Tipranavir inhibits broadly protease inhibitor-resistant HIV-1 clinical samples

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Objective: Although the use of HIV-1 protease inhibitors (PI) has substantially benefited HIV-1-infected individuals, new PI are urgently needed, as broad PI resistance and therapy failure is common.

Methods: The antiviral activity of tipranavir (TPV), a non-peptidic PI, was assessed in *in vitro* culture for 134 clinical isolates with a wide range of resistance to currently available peptidomimetic PI. The susceptibility of all 134 variants was then re-tested with the four PI simultaneously with TPV, using the Antivirogram™ assay.

Results: Of 105 viruses with more than tenfold resistance to three or four PI and an average of 6.1 PI mutations per sample, 95 (90%) were susceptible to TPV; eight (8%) had four- to tenfold resistance to TPV and only two (2%) had more than tenfold resistance.

Conclusions: The substantial lack of PI cross-resistance to TPV shown by highly PI-resistant clinical isolates makes TPV an attractive new-generation HIV inhibitor.

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Introduction

The widespread use of HIV-1 protease inhibitors (PI) in multidrug combinations has been a major factor in the current success of antiretroviral therapy. Unfortunately, therapy failure remains common and is frequently attributed to the emergence of drug-resistant HIV-1 strains [1–3]. A feature of resistance to the PI has been the observation that HIV-1 highly resistant to one drug in this class is often cross-resistant to other approved PI [4–6]. For example, strains highly resistant to indinavir (IDV) are commonly cross-resistant to ritonavir (RTV), nelfinavir (NFV) and saquinavir (SQV) [7,8]. Currently, substantial numbers of HIV-1-infected individuals receiving antiretroviral therapy may harbor virus broadly cross-resistant to PI [9,10; for review see 11]. Consequently, there is an urgent clinical need to develop new PI which are able to inhibit these broadly resistant HIV-1 variants.

Tipranavir (TPV) is a novel non-peptidic HIV-1 protease inhibitor, currently under clinical investigation [12,13]. Unlike the approved PI that are modeled on peptidic backbones, TPV was developed from a non-peptidic coumarin template [14], the antiprotease activity of which was discovered by high-throughput screening [15]. Like other PI, TPV is a potent inhibitor of the HIV-1 protease activity *in vitro* (K_i 0.008 nmol/l) and in cell culture, with an average 50% inhibitory dose (IC_{50}) below 0.1 μmol/l [16]. Furthermore, phase II clinical trials have shown that TPV is safe and well tolerated in HIV-infected patients [17,18].

It was anticipated from the non-peptidic nature of TPV and the crystallographic analysis of TPV-binding interactions with the protease that this inhibitor might have activity against HIV-1 strains that have become resistant to the current peptidomimetic PI. Indeed, preliminary data have suggested that TPV can block

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the replication of a number of viruses resistant to the current range of PI [3]. In this study, TPV was screened against a substantial panel of highly PI-resistant clinical isolates in order to determine the spectrum of activity of this inhibitor. Extensive genotypic analysis was performed to identify the major amino acid substitutions in HIV-1 protease associated with TPV sensitivity and resistance.

Materials and methods

Samples obtained from patients were submitted for routine assessment of drug susceptibility in order to identify samples resistant to PI. Overall, 127/134 of the samples had been taken from different individuals (multiple samples from the same individuals were from different sampling times). Although antiretroviral therapy histories were unavailable, the phenotypic resistance patterns and genotypes suggested that the patients had received extensive PI therapy. Subsequently, the *in vitro* susceptibility to IDV, RTV, NFV and SQV was determined and a group of 105 samples was selected with tenfold or greater increases in IC_{50} (relative to a wild-type control virus) to at least three of these four inhibitors (as this study was performed prior to the approval of amprenavir, this PI was not included in the analysis). A further group of 29 recombinant viruses had tenfold or greater resistance individually to RTV, NFV or SQV. The susceptibility of all 134 variants was then re-tested to the four PI simultaneously with TPV.

Sample preparation

Viral RNA was extracted from 200 µl patient plasma using the QIAamp Viral RNA Extraction Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. cDNA encompassing part of *pol* was produced using Expand reverse transcriptase (RT) (Boehringer Mannheim, Mannheim, Germany) as described previously [19]. A 2.2 kb fragment encoding the protease and RT regions was then amplified by nested polymerase chain reaction (PCR) using PCR primers and conditions as described [20]. This genetic material was subsequently used in both phenotyping and genotyping experiments.

Phenotypic susceptibility testing

MT-4 cells [21] were co-transfected with *pol* PCR fragments and a HIV-1 molecular clone with deletion of *gag*–protease–RT, as described [20,22]. This resulted in viable recombinant viruses containing part of *gag*, plus protease and RT (up to codon 400) from the donor PCR fragment. Phenotypic susceptibility to PI was determined using an MT-4 cell assay the Antivirogram™ [20,22]. Resistance values were derived by dividing the mean IC_{50} for a patient's recombinant virus by the mean IC_{50} for wild-type control virus (strain HXB2-D).

Genetic sequencing

The PCR products obtained from patient plasma samples were genotyped by dideoxynucleotide-based sequence analysis. Samples were sequenced using the Big Dye terminator kit (Applied Biosystems, Foster City, California, USA) and resolved on an ABI 377 DNA sequencer [23].

Results

The results of the 105 highly cross-resistant panel are shown in Fig. 1 and the susceptibilities of the 29 individually resistant variants (defined as having a greater than tenfold increase in IC_{50} value to a single PI) are shown in Table 1. The majority of broadly PI cross-resistant isolates were TPV sensitive (95/105; 90%); 8/105 (8%) had a four- to tenfold increase in TPV IC_{50} and only 2/105 (2%) had greater than a tenfold increase in TPV IC_{50} value. The mean increases in IC_{50} (SE in parentheses) were as follows: IDV, 44-fold (2.8); RTV, 87-fold (6.0); NFV, 45-fold (2.1); SQV, 46-fold (2.8); and TPV, 2-fold (0.23). All of the 29 variants that were individually resistant to RTV, NFV, or SQV remained fully sensitive to TPV or were even hypersensitive relative to the wild-type control (Table 1). Therefore, our data established that TPV was extremely effective at inhibiting a substantial range of PI-resistant clinical strains.

Genotypic analysis of all 134 samples revealed complex patterns of multiple mutations in the protease coding regions. Figure 2a illustrates the relative frequencies of recognized primary and secondary mutations in the 105 highly PI-resistant samples. The average number of documented PI resistance mutations per sample in this group was 6.1. Substitutions at the following 19 HIV-1 protease amino acid residues were considered to be associated with PI resistance: 10, 20, 24, **30**, 32, 33, 36, 46, 47, **48**, **50**, 54, 71, 73, 77, **82**, **84**, 88, and **90** (major or 'primary' mutations are shown in bold type). As anticipated from previous studies [10,11], there was a predominance of major or 'primary' mutations at codons 82, 84, 90 and secondary mutations at codons 10, 36, 46, 54, 71 and 77. The frequencies of PI mutations seen in the RTV-, NFV- or SQV-resistant strains are also shown in Fig. 2. This analysis revealed, as expected, marked differences in PI mutation patterns between the different groups. For example, there was a predominance of characteristic codon 30 and 88 mutations in the group resistant only to NFV and a predominance of characteristic codon 48 and 90 mutations in the group resistant only to SQV. This genotypic analysis demonstrated that the 134 samples studied contained a wide spectrum of different PI resistant genotypes, the vast majority of which remained TPV susceptible.

	INV	RTV	NFV	SQV	TPV
1	38	172	44	64	15
2	93	71	74	27	14
3	62	68	ND	16	9.7
4	91	174	74	89	8
5	62	149	48	46	7
6	52	253	73	75	6
7	37	86	52	89	5
8	75	143	45	33	5
9	70	188	48	46	5
10	43	94	29	82	5
11	19	11	61	82	4
12	5	24	24	30	3
13	61	120	48	46	3
14	81	196	79	95	3
15	37	62	39	32	3
16	86	147	68	82	3
17	17	65	48	46	2
18	8	89	23	28	2
19	59	88	34	8.6	2
20	51	175	44	73	2
21	85	102	69	52	2
22	70	102	48	46	2
23	44	78	39	32	2
24	41	145	43	13	2
25	63	176	54	21	2
26	13	73	19	5	2
27	7	43	27	32	2
28	76	107	38	12	2
29	36	70	44	52	2
30	65	93	68	82	2
31	69	67	52	82	2
32	45	176	41	56	2
33	11	56	13	5	2
34	15	42	40	39	2
35	81	114	79	95	2
36	32	60	64	44	1
37	18	167	27	11	1
38	31	55	48	82	1
39	42	133	43	16	1
40	86	9.7	68	59	1
41	8.5	16	28	42	1
42	85	147	69	52	1
43	100	221	91	125	1
44	51	156	68	82	1
45	13	13	21	14	1
46	20	24	32	6	1
47	100	175	91	38	1
48	13	82	9.3	32	1
49	28	168	39	32	1
50	105	64	68	82	1
51	14	27	46	55	1
52	29	22	19	82	1
53	28	164	21	2	1
54	24	62	54	42	1
55	12	38	14	24	1
56	100	278	91	125	0.9
57	85	236	69	52	0.9
58	8	48	16	32	0.9
59	55	186	73	42	0.9
60	13	131	39	32	0.9
61	26	78	35	32	0.9
62	9.7	23	19	15	0.9
63	44	116	39	32	0.9
64	44	168	39	20	0.9
65	44	54	39	32	0.8
66	35	73	46	24	0.8
67	26	74	39	32	0.8
68	85	83	56	52	0.8
69	35	28	72	18	0.8
70	81	29	63	95	0.7
71	37	24	77	57	0.7
72	18	78	24	4	0.7
73	60	29	40	62	0.6
74	21	58	27	14	0.6
75	23	45	22	27	0.6
76	27	24	39	32	0.6
77	35	114	29	13	0.6
78	35	20	48	9.6	0.6
79	27	20	57	19	0.6
80	105	90	68	82	0.6
81	66	55	72	42	0.6
82	12	26	35	42	0.6
83	17	16	18	16	0.6
84	19	40	39	32	0.5
85	67	24	45	82	0.5
86	53	100	72	42	0.5
87	82	99	91	125	0.4
88	38	113	74	95	0.4
89	30	91	35	13	0.4
90	41	28	38	82	0.4
91	90	70	68	82	0.4
92	23	9.6	7	46.0	0.4
93	80	152	91	67	0.3
94	66	143	53	44	0.3
95	59	25	48	42	0.3
96	9.3	62	13	32	0.2
97	44	55	39	32	0.2
98	7	16	18	18	0.2
99	5	NA	11	32	0.2
100	20	35	8.7	82	0.2
101	8.6	16	14	10	0.2
102	11	11	22	52	0.2
103	19	44	15	52	0.2
104	18	26	25	29	0.1
105	17	24	27	34	0.1

Fig. 1. Phenotypic susceptibility of HIV-1 clinical isolates to protease inhibitors. Recombinant clinical strains of HIV-1 previously shown to have at least a tenfold increase in IC_{50} value (50% inhibitory dose) to three or all four of indinavir (IDV), ritonavir (RTV), nelfinavir (NFV) and saquinavir (SQV) were chosen for susceptibility testing. Resistance values were derived by dividing the mean IC_{50} for a patient's recombinant virus by the mean IC_{50} for wild-type control virus (strain HXB2-D). All determinations were done in four replicate wells per drug concentration and in duplicate microtiter plates. Samples that showed a greater than fourfold increase in IC_{50} to TPV were tested independently at least twice and the mean fold relative increase in IC_{50} value is shown: green, less than fourfold; yellow, four to tenfold; red, greater than tenfold.

In an attempt to define those mutations responsible for TPV resistance and mutational patterns that did not confer resistance, two subsets of samples from the 105 highly resistant group were analysed. Since there were only two samples with greater than tenfold increase in IC_{50} values, all samples with a greater than fourfold increase in TPV IC_{50} were grouped together ($n = 10$); the mean increases in IC_{50} (SE in parentheses) were: TPV, 8-fold (1.1); IDV, 62-fold (6.4); RTV, 140-fold (18.7); NFV, 54-fold (5.0); and SQV, 57-fold (8.3). The mutational patterns were also examined in 19 samples with > 2.5-fold increased TPV sensitivity ('hypersensitivity') relative to the wild-type control. The mean fold *decrease* in IC_{50} (SE in parentheses) for TPV was 3 (0.02); mean increases in IC_{50} for the other PI were: IDV, 35-fold (6.3); RTV, 57-fold (10.4); NFV, 37-fold (6.2); and SQV, 51-fold (7.0). The PI mutation frequencies in these two subsets are illustrated in Fig. 3a. The average number of documented PI resistance mutations per sample was 6.8 in the TPV-

Table 1. Tipranavir susceptibility of HIV-1 variants resistant to individual protease inhibitors.

	Resistance value[b] (SE)				
Resistance group[a]	IDV	RTV	NFV	SQV	TPV
RTV (n = 14)	5 (0.5)	31 (7.9)	5 (0.69)	2 (0.54)	1 (0.12)
NFV (n = 10)	1 (0.13)	1 (0.1)	33 (10.5)	1 (0.13)	1 (0.1)
SQV (n = 5)	2 (0.19)	3 (0.73)	2 (0.25)	10 (1.7)	0.4 (0.03)

[a]Resistance group refers to the specific single inhibitor to which the viruses are resistant.
[b]Resistance values were derived by dividing the mean IC_{50} (50% inhibitory dose) for a patient's recombinant virus by the mean IC_{50} for wild-type control virus (strain HXB2-D).

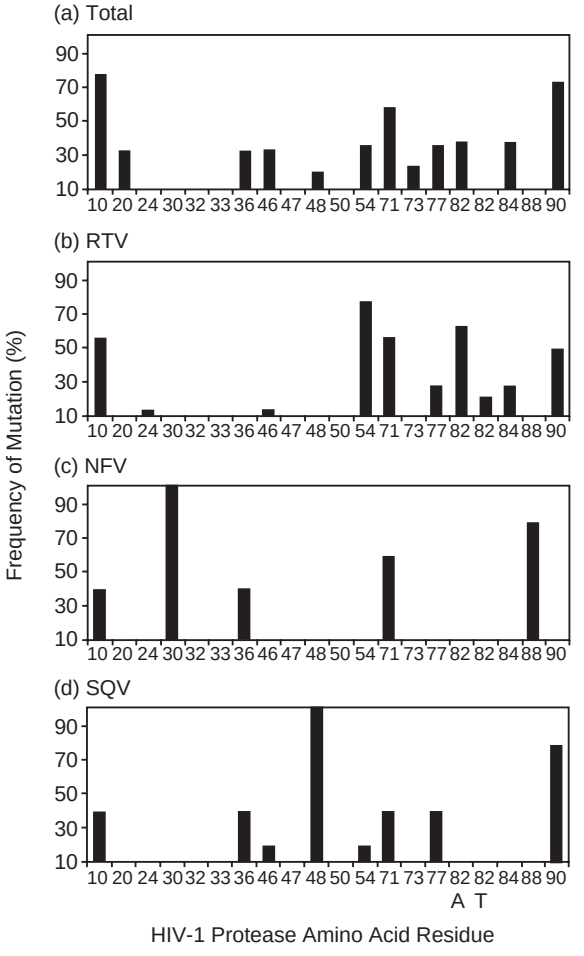

Fig. 2. Frequency of mutations affecting resistance to protease inhibitors (PI) in resistant HIV-1 varients. RTV, ritonavir; NFV, nelfinavir; SQV, saquinavir. Resistance-associated mutations present at a frequency of 10% or greater are shown. For this analysis a mutant was defined as the presence of a fully mutated residue or a mixture of mutant and wild type (where the mutant constituted at least 20% of the mixed base). Substitutions of V82A (A) or V82T (T) are indicated as separate mutation frequencies. (a) Highly PI-resistant group of 105 samples. (b) Samples only resistant to ritonavir (RTV; n = 14). (c) Samples only resistant to nelfinavir (NFV; n = 10); (d) Samples only resistant to saquinavir (SQV; n = 5).

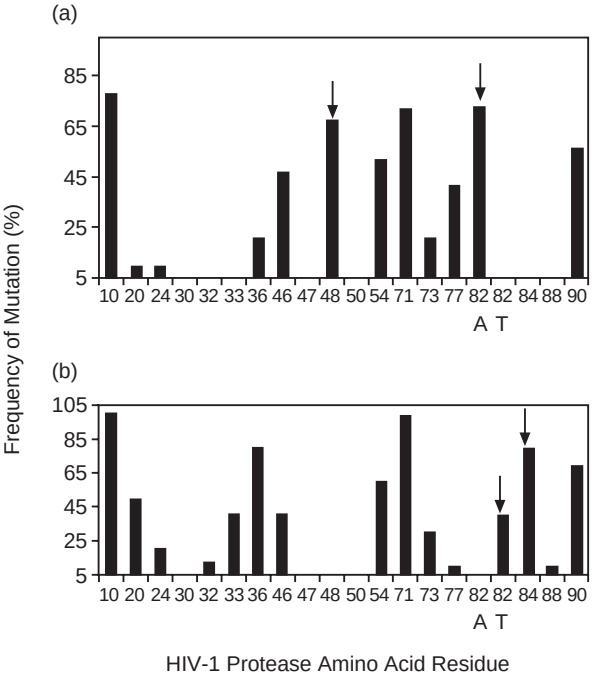

Fig. 3. Frequency of mutations affecting resistance to protease inhibitors in HIV-1 clinical isolates hypersensitive or resistant to tipranavir. Mutations present at a frequency of 10% or greater are shown for both groups of resistant viruses. The mutant definition was as described in Fig. 3. Substitutions of V82A (A) or V82T (T) are indicated as separate mutation frequencies. (a) Tipranavir-hypersensitive samples (n = 19). This virus group had a decrease in TPV IC_{50} value (relative to the wild-type control) of 2.5-fold or greater. The arrows indicate the frequent G48V and V82A mutations in these samples. (b) Tipranavir-resistant samples (n = 10). This virus group had an increase in TPV IC_{50} value (relative to the wild-type control) of fourfold or greater. The arrows indicate the frequent V82T and I84V mutations in these samples.

resistant group and 6.3 in the 'hypersensitive' group (although there were numerous additional polymorphisms in both groups relative to the laboratory reference strain HXB2-D). It was striking that the TPV-hyper-

sensitive group had a high frequency of G48V and V82A mutations (Fig. 3a). By contrast, the TPV-resistant group had a high frequency of the relatively rare mutation V82T, plus I84V (Fig. 3b). Examination of individual samples with reduced TPV susceptibility revealed two clusters of mutation patterns, either 82T with 84V or 84V with 90M (both with numerous secondary mutations) (data not shown). However, we also identified TPV-sensitive samples that had combinations of mutations including 84V and 90M. Therefore, the precise combinations of PI resistance mutations that dictate resistance to TPV remain to be elucidated. It is possible that novel mutation patterns could confer TPV resistance since the samples with reduced TPV susceptibility contained an average of 9.2 polymorphisms in the protease, in addition to recognized sites of PI resistance.

Discussion

Previous studies have provided clues as to why TPV might be less affected by the mutations described above that give rise to resistance to peptidomimetic PI. Specifically, peptidomimetic inhibitors bind to the protease via an extensive hydrogen bonding network, resulting in the main chain of the inhibitor being extended and rigidly constrained in the binding pocket [12]. Hydrogen bonds are highly directional, allowing for little flexibility. In contrast, TPV binds to the protease with far fewer key hydrogen bonds and is more dependent on hydrophobic interactions [12]. The notion of flexibility of the inhibitor to adjust to amino acid changes in the active site is supported by the fact that a range of different hydrophobic groups at C-6 and C-3 positions are well tolerated. In addition, X-ray derived structures of different protease–inhibitor complexes revealed subtle differences in the conformation of different ligands in the active site [12]. These observations provided an interesting rationale for testing the susceptibility of TPV against a large range of PI-resistant clinical isolates.

In view of the IC_{50} value (about 0.1 μmol/l) that is typically seen with TPV against wild-type HIV-1 (somewhat higher than the US Food and Drug Administration approved PI), the potential clinical efficacy of this inhibitor was of obvious interest. To address this issue, a recent phase II study in antiretroviral drug-naive subjects involved treatment with TPV with or without low-dose RTV [24]. Median decreases of HIV-1 plasma RNA of 0.8–1.6 $\log_{10}$ copies/ml were achieved with median TPV trough concentrations of 0.76–67 μmol/l [24]. These data clearly demonstrated that TPV has clinical activity against susceptible HIV-1 strains.

In summary we have used an *in vitro* culture approach to define the antiviral activity of TPV against a wide spectrum of clinically relevant, highly PI-resistant strains of HIV-1. This extensive assessment demonstrated a substantial and surprising lack of cross-resistance. Mutational patterns could be identified that differentiated TPV-resistant strains from those hypersensitive to the inhibitor. However, extensive site-directed mutagenesis and *in vitro* drug-selection studies will be required to define the precise genetic nature of TPV resistance, which is likely to be relatively complex. Since TPV has already shown significant clinical activity in antiretroviral drug-naive patients [17,18,24], it will be important to establish if the promising *in vitro* activity reported here translates into activity in patients who harbor PI-resistant strains. Such studies in patients with a history of therapy with other PI are currently in progress. Finally, pre-defining an individual's resistance status should facilitate the selection of potentially active partner drugs that can be used in combination with TPV to protect its activity and optimize viral suppression.

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