# Mechanism of resistance to U-90152S and sensitization to L-697,661 by a proline to leucine change at residue 236 of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase

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**Abstract Bisheteroarylpiperazines (BHAPs) are highly specific inhibitors of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT). BHAP-resistant HIV-1 is sensitized to other classes of nonnucleoside RT inhibitors and this has been primarily attributed to a proline-to-leucine substitution at amino acid 236 (P236L) of HIV-1 RT. To understand the basis for the in vitro sensitization-resistance phenomenon, single base pair mutations at amino acid P236 in HIV-1 RT were introduced to obtain P236L, P236T, P236H, P236R, and P236A HIV-1 RT mutants. Active HIV-1 RT mutants H235W, D237T, and H235W/D237T/T240K, containing substitutions from HIV-2 RT, were also cloned, expressed, and purified. Three BHAPs (U-88204E, U-87201E, and U-90125S) and the pyridinone L-697,661 were selected to quantitatively assess the effects of these amino acid substitutions on sensitization to L-697,661 and resistance to the BHAPs. The HIV-1 RT mutants bearing single (H235W; D237T) or multiple (H235W/D237T/T240K) HIV-2 RT substitutions around the conserved P236 conferred little resistance or sensitization to these RT inhibitors. The inhibition profiles of the P236 HIV-1 RT mutants demonstrated a direct correlation between sensitization to L-697,661 and resistance to the BHAPs. These results suggest alterations in the shape of the binding pocket as the mechanism by which the P236L mutation confers resistance to the BHAPs and sensitization to L-697,661.**

*Key words:* HIV-1 reverse transcriptase; HIV-1 RT mutant; Resistance to U-90152S; Sensititization to L-697,661; HIV-2 RT

## 1. Introduction

Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase is a well known target for chemotherapeutic intervention of AIDS. Recently, several classes of nonnucleoside reverse transcriptase inhibitors (NNRTIs), which are also potent inhibitors of HIV-1 replication in cell culture, have been identified. These include the pyridinone L-697,661 [1], nevirapine [2,3], the TIBO derivatives [4], TSAO [5], HEPT [6], inophyllums [7], and the BHAPs [8,9]. Despite their structural dissimilarity, they are all relatively inactive against HIV-2 RT and, in general, are non-competitive with respect to substrates.

The three-dimensional structure of HIV-1 RT complexed with nevirapine revealed that it binds to the enzyme in a hydrophobic pocket which is close to the polymerase active site of the p66 subunit [10]. This is consistent with the earlier affinity labeling [11,12] and mutational studies [12,13] which implicated interaction of nevirapine with the tyrosines, at positions 181 and 188 of HIV-1 RT. Moreover, HIV-1 RT enzymes mutated at either of these positions, with the corresponding amino acids from HIV-2 RT, show high resistance to TIBO R82150, L-697,661, and nevirapine [14]. When HIV-1 was grown in the presence of L-697,661, viral variants were resistant to all these three classes of nonnucleosides [15]. These [14,15] and other studies [1,7,16–21] suggest that most classes of NNRTIs appear to share the same binding pocket on the enzyme [10].

Recently, a BHAP (U-90152S)-resistant HIV-1 was found to contain a proline to leucine mutation at 236 (P236L) of HIV-1 RT that conferred increased sensitivity to L-697,661 [22]. How the P236L mutation in HIV-1 RT triggers both resistance to U-90152S and sensitization to L-697,661, is not known. To address this question, we constructed point mutants by site-directed mutagenesis at and around P236 of HIV-1 RT and studied their inhibition profiles in the presence of these NNRTIs. Our results suggest alteration in the shape of the binding pocket as the mechanism for the P236L-mediated resistance to the BHAPs and sensitization to L-697,661.

## 2. Materials and methods

### 2.1. HIV-1 RT inhibitors and other chemicals

L-697,661, U-88204E, U-87201E, and U-90152S were kindly supplied by Drs. J.R. Palmer, D.L. Romero and H.W. Smith of Upjohn Laboratories. General laboratory chemicals were obtained from Sigma. Protein reagents, SDS, Tris, ammonium persulfate, acrylamide and bis-acrylamide were purchased from Bio-Rad. Molecular weight markers, low melting point agarose, Taq polymerase, T4 ligase, restriction enzymes *Eco*RI and *Hin*dIII were from GIBCO/BRL. Glass microfiber filter paper was from Whatman. Poly(rA):oligo(dT), deoxythymidine and Chelating Sepharose were obtained from Pharmacia/LKB Biotechnology Inc. The radiolabled [$^3$H]dTTP was purchased from DuPont

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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; BSA, bovine serum albumin; TCA, trichloroacetic acid; TIBO R82150, (+)-(5*S*)-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)imidazo[4,5,1-j,k][1,4]-benzodiazepin-2(1H)- thione; TSAO, [2′,5′-bis-*O*(tert-butyl-dimethylsilyl)]-3′-spiro-5″-(4-amino-1″, 2″-oxathiole-2″,2″-dioxide); HEPT, 1-[(2-hydroxyethoxy)-methyl]-6-(phenylthio) thymine; BHAP, bisheteroarylpiperazine; NNRTIs, nonnucleoside reverse transcriptase inhibitors; DMSO, dimethylsulfoxide; PCR, polymerase chain reaction, IPTG, isopropyl-β-D-thiogalactopyranoside.


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NEN and was diluted with unlabeled dTTP to a specific activity of 200 cpm/pmol. The Ultima Gold scintillation fluid was from Packard. Samples were counted on a Packard 1900TR liquid scintillation counter using 4 ml Omni-vials (Wheaton). Sequencing grade [$^{35}$S]dATP was purchased from Dupont NEN and Sequenase Version 2.0 sequencing kit was obtained from USB. Oligonucleotide primers were purchased from Genosys Biotechnologies Inc.

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

Site-specific point mutations were generated by the 'megaprimer' method [23] using the hexa-His tagged HIV-1 RT expression clone DE-5,2 as the starting material [24]. A list of synthetic oligonucleotide primers used to construct mutated RT genes is shown below.

| Designation of primers | Sequence (5′–3′) |
|---|---|
| Upstream RT | 5′ AAACAGAATTCATGCCCATT |
| Downstream RT | 5′ CCAAAACAGAAGCTTCATAGTATT |
| H235W | 5′ TGTCCATTTATCAGGCCAGAGTTCATA |
| D237T | 5′ CTGTACTGTCCATTTAGTAGGATGGAG |
| H235W/D237T/T240K | 5′ TACTTTCCATTTAGTAGGCCAGAGTTCATAACC |
| P236L | 5′ CCATTCATCAAGATGGAGTT |
| P236T | 5′ CCATTCATCAGTATGGAGTT |
| P236R | 5′ CCATTCATCACGATGGAGTT |
| p236H | 5′ CCATTCATCATGATGGAGTT |
| p236A | 5′ CCATTCATCAGCATGGAGTT |

The megaprimer method utilizes three oligonucleotide primers and two rounds of PCR on the DE-5,2 plasmid DNA coding for HIV-1 RT. The desired mutation is introduced into the middle of a synthetic oligonucleotide matching the HIV-1 RT gene at both 3′ and 5′ to the desired mutation. This oligonucleotide is then used as one of the two primers in a PCR amplification reaction. The 'flanking' primer used contains the sequence in the junction between the 5′ region of the HIV-1 RT gene and the prokaryotic expression plasmid pKK223-3 and an *Eco*RI site. Twenty cycles of PCR were performed, and the appropriately amplified RT gene fragment was purified on a low melting agarose gel. In a second PCR, the purified DNA fragment was used as a megaprimer, together with a 'flanking' primer which contained sequences in 3′ end of the RT gene and a *Hin*dIII site. The amplified HIV-1 RT gene segment was cleaved with both *Eco*RI and *Hin*dIII. The 1.7 kb RT gene fragment obtained was subcloned into expression vector pKK223-3 that had been digested with *Eco*RI and *Hin*dIII at the multiple cloning site [24]. Competent *E. coli* strain JM 109 was transformed with the mutated RT construct and selected for ampicillin resistance. Resulting colonies were screened for expression of the p66 HIV-1 RT protein upon induction with 1 mM IPTG at 0.4 $OD_{600}$ nm as described elsewhere [24]. The *E. coli* cells were pelleted, lysed by SDS, and the cell lysate was analyzed by 12% SDS-PAGE. 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. Purification and characterization of HIV-1 RT mutants*

The crude *E. coli* extract was prepared by processing 10 g of cell paste [25] and adjusting the pH to 8.0 with 2 M Tris-HCl. The IMAC purification of the desired mutants containing the hexa-histidine tag was carried out on $Ni^{2+}$ charged columns equilibrated with 20 mM Tris, 0.5 M NaCl, pH 8.0, containing 10 μg/ml bestatin, leupeptin, aprotinin, and 1 mM phenylmethylsulfonyl fluoride and benzamidine. After loading, the column (8 ml bed volume) was washed with 10 column volumes of buffer containing 20 mM Tris, 1 M NaCl, pH 8.0, followed by 10 column volumes of the same buffer containing 0.5 M NaCl. The undesirable, bound proteins were washed away with buffer containing 75 mM imidazole. The HIV-1 RT mutants were eluted in the same buffer containing 300 mM imidazole. One ml fractions were collected and monitored for absorbance at 280 nm. Fractions containing protein, after analysis by a 12% SDS-polyacrylamide gel [26], were pooled and dialyzed into 200 mM Tris, 1 M NaCl, pH 7.4, and stored at −80°C.

*2.4. RNA-dependent DNA polymerase RT activity assay*

Reverse transcriptase activity of HIV-1 RT and its mutants was determined based on a standard method [24,25,27]. The assay solution (50 μl) contained poly(rA):oligo(dT) at a final concentration of 7.5 μM based on deca(dT) and 75 μM [$^3$H]dTTP in a buffer containing 25 mM Tris, pH 8.5, 5 mM $MgCl_2$, 1.5% glycerol, 50 μg/ml BSA and 0.01% NP-40. 10 μl of the RT enzyme or its mutants (4–6 nM) was incubated with the assay solution for 10 min at 370C. The specific HIV-1 RT activity (units/mg) was determined as described elsewhere [25].

*2.5. $IC_{50}$ 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. The final DMSO concentration was maintained at <5% in the assay mixture. All assays were performed in triplicate and the concentration of inhibitors that produced 50% inhibition ($IC_{50}$) were mean values from three independent assays. 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 [28]. The $IC_{50}$ values were used as a measure of the relative binding affinities of these inhibitors versus the HIV-1 RT mutants.

## 3. Results and discussion

*3.1. Rationale for selection of 235, 237, and 240 HIV-1 RT mutants*

Recently we reported a BHAP(U-90152S)-resistance mutation (P236L) in HIV-1 RT that confers increased sensitivity to the pyridinone L-697,661 [22]. Neither of these compounds inhibit RNA-dependent DNA polymerase activity of HIV-2 RT. It was noted from a comparison of reverse transcriptase amino acid sequences from HIV-1 and HIV-2 that P236 is conserved while amino acids at 235, 237, and 240 are non-conserved, as shown below.

HIV-1 RT 225-PPFLWMGYELHPDKWT-**240**
HIV-2 RT PPFQWMGYELWPTKW**K**

This comparison suggested that if the non-conserved amino acids surrounding the conserved proline are directly involved in binding to the BHAP U-90152S, then changing these residues to the corresponding amino acids from HIV-2 RT should result in resistant HIV-1 RT mutants. To test this possibility, we

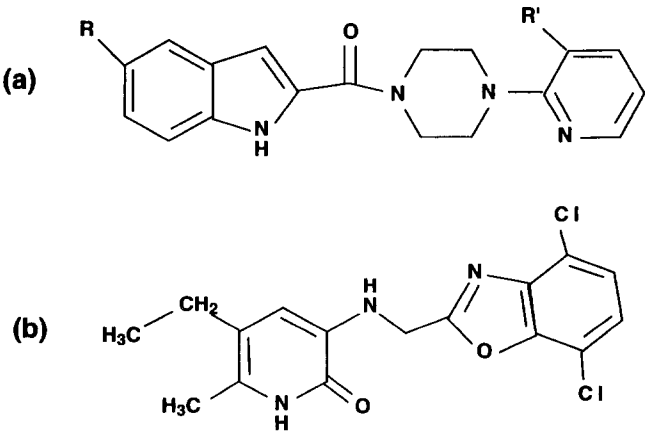


Fig. 1. Chemical structures of the BHAPs (a) and L-697,661 (b). Substituents for the BHAPs are as follows: for U-88204, R = H and R′ = NH-CH($CH_3$)$_2$; for U-87201, R = $OCH_3$and R′ = NH-$CH_2$-$CH_3$; for U-90152, R = NH-$SO_2$-$CH_3$ and R′ = NH-CH($CH_3$)2. These structures were published in the following references: L-697,661 in [14]; U-88204E and U-87201E in [8]; U-90152S in [9].

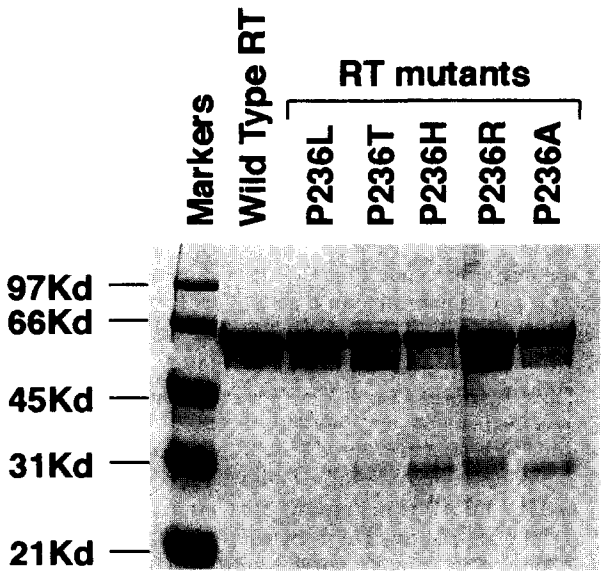


Fig. 2. A 12% SDS-polyacrylamide gel electrophoresis of IMAC purified HIV-1 RT mutants.

cloned, expressed, and purified a number of HIV-1 RT mutants: H235W; D237T; and H235W/D237T/T240K. In order to facilitate rapid isolation, we engineered these mutants with an hexa histidine-containing affinity tag that confers specificity for their purification by IMAC.

*3.2. Inhibition studies of HIV-1 RT 235, 237, and 240 mutants*

The p66 homodimers of HIV-1 RT are convenient to obtain and are useful for rapid in vitro evaluation of HIV-1 RT inhibitors [22,25]. The specific RNA-dependent DNA polymerase activity of the wild type p66 homodimer is similar to the specific activity of the p66/p51 heterodimer [25]. Moreover, incubation of the p66 homodimer of the P236L mutant with HIV-1 protease produced a stable heterodimeric RT (p66/p51). The heterodimer was indistinguishable from the P236L homodimer [22] with regard to resistance to U-90152S and sensitization to L-697,661 (data not shown). In the present work, therefore, all the mutant enzymes were purified as p66 homodimers in a single step from crude *E. coli* extracts, as described for the wild type HIV-1 RT [22,24,25]. As controls, three other mutants bearing single (H235L; D237N) and double (H235L/D237G) substitutions unrelated to HIV-2 RT were also obtained (data not shown).

Fig. 1 shows structures of the BHAPs and the L-697,661 used for evaluation of their inhibitory potential versus all the mutants of HIV-1 RT. Table 1 shows a comparison of the inhibition of HIV-1 RT mutants, bearing single and multiple mutations derived from HIV-2 RT, with the wild type enzyme. These mutant enzymes were found to be essentially indistinguishable from the wild type enzyme in terms of their inhibition by U- 90152S and L-697,661. Thus, HIV-1 RT can accommodate these HIV-2 RT amino acids without becoming resistant to these drugs. Similar results were obtained with the control mutants (H235L; D237N; H235L/D237G) containing substitutions which were not derived from HIV-2 RT (data not shown). These results suggested that the conserved proline at 236 and the surrounding residues are unlikely to be involved in direct interaction with U-90152S or L-697,661.

Table 1
In vitro inhibition of recombinant HIV-1 RT mutants by U-90152S and L-697,661

| HIV-1 RT | $IC_{50}$ ($\mu$M)[a] | |
|---|---|---|
| | U-90152S | L-697,661 |
| Wild type | 0.30 ± 0.01 | 0.23 ± 0.01 |
| H235W | 0.10 ± 0.003 | 0.10 ± 0.003 |
| D237T | 0.31 ± 0.01 | 0.15 ± 0.005 |
| H235W/D237T/T240K | 0.20 ± 0.006 | 0.65 ± 0.02 |

[a] Data represent mean ± S.D. ($n$ = 3).

*3.3. Inhibition studies with HIV-1 RT mutants bearing single base mutations at 236*

The above studies led us to consider the possibility of P236L-mediated alterations in the shape of the binding pocket as the mechanism for the observed drug resistance-sensitization phenomenon. If this hypothesis is correct, then changes at P236 should result in differential alterations whose magnitude can be

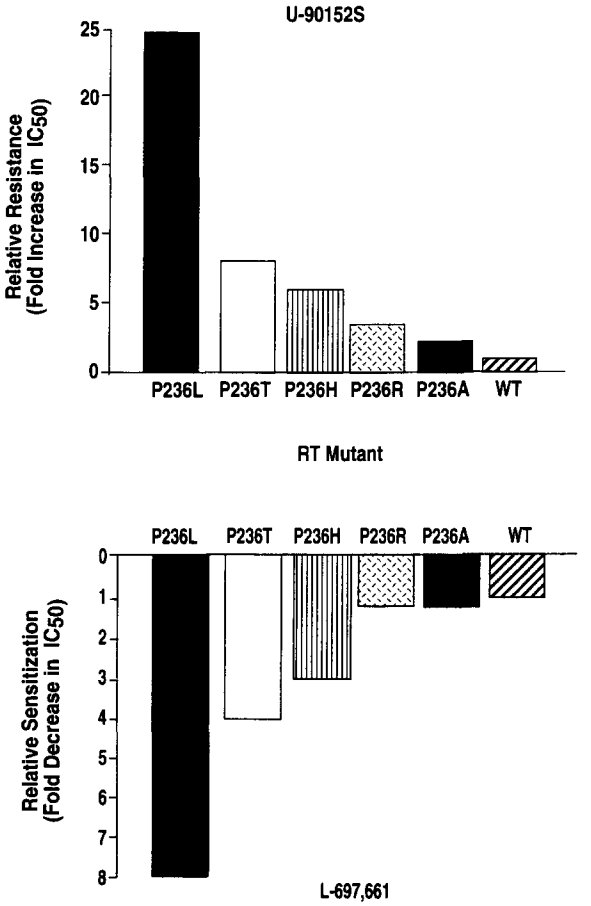


Fig. 3. Comparison of relative resistance and sensitization of P236 HIV-1 RT mutants to U-90152S and L-697,661, respectively. Relative resistance was calculated as fold-increase in $IC_{50}$, by dividing the $IC_{50}$ for each mutant with the $IC_{50}$ for the wild type enzyme. Relative sensitization was calculated as fold-decrease in $IC_{50}$, by dividing the $IC_{50}$ of the wild type enzyme with the $IC_{50}$ of the P236 mutant. The data are presented relative to the wild type HIV-1 RT, which is arbitrarily assigned a value of 1. The variation in the triplicate experiments was ≤3%. For other details see section 2.

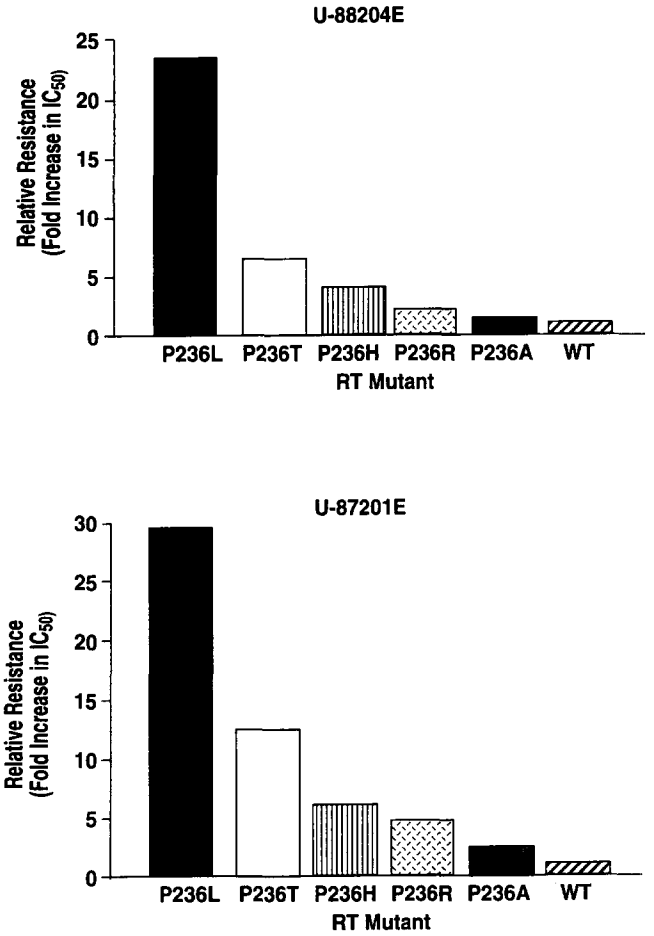


Fig. 4. Resistance profiles of P236 HIV-1 RT mutants in the presence of the BHAPs U-88204E and U-87201E. Other details are the same as in Fig. 3.

monitored in terms of resistance to U-90152S and sensitization to L-697,661. In order to test this possibility, site-specific mutations requiring a single base pair substitution at P236 were targeted. Specifically, five HIV-1 RT mutants (P236L, P236T, P236H, P236R, P236A) were cloned and expressed in *E. coli*. Fig. 2 shows a SDS-PAGE of the IMAC-purified P236 HIV-1 RT mutants. The specific RNA-dependent DNA polymerase activities of these IMAC-purified HIV-1 RT mutants is shown in Table 2. These mutant enzymes had relative specific activities comparable to the wild type HIV-1 RT, indicating that these mutations had not affected the functional characteristics of these enzymes.

We have shown previously that the P236L-mediated resistance to U-90152S and sensitization to L-697,661 can be quantitatively assessed in terms of increase and decrease in $IC_{50}$, respectively [22]. The $IC_{50}$ of each inhibitor was determined against each mutant enzyme and compared with the wild type HIV-1 RT containing P236. The increase in $IC_{50}$ (resistance) or decrease in $IC_{50}$ (sensitization), relative to the wild type HIV-1 RT, was calculated in terms of fold differences. Fig. 3 shows a direct comparison of U-90152S with L-697,661. In contrast to the observed resistance to U-90152S, all the P236 mutants showed sensitization to L-697,661 (Fig. 3). The largest increase in sensitization to L-697,661 was observed with the P236L mutant. These results show that the magnitude of resistance to U-90152S and sensitization to L-697,661 depends upon the substitution at P236. If the relative resistance of a mutant RT towards U-90152S is the lowest, the relative sensitization of that mutant to L-697,661 is also the lowest (Fig. 3). This direct correlation between sensitization to L-697,661 and resistance to U-90152S supports the previous evidence [20,21] that these structurally dissimilar RT inhibitors share the same binding pocket [10].

It is interesting to note (Fig. 3) the nature of the amino acids substituted for the proline in the HIV-1 RT mutants and their effect on resistance/sensitization. At the extreme of the resistance profile are amino acids with lipophilic sidechains; Leu, Ala, and the parent Pro. Intermediate levels of resistance are associated with the more polar amino acids Thr, His, and Arg. The observed resistance trend (Fig. 3) does not correlate well with the polarity or size of the amino acids present at position 236 of HIV-1 RT. This suggests that binding of the BHAP U-90152S does not involve a specific interaction between the drug and the sidechain of the 236 residue of HIV-1 RT. Rather, these studies led us to conclude that substitution of alternative amino acids for the proline found at the $\beta13$-$\beta14$ reverse turn [29] of HIV-1 RT alters the geometry of the inhibitor-binding pocket. The net result is that the relative binding affinity of the enzyme's altered pocket is increased for L-697,661 (sensitization) and decreased for the BHAPs (resistance).

Fig. 4 shows resistance profiles for the BHAPs U-88204E and U-87201E. The binding affinities determined in terms of relative $IC_{50}$'s followed the same trend. Relative to the wild type, the $IC_{50}$ was highest for the P236L mutant and lowest for the P236A mutant. The preferential resistance of the P236 mutants to all these BHAPs was in the order P236A < P236R < P236H < P236T < P236L.

It is concluded that the resistance profiles versus the P236 HIV-1 RT mutants do not depend upon the nature of the BHAP used.

Different classes of HIV-1 RT inhibitors display significant differences in the pattern of resistance mutations [7,14,22,30–33], suggesting that they interact with different, perhaps in some cases overlapping, sites on the binding pocket [10]. The resistance mutations observed during cell culture experiments with L-697,661 or other NNRTIs are clustered around this binding pocket. The Y181C and P236L mutations require a single base pair change and are specifically selected in the presence of L-697,661 [15] and U-90152S [22], respectively. This preference may be based on a number of factors such as: (a) degree of

Table 2
Relative specific reverse transcriptase activity of HIV-1 RT and its P236 mutants

| HIV-1 RT | Specific RT activity (Units/mg protein)[a,b] |
|---|---|
| Wild type | 40,909 ± 1148 |
| P236L | 44,675 ± 1676 |
| P236T | 38,702 ± 2036 |
| P236H | 21,445 ± 554 |
| P236R | 39,373 ± 563 |
| P236A | 37,181 ± 4236 |

[a] One unit is defined as 1 nmol of [$^3$H]dTMP incorporated into poly(rA):oligo(dT) in 1 h at 37°C.

[b] Data represent mean ± S.D. ($n$ = 5).

change in susceptibility to the drug; (b) single base pair change as opposed to two base pair changes (e.g. Y181I); (c) unaltered kinetic parameters of the resulting mutated enzyme [34].

One of the common characteristics of all the known NNRTIs is that they do not affect HIV-2 RT activity and replication. The replacement of $Tyr^{181}$ with Ile or Cys resulted in >100-fold resistance to L-697,661 [14]. The Ile is found in the 181 position in HIV-2 RT and, therefore, is believed to be primarily responsible for insensitivity of HIV-2 RT towards L-697,661 [14]. Since the BHAPs are relatively active versus the Y181C mutant [22] and select for the P236L resistance mutation [22], we looked into the possible role of non-conserved residues at 235, 237, and 240 in the insensitivity of BHAPs to HIV-2 RT. We show here (Table 1) that HIV-2 RT resistance to U-90152S appears to be unrelated to the simultaneous presence of W235, T237, and K240 in this enzyme. Thus, the structural basis for the HIV-2 RT resistance to the BHAPs remains unknown.

The exact molecular basis for resistance to NNRTIs is largely unknown. Three possible related mechanisms have been proposed for resistance to NNRTIs as a result of specific mutations in HIV-1 RT [35]. These include: lack of binding between the inhibitor and the mutated site, steric hinderance, and changes in geometry of the inhibitor-binding pocket [35]. Recent structural studies with nevirapine suggest that most resistant mutations work by changing the geometry of the binding pocket that is in contact with the inhibitor [36]. It has been proposed that the broad spectrum of Y181C-mediated resistance may be the result of an increase in the volume of the hydrophobic pocket [36]. Accordingly, the Y181C resistance to L-697,661 is decreased in the presence of a second mutation (P236L) which results in an overall decrease in pocket volume [36]. Consistent with this hypothesis [36], we have provided here biochemical data which support the view that the P236L-mediated resistance/sensitization phenomenon is the result of an alteration in the geometry of the inhibitor binding pocket.

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