Susceptibility of Human Immunodeficiency Virus Type 1 to the Maturation Inhibitor Bevirimat Is Modulated by Baseline Polymorphisms in Gag Spacer Peptide 1[▽]

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In this study, we evaluated baseline susceptibility to bevirimat (BVM), the first in a new class of antiretroviral agents, maturation inhibitors. We evaluated susceptibility to BVM by complete *gag* genotypic and phenotypic testing of 20 patient-derived human immunodeficiency virus type 1 isolates and 20 site-directed mutants. We found that reduced BVM susceptibility was associated with naturally occurring polymorphisms at positions 6, 7, and 8 in Gag spacer peptide 1.

Bevirimat (BVM; formerly known as PA-457) [3-*O*-(3′,3′-dimethylsuccinyl) betulinic acid] is the first of a new class of antiretroviral agents (maturation inhibitors) that is being developed for the treatment of human immunodeficiency virus type 1 (HIV-1) infection (8). BVM inhibits HIV-1 infectivity by blocking a late stage in protease (PR)-mediated Gag processing, specifically, the release of spacer peptide 1 (SP1) (also referred to as p2) from the C terminus of capsid (CA) (4). Blocking CA-SP1 cleavage results in defective core condensation and the release of noninfectious virus particles, inhibiting the spread of the infection to new cells. BVM shows potent activity against HIV-1 strains that are resistant to approved classes of antiretroviral agents (3, 4).

In vitro testing has demonstrated that BVM is a potent inhibitor of virus replication, with a mean 50% inhibitory concentration (IC_{50}) of approximately 10 nM against primary HIV-1 isolates (4). Serial passage experiments identified six single amino acid substitutions that independently conferred resistance to BVM: three at or near the C terminus of CA (H226Y, L231F, and L231M) and three at the first and third residues of SP1 (A1V, A3T, and A3V) (1, 4, 11). However, population genotyping of patient isolates from BVM phase 1/2 and 2a clinical trials did not reveal any isolates that contained known in vitro resistance mutations (2, 9).

Recently, a multicenter phase 2b dose escalation study was completed in which baseline factors that predict response to BVM were discovered (6). The predictors of response to BVM were assigned to amino acid changes at codon positions 369 to 371 in Gag (positions 6 to 8 in SP1). Patients who had virus with the most commonly occurring amino acids glutamine-valine-threonine (QVT) at these positions were much more likely to respond to BVM treatment, while patients whose virus had polymorphisms at these positions were less likely to respond to BVM (6).

In the current study, we developed a multicycle Gag-PR phenotypic recombinant virus assay (Fig. 1) to assess BVM susceptibility of 20 viral isolates derived from BVM-naïve subjects. An HXB2-based HIV backbone was first constructed in which the Gag-PR region was deleted (pHXB2-ΔGag-PR). Gag-PR amplicons were then recombined intracellularly with pHXB2-ΔGag-PR by MT4 nucleofection. Replication-competent recombinant viruses were titrated and subjected to antiviral testing in MT4-LTR-eGFP cells using BVM at concentrations from 0.1 nM to 8 μM. HIV-1 IIIB was used as a reference to calculate the change in IC_{50}. To determine the role of individual amino acids on susceptibility to BVM, mutations at the CA-SP1 cleavage site (in vitro resistance mutation controls) and at positions 6 to 8 in SP1 were introduced into both pNL4.3 and pHXB2 plasmids by site-directed mutagenesis. Gag-PR amplicons (1,980 bp in HXB2; GenBank accession number K03455) were generated by nested PCR using primers EF1 (annealing positions 550 to 571 in HXB2), IF1 (positions 619 to 640), Gagprout-R3 (positions 2597 to 2621), and Gagprin-R1 (positions 2574 to 2598) and sequenced using ABI technology. In addition, predictive phenotypic analysis of PR- and reverse transcriptase (RT)-derived sequences from each virus isolate was performed in parallel using vircoTYPE HIV-1 analysis (10).

The Gag-PR region was successfully amplified, sequenced, and phenotyped from 20 baseline virus isolates derived from two BVM clinical trials: study 201 (true monotherapy) and study 203 (functional monotherapy). All viral strains belonged to clade B.

In the Gag-PR phenotypic analysis, IIIB wild-type virus (control) showed a mean IC_{50} for BVM of 65 ± 9 nM, which was in concordance with previously reported data (4). In accordance with previous reports (1, 4), BVM-selected resistance mutations L231M (CA) and A1V (SP1) decreased BVM susceptibility by 37.6-fold and more than 77.5-fold, respectively (Fig. 2). Although biological cutoff values still have to be determined, BVM baseline susceptibility testing of 20 clinical isolates revealed 9 isolates with fully susceptible viruses (change in IC_{50}, <10-fold), while 11 viruses showed reduced

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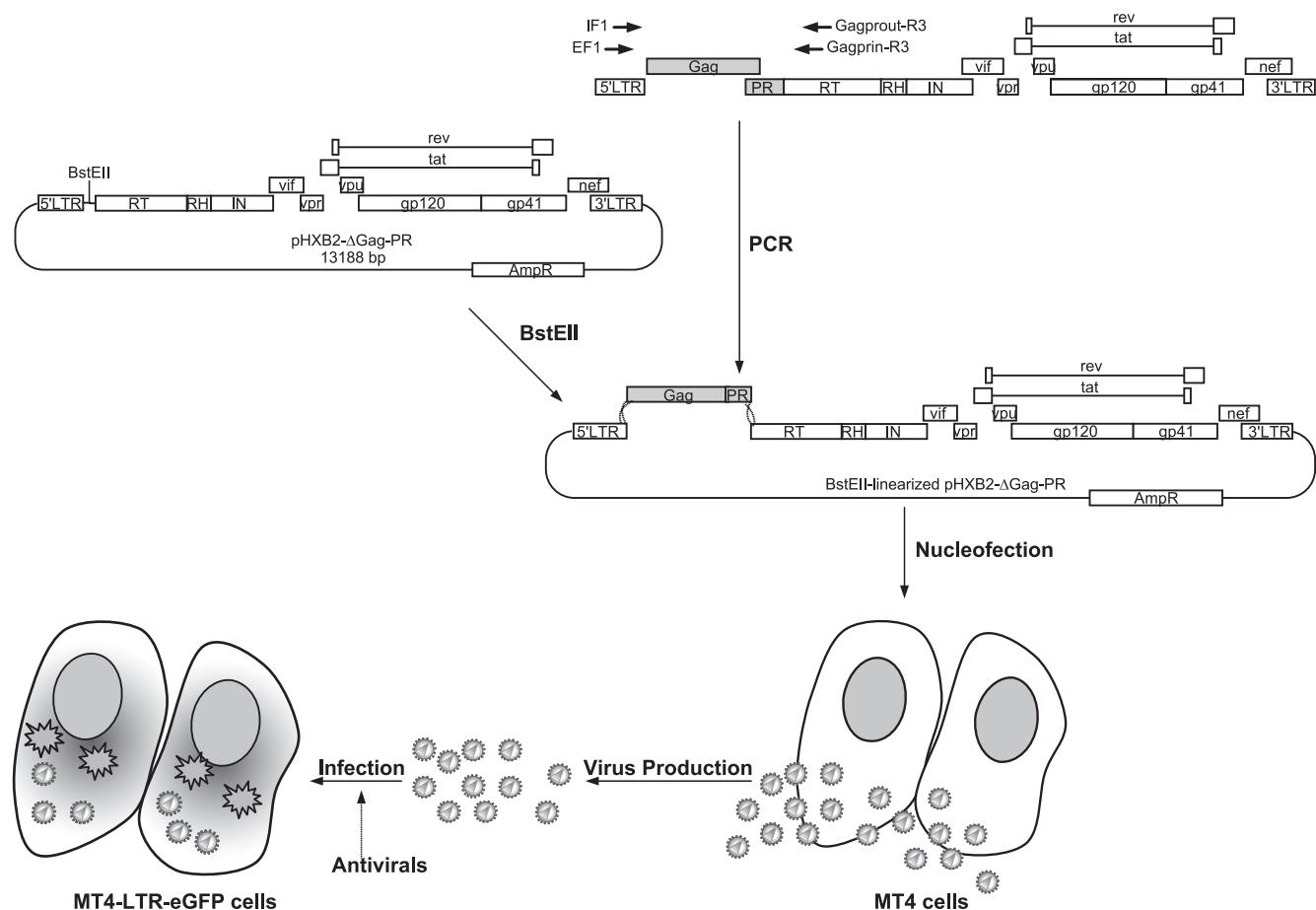
FIG. 1. Schematic representation of the Gag-PR phenotyping assay. Gag-PR amplification primers are indicated by arrows. Abbreviations: 5′LTR, 5′ long terminal repeat; IN, integrase; RH, RNaseH.

BVM susceptibility with changes in IC_{50}s ranging from >76.1- to >125.4-fold (Fig. 2).

Gag-PR genotyping did not reveal any isolates that contained reported in vitro selected resistance mutations (H226Y, L231F, and L231M in CA and A1V, A3T, and A3V in SP1; Fig. 2). By contrast, a "wild-type" (HXB2) QVT amino acid sequence at positions 6 to 8 in SP1 was observed in all 9 susceptible viruses, whereas all 11 of the viruses with reduced susceptibility contained polymorphisms in this region (Q6H, V7M, V7A, and T8Δ; Fig. 2). The PR-RT genotyping by vircoTYPE analysis did not show a clear correlation between reduced/minimal response to PR and RT inhibitors and susceptibility to BVM (Fig. 2).

Although deletions at positions 6, 7, and 8 in SP1 have previously been investigated in vitro (5), point mutations have not. Using both Gag-processing assays as well as cell-based replication assays, Q6Δ and V7Δ mutants (in a pNL4.3 backbone) were found to be less susceptible to BVM, while the T8Δ mutant had less dramatic effect on BVM activity (5, 7). In the current study, single, double, and triple amino acid substitutions and deletions were introduced at positions 6 to 8 in SP1 in both pNL4.3 and pHXB2 backbones (Fig. 2). Phenotyping of these mutants demonstrated that specific polymorphisms at positions 7 and 8 in SP1 (V7A, V7M, T8Δ, and T8N) were

sufficient to confer decreased BVM susceptibility (change in IC_{50} ranging from 10.9- to >151.4-fold), while other mutations at positions 6 and 8 (Q6A, Q6H, and T8A) retained full sensitivity to BVM (Fig. 2). Double and triple mutations at these positions resulted in changes in IC_{50}s of >111.0- to >157.2-fold (IC_{50} greater than the highest BVM concentration tested [8 μM]). The impact of these mutations on replication fitness needs further investigation.

The individual contribution of each amino acid at position 6, 7, and 8 on the phenotypic behavior of BVM needs further confirmation in a larger set of samples. Likewise, amino acid changes outside the QVT domain need to be addressed. In order to have a preliminary understanding of the genetic variability, we further investigated the Los Alamos database to determine the prevalence of the QVT motif in subtype B and non-B subtypes. Subtypes with more than 50 representative Gag sequences were retrieved and analyzed for the frequency of Q6, V7, and T8 as well as of the complete QVT motif (Table 1). Only subtype B contained QVT at a high prevalence (66.0%), while the consensus sequence at these positions differed in other subtypes (Table 1). The V7A polymorphism described in this study was present at >25% frequency in all subtypes except subtype B, and T8N was present in 81.4% of subtype C samples (Table 1). Since some mutations at posi-

FIG. 2. CA-SP1-NC amino acid alignment and changes in IC$_{50}$s for BVM. Sequences from clinical isolates are indicated as PA100 to PA119. Site-directed mutants are indicated according to the reference strain, being NL4.3 or HXB2. The change in IC$_{50}$ for BVM is presented as the average (Avg) and standard deviation (StDev) for six independent experiments. Sequences were aligned relative to HXB2 (top line). The numbers above the HXB2 sequence represent amino acid positions. Positions 6 to 8 in SP1 are marked in gray. Identical amino acids (hyphens) and deletions (Δ) are indicated. Drugs are shown as numbers in the vircoTYPE columns as follows: 1, zidovudine; 2, lamivudine; 3, didanosine; 4, stavudine; 5, abacavir; 6, emtricitabine; 7, tenofovir DF; 8, nevirapine; 9, indinavir; 10, nelfinavir; 11, saquinavir; 12, amprenavir; 13, lopinavir; 14, atazanavir; 15, tipranavir; 16, ritonavir; 17, darunavir. Maximal, reduced, and minimal responses are the response categories for protease and RT inhibitors as available on vircoTYPE HIV-1 analysis.

TABLE 1. Conservation of the QVT motif in HIV-1 subtype B, C, A, D, 01_AE, and 02_AG sequences retrieved from the Los Alamos database

Subtype	Prevalence[a] of:			Frequency (%) of QVT motif[b]
	Q	V	T	
C ($n = 553$)	$Q_{96.0}$	$V_{11.6}$	$T_{3.1}$	0.5
	$H_{2.0}$	$A_{65.8}$	$N_{81.4}$	
B ($n = 265$)	$Q_{97.4}$	$V_{74.3}$	$T_{86.8}$	66.0
	$H_{1.1}$	$A_{15.5}$	$N_{8.3}$	
A ($n = 134$)	$Q_{96.3}$	$V_{64.2}$	$T_{0.7}$	0.0
	$H_{3.0}$	$A_{27.6}$	$Q_{91.0}$	
D ($n = 76$)	$Q_{97.4}$	$V_{13.2}$	$T_{73.7}$	9.2
	$H/I_{1.3}$	$A_{85.5}$	$S_{21.1}$	
01_AE ($n = 72$)	$Q_{62.5}$	$V_{33.3}$	$T_{0.0}$	0.0
	$H_{23.6}$	$A_{63.9}$	$Q_{98.6}$	
02_AG ($n = 59$)	$Q_{100.0}$	$V_{69.5}$	$T_{0.0}$	0.0
		$A_{27.1}$	$Q_{94.9}$	

[a] The prevalence of Q, V, and T as a percentage is given as the subscript. Also, the most prevalent amino acid (or secondmost prevalent amino acid when Q, V, or T was the most prevalent) is presented. The most prevalent motif per subtype is indicated in boldface type.
[b] Frequency of the complete QVT motif.

tions 6 and 8 had little effect on BVM susceptibility in this study, further phenotypic testing will be needed to gain more insight into the role of specific polymorphisms in non-B subtypes.

While the current observation that polymorphisms at positions 6 to 8 in SP1 reduce BVM susceptibility was made in vitro, this result is consistent with recent clinical studies. Phase 2b trial results found that a subset of patients responded poorly to BVM despite the presence of high plasma concentrations of BVM and the absence of known in vitro resistance mutations. Further genotypic analysis suggested that poor response was statistically associated with baseline polymorphisms at positions 6 to 8 in SP1 (6). In the group of patients that lacked these Gag polymorphisms, more than 90% responded to BVM with a mean viral load reduction of 1.26 log. In conclusion, using a Gag-PR phenotypic and genotypic assay, baseline susceptibility to BVM was evaluated for a set of 20 patient-derived virus isolates. Reduced susceptibility was found to correlate with the presence of polymorphisms at Gag SP1 residues 6, 7, and 8. Site-directed mutagenesis confirmed that specific mutations at position 7 or 8 were sufficient to confer reduced susceptibility, whereas the effect of polymorphisms at position

* tested only once

6 may be dependent on the context of the Gag backbone. Testing of additional virus isolates should help to further clarify the role of individual polymorphisms in BVM susceptibility.

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