BIOLOGICAL CHARACTERIZATION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 SUBTYPE C PROTEASE CARRYING INDIANAVIR DRUG-RESISTANCE MUTATIONS

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Human immunodeficiency virus type 1 subtype C isolates belong to one of the most prevalent strains circulating worldwide and are responsible for the majority of new infections in the sub-Saharan region and other highly populated areas of the globe. In this work, the impact of drug-resistance mutations in the protease gene of subtype C viruses was analysed and compared with that of subtype B counterparts. A series of recombinant subtype C and B viruses was constructed carrying indinavir (IDV)-resistance mutations (M46V, I54V, V82A and L90M) and their susceptibility to six FDA-approved protease inhibitor compounds (amprenavir, indinavir, lopinavir, ritonavir, saquinavir and nelfinavir) was determined. A different impact of these mutations was found when nelfinavir and lopinavir were tested. The IDV drug-resistance mutations in the subtype C protease backbone were retained for a long period in culture without selective pressure when compared with those in subtype B counterparts in washout experiments.

INTRODUCTION

The activity of the human immunodeficiency virus type 1 (HIV-1) protease (PR) is of paramount importance in the maturation of the viral particles after virus budding (Lillehoj et al., 1988). A series of synthetic drugs has been developed to inhibit HIV-1 PR. Seven different PR inhibitors (PIs) are FDA-approved and available for clinical use: saquinavir (SQV), ritonavir (RTV), indinavir (IDV), nelfinavir (NFV), amprenavir (APV), lopinavir (LPV) and, more recently, atazanavir (ATV) (Carpenter et al., 1996; Hammer et al., 1997; Gulick et al., 1998; Ruiz et al., 1998; Piliero, 2002; Haas et al., 2003; Sanne et al., 2003). Despite the great biological and clinical potency of highly active antiretroviral therapy (HAART), including at least one PI, resistant strains can emerge from the viral population circulating in the patients, causing therapy failure (Condra et al., 1995; Iversen et al., 1996; Schmit et al., 1996; Hirsch et al., 2000). These resistant strains harbour PR and reverse transcriptase (RT) enzymes with amino acid substitutions that confer resistance to PI and RT inhibitors (RTIs), respectively (Condra et al., 1996; Molla et al., 1996; Boden & Markowitz, 1998; Patick et al., 1998; Shafer et al., 1999).

IDV is one of the first potent and selective PIs of HIV-1 and mutations at positions 82 and 46 are the most commonly found in patients failing IDV-containing regimens. In addition, positions 54, 90, 77, 71 and 20 are named secondary substitutions, due to their low impact on the IC50 for IDV (Condra et al., 1996). Normally, these drug-resistance mutations (DRMs) alter PR catalytic activity, decreasing viral fitness (Larder et al., 1995; Sharma & Crumpacker, 1997; Harrigan et al., 1998; Martinez-Picado et al., 1999). Consequently, when the drug treatment is interrupted, these mutations are selected against and disappear rapidly from the viral genome. This phenomenon, commonly referred to as mutation washout, can be observed in vivo during drug-holiday trials (Goudsmit et al., 1996, 1997; Miller et al., 2000).

The HIV-1 pandemic is characterized by an enormous genetic diversity. During the spread of HIV-1 among humans, group M viruses evolve rapidly and, presently, HIV-1 isolates can be segregated into nine pure subtypes and several commonly recognized circulating recombinant forms (Robertson et al., 1995a, b). HIV-1 subtype B is the predominant subtype in North America, western Europe and Australia. However, HIV-1 subtype B viruses account for only approximately 12% of the global HIV pandemic.
mutations in the subtype C PR background by comparing In this work, we have shown the impact of the IDV-related algorithms for drug-resistance genotyping were established based on both phenotyping assays and clinical correlations through therapeutic failure and viral strains carrying mutations (Hirsch et al., 2000; Schmidt et al., 2000, 2002; Kantor et al., 2001; Kijak et al., 2003). The algorithms are nevertheless based on observations made for subtype B strains of HIV-1 (Kantar & Katzenstein, 2003) and there is a lack of information for subtype C and other non-B clades (Pieniazek et al., 2000; Jülg & Goebel, 2005). The usefulness of these algorithms for isolates of non-B subtype C needs to be further validated. Substitutions found frequently in PRs of non-B isolates, so-called molecular signatures, may also interfere with the PI-resistance phenotype of other mutations that have been well characterized for subtype B isolates (Loemba et al., 2002; Calazans et al., 2005). Moreover, the genetic barrier in non-B subtypes can be smaller or higher than that in their subtype B counterparts (Weber et al., 2003; Dumans et al., 2004), as well the impact on viral fitness provoked by these IDV DRMs (Gonzalez et al., 2004).

In this work, we have shown the impact of the IDV-related mutations in the subtype C PR background by comparing their phenotypic and biological characteristics, such as IC50, viral replicative capacity (RC) and mutation washout, with those of prototypic subtype B viruses.

**METHODS**

**Molecular clones.** The PR gene from infectious clone pNL4-3 (Maschera et al., 1995) was used as the prototypic subtype B strain and pNL43-C6 was used as the subtype C prototypic clone. The subtype C clone (C6) used in this work is a modified pNL4-3 infectious clone in which the six amino acid differences found between the subtype C and B consensus sequences (I15V, M36I, R41K, H69K, L89M and 193L) were introduced by site-directed mutagenesis (Gonzalez et al., 2003).

**Site-directed mutagenesis.** In order to introduce the DRMs into the PR of subtype B and C clones, the prototypic infectious clones pNL4-3 (subtype B) and C6 (subtype C) were used as PCR targets and subjected to site-directed mutagenesis in order to introduce IDV DRMs (M46I, I54V, V82A and L90M) by using a PCR-mutagenesis procedure as described by the manufacturer (QuikChange kit; Stratagene). Clones were confirmed by DNA sequencing of plasmids extracted from the bacterial colonies grown after transformation, using a standard colony-PCR procedure. PCR fragments were sequenced in both directions in an ABI 310 automated sequencer (Applied Biosystems) with the same primers as were used in the amplifications to confirm the presence of the mutations (for primer sequences utilized in this mutagenesis, see the supplementary material in JGV Online).

**HIV-1 PR phenotyping.** Recombinant virus assay technology was used to measure the IC50 of wild-type and mutant PR using FDA-approved PI compounds (Gonzalez et al., 2003). Briefly, a 481 bp fragment containing the entire HIV-1 PR-coding sequence was PCR-amplified by using wild-type subtype B and C clones, as well as the in vitro mutant ones. These fragments were then co-transfected into CD4+ T lymphocytes (MT4) with the pGEM-T3/HXB2/APR BstEII-linearized plasmid, carrying the defective (APR) HIV HXB2 genomic cDNA (Mascher et al., 1995). A chimeric virus containing the HXB2/pNL4-3 PR was also produced as a substitute B control virus to obtain the reference IC50 values in the phenotyping assay. The susceptibility of chimeric viruses to all of the FDA-approved PI compounds (APV, IDV, LPV, RTV, SQV and NFV) was determined in three independent assays in MT4 cells by using a MTT-based cell-viability assay as described previously (Hertogs et al., 1998; García-Lerma et al., 2001). All statistical treatments to calculate the IC50 of the clones and isolates were performed by using Analyse-It v. 1.62 for Microsoft Excel and SigmaPlot software.

**RC from recombinant viruses in MT4 cells.** Recombinant viruses generated in the recombinant assay had their TCID50 measured in MT4 cells and 600 TCID50 was used to infect 1 × 10^6 MT4 cells (m.o.i. = 0.001). After 2 h incubation, the cells were washed twice in PBS and resuspended in complete medium (RPMI 1640, 10% fetal bovine serum, 1% penicillin/streptomycin and 1% sodium bicarbonate). The cell-culture supernatant was collected every day for 12 consecutive days. The amount of virus produced in the cell supernatants was evaluated through p24 antigen content by using a p24 Vironostika kit (BioMérieux). For the estimation of RC, the time taken (in days) to reach a level of 1000 ng p24 antigen ml^-1 in culture supernatants (replication constant K) was calculated using Hill’s three-parameter non-linear regression modelling (SigmaPlot, version 8.02). The relative RC of the primary isolates was determined from ratios of resistant strain K (Kres) to wild-type strain K (Kwt) multiplied by 100 and was expressed as a percentage of the Kres. For the evaluation of Kres, each drug-sensitive clone from either subtype C or B was used as reference for their respective mutated clones. The Kres and Kres used in the determination of the relative RC of HIV isolates were the mean K values of the duplicate experiments of each virus.

**Mutation-washout experiments.** In order to assess the genetic stability of the IDV DRMs in the absence of selective pressure, viral supernatants from subtype B and C clones carrying all mutations (M46I + I54V + V82A + L90M) were used to infect 1 × 10^6 MT4 cells in exponential growth at an m.o.i. of 0.001. Infected MT4 cells were cultivated in RPMI medium without IDV. After 4 days, 200 µl viral supernatant was used to infect 1 × 10^6 fresh MT4 cells. Twelve passages were done and aliquots were collected at each passage for genetic characterization. The relative number of each IDV DRM at each virus time point in the culture was evaluated through RT-PCR followed by cycle sequencing using a BigDye Terminator kit (Applied Biosystems) as described by García-Lerma et al. (2001). This methodology is able to detect at least 10% of a mutant sequence in a mixture.

**RESULTS**

In order to better explore biological characteristics of IDV DRMs in these two HIV-1 subtypes, PR-coding sequences from pNL4-3 and C6 clones were cloned into a pCR TA vector (Invitrogen). Both PR clones were then mutated
Characterization of mutations in subtype C HIV-1 PR

The recovered viruses from subtype B transfections did not accumulate any secondary mutations in PR. In contrast to that, the subtype C clone carrying all four IDV DRMs accumulated a new mutation solely at position 20 (K20R) of PR immediately after transfection that was maintained throughout the passage experiments. This secondary mutation has appeared in three independent experiments (data not shown). The Gag cleavage sites (p2/NC and p1/p6) were also sequenced and we could not find any secondary mutations in viral stocks from transfections.

The RC of the subtype B and C recombinant viruses was further evaluated in MT4 cells. These results are shown in Fig. 1. We could observe that the RCs of subtype B and C clones carrying comparable IDV DRM patterns were similar, with no significant differences. As expected, subtype B and C clones carrying four and three IDV DRMs showed a lower RC when compared with their wild-type counterparts (Fig. 1).

To assess changes in phenotypic profiles (IC50) for IDV and other FDA-approved PIs in subtype B and C clones generated previously, a phenotypic test was performed. Table 1 shows the fold resistance of all subtype B and C clones generated, compared with the IC50 of the subtype B prototypic clone. The level of resistance to IDV was high in the clones carrying combined-mutation patterns when compared with single-mutation counterparts, regardless of subtype. We could also observe a noticeable cross-resistance to

Table 1. Phenotypic analysis of subtype B and C clone and reference HXB2/ NL4-3 PRs to PIs

<table>
<thead>
<tr>
<th>Clone</th>
<th>APV (±sd)*</th>
<th>SQV</th>
<th>IDV</th>
<th>RTV</th>
<th>NFV</th>
<th>LPV</th>
</tr>
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<tbody>
<tr>
<td>HXB2/NL4-3 IC50 (nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subtype B</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>M46I</td>
<td>1.00±0.06†</td>
<td>0.60±0.02</td>
<td>1.83±0.15</td>
<td>0.60±0.06</td>
<td>0.90±0.05</td>
<td>0.90±0.12</td>
</tr>
<tr>
<td>I54V</td>
<td>1.15±0.08</td>
<td>1.25±0.00</td>
<td>1.40±0.10</td>
<td>2.42±0.19</td>
<td>1.32±0.07</td>
<td>1.90±0.27</td>
</tr>
<tr>
<td>V82A</td>
<td>0.94±0.05</td>
<td>0.41±0.03</td>
<td>4.40±0.12‡</td>
<td>2.51±0.09</td>
<td>0.80±0.03</td>
<td>2.20±0.16</td>
</tr>
<tr>
<td>L90M</td>
<td>1.28±0.13</td>
<td>3.75±0.52</td>
<td>2.91±0.18</td>
<td>2.72±0.15</td>
<td>3.20±0.13</td>
<td>0.86±0.09</td>
</tr>
<tr>
<td>I54V + V82A</td>
<td>0.65±0.04</td>
<td>1.29±0.19</td>
<td>0.90±0.19</td>
<td>10.00±0.66</td>
<td>1.49±0.05</td>
<td>3.75±0.38</td>
</tr>
<tr>
<td>M46I + I54V + V82A</td>
<td>0.66±0.04</td>
<td>2.47±0.25</td>
<td>45.80±0.87</td>
<td>121.00±4.82</td>
<td>3.34±0.31</td>
<td>43.00±3.25</td>
</tr>
<tr>
<td>M46I + I54V + V82A + L90M</td>
<td>1.00±0.07</td>
<td>11.70±0.67</td>
<td>145.00±4.15</td>
<td>240.00±6.95</td>
<td>7.13±0.35</td>
<td>12.80±0.62</td>
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<tr>
<td>Subtype C</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>M46I</td>
<td>0.49±0.03</td>
<td>0.60±0.02</td>
<td>3.60±0.18</td>
<td>0.60±0.01</td>
<td>0.46±0.01</td>
<td>0.25±0.01§</td>
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<tr>
<td>I54V</td>
<td>0.30±0.01</td>
<td>0.70±0.29</td>
<td>1.53±0.09</td>
<td>2.87±0.13</td>
<td>0.90±0.02</td>
<td>1.00±0.08</td>
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<tr>
<td>V82A</td>
<td>0.40±0.03</td>
<td>0.99±0.02</td>
<td>3.50±0.17</td>
<td>0.72±0.03</td>
<td>0.60±0.07</td>
<td>0.50±0.11</td>
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<tr>
<td>L90M</td>
<td>1.60±0.11</td>
<td>4.27±0.54</td>
<td>4.25±0.34</td>
<td>3.10±0.18</td>
<td>4.60±0.18</td>
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<tr>
<td>I54V + V82A</td>
<td>0.20±0.02</td>
<td>0.45±0.22</td>
<td>3.00±0.02</td>
<td>17.57±0.67</td>
<td>1.40±0.10</td>
<td>3.06±0.14</td>
</tr>
<tr>
<td>M46I + I54V + V82A</td>
<td>0.60±0.04</td>
<td>4.79±0.48</td>
<td>89.90±5.37</td>
<td>197.00±6.69</td>
<td>13.00±0.63</td>
<td>98.00±3.03</td>
</tr>
<tr>
<td>M46I + I54V + V82A + L90M</td>
<td>1.10±0.06</td>
<td>14.30±0.87</td>
<td>182.00±2.58</td>
<td>303.00±9.73</td>
<td>34.00±1.98</td>
<td>189.00±8.06</td>
</tr>
</tbody>
</table>

*Abbreviations: APV, amprenavir; SQV, saquinavir; IDV, indinavir; RTV, ritonavir; NFV, nelfinavir; LPV, lopinavir. sd, Standard deviation of six independent experiments.
†Values represent fold resistance compared with HXB2/NL4-3 PR.
‡Bold type indicates fold resistances significantly different from the HXB2/NL4-3 PR reference clone (t-test with P<0.05) and higher than the biological cut-off for this methodology (Harrigan et al., 2001).
§Clones showing hypersusceptibility are depicted in italics.
other PIs, such as LPV, RTV, NFV and SQV, in the multidrug-resistant clones from both subtypes evaluated. The only exception to these findings was APV, which continued to inhibit all mutants generated from both clades. In order to better study the correlation of each mutation pattern with the level of resistance encoded for each drug, the variation of fold resistance compared with wild-type IC50 was plotted for each clone from both subtypes. We observed a comparable impact on the level of IC50 for subtype B and C clones when IDV, APV, SQV and RTV were analysed (Table 1). In contrast to that, subtype C clones showed a more notable impact in the fold resistance for LPV and NFV through the accumulation of IDV DRMs when compared with their subtype B counterparts, particularly in the isolates carrying multiple DRMs (Fig. 2a, b).

In order to explore the capacity of each subtype to retain IDV DRMs with no PI selective pressure, we have set two time-course cultures infected with subtype B and C isolates carrying four IDV DRMs (M46I+I54V+V82A+L90M). Viruses obtained from supernatants from different days of culture were isolated and sequenced. The PI DRM-clearance kinetics throughout the time in culture are depicted in Fig. 3. There are distinct kinetics of IDV DRM washout when both clades were compared. The subtype B virus lost three out of four IDV DRMs during the 65 days in continuous culture. The first mutation to be lost completely was V82A after 25 days in culture, followed by M46I and I54V, which were lost after 65 days in culture. In contrast, mutation L90M remained after 65 days in culture. The four IDV DRMs in subtype C viruses were not lost at day 65 in culture. The only exception was V82A, which started to revert and, at the end of the experiment, still accounted for 80% of the isolates found in the culture supernatant. All mutation reversions used the original codon usage in pNL4-3.

**DISCUSSION**

Recently, there has been an increase in the number of studies showing a different accumulation of DRMs in non-B subtypes compared with subtype B isolates from patients under HAART (Kantor et al., 2005). Grossman et al. (2004), analysing subtype C-infected individuals from Israel failing IDV-containing regimens, found a lower prevalence of I54V. Additionally, patients infected with subtype C isolates using NFV select for mutation L90M in preference to D30N, a mutation normally selected for in subtype B (Cane et al., 2001; Gonzalez et al., 2004). In order to gain more knowledge about PI DRMs in subtype B and C isolates, we have explored the phenotypic impact of IDV-specific mutations in these two HIV-1 variants.

It is clear from our results that IDV DRM impact on the RC of the recombinant clones was independent of subtype, and the clones most affected were those with multiple mutations. Our results in subtype B are in accordance with data presented previously (Picchio et al., 2000) and we have extended these findings to subtype C. However, we could observe a selection of a secondary mutation (K20R) in virus C M46I+I54V+V82A+L90M during the recombination assay. Apparently, in our experiments, this mutation was probably selected to restore the fitness of a subtype C multidrug-resistant clone, and this might be a favourable genetic route in vitro, as we could observe the same mutation in three different recombination assays performed with this clone. Interestingly, clone B M46I+I54V+V82A+L90M did not accumulate secondary mutations in the PR gene. The selection of K20R could also potentially contribute to elevating the RC of clone C M46I+I54V+V82A+L90M and, in fact, this variant has a lower RC than its clone B counterpart.

Resistance to six FDA-approved PIs (SQV, RTV, IDV, APV, LPV and NFV) was studied through phenotyping assays and showed clearly that recombinant viruses carrying single IDV DRMs have very little impact on the level of resistance to all

![Fig. 2. Relative impact of IDV DRMs alone and in combination profiles on phenotypic cross-resistance to NFV (a) and LPV (b). Results are shown as fold resistance compared with wild-type virus pNL4-3. Shaded bars, subtype B; filled bars, subtype C.](image_url)
PIs studied. The only exception was observed for mutation V82A in both subtypes and mutations M46I and L90M for subtype C clones. Mutation V82 encoded resistance to IDV in clones of both subtypes, and mutations M46I and L90M conferred a significant increase in IC_{50} for IDV solely in subtype C PR.

We could notice a hypersusceptibility to APV (P<0.001, t-test) compared with the NL4-3 genome when mutations I54V and V82A were included alone or in combination in subtype C clones. This phenotype has been reported previously in subtype B viruses carrying the NFV mutation N88S (Ziermann et al., 2000). There was no in vitro cross-resistance between IDV and APV in our experiments, regardless of subtype. This points to possible usefulness of APV to treat individuals failing IDV-containing regimens.

There is a noticeable pattern of cross-resistance between viruses carrying multiple IDV DRMs (three or four) with SQV, RTV, NFV and LPV, regardless of the subtype analysed. However, we could identify a higher impact of these mutations on the cross-resistance to NFV and LPV in subtype C clones (see Fig. 3 for details). This fact points to a more dramatic effect of these mutations in the subtype C PR than in its subtype B counterpart. In the case of LPV, it has been shown previously that subtype C isolates from drug-naive individuals naturally carry a hypersusceptibility to this PI (Gonzalez et al., 2003) and the addition of three or four IDV DRMs has led to a highly resistant virus compared with its subtype B counterpart. The same phenomenon was observed with NFV. This means that the IDV DRM impact was much more noticeable in subtype C than in subtype B viruses. Differences in behaviours between the clade C and clade B viruses observed here in vitro may have a clinical relevance, suggesting a lower genetic barrier in subtype C viruses than in subtype B isolates. In fact, both subtypes could surpass the IC_{50} values for biological cut-off for both drugs (Harrigan et al., 2001), with fewer mutations accumulated. However, the use of RTV-boosted regimens can elevate the PI concentration and surpass the IC_{50} of these mutant viruses.

The major difference between the biological properties of subtype B and C viruses was the capability of these viruses to retain the IDV DRMs in culture without IDV selective pressure. Subtype B virus carrying four IDV DRMs lost mutations A82, I46 and V54, in this order, after 65 days in culture. Mutation M90 could be found in subtype B PR throughout all time in culture, showing the low impact on virus fitness promoted by this substitution. In fact, L90M is one of the most commonly found PI DRMs in recently infected individuals in drug-resistance surveys done in developed countries. In contrast to that, subtype C virus could maintain all of the mutations studied here, although our findings cannot be generalized for all subtype C isolates around the globe. As we have used mutated subtype B and C recombinant clones, our study points to an interesting biological difference between these two HIV-1 variants. These findings need confirmation in longitudinal clinical studies in areas where subtype C is prevalent.

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