were 4.41 nM for CCR5-tropic viruses, 20.2 nM for CXCR4-tropic viruses, and 2.38 nM for dual-tropic viruses. Excluding the resistant AE and group O viruses, the median EC_{50}s were 3.65 nM for CCR5-tropic viruses, 3.66 nM for CXCR4-topic viruses, and 2.38 nM for dual-tropic viruses.

BMS-626529 has a higher affinity for gp120 than BMS-488043. Evaluation of BMS-626529 against a cohort of laboratory strains and clinical isolates indicated that optimization of the inhibitory potency against a specific target envelope enhanced the inhibitory potency and increased the spectrum of activity against most viruses compared with BMS-488043. In an effort to better understand the mechanism of this improved inhibitory potency, direct binding affinities and off-rates of radiolabeled compounds to gp120 were determined. Purified gp120 and tritium-labeled compounds were examined using a spin column method. At room temperature, the K_d of BMS-626529 for soluble $gp120_{JRFL}$ was 0.83 ± 0.08 nM, compared with 19 ± 1 nM for BMS-488043. This represented a 23-fold improvement in affinity for gp120 with BMS-626529 over BMS-488043 (Table 3). The difference in the EC_{50} measured through the inhibition of replication of the JRFL virus in cell culture was 7.5-fold (Table 1). In a second set of experiments, the dissociative off-rates of these two compounds from soluble gp120 were determined. The data are presented in Fig. 5 and summarized in Table 3. The dissociation kinetics for BMS-488043 fit best to a simple mono-exponential decay equation, with a half-life of 43 min. By comparison, BMS-626529 dissociated from gp120 more slowly, with a half-life of 458 ± 67 min,

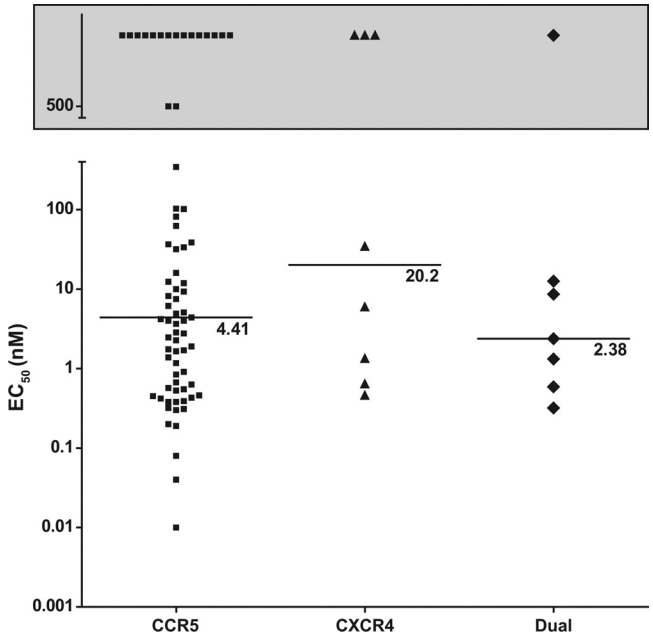

FIG 4 Distribution of EC_{50}s for BMS-626529 against HIV-1 clinical isolates according to tropism in the PBMC assay. The gray area represents concentrations above the highest concentration tested in the assay; thus, the determined values were greater than the indicated EC_{50}. The horizontal lines represent the median EC_{50}s for each cohort.

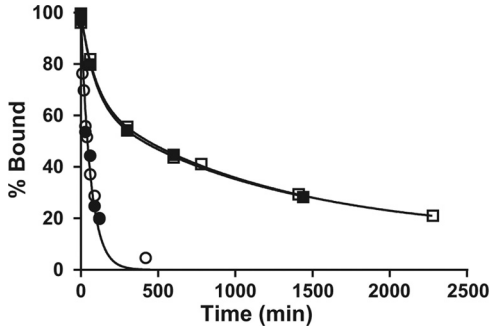

FIG 5 Dissociation of radiolabeled BMS-626529 and BMS-488043 from $gp120_{JRFL}$. Combined data from two experiments were fit to exponential decay equations as described in the text. Squares, [^{3}H]BMS-626529; circles, [^{3}H]BMS-488043. Open and closed symbols represent data from the two independent experiments.

or 11.6-fold more slowly than BMS-488043. Interestingly, the dissociation data for BMS-626529 fit best to a biphasic exponential dissociation equation, in which the overall half-life of the [^{3}H]BMS-626529 was 7.6 h. The initial dissociative half-life was faster than the final secondary half-life (23 h). This suggests that the improved inhibitory potency of BMS-626529 is a result of the longer dissociative half-life of the compound, which may be a consequence of more complex binding kinetics.

BMS-626529 demonstrates activity against the majority of HIV-1 clinical isolates in the PhenoSense Entry assay. The structural diversity within gp120 and the variability in activities observed against clinical isolates and laboratory strains mean that it is vital to understand the activity of BMS-626529 against different viral subtypes from around the world. In order to investigate this further in a greater number of HIV-1 isolates, envelopes from viruses isolated from subjects who participated in Bristol-Myers Squibb-sponsored clinical trials (n = 134) and from the Monogram collection (n = 108) were tested in the PhenoSense Entry assay. The majority of isolates were subtype B; however, envelopes from viruses of subtypes A, C, AE, F, F1, BF, and AG were also tested. As previously observed using the PBMC assay, the results demonstrated a wide range of susceptibilities to BMS-626529 (IC_{50}, 0.05 to >100 nM), although the compound displayed activity against almost all subtypes tested (Fig. 6A and B; see also Table S2 in the supplemental material). Similar to the data obtained from clinical isolates in PBMCs, none of the five subtype AE viruses examined were susceptible to BMS-626529, thus suggesting that this subtype of HIV-1 may be resistant to the attachment inhibitor. There did not appear to be any effect of region of origin on the susceptibility of viruses to BMS-626529 (see Table S2). Sufficient numbers of subtype A (n = 41), B (n = 133), and C (n = 36) isolates were examined to allow estimation of the IC_{50} percentiles; these data that showed that the majority of isolates were inhibited at low drug concentrations (Table 4). Calculations from this cohort suggested that a BMS-626529 concentration of 0.34 nM would exceed the IC_{50} for 50% of subtype B viruses, while for subtypes A and C the calculated values were slightly higher, at 2.26 and 1.30 nM, respectively. The IC_{50}s differentiate more at the higher percentiles. BMS-626529 would be expected to exhibit an IC_{50} below 4.59 nM for 90% of subtype B viruses, while for coverage of 90% of subtype A and C viruses the corresponding values would be 87.9 and 7.09 nM, respectively. This again suggests that subtype A viruses are generally susceptible to BMS-626529, but less so than subtype B viruses, while the susceptibility of subtype C viruses is only slightly less than that of subtype B isolates. This could be related to the use of a subtype B gp160 (LAI) as the primary target during the compound optimization phase. Analysis of the IC_{50} distribution across subtypes A, B, and C showed that BMS-626529 exhibited IC_{50}s below 1 nM against a greater percentage of subtype B virus envelopes (73%) than subtype A (22%) or C (42%) (Table 4; Fig. 7). A drug concentration of 10 nM exceeded the IC_{50} for 94% of subtype B and C viruses and 73% of subtype A viruses. A BMS-626529 concentration of 100 nM is expected to exceed the IC_{50} for 99% of subtype B, 94% of subtype C, and 90% of subtype A viruses (Table 4). Thus, although the results suggest that subtype B envelopes are generally more susceptible to BMS-626529 than subtype C envelopes, the total percentage of isolates with IC_{50}s below a relatively low cutoff (100 nM) was very similar. Even though subtype A envelopes seem to be less sensitive as a whole than subtype B envelopes, BMS-626529 exhibits IC_{50}s below 100 nM for the vast majority of subtype A, B, and C viruses.

TABLE 3 *In vitro* K_d and dissociative half-lives of BMS-488043 and BMS-626529 to purified JRFL envelope[a]

Drug	K_d (nM)	Dissociative $t_{1/2}$ (min)	EC_{50} (nM)
BMS-488043	19 ± 1	43 ± 2	3.0 ± 0.9
BMS-626529	0.83 ± 0.08	458 ± 67	0.4 ± 0.1

[a] Values are means ± standard deviations.

BMS-626529 is active against recombinant viruses expressing envelopes from NRTI-, NNRTI-, and PI-resistant isolates: comparison with maraviroc. Out of the 133 subtype B envelopes

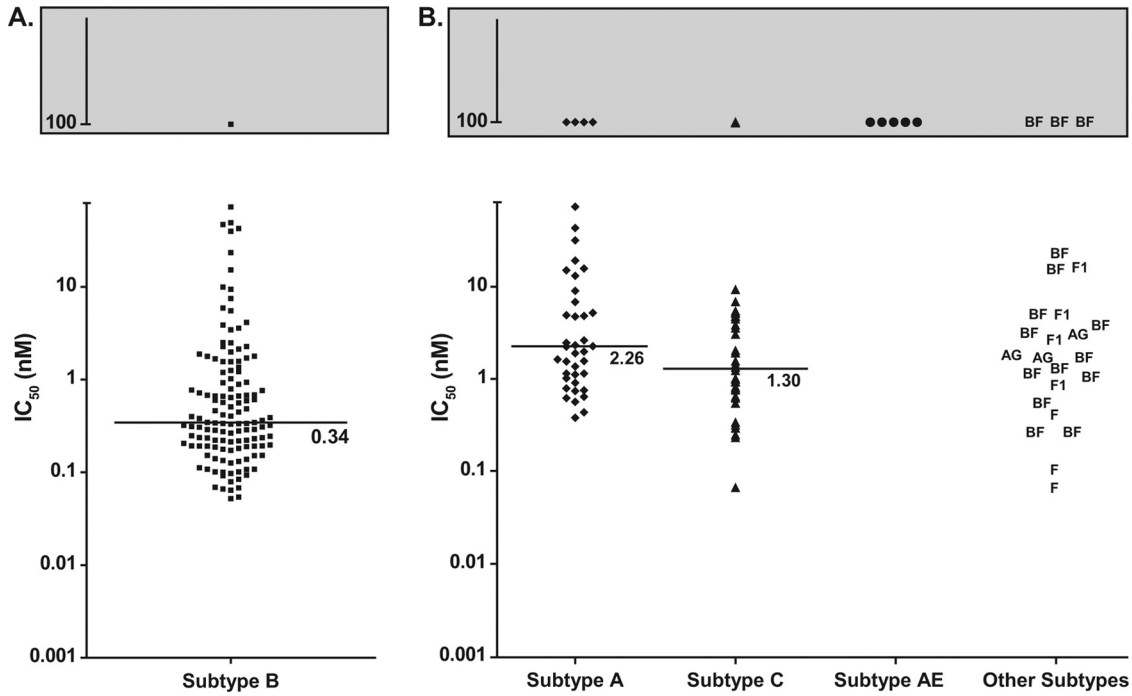

FIG 6 (A) Distribution of IC_{50}s for BMS-626529 against HIV-1 subtype B envelopes, determined using the PhenoSense Entry assay. The gray area represents concentrations above the highest concentration tested in the assay; thus, the determined values were greater than the indicated IC_{50}. The horizontal line represents the median IC_{50} for the cohort. (B) Distribution of IC_{50}s for BMS-626529 against HIV-1 non-subtype B envelopes as determined with the PhenoSense Entry assay. The gray area represents concentrations above the highest concentration tested in the assay; thus, the determined values were greater than the indicated IC_{50}. The horizontal lines represent the median IC_{50} for the subtype A and C viruses.

tested in the PhenoSense Entry assay, a total of 50 were isolated from subjects who had developed resistance to a variety of antiretroviral regimens consisting of different NRTIs, NNRTIs, and PIs. These pseudoviruses were sourced from Monogram and were tested in the PhenoSense Entry assay for their susceptibilities to BMS-626529. As a control, the viruses were also tested for susceptibility to maraviroc (Fig. 8; see also Table S2 in the supplemental material). There was little variation in susceptibility to maraviroc, with IC_{50}s ranging from 0.85 to 6.12 nM. In contrast, there was a wide range of susceptibility to BMS-626529 (IC_{50}, 0.05 to 72.6 nM), which is consistent with the variability seen previously with the attachment inhibitor. The three least susceptible pseudotyped viruses had IC_{50}s of 42.5, 46.9, and 72.6 nM, with two of the envelopes originating from NNRTI-resistant viruses, while the least susceptible envelope came from an NRTI-resistant virus. However, many NRTI-, NNRTI-, and multidrug-resistant isolates were highly sensitive to BMS-626529, indicating, as expected, that there was no link between class resistance and susceptibility to this compound (Fig. 8).

BMS-626529 demonstrated additive or synergistic interactions with antiretroviral drugs from different classes. The potential for BMS-626529 to be used in drug regimens consisting of antiretroviral agents from different classes necessitated an understanding of the interactions between the different inhibitory mechanisms. Consequently, two-drug combination studies were performed with a variety of approved and preclinical compounds. The combination indices and asymptotic confidence intervals, which represent a measure of the variability in the data, for selected combinations are shown in Table S3 of the supplemental material. Synergistic or additive to synergistic effects were observed for all combinations tested, including with other entry inhibitors. No antagonistic effects were observed for any of the combinations tested. In addition, no cytotoxicity was observed at the highest concentrations used in any of the combination assays.

DISCUSSION

BMS-488043 was the first HIV-1 attachment inhibitor to progress to proof-of-concept clinical trials (14, 15). Although this proto-

TABLE 4 Calculated percentile IC_{50}s for BMS-626529 against HIV-1 subtype A, B, and C envelopes derived from clinical isolates as determined with the PhenoSense Entry assay

	Percentile IC_{50} (nM)					% with IC_{50} below[a]:		
Subtype (*n*)	50th	75th	80th	85th	90th	1 nM	10 nM	100 nM
A (41)	2.26	13.8	17.2	37.4	87.9	22	73	90
B (133)	0.34	1.23	1.71	2.27	4.59	73	94	99
C (36)	1.30	4.69	5.02	5.35	7.09	42	94	94

[a] Rounded to the nearest whole number. The number (*n*) of isolates used for each calculation is shown.

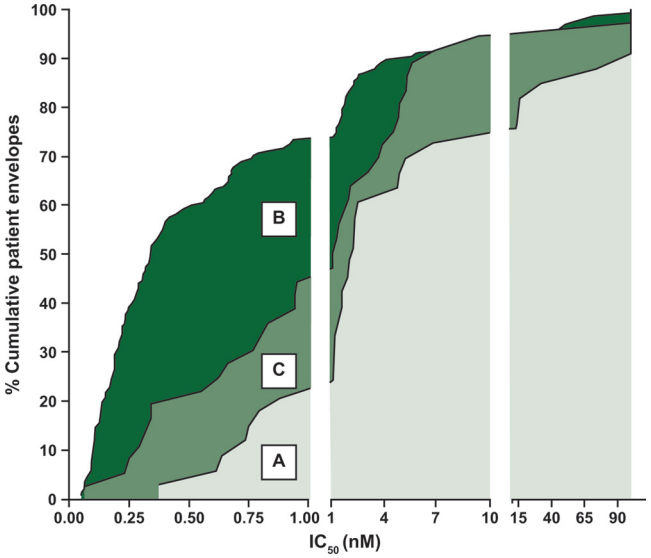

FIG 7 Spectrum of activities of BMS-626529 against HIV-1 subtype A, B, and C envelopes derived from clinical isolates in the PhenoSense Entry assay. The graph shows the estimated percentages of subtype A, B, and C viruses (shown on the *y* axis) with an IC_{50} below a specific concentration (shown on the *x* axis). Thus, the higher the IC_{50}, the higher the percentage of viruses covered. The overall coverage of subtype A virus by the IC_{50} is shown in pale green, subtype C is shown in medium green, and subtype B is shown in dark green. For instance, the graph shows that at low IC_{50}s, a higher percentage of subtype B viruses were covered, but at higher IC_{50}s (>7 nM), the percentages of subtype B and C viruses that were covered were similar.

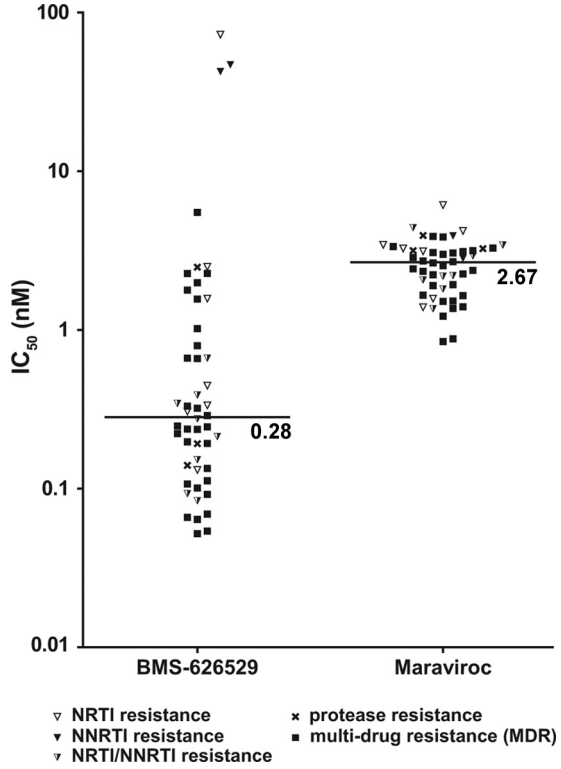

FIG 8 Distribution of BMS-626529 EC_{50}s against subtype B envelopes from HIV-1 viruses isolated from subjects with resistance to NRTIs, NNRTIs, or PIs as determined using the PhenoSense Entry assay. The horizontal line represents the median IC_{50} for each cohort.

type demonstrated clinical antiviral activity, the profile revealed that improvements in inhibitory potency and spectrum of activity were needed. We hypothesized that increasing the inhibitory potency against a specific target envelope (LAI) would boost the inhibitory potency against the broader spectrum of HIV-1 viruses. The data presented herein are consistent with this hypothesis, as in almost all cases the EC_{50} for BMS-626529 against a laboratory strain or clinical isolate were notably lower than those for BMS-488043. The magnitude of the increase in inhibitory potency with BMS-626529 varied for each isolate, from relatively modest to a greater than 2-log_{10} increase, and these *in vitro* data revealed that BMS-626529 is active against the majority of HIV-1 isolates. The increased inhibitory potency may be due to the significantly increased dissociative half-life of BMS-626529 compared with BMS-488043, shown by the 10-fold-slower dissociation rate we observed with purified gp120 sourced from the JRFL strain. Additionally, the dissociation curve for BMS-626529 fit best to a biphasic exponential dissociation equation in which the remainder of the compound was released more slowly, with a secondary half-life of ~23 h. Our interpretation is that the biphasic kinetics are a result of multistep binding kinetics, possibly involving an initial binding event, followed by conformational changes which lead to the final, more stable complex. This kind of complex binding has been described for small-molecule HIV integrase inhibitors (23). In addition, the binding of CD4 itself to gp120 is reported to be a slow complex process (30). The EC_{50} of BMS-626529 against JRFL virus is 3 nM; however, much lower EC_{50}s were recorded against many of the viruses examined in PBMCs or the PhenoSense Entry assay, in some cases >100-fold. Given the hypothesis that inhibitory potency is driven by the off-rate of BMS-626529 from gp120, it is reasonable to speculate that the off-rate from these highly susceptible isolates may be much longer than that measured for JRFL.

A clinical study of the prodrug of BMS-626529 (BMS-663068) has demonstrated good antiviral activity in subjects infected with virus shown to be susceptible (IC_{50}, <100 nM) to the agent (31a). Interestingly, there was no evidence of emerging resistance to BMS-626529 after 8 days of monotherapy in the only *in vivo* study completed to date of subjects with HIV-1 infection (34). This is in contrast to an earlier study of BMS-488043, in which viruses from 4/30 subjects were considered to have emergent resistance (defined as a >10-fold change in susceptibility from baseline) (41). This difference may indicate that the increased inhibitory potency of BMS-626529 over BMS-488043 translated into higher inhibitory quotients for BMS-626529, which in turn increased the barrier for selecting resistance to BMS-626529.

The EC_{50}s observed for BMS-626529 showed considerable variation within each coreceptor usage group and HIV-1 subtype. However, the overlap between EC_{50}s across the groups suggests that neither tropism nor subtype (with the exception of subtype AE and possibly group O) predetermines susceptibility to BMS-626529. It should be noted that the sample sizes of CXCR4- and dual-tropic viruses, as well as some non-B subtypes, were not large enough for definitive conclusions to be drawn. It must also be remembered that interpretation of data from *in vitro* experiments performed in PBMCs is complicated by the selection of viruses adapted to the cultured cells, and therefore such data are not necessarily representative of *in vivo* viral diversity (40). However,

these findings would be expected to translate into a clinical benefit for BMS-626529 as an antiretroviral agent and further distinguish the mechanism of action of the compound. Furthermore, BMS-626529 was shown to be active against pseudoviruses containing envelopes from viruses with resistance to other antiretroviral classes. The variability in EC_{50}s observed with BMS-626529 is a notable difference with respect to other antiretroviral classes and presumably reflects the heterogeneity inherent to gp120.

Consistent with observations of variability in the activity of BMS-626529 within coreceptor usage groups, BMS-626529 also demonstrated a range of EC_{50} values across HIV-1 subtypes. The CASTLE study was a large trial with worldwide recruitment of HIV-1-infected treatment-naïve subjects (29) that provided an opportunity to examine envelopes from clinical isolates from several continents and of multiple subtypes. BMS-626529 displayed excellent activity against almost all subtypes, with no evidence of any regional differences, suggesting that it would be suitable for use in most geographic areas. However, a notable exception to this is subtype AE, the predominant subtype found in parts of Southeast Asia, which appears from these results to be inherently insensitive to BMS-626529. Comparison of the amino acid sequences of 112 subgroup AE viruses (27) with subtype B viruses identified two loci that may be associated with this reduced susceptibility to BMS-626529. A total of 111 of the AE viruses had a histidine at position 375 rather than a serine. The substitution S375I/N in subtype B virus was found to be responsible for resistance to an earlier attachment inhibitor, BMS-488043, in a phase IIa clinical study (41). In addition, 82 of the subgroup AE viruses had an isoleucine at position 475, which has been implicated as a potential resistance marker against BMS-626529 *in vitro* (32).

Although the majority of clinical isolates and envelopes examined (except for subtype AE and possibly group O) were susceptible to BMS-626529, the observed ~6-$\log_{10}$ variation in EC_{50}s and the identification of viruses with inherent resistance to the agent suggest that there may be a need to screen virus for susceptibility to BMS-626529 prior to treatment. This was illustrated by the results of the phase IIa study with the prodrug BMS-663068 (31a). In that study, 7 of 49 subjects exhibited a reduced susceptibility to BMS-626529 at baseline (IC_{50}, >100 nM), and this finding correlated with a weaker antiviral response (the majority of these subjects failed to achieve a >1-$\log_{10}$ decline in plasma HIV-1 RNA) (31a). Consequently, a diagnostic test may be useful to screen out individuals who may not benefit from the use of BMS-626529.

The results of two-drug combination studies revealed no antagonistic interactions between BMS-626529 and members of any class of antiretrovirals, including other entry inhibitors. This suggests that BMS-626529 would be suitable for future use in combination regimens. Furthermore, as well as direct inhibition of virus attachment to CD4, the attachment inhibitor mechanism of action may have additional benefits not necessarily observed with agents that target other steps in HIV infection. *In vitro* studies have suggested that attachment inhibitors may inhibit apoptosis of $CD4^+$ T cells (1, 25). This may be of benefit through inhibition of the bystander effect (2, 24), similar to that suggested for HIV-1 entry inhibitors which act by alternative mechanisms (5, 7). In addition, the facts that BMS-626529 binds to purified gp120 and inhibits binding to CD4 suggest that it could act on the virus-free gp120 found in an infected individual. These and additional benefits associated with attachment inhibitors, including prevention of HIV-1 infection of dendritic cells (11), will need to be defined in longer-term clinical trials. These data support the continued clinical development of BMS-626529, and a phase IIb study of BMS-663068 in HIV-1-infected treatment-experienced subjects is ongoing (NCT01384734).

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