In Vitro Hypersusceptibility of Human Immunodeficiency Virus Type 1 Subtype C Protease to Lopinavir

Luis M. F. Gonzalez, Rodrigo M. Brindeiro, Michelle Tarin, Alexandre Calazans, Marcelo A. Soares, Sharon Cassol, and Amilcar Tanuri

Laboratório de Virologia Molecular, Departamento de Genética, Universidade Federal do Rio de Janeiro, CCS, Bloco A, Cidade Universitária, Ilha do Fundão, 21944-970 Rio de Janeiro, RJ, Brazil, and HIV-1 Molecular Virology and Bioinformatics Unit, Africa Center/Nelson Mandela School of Medicine, University of Natal, Durban, South Africa

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In order to characterize the impact of genetic polymorphisms on the susceptibility of subtype C strains of human immunodeficiency virus type 1 to protease inhibitors (PIs), a subtype B protease that originated from an infectious clone was modified through site-directed mutagenesis to include the amino acid residue signatures of subtype C viruses (I15V, M36I, R41K, H69K, L89M) with (clone C6) or without (clone C5) an I93L polymorphism present as a molecular signature of the worldwide subtype C protease. Their susceptibilities to commercially available PIs were measured by a recombinant virus phenotyping assay. We could not detect any differences in the 50% inhibitory concentration (IC50s) of amprenavir, indinavir, ritonavir, saquinavir, and nelfinavir for the clones analyzed. However, we did observe hypersusceptibility to lopinavir solely in clone C6, which includes the I93L substitution (a 2.6-fold decrease in the IC50 compared to that for the subtype B reference strain). The same phenotypic behavior was observed for 11 Brazilian and South African clinical isolates tested, in which only subtype C isolates carrying the I93L mutation presented significant hypersusceptibility to lopinavir.

Initially characterized in developed countries such as the United States and countries in Western Europe, subtype B of human immunodeficiency virus (HIV) type 1 (HIV-1) was considered the major variant outside Africa and in the rest of the world. As the HIV-1 pandemic has extended around the globe, strain C was shown to be the most prevalent subtype, accounting for 56% of the infections worldwide (4). Subtype C was first detected in South Africa and Ethiopia in retrospectively analyzed samples from 1984 and 1986, respectively (9, 36); and it has been found in the majority of sub-Saharan African countries, such as South Africa, Botswana, Tanzania, and Kenya (3, 15, 16, 19, 20, 31, 32). Recombinants of subtype C strains and strains of subtypes previously prevalent in these countries have also been documented in Zambia (23) and Tanzania (11). Outside Africa, India has the largest population infected with subtype C viruses (25), and it is estimated that by 2010 India will have the highest number of HIV-1-infected people in the world (2). Finally, China also has a high prevalence of subtype C strains, and notoriously, these strains have subtype B and subtype C recombinant genomes (21).

Drugs targeting the reverse transcriptase (RT) and protease (PR) of HIV-1, such as nucleoside RT inhibitors, nonnucleoside RT inhibitors, and PR inhibitors (PIs), have revolutionized the treatment of HIV-1-infected individuals (5, 17). However, not all patients respond to highly active antiretroviral therapy, and in many patients the virus develops drug resistance, one of the most serious obstacles to sustained suppression of HIV-1 (12, 22, 34). The emergence of amino acid mutations associated with resistance to RT inhibitors and PIs has been extensively characterized (8, 24). The primary mutations found in the PR gene lead to a severalfold decrease in sensitivity to one or more PIs (8, 24). Compensatory mutations may not result in a significant decrease in drug sensitivity but are associated with restoration of the original viral fitness in the presence of existing inhibitors (8, 24). The PR genes of the non-subtype B isolates found in drug-naïve patients have amino acid signatures different from those found in their subtype B counterparts. The high prevalence of the L10I, K20R, M36I, and L89M mutations in non-subtype B strains is largely known (18, 29). Likewise, the L63P and V77I substitutions are more frequent in subtype B isolates (18). Some of these substitutions are reported to be implicated in drug resistance in subtype B strains. Furthermore, almost all studies of HIV-1 drug susceptibility have been performed in developed countries, where subtype B still dominates the epidemic. Similarly, the resistance mutations selected during antiretroviral treatment were mostly studied with subtype B-infected patients.

The biochemical and phenotypic roles of the natural polymorphisms found in the PRs of non-subtype B strains are poorly understood. Velazquez-Campoy and colleagues (33) observed a 2.7-fold increased fitness of the clade C PR compared to that of the clade B PR. Whether this PR kinetics parameter influences the efficiency of virus replication still needs to be addressed. Given the increasing genetic heterogeneity of HIV-1 isolates worldwide and the intention to expand the use of PIs and other antiretroviral drugs to developing countries, it is important to characterize the PI susceptibility profiles of the subtype C isolates occurring among drug-naïve populations. It is important to check for the specific characteristics of these isolates to guide better, longer-lasting, and
efficient drug regimens. This work describes the results of a phenotypic study of HIV-1 subtype C PR genetically reconstructed from a prototypic clade B PR backbone (infectious clone pNL4-3 PR) into which the molecular signatures of clade C were introduced. These polymorphisms of clade C PRs are commonly found in isolates from Brazilian and South African drug-naïve individuals. Also, some clinical isolates of both clades from Brazil and South Africa were phenotypically tested.

MATERIALS AND METHODS

Clones and clinical samples. The PR gene from infectious clone pNL4-3 (1), a subtype B strain, was used as the prototypic clade B PR backbone for most of the site-direct mutagenesis studies. Plasma was obtained from 11 HIV-1-positive antiretroviral drug-naïve individuals, as confirmed by serology, at different Brazilian and South African AIDS clinics and voluntary counseling and testing centers. These samples were generated from a study previously approved by a Brazilian institutional review board (project 526-CONEP).

RNA isolation, PCR amplification, and sequencing. Viral RNA was isolated from plasma by using QIAamp Viral RNA kit (QIAGEN, Hilden, Germany), as described previously (27). Following cDNA generation with random primers, nested PCR was conducted for amplification of individual PRs (whole region) by using outer primers RPV5’ (5’-GGGAAAGATGTTGCTTCCTGCAAGG-3’), and RVP3’ (5’-GCCAACATCTGGAATATGTAAATG-3’), and inner primers K1 (5’-CACAGGCAAACAGGCCACCA-3’) and MOP2 (5’-GGTCTCATCCATTCCTGGTTT-3’) with the PCR conditions described elsewhere (27). The PCR fragments were sequenced in both directions with an ABI 310 automated sequencer (Applied Biosystems, Foster City, Calif.) with the same primers used in the second round of the amplifications.

Sequence analysis and subtyping. The sequences generated were aligned and manually edited with the ClustalW program (30) by using the Genetic Data Environment package for data exchange (26). A reference set for HIV-1 subtyping analysis from the Los Alamos HIV sequence database (http://hiv-web.lanl.gov) was included in the alignment. Phylogenetic inferences were deduced by the neighbor-joining method with the F84 model of substitution in the PAUP package (version 4.0b2a) (28).

A subtype C consensus sequence was deduced by using all subtype C sequences deposited in the Los Alamos HIV sequence database; multiple sequences from Brazil (n = 22) (27), South Africa, Tanzania, and Zambia (n = 53), and eastern India (n = 5) (25) were included in the alignment to generate country-based consensus sequences by using the VESPA program (10).

Site-directed mutagenesis. In order to introduce the molecular signatures of the subtype C PR into the PR of the prototypic infectious clone pNL4-3, a PR fragment was generated by PCR with the outer primers described above and cloned into the pcRT4 TOPO vector with the TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.) according to the protocol of the manufacturer. This gene was then subjected to site-directed mutagenesis in order to modify the target codons by a PCR mutagenesis procedure described by the manufacturer (Quick Change kit; Stratagene, La Jolla, Calif.). Briefly, the procedure induces the polymerization of a new strand from the original target plasmid by use of a set of complementary oligonucleotides as primers for the reaction for each strand. Those primers anneal at the same position in the plasmid and introduce the desired point mutation (or mutations) by mismatching. An Pfu DNA polymerase (Invitrogen) is used to complete the strands. To optimize the efficiency of mutagenesis, the parental methylated strands are digested with BspI, an endonuclease specific for methylated DNA that allows the new mutagenized strands to reanneal before being transformed into competent bacterial cell (Escherichia coli DH5α). Table 1 shows the primers used for mutagenesis. Six differences were found between the subtype C and subtype B consensus sequences (115V, M36I, R41K, H69K, L89M, and I93L). However, some investigators have excluded the I93L substitution from the subtype C consensus sequence (33). These six substitutions were then inserted into a prototypic subtype B infectious clone, pNL4-3, by site-directed mutagenesis. In order to specifically study the role of the I93L substitution in the subtype C backbone, two different clones were generated: clone C6 included all six signatures found in the subtype C PR consensus sequence, and clone C5 lacked the I93L substitution (see the Fig. 1 legend for details).

RESULTS

In order to characterize the genetic profiles of the PRs from subtype C viruses, the consensus PR amino acid sequences of subtype C isolates from Brazil, India, and Africa were obtained by use of the subtype C sequence from the Los Alamos HIV sequence database and were aligned with the subtype B PR consensus sequence (Fig. 1). Six differences were found between the subtype C and the subtype B consensus sequences (115V, M36I, R41K, H69K, L89M, and I93L). However, some investigators have excluded the I93L substitution from the subtype C consensus sequence (33). These six substitutions were then inserted into a prototypic subtype B infectious clone, pNL4-3, by site-directed mutagenesis. In order to specifically study the role of the I93L substitution in the subtype C backbone, two different clones were generated: clone C6 included all six signatures found in the subtype C PR consensus sequence, and clone C5 lacked the I93L substitution (see the Fig. 1 legend for details).

The susceptibilities of these clones to all commercially available PIs were determined, and the results are depicted in Table 2. The IC50s of lopinavir was 2.9 times lower for subtype C clone
C6 than for reference strain HXB2/NL4-3-PR \((P < 0.001\) by the two-tailed \(t\) test). Interestingly, this effect was not observed when clone C5 was tested \((P / H11021 0.4\) by the two-tailed \(t\) test).

This hypersusceptibility was observed only when lopinavir was used in the phenotypic test.

In order to see if this hypersusceptibility phenotype observed in these two artificial clones could be found in HIV-positive drug-naive patients infected with subtype C isolates, the PCR products directly generated from the PR genes of five isolates from Brazil and six isolates from South Africa by RT-PCR were selected. After phylogenetic analysis, eight clones with a clear assignment to clade C were selected (see the Fig. 1 legend for details). Isolate Br-SC1812 belonged to subtype C and did not carry the I93L mutation, and isolate Br-RS2172, which clustered with clade B and which carried the I93L substitution, was not lower than that for reference strain HXB2/NL4-3-PR.

All the other clade C isolates showed clear hypersusceptibility to lopinavir, with the IC50s for those isolates being from 16.2 to 2.6 times lower than that for reference strain HXB2/NL4-3-PR. The majority of the African isolates had higher levels of hypersusceptibility to lopinavir than their Brazilian counterparts. The results of phenotypic analysis of four selected South African isolates and reference strain HXB2/NL4-3-PR for susceptibility to each commercially available PI except lopinavir is depicted in Table 3. Hypersensitivity was observed only when lopinavir was tested.

**DISCUSSION**

Hypersusceptibility to antiretroviral compounds is found in several virus isolates from treated and untreated individuals. This feature is biologically defined by a lower IC50 of a certain drug compared to the IC50 for a reference strain (usually less

<table>
<thead>
<tr>
<th>Isolate</th>
<th>APV</th>
<th>SQV</th>
<th>IDV</th>
<th>RTV</th>
<th>NFV</th>
<th>LPV</th>
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<tr>
<td>pNL43</td>
<td>72.8 ± 6.2 (1)</td>
<td>3.4 ± 0.45 (1)</td>
<td>7.3 ± 0.45 (1)</td>
<td>44.0 ± 3.5 (1)</td>
<td>48.3 ± 5.8 (1)</td>
<td>27.13 ± 6.9 (1)</td>
</tr>
<tr>
<td>Clone C5</td>
<td>68.5 ± 5.16 (0.94)</td>
<td>2.9 ± 0.4 (0.85)</td>
<td>6.6 ± 0.74 (0.9)</td>
<td>46.2 ± 3.4 (1)</td>
<td>39.2 ± 2.9 (0.81)</td>
<td>19.0 ± 5.2 (0.69)</td>
</tr>
<tr>
<td>Clone C6</td>
<td>70.7 ± 4.3 (0.97)</td>
<td>2.6 ± 0.37 (0.75)</td>
<td>6.4 ± 0.37 (0.87)</td>
<td>42.0 ± 3.5 (0.95)</td>
<td>32.2 ± 4.2 (0.66)</td>
<td>9.4 ± 0.52 (0.34)</td>
</tr>
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</table>

*The values represent the means ± standard deviations of six independent experiments. The values in parentheses are the fold resistance compared to that of strain HXB2/NL4-3-PR abbreviations: APV, amprenavir; SQV, saquinavir; IDV, indinavir; RTV, ritonavir; NFV, nelfinavir; LPV, lopinavir.*
The IC₅₀s for these two clones were compared to those for strain HXB2/NL4-3-PR.

The same phenotypic behavior was observed when clinical isolates were phenotyped by a similar methodology. In this case, the IC₅₀s for all isolates belonging to subtype C and carrying the I93L mutation, regardless of the country from which they were isolated, ranged from 1.6 to 2.6 times lower than that for the reference strain (P < 0.0001 by the two-tailed t test). However, when subtype B or C isolates missing this specific substitution were analyzed, we observed IC₅₀s that were from 1.2 to 0.7 times lower than that for reference strain HXB2/NL4-3-PR (P < 0.77 by the two-tailed t test). Interestingly, it was not possible to observe the same phenotypic effect when I93L was naturally present in subtype B (isolate Br-RS2172). This hypersensitivity was notable only when lopinavir was tested with our clinical isolates and clone C6. These findings provide strong evidence for the role of the I93L mutation in the hypersusceptibility to lopinavir in the presence of a subtype C PR backbone. The molecular interactions between the specific substitutions of PR in the different clades may account for the different behaviors of subtype variants carrying the same I93L substitution. Harrigan et al. (6) have used a similar phenotyping technique to test several isolates obtained from antiretroviral drug-naïve individuals, including 358 South African patients. They have not found any significant differences in the IC₅₀ for subtype B and subtype C isolates with any drug tested. Other investigators have not included lopinavir among the antiretroviral compounds tested in phenotypic assays (35). However, in consonance with our results, those investigators could not show any differences in the IC₅₀ for subtype B and subtype C strains when the other PIs were tested. In another study, Velazquez-Campoy et al. (33) expressed HIV-1 protease in E. coli and purified recombinant PR enzymes from subtype B and subtype C isolates to determine their kinetic parameters by in vitro enzymatic activity assays with fluorogenic peptides. They demonstrated that the Kᵢ for all PIs tested (amprenavir, ritonavir, saquinavir, indinavir, and nelfinavir) were similar. However, again, lopinavir was not included in that analysis, and they did not include the I93L substitution in the subtype C recombinant PR enzyme. They also demonstrated that the vitality (kₑᵤ/kᵥₘₐₓ) of the PRs from subtype C isolates was 2.5 times higher than that of the PRs from subtype B isolates.

HIV infection and AIDS is the leading cause of death in sub-Saharan Africa and the fourth leading cause of death worldwide. A dramatic drop in the mortality rate was observed in AIDS patients in developed countries after the implementation of highly active antiretroviral therapy. However, few people from countries with limited resources have access to antiretroviral drugs. In fact, the World Health Organization conservatively estimated that in 2002, some 6 million people in resource-limited settings are in need of antiretroviral therapy (http://www.unaids.org). The introduction of innovative and affordable antiretroviral combinations in these countries is urgently needed. As subtype C strains are responsible for the majority of new infections in the HIV infection and AIDS epidemic, it is critical that the first drug treatment regimen be potent and tailored to yield long-lasting results without therapeutic failures. The findings reported here indicate that subtype C isolates are hypersusceptible to lopinavir in vitro. We do
not know if this in vitro phenotypic hypersensitivity to this PI would result in different clinical outcomes. It is known that the combination of lopinavir and ritonavir found in Kaletra has a high inhibitory quotient, but this hypersensitivity may not influence clinical outcomes. However, these findings must be further tested in controlled clinical trials comparing the virological responses of patients infected with subtype B HIV isolates and those infected with subtype C HIV isolates receiving lopinavir.

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