0026-895X/93/040521-06$03.00/0

MOLECULAR PHARMACOLOGY, 43:521-526

Kinetics of Different Human Immunodeficiency Virus Type 1 Reverse Transcriptases Resistant to Human Immunodeficiency Virus Type 1-Specific Reverse Transcriptase Inhibitors

Z. DEBYSER, K. DE VREESE, P. P. KNOPS-GERRITS, V. BAEKELANDT, R. BHIKHABHAI, B. STRANDBERG, R. PAUWELS, J. ANNÉ, J. DESMYTER, and E. DE CLERCQ

Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium (Z.D., K.D.V., P.P.K.-G., R.P., J.A., J.D., E.D.C.), Institute for Neuroendocrinology and Immunological Biotechnology, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium (V.B.), and Department of Molecular Biology, Uppsala University, Uppsala Biomedical Center, S-75124 Uppsala, Sweden (R.B., B.S.)

Received September 23, 1992; Accepted January 13, 1993

SUMMARY

The human immunodeficiency virus type 1 (HIV-1)-specific reverse transcriptase (RT) inhibitors [tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-one and -thione (TIBO), 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine, nevirapine, pyridinone, bis(heteroaryl)piperazine, etc.] are potent inhibitors of HIV-1 replication in cell culture. The rapid emergence of drug-resistant escape mutants *in vitro* (cell culture) and *in vivo* (patients) is predominantly linked to the $Y_{181}C$ mutation. Because amino acids Y_{181} and Y_{188} appear to be located within the drug binding site of the enzyme, we studied the impact of mutations of both amino acids on the enzyme kinetics and on the susceptibility of the enzyme to different HIV-1-specific RT inhibitors. Mutations $Y_{181}C$, $Y_{181}I$, and $Y_{188}L$ led to reduced sensitivity, albeit of varying extents, to all HIV-1-specific RT inhibitors. No resistance was observed to 2′,3′-dideoxyguanosine 5′-triphosphate or phosphonoformic acid. The k_{cat} of the $Y_{181}C$ mutant was similar to that of the wild-type RT (18 $sec^{-1} \times 10^{-3}$). The catalytic constant of the $Y_{181}I$ mutant was 6-fold higher and that of the $Y_{188}L$ mutant was 6-fold lower. Whereas TIBO displayed a linear mixed-type (noncompetitive) inhibition with respect to the deoxynucleotide substrate when wild-type p66/p51 was used, the pattern of inhibition became competitive or uncompetitive with $Y_{181}C$ or $Y_{181}I$, respectively. Thus, the TIBO binding site of HIV-1 RT seems to be functionally and/or spatially related to the natural deoxynucleoside triphosphate binding site.

The potential usefulness of the HIV-1-specific RT inhibitors (i.e., TIBO, HEPT, nevirapine, pyridinone, and bisheteroarylpiperazine) (1–6) is confounded by the rapid emergence of drug-resistant mutants in cell culture. Drug resistance has been reported after serial passage of virus in the presence of pyridinone (7) and nevirapine (8). Cross-resistance has been observed to TIBO (7, 8), nevirapine (7), and E-EPU (8). The selected HIV-1 variants retained their sensitivity to dideoxynucleosides (i.e., azidothymidine) and PFA and remained stable with continued passage in the absence of drug.

This research was supported in part by the AIDS Basic Research Programme of the European Community, by the Janssen Research Foundation, and by grants from the Belgian Geconcerteerde Onderzoeksacties (Project 90/94-2) and the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek (Project 3.0097.87). Z.D. is a Research Assistant of the Belgian National Fund for Scientific Research.

Substitution of cysteine for tyrosine at position 181 of HIV-1 RT is apparently the single most important mutation conferring reduced sensitivity to TIBO and TIBO-like compounds. The amino acids Y_{181} and Y_{188}, both flanking the highly conserved QYMDDL (182–187) locus, are critically involved in the binding of the HIV-1 RT inhibitors. Lysine at position 103 seems also to be important (7). These findings have been demonstrated independently by affinity labeling and site-directed mutagenesis (9–12) and were confirmed recently by the elucidation of the three-dimensional structure of HIV-1 RT, co-crystallized with nevirapine (13). Because the HIV-2 RT lacks both tyrosine residues, the specificity of the HIV-1-specific RT inhibitors may be at least partially attributed to these amino acids. In fact, an HIV-2 RT chimera with amino acids 176–190 from HIV-1 RT has proved to be sensitive to these inhibitors (10). Surprisingly, the RT of two SIV_{agm} strains

ABBREVIATIONS: HIV, human immunodeficiency virus; RT, reverse transcriptase; ddGTP, 2′,3′-dideoxyguanosine 5′-triphosphate; TIBO, tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-one and -thione; R82150, (+)-(*S*)-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)imidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-thione; HEPT, 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine; E-EPU, 5-ethyl-1-ethoxymethyl-6-(phenylthio)uracil; α-APA, α-anilinophenylacetamide; TSAO, 2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)pyrimidine nucleoside; IC_{50}, 50% inhibitory concentration; RDDP, RNA-dependent DNA polymerase; DDDP, DNA-dependent DNA polymerase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; dNTP, deoxynucleoside triphosphate; SIV, simian immunodeficiency virus; PFA, phosphonoformic acid.

(agm3 and TYO-1) is relatively sensitive to TIBO and E-EPU. These SIV RTs contain a valine at position 181 and tryptophan at position 188 (14).

Apparently, resistant variants are present in the genetic pool of a particular virus strain and are merely selected by serial passage in the presence of the drug (8). The question, however, arises of whether these variants are as pathogenic as the wild-type virus. From this perspective, we investigated the kinetic properties of three TIBO-resistant HIV-1 RTs ($Y_{181}C$, $Y_{181}I$, and $Y_{188}L$) obtained by site-directed mutagenesis (11). By using a "one-step" chromatographic purification procedure, RT was purified to 80–90%. The inhibitory effects of the different HIV-1-specific RT inhibitors on the mutated enzymes were then compared.

Materials and Methods

Compounds. The origin of TIBO R82150 (M_r 287.4) has been described previously (1). For E-EPU (M_r 306), see Ref. 3. Nevirapine (BI-RG-587; 11-cyclopropyl-5,11-dihydro-4-methyl-6*H*-dipyrido[3,2-*b*:2′,3′-*e*][1,4]diazepin-6-one) (M_r 266.3) (4) was obtained from Boehringer Ingelheim Pharmaceuticals. L-697,661 [3-[[(4,7-dichloro-1,3-benzoxazol-2-yl)methyl]amino]-5-ethyl-6-methylpyridin-2(1*H*)-one] (M_r 340) (5) was obtained from Merck Sharp & Dohme Research Laboratories. The α-APA derivatives (prototype, R89439) (M_r 351) represent a new series of HIV-1-specific RT inhibitors recently developed in our laboratory in collaboration with the Janssen Research Foundation (15). The prototypical compounds investigated in this report are presented in Fig. 1.

Stock solutions (20 mg/ml) of all compounds were made in dimethylsulfoxide. Under these dilution conditions, only L-697,661 was poorly soluble at high concentrations. The final dimethylsulfoxide concentration in the RT assays was <5%. ddGTP (Pharmacia) and PFA (Sigma) were dissolved directly in reaction buffer.

RT sources. The following pure recombinant RT preparations were used: p66/66 homodimer, produced in yeast, a kind gift from P. Barr (Chiron Corporation) (16), and p66/p51 heterodimer, produced in *Escherichia coli* as described previously (17). In the latter expression system the entire HIV-1 *pol* gene, encoding for protease, RT, and integrase, is expressed (18). Both bacterial and HIV-1 protease are responsible for the cleavage of p66 into p51. The viral protease cleaves the p66 subunit at F_{440} (16). Wild-type RT and three mutants derived from it were expressed and purified as described below.

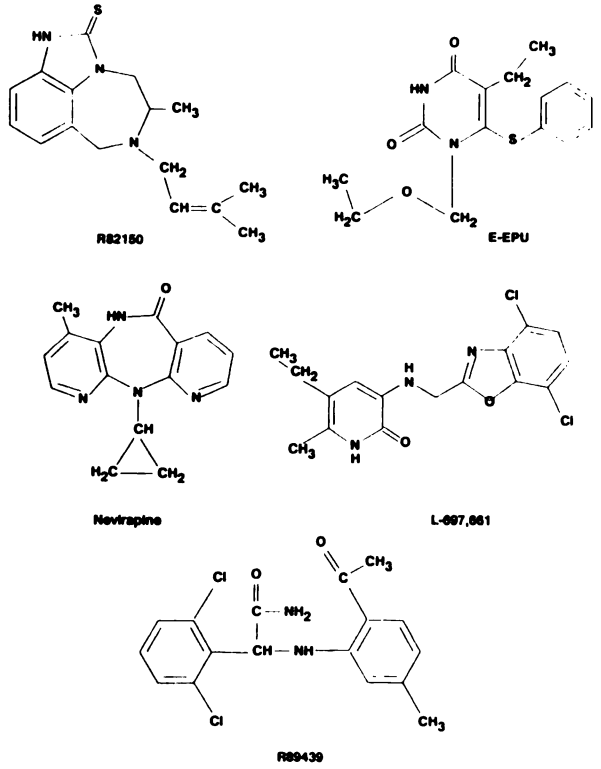

Fig. 1. Chemical structures of TIBO R82150, the HEPT congener E-EPU, nevirapine (BI-RG-587), pyridinone L-697,661, and α-APA R89439.

Site-directed mutagenesis. The expression vector pKRT2, containing the RT-encoding fragment of HIV-1 under the control of the *trc* promoter, was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS (National Institute of Allergy and Infectious Diseases, National Institutes of Health), from R. D'Aquila and W. C. Summers (Yale University School of Medicine, New Haven, CT) (19). Oligonucleotide-directed mutagenesis of RT was performed with the Altered Sites *in vitro* mutagenesis system from Promega. Oligonucleotide primers were synthesized on a Gene Assembler Plus (Pharmacia) by A. Van Aerschot, Laboratory of Pharmaceutical Chemistry (Rega Institute). Mutations were verified by DNA sequence analysis using a United States Biochemicals Sequenase kit (version 2.0) and [α-^{35}S]dATP from Amersham.

Expression and partial purification of recombinant RT. *E. coli* JM109, containing wild-type pKRT2 or mutants derived from it, were grown to late logarithmic phase (4 hr) at 37° in Lennox L. broth base with 500 mg/liter ampicillin. Transcription of the RT gene was induced from the *trc* promoter with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 6 hr. Pelleted cells (6000 rpm for 10 min at 4°) were washed with buffer (20 mM Tris·HCl, pH 8.2, 1 mM EDTA, 75 mM NaCl) and frozen at −20°. Pellets of 1.6 liters of cell culture (dry weight, 4–6 g) were lysed in a solution containing 20 mM Tris·HCl, pH 8.2, 75 mM NaCl, 5 mM EDTA, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 1 mM $MgSO_4$, RNase A, and DNase I. Lysates were sonicated and centrifuged for 20 min at 12,000 rpm at 4°. The supernatant was precipitated with 70% saturated ammonium sulfate, and the pellet was dissolved and dialyzed against buffer A (20 mM Tris·HCl, pH 7.8, 1 mM EDTA, 1 mM DTT, 2% glycerol) for 2 hr at 4°. Samples were then applied to an anion exchange column packed with DEAE-Sepharose (5 ml), connected in series with a 5-ml HiTrap affinity column packed with heparin-Sepharose. After application the columns were washed with 40 ml of buffer A. The column packed with DEAE-Sepharose was disconnected and the heparin-Sepharose column was eluted with a linear gradient from 0 to 1 M NaCl in buffer A. RT typically elutes at 0.35 M NaCl. Peak fractions, based on RT activity and analysis by SDS-PAGE, were pooled and stored at −80°. All chromatographic procedures were performed with a FPLC system (Pharmacia) in a Maxi Cold Lab (LKB) at 4°. DEAE-Sepharose and HiTrap heparin-Sepharose were purchased from Pharmacia. SDS-PAGE analysis was done with a PhastSystem (Pharmacia). Proteins were detected by silver staining.

Determination of RT concentration. The p66/p51 heterodimer of HIV-1 RT was obtained as reported previously (17). This enzyme was used to determine an ϵ_{280} of 400,000 M^{-1} cm^{-1}. Protein concentrations of pooled peak fractions of wild-type and mutant RT preparations were subsequently determined by absorbance. Because our enzyme preparations were only 80–90% pure (as judged by SDS-PAGE), the RT concentrations are given with an experimental error of 10–20%.

RT assays. The RT reaction mixture (50 μl) contained 50 mM Tris·HCl, pH 8.1, 10 mM $MgCl_2$, 100 mM KCl, 2.2 mM DTT, and 0.05% (w/v) Triton X-100. The template/primers [poly(C)·oligo$(dG)_{12-18}$ and poly(dC)·oligo$(dG)_{12-18}$] (Pharmacia) were used at a concentration of 65 μg/ml. Tritium-labeled dGTP was purchased from Amersham and used at a final concentration of 2.5 μM. Specific activity was 11 Ci/mmol. After addition of the inhibitors at various concentrations and of 10 μl of the different enzyme preparations, the reaction mixture was incubated for 1 hr at 37°. The final enzyme concentration was 20 nM, except for the p66/p66 (yeast) and p66/p51 (*E. coli*) preparations, which were used at a final concentration of 0.5 nM. The incorporation

rate was determined by a standard trichloroacetic acid precipitation procedure, using Whatman GF/C glass fiber filters (Whatman) and liquid scintillation counting (Ready-Protein; Beckman).

Kinetic studies. Kinetic studies were performed with varying substrate (dGTP) and template/primer [poly(C)·oligo(dG)] concentrations. ^{3}H-Labeled nucleotides dissolved in ethanol/water (1:1) were dried (Speedvac; Savant) and dissolved in water to avoid interference from ethanol in the kinetics studies. The rate of incorporation of ^{3}H-labeled nucleotides by wild-type and mutant RT was linear for 45 min. Studies were performed under steady state conditions in 15-min assays. Experimental data were analyzed by a nonlinear regression analysis software program (Enzfitter; Elsevier-Biosoft) based on Michaelis-Menten and Hill equations.

Results

Partial purification of wild-type and mutant RTs. Because the *E. coli* lysate RT preparations are unstable, mainly due to the presence of bacterial proteases, RT was purified based on a method described by Bhikhabhai *et al.* (17). This is a one-step purification procedure in which columns packed with DEAE-Sepharose and heparin-Sepharose are connected in series, thus combining anion exchange and affinity chromatography. All forms of RT at pH 7.8 adsorbed to heparin-Sepharose but did not bind to DEAE-Sepharose. The RT thus obtained was 80% pure as determined by SDS-PAGE.

On SDS-PAGE RT appeared as an equimolar mixture of p66 and p51, the latter resulting from proteolytic cleavage of p66 by contaminating bacterial proteases. Wild-type RT and the RT mutants $Y_{181}C$, $Y_{181}I$, and $Y_{188}L$ eluted at a similar salt concentration (0.35 M NaCl) from the heparin-Sepharose column. The p66 and p51 bands from different forms of RT displayed an identical electrophoretic mobility on SDS-PAGE (Fig. 2) and were recognized by a monoclonal antibody against p66 in a Western blot (data not shown). The partially purified RT preparations were stable for weeks at 4° and for months at −80°.

Inhibition studies with mutant RTs. The different enzymes were evaluated for their susceptibility to inhibition by the HIV-1-specific RT inhibitors TIBO (R82150), HEPT (E-EPU), nevirapine (BI-RG-587), pyridinone (L-697,661), and α-APA (R89439). The results are shown in Table 1. Diminished susceptibility of the RT mutants to all HIV-1-specific RT inhibitors was noted, albeit to varying extents. PFA, a pyrophosphate analogue, and ddGTP, a chain terminator, did not show diminished potency against any of the RT mutants.

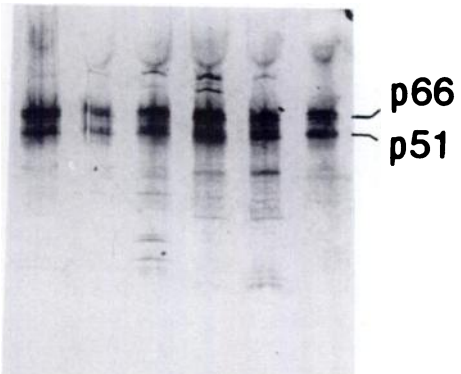

Fig. 2. Electrophoretic analysis of purified RT. SDS-PAGE was performed on an 8–25% PhastGel gradient. Proteins were detected by silver staining. *Lanes 2–5*, pKRT2-derived enzyme preparations (20 nM) of $Y_{188}L$ (*lane 2*), $Y_{181}I$ (*lane 3*), $Y_{181}C$ (*lane 4*), and wild-type RT (*lane 5*). *Lanes 1* and *6*, control enzyme preparations of p66/p51 (yeast) (*lane 1*) and p66/p51 (*E. coli*) (*lane 6*).

In general, resistance was lowest with the $Y_{181}C$ mutant and highest with the $Y_{188}L$ mutant. As for the $Y_{181}C$ mutant, this was more resistant to nevirapine and α-APA than to the other compounds. Only E-EPU, and to a lesser extent TIBO, retained some inhibitory potency against the $Y_{181}I$ mutant. All compounds lost activity when Y_{188} was replaced by leucine.

Kinetic analysis. Kinetic studies were performed with partially purified mutant RTs, in comparison with wild-type RT. Studies were performed under steady state conditions in 15-min assays with a saturating template/primer [poly(C)·oligo(dG)] concentration (65 μg/ml). The final enzyme concentration was always 20 nM. Incorporation of ^{3}H-labeled nucleotides by the different enzymes was linear for 45 min. Table 2 presents the kinetic parameters obtained with varying substrate (dGTP) concentrations. The purified wild-type RT preparation displayed a lower k_{cat} value than did the heterodimeric p66/p51 (19) and homodimeric p66/p66 (16). The K_m value was similar to that obtained with the heterodimeric p66/p51 RT but higher than that of the homodimeric p66/p66. Based on the Hill coefficients ($n_H = 1$), no positive cooperativity for dGTP was detected with the wild-type RT preparation. However, cooperativity was observed with the heterodimeric p66/p51 preparation ($n_H = 1.3$).

As for the mutants, the kinetic characteristics of $Y_{181}C$ were similar to those of wild-type RT. The catalytic constant was 6-fold higher for $Y_{181}I$ and 6-fold lower for $Y_{188}L$. The K_m value was lower for $Y_{188}L$.

The DDDP function of the enzymes, assayed with poly(dC)·oligo$(dG)_{12-18}$, was compared with the RDDP activity, tested with poly(C)·oligo$(dG)_{12-18}$ under typical RT reaction conditions (2.5 μM dGTP and 65 μg/ml template/primer) (Table 3). RDDP activities, measured with a subsaturating dGTP concentration, are consistent with previous estimates of RT activities in *E. coli* lysate (11). $Y_{188}L$ was about 2.5-fold less active than the wild-type enzyme in terms of RDDP activity, whereas its DDDP activity was about 6-fold lower than that of the wild-type enzyme.

Next, we studied the kinetics of inhibition of the enzymes by R82150 at its IC_{50} for each enzyme (Table 4). Kinetic parameters were calculated with a nonlinear regression analysis software program, based on the Michaelis-Menten equation. Whereas TIBO displayed a linear mixed type of inhibition (with respect to the substrate) when wild-type pKRT2-derived p66/p51 was used, the pattern of inhibition was competitive and uncompetitive for $Y_{181}C$ and $Y_{181}I$, respectively. The K_i values, determined from Dixon plots, were 0.25 μM (wild-type enzyme), 1.75 μM ($Y_{181}C$), and 28.4 μM ($Y_{181}I$). The K_i value of R82150 for $Y_{181}I$ was calculated at an infinitely high concentration of substrate, according to the formula $K_{i_{app}} = K_i(1 + K_m/[S])$.

Kinetic studies were also performed with varying template/primer [poly(C)·oligo$(dG)_{12-18}$] concentrations (Table 5). In these studies the substrate concentration was kept constant at 2.5 μM (subsaturation). The K_m value of the wild-type enzyme (1.8 μg/ml) was comparable to that of p66/p66 (yeast) (1 μg/ml), whereas the K_m of $Y_{181}I$ was considerably higher (10 μg/ml). Also, the k_{cat} of $Y_{181}I$ was considerably higher than those of the other RT preparations. In the presence of the inhibitor, the highest K_m was observed for $Y_{181}C$, whereas, again, $Y_{181}I$

TABLE 1

Sensitivity of HIV-1 mutant RTs to HIV-1-specific RT inhibitors

Data represent mean values ± standard deviations for three separate experiments.

Inhibitor	IC_{50}[a]			
	Wild-type RT	$Y_{181}C$	$Y_{181}I$	$Y_{188}L$
		μM		
TIBO R82150	0.16 ± 0.03	2.6 ± 1.4 (16)	44.9 ± 0.7 (273)	>350 (>2121)
HEPT congener E-EPU	0.17 ± 0.06	3.4 ± 1.4 (20)	14.8 ± 1.2 (88)	>340 (>2024)
Nevirapine (BI-RG-587)	0.34 ± 0.04	30 ± 1 (88)	>78 (>230)	>390 (>1147)
Pyridinone L-697,661	0.056 ± 0.003	2.0 ± 0.6 (36)	55 ± 4 (987)	>294 (>5250)
α-APA R89439	0.100 ± 0.013	22.0 ± 8.2 (220)	>55 (>550)	>274 (>2740)
PFA	6 ± 3	9.47 ± 1.32 (1.6)	35.8 ± 0.5 (6)	1.9 ± 0.7 (0.3)
ddGTP	0.025 ± 0.006	0.012 ± 0.008 (0.5)	0.029 ± 0.005 (1.1)	0.012 ± 0.003 (0.5)

[a] Concentration of compound required to inhibit RT activity by 50% in a poly(C)·oligo(dG)$_{12-18}$-directed assay. Fold increase in IC_{50} of mutants compared with wild-type enzyme, is indicated in parentheses.

TABLE 2

Kinetic parameters of HIV-1 mutant RTs (substrate)

The kinetic parameters k_{cat} (catalytic constant), K_m, and n_H were calculated by nonlinear regression analysis (Enzfitter) based on Michaelis-Menten and Hill equations. Data represent mean values ± standard deviations for at least two separate experiments.

Enzyme	k_{cat}	K_m	n_H
	$sec^{-1} \times 10^{-3}$	μM	
Wild-type	18.0 ± 1.0	16 ± 4	1.0 ± 0.1
$Y_{181}C$	18 ± 1	22 ± 2	1.0 ± 0.1
$Y_{181}I$	116 ± 11	26 ± 6	0.96 ± 0.01
$Y_{188}L$	3.0 ± 0.6	8.2 ± 1.8	1.0 ± 0.2
p66/p66 (yeast)	319 ± 61	6 ± 2	1.05 ± 0.05
p66/p51 (*E. coli*)	760 ± 10	17 ± 2.4	1.3 ± 0.4

TABLE 3

RDDP and DDDP functions of the HIV-1 mutant RTs

Enzymatic activity is defined as the amount (μmol) of dGMP incorporated per sec and per μmol of enzyme at 37° under the reaction conditions used (dGTP at 2.5 μM, template/primer at 65 μg/ml).

Enzyme	Enzymatic activity	
	Poly(C)·oligo(dG)$_{12-18}$	Poly(dC)·oligo(dG)$_{12-18}$
	$sec^{-1} \times 10^{-3}$	
Wild-type	2.4 ± 0.2	15 ± 3
$Y_{181}C$	1.6 ± 0.1	9.7 ± 0.3
$Y_{181}I$	6 ± 3	27 ± 6
$Y_{188}L$	1.0 ± 0.1	2.6 ± 0.4

TABLE 4

Kinetics of HIV-1 mutant RT inhibition with respect to the substrate (dGTP)

The kinetic parameters K_m and k_{cat} are given for the reactions without and with 0.35 μM (wild-type enzyme), 3.5 μM $Y_{181}C$), or 70 μM ($Y_{181}I$) R82150. Data represent mean values ± standard deviations for three independent assays.

Enzyme	Without inhibitor		With inhibitor (R82150)		Type of inhibition[a]
	K_m	k_{cat}	K_m	k_{cat}	
	μM	$sec^{-1} \times 10^{-3}$	μM	$sec^{-1} \times 10^{-3}$	
Wild-type	16 ± 4	21 ± 2	33 ± 9	14 ± 3	M
$Y_{181}C$	22 ± 2	19 ± 1	53 ± 14	18 ± 1	C
$Y_{181}I$	26 ± 6	137 ± 14	13.8 ± 0.2	53 ± 11	UC

[a] Type of inhibition: M, linear mixed type; C, competitive; UC, uncompetitive.

showed the highest k_{cat}. The pattern of inhibition of the wild-type RT by TIBO was uncompetitive with regard to the template/primer, which is in accord with previous observations for the p66/p66 (yeast) RT (2). For the $Y_{181}I$ mutant inhibition was uncompetitive, whereas for the $Y_{181}C$ mutant it was of the linear mixed type.

TABLE 5

Kinetics of HIV-1 mutant RT inhibition with respect to the template/primer [poly(C)·oligo(dG)$_{12-18}$]

The kinetic parameters K_m and k_{cat} are given for the reactions without and with inhibitor at 0.35 μM (wild-type enzyme and p66/p66), 3.5 μM ($Y_{181}C$), or 70 μM ($Y_{181}I$). dGTP was at a subsaturating concentration of 2.5 μM. Data represent mean values ± standard deviations for three independent assays.

Enzyme	Without inhibitor		n_H	With inhibitor (R82150)		Type of inhibition[a]
	K_m	k_{cat}		K_m	k_{cat}	
	μg/ml	$sec^{-1} \times 10^{-3}$		μg/ml	$sec^{-1} \times 10^{-3}$	
Wild-type	1.8 ± 0.2	2.3 ± 0.5	1.0 ± 0.1	0.8 ± 0.3	0.7 ± 0.2	UC
$Y_{181}C$	7.0 ± 2.8	1.3 ± 0.2	0.9 ± 0.1	15 ± 6	0.7 ± 0.1	M
$Y_{181}I$	10.0 ± 0.5	14 ± 2.6	0.8 ± 0.1	4.0 ± 0.7	7 ± 1	UC
$Y_{188}L$	2.0 ± 1.3	0.5 ± 0.1	1.0 ± 0.1			
p66/p66	1.0 ± 0.3	89 ± 6	1.1 ± 0.1	0.4 ± 0.2	13 ± 2	UC

[a] Type of inhibition: UC, uncompetitive; M, linear mixed type.

Discussion

Rapid emergence of virus drug resistance *in vitro* has recently been reported for the HIV-1-specific inhibitors pyridinone and nevirapine (7, 8). Similar observations have been made for TIBO compounds (15).[1] Resistance to pyridinone and nevirapine has been linked to a $Y_{181}C$ mutation. Preliminary findings with pyridinone and nevirapine in clinical trials point to a rapid selection of HIV-1 variants carrying the $Y_{181}C$ mutation. Both Y_{181} and Y_{188} are located within the binding site for the HIV-1-specific RT inhibitors (9–12). Their counterparts in HIV-2 are I_{181} and L_{188}. Previously (11), we constructed three TIBO-resistant RTs by site-directed mutagenesis, i.e., $Y_{181}C$, $Y_{181}I$, and $Y_{188}L$. These enzymes are expressed in *E. coli*. We have now performed extensive kinetic studies with the purified enzymes.

First we compared the various HIV-1-specific RT inhibitors for their inhibitory effects on wild-type and resistant RTs. The mutated enzymes showed reduced sensitivity ("resistance") to the inhibitors tested, including TIBO, E-EPU, nevirapine, pyridinone, and α-APA, as well as to the TSAO derivatives, another class of HIV-1-specific inhibitors (20) (data not shown). From these findings, three conclusions can be drawn, (i) tyrosine residues 181 and 188 are essential for the interaction of the HIV-1-specific RT inhibitors with their target, (ii) all HIV-1-specific RT inhibitors bind to the same target on HIV-1 RT and thus belong to the same pharmacological class, and (iii) the specificity of these RT inhibitors for HIV-1 depends on the presence of the two tyrosine residues.

Marked differences were noted in the sensitivity of the RT

mutants to the various RT inhibitors tested. TIBO and E-EPU were only 16–20-fold less active against the $Y_{181}C$ mutant (compared with the wild-type enzyme). This may imply that $Y_{181}C$ mutants that are resistant to nevirapine may still respond to TIBO or E-EPU, albeit at higher concentrations than required for inhibition of the wild-type strains. The differential extent of resistance with various HIV-1-specific RT inhibitors is indicative of subtle differences in their molecular interactions with the enzyme. These findings are of interest for the development of new HIV-1 RT inhibitors in attempts to overcome the problem of resistance.

Our kinetic studies revealed lower enzymatic activity for the wild-type pKRT2-derived enzyme than for the previously obtained pure RT preparations, namely p66/p66 (expressed in yeast) and p66/p51 (expressed in *E. coli* and at least partially cleaved by the coexpressed viral protease). The lower activity may be due to impurities in our pKRT2 enzyme preparations or may be inherent to the construct. We carried out the following experiment to rule out the presence of inhibitory contaminants in our purified RT preparations. A p51 preparation (from a novel pKRT2-derived construct encoding only p51) was expressed in *E. coli* and purified following the same procedure as for p66/p51. Addition of this p51 preparation to pure p66/p51 (*E. coli*) did not result in any significant decrease of enzymatic activity. The low specific activity of the pKRT2-derived enzymes may be inherent in the construct and/or expression system. The pKRT2 construct, expressed in *E. coli*, results in heterodimeric RT, because one of the p66 subunits is processed by bacterial proteases. Whether differential cleavage by bacterial versus viral proteases may result in altered protein folding and RT activity is presently being studied in our laboratory. Because the pKRT2-derived wild-type and mutant enzymes are expressed and purified in the same way, we were able to directly compare their kinetic properties.

Allosteric properties have been recently reported for the p66/p51 preparation, with both subunits being separately expressed in yeast (21). These allosteric kinetics were not observed with the wild-type (pKRT2-derived) enzyme (see Hill coefficients), although they were again found for the p66/p51 enzyme. It is known that cleavage by bacterial proteases results in a p51 subunit that is seven amino acids shorter than the natural counterpart (22). The question remains once again whether the p51 processed by bacterial protease has biochemical characteristics different from those of the p51 cleaved by viral protease.

Next, we compared the pKRT2-derived wild-type enzyme and the mutants derived from it for their kinetic parameters. Whereas the $Y_{181}C$ mutant could hardly be distinguished from the wild-type enzyme, the two HIV-2-chimeric mutants $Y_{181}I$ and $Y_{188}L$ displayed markedly altered kinetic properties. The fact that the $Y_{181}C$ mutant, but not the $Y_{188}L$ mutant, has been readily selected upon cell culture passage of the virus in the presence of inhibitors (nevirapine and pyridinone) may be related to these altered enzymatic properties.

The TIBO binding site of the HIV-1 RT may be functionally, or even spatially, related to the normal dNTP substrate binding site (23, 24). TIBO R82150 is a potent noncompetitive inhibitor of HIV-1 RT with respect to the dNTP substrate (2, 25). However, TIBO R82150 has a weak competitive inhibitory effect on SIV_{agm} RT (14), and inhibition of HIV-1 RT by HEPT (predecessor of the more potent E-EPU) has also proved to be competitive (23). Both of these competitive inhibitory effects on RT are characterized by a weak affinity of the inhibitors for the enzyme. Interestingly, substitution of tyrosine at position 181 of HIV-1 RT by a cysteine decreases the affinity of TIBO by about 15-fold and, concomitantly, reverses the noncompetitive inhibition to a competitive type of inhibition. These findings indicate that the type of inhibition with respect to the deoxynucleotide (dNTP) substrate is dependent on the affinity of the inhibitor for the enzyme. They point to the existence of a high and a low affinity binding site, resulting in noncompetitive and competitive inhibition, respectively. Whether these binding sites can be discriminated spatially or merely represent two distinct conformational forms of a single site needs to be elucidated. From our kinetic data, it appears that the TIBO binding site must be closely associated with, and may even be located within, the dNTP substrate binding site.

In conclusion, the single most important mutation of HIV-1 RT ($Y_{181}C$) responsible for *in vitro* and *in vivo* resistance to the whole class of HIV-1-specific RT inhibitors does not alter the kinetic parameters of the enzyme. To the extent that the RT kinetics play a role in the pathogenicity of the virus, $Y_{181}C$ mutation may be expected not to affect the pathogenicity of the resistant virus.

Acknowledgments

We thank H. Azijn and B. Van Remoortel for excellent technical assistance, Dr. F. Vandesande for advice, and C. Callebaut for fine editorial help. The expression vector pKRT2 was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, (National Institute of Allergy and Infectious Diseases, National Institutes of Health), from R. D'Aquila and W. C. Summers (Yale University School of Medicine, New Haven, CT).

References

1. Pauwels, R., K. Andries, J. Desmyter, D. Schols, M. J. Kukla, H. J. Breslin, A. Raeymaeckers, J. Van Gelder, R. Woestenborghs, J. Heykants, K. Schellekens, M. A. C. Janssen, E. De Clercq, and P. A. J. Janssen. Potent and selective inhibition of HIV-1 replication *in vitro* by a novel series of TIBO derivatives. *Nature (Lond.)* **343**:470–474 (1990).
2. Debyser, Z., R. Pauwels, K. Andries, J. Desmyter, M. Kukla, P. A. J. Janssen, and E. De Clercq. An antiviral target on reverse transcriptase of human immunodeficiency virus type 1 revealed by tetrahydroimidazo[4,5,1-*jk*][1,4] benzodiazepin-2(1*H*)-one and -thione derivatives. *Proc. Natl. Acad. Sci. USA* **88**:1451–1455 (1991).
3. Baba, M., E. De Clercq, H. Tanaka, M. Ubasawa, H. Takashima, K. Sekiya, I. Nitta, K. Umezu, H. Nakashima, S. Mori, S. Shigeta, R. T. Walker, and T. Miyasaka. Potent and selective inhibition of human immunodeficiency virus type 1 (HIV-1) by 5-ethyl-6-phenylthiouracil derivatives through their interaction with the HIV-1 reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **88**:2356–2360 (1991).
4. Merluzzi, V. J., K. D. Hargrave, M. Labadia, K. Grozinger, M. Skoog, J. C. Wu, C.-K. Shih, K. Eckner, S. Hattox, J. Adams, A. S. Rosenthal, R. Faanes, R. J. Eckner, R. A. Koup, and J. L. Sullivan. Inhibition of HIV-1 replication by a nonnucleoside reverse transcriptase inhibitor. *Science* (Washington D. C.) **250**:1411–1413 (1990).
5. Goldman, M. E., J. H. Nunberg, J. A. O'Brien, J. C. Quintero, W. A. Schleif, K. F. Freund, S. L. Gaul, W. S. Saari, J. S. Wai, J. M. Hoffman, P. S. Anderson, D. J. Hupe, E. A. Emini, and A. M. Stern. Pyridinone derivatives: specific human immunodeficiency virus type 1 reverse transcriptase inhibitors with antiviral activity. *Proc. Natl. Acad. Sci. USA* **88**:6863–6867 (1991).
6. Romero, D. L., M. Busso, C.-K. Tan, F. Reusser, J. R. Palmer, S. M. Poppe, P. A. Aristoff, K. M. Downey, A. G. So, L. Resnick, and W. G. Tarpley. Nonnucleoside reverse transcriptase inhibitors that potently and specifically block human immunodeficiency virus type 1 replication. *Proc. Natl. Acad. Sci. USA* **88**:8806–8810 (1991).
7. Nunberg, J. H., W. A. Schleif, E. J. Boots, J. A. O'Brien, J. C. Quintero, J. M. Hoffman, Jr., E. A. Emini, and M. E. Goldman. Viral resistance to human immunodeficiency virus type 1-specific pyridinone reverse transcriptase inhibitors. *J. Virol.* **65**:4887–4892 (1991).
8. Richman, D., C.-K. Shih, I. Lowy, J. Rose, P. Prodanovich, S. Goff, and J. Griffin. Human immunodeficiency virus type 1 mutants resistant to nonnucleoside inhibitors of reverse transcriptase arise in tissue culture. *Proc. Natl. Acad. Sci. USA* **88**:11241–11245 (1991).
9. Cohen, K. A., J. Hopkins, R. H. Ingraham, C. Pargellis, J. C. Wu, D. E. H. Palladino, P. Kinkade, T. C. Warren, S. Rogers, J. Adams, P. R. Farina, and P. M. Grob. Characterization of the binding site for nevirapine (BI-RG-587), a nonnucleoside inhibitor of human immunodeficiency virus type-1 reverse transcriptase. *J. Biol. Chem.* **266**:14670–14674 (1991).

10. Shih, C.-K., J. M. Rose, G. L. Hansen, J. C. Wu, A. Bacolla, and J. A. Griffin. Chimeric human immunodeficiency virus type 1/type 2 reverse transcriptases display reversed sensitivity to nonnucleoside analog inhibitors. *Proc. Natl. Acad. Sci. USA* **88**:9878–9882 (1991).
11. De Vreese, K., Z. Debyser, A.-M. Vandamme, R. Pauwels, J. Desmyter, E. De Clercq, and J. Anné. Resistance of human immunodeficiency virus type 1 reverse transcriptase to TIBO derivatives induced by site-directed mutagenesis. *Virology* **188**:900–904 (1992).
12. Sardana, V. V., E. A. Emini, L. Gotlib, D. J. Graham, D. W. Lineberger, W. J. Long, A. J. Schlabach, J. A. Wolfgang, and J. H. Condra. Functional analysis of HIV-1 reverse transcriptase amino acids involved in resistance to multiple nonnucleoside inhibitors. *J. Biol. Chem.* **267**:17526–17530 (1992).
13. Kohlstaedt, L. A., J. Wang, J. M. Friedman, P. A. Rice, and T. A. Steitz. Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* (Washington D. C.) **256**:1783–1790 (1992).
14. Debyser, Z., K. De Vreese, R. Pauwels, N. Yamamoto, J. Anné, E. De Clercq, and J. Desmyter. Differential inhibitory effects of TIBO derivatives on different strains of simian immunodeficiency virus. *J. Gen. Virol.* **73**:1799–1804 (1992).
15. Pauwels, R., K. Andries, Z. Debyser, P. Van Daele, D. Schols, A.-M. Vandamme, P. Stoffels, K. De Vreese, R. Woestenborghs, C. G. M. Janssen, J. Anné, G. Cauwenbergh, J. Desmyter, J. Heykants, M. A. C. Janssen, E. De Clercq, and P. A. J. Janssen. Potent and highly selective HIV-1 inhibition by a new series of α-anilinophenylacetamide (α-APA) derivatives targeted at HIV-1 reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **90**:1711–1715 (1993).
16. Bathurst, I. C., L. K. Moen, M. A. Lujan, H. L. Gibson, P. H. Feucht, S. Pichuantes, C. S. Craik, D. V. Santi, and P. J. Barr. Characterization of the human immunodeficiency virus type-1 reverse transcriptase enzyme produced in yeast. *Biochem. Biophys. Res. Commun.* **171**:589–595 (1990).
17. Bhikhabhai, R., T. Jackson, T. Unge, B. Strandberg, T. Carlsson, and S. Lövgren. Purification, characterization and crystallization of recombinant HIV-1 reverse transcriptase. *J. Chromatogr.* **604**:157–170 (1992).
18. Unge, T., H. Ahola, R. Bhikhabhai, K. Bäckbro, S. Lövgren, E. M. Fenyö, A. Honigman, A. Panet, J. S. Gronowitz, and B. Strandberg. Expression, purification, and crystallization of the HIV-1 reverse transcriptase (RT). *AIDS Res. Hum. Retroviruses* **6**:1297–1303 (1990).
19. D'Aquila, R. T., and W. C. Summers. HIV-1 reverse transcriptase/ribonuclease H: high level expression in *Escherichia coli* from a plasmid constructed using the polymerase chain reaction. *J. Acquired Immune Defic. Syndrome* **2**:579–587 (1989).
20. Balzarini, J., M.-J. Pérez-Pérez, A. San-Félix, D. Schols, C.-F. Perno, A.-M. Vandamme, M.-J. Camarasa, and E. De Clercq. 2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)pyrimidine (TSAO) nucleoside analogues: highly selective inhibitors of human immunodeficiency virus type 1 that are targeted at the viral reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **89**:4392–4396 (1992).
21. Debyser, Z., R. Pauwels, K. Andries, J. Desmyter, Y. Engelborghs, P. A. J. Janssen, and E. De Clercq. Allosteric inhibition of human immunodeficiency virus type 1 reverse transcriptase by tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-one and -thione compounds. *Mol. Pharmacol.* **41**:203–208 (1992).
22. Jacobo-Molina, A., and E. Arnold. HIV reverse transcriptase structure-function relationships. *Biochemistry* **30**:6351–6361 (1991).
23. Debyser, Z., R. Pauwels, M. Baba, J. Desmyter, and E. De Clercq. Common features in the interaction of tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-one and -thione and 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine derivatives with the human immunodeficiency virus type 1 reverse transcriptase. *Mol. Pharmacol.* **41**:963–968 (1992).
24. Dueweke, T. J., F. J. Kézdy, G. A. Waszak, M. R. Deibel, Jr., and W. G. Tarpley. The binding of a novel bisheteroarylpiperazine mediates inhibition of human immunodeficiency virus type 1 reverse transcriptase. *J. Biol. Chem.* **267**:27–30 (1992).
25. Frank, K. B., G. J. Noll, E. V. Connell, and I. S. Sim. Kinetic interaction of human immunodeficiency virus type 1 reverse transcriptase with the antiviral tetrahydroimidazo[4,5,1-*jk*][1,4]-benzodiazepine-2-(1*H*)-thione compound, R82150. *J. Biol. Chem.* **266**:14232–14236 (1991).

Send reprint requests to: Prof. Dr. E. De Clercq, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroeders straat 10, B-3000 Leuven, Belgium.