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# Tissue culture drug resistance analysis of a novel HIV-1 protease inhibitor termed PL-100 in non-B HIV-1 subtypes☆

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ABSTRACT

PL-100 is a novel HIV-1 protease inhibitor (PI) that maintains activity against viruses that are resistant to other PIs. To further characterize this compound, we used it to select for drug resistance in tissue culture, using two non-B HIV-1 subtypes, viz. subtype C and a CRF01_AE recombinant virus. PL-100 selected for both minor and major PI resistance mutations along either of two distinct pathways. One of these involved the V82A and L90M resistance mutations while the other involved a mutation at position T80I, with other mutations being observed at positions M46I/L, I54M, K55R, L76F, P81S and I85V. The resistance patterns in both subtype C and CRF01_AE were similar and an accumulation of at least three mutations in the flap and active sites were required in each case for high-level resistance to occur, demonstrating that PL-100 has a high genetic barrier against the development of drug resistance.



## 1. Introduction

The protease (PR) enzyme of human immunodeficiency virus type 1 (HIV-1) is an excellent therapeutic target since its inhibition prevents the proteolytic processing of the Gag and Gag-Pol polyproteins (Ventoso et al., 2005). Protease inhibitors (PIs) are routinely prescribed for both treatment-naïve and experienced patients and have had a profound impact on HIV-associated disease progression, transmission, and morbidity and mortality (Palella et al., 1998).

Drug resistance is a frequent complication in patients who fail therapy (Descamps et al., 2009; Luis Jimenez et al., 2005; Turner et al., 2005) and many studies have shown that drug-resistant viruses can be sexually transmitted (Aghokeng et al., 2009; Brenner et al., 2008). The problem of drug resistance is compounded by the worldwide dissemination of multiple different subtypes of HIV-1 and the fact that natural polymorphisms in both HIV-1 and HIV-2 can affect the emergence of drug resistance to currently approved drugs (Kantor and Katzenstein, 2003; Ntemgwa et al., 2007; Stranix et al., 2004). Furthermore, polymorphisms within the PR enzyme may not themselves be responsible for resistance but can contribute to the development of high-level resistance if other mutations are present (Bessong, 2008; Kantor and Katzenstein, 2003; Liu et al., 2007; Velazquez-Campoy et al., 2003; Vergne et al., 2000). It is therefore important to understand antiviral activity and drug resistance profiles in viruses of different subtypes.

Recently, a novel PI termed PL-100 was shown to be active against both wild-type and drug-resistant forms of HIV-1 of subtype B origin (Dandache et al., 2007). PL-100 is a lysine-based sulphonamide that was designed on the basis of subtype B PR structural data (Stranix et al., 2004). Although PL-100 demonstrates a high genetic barrier for the development of drug resistance to subtype B viruses (Dandache et al., 2008), little is known about the role that individual polymorphisms in some non-B subtypes might play in regard to the development of drug resistance (Kantor and Katzenstein, 2003; Liu et al., 2007; Ntemgwa et al., 2007). Here we describe *in vitro* development of resistance and the antiviral properties of PL-100 against a panel of wild-type and drug-resistant non-B viruses. As examples of non-B subtypes we used subtype C and the circulating recombinant form (CRF) CRF01_AE that represent the most prevalent subtype and CRF in circulation at this time (Hemelaar et al., 2006).

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## 2. Materials and methods

### 2.1. Virus isolates, cells and plasmids

Three HIV-1 CRF01_AE clinical isolates (NI1052, M02138 and NP1525) were obtained from the AIDS Research and Reference Reagent Program of the National Institutes of Health (NIH), Bethesda, MD. Five HIV-1 subtype C (7208, 8032, 8947, 7906 and HB-1) clinical isolates were obtained with informed consent from drug-naive individuals at our clinics in Montreal, Canada. The CRF01_AE isolates have been previously characterized as X4-tropic viruses (Brown et al., 2005) while the subtype C isolates were non-syncytium inducing and were considered likely to be R5 viruses. MT-2 cells were obtained from the NIH AIDS Research and Reference Reagent Program. Cord blood mononuclear cells (CBMCs) were obtained through the Department of Obstetrics, Jewish General Hospital, Montreal, Canada. The AG plasmid (p97GH-AG2) was kindly provided by Dr. Masashi Tatsumi, National Institute of Infectious Diseases, Tokyo, Japan.

### 2.2. Drugs

Nelfinavir (NFV), amprenavir (APV), lopinavir (LPV), atazanavir (ATV), PL-100, and saquinavir (SQV) were gifts from Pfizer, Inc., (San Diego, CA), GlaxoSmithKline (Research Triangle Park, NC), Abbott Laboratories (North Chicago, IL), Bristol-Myers Squibb (BMS), Inc., Ambrilia Biopharma Inc., (Montreal, Quebec, CA) and Roche, Inc., respectively. Efavirenz (EFV), a non-nucleoside reverse transcriptase inhibitor, was used as a control drug and was obtained courtesy of BMS Inc.

### 2.3. In vitro selection of resistance mutations in CBMCs and MT-2 cells

PHA-stimulated CBMCs or MT-2 cells were infected with viruses (multiplicity of infection of 0.1 for CBMCs and 0.01 for MT-2 cells) for 2 h, incubated at 37 °C, and subsequently washed with RPMI 1640 media (Invitrogen) supplemented with 10% fetal bovine serum and seeded into a 24-well plate at a density of $2.5 \times 10^5$ cells per well (Gao et al., 1993). Selection for resistance in CBMCs and MT-2 was performed using increasing concentrations of drugs (PL-100 and APV) at a starting concentration that was below the 50% effective concentration ($EC_{50}$) of the drugs (Oliveira et al., 2009). As controls, all viruses were simultaneously passaged without drugs. With CBMCs, drug concentrations were increased at subsequent passages based on RT levels in culture fluids (Loemba et al., 2002; Petrella et al., 2004). With MT-2 cells sub-culturing was performed every 3–4 days and the cells studied for cytopathic effect (Vingerhoets et al., 2005). Virus-containing culture fluids were harvested and kept at −80 °C for subsequent genotypic analysis at the same time that drug concentrations were increased.

### 2.4. Nucleic acid extraction, amplification and sequencing analysis

Viral RNA was extracted from culture supernatants using the Qiagen QIAamp viral extraction kit (Mississauga, Ontario, Canada). PCR amplification was performed using a previously published protocol (Virco BVBA, Mechelen, Belgium). The resulting PCR-amplified DNA fragments were purified using the QIAquick PCR purification kit and products were used as templates for nucleotide sequencing analysis. Genotyping was performed by sequencing a 325-bp fragment of HIV *pol* (position 2253–2578) spanning the entire protease (PR) using Virco primers (Virco BVBA Mechelen, Belgium) with a BigDye Terminator sequencing kit (Version 1.1; Applied Biosystems, Forter City, CA) and automated sequencer (ABI Prism 3130 genetic analyzer; Applied Biosystems). The sequence of each sample was compared to that of wild-type (wt) subtype B consensus virus and to data in the Stanford database.

### 2.5. Site-directed mutagenesis and virus production

The K55R, I85V and K55R/I85V mutations were introduced into the p97GH-AG2 plasmid by site-directed mutagenesis (SDM) using a QuikChange II XL site-directed mutagenesis kit (Stratagene, LaJolla, CA). For SDM of K55R, the forward primer 5′-GGG GGA ATT GGA GGT TTT ATC AGG GTA AGA CAG TAT GAC CAG-3′ and reverse primer 5′-CTG GTC ATA CTG TCT TAC CCT GAT AAA ACC TCC AAT TCC CCC-3′ were used while for I85V the forward primer 5′-GGA CCT ACA CCT GTC AAC ATA GTT GGA CGA AAT ATG-3′ and reverse primer 5′-CAT ATT TCG TCC AAC TAT GTT GAC AGG TGT AGG TCC-3′ were employed. Introduction of the various mutations into the plasmid was confirmed by sequencing and DNA ultimately transformed into DH5α cells (Invitrogen) for high-yield of plasmid.

HEK 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and transfected with 16 μg of plasmid using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's protocols. Two days after transfection, supernatants of the transfected cells were clarified by centrifugation at 1500 rpm for 5 min and stored in aliquots at −80 °C. Virus production was confirmed by RT assay.

### 2.6. Phenotypic susceptibility to PIs in CBMCs and MT-2 cells

Drug susceptibility was measured in cell-culture based phenotypic assays as previously described (Loemba et al., 2002; Salomon et al., 1994), to determine the extent to which PIs (NFV, SQV, APV, LPV, ATV and PL-100) blocked HIV replication *in vitro*. Efavirenz (EFV), a non-nucleoside inhibitor of reverse transcriptase, was used as a control. In brief, cells were infected for 2 h with either wt or drug selected variants, washed to remove unbound virus and plated in duplicates into 96-well plates containing drugs or not and incubated at 37 °C. For MT-2 cells, cytopathic effect was confirmed on day 4 or 5 and supernatants harvested for RT activity. For CBMCs, cells were fed with fresh media containing appropriate drug dilutions and RT assays were performed at day 7. $EC_{50}$ concentrations of PIs were determined from RT values. A preliminary cut-off value had previously been calculated for PL-100 (Dandache et al., 2008; Dandache et al., 2007). The manner in which preliminary cut-off values are calculated is through access to a limited number of clinical samples that have not been exposed to PL-100 but that contain mutations associated with resistance against other members of the PI family of drugs. The $EC_{50}$ values of these clinical isolates were measured in phenotypic assays in regard to PL-100 and mean fold changes ± 2 standard deviations in $EC_{50}$ in comparison with laboratory wild-type viruses were calculated. The mean fold-change +2 standard deviations corresponds to the preliminary biological cut-off value.

## 3. Results

### 3.1. Selection of resistance mutations to PL-100 and APV in MT-2 cells

Polymorphisms are defined as naturally occurring amino acid variations from the subtype B consensus sequence (HXB2) that is present in the Los Alamos HIV database (http://www.hiv.lanl.gov). Three CRF01_AE viruses (NI1052, M02138 and NP1525) were used for selection in MT-2 cells (Table 1). Polymorphisms at positions I13V, E35D, M36I, R41K, H69K and L89M were present in all three CRF01_AE isolates. In addition, K20R and R57K were also present in the M02138 and NI1052 viruses, respectively (Table 1). Some of

**Table 1**
Development of resistance mutations to PL-100 and APV in MT-2 cells.

| Drug | Passage | CRF01_AE | | |
|---|---|---|---|---|
| | | NI1052 | M02138 | NP1525 |
| | 0 | I13V, E35D, M36I, R41K, R57K, H69K, L89M | I13V, K20R, E35D, M36I, R41K, H69K, L89M | I13V, E35D, M36I, R41K, H69K, L89M |
| PL-100 | 15–17 | K43L, K45T, L63V | L10I, K55R, I85V, Q92H | K14R, P79H |
| | 24–30 | M46I, V82A | L10I | M46L, K70R |
| | 35–66 | L10I, M46I, L76F, V82A | L10I, K45I, I62V, V82A | M46L, K70R, Q92L |
| APV | 45–66 | L10F, L33F, M46L, I93V | ND[a] | L10F, I15V, Q18E, L19M, K20R, M46L, 150V, I62V, L63S, C67Y, L89I |

[a] ND, not determined.

these polymorphisms are known to contribute to drug resistance for certain PIs (Kantor and Katzenstein, 2003).

In MT-2 cells, mutations emerged through the V82A pathway in the CRF01_AE isolates. The novel mutation K45I was observed with one A/E isolate and this mutation persisted until the end of the selection experiments. The L76F mutation emerged in one A/E isolate between weeks 35 and 66. This mutation was accompanied by other mutations known to be associated with resistance to PIs (Table 1). None of the mutations selected by PL-100 by passage 16 or 17 with isolate M02138 persisted with the exception of L10I. Two additional major PI mutations, L33F and I50V, were selected by APV but not by PL-100.

### 3.2. Genotypic analysis and selection of resistance mutations to PL-100 in CBMCs

Five subtype C clinical isolates were used for selection in CBMCs with each containing 6–9 polymorphisms at baseline (Table 2). After selection, all viruses passaged without drugs (controls) were sequenced to confirm the absence of new mutations. The results show that two distinct resistance pathways seem to be associated with PL-100 in subtype C in tissue culture drug selection. One pathway consists of active site mutations at either positions 82 and 90 or at both positions, and an alternate pathway, unique to PL-100, utilized a novel active site mutation at position 80 (Table 2). The T80I mutation occurred with additional changes at active sites 85 and 89 (Table 2). Flap mutations, most often at position 46, accompanied both of these pathway options.

### 3.3. Incidence of mutations selected with PL-100 in CBMCs and MT-2 cells

For both subtypes, mutations at positions 46 and 82 were the most prevalent, both being observed in 6 of 8 cases (Fig. 1). This was followed by the combinations of M46L/I together with

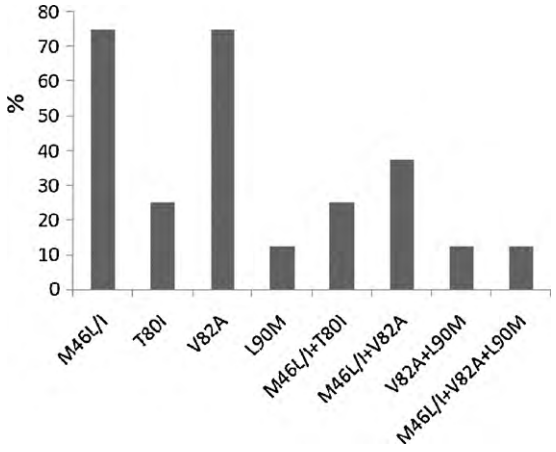


**Fig. 1.** Frequencies of mutations most commonly selected with PL-100. M46L/I and T82A were the most prevalent, being observed in 6 of 8 cases each, followed by the combinations M46L/I + V82A in 3 of 8 cases, T80I and the combination M46L/I + T80I in 2 of 8 cases and L90M, V82A + L90M and M46L/I + V82A + L90M in 1 of 8 cases each.

V82A in 3 of 8 cases. The T80I mutation or the combination M46L/I + T80I was present in 2 of 8 cases. The L90M or V82A + L90M or M46L/I + V82A + L90M was each found in one of 8 cases. In addition, the simultaneous presence of mutations at positions 46 and 82 was common in all subtypes. The novel T80I mutation was always found together with a change at position 46. With the exception of T80I, all of the other mutations described have frequently been observed in patients who have failed a variety of regimes containing PI drugs.

### 3.4. In vitro phenotypic susceptibility in MT-2 cells

We next evaluated the *in vitro* susceptibilities of selected viruses, site-directed mutants, and wt viruses by measuring $EC_{50}$s

**Table 2**
Baseline polymorphisms and resistance mutations associated with subtype C viruses in CBMCs.

| | Weeks | 7208 | 8032 | 8947 | 7906 | HB-1 |
|---|---|---|---|---|---|---|
| Baseline polymorphisms | 0 | T12S, I15V, L19I, E35D, M36I, R41K, L63P, H69K, I93L | I15V, L19T, M36I, N37N/K, R41K/N, L63P, H69K, K70K/R, I93L | L14R, I15V, L19I, M36I, R41K, H69K, I93L | I15V, L19I, M36L, R41K, H69K, L89M | T12S, L19I, M36I/L, R41K, I62I/V, L63P, H69KL89MI93L |
| PL-100 selected mutations | 31 | ND[a] | M46M/I, T74S, T80I, L89M | I64V, V82A | ND[a] | ND[a] |
| PL-100 selected mutations | 40 | M46I, V82A, L90M | M46I, T74S, T80I, V82I, I85I/V, L89M | I13I/V, I64V, V82A | K43N, M46L, T80I, I85V, L89I | L10R, M46L, V82A |

[a] ND, not determined.

**Table 3**
*In vitro* efficacy in MT-2 cells of various ARVs against CRF01_A/E viruses containing relevant PI resistance mutations.

| Viral isolate | Subtype | PI mutation | Mean $EC_{50}$ (nM) ± SD[a] and fold-change (FC) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | NFV | APV | LPV | ATV | PL-100 | EFV |
| Lower and upper clinical cut-offs[b] | | | 1.2–9.4 | 1.5–19.5 | 6.1–51.2 | 2.5–32.5 | 2.5[c] | 3.4 |
| NI1052 | A/E | wt | 16.5 ± 2 | 15.0 ± 3 | 10.1 ± 2 | 4.0 ± 0.2 | 9.0 ± 1.8 | 3.1 ± 0.3 |
| NI1052 | A/E | L10I, M46I, L76F, V82A | 35.5 ± 7 (2.1) | 55.0 ± 3.2 (3.7) | 82.0 ± 6 (8.1) | 15.0 ± 2 (3.7) | 262.0 ± 2 (29.1) | 6.2 ± 0.1 (2.0) |
| M02138 | A/E | wt | 18.5 ± 0.7 | 4.7 ± 0.67 | 2.95 ± 0.3 | 0.46 ± 0.03 | 9.03 ± 2 | 2.9 ± 0.7 |
| M02138 (P 17)[d] | A/E | L10I, K55R, I85V, Q92H | 36.0 ± 6 (1.94) | 32.7 ± 3 (6.9) | 5.5 ± 2 (1.86) | 3.9 ± 0.3 (8.5) | 30.1 ± 1 (3.3) | ND |
| M02138 | A/E | L10I, K45I, I62V, V82A | 81 ± 12 (4.5) | 120.0 ± 40 (25.5) | 95.5 ± 0.0.7 (32) | 19.0 ± 1.4 (41) | 289.0 ± 4 (32) | 2.5 ± 0.6 (0.86) |
| NP1525 | A/E | wt | 15.0 ± 4 | 10.2 ± 1.7 | 6.0 ± 2 | 3.0 ± 0.7 | 11.0 ± 0.7 | 2.4 ± 0.1 |
| NP1525 | A/E | M46L, K70R, Q92L | 19.0 ± 5 (1.3) | 49.4 ± 1 (4.8) | 11.0 ± 1.1 (1.8) | 4.0 ± 2 (1.3) | 16.3 ± 6 (1.45) | 2.8 ± 0.2 (1.2) |

The shaded area indicates the wild-types of each isolate to differentiate the EC50 from that of selected variants.
[a] The values represent the means of two independent experiments, each performed in duplicate. Drug susceptibility was expressed as fold-change in $EC_{50}$, determined by calculating the ratio of $EC_{50}$ values for mutated and wt virus (values in parentheses).
[b] Van Houtte et al. (2009) and Winters et al. (2009).
[c] Arbitrarily chosen lower cut-off value of PL-100 for this analysis pending availability of clinical data.
[d] M02138 isolate containing resistance mutations selected at passage 17.

of several PIs in a cell-based phenotypic assay. In each case, a wt isolate was used as a reference for the drug susceptibility assays and PL-100 resistant variants were compared with their respective wt isolates to calculate fold-change (FC) for resistance. A lower biological cut-off of 2.5 for PL-100 (preliminary cut-off value for this analysis pending clinical availability of the drug) was used. Lower and upper clinical cut-offs were established as 1.2–9.4 for NFV, 1.5–19.5 for APV, 6.1–51.2 for LPV, 2.5–32.5 for ATV 3.1–22.6 for SQV and 3.4 for EFV (Van Houtte et al., 2009; Winters et al., 2009). As shown in Table 3, all of the selected variants displayed cross-resistance to NFV, APV and ATV with the exception of the CRF01_AE isolate (NP1525) containing mutations M46L/K70R/Q92L that was moderately resistant to NFV and APV but retained susceptibility to ATV.

With one CRF01_AE isolate (M02138), the mutations L10I, K55R, I85V and Q92H were observed by passage 17. This isolate displayed fold changes in drug susceptibility of 8.5, 6.82 and 3.34 for ATV, APV and PL-100, respectively and was susceptible to LPV and NFV (Table 3). To further confirm the biological relevance of the K55R and I85V mutations, site-directed mutants were created in a CRF02_AG plasmid. A very modest reduction in NFV susceptibility (1.9-fold) was seen with I85V (data not shown). Neither of these mutations affected susceptibility to other PIs since the FC in each case was less than the relevant clinical or biological cut-off. No significant differences were observed when the panel of protease inhibitors was used against the combination of the K55R/I85V mutations (data not shown). In the case of clinical isolate NP1525, selection with PL-100 did not result in obvious drug resistance.

### 3.5. Phenotypic susceptibility in CBMCs

Despite the presence of resistance mutations, all viral isolates retained susceptibility to SQV. All selected viruses showed decreased susceptibility to PL-100. Low and moderate level cross-resistance was observed with NFV, APV, LPV and ATV with higher level resistance being displayed against both NFV and APV (Table 4). The mutational combination of M46I/T74S/T80I/V82/I85IV/L89M (isolate 8032) possessed a FC of 20.8 for PL-100 and a FC of 28.7 and 10.2 for APV and ATV, respectively. This isolate was susceptible to LPV. The other two isolates with the combinations M46I/V82A/L90M and K43N/M46I/T80I/I85V/L89I were resistant to all PIs tested except SQV.

## 4. Discussion

In this study, we show that resistance to PL-100 can emerge via mutations at positions 82 and 90 or alternatively via a T80I substitution that appears to be unique to this drug. We demonstrate the possible role of mutations L76F, K55R and I85V. Although viruses containing K55R, I85V, or K55R/I85V are susceptible to PL-100, Table 3 indicates that viruses containing the mutations selected at passage 17 (i.e. L10I, K55R, I85V, Q92H) showed a 3.3 decrease in susceptibility to PL-100. This might have resulted from combinations of mutations together with secondary PI mutations such as L10I. The K55R and I85V substitutions might be considered to be secondary PI mutations. Our data confirm that PL-100 possess a very high genetic barrier for development of drug resistance.

The most common mutations selected by PL-100 were at positions 46, 82, 90 and 80 in decreasing order of frequency. Although T80I appears to be novel, M46I/L, V82A and L90M have been described in patients failing SQV, NFV, APV, LPV and ATV (Doualla-Bell et al., 2006; Marcelin et al., 2004a,b; Mo et al., 2005; Santoro et al., 2009; Svedhem et al., 2005). While the T80I mutation seems to be important, resistance can also occur along a second pathway that is common to other PIs that includes the V82A and L90M mutations. These two pathways appear to be mutually exclusive in all cases. This study demonstrates the importance of using a wide

**Table 4**
*In vitro* efficacy in CBMCs of various ARVs against subtype C viruses containing relevant PI resistance mutations.

| Viral isolate | Subtype | PI mutation(s) | Mean $EC_{50}$ (nM) ± SD[a] and fold-change (FC) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | NFV | APV | LPV | ATV | PL-100 | SQV | EFV |
| 7208 | C | wt | 13.0 ± 7.0 | 12.6 ± 0.1 | 11.2 ± 3.7 | 2.4 ± 0.5 | 9.2 ± 0.5 | 40.8 ± 0.6 | 0.31 ± 0.16 |
| 7208 | C | M46I, V82A, L90M | 222.8 ± 20 (17.1) | 27.3 ± 23 (2.2) | 98.6 ± 15 (8.8) | 16.4 ± 12 (6.8) | 168.8 ± 21 (18.4) | 31.0 ± 3.0 (0.8) | 0.03 ± 0.01 (0.1) |
| 7906 | C | wt | 2.8 ± 0.1 | 5.1 ± 1.1 | 1.5 ± 1.0 | 1.5 ± 1.0 | 2.2 ± 0.5 | 15.0 ± 2.0 | 1.28 ± 0.4 |
| 7906 | C | K43N, M46L, T80I, I85V, L89I | 11.9 ± 2.5 (4.3) | 173 ± 15.8 (34.6) | 20.8 ± 7.0 (13.9) | 14.1 ± 10 (9.4) | 129.3 ± 54 (58.8) | 22.5 ± 1.0 (0.9) | 1.1 ± 0.1 |
| 8032 | C | wt | 14.7 ± 1.0 | 39.0 ± 5.0 | 19.1 ± 19 | 6.7 ± 0.7 | 22.2 ± 2.0 | 46.7 ± 28 | 0.99 ± 0.08 |
| 8032 | C | M46I, T74S, T80I, V82L, I85IV, L89M | 33.0 ± 19 (2.3) | 1120 ± 327 (28.7) | 34.5 ± 3.0 (1.8) | 68.0 ± 8.0 (10.2) | 460.8 ± 121 (20.8) | 22.9 ± 9.8 (0.5) | 0.51 ± 0.21 (0.5) |

The shaded area indicates the wild-types of each isolate to differentiate the EC50 from that of selected variants.
[a] The values represent the means of two independent experiments, each performed in duplicate. Drug susceptibility was expressed as fold-change in $EC_{50}$, determined by calculating the ratio of $EC_{50}$ values for mutated and wt virus (values in parentheses).

range of viral isolates in drug selection to ensure that the potential for manifestation of a broad array of mutations is fully explored.

We observed similar patterns of resistance to PL-100 with both subtype C and CRF01_AE viruses. A recent *in vitro* selection study showed that resistance may develop slower under PL-100 compared with APV drug pressure in the case of the laboratory-adapted HIV/IIIb (subtype B) virus and that a unique T80I resistance pathway is involved (Dandache et al., 2008). The importance of this pathway is confirmed in the present study.

Our data follow a previous investigation that also identified a L76F mutation in PR (Bold et al., 1998). The role of L76F in PI resistance is yet to be explored. Mutations K55R and I85V were transiently present during PL-100 selection and have been previously observed in cases of PI failure (Descamps et al., 2009; Johnson et al., 2008; Margerison et al., 2008; Palma et al., 2009; Svicher et al., 2005). Site-directed mutagenesis demonstrated, however, that these mutations were susceptible to PL-100 but only impacted moderately on PL-100 susceptibility in the presence of other mutations. K55R and I85V are thought to be able to restore viral replicative capacity (Margerison et al., 2008). Previous site-directed mutagenesis studies of the M46I and T80I mutations showed no effect on susceptibility to PL-100 (Dandache et al., 2008) and neither mutation significantly affected viral replication capacity. Additional studies in our laboratory will evaluate whether other mutations, including L76F and V82A, either alone or in combination, might affect susceptibilty to PL-100 and other members of the PI family of drugs.

The genotype of virus 7208 is M46I, V82A and L90M. These are major mutations that decrease susceptibility to saquinavir. The mean fold-change observed in two independent experiments in our phenotypic assay indicated susceptibility to saquinavir. Phenotypic resistance might be of greater value than genotypic data to predict outcome of resistance in some cases. In support of our findings, Piketty et al. (1999) showed that patients with baseline mutations at positions 46, 82 and 90 had a decreased viral load of fewer than 50 copies/ml after 24 weeks of r/saquinavir therapy. In both MT-2 cells and CBMCs, levels of cross-resistance after selection with PL-100 followed a consistent pattern. First, both NFV and APV were significantly affected by the mutations selected by PL-100. Second, LPV and ATV were only partially affected by these mutations because the fold-change is just slightly above the lower clinical cut-off and SQV seemed to retain significant activity against viruses that were resistant to PL-100 since the fold-change of the selected viruses did not reach the lower clinical cut-off value. The fact that PL-100 has a distinct resistance profile from other PIs suggests that it might potentially be sequenced with ATV and LPV. Given its overall profile, PL-100 could very well be placed as a first line or second-line therapy for protease-naïve or experienced patients and be a valuable new addition to the HIV drug armamentarium.

## 5. Conclusion

Ultimately, similar patterns of resistance to PL-100 seem to exist in both subtype C and CRF01_AE, despite important differences in baseline polymorphisms among these viruses. High-level resistance to PL-100 was observed only in the presence of three or more PI resistance mutations demonstrating the high genetic barrier of this compound. Our observations have also confirmed the importance of a signature T80I mutation that is involved in PL-100 resistance.

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## References


Aghokeng, A.F., Vergne, L., Mpoudi-Ngole, E., Mbangue, M., Deoudje, N., Mokondji, E., Nambei, W.S., Peyou-Ndi, M.M., Moka, J.J., Delaporte, E., Peeters, M., 2009. Evaluation of transmitted HIV drug resistance among recently-infected antenatal clinic attendees in four Central African countries. Antivir. Ther. 14 (3), 401–411.

Bessong, P.O., 2008. Polymorphisms in HIV-1 subtype C proteases and the potential impact on protease inhibitors. Trop. Med. Int. Health 13 (2), 144–151.

Bold, G., Fassler, A., Capraro, H.G., Cozens, R., Klimkait, T., Lazdins, J., Mestan, J., Poncioni, B., Rosel, J., Stover, D., Tintelnot-Blomley, M., Acemoglu, F., Beck, W., Boss, E., Eschbach, M., Hurlimann, T., Masso, E., Roussel, S., Ucci-Stoll, K., Wyss, D., Lang, M., 1998. New aza-dipeptide analogues as potent and orally absorbed HIV-1 protease inhibitors: candidates for clinical development. J. Med. Chem. 41 (18), 3387–3401.

Brenner, B.G., Roger, M., Moisi, D.D., Oliveira, M., Hardy, I., Turgel, R., Charest, H., Routy, J.P., Wainberg, M.A., 2008. Transmission networks of drug resistance acquired in primary/early stage HIV infection. AIDS 22 (18), 2509–2515.

Brown, B.K., Darden, J.M., Tovanabutra, S., Oblander, T., Frost, J., Sanders-Buell, E., de Souza, M.S., Birx, D.L., McCutchan, F.E., Polonis, V.R., 2005. Biologic and genetic characterization of a panel of 60 human immunodeficiency virus type 1 isolates, representing clades A, B, C, D, CRF01_AE, and CRF02_AG, for the development and assessment of candidate vaccines. J. Virol. 79 (10), 6089–6101.

Dandache, S., Coburn, C.A., Oliveira, M., Allison, T.J., Holloway, M.K., Wu, J.J., Stranix, B.R., Panchal, C., Wainberg, M.A., Vacca, J.P., 2008. PL-100, a novel HIV-1 protease inhibitor displaying a high genetic barrier to resistance: an in vitro selection study. J. Med. Virol. 80 (12), 2053–2063.

Dandache, S., Sevigny, G., Yelle, J., Stranix, B.R., Parkin, N., Schapiro, J.M., Wainberg, M.A., Wu, J.J., 2007. In vitro antiviral activity and cross-resistance profile of PL-100, a novel protease inhibitor of human immunodeficiency virus type 1. Antimicrob. Agents Chemother. 51 (11), 4036–4043.

Descamps, D., Lambert-Niclot, S., Marcelin, A.G., Peytavin, G., Roquebert, B., Katlama, C., Yeni, P., Felices, M., Calvez, V., Brun-Vezinet, F., 2009. Mutations associated with virological response to darunavir/ritonavir in HIV-1-infected protease inhibitor-experienced patients. J. Antimicrob. Chemother. 63 (3), 585–592.

Doualla-Bell, F., Avalos, A., Gaolathe, T., Mine, M., Gaseitsiwe, S., Ndwapi, N., Novitsky, V.A., Brenner, B., Oliveira, M., Moisi, D., Moffat, H., Thior, I., Essex, M., Wainberg, M.A., 2006. Impact of human immunodeficiency virus type 1 subtype C on drug resistance mutations in patients from Botswana failing a nelfinavir-containing regimen. Antimicrob. Agents Chemother. 50 (6), 2210–2213.

Gao, Q., Gu, Z., Parniak, M.A., Cameron, J., Cammack, N., Boucher, C., Wainberg, M.A., 1993. The same mutation that encodes low-level human immunodeficiency virus type 1 resistance to 2′,3′-dideoxyinosine and 2′,3′-dideoxycytidine confers high-level resistance to the (-) enantiomer of 2′,3′-dideoxy-3′-thiacytidine. Antimicrob. Agents Chemother. 37 (6), 1390–1392.

Hemelaar, J., Gouws, E., Ghys, P.D., Osmanov, S., 2006. Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. AIDS 20 (16), W13–23.

Johnson, V.A., Brun-Vezinet, F., Clotet, B., Gunthard, H.F., Kuritzkes, D.R., Pillay, D., Schapiro, J.M., Richman, D.D., 2008. Update of the Drug Resistance Mutations in HIV-1. Top. HIV Med. 16 (5), 138–145.

Kantor, R., Katzenstein, D., 2003. Polymorphism in HIV-1 non-subtype B protease and reverse transcriptase and its potential impact on drug susceptibility and drug resistance evolution. AIDS Rev. 5 (1), 25–35.

Liu, J., Yue, J., Wu, S., Yan, Y., 2007. Polymorphisms and drug resistance analysis of HIV-1 CRF01_AE strains circulating in Fujian Province. China Arch. Virol. 152 (10), 1799–1805.

Loemba, H., Brenner, B., Parniak, M.A., Ma'ayan, S., Spira, B., Moisi, D., Oliveira, M., Detorio, M., Wainberg, M.A., 2002. Genetic divergence of human immunodeficiency virus type 1 Ethiopian clade C reverse transcriptase (RT) and rapid development of resistance against nonnucleoside inhibitors of RT. Antimicrob. Agents Chemother. 46 (7), 2087–2094.

Luis Jimenez, J., Resino, S., Martinez-Colom, A., Bellon, J.M., Angeles Munoz-Fernandez, M., 2005. Mutations at codons 54 and 82 of HIV protease predict virological response of HIV-infected children on salvage lopinavir/ritonavir therapy. J. Antimicrob. Chemother. 56 (6), 1081–1086.

Marcelin, A.G., Affolabi, D., Lamotte, C., Mohand, H.A., Delaugerre, C., Wirden, M., Voujon, D., Bossi, P., Ktorza, N., Bricaire, F., Costagliola, D., Katlama, C., Peytavin, G., Calvez, V., 2004a. Resistance profiles observed in virological failures after 24 weeks of amprenavir/ritonavir containing regimen in protease inhibitor experienced patients. J. Med. Virol. 74 (1), 16–20.

Marcelin, A.G., Dalban, C., Peytavin, G., Lamotte, C., Agher, R., Delaugerre, C., Wirden, M., Conan, F., Dantin, S., Katlama, C., Costagliola, D., Calvez, V., 2004b. Clinically relevant interpretation of genotype and relationship to plasma drug concentrations for resistance to saquinavir–ritonavir in human immunodeficiency virus type 1 protease inhibitor-experienced patients. Antimicrob. Agents Chemother. 48 (12), 4687–4692.

Margerison, E.S., Maguire, M., Pillay, D., Cane, P., Elston, R.C., 2008. The HIV-1 protease substitution K55R: a protease-inhibitor-associated substitution involved in restoring viral replication. J. Antimicrob. Chemother. 61 (4), 786–791.

Mo, H., King, M.S., King, K., Molla, A., Brun, S., Kempf, D.J., 2005. Selection of resistance in protease inhibitor-experienced, human immunodeficiency virus type 1-infected subjects failing lopinavir- and ritonavir-based therapy: mutation patterns and baseline correlates. J. Virol. 79 (6), 3329–3338.

Ntemgwa, M., Brenner, B.G., Oliveira, M., Moisi, D., Wainberg, M.A., 2007. Natural polymorphisms in the human immunodeficiency virus type 2 protease can accelerate time to development of resistance to protease inhibitors. Antimicrob. Agents Chemother. 51 (2), 604–610.

Oliveira, M., Brenner, B.G., Wainberg, M.A., 2009. Isolation of drug-resistant mutant HIV variants using tissue culture drug selection. Methods Mol. Biol. 485, 427–433.

Palella Jr., F.J., Delaney, K.M., Moorman, A.C., Loveless, M.O., Fuhrer, J., Satten, G.A., Aschman, D.J., Holmberg, S.D., 1998. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. N. Engl. J. Med. 338 (13), 853–860.

Palma, A.C., Abecasis, A.B., Vercauteren, J., Carvalho, A.P., Cabanas, J., Vandamme, A.M., Camacho, R.J., 2009. Effect of human immunodeficiency virus type 1 protease inhibitor therapy and subtype on development of resistance in subtypes B and G. Infect. Genet. Evol..

Petrella, M., Oliveira, M., Moisi, D., Detorio, M., Brenner, B.G., Wainberg, M.A., 2004. Differential maintenance of the M184V substitution in the reverse transcriptase of human immunodeficiency virus type 1 by various nucleoside antiretroviral agents in tissue culture. Antimicrob. Agents Chemother. 48 (11), 4189–4194.

Piketty, C., Race, E., Castiel, P., Belec, L., Peytavin, G., Si-Mohamed, A., Gonzalez-Canali, G., Weiss, L., Clavel, F., Kazatchkine, M.D., 1999. Efficacy of a five-drug combination including ritonavir, saquinavir and efavirenz in patients who failed on a conventional triple-drug regimen: phenotypic resistance to protease inhibitors predicts outcome of therapy. AIDS 13 (11), F71–F77.

Salomon, H., Belmonte, A., Nguyen, K., Gu, Z., Gelfand, M., Wainberg, M.A., 1994. Comparison of cord blood and peripheral blood mononuclear cells as targets for viral isolation and drug sensitivity studies involving human immunodeficiency virus type 1. J. Clin. Microbiol. 32 (8), 2000–2002.

Santoro, M.M., Bertoli, A., Lorenzini, P., Ceccherini-Silberstein, F., Gianotti, N., Mussini, C., Torti, C., Di Perri, G., Barbarini, G., Bini, T., Melzi, S., Caramello, P., Maserati, R., Narciso, P., Micheli, V., Antinori, A., Perno, C.F., 2009. Two different patterns of mutations are involved in the genotypic resistance score for atazanavir boosted versus unboosted by ritonavir in multiple failing patients. Infection 37 (3), 233–243.

Stranix, B.R., Sauve, G., Bouzide, A., Cote, A., Sevigny, G., Yelle, J., Perron, V., 2004. Lysine sulfonamides as novel HIV-protease inhibitors: nepsilon-disubstituted ureas. Bioorg. Med. Chem. Lett. 14 (15), 3971–3974.

Svedhem, V., Lindkvist, A., Bergroth, T., Knut, L., Sonnerborg, A., 2005. Diverse pattern of protease inhibitor resistance mutations in HIV-1 infected patients failing nelfinavir. J. Med. Virol. 76 (4), 447–451.

Svicher, V., Ceccherini-Silberstein, F., Erba, F., Santoro, M., Gori, C., Bellocchi, M.C., Giannella, S., Trotta, M.P., Monforte, A., Antinori, A., Perno, C.F., 2005. Novel human immunodeficiency virus type 1 protease mutations potentially involved in resistance to protease inhibitors. Antimicrob. Agents Chemother. 49 (5), 2015–2025.

Turner, D., Brenner, B., Mosis, D., Liang, C., Wainberg, M.A., 2005. Substitutions in the reverse transcriptase and protease genes of HIV-1 subtype B in untreated individuals and patients treated with antiretroviral drugs. MedGenMed 7 (1), 69.

Van Houtte, M., Picchio, G., Van Der Borght, K., Pattery, T., Lecocq, P., Bacheler, L.T., 2009. A comparison of HIV-1 drug susceptibility as provided by conventional phenotyping and by a phenotype prediction tool based on viral genotype. J. Med. Virol. 81 (10), 1702–1709.

Velazquez-Campoy, A., Vega, S., Fleming, E., Bacha, U., Sayed, Y., Dirr, H.W., Freire, E., 2003. Protease inhibition in African subtypes of HIV-1. AIDS Rev. 5 (3), 165–171.

Ventoso, I., Navarro, J., Munoz, M.A., Carrasco, L., 2005. Involvement of HIV-1 protease in virus-induced cell killing. Antiviral Res. 66 (1), 47–55.

Vergne, L., Peeters, M., Mpoudi-Ngole, E., Bourgeois, A., Liegeois, F., Toure-Kane, C., Mboup, S., Mulanga-Kabeya, C., Saman, E., Jourdan, J., Reynes, J., Delaporte, E., 2000. Genetic diversity of protease and reverse transcriptase sequences in non-subtype-B human immunodeficiency virus type 1 strains: evidence of many minor drug resistance mutations in treatment-naive patients. J. Clin. Microbiol. 38 (11), 3919–3925.

Vingerhoets, J., Azijn, H., Fransen, E., De Baere, I., Smeulders, L., Jochmans, D., Andries, K., Pauwels, R., de Bethune, M.P., 2005. TMC125 displays a high genetic barrier to the development of resistance: evidence from in vitro selection experiments. J. Virol. 79 (20), 12773–12782.

Winters, B., Van Craenenbroeck, E., Van der Borght, K., Lecocq, P., Villacian, J., Bacheler, L., 2009. Clinical cut-offs for HIV-1 phenotypic resistance estimates: update based on recent pivotal clinical trial data and a revised approach to viral mixtures. J. Virol. Methods 162 (1–2), 101–108.