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Selection and characterization of HIV-1 showing reduced susceptibility to the non-peptidic protease inhibitor tipranavir

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Abstract

Tipranavir is a novel, non-peptidic protease inhibitor, which possesses broad antiviral activity against multiple protease inhibitor-resistant HIV-1. Resistance to this inhibitor however has not yet been well described. HIV was passaged for 9 months in culture in the presence of tipranavir to select HIV with a drug-resistant phenotype. Characterization of the selected variants revealed that the first mutations to be selected were L33F and I84V in the viral protease, mutations which together conferred less than two-fold resistance to tipranavir. At the end of the selection experiments, viruses harbouring 10 mutations in the protease (L10F, I13V, V32I, L33F, M36I, K45I, I54V, A71V, V82L, I84V) as well as a mutation in the CA/SP1 gag cleavage site were selected and showed 87-fold decreased susceptibility to tipranavir. In vitro, tipranavir-resistant viruses had a reduced replicative capacity which could not be improved by the introduction of the CA/SP1 cleavage site mutation. Tipranavir resistant viruses showed cross-resistance to other currently approved protease inhibitors with the exception of saquinavir. These results demonstrate that the tipranavir resistance phenotype is associated with complex genotypic changes in the protease. Resistance necessitates the sequential accumulation of multiple mutations.

Keywords: Tipranavir; HIV; Resistance

1. Introduction

Protease inhibitors (PIs) are inhibitors of human immunodeficiency virus (HIV) replication with proven clinical efficacy (Randolph and DeGoey, 2004). As for all other classes of inhibitors, however, the efficacy of PIs can be significantly decreased by the development of resistance, a process during which viruses containing mutations in the target protein outgrow wild type (WT) HIV (De Mendoza and Soriano, 2004). Development of resistance has been characterized for all currently approved PIs (atazanavir (ATV), amprenavir (APV), fosamprenavir (fAPV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV) and saquinavir (SQV)) and is generally associated with the presence of one or multiple mutations in the protease gene (Johnson et al., 2004). In addition, mutations outside of the protease genetic locus have been identified in PI-resistant viruses, namely in gag and gag-pol cleavage sites (Doyon et al., 1996; Zhang et al., 1997; Carillo et al., 1998; Mammano et al., 1998; Bally et al., 2000; Gong et al., 2000; Côté et al., 2001; Maguire et al., 2002; Watkins et al., 2003). These mutations have been shown to improve viral replication of viruses containing mutant proteases (Doyon et al., 1996; Zhang et al., 1997; Carillo et al., 1998; Mammano et al., 1998; Robinson et al., 2000; Maguire et al., 2002) and, in one study, to increase resistance to the selecting inhibitor (Maguire et al., 2002). As has been observed for other classes of antiretrovirals, resistance to one PI can confer cross-resistance to other PIs (Condra et al., 1995; Tisdale et al., 1995; Palmer et al., 1999; Schapiro et al., 1999; Hertogs et al., 2000; Dronda et al., 2001; Race, 2001), significantly limiting the therapeutic alternatives upon treatment failure (Lorenzi et al., 1999; Piketty et al., 1999; Karmochkine et al., 2000; Paolucci et al., 2000; Ross et al., 2001). The development of new PIs with non-overlapping resistance profiles or with higher genetic barriers

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to the development of resistance would, therefore, be beneficial for the treatment of HIV infections.

Tipranavir (TPV) is a non-peptidic PI which retains potent antiviral activity against a wide range of patient-derived HIV isolates resistant to multiple PIs (Poppe et al., 1997; Back et al., 2000; Larder et al., 2000; Rusconi et al., 2000; Schwartz et al., 2002). The mutational pattern conferring resistance to TPV, however, is poorly understood, in part due to the limited number of available clinical isolates exhibiting reduced susceptibility to this inhibitor and to the diverse and complex genotypic changes present in such isolates. In addition, initial attempts to select viruses with decreased susceptibility to TPV in vitro met with limited success, as only low levels of resistance could be selected and resistance could not be transferred by genetic methods (Poppe et al., 1997; Kemp et al., 2000). Although these preliminary observations suggest a high genetic barrier for the development of resistance to TPV, it is necessary to more precisely define the genotype–phenotype correlates of viruses possessing decreased susceptibility to TPV. This information will lead to a better understanding of the antiviral activity of TPV and will be of assistance in developing an ability to predict the tipranavir sensitivity phenotype of pre- or post-treatment clinical isolates based upon genotypic information. In order to select viruses showing decreased susceptibility to TPV, HIV was serially passaged in vitro, in the presence of increasing concentrations of the inhibitor. Viruses selected to grow in these conditions were characterized in terms of their genotype and drug-sensitivity phenotype.

2. Materials and methods

2.1. Protease inhibitors

APV, ATV, IDV, RTV and SQV were synthesized in house at Boehringer Ingelheim (Canada) Ltd., Research and Development, LPV and NFV were extracted from Kaletra® and Viracept® capsules, respectively. The purity of the latter two inhibitors after extraction was 100%, as determined by reverse-phase HPLC. TPV was received from Boehringer Ingelheim Pharmaceuticals Inc., USA.

2.2. Construction of recombinant HIV-1

The proviral DNA used to produce WT HIV-1 was plasmid 2.12, an HIV-1 encoding pNL4.3 plasmid (NIH AIDS Research and Reference Reagent Program) (Adachi et al., 1986), modified to contain a unique Bst1107I site at codon 125 of the RT open reading frame (Doyon et al., 1996). Viral stocks were produced by transfecting p2.12 into human kidney 293 cells (American Type Culture Collection) by the calcium phosphate method (Graham and van der Eb, 1973). After 3 days of incubation at 37 °C, supernatant was collected and the 50% cell culture infective dose ($CCID_{50}$) determined by monitoring the microscopic formation of syncytia using the C8166 cell line (obtained from J. Sullivan, University Massachusetts Medical Centre).

To construct protease mutant viruses, site directed mutagenesis was carried out using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). All mutations were confirmed by sequencing on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). Mutant viral stocks were produced by transfection as described above.

2.3. Selection of HIV-1 variants resistant to tipranavir

C8166 cells were infected at a multiplicity of infection (MOI) of 0.5 with WT HIV-1 and cultures maintained in complete RPMI 1640 (RPMI 1640 medium + 10% fetal bovine serum + 10 μg/ml gentamycin + 10 μM β-mercaptoethanol) initially containing 400 nM of TPV. After 3–4 days (one passage), microscopic evaluation of the cytopathic effect (CPE) was determined and culture supernatant was used to infect fresh C8166 which were maintained at the same or increasing concentrations of TPV.

2.4. Protease gene amplification, cloning and sequencing

At specific passages, genomic DNA of infected cells was isolated using the DNeasy® Tissue Kit (Qiagen Inc., Valencia, CA). Protease and cleavage site gene sequences were then amplified by polymerase chain reaction (CombiPol polymerase kit, InViTek, Berlin, Germany) using specific oligonucleotides. PCR fragments were cloned into the Zero Blunt® TOPO® PCR Cloning Kit (In vitrogen Corp., Carlsbad, CA) and sequenced by automated sequencing.

2.5. Antiviral activity (EC_{50}) determinations

Inhibitors were initially prepared as 10 mM stocks in 100% dimethyl sulfoxide (DMSO) before being serially diluted in complete RPMI 1640. C8166 cells were infected at a multiplicity of infection of 0.001 with HIV-1 for 1.5 h, then washed and plated at 5×10^4 cells per well, in wells already containing inhibitors. Final concentrations of inhibitors ranged from 0.1 to 25,000 nM. Plates were incubated for 3 days at 37 °C in a 5% CO_2 incubator and extracellular p24 levels were determined using the Coulter® HIV-1 p24 Antigen Assay (Beckman Coulter, Mississauga, Ont., Canada). Percent inhibition of viral replication was determined using the SAS NLIN procedure.

2.6. Replication capacity assay

Jurkat cells containing an integrated HIV-1 LTR-luciferase reporter construct were generated in house by stably transfecting an HIV-1 LTR-luciferase containing plasmid into Jurkat clone E6-1 cells (ATCC #TIB-152). To construct the HIV-1 LTR-luciferase plasmid, an HIV-1 LTR fragment

(nucleotides 3165-536 of the HXB2 sequence) was amplified by PCR and cloned into the luciferase expression vector pGL3-Basic (Promega, #E1751). Jurkat HIV-1 LTR-luciferase cells were infected with HIV (10 ng p24 of virus per 10^6 cells) for 2 h at 37 °C, washed and incubated in 96-well clear bottom Costar black plates (1×10^5 cells per well, in 200 μL), in complete RPMI medium lacking phenol red. Eight replicate wells were seeded for each time point. Every 3–4 days, 100 μL of cells was removed and replaced with fresh RPMI medium. Luciferase readout was determined by adding 50 μL of substrate (Bright-Glo®, from Promega) per well at days 7, 10, 12 and 13 post-infection. Luminescence (relative luminescence units, RLU) was measured using a LUMIstar Galaxy microplate luminescence reader (BMG). Background RLU (from uninfected cells cultured in the same conditions) was substracted from all samples and means of eight replicate values were determined.

2.7. P24 western blot analysis of viral proteins

Proviral DNA was transfected in 293 cells as described above. Culture supernatants containing virions were harvested 3 days after transfection, low speed centrifuged ($800 \times g$, 5 min) and filtered on 0.22 μM MillexTM-GV Millipore membranes. Supernatants were then centrifuged at 20,000 × *g* for 2 h to pellet virus particles. Pellets were resuspended in 1X Laemmli sample buffer (0.06M Tris, 2% SDS, 10% sucrose and 5% β-mercaptoethanol) and heated at 100 °C for 5 min. Samples were run on 10% SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes and hybridized with a monoclonal antibody to p24 (ID Labs Inc. #39/5.1.23) followed by a horseradish peroxydase-coupled goat anti-mouse IgG antibody (Sigma #A-2554). Blots were revealed using the ECL Plus Western Blotting Detection System (#RPN2132, Amersham Biosciences, UK) analyzed by autoradiography. Similar amounts of p24 protein were detected by ELISA in all samples upon transfection.

3. Results

3.1. Selection of TPV-resistant viruses

A wild type clone of HIV-1 was serially passaged in C8166 cells in the presence of increasing concentrations of TPV, in order to select for viruses showing reduced susceptibility to this inhibitor. In an acute model of C8166 cell infection by wild type HIV-1, TPV showed an $EC_{50} = 60 \pm 13$ nM and an $EC_{95} = 520 \pm 200$ nM. In vitro, the development of resistance to TPV occurred in several incremental steps and over several months in culture so that after 73 passages (or 9 months), HIV-1 variants capable of growing in the presence of 20 μM of TPV were selected. This viral population (passage 73) showed an 87-fold decrease in susceptibility to TPV when compared to WT virus (EC_{50} = 5231 nM versus 60 nM, respectively, Table 1).

3.2. Characterization of viral populations selected in culture

Table 1 describes the genotypic and phenotypic characteristics of the viral populations selected during these experiments. The first viral population to replicate in concentrations of TPV exceeding the EC_{95} of wild type virus was selected after 16 passages in culture (p16), in the presence of 800 nM of TPV. Viruses from this population contained the protease mutations L33F and I84V, which were simultaneously present in 5/8 clones sequenced. WT virus was however still detected at this passage, as well as viruses with the genotypes L33F and L33F/I57K/I84V. However, although this population was selected at a concentration of TPV which completely inhibited wild type virus replication, p16 viruses showed no measurable decrease in susceptibility to TPV (P16 EC_{50} = 56 versus WT EC_{50} = 60 nM). Viruses from passage 33, growing in 1000 nM of TPV, also showed only marginal resistance (1.4-fold decreased susceptibility) possibly associated with the selection of the mutation K45I, which, although only present in a subset of clones at passage 33, was subsequently maintained throughout the experiment. A significant increase in resistance was observed at passage 39 (10-fold resistance) concomitant with the selection of mutations I13V and V32I. Interestingly, p49 viruses, a seemingly clonal viral population harbouring the newly selected active site mutation V82L, showed a higher level of resistance than the more heterogeneous population from the subsequent passage p68 which in addition to V82L also contained the non-active site mutations M36I and A71V (p49 = 34-fold, p68 = 25-fold resistance). Finally, viruses selected to grow in the presence of the highest concentration of TPV used in this study (20 μM) showed 87-fold resistance to this inhibitor and the dominant genotype from this passage was composed of 10 mutations: L10F/I13V/V32I/L33F/M36I/K45I/I54V/A71V/V82L/I84V. This genotype was conserved even upon removal of the inhibitor for 12 further passages in culture (passage 85). Altogether, these results demonstrate that the development of resistance to TPV in vitro is initially very slow and high level resistance is only selected after multiple passages in the very permissive C8166 cell line. In most instances the increase in resistance was associated with the accumulation of two new mutations in the protease gene.

3.3. Reconstitution of the TPV resistance phenotype

To confirm that the mutations selected in culture indeed contributed to the resistance phenotype to TPV, viral molecular clones containing combinations of the 10 dominant mutations observed during the selection experiments (L10F, I13V, V32I, L33F, M36I, K45I, I54V, A71V, V82L, I84V) were constructed and viruses tested for their susceptibility to TPV (Table 2). In these experiments, none of the mutations alone conferred decreased susceptibility to TPV (data not shown). When tested in antiviral assays, clone TPV.16,

Table 1
In vitro selection of HIV-1 showing decreased susceptibility to TPV

Passage	Protease mutations	[TPV] (nM)	$EC_{50} \pm$ S.D. (nM)	Fold
P0	NA	NA	60 ± 13	1
P16	WT (1/8) L33F (1/8) **L33F, I84V** (5/8) L33F, I57K, I84V (1/8)	800	56 ± 30	1
P33	L33F, I84V (7/12) L33F, **K45I**, I84V (2/12) L33F, K45I, I84V, L89M (1/12) L33F, C67Y, I84V (1/12) K45I, I84V (1/12)	1000	85 ± 4	1.4
P39	**I13V, V32I**, L33F, K45I, I84V (15/19) I13V, V32I, L33F, I84V (1/19) I13V, V32I, L33F, K45I, I84V, N88D (1/19) V32I, L33F, I84V (1/19) I13V, A28T, V32I, L33F, K45I, I84V (1/19)	2000	628 ± 147	10
P49	I13V, V32I, L33F, K45I, **V82L**, I84V (19/19)	5000	2072 ± 327	34
P68	I13V, V32I, L33F, **M36I**, K45I, **A71V**, V82L, I84V (4/6) I13V, V32I, L33F, M36I, K45I, I54V, A71V, V82L, I84V (1/6) L10F, I13V, V32I, L33F, M36I, K45I, I54T, A71V, V82L, I84V (1/6)	10000	1473 ± 165	25
P73	**L10F**, I13V, V32I, L33F, M36I, K45I, **I54V**, A71V, V82L, I84V (6/11) I13V, V32I, L33F, M36I, K45I, I54V, A71V, V82L, I84V (3/11) L10F, I13V, K14R, V32I, L33F, M36I, K45I, I54V, A71V, V82L, I84V (1/11) I3V, L10F, I13V, V32I, L33F, M36I, K45I, G49E, I54V, A71V, V82L, I84V 1/11)	20000	5231 ± 428	87
P85-r	L10F, I13V, V32I, L33F, M36I, K45I, I54V, A71V, V82L, I84V (7/12) I13V, V32I, L33F, M36I, K45V, I54T, A71V, V82L, I84V (1/12) L10F, I13V, V32I, L33F, M36I, K45I, I54V, A71V, V82L, I84V, G86E (1/1/2) L10F, I13V, V32I, L33F, M36I, K45I, I54V, A71V, V82L, I84V (2/12) L10F, I13V, V32I, L33F, K45I, I54V, A71V, V82L, I84V (1/12)	0	ND	ND

Protease sequences were obtained from clones amplified from each passage. Numbers in parentheses represent the frequency of the genotypes among the clones sequenced. Mutations in bold are new to that passage and maintained throughout the selection experiment. [TPV] is the concentration of TPV present in the culture at that passage. EC_{50}s represent means of two to three experiments. Fold resistance is mutant EC_{50}/WT EC_{50}. S.D., standard deviation; NA, not applicable; ND, not determined. P85-r is virus cultured in the absence of inhibitor for the last 12 passages.

representing variants selected at passage 16 (L33F, I84V) showed only 1.7-fold resistance compared to the WT virus (EC_{50}s of 101 nM versus 60 nM, respectively), whereas the addition of mutation K45I increased the resistance to 2.1-fold (clone TPV.33). These results confirm the marginal level of resistance obtained when using viral populations from early passages. The presence of mutations I13V and V32I in clone TPV.39 increased resistance to seven-fold and resistance reached levels greater than 10-fold when six mutations were present in the protease of viral clones (I13V, V32I, L33F, K45I, V82L, I84V, TPV.49). When reintroduced into viral clones, the genotypes observed at passages 49 and 68 conferred a progressive increase in resistance from 16- to 28-fold, respectively, in contrast to what was observed with the susceptibility of viral populations. The bulk of the resistance phenotype observed during the selection experiments was reconstituted when the full 10-mutations genotype was introduced in viral clone TPV.73 which showed a 69-fold reduction in susceptibility to TPV. These experiments therefore confirm that the mutations selected in culture are involved in

Table 2
Susceptibility to TPV of viral clones constructed to contain the protease mutations observed in viruses selected in culture

Mutant virus	Protease mutations	$EC_{50} \pm$ S.D. (nM)	Fold
WT	Wild type	60 ± 13	1
TPV.16	L33F, I84V	101 ± 43	1.7
TPV.33	L33F, K45I, I84V	124 ± 43	2
TPV.39	I13V, V32I, L33F, K45I, I84V	407 ± 206	7
TPV.49	I13V, V32I, L33F, K45I, V82L, I84V	967 ± 434	16
TPV.68	I13V, V32I, L33F, M36I, K45I, A71V, V82L, I84V	1687 ± 178	28
TPV.73	L10F, I13V, V32I, L33F, M36I, K45I, I54V, A71V, V82L, I84V	4156 ± 1112	69

EC_{50} values are means of two to five independent experiments. The fold resistance is mutant EC_{50}/WT EC_{50}. S.D., standard deviation.

Table 3
Susceptibility of TPV-resistant mutant clones to protease inhibitors

Mutant virus	Fold resistance							
	TPV	APV	ATV	IDV	LPV	NFV	RTV	SQV
WT	1 (60)	1 (15)	1 (4)	1 (6)	1 (8)	1 (9)	1 (26)	1 (3)
TPV.33	2	5	2	6	4	1	6	3
TPV.49	16	79	37	28	24	5	61	0.6
TPV.73	69	29	134	90	59	22	164	3

Fold resistance (mutant EC_{50}/WT EC_{50}) values represent means of two to five independent experiments. WT virus EC_{50} values for all PIs (in nM) are indicated in parenthesis. APV, amprenavir; ATV, atazanavir; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; RTV, ritonavir; SQV, saquinavir.

the development of resistance to TPV and further emphasize that there is a slow progression to resistance associated with incremental phenotypic changes.

3.4. Cross-resistance of TPV-resistant viruses to other protease inhibitors

The susceptibility of three TPV-resistant clones: TPV.33 (L33F/K45I/I84V), TPV.49 (I13V/V32I/L33F/K45I/V82L/I84V) and TPV.73 (10 mutations in the protease) to other PIs was tested. Table 3 illustrates that clone TPV.33, while showing limited resistance to TPV (two-fold) showed low level resistance to the other PIs. Nonetheless, TPV.33 was not more than six-fold resistant to any of the tested PIs, the highest cross-resistance being to RTV and IDV (six-fold) and to APV (five-fold). Notably, TPV.49 had higher levels of resistance to most PIs compared to TPV itself (16-fold), although only five-fold resistance was observed for NFV, and no cross-resistance (0.6-fold) could be detected for SQV. Similarly, TPV.73 showed cross-resistance (>10-fold) to all PIs except SQV. During these studies, the highest levels of cross-resistance were observed for RTV (154-fold) and ATV (134-fold). Throughout the selection study no significant cross resistance to SQV was observed, the TPV.73 virus exhibiting only three-fold reduced susceptibility compared to WT virus.

3.5. Replication capacity of TPV-resistant viruses

PI-resistant HIV was shown in several instances to have impaired replication capacity (Croteau et al., 1997; Zhang et al., 1997; Carillo et al., 1998; Mammano et al., 1998; Martinez-Picado et al., 1999; Berkhout, 1999; Nijhuis et al., 1999; Robinson et al., 2000; Nijhuis et al., 2001; Maguire et al., 2002; Watkins et al., 2003). To determine if TPV-resistant viruses also showed diminished replicative capacity, viral clones were studied in replication capacity assays using Jurkat T-cells containing an HIV-LTR driven luciferase gene as a reporter. Infection of these cells by WT HIV caused a 10-fold transactivation of the reporter gene after 10–13 days (not shown). Table 4 shows that when infecting Jurkat cells, clone TPV.16 (L33F/I84V), although replicating with kinetics similar to WT virus, only gave a maximum of 55% of the WT luciferase signal at day 13 post-infection. In contrast, viruses containing 3–10 mutations in the protease (TPV.33, TPV.39, TPV.49, TPV.68, TPV.73) barely gave detectable luciferase signals at any time point during these experiments. The levels of luciferase signal observed with these viruses at day 7 is attributable to remaining input virus, as this signal decreased over time consequently to culture medium replacements. Parallel studies using a derivative of the very permissive C8166 cell line, also containing a luciferase reporter gene, showed that all viral clones tested were replication competent in C8166 cells, giving low but detectable luciferase signals within 3 days of infection (not shown).

3.6. Cleavage site mutations in TPV-resistant viruses

Cleavage site sequences have previously been demonstrated to mutate during the development of resistance to PIs and to improve the replication capacity of protease mutant viruses (Doyon et al., 1996; Zhang et al., 1997; Carillo et al., 1998; Mammano et al., 1998; Robinson et al., 2000; Maguire et al., 2002). The nine cleavage sites of Gag and Gag/Pol in HIV variants selected in culture with TPV were therefore sequenced to determine if any mutations had occurred in these

Table 4
Replication capacity of TPV-resistant clones in infected Jurkat-luciferase cells

Days Pi	Luciferase signal								
	Mutant virus								
	WT	TPV.16	TPV.33	TPV.39	TPV.49	TPV.68	TPV.73	TPV.49_CA	TPV.73_CA
7	7843 ± 2497	3207 ± 1289	3006 ± 1235	1060 ± 340	4799 ± 1345	915 ± 602	2676 ± 1415	4478 ± 1287	1422 ± 444
10	21790 ± 7963	6878 ± 33995	BDL	BDL	1609 ± 464	300 ± 284	715 ± 224	705 ± 423	588 ± 208
12	68863 ± 20739	28574 ± 13981	BDL	BDL	BDL	BDL	BDL	BDL	BDL
13	106652 ± 19756	58991 ± 34978	BDL	BDL	BDL	373 ± 1341	1101 ± 910	305 ± 600	768 ± 1332

Results are expressed as Relative Luminescence Units (RLU) and represent means of eight replicate values. Typical results from one of four independent experiments are shown. Days Pi, days post-infection; BDL, below detectable limit.

regions. A mutation in the P2 residue of the CA/SP1 cleavage site (KARVL-AEAMS mutated to KAR ***I*** L-AEAMS, also designated as V362I) was observed in TPV-resistant viruses, while all other cleavage sites remained unchanged. This V362I mutation was first observed in variants from passage 39 (2/10 clones sequenced) and it was present in 24/25 clones sequenced at later passages, even upon removal of the inhibitor for 12 passages in culture. Although V362I results in a conservative amino acid change in sequence, its genetic stability during the selection experiments suggested that it could have an important role in virus survival. The V362I mutation was therefore introduced into TPV.49 and TPV.73 clones and their replication capacity compared to that of parental clones containing WT cleavage sites. Table 4 shows that the presence of the CA/SP1 cleavage site mutation in clones TPV49_CA and TPV.73_CA did not lead to any gains in replication capacity as luciferase signals still remained at the limit of detection.

A different measure of the effects of cleavage site mutations on viral fitness is the study of virion-associated polyprotein processing. Cleavage site mutations can indeed partially or completely reverse the altered patterns of precursor processing caused by the presence of mutations in the viral protease (Doyon et al., 1996; Maguire et al., 2002). Therefore, capsid processing was studied in TPV.49 and TPV.73 viruses that either contained WT or mutant cleavage site sequences (Fig. 1A). Results show that in TPV-resistant clones containing WT cleavage sites, processing of the capsid protein occurred quite efficiently as very little accumulation of intermediate precursors was observed. Except for the presence of a faint band of molecular weight consistent with the presence of the MA–CA precursor in TPV.49 viruses, capsid processing in these TPV-resistant viruses was indeed comparable to that observed in WT virus. The addition of the CA/SP1 mutation in TPV.49 CA, however, led to the disappearance of the MA–CA precursor band, whereas it led to the accumulation of precursors in both WT and TPV.73 CA viruses. These results suggest that the CA/SP1 cleavage site mutation has differential effects on CA processing depending on the genotype of the protease present in the virus.

Finally, to determine if the CA/SP1 mutation affected the susceptibility of viruses to TPV, EC_{50}s were determined (Fig. 1B). The presence of the CA/SP1 mutation did not alter the susceptibility to TPV of WT CA, TPV.49 CA or TPV.73 CA viruses compared to their parental strains containing a wild type CA/SP1 cleavage site.

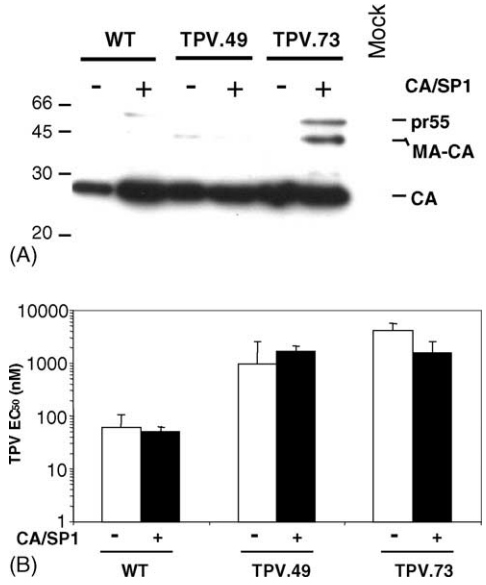

Fig. 1. Effects of the CA/SP1 cleavage site mutation on precursor processing and viral susceptibility to TPV. (A) Analysis of capsid precursor processing in WT and mutant virions by western blot. Supernatants from HIV-transfected cells were harvested and virion-associated proteins were analyzed by western blot using a CA-specific antibody. Bands corresponding to the mature CA protein as well as the precursors MA-CA and $p55^{gag}$(pr55) are identified on the right of the Figure. Clones containing the CA/SP1 mutation (WT CA, TPV.49 CA and TPV.73 CA) are in the "+" lanes. Results typical of those obtained in three independent experiments are shown. (B) Susceptibility to TPV of viruses containing a CA/SP1 cleavage site mutation. Mutant viral clones containing WT or mutant proteases and CA/SP1 cleavage sites were tested in EC_{50} assays to determine their susceptibility to TPV. Clones containing WT CA/SP1 sequences are identified by white bars, whereas clones containing a mutant CA/SP1 (WT CA, TPV.49 CA and TPV.73 CA) are identified by black bars. Mean EC_{50}s from two to three determinations are shown.

4. Discussion

In this study, viruses with reduced susceptibility to the antiviral effects of the non-peptidic protease inhibitor TPV were selected by in vitro passaging. The development of resistance to TPV was slow, requiring 9 months of sequential passage to select viruses with 87-fold resistance to TPV. Sequential isolates exhibited <2–7-fold increases in resistance to TPV and were often (four of the six sequential isolates) associated with the acquisition of two new mutational changes in the protease gene. This is in contrast to observations with most other PIs for which high level resistance is often selected in less than 30 passages in vitro and increases of >10-fold in resistance are frequently observed between passages (Jacobsen et al., 1995; Markowitz et al., 1995; Partaledis et al., 1995; Patick et al., 1996; Carillo et al., 1998; Gong et al., 2000; Mo et al., 2003).

The first mutations to be selected in the presence of TPV were L33F and I84V at passage 16. It was however impossible to determine with certainty which of these two mutations was selected first since only one clone sequenced from passage 16 contained a single mutation (L33F) and PCR amplification of viruses growing at earlier passages failed to produce amplicons due to the very low viral replication in these cultures. In addition viral clones containing either mutation L33F or I84V alone or in combination failed to show significant levels of resistance. The relevance of these mutations for the development of reduced susceptibility to TPV was confirmed in vivo following TPV therapy (Mayers et al., 2004). Significant levels of resistance to TPV in vitro were not observed before passage 39 when the concentrations of TPV in culture

reached 2 μM and when viruses were shown to harbour five or six mutations in the protease. This was confirmed using reconstituted viral clones and suggests that the genetic barrier to resistance to TPV is relatively high. Some discrepancy was observed in the progression of resistance of viruses from passage 49 to 68. Whereas resistance seemed to decrease when analysing viral populations, the study of reconstituted viral clones showed a two-fold increased in resistance between these passages. This discrepancy is thought to be due to the limitations of working with viral populations where the amplification of minor genotypes during assays can significantly influence the results.

The results presented in this study demonstrate that 10 mutations are involved in the in vitro development of resistance to TPV: L10F, I13V, V32I, L33F, M36I, K45I, I54V, A71V, V82L, I84V. All of these mutations have been reported to be selected in the presence of other protease inhibitors except for the active site mutation V82L which seems to be unique to TPV (Johnson et al., 2004). HIV protease mutation of Val82 to Ala, Phe, Thr, or Ser has previously been implicated in resistance to PIs. However, this is the first description of a Leu substitution at the V82 position (Johnson et al., 2004). Interestingly, initial attempts to raise resistance to TPV also identified V82L in the protease of emerging viruses; however, the investigators were unable to reconstitute a resistance phenotype by reintroducing this mutation into a wild type background (Kemp et al., 2000). The present study confirms that V82L alone does not confer resistance to TPV, but V82L contributes to resistance when selected in the context of the complex genotype containing five pre-existing mutations. V82L was responsible for a 2.4-fold increase in TPV resistance in this context.

These results also highlight the requirement for the presence of six mutations in the protease (I13V, V32I, L33F, K45I, V82L, I84V, including three in the active site of the enzyme) to confer >10-fold resistance to TPV. Therefore these results suggest that although the mutations selected by this non-peptidic PI do not significantly differ from those selected by peptidic PIs, the genetic barrier for the development of resistance to TPV is higher than for most other PIs. This could explain why TPV has such a broad activity against PI-resistant clinical isolates and why significant resistance to TPV is so slow to develop in cell culture.

Viruses showing >10-fold resistance to TPV also showed high levels of cross-resistance to other PIs. Cross-resistance levels ranged from 2- to 154-fold but susceptibility to SQV was largely unaffected. Most probably this is attributed to the fact that TPV failed to select the SQV primary mutations G48V and L90M in these studies (Jacobsen et al., 1995; Johnson et al., 2004). Cross-resistance was most significant towards the PIs ATV and RTV, inhibitors for which in vitro resistance was previously reported to be associated with mutations at positions V32I/L33F/A71V/I84V (ATV) (Gong et al., 2000) or V82F/I84V (RTV) (Markowitz et al., 1995). These mutant genotypes contain subsets of the mutations necessary for resistance to TPV.

The genetic events leading to reduced susceptibility to the antiviral effects of TPV were also accompanied by a novel mutation in the CA/SP1 gag cleavage site. This V362I mutation did not increase resistance to TPV nor did it improve the significantly impaired replication capacity of resistant viruses. This is in contrast with other cleavage site mutations, namely A431V and L449F, which have been shown to improve the replicative capacity of PI-resistant viruses harbouring multiple mutations in the protease gene (Doyon et al., 1996; Zhang et al., 1997; Carillo et al., 1998; Mammano et al., 1998; Robinson et al., 2000; Maguire et al., 2002) and, in one study, to increase resistance to APV (Maguire et al., 2002).

Interestingly, the CA/SP1 mutation did affect virion-associated CA processing under certain circumstances, however, the effects were dependent on the protease genotype. Indeed, for isolate TPV.49 containing six resistance mutations, virion-associated CA processing was more efficient in the presence of the CA/SP1 mutation than in the presence of the WT CA/SP1 cleavage site. In contrast, CA processing in WT and TPV.73 viruses was more efficient without the CA/SP1 mutation. One explanation for these results could be that the CA/SP1 mutation was initially selected in viruses poorly processing this cleavage site and remained fixed in the population at later passages despite not being beneficial to CA/SP1 processing in TPV.73 viruses, whereas the accumulation of additional mutations in the protease at further passages shifted the specificity of the enzyme to favor the WT cleavage site lacking the V362I mutation. It is also possible that this mutation could represent a purely stochastic mutational event co-selected with a resistance mutation but providing no biologically relevant effect on viral fitness. Such a mutation might be susceptible to fixation in the viral population and may be maintained through the selection experiment if it contributes no detrimental effect to viral fitness. Recent reports of selection of mutations in residues adjacent to V362 during the development of resistance to betulinic acid derivatives, however, suggest that this region can be involved in drug evasion when virus is cultured under selective pressure with inhibitors which influence capsid processing/assembly (Li et al., 2003; Zhou et al., 2004a,b).

In conclusion, resistance to TPV is not associated with a limited number of dominant signature mutations with high impact but requires the accumulation of multiple mutational changes each of which incrementally effects TPV sensitivity. These observations highlight the potential of TPV to exhibit a high genetic barrier to the development of drug resistance in the clinic. Moreover, the large number of mutational changes necessary to observe significant levels of resistant suggests that TPV will be a useful protease inhibitor in patients with complex PI resistance genotypes. The identification of combinations of 2–10 mutations in the protease associated with reduced susceptibility to TPV begin to provide a basis for the prospective identification of complex genotypes which may be predictive of treatment response to this new non-peptidic protease inhibitor.

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