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Inhibition of reverse transcriptase of human immunodeficiency virus type 1 and chimeric enzymes of human immunodeficiency viruses types 1 and 2 by two novel non-nucleoside inhibitors

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Abstract

We have studied the effects of two non-nucleoside reverse transcriptase inhibitors (NNRTI), nitrophenyl phenyl sulfone (NPPS) and a potent derivative of oxathiin carboxanilide (UC-38), on enzymatically active molecular chimeras composed of complementary segments of the reverse transcriptases (RTs) of human immunodeficiency virus type 1 (HIV-1) and -2 (HIV-2). The substances inhibit only the DNA polymerase activity of HIV-1 RT with no effect on HIV-2 RT. The results suggest that there is a protein segment located between residues 158 and 190 that is critical for the inhibition by both compounds. However, there is probably a second segment that resides between residues 192 and 202, as in the case of NPPS, or residues 203 and 224, as in the case of UC-38, that is also crucial for the sensitivity of HIV-1 RT to both inhibitors.

Key words: Reverse transcriptase; HIV-1; HIV-2; UC-38; Nitrophenyl phenyl sulfone

1. Introduction

Acquired immunodeficiency syndrome (AIDS) is caused by human immunodeficiency virus type 1 (HIV-1) or -2 (HIV-2). As is true for all retroviruses, a key process in the life cycle of the virus is the reverse transcription of the viral single-stranded RNA into double-stranded DNA. This process does not appear to be required for normal cellular processes. First, the viral RNA is reverse transcribed into single-stranded DNA by the RNA-dependent DNA polymerase (RDDP) activity of reverse transcriptase (RT). Next, the RNA template is hydrolyzed by the ribonuclease H (RNase H) activity of the enzyme. Finally, the DNA serves as a template for the synthesis of the second DNA strand, catalyzed by the DNA-dependent DNA polymerase (DDDP) activity of the RT.

Numerous compounds that inhibit the reverse transcription process have been found; some of them serve as anti-HIV drugs for the treatment of AIDS patients. Most of these inhibitors can be grouped into two distinct classes. The first group consists of nucleoside analogs that, when incorporated into the nascent DNA by the RT cause premature chain terminations. Among the drugs included in this class are 3′-azido-2′,3′-dideoxythymidine (AZT), 2′,3′ dideoxycytidine (ddC) and 2′,3′ dideoxyinosine (ddI). These substances inhibit HIV-1 RT and HIV-2 RT to about the same extent [1].

A second class of inhibitors are complex aromatic substances that are potent inhibitors of HIV-1 RT but have no effect on HIV-2 RT. These non-nucleoside inhibitors are structurally diverse and include nevirapine [2], pyridinone derivatives [3], tetrahydroimidazo [4,5,1-jk] [1,4]-benzo diazepin-2(1H) one and-thione (TIBO) derivative [4], and HEPT [5,6]. Surprisingly, these inhibitors share functional similarities, despite their chemical diversity. Thus, HIV-1 RT mutants show a cross-resistance to several members of this group of inhibitors (see [7] for review). More recently, the calanolides have been identified as a second type of non-nucleoside inhibitors of HIV-1 RT (NNRTI). Hence, a TIBO- and nevirapine-resistant mutant strain of HIV-1 was found to be inhibited by calanolide A [8].

Several studies have shown that Tyr^{181} and Tyr^{188} in HIV-1 RT are the main amino acid residues that interact with the first group of NNRTI (e.g. TIBO and nevirapine), whereas Lys^{103} is involved also to a certain extent with the inhibition [7,9]. These results are based mainly on the behavior of HIV-1 RT mutants derived from either clinical isolates of drug-resistant virions or from in vitro mutagenesis. Another approach for localizing the interaction sites with the inhibitors is to use enzymatically active molecular chimeras composed of complementary segments of the inhibitor-sensitive HIV-1 RT and the inhibitor-resistant HIV-2 RT. The inhibition pattern with calanolide A provided evidence that there are two regions in HIV-1 RT molecules that are critical for the inhibition. The first domain is located between amino acid residues 94 and 157 and the second between residues 227 and 427 [10]. In the same study, two other non-nucleoside inhibitors of HIV-1 RT, thiazolobenzim-

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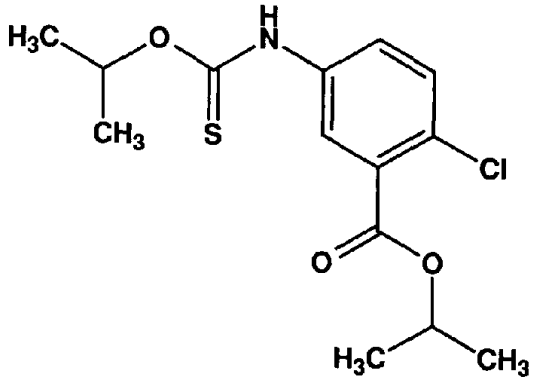
idazole (TBZ) [11] and sulfoxamine [12], led to the suggestion that two other possible regions are crucial for the inhibition of both compounds. The first domain resides between amino acid residues 158 and 190 and the second between residues 203 and 224 [10].

In the current study, we have investigated the pattern of inhibition of two new compounds by a variety of enzymatically active chimeric HIV-1/HIV-2 RT molecules, in order to identify the regions in HIV-1 RT responsible for inhibition. The compounds were identified through the US National Cancer Institute's high capacity screening program [13]. Through extensive structure–activity relationship (SAR) studies, a simple derivative of oxathiin carboxanilide (OC, NSC 615985) [14] was identified. The compound, designated UC-38 (NSC 629243) had improved activity and solubility characteristics over OC (McMahon et al., in preparation). The second compound, 2′-nitrophenyl phenyl sulfone, (NPPS, NSC 624231), was identified through SAR analysis of a large group of biologically active diarylsulfones [15].

NPPS
(NSC 624231)



UC38
(NSC 629243)

Fig. 1. The chemical structures of the non-nucleoside inhibitors of HIV-1 RT used in the current study.

2. Materials and methods

2.1. Reagents

The two compounds used in this study were obtained through the Drug Synthesis and Chemistry Branch, National Cancer Institute. The chemical structures of the UC-38 (NSC 629243) and NPPS (NSC 624231) are shown in Fig. 1.

2.2. Enzymes

HIV-1 and HIV-2 RTs were recombinant enzymes expressed in *E. coli* as described previously [16,17]. The enzymes were purified to homogeneity by the method of Clark et al. [18].

2.3. Construction of HIV-1/HIV-2 RT chimeric proteins

The construction of the chimeras designated A1, A4, A6, A6/B6, B1, B2, B6, C1 and C2 was described in detail previously [10,19]. The new chimeras C4, C5, C8 and C9 were constructed basically as chimeras C1 and C2 using the BspMI cassette mutagenesis system as described in detail elsewhere [20]. In all cases, in the expression plasmids we have replaced the DNA segments of the HIV-1 RT gene by the DNA segments coding for the appropriate peptide sequences of HIV-2 RT. The plasmids encoding either the wild-type HIV-1 RT and HIV-2 RT or the chimeric HIV-1/HIV-2 RTs were all introduced into the DH5α strain of *E. coli*. Extracts of the bacterial strains expressing the wild-type or chimeric RTs were prepared as described previously [10,19].

2.4. Enzymatic assays

The enzymatic assays for the RNA- and DNA-dependent DNA polymerase and RNase H activities of both wild-type and chimeric RTs were performed as described in detail previously [10]. In the inhibition studies, the IC_{50} values (concentrations of inhibitors leading to a 50% inhibition of the initial DNA polymerase activity) were calculated from the dose–response inhibition curves for each recombinant enzyme from duplicate determinations.

3. Results and discussion

3.1. The effects of UC-38 and NPPS on enzymatic activities of purified HIV-1 RT and HIV-2 RT

We have confirmed that both UC-38 and NPPS are specific inhibitors of HIV-1 RT. To this aim, we have analyzed the effect of both compounds on the catalytic activities of HIV-1 RT as compared with HIV-2 RT (Fig. 2). The enzymatic activities analyzed were the RNA-dependent DNA polymerase (RDDP) activity (using poly(rA)·olido(dT) or poly(rC)·oligo(dG) as substrates), the DNA-dependent DNA polymerase (DDDP) function (with activated DNA as a template primer) and the RNase H activity. The purified enzymes were incubated with increasing concentrations of the inhibitors and the residual enzymatic activities were assayed and compared with the activities of control enzymes. Both the RDDP and DDDP activities of the HIV-1 RT are very sensitive to UC-38 and NPPS. The IC_{50} values calculated for UC-38 were about 2.5 μM and 0.5 μM for the RDDP activity using poly(rA)·oligo (dT) and poly(rC)·oligo(dG), respectively, and 6 μM for the DDDP activity of HIV-1 RT. The IC_{50} values for the inhibition of these activities by NPPS were about 19 μM and 9.5 μM (with poly(rA)·oligo(dT) and poly(rC)·oligo(dG), respectively) for RDDP and 10 μM for DDDP. This indicates that with both inhibitors the poly(rC)·oligo(dG)-directed RDDP activity is more susceptible to inhibition than the poly(rA)·oligo(dT)-directed reaction. As expected, the two inhibitors did not have any apparent effect on the

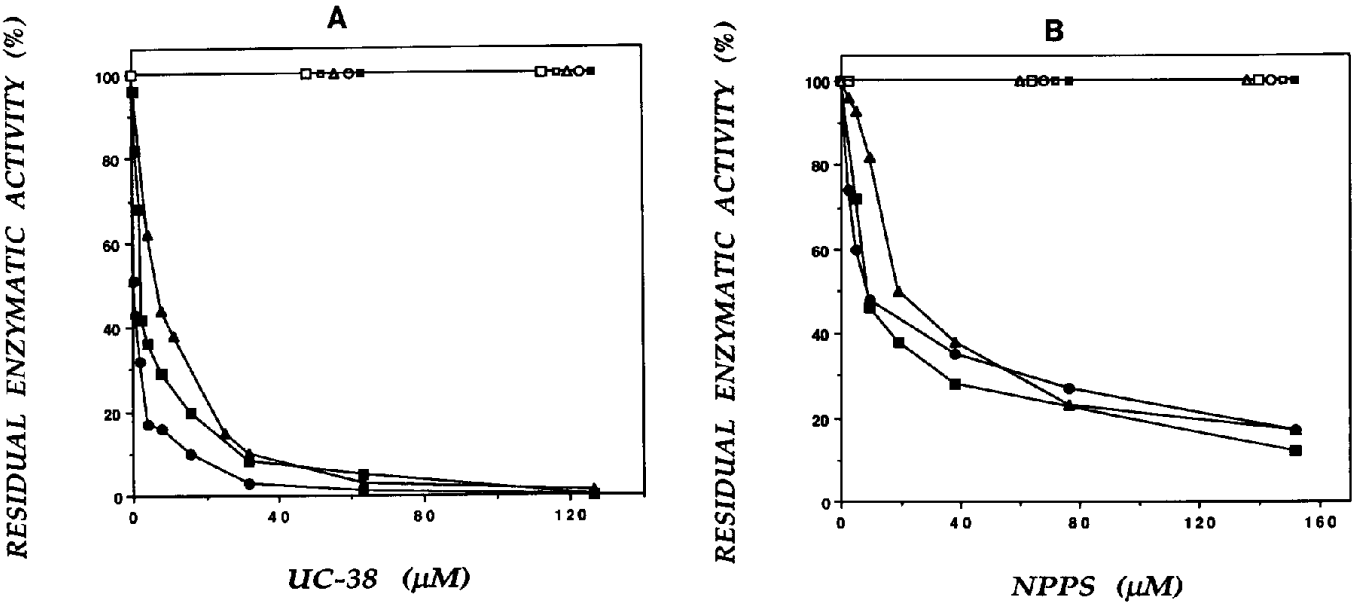


Fig. 2. The effects of UC-38 (A) and NPPS (B) on the DNA-dependent DNA polymerase, RNA-dependent DNA polymerase and RNase H activities of purified recombinant HIV-1 RT and HIV-2 RT. Increasing amounts of the inhibitors were added to the two enzymes in a solution of dimethylsulfoxide at a final concentration of 1%. The levels of enzymatic activities inhibited, as expressed in dNMP incorporated (for the DNA polymerase activities) or dAMP hydrolyzed (for the RNase H activity) in 30 min under the standard assay conditions [25] are as follows: for HIV-1 RT, 235, 64 and 41 pmol incorporated with poly(rA)·oligo(dT), poly(rC)·oligo(dG) and activated DNA as substrates, respectively, for the DNA polymerase activities and 36 pmol for the RNase H activity. For HIV-2 RT, 180, 61 and 42 pmol for the poly(rA)·oligo(dT), poly(rC)·oligo(dG) or activated DNA-directed DNA polymerase activities, respectively, and 30 pmol for the RNase H activity. The residual activities in the presence of the inhibitors were calculated relative to the enzymatic activities of control enzymes that underwent similar treatments with no inhibitors. Filled symbols refer to HIV-1 RT and open symbols to HIV-2 RT. Poly(rC)·oligo(dG)-directed RDDP activity (circles); poly(rA)·oligo(dT)-directed RDDP activity (squares); DNA-dependent DNA polymerase activity assayed with gapped-activated herring sperm DNA, prepared as described [16] (triangles); RNase H activity assayed with [^{3}H]poly(rA)·poly(dT) (squares). Each value represents the mean of the results of two independent experiments.

DNA polymerase function of HIV-2 RT, even at concentrations above 300 μM (Figs. 2 and 3). Neither the RNase H activity of HIV-1 RT nor that of HIV-2 RT were affected to any detectable extent by any of the two substances tested (Fig. 2). This is not surprising, since none of the NNRTI or the nucleoside analogs of HIV RTs described so far had substantial effects on the RNase H activity of the RTs [21].

3.2. *Inhibition of chimeric HIV-1/HIV-2 RT molecules by UC-38 and NPPS*

We have taken advantage of the total insensitivity of HIV-2 RT to a variety of non-nucleoside inhibitors to investigate the protein segments in HIV-1 RT that are pivotal for the inhibitory activity of these substances. This was done by analyzing the relative sensitivity of a series of enzymatically active recombinant HIV-1/HIV-2 RT molecules constructed from complementary protein segments from the two wild-type RTs. The sensitivity of the chimeric RTs is expected to result from the segments derived from only HIV-1 RT. We have previously used this approach to study TIBO, calanolide A, TBZ and sulfoxamine [10].

As shown in Fig. 2, there are basically few differences in the response of the DNA polymerase activity to either one of the primer templates used. Therefore, we have proceeded by analyzing the chimeric RTs with only poly(rC)·oligo(dG) for assaying the DNA polymerase activity. The results with UC-38 and NPPS indicate that the chimeric HIV-1/HIV-2 RTs do not respond to these two novel inhibitors in a similar fashion (Fig. 3). A systematic inspection of the inhibition pattern of the hybrid HIV-1/HIV-2 RTs by UC-38 indicates a somewhat complex pattern. The chimeras designated A6 and B2 are fully resistant to UC-38, whereas B6 is fully sensitive, suggesting that the specific interaction segments are located between residues 203 and 226. Since chimera C2 is not susceptible to UC-38, this segment cannot span beyond residue 224. Hence, it is located between residues 203 and 224. However, the fact that chimera C1 is also resistant, strongly suggests the possibility that there is a second segment between residues 158 and 190 that is also crucial to the interaction with the inhibitor. These results are similar to those obtained previously by us with two other non-nucleoside inhibitors, TBZ and sulfoxamine [10].

A similar examination of the response pattern of the same chimeric RTs to NPPS reveals a difference in the putative interaction segments (Fig. 3). Unlike UC-38, the chimera B2 is fully sensitive to NPPS. All other chimeric

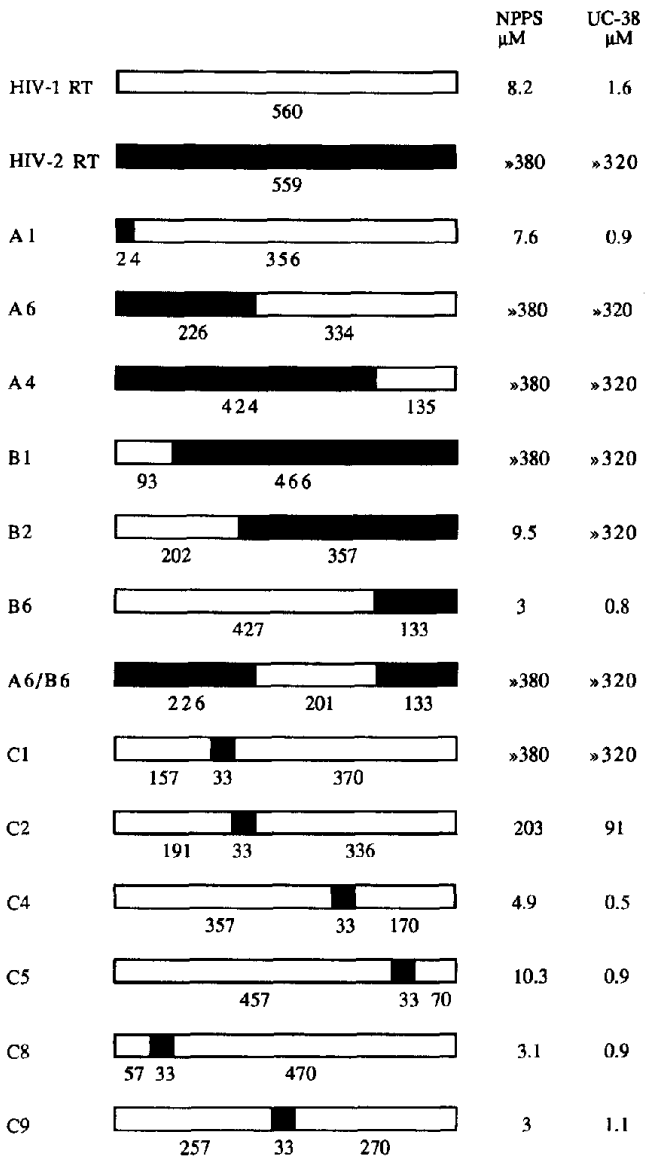


Fig. 3. The structures of the chimeric HIV-1/HIV-2 RTs and their sensitivity to UC-38 and NPPS. The construction of all plasmids used was described in section 2. Open boxes represent protein sequences derived from HIV-1 RT and filled boxes sequences of HIV-2 RT. The extracts of the bacterial strains expressing the various proteins were diluted 5-fold in a solution containing increasing concentrations of the inhibitors in a final concentration of 1% dimethylsulfoxide and incubated with the appropriate substrates. The residual poly(rC)·oligo(dG)-directed DNA synthesis was determined as described [10,20] and the IC_{50} values were calculated as described in section 2.

RTs respond to the two structurally diverse inhibitors in a similar manner. A comparison of the responses of chimeras B1 and B2 to NPPS implies that the interaction sites reside between residues 93 and 202 in HIV-1 RT. Since both chimeras C1 and C2 are resistant to NPPS, the most likely explanation is that, as in the case of UC-38, there are two separate segments crucial for the inhibition. The first one resides in the same region as the comparable one inthe case of UC-38, namely between amino acid residues 158 and 190. However, the second one is probably different for NPPS and UC-38, being located between residues 192 and 202 for NPPS. It is interesting to note that the chinmera designated C2 is somewhat more sensitive to both inhibitors than are HIV-2 RT and other drug-resistant chimeric RTs (see Fig. 3). This may imply that the first putatative protein segment, that is required for the inhibition by the two compounds, is more important for inhibition than the second segment involved in this inhibition.

It should be emphasized that the segments postulated for the interaction of UC-38 and NPPS with HIV-1 RT exhibit some similarity with the complementary ones in HIV-2 RT. Thus, in the first protein fragment important to the interaction of both inhibitors (residues 158–190), only 15 amino acid residues out of a total of 33 residues are different (suggesting that only one or several of these 15 residues are candidates for interaction). Likewise, the second segment required for the inhibition by UC-38 (residues 203–224) is comprised of only 12 amino acids (out of 22 residues) that are different. In the case of NPPS, the second putative peptide for the interaction (residues 192–202) contains 9 different residues (out of 11 residues).

The difference in the position of the second putative segment for specifying the susceptibility to either UC-38 or NPPS may explain the slight disparity observed in the response of the DNA polymerase activities of HIV-1 RT to the inhibitors (Fig. 2). Generally, UC-38 is a better inhibitor of both RDDP and DDDP activities, as apparent from the lower IC_{50} values (Fig. 3). The inhibition of the RDDP activity by UC-38 is obtained at lower concentrations relative to the DDDP function. On the other hand, NPPS inhibits the DDDP activity of HIV-1 RT slightly better, or to about the same extent, compared to RDDP activity. Taken together, it is possible that the difference in the response of HIV-1 RT to the two compounds stems from the putative interaction sites in the region that spans residues 192–224 (residues 192–202 for NPPS and residues 203–224 for UC-38).

Recent X-ray crystallographic studies have resulted in the three-dimensional structure determination of HIV-1 RT [22–24]. This has led to the conclusion that nevirapine, a typical NNRTI (that interacts with mainly Tyr^{181} and Tyr^{188} in HIV-1 RT) is buried in a deep hydrophobic pocket at the base of the 'palm' subdomain of the protein, near but not overlapping the DNA polymerase active site. The putative protein segments crucial for the susceptibility to both UC-38 and NPPS are also located in the same 'palm' subdomain. Thus, the segment common for the interaction of both inhibitors (residues 158–190) spans the α-helix designated αE and the β sheets, β9 and β10. The second segment important for the inhibition by UC-38 (residues 203–224) comprises the carboxyl-terminal part of αF, β11a and β11b, whereas the second site for the interaction with NPPS (residues

192–202) is located in the amino-terminal portion of αF. For both inhibitors it may be possible that the compounds form a bridge between the two putative domains required for the inhibition by each compound. This interaction may inactivate the DNA polymerase catalytic site by mechanisms such as blocking the access of the template primer, preventing its binding to the protein, or by interfering with the incoming dNTP.

The relatively complex interactions between various protein segments and UC-38 and NPPS were seen in the present study based on the pattern of inhibition of the chimeric HIV-1/HIV-2 RTs. Further detailed analysis of selected drug-resistant mutants will complement this study and identify the specific amino acid residues that are directly involved in the inhibition. It is also apparent that analysis of specific NNRTI-resistant mutants without screening chimeric HIV-1/HIV-2 RTs might result in overlooking other non-mutated regions that may also be pivotal for enzyme inhibition. This is based on the assumption that the resistance is usually not an additive phenomenon (when more than one protein domain is involved) and that modifying each domain independently can lead to full resistance. The future availability of HIV-1 RT variants resistant to UC-38 and NPPS will allow a more precise localization of the amino acid residues that interact with these two novel inhibitors.

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