0026-895X/92/050963-06$03.00/0

MOLECULAR PHARMACOLOGY, 41:963-968

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

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Received July 11, 1991; Accepted February 4, 1992

SUMMARY

Recently, several classes of compounds have been shown to be potent, selective, and specific inhibitors of human immunodeficiency virus type 1 replication *in vitro*. These include the tetrahydroimidazo[4,5,1-*jk*][1,4]-benzodiazepin-2(1*H*)-one and -thione (TIBO) and the 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) derivatives. Both the TIBO and HEPT derivatives specifically inhibit human immunodeficiency virus type 1 reverse transcriptase (RT). From a comparative study of the characteristics of RT inhibition by TIBO and HEPT, and from the competition between TIBO and HEPT for RT inhibition, we infer that both classes of compounds, although structurally unrelated, are targeted at the same site of the enzyme. Detailed functional and kinetic analyses indicate that this target site is functionally and possibly also spatially related to the substrate binding site.

Although the amino acid sequences of the RTs of HIV-1 and HIV-2 are only partially homologous (40–60%) (1), the "classical" HIV RT inhibitors (2′,3′-dideoxynucleoside 5′-triphosphates) are equally effective against both virus types and their RTs (as reviewed in Refs. 2 and 3). However, some 6-substituted acyclouridine derivatives have been reported to be specifically inhibitory to HIV-1. The prototype of this class of compounds, HEPT, has been shown to inhibit virus replication in acutely HIV-1-infected T cells at a 50% effective concentration of 7 μM, with the 50% cytotoxic concentration being 740 μM (4). In contrast to 2′,3′-dideoxynucleoside analogues, which need to be phosphorylated to their 5′-triphosphates before they are able to interact with the HIV-1 RT, the triphosphate of HEPT does not interfere with HIV-1 RT. Structural modification of HEPT led to new congeners that inhibit HIV-1 replication at nanomolar concentrations that are up to 5 orders of magnitude below the cytotoxic concentration (5, 6). The target for the anti-HIV-1 action of the HEPT derivatives appears to be the HIV-1 RT (5, 6).

Concomitantly with and independently of the HEPT derivatives, we have identified the TIBO derivatives as potent, selective, and specific inhibitors of HIV-1 replication (7). The prototype TIBO compound, R82150, inhibits HIV-1 replication at nanomolar concentrations that are, again, up to 5 orders of magnitude below the cytotoxic concentration. The anti-HIV action of the TIBO derivatives is due to an inhibitory effect on HIV-1 RT, which is apparently inhibited by these compounds by a mechanism that is different from that of the classical RT inhibitors (7, 8).

Dipyridodiazepinone and pyridinone derivatives, which represent structurally distinct classes of compounds, have also proved to be specific inhibitors of HIV-1 RT (9, 10). The prototypes of these series of compounds, BI-RG-587 and L-697,639, respectively, inhibit HIV-1 RT noncompetitively with respect to dGTP.

What the TIBO, HEPT, dipyridodiazepinone, and pyridinone derivatives have in common is a marked potency, selec-

This work was supported by the AIDS Basic Research Programme of the European Community and by grants from the Belgian "Fonds voor Geneeskundig Wetenschappelijk Onderzoek," the Belgian "Geconcerteerde Onderzoeksacties," and the Janssen Research Foundation. Z.D. is a Research Assistant of the Belgian National Fund for Scientific Research.

ABBREVIATIONS: RT, reverse transcriptase; HIV, human immunodeficiency virus; TIBO, tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-one and -thione; HEPT, 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine; E-EPU, 5-ethyl-1-ethoxy-methyl-6-(phenylthio)uracil; PLP, pyridoxal 5′-phosphate; AZT-TP, 3′-azido-3′-deoxythymidine 5′-triphosphate; ddGTP, 2′,3′-dideoxyguanosine 5′-triphosphate; IC_{50}, 50% inhibitory concentration; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DDDP, DNA-directed DNA polymerase; RDDP, RNA-directed DNA polymerase.

tivity, and specificity for HIV-1 and its RT. Photoaffinity labeling experiments suggest that R82150 and BI-RG-587 may interact with a common target site on the HIV-1 RT (11). Displacement of ^{3}H-labeled pyridinone from HIV-1 RT by BI-RG-587 and TIBO R82150 is indicative of a common target. However, TIBO and HEPT, two classes of compounds that are structurally unrelated, have so far not been the subjects of any comparative studies aimed at deciphering the molecular basis of their mode of anti-HIV-1 action.

We now present conclusive evidence that TIBO and HEPT interact with the same target site at HIV-1 RT. From a detailed analysis of the kinetic data, we postulate that this target site may be related functionally and/or spatially to the substrate binding site of the enzyme.

Experimental Procedures

Compounds. The origin of the prototype TIBO compound, 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], has been described previously (7) (Fig. 1). HEPT was prepared according to a procedure that has also been described previously (12) (Fig. 1), and E-EPU has been reported as a potent HEPT derivative (5).

Stock solutions of the compounds were prepared in dimethylsulfoxide. Final dimethylsulfoxide concentrations in the RT assays were <1%. ddGTP (Pharmacia), AZT-TP (13), and dextran sulfate (Sigma) were dissolved in RT reaction buffer.

RT sources. Recombinant HIV-1 RT (p66) was kindly provided by P. J. Barr (Chiron). It was derived from an HIV-1 (SF2 strain) *pol* gene fragment coding for RT (Pro-156 to Leu-715), expressed in yeast (14). The final concentration was 1.1 nM. The incorporation rate was estimated at 30 pmol of dTMP/hr in the poly(A)·oligo(dT)-directed assay (specific activity, 0.15 sec^{-1}). Whereas the incorporation rate was 10 pmol of dGMP/hr in the poly(C)·oligo(dG)-directed assay (specific activity, 0.05 sec^{-1}), the poly(dC)·oligo(dG)-directed polymerization was 3 times as efficient (30 pmol of dGMP/hr; specific activity, 0.15 sec^{-1}), according to previous reports for Moloney murine leukemia virus RT (15). Recombinant HIV-2 RT (p68) was a kind gift from S. Hughes. The final concentration was 3 nM. The incorporation rate was estimated at 7.5 pmol of dGMP/hr in the poly(C)·oligo(dG)-directed assay (specific activity, 0.0125 sec^{-1}).

Virion-derived RT was obtained from the culture fluids of CD4$^+$ T cells persistently infected with HIV-1 or HIV-2. The culture fluids from HUT-78/HTLV-III$_B$, MOLT/HTLV-III$_{RF}$, and MOLT/HIV-2$_{ROD}$ cells were clarified by low speed centrifugation (10 min at 140 × *g*). Supernatants were filtered through a 0.22-μm Millex GV filter (Millipore). Virus particles were subsequently pelleted by centrifugation at 100,000 × *g* for 2 hr. Pellets were resuspended in a solution containing 5 mM Tris·HCl at pH 8.1, 1 mM DTT, 0.1% (w/v) Triton X-100, and 0.5 M KCl. The final total protein concentration in the RT reaction mixture was 13 μg/ml, as determined by the Bradford method (Bio-Rad). Resuspended pellets were stored in aliquots at −70° until used.

RT assays. The standard RT reaction mixture contained 50 mM Tris·HCl at pH 8.1, 10 mM $MgCl_2$, 100 mM KCl, 2.2 mM DTT, and 0.05% (w/v) Triton X-100. The template [poly(C), poly(A), or poly(I)] and the primer [$(dG)_{12-18}$, $(dT)_{12-18}$, or $(dC)_{12-18}$] were used at a concentration of 40 and 6 μg/ml, respectively. The DNA-directed DNA polymerase activity of RT was measured with poly(dC) as the template (final concentration, 40 μg/ml) and with $(dG)_{12-18}$ as the primer (final concentration, 6 μg/ml). Templates and primers were purchased from Pharmacia. Tritium-labeled deoxyribonucleotides (dGTP, dTTP, and dCTP) were obtained from Amersham and used at a concentration of 2.5 μM. Their specific activity was 15.6, 46, and 19.4 Ci/mmol, respectively.

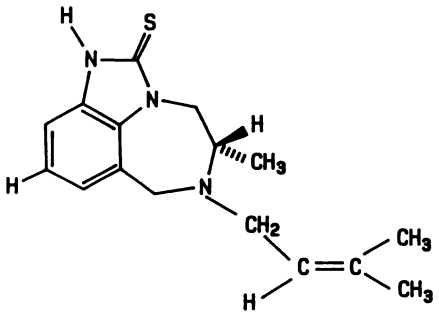
After addition of the enzyme and various concentrations of the inhibitors, the reaction mixture was incubated for 1 hr at 37°. The incorporation rate was determined by a standard trichloroacetic acid precipitation procedure, using Whatman GF/C glass fiber filters (Whatman) and liquid scintillation counting.

Kinetic studies. Kinetic studies were performed with varying substrate (dGTP and dTTP) and template/primer [poly(C)·oligo(dG)] concentrations. ^{3}H-Labeled nucleotides dissolved in ethanol/water (1:1) were dried (Speedvac; Savant) and then dissolved in water, to avoid interference from ethanol in the kinetics studies. The incorporation rate of ^{3}H-labeled nucleotides was linear for at least 1 hr. Studies were performed under steady state conditions (i.e., the amount of substrate incorporated was <2.5% of the amount available in the reaction mixture) in 15-min assays. Experimental data were analyzed by a nonlinear regression analysis software program (Enzfitter; Elsevier-Biosoft).

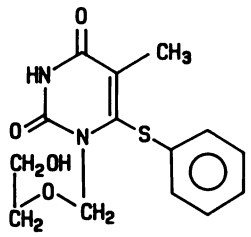
RT inactivation assay. Recombinant HIV-1 RT (adjusted to 0.5 μM) or HIV-2 RT (0.3 μM) was incubated in the dark in a reaction mixture containing 50 mM HEPES at pH 7.8, 20% glycerol (v/v), 5 mM $MgCl_2$, 10 mM NaCl, 65 μg/ml template/primer, and a dilution series of PLP. After 10 min at 37°, the reaction mixture was immediately cooled to 4°, and a fresh, ice-cold solution of $NaBH_4$ in 5 mM NaOH was added, to a final concentration of 10 mM. After neutralization of all PLP, as judged by the disappearance of the yellow color, the reaction mixture was incubated on ice for 10 min. Residual enzymatic activity was estimated by diluting PLP-treated enzyme 10-fold in a solution of 5 mM Tris·HCl at pH 8.1, 1 mM DTT, 0.1% (w/v) Triton X-100, and 0.5 M KCl, followed by assessment of RT activity with poly(C)·oligo(dG) as template/primer. For the competition experiments, desired concentrations of R82150 or HEPT were added to the PLP-containing reaction mixture before incubation at 37°.

Results

Characteristics of RT inhibition by TIBO and HEPT. Virion-derived HIV-1 (RF strain) RT activity was inhibited by 50% at an R82150 concentration of 4.9 μM and at a HEPT concentration of 280 μM, in a poly(A)·oligo(dT)-directed assay



R82150



HEPT

Fig. 1. Structural formulae of TIBO (R82150) and HEPT.

TABLE 1
RT inhibition by different compounds

RT source	IC_{50}[a]			
	R82150	HEPT	AZT-TP	Dextran sulfate
	μM	μM	μM	μg/ml
HIV-1 (RF) virion-derived	4.9 ± 0.1	280 ± 15	0.07 ± 0.03	12 ± 1
HIV-1 recombinant p66	6 ± 0.15	240 ± 20	0.05 ± 0.03	0.7 ± 0.1

[a] Concentration of compound that inhibits RT activity by 50%, using poly(A)·oligo(dT)$_{12-18}$ as template/primer. Data represent mean values ± standard deviations for at least two separate experiments.

(Table 1). Similar IC_{50} values were obtained using recombinant HIV-1 RT (p66). Dextran sulfate has a 17-fold lower IC_{50} value for the recombinant than for the virion-derived enzyme, which is indicative of nonspecific RT inhibition (16). Whereas ddGTP was equally inhibitory to HIV-1 RT and HIV-2 RT, TIBO and HEPT did not inhibit HIV-2 RT at the highest concentrations tested (140 and 1200 μM, respectively) (Table 2).

The dideoxynucleotides AZT-TP and ddGTP inhibit HIV-1 RT activity only if complementary templates [i.e., poly(A) and poly(C), respectively] are used (Table 2). This contrasts with R82150 and HEPT, which inhibit HIV-1 RT activity with different templates. The most pronounced inhibition was observed if poly(C)·oligo(dG) was used as the template/primer. In this case, the IC_{50} of R82150 was 20-fold lower than if poly(A)·oligo(dT) served as the template/primer. For HEPT, the IC_{50} was 14-fold lower with poly(C)·oligo(dG) than with poly(A)·oligo(dT) as template/primer. When R82150 was studied for its inhibitory effect on the DDDP activity of HIV-1 RT with poly(dC)·oligo(dG) as a template/primer, it appeared that this polymerization function of HIV-1 RT was 25-fold less susceptible to inhibition by R82150 than was the RDDP activity of the enzyme. HEPT exhibited a 16-fold lesser activity against the DDDP function of HIV-1 RT (Table 2). In contrast, ddGTP showed a markedly higher inhibition of the DDDP function than of the RDDP function of HIV-1 RT. E-EPU, an HEPT congener with an inhibitory effect on HIV-1 replication in cell culture similar to that of R82150 (5), displayed identical properties of specificity and template preference as TIBO and HEPT (Table 2).

Kinetic studies on inhibition of HIV-1 RT by TIBO and HEPT. We have previously reported on the kinetics of HIV-1 RT inhibition by TIBO (8); R82150 behaves as a noncompetitive inhibitor with respect to the natural substrate dGTP (K_i, 0.65 μM) and as an uncompetitive inhibitor toward the template/primer [poly(C)·oligo(dG)] (K_i at infinite template/primer concentration is 0.3 μM). However, kinetics for HEPT have not been reported so far.

We performed kinetic studies with HIV-1 recombinant RT (p66) under steady state conditions. With varying dGTP concentrations, HEPT behaved as a competitive inhibitor. A Dixon transformation plot is shown in Fig. 2. Under these reaction conditions, the Michaelis-Menten constant (K_m) for dGTP was 7 μM, and the inhibition constant (K_i) for HEPT was 30 μM. R82150 and ddGTP, run in parallel, behaved as noncompetitive and competitive inhibitors, respectively (data not shown). With varying template/primer [poly(C)·oligo(dG)] concentrations, both TIBO and HEPT behaved as uncompetitive inhibitors. A second Dixon transformation plot is shown for HEPT in Fig. 3. The K_m for poly(C)·oligo(dG) was 8 μg/ml, and the K_i for HEPT at an infinitely high template/primer concentration was estimated as 36 μM. With varying dTTP concentrations in a poly(A)·oligo(dT)-directed assay, similar results were obtained; again, R82150 behaved noncompetitively (K_i, 4 μM), whereas HEPT was competitive (K_i, 80 μM) (data not shown).

Antagonism of the inhibitory effects of TIBO and HEPT on HIV-1 RT. When the inhibitory effect of the TIBO R82150 on HIV-1 RT was assessed in the presence of HEPT, or its more potent congener E-EPU, the TIBO derivative lost some of its anti-RT activity (Fig. 4). The IC_{50} of R82150 increased by 3-fold upon addition of HEPT and by 7-fold upon addition of E-EPU. HEPT and E-EPU were added at concentrations that inhibited RT activity by 50%. These results point to antagonism in the inhibitory effects of the TIBO and HEPT derivatives on HIV-1 RT.

Antagonism of PLP-mediated inactivation of HIV-1 RT by TIBO and HEPT. The RT of HIV-1 has been shown to be susceptible to inhibition by PLP, a substrate binding site-directed reagent for DNA polymerases. Schiff base formation between PLP and a free amino group, followed by sodium borohydride reduction, results in irreversible inactivation of HIV-1 RT. Because deoxynucleoside triphosphates in the presence of appropriate template/primer can block PLP reactivity, the PLP-reactive site has been localized to the substrate-binding domain of HIV-1 RT (17). In order to investigate the impact of TIBO and HEPT derivatives on the substrate-binding domain of HIV-1 RT, we performed RT inactivation studies, according to the method of Basu *et al.* (17). We confirmed the concentration-dependent inactivation of HIV-1 RT by PLP (IC_{50} = 284 μM). We also found that HIV-2 RT was susceptible to PLP inactivation (IC_{50} = 252 μM). PLP reactivity with HIV-1 RT was prevented, in a concentration-dependent way, upon addition of R82150 (Fig. 5). We observed a 3- and 6-fold increase, respectively, in the IC_{50} values of PLP in the presence

TABLE 2
Specificity and template dependence of RT inhibition by R82150, HEPT, and E-EPU

RT	Template/primer[a]	IC_{50}[b]				
		R82150	HEPT	E-EPU	AZT-TP	ddGTP
				μM		
HIV-1 recombinant p66	Poly(C)·(dG)$_{12-18}$	0.31 ± 0.08	17 ± 3	0.2 ± 0.02	>5	0.04 ± 0.005
	Poly(A)·(dT)$_{12-18}$	6 ± 0.15	240 ± 20	2.2 ± 0.2	0.05 ± 0.03	>5
	Poly(I)·(dC)$_{12-18}$	10 ± 0.6	>325	5.2 ± 0.5	>5	>5
	Poly(dC)·(dG)$_{12-18}$	8 ± 1	515 ± 50	1.8 ± 0.2	>5	0.004 ± 0.001
HIV-2 recombinant p68	Poly(C)·(dG)$_{12-18}$	>140	>1200	>100	>5	0.055 ± 0.01

[a] Templates were used at a concentration of 40 μg/ml, and the oligonucleotide primers were at 6 μg/ml.
[b] The IC_{50} is the concentration that inhibits RT activity by 50%. Data represent mean values ± standard deviations for at least two separate experiments.

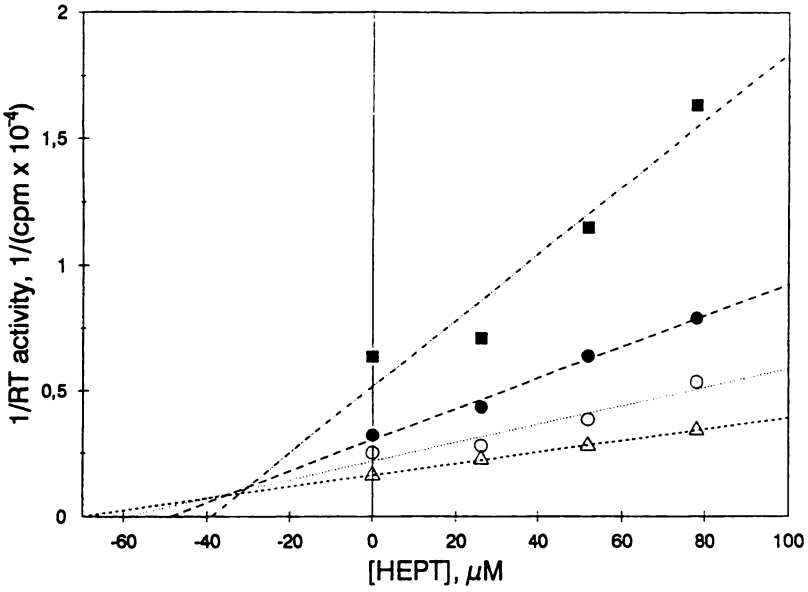


Fig. 2. Kinetics of inhibition of HIV-1 RT activity by HEPT in the presence of varying concentrations of the substrate dGTP. Kinetic studies with HIV-1 recombinant RT were carried out under steady state conditions, with a fixed concentration (65 μg/ml) of template/primer [poly(C)·$(dG)_{12-18}$] and varying concentrations of substrate ([^{3}H] dGTP). Data are presented as Dixon transformation plots; 1/*v* (expressed as 1/cpm in a 15-min assay) is plotted against inhibitor (HEPT) concentration at different substrate concentrations. *Lines* were drawn by linear regression analysis. Concentrations of dGTP were 1.4 μM (■), 2.8 μM (●), 5.6 μM (○), and 11.2 μM (△). K_i was calculated to be 30 μM.

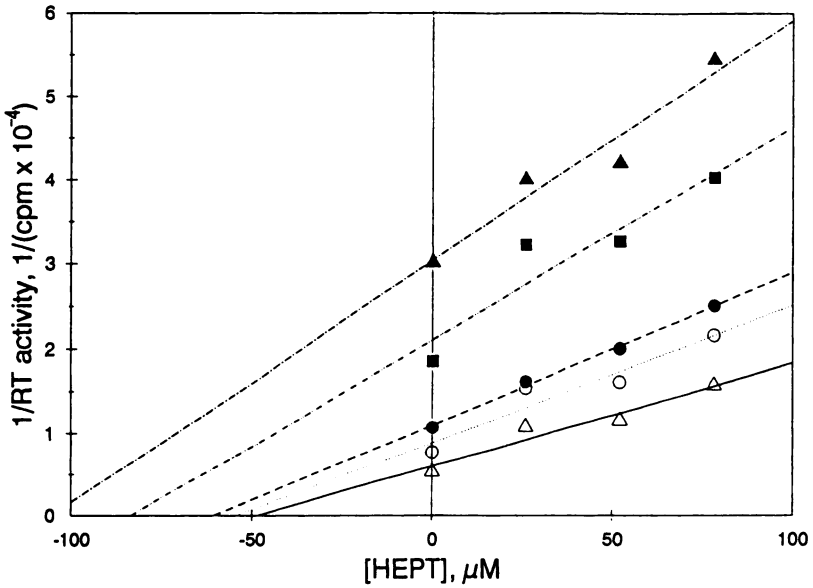


Fig. 3. Kinetics of inhibition of HIV-1 RT activity by HEPT with respect to varying concentrations of the template/primer poly(C)·$oligo(dG)_{12-18}$. Kinetic studies with HIV-1 recombinant RT were carried out under steady state conditions, with a fixed concentration of substrate (2.5 μM [^{3}H] dGTP) and varying concentrations of the template/primer poly(C)·$oligo(dG)_{12-18}$. Data are presented as Dixon transformation plots; 1/*v* (expressed as 1/cpm in a 15-min assay) is plotted against inhibitor (HEPT) concentration at different template/primer concentrations. *Lines* were drawn by linear regression analysis. Concentrations of poly(C)·oligo(dG) were 66 μg/ml (△), 16.5 μg/ml (○), 8.25 μg/ml (●), 4.125 μg/ml (■), and 2.06 μg/ml) (▲). The K_i is highly dependent on the template/primer concentration. From the formula $K_{i_{app}} = K_i(1 + K_m/[S])$, K_i was estimated at 36 μM.

of 7 and 35 μM R82150. Similar results were obtained with HEPT. The antagonistic effect of R82150 on HIV-2 RT inactivation by PLP was marginal. Antagonism was higher with poly(C)·oligo(dG) than with poly(A)·oligo(dT) as the template/primer (data not shown).

Discussion

Two conclusions can readily be drawn from the results of our studies, (i) TIBO and HEPT are targeted at the same site of HIV-1 RT and (ii) this site is functionally related to a substrate binding site on the enzyme. Previously, we attributed the following characteristics to TIBO derivatives (8): (i) specific inhibition by TIBO of HIV-1 RT but not of HIV-2 RT; (ii) preference of TIBO for poly(C)·oligo(dG) as template/primer, although TIBO is also inhibitory to the RT activity directed by other template/primers; and (iii) much lower susceptibility to inhibition by TIBO for DDDP activity than for RDDP activity. Here we report on the same characteristics for HEPT derivatives when assayed under identical reaction conditions.

The similar properties shown by the TIBO and HEPT derivatives, as well as their antagonistic effect when combined, suggest that they interact with the same site at the HIV-1 RT. Conclusive evidence for a common antiviral target site is provided by induction of cross-resistant mutant RTs by means of site-directed mutagenesis. Mutations $Y_{181}I$ and $Y_{188}L$ confer partial to complete resistance to TIBO and HEPT derivatives, without affecting the enzymatic activity.[1]

Kinetic and functional analysis was performed in order to identify the common antiviral target on HIV-1 RT. Kinetic studies revealed that both TIBO and HEPT are uncompetitive with respect to the template/primer poly(C)·oligo(dG). This is indicative of an ordered mechanism of inhibition, whereby binding of the inhibitor is preceded by binding of the template/primer to the enzyme. Also the natural (deoxynucleotide) substrate is assumed to bind only after the template/primer has

[1] K. De Vreese, Z. Debyser, 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. Submitted for publication.

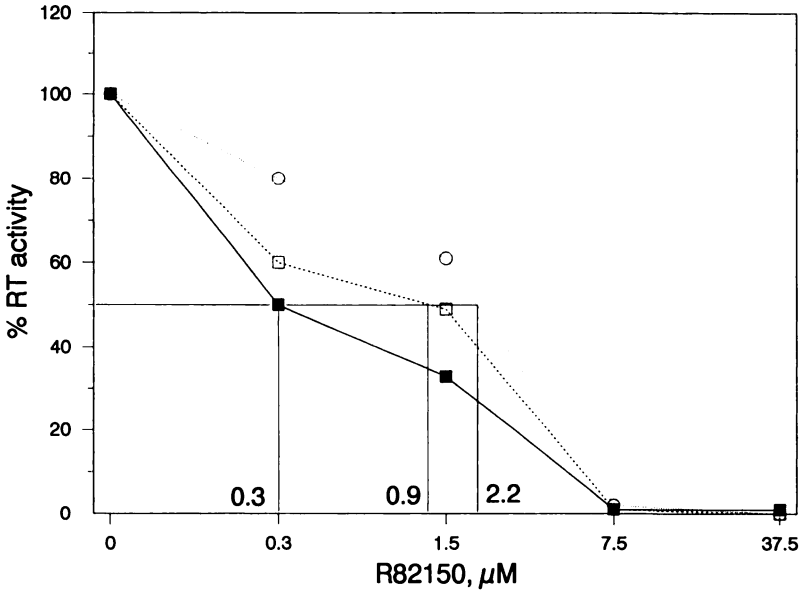


Fig. 4. Antagonistic effect of HEPT and E-EPU on inhibition of HIV-1 RT by TIBO (R82150). Inhibition of HIV-1 recombinant RT by R82150 was measured in a poly(C)·oligo(dG)-directed assay, in the absence of HEPT (■) or in the presence of 17 μM HEPT (□) or 0.2 μM E-EPU (○). These antagonists were added at IC_{50} concentrations. RT activity is presented as a percentage of the activity observed in the absence of R82150, with 100% corresponding to the RT activity measured in the absence of any compound or in the presence of 17 μM HEPT or 0.2 μM E-EPU. The IC_{50} values of R82150 obtained in the absence of HEPT or in the presence of 17 μM HEPT or 0.2 μM E-EPU are indicated on the *x*-axis.

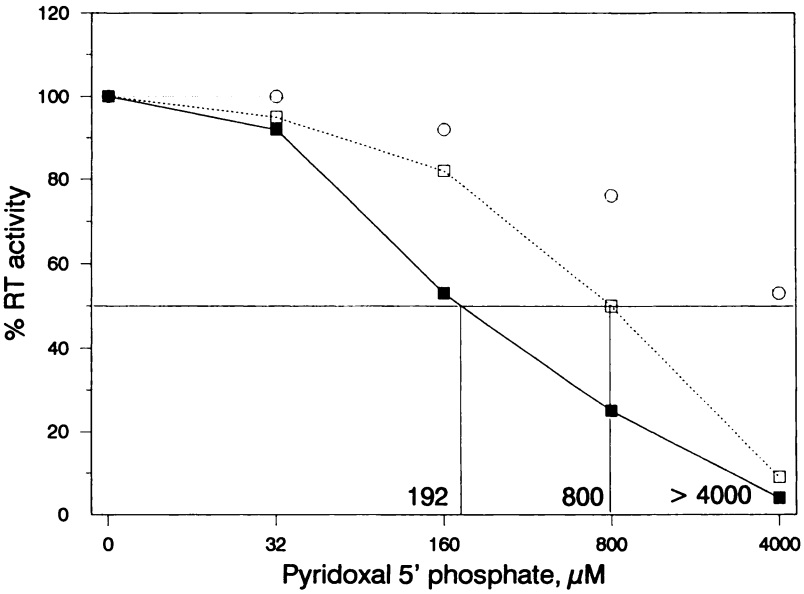


Fig. 5. Antagonism by R82150 of PLP-mediated inactivation of HIV-1 RT. HIV-1 RT was incubated with the indicated concentrations of PLP at 37° for 10 min, treated with $NaBH_4$, and tested for remaining RT activity (■). Inactivation by PLP was also carried out in the presence of 7 (□) and 35 (○) μM R82150. All PLP incubation mixtures contained poly(C)·oligo$(dG)_{12-18}$ at a final concentration of 65 μg/ml. The remaining RT activity is presented as a percentage of the RT activity observed in the absence of PLP, with 100% corresponding to the RT activity measured in the absence of any compound or in the presence of 7 or 35 μM R82150. The IC_{50} values of PLP obtained in the absence of R82150 or in the presence of 7 or 35 μM R82150 are indicated on the *x*-axis.

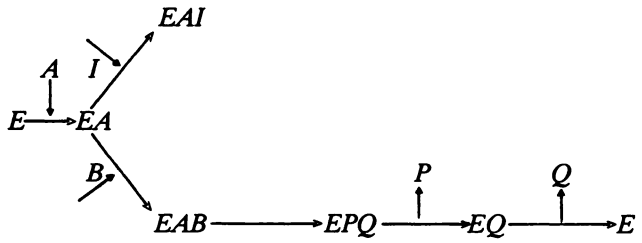


Fig. 6. Cleland scheme for an ordered bireactant biproduct system. The enzyme (*E*) can combine with template/primer (*A*) or template/product (*Q*). Binding of inhibitor (*I*) leads to formation of a ternary dead-end inhibitor complex (*EAI*), whereas binding of substrate (*B*) (deoxynucleotide) finally leads to formation of product (*Q*). Before product *Q* (DNA chain) is formed, product *P* (PP_i) is released.

been bound to the enzyme (17). In agreement with this kinetic observation are recent binding studies with the TIBO-like ^3H-labeled pyridinone, which have shown that no binding of the inhibitor occurs in the absence of template/primer (10). The ordered type of inhibition may be based on a conformational shift upon template/primer binding, thus enabling the inhibitor to interact with an unblocked amino acid sequence. Because the template base, present in the catalytic center, is generally believed to mold the unique (deoxynucleotide) substrate binding site in favor of its complementary base (18), an ordered type of inhibition may be related to a differential binding of the inhibitor to the enzyme, depending on the actual conformation of the versatile substrate-binding pocket. This hypothesis may explain why RT inhibition by TIBO and HEPT is dependent on the type of template/primer used. It does not even exclude a direct interaction between the inhibitor and the template base, once the template is niched in the catalytic center.

From the kinetic studies with varying substrate concentrations, it appeared that TIBO behaves as a noncompetitive inhibitor with respect to the natural substrates dGTP and dTTP, whereas HEPT is competitive with both deoxynucleotides. Interestingly, some of the more potent HEPT derivatives were found to have kinetic properties that are intermediate

between those of TIBO and HEPT; they proved to be competitive with dTTP but noncompetitive with dGTP (5, 6).

The common TIBO/HEPT binding site at the HIV-1 RT seems to be functionally related to the deoxynucleotide substrate binding site, according to the following findings: (i) competitive type of inhibition of HEPT with respect to dGTP, (ii) antagonism by TIBO and HEPT of PLP-mediated inactivation of HIV-1 RT, (iii) ordered type of inhibition with respect to the template/primer, and (iv) template preference for inhibition. Interestingly, the recently resolved crystal structure of TIBO (R82913) resembles that of dAMP (19). The competitive behavior of HEPT with respect to the natural substrate (dGTP) may suggest that they bind to the substrate binding site. One should, however, bear in mind that a competitive kinetic behavior between substrate and inhibitor does not provide unequivocal proof for an identical binding site. Two independent binding sites may elicit competitive behavior as well.

The paradoxical kinetic behavior of R82150 and HEPT toward the natural substrate dGTP can be resolved by postulating the existence of a low and a high affinity binding site on the enzyme. Enzymatic activity of HIV-1 RT is restricted to the dimeric enzyme form (20), both subunits of which may have TIBO binding sites. If binding of low potency molecules (e.g., HEPT) were restricted to a high affinity binding site, closely related to the substrate binding site, HEPT would elicit competitive kinetics, whereas binding of the high potency molecules E-EPU and R82150 to a second binding site would provoke noncompetitive kinetics. In fact, the existence of a second low affinity binding site has been suggested previously by Merluzzi *et al.* (9). In support of this hypothesis is our finding that low potency TIBO derivatives also raise the K_m for dGTP.[2]

The dipyridodiazepinones (i.e., BI-RG-587), like the TIBO derivatives, also act as noncompetitive inhibitors with regard to dGTP (9). These compounds are assumed to bind to a nonsubstrate binding site, because the natural substrate (dGTP) could not protect the RT from photoinactivation by photoaffinity labeling with an azido analogue of BI-RG-587. Although this lack of protection could be attributed to the absence of template/primer in that particular experiment (11), the data are compatible with BI-RG-587 binding to a modulatory site that is somehow linked to the substrate binding site.

The question of whether the binding sites of TIBO, HEPT, and the dipyridodiazepinones are only functionally or are also spatially related to the substrate binding site cannot be resolved without further experiments. Amino acid alignments and site-directed mutagenesis of HIV-1 RT and HIV-2 RT may help in understanding the reason for the unique specificity of HEPT, TIBO, and dipyridodiazepinone for the HIV-1 RT and may, for example, provide an answer to the question of whether HIV-2 RT is not susceptible to TIBO because of steric hindrance or absence of the putative TIBO-binding pocket.

In conclusion, we present a scheme for the mechanism of RT inhibition by TIBO and HEPT. This scheme (Fig. 6) is modified from the Cleland (21) scheme for an ordered bireactant biproduct reaction mechanism and is based on the finding that TIBO and HEPT achieve uncompetitive inhibition with respect to the template/primer. The interaction of the inhibitor (I) with the enzyme/template/primer complex may lead to formation of a ternary dead-end inhibitory complex.

[2] Z. Debyser and E. De Clercq, unpublished observations.

Acknowledgments

We thank H. Azijn for excellent technical assistance and C. Callebaut for fine editorial help. HIV-2 recombinant RT (p68) was kindly provided by S. Hughes (ABL-Basic Research Programme-NCI Frederick Cancer Research Facility).

References

1. Clavel, F. Virological and clinical features of HIV-2. *Curr. Top. AIDS* **2**:69–80 (1989).
2. De Clercq, E. New acquisitions in the development of anti-HIV agents. *Antiviral Res.* **12**:1–20 (1989).
3. De Clercq, E. Basic approaches to anti-retroviral treatment. *J. Acquired Immune Deficiency Syndrome* **4**:207–218 (1991).
4. Baba, M., H. Tanaka, E. De Clercq, R. Pauwels, J. Balzarini, D. Schols, H. Nakashima, C.-F. Perno, R. T. Walker, and T. Miyasaka. Highly specific inhibition of human immunodeficiency virus type 1 by a novel 6-substituted acyclouridine derivative. *Biochem. Biophys. Res. Commun.* **165**:1375–1381 (1989).
5. Baba, M., E. De Clercq, H. Tanaka, M. Ubasawa, H. Takashima, K. Sekiya, I. Nitta, K. Umezu, H. Nakahsima, 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 its interaction with the HIV-1 reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **88**:2356–2360 (1991).
6. Baba, M., E. De Clercq, H. Tanaka, M. Ubasawa, H. Takashima, K. Sekiya, I. Nitta, K. Umezu, R. T. Walker, S. Mori, M. Ito, S. Shigeta, and T. Miyasaka. Highly potent and selective inhibition of human immunodeficiency virus type 1 by a novel series of 6-substituted acyclouridine derivatives. *Mol. Pharmacol.* **39**:805–810 (1991).
7. 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).
8. 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).
9. Merluzzi, V. J., K. D. Hargrave, M. Labadia, K. Groringer, M. Skoog, J. C. Wu, C.-K. Shih, K. Eckner, S. Hattox, J. Adams, A. S. Rosenthal, R. Faones, 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).
10. Goldman, N. 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. N. 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).
11. Wu, J. C., T. C. Warren, J. Adams, J. Roudfoot, J. Skiles, P. Raghavan, C. Perry, J. Potocki, P. R. Farina, and P. M. Grob. A novel dipyridodiazepinone inhibitor of HIV-1 reverse transcriptase acts through a nonsubstrate binding site. *Biochemistry* **30**:2022–2026 (1991).
12. Miyasaka, T., H. Tanaka, M. Baba, H. Hayakawa, R. T. Walker, J. Balzarini, and E. De Clercq. A novel lead for specific anti-HIV-1 agents: 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine. *J. Med. Chem.* **32**:2507–2509 (1989).
13. Herdewijn, P., J. Balzarini, E. De Clercq, R. Pauwels, M. Baba, S. Broder, and H. Vanderhaeghe. 3′-Substituted 2′,3′-dideoxynucleoside analogues as potential anti-HIV (HTLV-III/LAV) agents. *J. Med. Chem.* **30**:1270–1278 (1987).
14. Barr, P. J., M. D. Power, C. T. Lee-Ng, H. L. Gibson, and P. A. Luciw. Expression of active human immunodeficiency virus reverse transcriptase in *Saccharomyces cerevisiae*. *Bio/Technology* **5**:486–489 (1987).
15. Baltimore, D., and D. Smoler. Primer requirement and template specificity of the DNA polymerase of RNA tumor viruses. *Proc. Natl. Acad. Sci. USA* **68**:1507–1511 (1971).
16. Schinazi, R. F., B. F. H. Eriksson, and S. Hughes. Comparison of inhibitory activities of various antiretroviral agents against particle-derived and recombinant human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob. Agents Chemother.* **31**:1613–1617 (1987).
17. Basu, A., R. S. Tirumalai, and M. S. Modak. Substrate binding in human immunodeficiency virus reverse transcriptase. *J. Biol. Chem.* **264**:8746–8752 (1989).
18. Majumdar, C., J. Abbotts, S. Broder, and S. H. Wilson. Studies on the mechanism of human immunodeficiency virus reverse transcriptase. *J. Biol. Chem.* **263**:15657–15665 (1988).
19. Liow, Y.-C., Y.-G. Ao, H. Robinson, and A. H.-J. Wang. Molecular structure of a potent HIV-1 inhibitor belonging to the TIBO family. *J. Am. Chem. Soc.* **113**:1857–1859 (1991).
20. Restle, T., B. Müller, and R. S. Goody. Dimerization of human immunodeficiency virus type 1 reverse transcriptase. *J. Biol. Chem.* **265**:8986–8988 (1990).
21. Cleland, W. W. The kinetics of enzyme-catalyzed reactions with two or more substrates or products. I. Nomenclature and rate equations. *Biochim. Biophys. Acta* **67**:104–137 (1963).

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