

Mutational Analysis of Trp-229 of Human Immunodeficiency Virus Type 1 Reverse Transcriptase (RT) Identifies This Amino Acid Residue as a Prime Target for the Rational Design of New Non-Nucleoside RT Inhibitors

HEIDI PELEMANS, ROBERT ESNOUF, ERIK DE CLERCQ, and JAN BALZARINI

Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium

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ABSTRACT

Trp-229 is part of the non-nucleoside reverse transcriptase inhibitor (NNRTI)-binding pocket of HIV type 1 (HIV-1) reverse transcriptase (RT), and is also part of the "primer grip" of HIV-1 RT. Using site-directed mutagenesis, seven RT mutants were constructed bearing the mutations 229Phe, 229Tyr, 229Ile, 229His, 229Lys, 229Cys, and 229Gln. We found that all of the mutants showed severely compromised RNA- and DNA-dependent DNA polymerase activities (<2% of wild-type activity). The recombinant 229Phe and 229Tyr RT enzymes were among the mutant enzymes with the highest activity (0.7 and 1.1% of wild-type activity, respectively) and we evaluated these for resistance against several NNRTIs. No resistance was found for

the 229Phe RT, but the 229Tyr RT showed a ~20-fold resistance against UC-781 and lower resistance against emivirine and nevirapine. Attempts to make recombinant virus strains bearing the single 229Phe or 229Tyr RT mutation failed. Experiments in which we varied the pentenyl ether substituent of the thiocarboxanilide UC-781 revealed that Trp-229 can be specifically targeted by NNRTIs and that an alkenyloxy group length of five atoms assures an optimal interaction of the thiocarboxanilides with Trp-229. Our findings indicate that Trp-229, when combined with other crucial immutable amino acids (i.e., Tyr-318), is an appropriate candidate for the targeted design of new NNRTIs.

The reverse transcriptase (RT) of HIV type 1 (HIV-1) is an important and extensively studied antiviral target for the chemotherapy of AIDS because of its key role in virus replication. Four major categories of RT inhibitors can be distinguished: 1) 2',3'-dideoxynucleoside analogs (designated nucleoside RT inhibitors); 2) acyclic nucleoside phosphonate analogs; 3) non-nucleoside reverse transcriptase inhibitors (NNRTIs); and 4) phosphonoformic acid (for an overview see De Clercq, 1994, 1996; Balzarini and De Clercq, 1996).

The NNRTIs represent a wide range of specific and potent inhibitors of HIV-1 RT. Despite their potency and generally low toxicity, the relatively rapid emergence of resistant viral variants has initially limited their widespread use. Drug resistance is primarily associated with mutations of the amino acids lining the lipophilic NNRTI-binding pocket in the p66 subunit of the RT. Mutations against one NNRTI often give cross-resistance to other NNRTIs, thus compro-

promising the potential of therapies based on different NNRTI combinations. Four residues (Phe-227, Trp-229, Leu-234, and Tyr-318) lining the NNRTI-binding pocket (Smerdon et al., 1994; Ren et al., 1995) have been identified as highly conserved amino acid residues among lentiviral RTs. Recently, the Phe227Leu mutation has been discovered in vitro on *N*-[4-chloro-3-(3-methyl-2-butenyloxy)phenyl]-2-methyl-3-furan-carbothioamide (UC-781) treatment (Balzarini et al., 1998), and treatment of HIV-1-infected cell cultures with the NNRTI S-1153 (AG1549) has been reported to select for the Leu234Ile mutation (Fujiwara et al., 1998).

In a previous study, we demonstrated that mutation of residue Tyr-318 of HIV-1 RT resulted in a severe drop of catalytic activity of the enzyme, with the exception of the mutations Tyr318Phe and Tyr318Trp. However, these latter mutations did not markedly alter the sensitivity of the RT to most NNRTIs. Thus, we concluded that it may be unlikely that treatment of HIV-1-infected cells with NNRTIs would result in the selection of RT-Tyr-318-mutated virus strains (Pelemans et al., 1998).

In this study we constructed seven mutations, five of which

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ABBREVIATIONS: RT, reverse transcriptase; NNRTI, non-nucleoside reverse transcriptase inhibitor; UC-781, *N*-[4-chloro-3-(3-methyl-2-butenyloxy)phenyl]-2-methyl-3-furan-carbothioamide; RDDP, RNA-dependent DNA polymerase; DDDP, DNA-dependent DNA polymerase; Ni-NTA, nickel-nitrilotriacetic acid.

have never been reported so far, at amino acid position Trp-229 of HIV-1 RT by site-directed mutagenesis and investigated its potential role as a target for NNRTI drug design. This amino acid residue is: 1) highly conserved among all known lentiviruses; 2) part of the primer grip region (Jacobo-Molina et al., 1993); 3) part of the NNRTI-binding pocket; and 4) not reported as a characteristic NNRTI resistance mutation. We could demonstrate that the RT enzymes that contained the 229Phe and 229Tyr mutations kept high sensitivity to NNRTIs, whereas the RT enzymes that contained other mutations at amino acid position 229 were not catalytically active. Therefore, we concluded that amino acid position 229 should be considered as a prime target amino acid for interaction with novel NNRTIs.

Materials and Methods

Test Compounds. The thiocarboxanilide UC-781 and its derivatives were obtained from Uniroyal Chemical Ltd. (Middlebury, CT, and Guelph, Ontario, Canada). Nevirapine (BI-RG-587; dipyrindodiazepinone) was provided by Dr. P. Ganong (Boehringer Ingelheim, Ridgefield, CT). Delavirdine [U-90152; bis(heteroaryl)piperazine; BHAP], quinoxaline [HBY097; (S)-4-isopropoxycarbonyl-6-methoxy-3-(methylthiomethyl)-3,4-dihydroquinoxaline-2(1H)-thione], and efavirenz (DMP 266) were provided by Dr. R. Kirsch (Hoechst AG, Frankfurt, Germany). The HEPT [1-(2-hydroxyethoxymethyl)-6-(phenylthio)thymine] derivative MKC-442 (emivirine) was provided by Dr. M. Baba (Fukushima Medical College, Fukushima, Japan) and Dr. P. Furman (Triangle Pharmaceuticals, Research Triangle Park, NC). GW420867 was provided by Dr. J.-P. Kleim (Glaxo Wellcome, Stevenage, UK). 2',3'-dideoxyguanosine-5'-triphosphate (ddGTP) was obtained from Sigma Chemical Co. (St. Louis, MO).

Cells. CEM cells were obtained from the American Tissue Cell Culture Collection (Manassas, VA). MT4 cells were provided by Dr. N. Yamamoto (Tokyo Medical School and Dental University School of Medicine, Tokyo, Japan).

Activity Assay for Thiocarboxanilides against Wild-Type HIV-1 in CEM Cell Cultures. CEM cells were suspended at approximately 200,000 cells/ml of culture medium and infected with wild-type HIV-1. Then, 100 μ l of the infected cell suspensions were added to 200- μ l microtiter plate wells containing 100 μ l of an appropriate dilution of the test compounds. After 4 days of incubation at 37°C, the cell cultures were microscopically examined for syncytium formation. The EC₅₀ (50% effective concentration) was determined as the compound concentration required to inhibit syncytium formation by 50%.

Site-Directed Mutagenesis of HIV-1 RT. Mutant RT enzymes containing the 229Tyr, 229Phe, 229His, 229Gln, 229Ile, 229Cys, and 229Lys mutations were derived from the RT sequence cloned in pKRT2His (D'Aquila and Summers, 1989; Pelemans et al., 1998).

Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene, Westburg, The Netherlands). Briefly, supercoiled double-stranded pKRT2His DNA and two synthetic oligonucleotide primers containing the desired mutation at amino acid position 229 of HIV-1 RT and a silent mutation that creates a unique *Bsi*I restriction site were used. The two primers, each complementary to opposite strands of the vector, were extended during temperature cycling by means of *Pfu* DNA polymerase leading to a mutated plasmid containing staggered nicks. After temperature cycling, the product was treated with *Dpn*I. The *Dpn*I endonuclease is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. The nicked vector DNA containing the desired mutations was then transformed into *Escherichia coli* XL1-Blue. The presence of the desired mutations was determined by restriction with *Bsi*I and confirmed by sequencing of the RT gene on

an ABI Prism 310 sequencer (PE Biosystems, Foster City, CA) using the dRhodamine terminator cycle sequencing ready reaction kit (PE Biosystems).

Expression of Mutant Recombinant HIV-1 RT. Recombinant HIV-1 RT enzymes were expressed from a two-plasmid coexpression system described by Jonckheere et al. (1996). The p66 subunit of RT is expressed from pACYC66His and the p51 subunit from pKRT51. To construct wild-type and 229-mutated pACYC66His, wild-type and 229-mutated pKRT2His was digested with *Mst*I and *Eco*RI and the RT-containing fragment was ligated into pACYC184 digested with *Sca*I and *Eco*RI. To construct wild-type and 229-mutated pKRT51, wild-type and 229-mutated pKRT2His was digested with *Nco*I and *Kpn*I and the RT-containing fragment was ligated into pKRT51 digested with *Nco*I and *Kpn*I. Expression of recombinant RT was performed as described previously (Pelemans et al., 1998).

Purification of Mutant Recombinant HIV-1 RT. The purification was performed as described previously (Pelemans et al., 1998). Briefly, the supernatants of the lysed bacterial cell culture were incubated with nickel-nitrilotriacetic acid (Ni-NTA) resin. After sedimentation of the Ni-NTA resin with the bound (His)₆-tagged proteins, the column was formed and washed with wash buffer (a sodium phosphate buffer with 25 mM imidazole). Then, the RT was eluted with the same phosphate buffer containing 125 mM imidazole. The imidazole-containing buffer was exchanged by the heparin buffer (a Tris-HCl buffer with 0.05 M NaCl) and the eluate was concentrated to 2 ml using Ultrafree-15 centrifugal filtration devices (Millipore, Brussels, Belgium). The (His)₆-tagged RT was further purified to about 98% purity over a heparin column (Hitrap heparin column; Amersham Pharmacia Biotech, Roosendaal, the Netherlands). Bound RT was eluted with a linear salt gradient of 0.05 to 1 M NaCl. All fractions containing heterodimer RT were pooled and stored in a 50% glycerol buffer at -20°C. Protein concentrations in the stock solutions were determined with the Bio-Rad protein assay using BSA (Bio-Rad, Hercules, CA) as a standard.

RT Assay. For determination of the 50% inhibitory concentration (IC₅₀) of the test compounds, the RT assay was performed as described previously (Balzarini et al., 1992). A fixed concentration of the labeled substrate [2,8-³H]dGTP (specific radioactivity: 3.6 Ci/mmol) (5.6 μ M, 1 μ Ci; Amersham Pharmacia Biotech) and a fixed concentration of the template-primer poly(C)-oligo(dG₁₂₋₁₈) (0.1 mM; Amersham Pharmacia Biotech) were used. The IC₅₀ for each test compound was determined as the compound concentration that inhibited recombinant RT activity by 50%. The RNA- and DNA-dependent DNA polymerase (RDDP; DDDP) activities of the amino acid 229-mutated recombinant RTs were determined in a similar way.

Recombinant Virus Assay. The recombinant virus assay as described by Kellam and Larder (1994) was used. Briefly, recombinant viruses were obtained through homologous recombination of RT with RT gene-deleted proviral DNA, which was propagated in pHIV Δ RTBstEII. The RT gene DNA used in the recombination came from a polymerase chain reaction performed on the 229-mutated pACYC66His. MT4 cells were electroporated with ~2 μ g of RT gene DNA and ~5 μ g of pHIV Δ RTBstEII DNA. On successful homologous recombination, viable recombinant virus could be recovered from the cell cultures.

Results

Site-Directed Mutagenesis and Enzymatic Activities of HIV-1 RTs Mutated at Position 229. To investigate the influence of changes to the amino acid residue Trp-229 on HIV-1 RT activity, we constructed seven recombinant RTs by site-directed mutagenesis: 229Phe, 229Tyr, 229His, 229Ile, 229Cys, 229Gln, and 229Lys. Only the closely related 229Phe and 229Tyr have been reported before (Jacques et al., 1994; Ghosh et al., 1997). In this way, different types of amino acid side chains were represented: neutral aromatic side chains

(Tyr and Phe), a positively charged aromatic side chain (His), a small side chain (Cys), a large polar side chain (Gln), an aliphatic hydrophobic side chain (Ile), and a positively charged aliphatic side chain (Lys). The mutations were introduced in both subunits (p66 and p51) of the heterodimer and all mutant recombinant RTs were purified to $\geq 98\%$ homogeneity through the two successive affinity columns (a Ni-NTA column followed by a heparin column).

Analysis of RDDP activity revealed severely impaired activities for all seven of the recombinant RTs mutated at amino acid position 229 (Fig. 1). The most active mutant RTs were 229Tyr RT (residual RDDP activity: 1.12%), 229Phe RT (0.71%), and 229Gln RT (0.88%). 229Lys RT showed no activity at all, whereas 229His RT, 229Ile RT, 229Gln RT, and 229Cys RT had activities ranging from 0.16 to 0.05% (Fig. 1). Thus, mutating Trp-229 of the HIV-1 RT to other amino acids resulted in a severely compromised RT endowed with only marginal RDDP catalytic activity.

The DDDP activities were quite similar as the RDDP activities (Fig. 1). The 229His-, 229Ile-, 229Lys-, and 229Cys-mutated RTs showed severely impaired DDDP activities, similar to their RDDP activities. The 229Tyr- and 229Phe-mutated RTs showed DDDP activities that were 3- to 5-fold higher than the corresponding RDDP activities (Fig. 1).

Inhibitory Activities of NNRTIs and ddGTP against Wild-Type, 229Tyr- and 229Phe-Mutant Recombinant HIV-1 RTs. The 229Phe RT and 229Tyr RT were evaluated for their sensitivities to a variety of NNRTIs and ddGTP (Table 1). The 229Phe-mutant RT kept full sensitivity to all of the NNRTIs tested and to ddGTP. In contrast, the 229Tyr-

mutant RT showed marginal resistance to delavirdine (2.4-fold) and quinoxaline GW420867 (4-fold), it showed more substantial resistance to nevirapine (8.7-fold), emivirine (8.5-fold), and, especially, the thiocarboxanilide UC-781 (21-fold). The quinoxaline HBY 097, efavirenz, and ddGTP retained full inhibitory activity against the 229Tyr-mutated RT.

Generation of Recombinant HIV-1 Strains Mutated at Amino Acid Position 229. Because 229Phe and 229Tyr RT displayed the highest activity among the mutant enzymes, several attempts to construct recombinant viruses with the 229Phe or 229Tyr mutation in the *pol* gene were made. Under the experimental conditions used, wild-type recombinant virus was easily generated. However, three independent attempts to generate 229Phe or 229Tyr RT recombinant viruses failed to recover viable mutant virus strains except in one case. The recombinant virus strain that emerged approximately 1 month after the initiation of the experiment contained the 229Tyr mutation in the presence of two additional amino acid changes (i.e., Ile63Met and Val189Ile). Most likely, the amino acid mutations that were added to the 229Tyr RT mutant genetic background represent compensatory mutations that may have allowed the 229Tyr RT HIV-1 to emerge by increasing the fitness of the virus. In all other cases, there was no sign of virus breakthrough, either microscopically (cytopathogenicity) or by p24 measurement.

Effect of Altering the Pentenyl Ether Moiety of Thiocarboxanilide UC-781. Our previous modeling studies on the RT-UC-781 complex indicated that the pentenyl ether group of the inhibitor points toward the functional group of

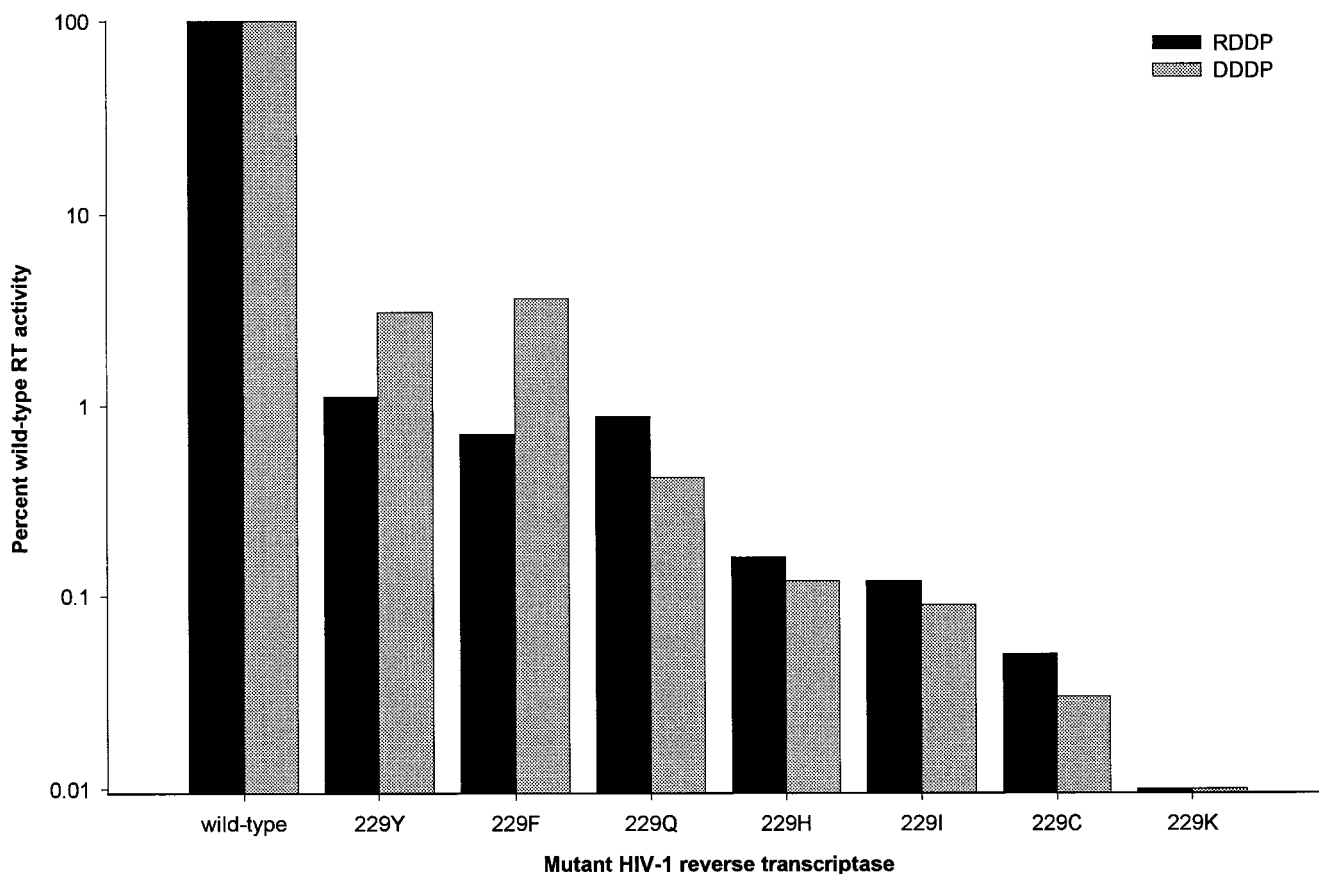


Fig. 1. RDDP and DDDP activities of HIV-1 RTs mutated at position 229. Wild-type (Trp-229) RT activity is designated as 100%.

amino acid Trp-229 with which it interacts in an optimal way with regard to distance and positioning of the methyl groups of UC-781 with the aromatic group of tryptophan (Esnouf et al., 1997). With small differences, this model was confirmed by crystallographic analysis (Ren et al., 1998) (Fig. 2). To investigate the significance of the interaction between Trp-229 and UC-781, we investigated the effects of varying the pentenyl ether moiety at the 3-position of the thiocarboxanilide ring in UC-781 on the antiviral properties of the drug by determining the IC_{50} and EC_{50} of the modified thiocarboxanilides against wild-type RT and wild-type HIV-1, respectively (Table 2). From a log-log plot of these values (Fig. 3) it is obvious that: 1) there is a close linear relationship between the anti HIV-1 RT activity and the anti-HIV-1 potency of the compounds and 2) that the most potent inhibitors of HIV-1 RT activity and HIV-1 replication are those with 3-substituents of five atoms length. Shortening or lengthening this substituent of UC-781 by only one atom raised the IC_{50} values by approximately 100-fold and the EC_{50} by approximately 20-fold (Table 2). The large difference in activities between the thiocarboxanilides with 5-atom substituent lengths on the one hand and the ones with 4- or 6-atom substituent lengths on the other (as clearly evident from Fig. 3) demonstrates that it is the length of this substituent, rather than its chemical identity, that is important for optimal activity of this thiocarboxanilide series. From these data, and the structural/modeling work, it appears that the interaction between the thiocarboxanilides and Trp-229 in HIV-1 RT is of crucial importance for the antiviral potency of thiocarboxanilides such as UC-781. Varying the nature of the five-membered ring from a furanyl to a thienyl in UC-781 did not affect the IC_{50} or EC_{50} (Table 2).

Discussion

NNRTIs are highly specific and potent inhibitors of HIV-1 RT, and they do not interfere with cellular or mitochondrial DNA synthesis. However, the rapid emergence of resistant virus variants and the problem of cross-resistance have limited their clinical use. Several NNRTIs have already been approved for the treatment of AIDS (i.e., nevirapine, delavirdine, and efavirenz).

In this study, we focused on Trp-229 as a possible candidate amino acid for targeted drug design of NNRTIs. Trp-229 is part of the primer grip (residues Phe-227–His-235) (Jacobo-Molina et al., 1993), which appears to maintain the

primer terminus in the appropriate orientation for the nucleophilic attack on the incoming dNTP. Several investigators have studied Trp-229 with regard to its role in the primer grip (Jacques et al., 1994; Ghosh et al., 1996, 1997; Palaniappan et al., 1997; Wöhrle et al., 1997). Jacques et al. (1994), Ghosh et al. (1996), and Wöhrle et al. (1997) showed that the Trp229Ala mutation provoked severely reduced RDDP and DDDP activities, probably resulting from a reduced affinity for the template primer, whereas the RNaseH function stayed intact. Additionally, Wöhrle et al. (1997) demonstrated that the Trp229Ala mutation affected the stability of the RT heterodimer. Jacques et al. (1994) also showed that virus harboring the Trp229Ala mutation in its RT lost its infectivity. Ghosh et al. (1997) studied HIV-1 RT bearing the 229Phe and 229Tyr mutations and found that the mutant RT enzymes had diminished DNA polymerase activity but retained the RNaseH activity. However, no information on the sensitivity of such enzyme mutants to NNRTIs is available. Also, because no data are available on any other Trp-229 mutated RTs and their associated potential resistance spectrum and because Trp-229 forms an important part of the hydrophobic NNRTI-binding pocket, we studied the effect on both the catalytic activity and potential resistance to NNRTIs of a variety of rationally chosen mutations at position 229. All of the 229-mutated recombinant RT enzymes (229Phe, 229Tyr, 229His, 229Ile, 229Cys, 229Lys, and 229Gln) showed severely compromised or even undetectable RDDP and DDDP activities. Because recombinant RT enzymes bearing the 229Phe and 229Tyr mutation showed higher catalytic activities, we evaluated them for resistance against several NNRTIs. No resistance was found for the 229Phe RT enzyme, but the 229Tyr RT enzyme showed a pronounced resistance profile (21-fold) against UC-781 and a lower resistance profile against emvirine (8.5-fold) and nevirapine (8.7-fold).

Jacques et al. (1994) were unable to produce infectious HIV-1 containing the 229Ala mutation. We also made several attempts to make recombinant virus bearing either the 229Phe or the 229Tyr mutation. Only in one attempt, a mutant 229Tyr RT HIV-1 strain emerged after a prolonged incubation time but it contained two additional amino acid mutations. Presumably, additional mutations represent compensatory mutations to increase the fitness and replication competence of the mutant virus. A similar phenomenon has been observed for Gly190Glu RT mutant virus, that has been shown to emerge in the presence of quinoxalines (Kleim et al., 1996). This virus contains an RT that displays only $\approx 5\%$ of the catalytic activity of wild-type RT. On prolonged exposure of this mutant virus to quinoxaline, additional mutations appeared that did not further enhance the level of drug resistance, but markedly increased the catalytic efficacy of the enzyme and concomitant fitness of the virus (Kleim et al., 1996; Boyer et al., 1998).

Taken together, these data show that any change at residue 229 of the HIV-1 RT has a catastrophic effect on its catalytic activity and appears to render these virus mutants incapable of productive replication. Thus, it is highly unlikely that such single-mutant virus strains will arise on exposure of HIV-1 to drugs that are targeted at this amino acid. The fact that a mutation at Trp-229 of HIV-1 RT has never been observed either in vitro or in vivo under any drug pressure

TABLE 1
Sensitivity of HIV-1 wild-type Trp-229 RT, 229Phe RT, and 229Tyr RT to the inhibitory effects of NNRTIs and ddGTP

NNRTI	IC_{50}^a		
	Trp-229	229Phe	229Tyr
	μM		
Nevirapine	6.16 ± 0.3	8 ± 4	16.7 ± 0.3
Delavirdine	0.66 ± 0.04	0.6 ± 0.3	1.6 ± 0.9
Efavirenz	0.02 ± 0.01	0.025 ± 0.003	0.02 ± 0.00
Emvirine	0.122 ± 0.007	0.13 ± 0.02	1.03 ± 0.09
HBV 097	0.014 ± 0.002	0.0162 ± 0.0002	0.020 ± 0.006
GW 420867	0.04 ± 0.02	0.08 ± 0.05	0.19 ± 0.09
UC-781	0.022 ± 0.001	0.035 ± 0.009	0.48 ± 0.38
ddGTP	0.42 ± 0.04	0.68 ± 0.09	0.57 ± 0.12

^a IC_{50} , or 50% inhibitory concentration required to inhibit the enzyme activity by 50%, using poly(C) · oligo(dG) as the template-primer and (2,8-³H)dGTP as the radiolabeled substrate. The data are means of at least two independent experiments.

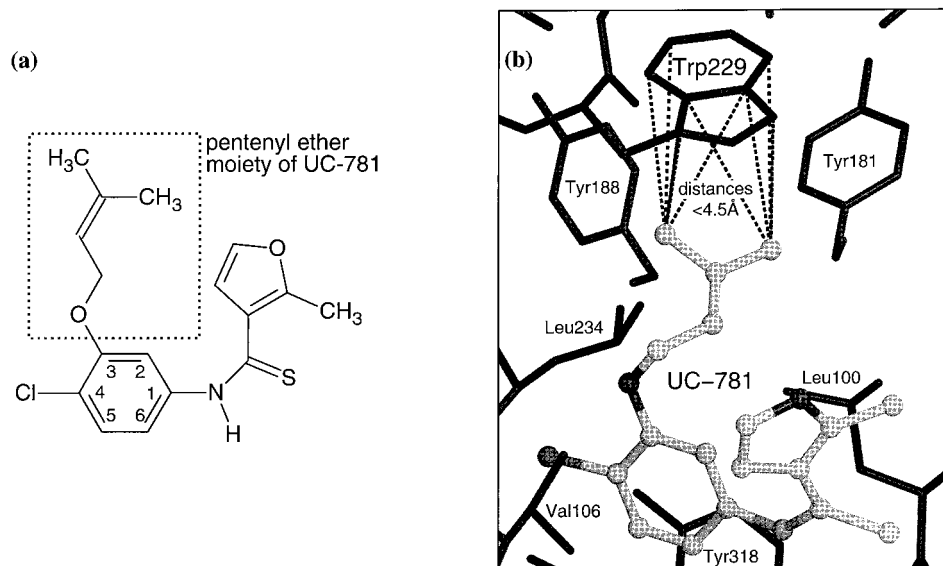


Fig. 2. Thiocarboxanilide UC-781 and its interactions with residue Trp-229 of HIV-1 RT. (a), chemical structure of UC-781, highlighting the pentenyl ether moiety in the 3-position. (b), structure of the complex between RT and UC-781 (Ren et al., 1995). UC-781 is shown as a ball-and-stick model and the surrounding residues of HIV-1 RT are shown by dark gray sticks. Distances less than 4.5 Å between the terminal methyl groups of the pentenyl ether moiety of UC-781 and atoms of Trp-229 are shown by dashed lines. Figure produced using Bobscript (Esnouf, 1999) and rendered with Raster3D (Merritt and Murphy, 1994).

TABLE 2
Inhibitory effects of a variety of UC-781 derivatives on HIV-1 replication (EC_{50}) and HIV-1 RT activity (IC_{50})

Substituent at 3-position	Length of 3-substituent atoms	EC_{50} HIV-1	IC_{50} HIV-1 RT
		nM	nM
O-CH ₂ -C=CH	4	196	3039
O-CH ₂ -C-(CH ₃) ₃ ^a	4	395	14520
O-CH ₂ -CH=CH ₂ ^a	4	108	2531
O-CH ₂ -C-(CH ₃) ₃	4	450	16775
O-CH ₂ -CH=CH-CH ₃	5	18	233
O-CH ₂ -CH=C(CH ₃) ₂ (UC-781)	5	9	21
O-CH ₂ -CH=C(CH ₃) ₂	5	9	54
O-CH ₂ -CH ₂ -CH(CH ₃) ₂	5	19	133
S-CH ₂ -CH=CH-CH ₃	5	17	118
S-CH ₂ -(C ₆ H ₅)-CH ₃	6	286	8586
S-CH ₂ -C(=O)-O-C(CH ₃) ₃	6	312	1658
S-CH ₂ -C(=O)-CH ₂ -CH ₃	6	229	1811
O-CH ₂ -C(=O)-O-C(CH ₃) ₃	6	757	9476

EC_{50} , 50% effective concentration or the compound concentration required to inhibit HIV-1-induced cytopathicity in CEM cell cultures by 50%; IC_{50} , concentration required to inhibit the RT reaction by 50%.

^a The 3-(2-methylfuran-2-yl) moiety of the thiocarboxanilide derivative is replaced by a 3-(2-methylthien-2-yl) moiety.

strongly supports our notion on the potential role of Trp-229 as a target in rational drug design.

Crystal structures of complexes between HIV-1 RT and diverse NNRTIs have shown that the NNRTI-binding pocket has a well defined shape (with specific exceptions in the cases of HEPT and delavirdine). In all RT-NNRTI

complexes the position of the side chain of Trp-229 is well conserved. However, for certain NNRTIs (thiocarboxanilides and, to a lesser extent, emivirine and delavirdine) the main chain for the primer grip residues is somewhat repositioned. Although the rings of Trp-229 still occupy a similar position, they are "flipped over" by approximately 180° around the C β -C γ bond (Ren et al., 1998). In unliganded RT structures, Trp-229 is reoriented and partly exposed to the polymerase active site. In the structure of the "trapped" catalytic complex between RT, dsDNA, and dTTP (Huang et al., 1998), Trp-229 is displaced by approximately 4 Å away from the polymerase active site into the space normally considered part of the NNRTI-binding pocket, and its original position is occupied by Met230. Thus, Trp-229 is in different positions and environments in different states of the RT, making a detailed understanding of the effects of mutations very difficult.

Given that Trp-229 can occupy a variety of positions and that it does not contact the template-primer directly (Huang et al., 1998), it is remarkable that all the mutations (even 229Phe and 229Tyr) reduced RT activity to <2% (Fig. 1). Possibly, Trp-229 is vital for correct protein folding or for stabilizing the complex between RT and the template-primer. The marginal activity of the 229Gln mutant found in our study may be due either to the size of the side chain (in which case the 229Glu and 229Leu mutants might share similar activity) or to the polar nitrogen atom that is at the same distance on the side chain for both Trp and Gln. The side chain of residue 229 is positioned close to that of the catalytically important Tyr183, and thus changes in residue 229, which affect Tyr183 may have a large indirect impact on enzyme activity.

For the 229Phe and 229Tyr mutants it was possible to measure enzyme activity and to assess whether these mutations conferred resistance to NNRTIs. The 229Phe RT mutation conferred little if any resistance, but some resistance was conferred by the 229Tyr RT mutation, most notably to UC-781 and emivirine (Table 1). Interestingly, these are the only two NNRTIs in the test panel for which the RT-NNRTI structure shows repositioning of the primer grip residues (Ren et al., 1998). When the 229Tyr was modeled into a

variety of RT-NNRTI structures, we found that 229Tyr could be accommodated easily and there might be a favorable interaction (possibly a hydrogen bond) between the hydroxyl groups of Tyr183 and 229Tyr in the normal primer grip position. However, with the thiocarboxanilides and emivirine the altered primer grip position leads either to a clash of the 229Tyr ring with Pro95 or to a steric clash between the hydroxyl groups of Tyr183 and 229Tyr, resulting in the 229Tyr-mutated RT displaying resistance to UC-781 and emivirine.

The crystal structure of RT complexed with UC-781 shows Trp-229 in a conformation characteristic for thiocarboxanilides (inverted ring rotation), the end of the UC-781 pentenyl ether group being positioned about 4 Å from the face of the Trp-229 ring system (Ren et al., 1998). We could now demonstrate that an equivalent interaction is expected for all thiocarboxanilides where the pentenyl ether moiety is replaced by a group of the same length (five atoms), whereas the nature of the atoms in the group is less important. Shorter or longer substituents would be unable to make such an interaction or clash sterically with Trp-229, and result in much less potent inhibitors (Table 2, Fig. 3). Optimal interactions with Trp-229 can, of course, also be made by other inhibitors, and several examples of such optimization with HEPT and PETT derivatives are reported in the literature (Balzarini et al., 1995; Cantrell

et al., 1996). However, this report highlights the structural basis underlying these optimizations. The experiments in which we varied the pentenyl ether substituent of UC-781 clearly indicate that it is realistic to specifically target Trp-229 of RT by a NNRTI and that an alkenyloxy group length of five atoms is ideal for interactions between thiocarboxanilides and Trp-229. Our study now also revealed that resistance mutations at position 229 have never been observed under UC-781 pressure due to the fact that the 229Tyr mutation (which confers 21-fold resistance to UC-781) and any other possible mutation at this position is virtually lethal for the virus. NNRTIs such as some HEPT and PETT derivatives whose crystal RT complex structure have shown a relatively close interaction with Trp-229, have also never been reported to select for a mutation at amino acid position 229. It is interesting to note that a mutation at amino acid position 229 has also never been observed in combination with other mutations, indicating that compensatory mutations to restore RT activity of Trp-229 mutated enzyme will not easily occur.

In conclusion, our results indicate that Trp-229 is a prime amino acid candidate within the HIV-1 RT for targeted design of NNRTIs because: 1) it is not possible to mutate Trp-229 without severe loss of RT activity and virus infectivity; 2) mutating Trp-229 does not result in a high resistance profile to NNRTIs; and 3) it is feasible to

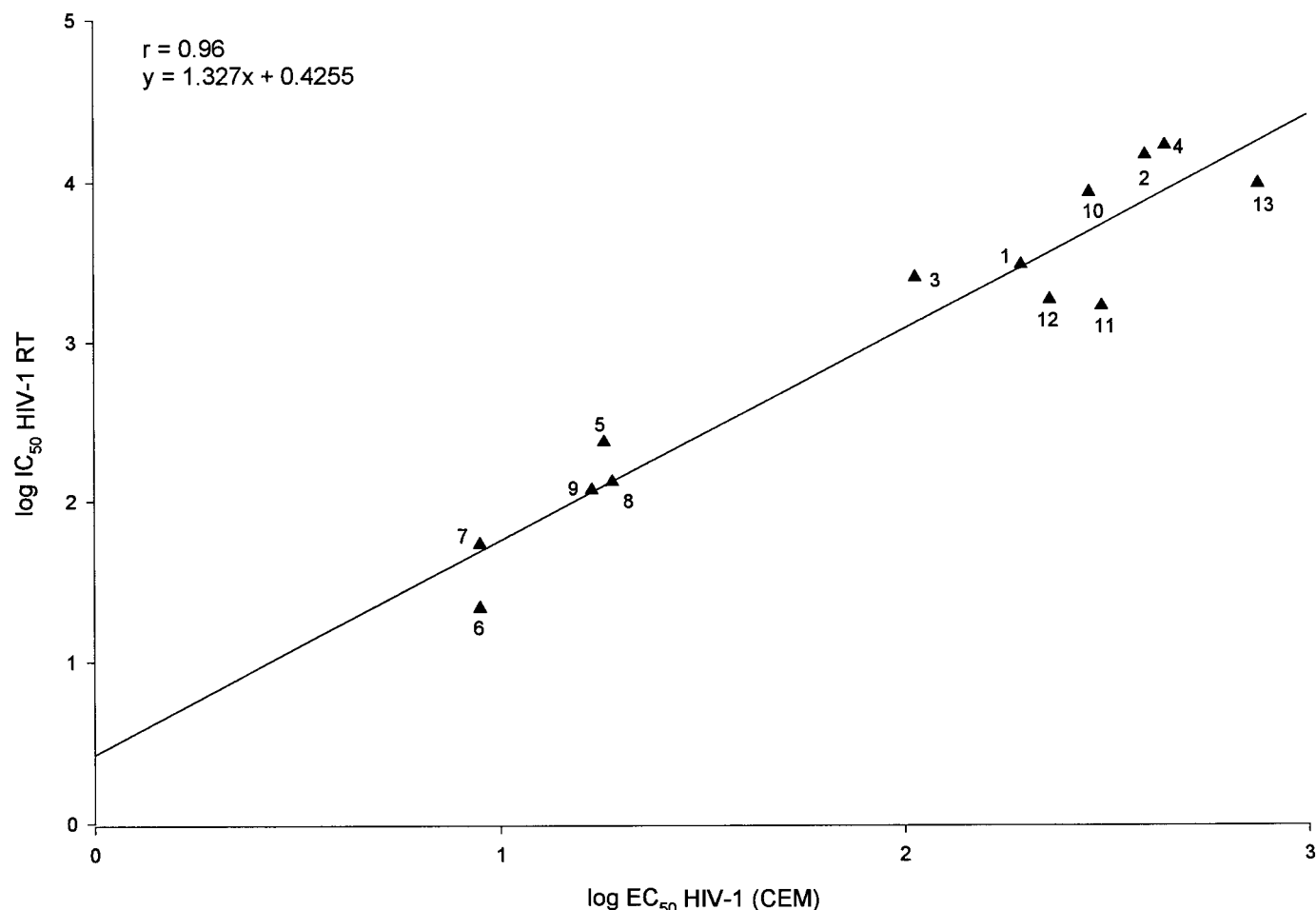


Fig. 3. Correlation between the log EC₅₀ values of 13 different thiocarboxanilide analogs against wild-type HIV-1 replication in CEM cell cultures and their log IC₅₀ values against wild-type recombinant HIV-1 RT. The test compounds are described in Table 2.

target Trp-229 with NNRTIs (as exemplified by UC-781) because of its physical participation in creating the NNRTI-characteristic binding pocket. Because targeting one crucial amino acid in the RT is insufficient to afford efficient resistance suppression, we believe that designing new drugs should be concomitantly targeted at different immutable amino acids, like Trp-229 (as shown in this study) and Tyr-318 (Pelemans et al., 1998). This would be a rational strategy to be pursued in an attempt to further potentiate the antiviral activity of existing or novel NNRTIs and to more efficiently suppress resistance development.

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Send reprint requests to: Jan Balzarini, Rega Institute for Medical Research, Minderbroedersstraat 10, B-3000 Leuven, Belgium. E-mail: Jan.Balzarini@rega.kuleuven.ac.be