Simultaneous mutations at Tyr-181 and Tyr-188 in HIV-1 reverse transcriptase prevents inhibition of RNA-dependent DNA polymerase activity by the bisheteroarylpiperazine (BHAP) U-90152s

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Abstract The replacement of either Tyr-181 or Tyr-188 of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) by the corresponding HIV-2 RT amino acids Ile-181 or Leu-188 is known to result in active mutant enzymes (Y181I; Y188L) with virtual loss of sensitivity towards three structural classes of nonnucleoside RT inhibitors; L-697,661, nevirapine, and TIBO R82913. The bisheteroarylpiperazine (BHAP) U-90152S, a highly specific inhibitor (IC_{50}, 0.29 ± 0.01 μM) of HIV-1 RT, inhibited the recombinant Y181I and Y188L HIV-1 RT mutants with IC_{50} values of 3.6 ± 0.15 μM and 0.71 ± 0.02 μM, respectively. Construction and in vitro analysis of double mutants Y181I/Y188L and Y181C/Y188L of HIV-1 RT showed >150-fold resistance to U-90152S. An HIV-2 RT mutant containing amino acids 176–190 from HIV-1 RT acquired full sensitivity to U-90152S (IC_{50}, 0.26 ± 0.01 μM). It is concluded that simultaneous mutations at Tyr-181 and Tyr-188 of HIV-1 RT promotes resistance to U-90152S.

Key words: HIV-1 reverse transcriptase; HIV-1 RT mutant; Resistance to U-90152S; HIV-2 RT; Double mutant

1. Introduction

The human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is a well established target for therapeutic intervention of AIDS [1]. A number of different classes of highly specific nonnucleoside inhibitors of the HIV-1 RT have been recently described [2–7]. The most widely studied classes include the pyridinone L-697,661 [2], nevirapine [3,4], TIBO R82913 [5] and the BHAPs [6,7]. However, potential usefulness of some of these compounds is confounded by the accumulation of resistant viral variants both in cell culture as well as in HIV-1 infected patients [8–15].

A three-dimensional co-crystal-structure of HIV-1 RT and nevirapine shows that the side chains of Tyr-181 and Tyr-188 are in contact with nevirapine [16]. The structural data are consistent with the previous chemical cross-linking studies implicating the involvement of these residues in drug binding [17–19]. The replacement of Tyr-181 or Tyr-188 with the corresponding HIV-2 RT amino acids (Ile-181 or Leu-188) resulted in HIV-1 RT mutants which were insensitive to L-697,661, nevirapine, and TIBO 82913 [20]. It is believed that insensitivity of HIV-2 RT to these NNRTIs is primarily due to the presence of Ile-181 or Leu-188 [17,20–22]. Subsequently, there were differences in the pattern of resistance mutations observed in the presence of NNRTIs [11,23–27]. Taken together, these studies suggest that there is a common binding pocket [16] and overlapping binding sites for different inhibitors.

In contrast to other classes of NNRTIs, the BHAP U-90152S selects for a resistance mutation (P236L) at a conserved site of RT with the consequence of conferring sensitization to other NNRTIs [11]. Recent site-directed mutagenesis studies at and around P236 have suggested that the P236L mutation triggers both resistance to U-90152S and sensitization to L-697,661 [28]. In this paper we investigate activity of U-90152S against the mutant HIV-1 RT enzymes bearing mutations at Tyr-181, Tyr-188, or Tyr-181/Tyr-188. Our studies show that U-90152S is active against the mutant RT enzyme Y181I or Y188L. Studies with the double mutants suggest a requirement for simultaneous mutations in HIV-1 RT at Y181 and Y188 for HIV-1 to escape U-90152S-mediated inhibition. Furthermore, we demonstrate that the U-90152S-binding requires the 176–190 domain from the β strands 9 and 10 of HIV-1 RT.

2. Materials and methods

2.1. HIV-1 RT inhibitors and other chemicals

L-697,661 and U-90152S were kindly supplied by Drs. H.W. Smith and D.L. Romero of Upjohn Laboratories. General laboratory chemicals were purchased from Sigma and Bio-Rad. Low melting point agarose, Taq polymerase, T4 ligase, restriction enzymes *Eco*RI and *Hin*dIII, and molecular weight markers were from Gibco/BRL. The substrates poly(rA):oligo(dT), poly(rC):oligo(dG), deoxythymidine triphosphate, deoxyguanosine triphosphate, and Chelating Sepharose for IMAC were purchased from Pharmacia/LKB Biotechnology Inc. The [^{3}H]dTTP and [^{3}H]dGTP were obtained from DuPont NEN and diluted with cold dTTP and dGTP, respectively.

2.2. Site-directed mutagenesis and expression of HIV-1 RT mutants

The hexa-His tagged HIV-1 RT expression plasmid called DE-5,2 [29] was used to generate site-specific point mutations by the 'mega-primer' method [30], as described elsewhere [28]. For each RT mutant, the 1.7 kb RT gene fragment obtained was subcloned into the expression vector pKK223-3 that had been digested with *Eco*RI and *Hin*dIII at the multiple cloning site [29]. Competent *E. coli* strain JM 109 was transformed with the mutated RT construct and resulting colonies were screened for expression of the p66 HIV-1 RT protein upon induction with 1 mM IPTG [29]. The *E. coli* cells were pelleted, lysed by SDS, and

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Abbreviations: HIV-1, HIV-2, human immunodeficiency virus types 1 and 2; RT, reverse transcriptase; AIDS, acquired immunodeficiency syndrome; IMAC, immobilized metal affinity chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; TCA, trichloroacetic acid; TIBO R82913, (+)-(5*S*)-4,5,6,7-tetrahydro-9-chloro-5-methyl-6-(3-methyl-2-butenyl)-imidazo[4,5,1-*j*,*k*][1,4]benzodiazepin-2(1*H*)-thione; BHAP, bisheteroarylpiperazine; NNRTIs, nonnucleoside reverse transcriptase inhibitors; PCR, polymerase chain reaction.

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the cell lysate was analyzed by 12% SDS-PAGE [31]. The mutated region of the selected clones was then sequenced by the dideoxy method using Sequenase Version 2.0 sequencing kit and ^{35}S.

2.3. Cloning and expression of HIV-2 RT containing the amino acid sequence 176–190 (PDIVIYQYMDDLYVG) from HIV-1 RT

Chimeric HIV-2 RT substituted with amino acids at position 176 to 190 from HIV-1 RT was constructed from plasmid NSF-20B-5. A hexa histidine linker was engineered into the N-terminal of HIV-2 RT (coding region 2381-4078, HIV-2_{rod} isolate, GenBank accession No. M15390) to facilitate purification by IMAC [28]. Cloning and expression of the chimeric HIV-2 RT protein was performed as described above.

2.4. Purification and characterization of HIV-1 RT mutants

The IMAC purification of the desired mutants, including the HIV-2 RT mutant, containing the hexa-histidine tag was carried out essentially as detailed elsewhere [32]. The p66/p51 heterodimers of the double mutants were obtained by in vitro processing of the corresponding p66 HIV-1 RT mutants with HIV-1 protease as described for the wild type p66 HIV-1 RT [32].

2.5. RNA-dependent DNA polymerase RT activity assay

Reverse transcriptase activity of HIV-1 RT and its mutants was determined using the previously described assay using poly(rA):oligo(dT) as the template primer [28,32]. Protein concentration was determined by the Bradford protein assay using BSA as the standard protein [32].

2.6. IC_{50} determinations

For inhibition studies, enzymatic assays were carried out in the presence and absence of HIV-1 RT inhibitors. All the inhibitors were prepared in 50% DMSO as 25× stock solutions and 2 μl of each inhibitor was added to give the desired inhibitor concentration. For determination of relative IC_{50} values with standard deviations, statistical analysis of the upper and lower confidence limits were calculated using the 3 parameter logistic model [33].

2.7. Kinetic studies

Kinetic studies were performed with varying template-primer [poly(rA):oligo(dT)] concentrations from 0.075 μM to 7.5 μM [32]. The rate of incorporation of [^{3}H] nucleotides by wild type and mutant enzymes (2 nM) was linear for 30 min. Studies were done under steady state conditions in 10 minassays as described above. The kinetic parameter K_m (μM) and the catalytic constant k_{cat} (nmol [^{3}H]dTTP incorporated/h) were determined using the non-linear regression analysis software program (Enzfitter; Biosoft), based on the Michaelis–Menten equation.

3. Results and discussion

3.1. Studies with HIV-1 RT mutants Y181I and Y188L

Significant homologies between HIV-1 RT and HIV-2 RT have been noted from an alignment of sequences in the β strands 5, 6, 9, 10, 12, 13, and 14 [17–21,28]. These β strands are important because they form the inhibitor binding pocket and are close to the polymerase active site [16,34–36]. We constructed a number of HIV-1 RT single and double mutants containing corresponding substitutions from HIV-2 RT. All the HIV-1 RT mutants were purified as p66 homodimers from crude *E. coli* extracts in the same manner by IMAC, as described previously for the wild type RT [28,32]. The chemical structures of the compounds used in the inhibition studies of these HIV-1 RT mutants are depicted in Fig. 1.

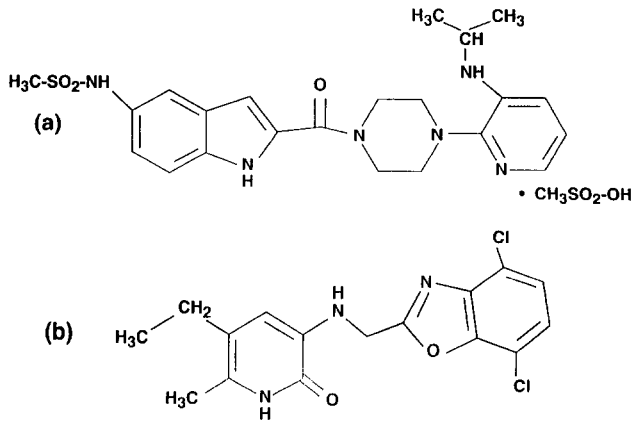

Fig. 1. Chemical structures of the BHAP U-90152S (a) and L-697,661 (b). These structures were published in the following references: L-697,661 in [20]; U-90152S in [7].

The RNA-dependent DNA polymerase activity of the HIV-1 RT single mutants was determined in the presence of U-90152S and the pyridinone derivative L-697,661 (Table 1). As shown, U-90152S and L-697,661 are potent inhibitors of the wild type enzyme with IC_{50} values of 0.29 ± 0.01 μM and 0.23 ± 0.01 μM, respectively. The single mutants Y181I and Y188L were highly resistant towards L-697,661, in agreement with an earlier report [20]. However, these HIV-1 RT mutants were inhibited by U-90152S with IC_{50} values of 3.60 ± 0.15 μM and 0.71 ± 0.02 μM, respectively. Similar results were obtained when poly(rC):oligo(dG) was the template-primer (data not shown). The V106I HIV-1 RT mutant, containing the corresponding HIV-2 RT amino acid from the β strand 6, was also included in the study. Both these compounds inhibited the single mutant V106I (Table 1), suggesting that V106 is not critical for inhibitory activity of U-90152S or L-697,661.

Table 1
Fifty percent inhibition of RNA-dependent DNA polymerase activity of mutant enzymes of HIV-1 RT by U-90152S and L-697,661

HIV-1 RT	IC_{50} (μM)[a]			
	U-90152S		L-697,661	
Wild type	0.29 ± 0.01	(1.0)	0.23 ± 0.01	(1.0)
Y181I	3.60 ± 0.15	(12.4)	>100	(>400)
Y188L	0.71 ± 0.02	(2.4)	35.2 ± 1.50	(153)
V106I	0.30 ± 0.01	(1.0)	0.21 ± 0.01	(1.0)

[a]The RT activity assays were carried out in the presence of poly(rA):oligo(dT) as the template primer. IC_{50} values greater than 100 μM cannot be determined accurately due to solubility limits. The data represent mean ± S.D. (n = 3). The fold differences in IC_{50} were calculated relative to the wild type HIV-1 RT and are shown in parenthesis.

Fig. 2 shows U-90152S-mediated inhibition profiles of HIV-1 RT mutants bearing substitutions from HIV-2 RT. For comparison purposes, U-90152S results with a HIV-1 RT mutant containing triple substitutions (H235W/D237T/T240K) from HIV-2 RT [28] are also presented. Fig. 2 also shows the effect of L-697,661 on the Y181I and Y188L HIV-1 RT mutants. In contrast to the three well known classes of NNRTIs [20], U-90152S is still relatively active versus the HIV-1 RT single mutants Y181I and Y188L. Our results suggest that these tyrosines alone interact very weakly with U-90152S.

3.2. Studies with the double mutants of HIV-1 RT

The above conclusions led to the view that perhaps simultaneous presence of two or more mutations at the non-conserved

sites in the β strands 9 and 10 might be required to produce HIV-1 RT that is highly resistant to U-90152S. Therefore, two double mutants were cloned, expressed, and purified. The double mutant Y181I/Y188L carried amino acid substitutions from HIV-2 RT and had been reported by others [18,19,27]. The other double mutant Y181C/Y188L has not been reported and contains amino acid substitutions based on the presence of individual mutations Y181C [12] or Y188L [14] in resistant viral isolates from patients treated with NNRTIs. The relative specific RT activities were 60990 ± 1252 units/mg and 53518 ± 924 units/mg for the Y181I/Y188L and Y181C/Y188L mutants, respectively. This was similar to specific RT activity of 48916 ± 633 units/mg for the wild type HIV-1 RT. The ratio of the kinetic parameters, k_{cat} and K_m, for the double mutants was also indistinguishable from the wild type HIV-1 RT (data not shown).

As shown in Table 2, the double mutants Y181I/Y188L and Y181C/Y188L showed >150-fold resistance to U-90152S, irrespective of the nature of the template primer used in the assay. Notably, these results were indistinguishable when compared with the results of the corresponding p66/p51 heterodimers of the double mutants (data not shown). A side by side comparison of the Y181I/Y188L mutant profile with the Y181I and Y188L single mutants is shown in Fig. 2. It is concluded that the simultaneous presence of both the mutations, which alone were inadequate to produce resistant enzymes (Fig. 2), resulted in a highly insensitive HIV-1 RT. Since both mutations in the Y181I/Y188L double mutant are derived from HIV-2 RT, it is likely that insensitivity of HIV-2 RT towards U-90152S, to some extent, is related to the simultaneous absence of tyrosines at 181 and 188 in this enzyme.

3.3. Studies with mutant HIV-2 RT containing the 176–190 amino acids from HIV-1 RT

It has been reported that an HIV-2 RT mutant with amino acid substitutions 176–190 from HIV-1 RT, called RT-2 (176–190), acquires sensitivity to nevirapine and TIBO R82913 [18,19]. We also constructed an HIV-2 RT substituted with the corresponding amino acids 176–190 (PDIVIYQYMDDLYV-G) from HIV-1 RT and showed that its U-90152S-sensitivity was indistinguishable from the wild type HIV-1 RT (Table 2). We conclude that the 176–190 domain in HIV-1 RT is involved in U-90152S binding. These results are consistent with our recent studies [28] which suggest that the P236L-mediated sensitization to L-697,661 and resistance to U-90152S are the consequence of alterations in the geometry of the inhibitor binding pocket [16,35].

Table 2
Sensitivity of multiple mutants of HIV-1 RT and HIV-2 RT towards U-90152S

Enzyme	U-90152S Inhibition (IC_{50}, μM)[a]			
	rA:dT		rC:dG	
Wild type HIV-1 RT	0.29 ± 0.01	(1.0)	0.12 ± 0.003	(1.0)
Y181I/Y188L HIV-1 RT	62 ± 2.50	(213)	26.3 ± 0.88	(219)
Y181C/Y188L HIV-1 RT	>100	(>300)	>100	(>300)
Wild type HIV-2 RT	>100	(>300)	>100	(>300)
HIV-2 RT (176–190)	0.27 ± 0.01	(1.0)	0.12 ± 0.004	(1.0)

[a]Fifty percent inhibition of RNA-dependent DNA polymerase activity was determined either using poly(rA):oligo(dT) or poly(rC):oligo(dG) as the template primer. IC_{50} values greater than 100 μM cannot be determined accurately due to solubility limits. The data represent mean ± S.D. (n = 3). The fold differences in IC_{50} were calculated relative to the wild type HIV-1 RT and are shown in parenthesis.

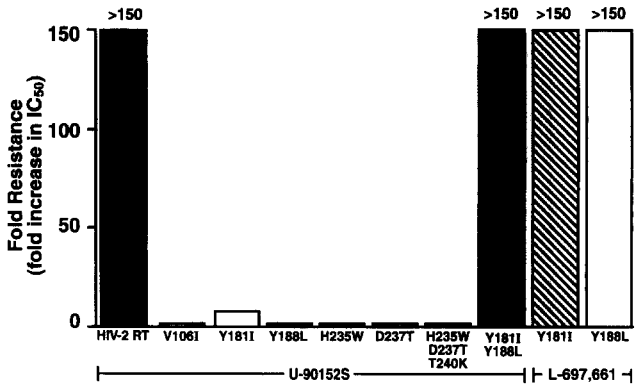

Fig. 2. Relative resistance of HIV-1 RT mutants and the wild type HIV-2 RT to U-90152S. Data for the H235W, D237T and T240K mutants are based on the results from [28]. Relative resistance was calculated as fold-increase in IC_{50}, by dividing the IC_{50} for each mutant with the IC_{50} for the wild type HIV-1 RT enzyme. The data are presented relative to the wild type HIV-1 RT, which is arbitrarily assigned a value of 1. For comparison purposes, resistance profiles of Y181I and Y188L HIV-1 RT mutants in the presence of L-697,661 are also included. The variation in the triplicate experiments was ≤5%. For other details, see section 2.

In addition to the well known Y181C and Y188L mutations, the Y181I mutation has also been observed in cell culture experiments [37]. The unique activity of U-90152S versus the Y181I or Y188L HIV-1 RT mutant (Fig. 2) distinguishes it from the other well known classes of NNRTIs [20–22,26]. The BHAP U-90152S is also active versus the Y181C HIV-1 RT mutant and selects for a P236L resistance mutation in cell culture experiments [25]. These characteristics of U-90152S are not shared by TIBO R82913, nevirapine, and L-697,661. However, the U-90152S-binding domain in HIV-1 RT seems to be also localized to residues 176–190 from β strands 9 and 10. Thus, it is tempting to speculate that U-90152S is targeted directly to the conserved amino acids of these β strands such that viral variants containing substitutions at these sites are incompatible with viral replication. Consequently, in the presence of U-90152S HIV-1 is forced to select for a resistant mutation (P236L) at the β13–β14 reverse turn [36], a site which does not appear to be in direct contact with the inhibitor [28].

The observed high level resistance (>150-fold) of the double mutants (Y181I/Y188L and Y181C/Y188L) toward U-90152S seems to be the consequence of changes in the shape of the inhibitor binding pocket [16]. The loss of inhibitory activity of U-90152S versus the double mutant Y181I/Y188L also implies that the simultaneous presence of both Ile 181 and Leu 188 in HIV-2 RT may play a role in its resistance to U-90152S. These results are consistent with the observation that sensitivity to U-90152S can be conferred onto HIV-2 RT by substitution of residues 176–190 from HIV-1 RT (Table 2).

It is becoming apparent that viral resistance during monotherapy with RT inhibitors is a serious concern. Thus, there is an urgent need for potent antiHIV agents for evaluation in combination with other active anti-HIV compounds. The chances of success of such an approach should increase if the compounds act on the same target with different mechanisms. Accordingly, in combination therapy, there is also the potential to derive mutually counteracting resistance mutations.

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