HIV-1 Protease Codon 36 Polymorphisms and Differential Development of Resistance to Nelfinavir, Lopinavir, and Atazanavir in Different HIV-1 Subtypes

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The amino acid at position 36 of the HIV-1 protease differs among various viral subtypes, in that methionine is usually found in subtype B viruses but isoleucine is common in other subtypes. This polymorphism is associated with higher rates of treatment failure involving protease inhibitors (PIs) in non-subtype B-infected patients. To investigate this, we generated genetically homogeneous wild-type viruses from subtype B, subtype C, and CRF02_AG full-length molecular clones and showed that subtype C and CRF02_AG I36 viruses exhibited higher levels of resistance to various PIs than their respective M36 counterparts, while the opposite was observed for subtype B viruses. Selections for resistance with each variant were performed with nelfinavir (NFV), lopinavir (LPV), and atazanavir (ATV). Sequence analysis of the protease gene at week 35 revealed that the major NFV resistance mutation D30N emerged in NFV-selected subtype B viruses and in I36 subtype C viruses, despite polymorphic variation. A unique mutational pattern developed in subtype C M36 viruses selected with NFV or ATV. The presence of I47A in LPV-selected I36 CRF02_AG virus conferred higher-level resistance than L76V in LPV-selected M36 CRF02_AG virus. Phenotypic analysis revealed a >1,000-fold increase in NFV resistance in I36 subtype C NFV-selected virus with no apparent impact on viral replication capacity. Thus, the position 36 polymorphism in the HIV-1 protease appears to have a differential effect on both drug susceptibility and the viral replication capacity, depending on both the viral subtype and the drug being evaluated.

The high rate of mutation of HIV-1 isolates belonging to group M permits the phylogenetic taxonomic division of viral variants into multiple subtypes, currently designated A1 to A4, B, C, D, F1, F2, G, H, J, and K (19, 37). Furthermore, genomic recombination between different HIV-1 subtypes (resulting from coinfection or superinfection) has led to the circulation of genetically mosaic HIV-1 conventionally termed “circulating recombinant forms” (CRFs) (19, 33, 37). Non-subtype B HIV-1 subtypes constitute >90% of the isolates in the current pandemic (14, 38), with over 50% of new infections now being characterized as subtype C HIV-1 (2, 3, 38). Given that most antiretroviral drug design and resistance research has utilized subtype B (5, 16, 24, 28, 30, 31, 35, 40), there is a paucity of information in regard to drugs and non-subtype B subtypes. Since most globally accessible first-line therapies consist of reverse transcriptase (RT) inhibitors, it is known that second-line regimens will commonly need to include protease inhibitors (PIs) to counteract the development of drug resistance (DR). However, the nucleotide sequences of the HIV-1 protease (PR) of subtype B and C HIV-1 are notably different (8, 18) due to polymorphisms that might affect the development of DR. Hence, we wished to define the role of a suspect PR polymorphism in the context of HIV-1 evolution under PI pressure.

An isoleucine at position 36 (I36) in PR is found in 13% of subtype B viruses but in over 80% of subtype C and CRF02_AG (A/G) viruses (28, 32). After PI treatment, the prevalence of this polymorphism increases to 35% and 90% in patients infected with subtype B and non-subtype B viruses, respectively (32), and the presence of I36 in PR is linked to a higher rate of treatment failure (29). Furthermore, biochemical studies with subtype A and C recombinant I36 PR revealed that the PR enzymes of non-subtype B viruses have a higher $K_i$ and greater catalytic efficiency than subtype B viruses (39) and that introduction of I36 into subtype B HIV-1 results in a higher virus replication capacity in both the absence and presence of PIs (15).

Accordingly, we hypothesized that the presence of methionine or isoleucine at position 36 within PR (M36 and I36, respectively) might impact the development of PI-selected resistance. To this end, we introduced I36 or M36 into full-length HIV-1 molecular clones of subtypes B, C, and CRF02_A/G and cultured both the wild-type and mutated resultant viruses for 35 weeks in cord blood mononuclear cells (CBMCs) under...
increasing PI drug concentrations. The drugs atazanavir (ATV) and nelfinavir (NFV) were chosen for study because they have demonstrated a low genetic barrier for resistance, while lopinavir (LPV) was used because of its high genetic barrier for resistance (25, 30). The genotypic profile, phenotypic susceptibility, and replication capacities of the drug-selected viruses were evaluated.

**MATERIALS AND METHODS**

**Molecular clones, virus production, and drugs.** ATV, NFV, and LPV were kind gifts from Bristol-Myers Squibb Inc., Pfizer Inc., and Abbott Laboratories Inc., respectively.

The pNL4-3 plasmid (catalogue number 114, GenBank accession number AF324493) was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIH, Bethesda, MD, courtesy of Malcolm Martin (1). The subtype B 36M and 36I polymorphic variants using the QuikChange II XL site-directed mutagenesis kit (Stratagene, LaJolla, CA), according to the manufacturer's instructions. The subtype B M36I clone was created using the directed mutagenesis (SDM) kit (Stratagene, LaJolla, CA), according to the manufacturer's instructions. The subtype C 36M and 36I clones were used to create I36 and M36 polymorphic variants using the QuikChange II XL site-directed mutagenesis kit (Stratagene), as specified by the manufacturer. The presence of the expected 1.7-kb PCR product was confirmed by running 5 μl of each product on a 1% agarose gel. The samples were directly sequenced with subtype-specific PR primers using the BigDye Terminator cycle sequencing kit (version 1.1; Applied Biosystems). The sequences were run on an ABI Prism 3130xl genetic analyzer (Applied Biosystems). The data were analyzed using SeqScape software (version 2.5), and the PR sequences were aligned using Bioedit software, version 7.0.

**Phenotypic drug susceptibility.** Wild-type (week 0) and virus variants selected with ATV, NFV, and LPV were phenotyped against the PIs ATV, NFV, and LPV at 0, 25, and 35 weeks using drug concentrations ranging from 0.04 nM to 10 μM in 96-well plates. CBMCs were infected for 2 h with week 0 or PI-selected viruses and plated at 5 × 10^6 cells per well in the presence of a PI. Following 3 days of incubation, the viral cultures were replenished with fresh RPMI 1640 complete medium (Invitrogen) containing serial drug dilutions to determine a dose-response. On day 7, the culture supernatants were collected to quantify RT activity as a reflection of viral replication. The data were analyzed using Prism software, version 5.0 (GraphPad, Inc.), to determine the EC_{50} for each PI tested.

**Viral replication assays.** TZM-bl cells (10^5 cells/well) (19, 29) were added to 96-well plates in 100 μl of Dulbecco modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin-streptomycin (Invitrogen), L-glutamine (Invitrogen), and interleukin-2 (IL-2; Roche) (34). RT activity was quantified as described elsewhere (10, 20), and the results were used to determine the amount of virus present in the infected samples. The virus-cell cultures were replenished with fresh RPMI 1640 medium (Invitrogen) containing serial drug dilutions to determine a dose-response. On day 7, the culture supernatants were collected to quantify RT activity as a reflection of viral replication. The data were analyzed using Prism software, version 5.0 (GraphPad, Inc.), to determine the EC_{50} for each PI tested.

**RESULTS**

**Generation and verification of subtype-specific HIV-1 M36 and I36 HIV-L.** To evaluate the impact of M36 and/or I36 on the development of PI resistance, genetically homogeneous viral variants coding for M36 and/or I36 were generated from full-length subtype-specific HIV-1 molecular clones. Site-directed mutagenesis was employed to introduce the less frequently occurring polymorphisms into subtype B, subtype C, and CRF02_AG clones to ultimately obtain six HIV-1 polymorphic variants. Each viral variant was sequenced to confirm the presence of the introduced amino acid (Fig. 1). The results show that the only differences within a given subtype were at PR position 36.
Baseline phenotypes in CBMCs and viral replication in the TZM-bl reporter cell line. Most commercially available EC_{50} tests use viruses generated from a standard laboratory-adapted backbone that contains an insert of a specific drug-targeted HIV-1 genomic region (e.g., backbone that contains an insert of a specific drug-targeted tests use viruses generated from a standard laboratory-adapted TZM-bl reporter cell line.

For example, in the presence of either NFV or LPV, M36 subtype B virus was approximately two times more resistant than the I36 polymorphic variant. Similarly, the I36 subtype C and CRF02_A2 variants appeared to be slightly more adept than their respective M36 counterparts at replicating in the presence of all tested PIs (Table 1). To assess the replication capacity of each of the parental viral variants, a short-term TZM-bl cell-based replication assay was employed. The presence of M36 or I36 in PR did not appear to compromise the replication capacity within subtype B (Fig. 2a), subtype C (Fig. 2b), or CRF02_AG (Fig. 2c) virus, although minor differences in replication capacity between subtypes were observed (C > B > A/G).

Impact of the M/136 PR polymorphism on resistance mutations, drug susceptibility, and viral replication. To better understand the patterns of PI resistance mutations in non-subtype B subtypes and the impact of the M36I polymorphism on drug resistance, full-length replication-competent clonal v

TABLE 1. Major mutations, polymorphisms, and EC_{50} values for wild-type and drug-selected HIV-1 isolates

<table>
<thead>
<tr>
<th>PI Wk</th>
<th>Subtype B</th>
<th>Subtype C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(M36)</td>
<td>(I36)</td>
</tr>
<tr>
<td></td>
<td>Mutations</td>
<td>EC_{50} (nM) ± SEM</td>
</tr>
<tr>
<td>NFV</td>
<td>D30N, A71T, V771</td>
<td>16.3 ± 0.87</td>
</tr>
<tr>
<td>0</td>
<td>35</td>
<td>1,139 ± 1.27 (70)</td>
</tr>
<tr>
<td>LPV</td>
<td>L10F, V82A</td>
<td>12.4 ± 0.81</td>
</tr>
<tr>
<td>0</td>
<td>35</td>
<td>196 ± 1.13 (16)</td>
</tr>
<tr>
<td>ATV</td>
<td>M46I, I50L, N88S</td>
<td>3.0 ± 1.33</td>
</tr>
<tr>
<td>0</td>
<td>35</td>
<td>183 ± 1.37 (61)</td>
</tr>
</tbody>
</table>

Adapted from Antimicrob. Agents Chemother. 2016, 60(6), 3095-3102.
spectively) (Table 1). The presence of the primary resistance mutation L90M in NFV-selected I36 subtype C (which emerged at week 25 but which was undetectable by week 35) may account for the higher level of NFV resistance. In contrast, ATV-selected M36 subtype C virus exhibited higher-level ATV fold resistance (Table 1) and withstood higher-level ATV pressure (Fig. 4c) than the I36 virus did (Table 1 and Fig. 4f, respectively). In the selections with both NFV and ATV, the viral variant that was best able to endure higher-level PI pressure and that exhibited higher-level resistance also possessed superior replication ability in the TZM-bl cell assay. With ATV-selected variants, the difference was marked (Fig. 2 h); however, for NFV-selected viruses, differences in replication capacity were modest (Fig. 2b).

The development of resistance mutations in LPV-selected I36 subtype C virus was slower than that with M36 virus. The I36 viral cultures selected with LPV contained the L76V major mutation at week 35, at which time a second mutation, TABLE 1—Continued

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Subtype C (I36)</th>
<th>CRF02_AG (M36)</th>
<th>(I36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D30N, E35G, (I36), I85V, L90L/M</td>
<td>3.0 ± 0.94</td>
<td>5.7 ± 0.75</td>
<td>8.7 ± 0.85</td>
</tr>
<tr>
<td>L33I, (M36), M46I, A71T</td>
<td>3.47 ± 1.12 (1,158)</td>
<td>202 ± 1.20 (35)</td>
<td>55 ± 1.30 (6)</td>
</tr>
<tr>
<td>L33I, (M36), M46I, L76V, 184V</td>
<td>6.0 ± 0.90</td>
<td>134 ± 0.82 (22)</td>
<td>14.5 ± 1.16</td>
</tr>
<tr>
<td>(I36), M46I, L76V, 184V</td>
<td>1.0 ± 1.33</td>
<td>2.0 ± 1.27</td>
<td>3.4 ± 1.6</td>
</tr>
<tr>
<td>L23I, (I36), M46I/M, L76V</td>
<td>1.01 (46)</td>
<td>2.3 ± 1.31</td>
<td>18.6 ± 1.2 (3.4)</td>
</tr>
</tbody>
</table>

FIG. 2. Replication capacity of parental HIV-1 variants compared to that of drug-selected progeny HIV-1. The results for parental isolates and subtype B, subtype C, and CRF02 AG HIV-1 isolates selected with NFV (a, b, and c, respectively), LPV (d, e, and f, respectively), and ATV (g, h, and i, respectively) are shown. Percent replication of preselected (---) and drug-selected (—) M36 (▲) and I36 (●) HIV-1 in TZM-bl cells are expressed as a function of HIV-1 RT activity (cpm). Data points depict the averages (mean ± standard deviation) of three independent experiments performed in duplicate.
M46I/M, was beginning to emerge (Table 1). In contrast, the LPV-selected M36 subtype C virus contained three major mutations after 35 weeks: M46I, I54V, and V82S (Table 1). Furthermore, higher concentrations of LPV could be applied to the M36 viruses than to the I36 viruses (Fig. 4b and e). Accordingly, the observed resistance to LPV (Table 1) was greater for LPV-selected M36 subtype C virus than I36 virus. However, the replication capacities for both LPV-selected subtype C polymorphic variants in the short-term replication assay were very similar (Fig. 2e).

(iii) CRF02_AG selections. NFV-selected M36 CRF02_AG virus withstood greater PI pressure over 35 weeks (Fig. 5a and d), developed more resistance mutations (Table 1), presented with a greater fold NFV resistance (Table 1), and had a replication capacity similar to that of parental virus, in contrast to NFV-selected I36 CRF02_AG virus, which did not (Fig. 2c). The accessory mutations detected in the NFV-selected M36 CRF02_AG virus, L33I and A71T, may have contributed to a higher-level NFV resistance phenotype than was seen with NFV-selected I36 CRF02_AG (Table 1). The opposite was
observed with LPV-selected CRF02_AG viruses. Even though similar LPV pressure was applied to both types of viral cultures over time (Fig. 5b and e), the I36 CRF02_AG virus developed greater LPV fold resistance (Table 1) and possessed a better replication capacity than the LPV-selected M36 CRF02_AG virus (Fig. 2f). The major and cross-resistance LPV resistance mutations, M46I and I84V, respectively, were detected in both M36 and I36 CRF02_AG LPV-selected viruses (Table 1). However, a rare I47A major LPV resistance mutation was detected in LPV-selected I36 CRF02_AG virus (Table 1). The simultaneous presence of I47A, together with M46I and I84V, may be the reason for the higher fold resistance to LPV seen in the I36 viruses than in the M36 viruses.

ATV-selected M36 CRF02_AG viruses developed the accessory mutation A71V and also started to develop a major resistance mutation at position 50 in PR (Table 1). In contrast, no resistance mutations were observed in the ATV-selected I36 CRF02_AG viruses (Table 1), which may be due, in part, to the modestly lower ATV pressure that was applied to the viral cultures over time (Fig. 5c and f). At week 35, a mixed signal was present at amino acid 50 at the first nucleotide position (XTT) of these viruses (Table 1). This probably represents a mutation that is only beginning to emerge but one for which the signal was not yet strong enough to be resolved by the chromatogram. The fold resistance to ATV (Table 1) and the replication capacity (Fig. 2i) were comparable between the I36 and M36 viruses; of note, these viruses did not replicate as well as the parental virus (Fig. 2i).

DISCUSSION

The impact of a single nucleotide polymorphism at position 36 in PR on the selection of NFV, LPV, and ATV resistance mutations in HIV-1 subtype B and non-subtype B subtypes was investigated in culture using CBMCs infected with genetically homogeneous virus containing defined polymorphisms. Although previous work has suggested that subtype B clonal virus encoding I36 in PR should display greater drug resistance and possess a higher replicative capacity than M36 virus (15, 40), this had not yet been assessed in other HIV-1 subtypes or studied in the context of drug resistance mutation development.

These studies are both labor-intensive and tedious, requiring viral cultivation over many months. Accordingly, it has not been possible to conduct all of this work on repeat occasions. However, certain of the selections were carried out in parallel in different cultures, yielding similar results on each occasion. The possibility that stochastic changes may also have affected viral susceptibility to the PIs used cannot be ruled out. We also cannot rule out a potential role for mutations within Gag, including cleavage site mutations, as has previously been shown (21, 25, 42). An investigation of this topic is beyond the scope of this paper.

At the baseline, we observed that only modest differences in NFV, LPV, and ATV resistance may be attributed to polymorphic variation at position 36 in PR (Table 1). I36 HIV-1 may have a slight natural competitive advantage over M36 HIV-1 that is independent of drug pressure. We also assessed viral replication capacity in the TZM-bl indicator cell line. The presence of M36 or I36 in PR did not compromise the replication capacity of subtype B, C, or CRF02_AG (Fig. 2i), although minor differences in replication capacity between subtypes were observed (C > B > A/G). It is possible that some evolutionary divergence may have resulted from the introduction of PI-containing antiretroviral therapy (ART) in developed countries. Since the majority of HIV-1 infections in developed countries are subtype B, it is possible that the clinical prevalence of M36 subtype B over I36 subtype B is a function of the selective pressure of PI-containing ART.
NFV pressure led to the development of mutations in I36 subtype B virus that resulted in a phenotype more resistant than that found with M36 (150-fold and 70-fold, respectively) (Table 1). This agrees with the concept that the I at position 36 in PR acts as a resistance mutation only when other resistance mutations are present (26). In addition, the combination of mutations L33I and N88D together with I36 may provide the virus with a resistance advantage over the combination of A71T and V77I together with M36 (Table 1); a negligible loss of replication capacity was observed for both viruses (Fig. 2a). In the case of subtype B and ATV pressure, the presence of M36 as opposed to I36 may have favored resistance development without compromising viral replication capacity (Table 1 and Fig. 2g).

The V82A mutation is known to emerge in subtype B HIV-1 following LPV therapy and to modestly decrease the level of binding between PIs and PR because of changes to the enzyme active-site cavity (17, 22, 23). Our results show that V82A results in a modest increase in the LPV fold resistance (Table 1) at great expense to viral replication capacity (Fig. 2d). Additional accessory and/or compensatory mutations are possibly needed to restore viral replication capacity in this context.

Gag and Gag-Pol cleavage site mutations have been described, but the frequency with which they occur in the context of non-subtype B subtypes is unknown. Both compensatory mutations that appear following the emergence of PI resistance and cleavage site mutations can partially restore replication capacity (11, 21, 25, 42). It is possible that cleavage site mutations may have been present in some of our viruses.

A unique mutational signature (L23I, M46I, and L89I) was present in subtype C M36 viruses grown under increasing NFV or ATV pressure. We also detected this mutational pattern in other non-subtype B drug-selected viruses that originated from clones or clinical isolates (data not shown). These PI-selected viruses displayed >150-fold resistance (Table 1) to the PIs used in the selections. However, this elevated level of resistance did not result in a replicative disadvantage (Fig. 2b and h). The conformational changes caused by these mutations possibly reduce the binding affinity between the PIs and the viral PR (8, 23).

After 35 weeks of NFV pressure, M36 CRF02_AG virus manifested a greater fold resistance and replication capacity than I36-encoding viruses (Table 1 and Fig. 2c). Conceivably, either or both the L33I and A71T mutations may help to restore replicative capacity and/or increase NFV resistance in M36-containing CRF02_AG viruses to an extent that exceeds that seen with I36. In contrast to the subtype B and I36 subtype C viruses, none of the CRF02_AG polymorphic HIV-1 variants developed the D30N mutation following NFV pressure, in agreement with previous findings (7, 9, 10, 13, 32). These results contribute to the increasing body of evidence that different HIV-1 subtypes develop different resistance mutations or develop them at differential rates following exposure to the same PI (4, 6, 10, 12, 13, 16).

Full-length non-subtype B subtype replication-competent molecular clones are scarce. Moreover, no work has previously investigated which polymorphisms affect resistance patterns in non-subtype B subtypes, a clinical impossibility given the paucity of non-subtype B-infected patients who have been treated with atazanavir and darunavir. Few such samples have ever been genotyped for resistance, the only exception being subtype C in the context of lopinavir.

We chose to focus on the I36 polymorphism, because it was found to be linked to a higher rate of treatment failure among treatment-naive patients infected with non-subtype B subtypes (15, 28, 29, 32). Our results could not ascertain whether I36 is the determining factor that predicts which resistance mutations will emerge; rather, it might contribute to the emergence of mutational patterns that eventually develop. This polymorphism appears to affect the resistance level to a different extent among viruses of assorted subtypes exposed to the same PIs. The effect of minor mutations or polymorphisms cannot be extrapolated from data generated for subtype B and applied to interpretations of resistance in non-subtype B subtypes until clinical responses or phenotypic validations have been completed.

In general, the D30N mutation seems to occur commonly in NFV-experienced individuals infected with subtype B viruses and in some subtype C-infected individuals but not in subjects infected with CRF02_AG viruses (7, 10, 13). Our polymorphic HIV-1 selection results are in agreement with these findings (Table 1). The levels of resistance to NFV following 35 weeks of comparable drug pressure were 6-, 70-, and 1,173-fold for CRF02_AG (I36), subtype B (M36), and subtype C (I36) viruses, respectively (Table 1); and the ability of the CRF02_AG polymorphic variants to replicate in TZM-bl cells was compromised.

Our observations suggest that different subtypes might adapt differently following the acquisition of resistance mutations. Subtype C LPV- and NFV-selected clones, in contrast to subtype B and CRF02_AG HIV-1 isolates grown under PI pressure, showed only minimal reductions in replication capacity, despite the presence of polymorphisms, resistance mutations, and increased levels of drug resistance. However, the replication capacity of ATV-selected subtype C virus, in contrast to subtype B and CRF02_AG virus grown under ATV pressure, was greatly reduced, despite the presence of one accessory resistance mutation, low drug pressure, and 3-fold resistance to ATV. In general, among the HIV-1 isolates tested, the rate of emergence of resistance mutations following ATV pressure appeared to be slower than that with selections using NFV or LPV. These findings may be important in the context of widespread NFV and LPV use as part of first-line and second-line PI-based antiretroviral therapy in developing country settings.

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REFERENCES


