Low Accumulation of L90M in Protease from Subtype F HIV-1 with Resistance to Protease Inhibitors Is Caused by the L89M Polymorphism

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Background. This work evaluates the role of subtype F human immunodeficiency virus type 1 (HIV-1) protease (PR) substitutions L89M and L90M in viral replication and resistance to PR inhibitors (PIs).

Methods. Subtype B and F PR genes were subjected to site-directed mutagenesis, to create and reverse the methionine at positions 89 and 90. Viruses were re-created in cell culture, and their replicative capacity was assessed by fitness assay. Generated viruses were also phenotyped for PI resistance.

Results. The subtype F clone (89M90L) showed a replicative capacity comparable to that of the PI-susceptible subtype B clone (89L90L) and was more fit than the L89M mutated subtype B clone (89M90L). Both 89M90M subtype B and F clones presented the lowest fitness values. The L89M mutation impacted phenotypic resistance to all PIs in half of the subtype F isolates but not in the subtype B isolates. Subtype F isolates presented a phenotypic profile similar to that of subtype B isolates when the M89L mutation was introduced.

Conclusion. The L89M mutation in subtype F viruses is a high genetic barrier to the accumulation of the L90M resistance mutation and can function as a resistance mutation, depending on the presence of other polymorphisms in the subtype F PR backbone.

HIV-1 protease (PR) inhibitors (PIs) are crucial components in combined antiretroviral therapy (or highly active antiretroviral therapy [HAART]) for patients with AIDS [1–4]. Seven different PIs have been approved by the United States Food and Drug Administration (FDA) and are available for clinical use: saquinavir (SQV), ritonavir (RTV), indinavir (IDV), nelfinavir (NFV), amprenavir (APV), lopinavir (LPV), and, more recently, atazanavir (ATV) [5–7]. Despite the great potency of HAART that includes at least 1 PI, resistant strains can emerge from the viral population circulating in the infected individual and cause therapy failure [8–11]. These resistant strains harbor the enzymes PR and reverse transcriptase (RT) with amino acid substitutions that confer resistance to the PIs and RT inhibitors (RTIs), respectively [1, 10, 12–16].

Drug-resistance mutations have been characterized extensively for subtype B strains of HIV-1 [10, 17]. The primary resistance mutations appear to modify the amino acids that interact directly with the PIs inside of the catalytic pocket of the enzyme [18–20], whereas the secondary, or compensatory, mutations occur mostly outside of the active cleft of the enzyme, restoring its plasticity and catalytic capacity, which is generally diminished by the presence of primary mutation(s) [12, 18, 21, 22].

Genotyping and phenotyping assays play an important role in the characterization of the mutational patterns responsible for drug resistance. Different interpretation algorithms for drug-resistance genotyping have been established on the basis of both phenotyping assays and clinical correlations between therapy failure...
Figure 1. Amino acid sequences of protease (PR) from viral clones. A, Consensus sequences of PR from HIV-1 subtypes B and F (sequences obtained from Los Alamos HIV sequence databank), illustrating the molecular signatures of subtype F PRs. B, The polymorphic regions of amino acids 89 and 90, generated from PR inhibitor–susceptible subtype B (pNL4-3) and F (F13) clones by the mutagenized clones. C, Amino acid sequences from 3 treatment-naive isolates of subtype F (F18, F17, and F22) with their respective mutants at amino acid 89. The amino acids at positions 89 and 90 are depicted to the right of the clone name.

and viral strains carrying mutations [23–27]. The algorithms are nevertheless based on observations made using subtype B strains of HIV-1, and there is little information on non–subtype B strains [6, 28–30]. The usefulness of these algorithms to the study of isolates of non–subtype B strains, as well as to the characterization of new, subtype-specific PI-resistance mutations, still needs to be addressed. Substitutions frequently found in PRs of non–subtype B isolates, so-called “molecular signatures,” may also interfere with the PI-resistance phenotype produced by other mutations that are well characterized in subtype B isolates.

Subtype F is the most prevalent (prevalence, 15%) non-B HIV-1 subtype found in Brazil [31–33]. Recently, we genotypically and phenotypically characterized Brazilian clinical isolates of HIV-1 subtype F and B infecting adults [34] and children [35] in whom antiretroviral therapy failed. Surprisingly, we observed discordant genotypic profiles among subtype F isolates that were phenotypically resistant to NFV. In total, 7 (53.8%) of 13 NFV-resistant subtype B isolates carried the L90M substitution, which was characterized as conferring in vitro and in vivo primary resistance to SQV [16, 36] and NFV [15]. Another 3 isolates (23%) carried the D30N mutation, which is also related to NFV resistance [37]. Of the 5 NFV-resistant subtype F isolates found, however, only 1 (20%) carried the L90M substitution (none carried the D30N mutation). All of the subtype F isolates carried a polymorphism at neighboring amino acid position 89 (L89M) identical to that found at position 90, which confers resistance to NFV. No subtype B isolate harbored this latter polymorphism. In the present study, the role of the L89M substitution in preventing the accumulation of L90M in subtype F isolates is demonstrated, by use of a series of recombinant viruses carrying different mutations in fitness experiments.

MATERIALS AND METHODS

Cells and viruses. MT-4 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (Invitrogen). The HIV-1 subtype B proviral clone pNL4-3 [38] was used as the reference virus. The cDNA of HIV-1 viral isolate TVGG13 [35] was used as the representative of HIV-1 subtype F (F13; GenBank accession number AY145824). This isolate carries 11 amino acid differences in comparison with the consensus subtype B PRs (figure 1), 7 of which are the molecular signatures of subtype F PRs (F1 consensus). In addition, 3 other subtype F clinical isolates from PI-experienced patients were used: F17 (TVGG17), F18 (TVGG18), and F22 (TVGG22) (GenBank accession numbers AY145825, AY145826, and AY145832, respectively) (figure 1C). The recombination vector pHXB-2-Δpro was used for the phenotyping assay [39].

Polymerase chain reaction (PCR), cloning, and site-directed mutagenesis. A 651-bp PR-coding sequence was amplified by PCR from both F13 cDNA and the pNL4-3 plasmid, by use of the primers RVP5 (5′-gggaagatctggccttcctacaaggg-3′) and RVP3 (5′-ggcaaatactggagtattgtatgg-3′), with a standard protocol [39]. The amplification products were cloned into the pCR4-TOPO vector as instructed by the TOPO TA Cloning Kit manual (In-
vitrogen). The cloned PR fragments were subjected to site-directed mutagenesis, by use of the Quick Change Mutagenesis Kit (Stratagene), to create or reverse the 89M polymorphism, as well as to introduce the L90M mutation (primers are shown in table A1 of the Appendix, which appears only in the electronic edition of the Journal). The other 3 PR genes from subtype F clinical isolates (F17, F18, and F22) were cloned and mutagenized at amino acid position 89, to revert the methionine to leucine, by use of primers 89L/90L F and R. The L89I mutation was also introduced into clones F13 and Bwt, which carry either leucine or methionine at PR position 90 (table A1 of the Appendix, which appears only in the electronic edition of the Journal). The putative mutagenized clones were confirmed through DNA sequencing in both directions, by use of the BigDye Terminator Sequencing Kit and an ABI 3100 automated sequencer (Applied Biosystems).

**Virus PR genotyping.** Viral RNA was extracted with Trizol Reagent (Invitrogen), and the PR gene was amplified and sequenced, as described elsewhere [33]. PR sequences were edited with DNASTar software and were submitted to the HIV-genotyping algorithm of the HIV RT and Protease Sequence Database (available at: http://hivdb.stanford.edu) [23].

**Phenotyping assay.** The determination of HIV-1 phenotypic resistance to FDA-approved PR inhibitors (except ATV) was performed using recombinant virus assay technology, as described elsewhere [40], by use of PCR fragments of the PR region of the clones generated by site-directed mutagenesis (figure 1). These fragments were cotransfected into CD4+ T lymphocytes (MT-4) with the linearized pHXB-2-pro plasmid [39]. Homologous recombination led to generation of chimerical virus containing the different PR sequences. The virus containing the Bwt 89L90L PR was used to obtain the reference 50% effective concentration (EC50) values during the phenotyping assay. The susceptibility of chimerical virus to all PIs was determined, in 2 independent assays with duplicates, by an MT-4-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)-based cell viability assay, as described elsewhere [40].

**Growth competition experiments (fitness assay).** Experiments were performed as described elsewhere [41, 42], in the absence of drugs in MT-4 cells. The assay consists of the coinfection of cultures with known amounts of the virus to be tested, together with a reference clone. These cocultures were subjected to repeated bottleneck passages [43]. The ratio of the 2 viruses was quantified by a genotypic assay as follows: the proportion of the competing variant with respect to the reference strain (Rn) is divided by its proportion in the initial mixture (Rn), and its value (Rn/Rn) is plotted against the competition passage, to derive the fitness vector [43, 44]. Each competition assay was performed in duplicate by infecting MT-4 cells with mixtures of the viruses to be tested (Bwt- and F13-derived mutagenesis clones) with Bwt 89L90L (the reference virus), at an initial ratio of 1:1. For each competition, 10⁵ MT-4 cells were infected with 10⁴ 50% cell culture infectious doses (CCID₅₀) of the virus tested and of the reference virus (MOI for each virus, 0.05). The culture supernatant was harvested when a cytopathic effect was evident (~3–7 days after infection). Fresh MT-4 cells were then infected with 50 μL of this supernatant. A total of 5 passages were performed, as described below, and aliquots were removed from the cultures at days 0, 7, 10, 13, 16, and 19 and were centrifuged; the supernatants were stored at −80°C for further quantitative analysis of genotypes.

We have developed a quantitative assay to determine the ratio of the different competing genotypes found in each culture passage by use of the ABI Prism Snapshot Multiplex Kit (Applied Biosystems). This kit is based on the dideoxy single-base extension of an unlabeled oligonucleotide (or a set of oligonucleotides; see table A1 of the Appendix, which appears only in the electronic edition of the Journal). Each primer binds to a complementary template in the presence of the 4 ddNTPs labeled with different fluorophores and Taq DNA polymerase. The polymerase extends the primer by 1 nt, adding a single labeled ddNTP to its 3' end. The sample reactions are electrophoresed in an ABI Prism 310 Genetic Analyzer, and data are analyzed with GeneScan Analysis Software (version 3.1; ABI). This methodology was validated using an artificial mixture of wild-type and mutant cloned material, and a linear regression curve was obtained for the different mixture ratios, with a coefficient of correlation (R²) >0.98.

**Segregation of fitness assay PRs and analysis of recombination.** Supernatant harvested on day 19 of the fitness assay (viral competitions Bwt 89L90L × F13 89L90L and Bwt 89L90L × F13 89M90M) were submitted to viral RNA extraction, followed by reverse-transcription PCR and cloning in a TOPO TA Cloning kit (Invitrogen). Colony PCR for the PR gene was performed and submitted to BclI endonuclease digestion. Restriction-negative clones were submitted to sequencing, followed by sequence alignment and analysis of recombination, by use of the RIP 1.9 beta test (http://www.hiv.lanl.gov/content/hiv-db/RIPPER/RIP.html).

**Clinical samples.** Analysis of clinical samples was performed with internal databases from the Laboratory of Retrovirology of the Federal University of São Paulo and the Molecular Virology Laboratory of the Federal University of Rio de Janeiro. These databases include clinical information on treated patients from different regions of Brazil, including treatment regimens, CD4+ T lymphocyte and viral load counts, and resistance genotyping profiles. For the viral isolates from infected individuals without PI exposure (treatment-naïve individuals), we used the database of these 2 laboratories from BRsNet [31]. The data on viral isolates from HIV-1–infected individuals with exposure to NFV and/or SQV was also obtained from Caride et al. [34] and Brindeiro et al. [35]. All data were collected
under the supervision of the Universities’ respective institutional review boards.

Statistical analysis. All statistical analyses were performed using Analyse-it (version 1.62) for the Microsoft Excel statistics package. Sigmaplot software (version 8.0) was used to perform linear regressions for the fitness assay and nonlinear regressions (Hill’s dose-response curve) for phenotyping. We established the significant values of resistance by use of a nonparametric Mann-Whitney U test, for comparison between the quadruplicates of EC_{50} for each mutagenized virus and the quadruplicates of EC_{50} for the Bwt L89L90 drug-susceptible clone.

RESULTS

Genotypic frequencies of the L89M and L90M polymorphisms in subtype B and F clinical samples. Seventy-six previously genotyped clinical isolates of virus from NFV- or SQV-treated individuals (54 from subtype B and 22 from subtype F) were analyzed for the presence of the L89M and L90M polymorphisms (table 1). Since L89M is a characteristic molecular signature of subtype F PRs, we also analyzed its frequency (as well as the frequency of L90M) in genotyped clinical samples from treatment-naive infected individuals (2021 from subtype B and 118 from subtype F), obtained from the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu/) [23].

To avoid misinterpretation of the L89M frequencies in different subtypes due to its presence as a natural polymorphism of subtype F, the frequencies were also analyzed in separate groups of treatment-naive and PI (NFV and SQV)–treated individuals. A significant difference was noted between the subtype B and F samples, respectively; \( p = .0001 \) (Fisher’s exact test), for contingency tables. Of note, the 89M90M PR genotype could not be found in subtype F isolates. Some other studies (R.S.D., un-published data) have found this genotype in the subtype F backbone, at a frequency of <1%. Nevertheless, the subtype F isolates from treated individuals described here as carrying the L90M resistance mutation have also accumulated the M89I polymorphism.

Relative replicative fitness of mutated viruses. After generating the viral stocks in cell culture by cotransfection of PR amplicons with a pHXB-2-Δpro full-length genome, we performed replicative competition experiments (fitness assay) between the PI-susceptible Bwt 89L90L viral clone and the other clones herein described, in the absence of any PI (figure 2). The Bwt 89M90L and Bwt 89M90M viruses replicate with a lower capacity than does the reference virus, whereas the Bwt 89L90M mutant had minimally increased replicative fitness, compared with that of Bwt 89L90L (figure 2A). The F13 89M90L and F13 89L90M clones had a replicative capacity similar to that of the Bwt reference virus, but the latter reached a positive fitness value (\( s \)) slightly higher than that of its F9M90L counterpart (figure 2B). Both the F13 89L90L and F13 89M90M mutants showed a significant decrease in their replicative capacity, compared with that of the Bwt reference virus (\( s = -0.072 \) and -0.1622, respectively). Surprisingly, after bottleneck passages 1 (F13 89M90M) and 2 (F13 89L90L), these clones recovered their replicative capacity and reached positive fitness values (\( s = 0.0459 \) and 0.0499, for F13 89L90L and F13 89M90M, respectively).

To assess possible genetic causes of the fitness shift of viruses F13 89L90L and F13 89M90M during the replicative competition experiment (fitness assay), we isolated and sequenced clones of subtype F PR genes amplified from the mixture of subtype B and F viruses at the last bottleneck event of the fitness assay (day 19 virus passage). After PCR amplification of cDNA obtained from viral RNA of culture supernatant, PR gene amplicons were cloned directly into a pCR4.3 vector. Bacterial colonies carrying segregated PR genes were submitted to colony PCR, followed by BclI enzyme digestion screening for subtype F PRs. A BclI cut site can be found at nucleotide position 177 of the subtype B—but not subtype F—PR genes.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Subtype B*</th>
<th>Subtype F*</th>
<th>( p^b )</th>
<th>Subtype B</th>
<th>Subtype F</th>
<th>( p^b )</th>
</tr>
</thead>
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<tr>
<td>89L</td>
<td>1997</td>
<td>49</td>
<td></td>
<td>50</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>89M</td>
<td>24</td>
<td>69</td>
<td>&lt;.0001</td>
<td>4</td>
<td>18</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>90L</td>
<td>2021</td>
<td>118</td>
<td></td>
<td>41</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>90M</td>
<td>0</td>
<td>0</td>
<td>&lt;.0001</td>
<td>13</td>
<td>1</td>
<td>.04</td>
</tr>
</tbody>
</table>

NOTE. Data are no. of isolates found with each mutation.

\( ^a \) Calculation based on data from the Stanford HIV sequence database (available at: http://hivdb.stanford.edu/).

\( ^b \) \( P \) (Fisher’s exact test), \( \alpha = 0.05 \), for 2 \times 2 contingency tables.
Figure 2. Fitness vectors and corresponding fitness values of HIV-1 Bwt (A) and F13 (B) 89L90L, 89L90M, 89M90L, and 89M90M and of 89I90L and 89I90M (C). In each competing passage, viral proportion ($R_n$) was compared with the ratio of the 2 viruses in the initial mixtures ($R_o$). The value in each passage was used to derive the fitness vectors by linear regression. Details of all procedures involved are given in Materials and Methods. For all lineages, the initial 1:1 viral competition rate was used in the calculation of the vectors. Vectors were drawn with data from 3 competing passages. Slopes of fitness vectors (fitness values, $s$) for the HIV-1 clones are indicated for each clone in the plotting areas. The pNL4-3 Bwt L89L90 clone was used as a reference, under the assumption of an $s$ value of 0.
The L89I mutation negatively impacted the replicative capacity of subtype B and F viruses carrying this mutation. This effect was more drastic in subtype B viruses (Bwt 89L90L and Bwt 89I90M) than in subtype F viruses (F13 89I90L and F13 89I90M), and it was also independent of the presence or absence of the L90M mutation (figure 2C). Both subtype F viruses carrying the L89I substitution had a replicative capacity coefficient (s) similar to that found for virus F 89M90M.

**Phenotyping of clones from subtypes B and F.** Recombinant viruses carrying the mutant PR genes were generated in MT-4 cells, and all of the constructs were phenotyped in quadruplicate for PI susceptibility by MT-4 cell–MTT-based cell viability assay. The resulting EC_{50} values are shown in table 2. To facilitate the comparison between the different clones from subtypes B and F, we chose to establish the statistical significance of resistance on the basis of the quadruplicate sampling comparing the EC_{50} in the Bwt L89L90 clone (subtype B wildtype clone) with those in the other clones tested. The L89M mutation conferred significant (5.6-, 4.7-, and 6.2-fold) increases in EC_{50} for IDV, NFV, and RTV, respectively, on F13 89M90L virus, compared with Bwt 89L90L virus. These values are comparable to the resistance values obtained with Bwt 89I90M virus: 2.8-, 3.8-, and 5.5-fold resistance, respectively. In contrast, the Bwt 89M90L clone behaved in a way that was phenotypically similar to the susceptible Bwt 89L90L virus.

The F13 89L90M clone showed a 6.4-, 6.5-, 7.5-, 23-, 5.1-, and 3.9-fold resistance increase for SQV, IDV, NFV, RTV, APV, and LPV, respectively. These values represent a 3.0-, 3.7-, 1.9-, 4.3-, 1.8-, and 2.6-fold increase over its counterpart, Bwt 89L90M, for the same drugs. The Bwt 89M90M and F13 89M90M clones showed divergent phenotypic behavior. There was a significant increase in resistance in the Bwt 89M90M clone, compared with that in the Bwt 89L90L clone (table 2), with the exception of resistance to LPV. In contrast, there was a significant decrease in resistance to RTV, NFV, and APV in the Fwt 89M90M clone, compared with that in the Fwt 89L90L clone. (P<.05, Mann–Whitney U test).

Virus clones of both subtypes carrying an isoleucine at amino acid position 89 were also phenotypically characterized (table 2). This mutation was not able to confer any significant level of resistance to the PIs analyzed. The coexistence of an L90M substitution with the 89I residue did not modify this pattern of PI susceptibility, for either subtype analyzed.

To explore the unexpected phenotype coded by the L89M polymorphism in subtype F PR, we cloned the PR genes of 3 other clinical isolates, and the 89M signature (wild-type subtype F signature) was reversed to 89L by site-directed mutagenesis. All 89L reversed clones were phenotyped for PI resistance (table 2).
and F18 showed a significant decrease in EC50 values for IDV, RTV, NFV, APV, and IDV alone when 89M was reversed to 89L. In contrast, isolates F17 and F22 showed no significant difference in EC50 values when 89M was reversed.

**DISCUSSION**

HIV-1 subtype F is found throughout South America [18], in Romania, and in Central Africa. Since the beginning of the 1990s, surveys conducted in Brazil have identified subtype F as the main non–subtype B isolate in that country. Subtype F strains, together with their recombinant forms with subtype B, account for 15% of HIV-1 infections in Brazil. The subtype F consensus sequence differs from the US and Brazilian subtype B consensus sequence at 8 positions (115V, E35D, M36I, R41K, R57K, Q61N, L63P, and L89M) [33].

Subtype F isolates are infrequently found to carry the L90M NFV and SQV–resistance mutation in HIV-1–infected individuals in whom therapy has failed [34, 35, 46]. The relative replicative capacity of viruses carrying subtype F PRs with M89L and L90M substitutions appears to be higher than those with the PI-susceptible prototypic subtype F PR genotype 89M90L, as shown here by fitness assay. However, the accumulation of L90M appears to be constrained to the change in the 89M subtype F polymorphism. This hypothesis is supported by the poorest relative replicative capacity being found for F13 89M90M, compared with that in the F13 89L90M and F13 89M90L virus clones (s = −0.1622, vs. 0.0165 and −0.0018, respectively; see figure 2B for details). This effect was more noticeable in subtype F clones, since the s values obtained for the subtype B clone counterparts did not show this difference (figure 2A). Furthermore, a detailed search of the PI-susceptibility data in the Stanford HIV sequence database [23] reveals the low clinical prevalence (0.36%, or 6/1682 samples analyzed) of viruses carrying the 89M90M polymorphisms. None of these isolates belonged to subtype F, and, of 109 subtype F isolates deposited, only 1 carried the L90M mutation together with a M89I polymorphism (isolate TVGG27 [35]).

Since the F13 89L90L and F13 89M90L viruses had low replicative fitness, the M89I mutation could represent the best in vivo alternative for the transient mechanism of generation of L90M PRs from the original 89M90L subtype F PRs [8, 34, 35, 47]. Nevertheless, the fitness assay showed both F13 89I90M and F13 89I90L viruses with no advantage over their counter-

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**Table 2. Results of protease inhibitor phenotyping assay for Bwt and F13 clones.**

<table>
<thead>
<tr>
<th>Virus, clone</th>
<th>SQV</th>
<th>IDV</th>
<th>RTV</th>
<th>NFV</th>
<th>APV</th>
<th>LPV</th>
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<tr>
<td>Bwt</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>89L90L</td>
<td>1.00 ± 0.127</td>
<td>1.00 ± 0.625</td>
<td>1.00 ± 0.124</td>
<td>1.00 ± 0.314</td>
<td>1.00 ± 0.192</td>
<td>1.00 ± 0.159</td>
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<tr>
<td>89M90L</td>
<td>1.00 ± 0.329</td>
<td>2.96 ± 0.095</td>
<td>0.77 ± 0.135</td>
<td>1.10 ± 0.069</td>
<td>1.47 ± 0.136</td>
<td>0.77 ± 0.240</td>
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<tr>
<td>89L90M</td>
<td>2.12 ± 0.480</td>
<td>2.80 ± 0.871</td>
<td>3.82 ± 0.819</td>
<td>5.46 ± 0.956</td>
<td>2.90 ± 0.173</td>
<td>1.48 ± 0.239</td>
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<tr>
<td>89M90M</td>
<td>1.67 ± 0.399</td>
<td>2.04 ± 0.020</td>
<td>2.71 ± 0.303</td>
<td>4.44 ± 0.410</td>
<td>2.57 ± 0.195</td>
<td>1.32 ± 0.150</td>
</tr>
<tr>
<td>89I90L</td>
<td>0.71 ± 0.085</td>
<td>0.90 ± 0.076</td>
<td>0.77 ± 0.136</td>
<td>0.93 ± 0.067</td>
<td>0.91 ± 0.075</td>
<td>ND^a</td>
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<tr>
<td>89I90M</td>
<td>1.31 ± 0.108</td>
<td>1.60 ± 0.070</td>
<td>2.56 ± 0.152</td>
<td>1.81 ± 0.210</td>
<td>1.23 ± 0.067</td>
<td>ND^a</td>
</tr>
<tr>
<td>F13</td>
<td></td>
<td></td>
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<tr>
<td>89L90L</td>
<td>0.51 ± 0.182</td>
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<td>0.33 ± 0.068</td>
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<td>0.74 ± 0.143</td>
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<td>89M90L</td>
<td>2.06 ± 0.237</td>
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<td>7.45 ± 1.901</td>
<td>23.39 ± 4.605</td>
<td>5.13 ± 1.880</td>
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<td>ND^a</td>
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<tr>
<td>89L90L</td>
<td>1.33 ± 0.126</td>
<td>0.60 ± 0.215</td>
<td>2.91 ± 0.681</td>
<td>4.78 ± 0.855</td>
<td>1.51 ± 0.278</td>
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<td>89M90L</td>
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<td>3.71 ± 0.832</td>
<td>5.84 ± 0.714</td>
<td>1.21 ± 0.644</td>
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<td>89L90L</td>
<td>0.79 ± 0.026</td>
<td>0.49 ± 0.020</td>
<td>1.32 ± 0.060</td>
<td>1.00 ± 0.106</td>
<td>0.44 ± 0.018</td>
<td>0.93 ± 0.688</td>
</tr>
<tr>
<td>89M90L</td>
<td>0.18 ± 0.032</td>
<td>0.36 ± 0.036</td>
<td>0.59 ± 0.056</td>
<td>0.38 ± 0.038</td>
<td>0.33 ± 0.024</td>
<td>0.51 ± 0.171</td>
</tr>
</tbody>
</table>

^a APV, amprenavir; IDV, indinavir; LPV, lopinavir; ND, not determined; NFV, nelfinavir; RTV, ritonavir; SQV, saquinavir. Fold resistance is calculated as (50% effective concentration (EC50) of sample virus)/(EC50 of reference virus Bwt 89L90L). Data represent the results of quadruplicate experiments. Cut-off values of fold resistance with clinical significance, according to Harrigan et al. [45], are as follows: SQV, 2.5; IDV, 3.0; RTV, 3.5; NFV, 4.0; APV, 2.5; LPV, 4.0. Fold resistance values higher than the cut-off value are highlighted in bold.

^b Phenotyping values for 89I clones were not determined for LPV.
part F13 89M90M in their fitness $s$ values (all surrounding $-0.16$). Moreover, there was no fold increase, in these 2 89I F13 clones, in resistance to any PI analyzed. Together, the fitness assay and PI-resistance data indicate the existence of L90M and M89I mutations occurring in subtype F PRs as independent genetic events. The emergence of an M89I mutation in the backbone of a subtype F 89M90M virus is favored by the low genetic barrier of a single G→A transition, which changes the ATG codon of methionine to the ATA codon of isoleucine, against a less probable A→T or A→C transversion, which could generate a highly adapted subtype F 89L90M virus. The common G→A hypermutation that occurs in HIV sequences and is caused by the activity of cellular enzymes such as APOBEC 3G [48–50] is another possible mechanism that could favor the ATG→ATA transition at codon 89 in subtype F PRs, generating the 89I mutation, although at a low frequency.

A recovery of relative replicative capacity for the F13 89M90M and 89I90L clones was seen after bottleneck passages 2 and 3, respectively, in the fitness assay. The sequences obtained from different segregated clones of PRs from these subtype F virus subpopulations revealed a recombination event between 2 viruses of different subtypes (B and F). These recombinant events have probably yielded more fit viruses, explaining the fitness recovery by consequent alteration of the PR structure and its residue interactions. The loss of some PR molecular signatures of the subtype F backbone through genetic recombination could be involved with the fitness recovery, but this hypothesis needs to be further analyzed.

The Bwt 89L90M virus was more fit than the PI-susceptible Bwt 89I90L, in contrast to the findings of Martinez-Picado et al. [44]. They found a low relative replicative fitness of subtype B viruses carrying L90M, compared with a wild-type virus (10% less fit than wild type), although their theoretical mathematical model used for fitness calculation may not adequately quantify relative growth rates of different HIV-1 mutants, as stated in their article. The L89M signature of F13 in its original sequence could confer a significant level of resistance to IDV, as expected [13, 51], but also, surprisingly, to RTV and NFV. The reversion of the L89M mutation in subtype F isolates was able to confer a significant decrease in EC$_{50}$ to all 6 PIs analyzed. The same phenotypic behavior was not observed for the subtype B clone carrying L89M, which remained susceptible to PIs, with the exception of IDV. The role of the L89M polymorphism in IDV resistance in the subtype B backbone has already been demonstrated by others [13, 49]. In contrast, the L→M change in PR amino acid position 89 was previously described to have no correlation with PI regimen failure [16], although only clinical samples from subtype B were analyzed.

Interestingly, this 89M-related resistance is observed in only half of the subtype F isolates (F13 and F18) analyzed in our work, which indicates that other polymorphisms are involved in this phenotypic behavior. Samples F17 and F22 did not present significant levels of resistance to IDV, RTV, or NFV, unlike their subtype F counterparts F13 and F18. This fact can be partially explained by other polymorphisms found to differ from those common subtype F signatures, such as the I72T mutation, present in both F17 and F22, or the V15I and K70R mutations (reversing the F molecular signature), present in F17 and F22, respectively. The question of whether the differences between the sequences of clones F17 and F22 and the sequences of clones F13 and F18 account for the phenotypic divergence found still remains to be addressed.

Several studies [28–30, 34–47] have already demonstrated different adaptive strategies of resistance-mutation accumulation between subtype B and non–subtype B viruses, although the amino acid substitutions described for non–subtype B viruses and conferring resistance to antiretrovirals were basically the same as those described for subtype B viruses. The L89M polymorphism in the subtype F PR backbone is mostly related to the maintenance of viral fitness, and its presence as a molecular signature in the subtype F PR backbone confers a higher genetic barrier to the accumulation of the L90M resistance mutation. The L89M polymorphism is the most prevalent signature among treatment-naïve non–subtype B isolates and accounts for 98% of all PR sequences in the Stanford HIV sequence database. The same genetic barrier posed by L89M could play a role in other non–subtype B isolates and needs to be addressed in specific studies.

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**References**

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