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Biochemistry

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

(acquired immunodeficiency syndrome/antiviral chemotherapy)

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ABSTRACT **Screening of pharmacologically acceptable prototype compounds has recently led to the discovery of a series of ultraselective inhibitors of human immunodeficiency virus (HIV)-1 replication, the tetrahydroimidazo[4,5,1-*jk*][1,4]-benzodiazepin-2(1*H*)-one and -thione (TIBO) derivatives. The TIBO compounds completely suppress the formation of proviral DNA in acutely infected cells, as revealed by polymerase chain reaction (PCR) analysis. TIBO derivatives are inhibitory to the reverse transcriptase (RT) of HIV-1 but not that of HIV-2 or other retroviruses. The inhibition is most effective with poly(C)·oligo(dG) as the template/primer, and it is selectively directed against the RNA-dependent DNA polymerase activity and not the accompanying DNA-dependent DNA polymerase and ribonuclease H activity of HIV-1 RT. Kinetic studies point to an uncompetitive inhibition with regard to the template/primer. TIBO compounds are active against HIV-1 replication through a unique interaction with HIV-1 RT. The experimental data indicate the existence of a target on HIV-1 RT that is responsible for the inhibition of replication and a mode of action unrelated to that of previously studied RT inhibitors.**

The AIDS epidemic has resulted in intensive efforts by several laboratories to develop effective inhibitors of human immunodeficiency virus (HIV)-1, the causative agent of the disease (1, 2). The only drug that has been formally approved for the treatment of AIDS is 3′-azido-3′-deoxythymidine (AZT, zidovudine, or retrovir) (3), which is efficacious in prolonging the life of AIDS patients (4). A recent report points to the benefit of zidovudine treatment of asymptomatic HIV carriers (5). Several other compounds with proven anti-HIV activity *in vitro* [i.e., 2′,3′-dideoxycytidine (DDC) and 2′,3′-dideoxyinosine (DDI)], have been recently submitted to clinical trials (6).

The long-term treatment of AIDS necessitates the development of anti-AIDS compounds with minimal or no toxicity (7). Furthermore, the emergence of drug-resistant virus strains should be carefully monitored (8). Ideally, new anti-HIV agents should have novel chemical structures and, if possible, attack HIV replication in a way different from the presently known anti-HIV agents.

Through rational screening and subsequent lead optimization we recently identified a series of compounds belonging to the class of the tetrahydro-1*H*-imidazo[4,5,1-*jk*][1,4]benzodiazepin-2-one and -thione (TIBO) derivatives which specifically inhibit HIV-1 replication at nanomolar concentrations, at least four orders of magnitude below their cytotoxic concentrations (9). This anti-HIV-1 activity has been demonstrated against different HIV-1 strains in different cell types.

Our initial studies on the mechanism of action of TIBO compounds indicated that they inhibited HIV-1 reverse transcriptase (RT), a virus-encoded enzyme that plays a key role in the replicative cycle of HIV-1. The mechanism of action of the TIBO derivatives has now been explored in more detail, and in particular their mode of interaction with HIV-1 RT has been examined.

MATERIALS AND METHODS

Virus. HIV-1 (strain HTLV-III$_B$; R. C. Gallo, National Institutes of Health) was obtained from the culture supernatant of a persistently infected HUT-78 cell line (HUT-78/HTLV-III$_B$). The virus titer of the supernatant was determined in MT-4 cells. The virus stock [100,000 CCID$_{50}$ (50% cell culture infective dose)/ml] was stored at −70°C until used.

Cell Culture. MT-4 cells and persistently HIV-1 infected MOLT cells (MOLT/HTLV-III$_B$) were grown in RPMI 1640 DM medium (Flow Laboratories), supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS) and gentamycin (Merck) at 20 μg/ml. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Every 3–4 days, cells were spun down and seeded at 200,000 cells per ml in new cell culture flasks.

Compounds. The origin of the TIBO compounds R82150, (+)-(5*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 and R82913, (+)-(5*S*)-4,5,6,7-tetrahydro-9-chloro-5-methyl-6-(3-methyl-2-butenyl)imidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-thione (Fig. 1), has been described previously (9). Stock solutions of the compounds were prepared in dimethyl sulfoxide. Final dimethyl sulfoxide concentrations in the RT assays were less than 1%. 2′,3′-Dideoxyguanosine 5′-triphosphate (ddGTP) (Pharmacia), AZT 5′-triphosphate (AZT-TP) (10), suramin (Bayer, Leverkusen, F.R.G.), and phosphonoformic acid (PFA) (Sigma) were dissolved in reaction buffer.

Detection of HIV-1 DNA by PCR. Exponentially growing MT-4 cells were centrifuged for 10 min at 140 × *g* and the supernatants were discarded. The pellet was resuspended in fresh RPMI medium in 25-cm^2 culture flasks (Falcon and Becton Dickinson) at a density of 200,000 cells per ml. The cell cultures were infected with 100 μl of the virus stock

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Abbreviations: HIV, human immunodeficiency virus; AMV, avian myeloblastosis virus; MMuLV, Moloney murine leukemia virus; AZT, 3′-azido-3′-deoxythymidine; AZT-TP, AZT 5′-triphosphate; ddGTP, 2′,3′-dideoxyguanosine 5′-triphosphate; PFA, phosphonoformic acid; TIBO, tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-one and -thione; RT, reverse transcriptase.

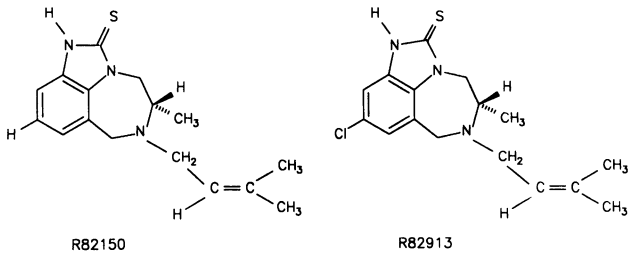

FIG. 1. Chemical structures of the prototype TIBO derivatives R82150 and R82913.

solution which had been filtered through a 0.22-μm Millex GV (Millipore) filter. The antiviral compounds (at different concentrations in RPMI medium) were added to cells. After 18 hr of incubation, 250,000 cells were put into centrifuge tubes. Cells were spun down (10 min at 140 × *g*) and washed twice in RPMI medium. Each pellet was resuspended in 200 μl of water. The samples were heated at 95°C for 20 min. Twenty microliters of each sample, representing the nucleic acid content of 25,000 cells, was added to the PCR reaction mixture, containing 10 mM Tris·HCl at pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, dATP, dCTP, dGTP, and dTTP at 200 μM each, and each primer at 1 μM. The 17-mer primers SKO1 and SKO2 (11) were synthesized by A. Van Aerschot and P. Herdewijn at the Rega Institute. These primers amplified a 105-base-pair (bp) fragment of the HIV-1 *gag* gene. The reaction mixture was overlaid with 20 μl of light mineral oil and heated at 94°C for 3 min, after which the reaction was started by adding 0.5 μl of AmpliTaq DNA polymerase (Perkin–Elmer/Cetus) (2.5 units per assay). The samples were subjected to 40 cycles consisting of a denaturation step (1 min, 94°C), primer annealing (2 min, 55°C), and DNA synthesis (1 min, 72°C). A final elongation step (7 min, 72°C) was followed by soaking at 4°C. PCR was carried out in GeneAmp reaction tubes in a DNA thermal cycler (both from Perkin–Elmer/Cetus). The length of the amplified DNA product was verified by electrophoresis of 10 μl of the PCR reaction product in a 4% agarose gel (3% NuSieve and 1% SeaKem GTG, FMC). Molecular weight markers, phage ΦX174 replicative form DNA digested by *Hae* III (BRL) were run in parallel.

RT Sources. A first recombinant HIV-1 RT preparation (p66-MGS) was obtained from MicroGeneSys (West Haven, CT). This enzyme was derived from an HIV-1 *pol* gene fragment which codes for the whole RT plus 13 amino acids of the C terminus of the protease and 34 amino acids of the N terminus of the endonuclease. It is produced in an insect cell/baculovirus expression system. The apparent molecular weight (by SDS/PAGE) is 71,000. Final concentration in the reaction mixture was 4 μg/ml (56 nM). Incorporation rate was estimated at 10 pmol of dTMP per hr with poly(A)·oligo(dT) as a template/primer.

A second recombinant HIV-1 RT preparation (p66-CC) was kindly provided by P. J. Barr (Chiron). It was derived from an HIV-1 (SF2 strain) *pol* gene fragment coding for the RT (Pro-156 to Leu-715). Final concentration was 72 ng/ml (1.1 nM). Incorporation rate was estimated at 60 pmol of dTMP per hr in the poly(A)·oligo(dT)-directed assay.

Virion particle-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, from 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 MillexGV filter. Virus particles were subsequently sedimented 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 dithiothreitol, 0.1% 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°C until used.

Avian myeloblastosis virus (AMV) RT and cloned Moloney murine leukemia virus (MMuLV) RT (both FPLC pure from Pharmacia) were used at a final concentration of 40 units/ml (1 μg/ml).

RT Assays. The RT reaction mixture contained 50 mM Tris·HCl at pH 8.4, 10 mM $MgCl_2$, 100 mM KCl, 2.2 mM dithiothreitol, and 0.03% 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 concentrations 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, dCTP) were obtained from Amersham and used at a concentration of 2.5 μM. Specific activities were 15.6, 46, and 19.4 Ci/mmol (1 Ci = 37 GBq), respectively.

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

Ribonuclease H Assay. RNase H activity of HIV-1 RT was determined according to a procedure described by Starnes and Cheng (12). The reaction rate, expressed as the amount of solubilized [^{3}H]AMP, was calculated to be 0.24 pmol/hr. Contamination with ribonucleases was excluded by lack of solubilization of [^{3}H]AMP in the absence of poly(dT).

RESULTS

Inhibition of HIV-1 DNA Formation in Acutely Infected MT-4 Cells. In our previous studies we showed that TIBO compounds interact with an HIV-1 RT-associated process (9). Hence, they should block the synthesis of viral DNA in acutely HIV-1- infected cells. Newly formed viral DNA was determined by PCR. PCR samples contained 25,000 HIV-1-infected MT-4 cells that were heated at 95°C for 20 min. The primer pair chosen, SKO1 and SKO2 (17-mers) (11), amplified a 105-bp *gag* gene fragment of the HIV-1 genome, as is shown by agarose gel electrophoresis analysis of the PCR product (Fig. 2). HIV-1-infected MT-4 cells that were exposed to R82150 at 17.5 or 3.5 μM did not allow the formation of any viral DNA, whereas at a lower TIBO concentration (0.7 μM) viral DNA production was not inhibited. The dose-dependent inhibition of HIV-1 DNA formation by R82150 was confirmed by hybridizing a Southern blot of this gel with a ^{32}P-labeled probe (SKO3) complementary to part of the 105-bp *gag* gene fragment (11) (data not shown). PCR studies with DNA extracted from HIV-1-infected MT-4 cells gave identical results (data not shown).

Inhibition of RT. Virion-particle derived HIV-1 (RT) was inhibited by 50% at R82150 concentrations of 3 μM (HTLV-III_B strain) and 4.9 μM (RF strain) in a poly(A)·oligo(dT)-directed assay. Similar IC_{50} values were obtained for the two different p66 (MicroGeneSys and Chiron) recombinant HIV-1 RT. Dose–response curves of HIV-1 RT inhibition by TIBO prototypes R82150 and R82913 are shown in Fig. 3. Whereas the four HIV-1 RT preparations tested were inhibited at similar TIBO concentrations, the RT derived from HIV-2 (strains ROD and EHO, both virion-particle derived) was not inhibited at all (Table 1 shows data for one HIV-1 and one HIV-2 RT). Similarly, AMV RT and MMuLV RT were not inhibited at TIBO concentrations up to 300 μM. Cellular

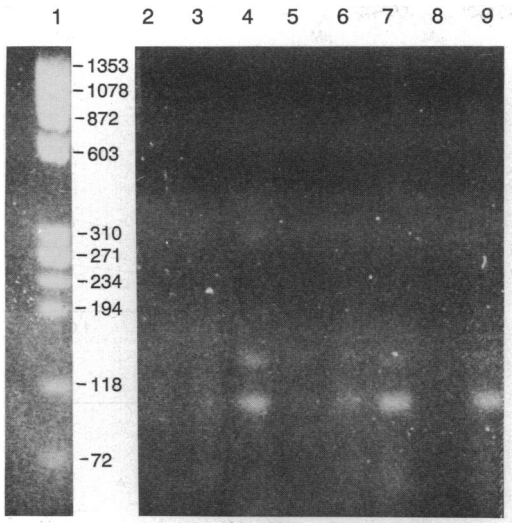

FIG. 2. Inhibition of HIV-1 DNA formation in acutely infected MT-4 cells by R82150. Total DNA of MT-4 cells, infected with HIV-1, was subjected to amplification with the primer pair SKO1 and SKO2, which amplifies an HIV-1 *gag* gene fragment of 105 bp (11). The PCR reaction products were analyzed by agarose gel electrophoresis; 10 μl of product was run on a 4% agarose gel and stained with ethidium bromide. Lane 1 contains 1 μg of ϕX174 replicative form DNA cut with *Hae* III, as size standards (bp). Lanes 2, 3, and 4 show the result for MT-4 cells infected with HIV-1(HTLV-III$_B$) and treated with R82150 at concentrations of 17.5, 3.5, and 0.7 μM, respectively. Lanes 5, 6, and 7 show the result of a treatment with AZT at concentrations of 1.875, 0.375, and 0.075 μM, respectively. Lane 8 contains DNA of mock-infected MT-4 cells, whereas lane 9 represents the result of amplification of the DNA of HIV-1-infected MT-4 cells.

DNA polymerases α, β, and γ were also resistant to inhibition by R82150 at concentrations up to 175 μM (K. Ono, H. Nakane, R.P., and E.D.C., unpublished data).

Comparison with Other RT Inhibitors. We compared the RT-inhibitory activity of TIBO compounds with that of other known RT inhibitors under the same assay conditions (Table 1). TIBO compounds are unique in their specificity as HIV-1 RT inhibitors. The other compounds tested (PFA, suramin, and AZT-TP) inhibited both HIV-1 RT (p66-MGS) and HIV-2$_{ROD}$ virion particle-derived RT (Table 1).

We also examined the template dependence of RT inhibition for the different compounds (Table 1). When poly(C)·oligo(dG) was used as a template/primer, the IC_{50} values of R82150 decreased about 10-fold as compared with a poly(A)·oligo(dT)-directed assay (0.34 μM and 5.9 μM, respectively). The same observation was made for all other TIBO compounds tested. Whereas RT inhibition by suramin was independent of the template used, the dideoxynucleotides AZT-TP and ddGTP inhibited RT only when a complementary base was used in the template. The IC_{50} values of PFA also varied according to the template/primer chosen. PFA was 8- and 20-fold less inhibitory in assays directed by poly(C)·oligo(dG) and poly(I)·oligo(dC), respectively. Since

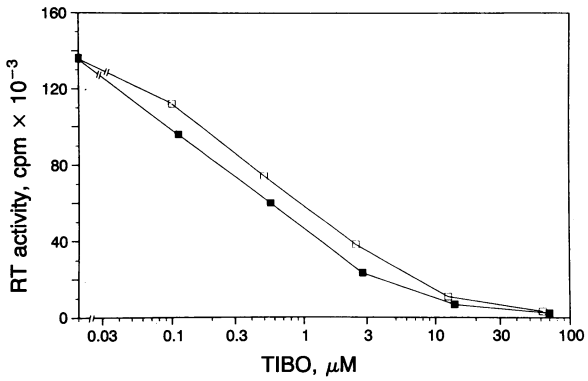

FIG. 3. RT activity, corresponding to the amount of radiolabeled dGMP incorporated in a poly(C)·(dG)$_{12-18}$-directed reaction, is plotted against concentration of R82150 (■) or R82913 (□). The recombinant enzyme in the assay was p66-CC. Samples were run twice and data presented are mean values. Background never exceeded 1000 cpm and was subtracted from each value. IC_{50} values for R82150 and R82913 calculated from these data were 0.36 and 0.6 μM, respectively.

the reaction velocity, as measured by the amount of radiolabeled deoxyribonucleotide incorporated, is dependent on the template used (13), the template dependence of RT inhibition was determined as a function of the reaction rate. The reaction rate was ⅓ or ⅙ as fast when poly(C)·oligo(dG) or poly(I)·oligo(dC) was used as template/primer instead of poly(A)·oligo(dT). The IC_{50} values for R82150 were similar in the poly(A)·oligo(dT) and poly(I)·oligo(dC)-directed assays (5.9 and 7.7 μM, respectively), which means that the template preference of TIBO inhibition is independent of the reaction rate. In contrast, PFA appeared to inhibit RT proportionally to the reaction rate.

Effect on Different Enzyme Functions of HIV-1 RT. We examined whether TIBO derivatives would inhibit DNA-directed DNA polymerase or RNase H activity of HIV-1 RT. The results for the DNA-directed DNA synthesis are shown in Table 2. Using poly(dC)·oligo(dG) as a template/primer and recombinant RT p66-CC as the enzyme, we found an IC_{50} value of 12 μM for R82150, which is 40-fold higher than its IC_{50} in a poly(C)·oligo(dG)-directed assay. In contrast, ddGTP had a slightly higher inhibitory effect on the DNA-dependent DNA polymerization reaction. As to RNase H, no inhibition by R82150 could be observed at concentrations up to 300 μM.

Kinetic Studies with HIV-1 RT. Kinetic studies were performed with recombinant HIV-1 RT (p66-MGS and p66-CC). The incorporation 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 less than 2.5% of the amount available in the reaction mixture). When the enzyme concentration was decreased, the maximum velocities (V_{max}) of the control reaction and the reaction in the presence of 0.35 μM R82150 diminished proportionally (Fig. 4). This points to a shift in the reaction equilibrium

Table 1. Specificity and template dependence of RT inhibition by R82150

RT	Template/primer	IC_{50}, μM				
		R82150	PFA	Suramin	AZT-TP	ddGTP
HIV-1 p66-MGS	Poly(A)·(dT)$_{12-18}$	5.9 ± 0.3	0.25 ± 0.06	3.2 ± 0.4	0.05 ± 0.03	>5
HIV-2$_{ROD}$		>300	0.14 ± 0.10	4.6 ± 2.0	0.05 ± 0.04	>5
HIV-1 p66-MGS	Poly(C)·(dG)$_{12-18}$	0.34 ± 0.07	1.6 ± 0.05	1.4 ± 0.6	>5	0.008 ± 0.001
HIV-1 p66-MGS	Poly(I)·(dC)$_{12-18}$	7.7 ± 2.0	4.9 ± 0.1	2.4 ± 0.4	>5	>5

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

Table 2. Inhibitory effect of R82150 on DNA- and RNA-dependent DNA polymerase activity of recombinant HIV-1 RT

Template/primer	IC_{50}, μM		
	R82150	R82913	ddGTP
Poly(dC)·oligo(dG)	12 ± 3	>60	0.003 ± 0.002
Poly(C)·oligo(dG)	0.30 ± 0.04	0.7 ± 0.2	0.008 ± 0.001

Poly(dC) and poly(C) were used at 40 μg/ml and oligo(dG) was 6 μg/ml. The RT was p66-CC. Data represent mean ± SD.

E + I ⇌ EI (where E stands for free enzyme, I for unbound inhibitor, and EI for enzyme–inhibitor complex) to the left, which is consistent with a reversible inhibition of RT by the TIBO compound. Similarly, IC_{50} values for inhibition of RT by TIBO were not influenced by the addition of excess dithiothreitol to the reaction mixture. Both these findings argue against the possible formation of a covalent disulfide bond between RT and the sulfur of the cyclic thiourea functionality of the TIBO compounds.

Kinetic studies were performed with various substrate (dGTP) and template/primer [poly(C)·oligo(dG)] concentrations to determine the mode of inhibition of R82150. As illustrated by the Dixon plots in Fig. 5, the inhibition of the HIV-1 RT reaction by R82150 appeared to be noncompetitive with regard to the substrate and uncompetitive with respect to the template/primer. Under these reaction conditions, the Michaelis–Menten constant (K_m) for dGTP was 5 μM and the inhibition constant (K_i) for R82150, read from the intercept with the abscissa, was about 0.65 μM. When the concentration of the template/primer was varied, a K_m of 8 μg/ml was calculated, whereas the K_i value for R82150 was highly dependent on the template/primer concentration. The K_i value for R82150 at an infinitely high concentration of poly(C)·oligo(dG) was estimated at 0.3 μM. For the poly(A)·oligo(dT)-directed reaction also the K_i values were dependent on the template/primer concentration (data not shown).

DISCUSSION

From our previous findings (9) we inferred that TIBO compounds interact in an RT-associated process. Here, we characterize the inhibitory effect of TIBO derivatives on HIV-1 RT and describe the unique properties of this inhibition.

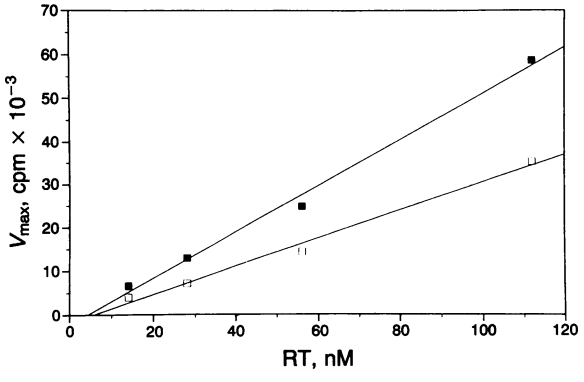

FIG. 4. Reversible inhibition of RT (p66-MGS). V_{max}, expressed as cpm in a 15-min assay, is plotted at various enzyme concentrations (■) and the effect of R82150 on V_{max} at different enzyme concentrations (□) is shown. V_{max} was measured at saturation concentrations of template/primer [poly(C)·oligo(dG) at 65 μg/ml] and substrate (10 μM dGTP). The concentration of R82150 was 0.35 μM. Lines were drawn by linear regression analysis.

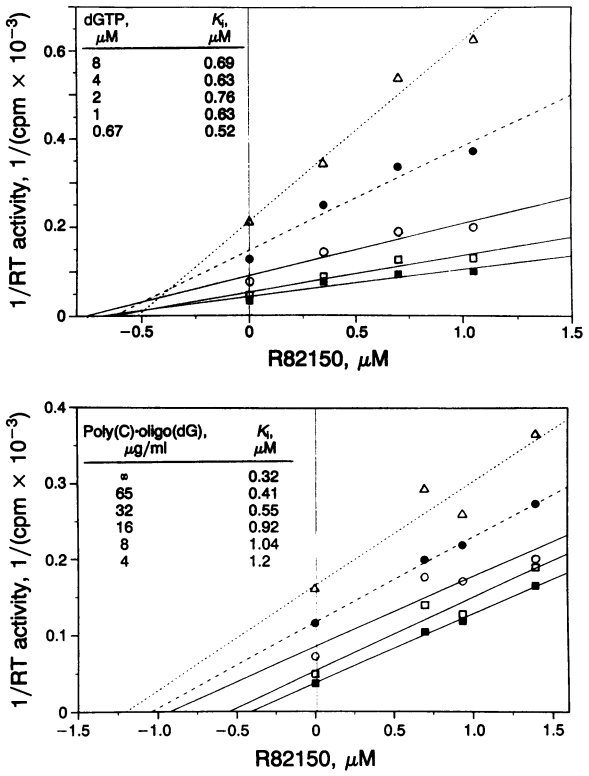

FIG. 5. Kinetic studies with HIV-1 recombinant RT (p66-CC) carried out under steady-state conditions. Data are presented in Dixon transformation plots: $1/v$ (expressed as 1/cpm in a 15-min assay) is plotted against inhibitor (R82150) concentration at different substrate concentrations. Lines are drawn by linear regression analysis. (*Upper*) Concentration of the substrate dGTP was varied: ■, 8 μM; □, 4 μM; ○, 2 μM; ●, 1 μM; and △, 0.67 μM. Poly(C)·$(dG)_{12-18}$ at 65 μg/ml was used as the template/primer. The noncompetitive mode of inhibition with regard to the substrate is illustrated by the table showing the K_i values. (*Lower*) The concentration of template/primer poly(C)·$(dG)_{12-18}$ was varied: ■, 65 μg/ml; □, 32 μg/ml; ○, 16 μg/ml; ●, 8 μg/ml; △, 4 μg/ml. dGTP was 2.5 μM. The uncompetitive mode of inhibition is illustrated by the table showing the apparent K_i values. The K_i value for R82150 with regard to the template/primer, as calculated from the formula $K_i app = K_i(1 + K_m/[S])$, was 0.32 μM.

R82150 inhibits HIV-1 DNA production in acutely HIV-1-infected T cells, as determined by PCR analysis. This observation, as well as the absence of any inhibitory effect in chronically HIV-1-infected T cells (9), indicates that the stage at which this class of compounds interacts with the replicative cycle of HIV-1 must be prior to proviral DNA formation. Several other observations (9) point to the RT as the target enzyme for the TIBO compounds: (*i*) virus binding to the cells is not affected; (*ii*) according to time of addition experiments, the drug-sensitive phase of intervention coincides with that of the 2′,3′-dideoxynucleoside analogues; and (*iii*) the ranking orders of TIBO derivatives in inhibition of HIV-1 replication and in cell-free RT inhibition are identical. The unique specificity of TIBO for HIV-1 replication in cell culture is also reflected at the RT level, since none of the RT preparations derived from HIV-2 (ROD or EHO), AMV, or MMuLV proved susceptible to inhibition by TIBO.

Using the exogenous template/primer complexes poly(A)·oligo(dT) and poly(C)·oligo(dG), we have demonstrated that the TIBO derivative R82150 inhibits in the same fashion virion ($HTLV\text{-}III_B$ and $HTLV\text{-}III_{RF}$)-derived and recombinant (p66-MGS and p66-CC) RT preparations. Thus, RT inhibition by TIBO has been confirmed with several HIV-1 strains and different RT preparations, and it does not seem to

be influenced by the presence of the virion proteins in the RT preparations.

From a comparison with the RT inhibitors PFA, suramin, AZT-TP, and ddGTP it is clear that TIBO compounds represent a different class of RT inhibitors: (*i*) they are inhibitory to HIV-1 but not any other RT; and (*ii*) they show a preference for poly(C)·oligo(dG), since RT inhibition with this template/primer is 10- to 20-fold higher than with other exogenous template/primers. TIBO compounds do not inhibit cellular polymerases. Moreover, they selectively inhibit the RNA-dependent DNA polymerase activity of the HIV-1 RT. In contrast, the 2′,3′-dideoxynucleotide ddGTP, when evaluated under identical conditions, is equally inhibitory to the RNA- and the DNA-dependent DNA polymerase activity of HIV-1 RT. Finally, TIBO compounds do not inhibit the RNase H function.

Kinetic studies with the TIBO compounds in the RT reaction have indicated that, unlike the 2′,3′-dideoxynucleotide analogues, the TIBO compounds do not compete with the natural substrate (dTTP or dGTP). They are uncompetitive with respect to the template/primer, with K_i values that highly depend on the template/primer concentration. This indicates that the putative TIBO-binding site must be affected by template binding. This phenomenon also explains why RT inhibition by TIBO is dependent on the type of template/primer used. Since these homopolymers have different structural conformations they may be recognized with different affinities by the RT and, in addition, induce different conformational shifts after binding to the enzyme. The molecular parameters of the binding of TIBO with HIV-1 RT remain to be identified. Our template/primer studies suggest that RT cocrystallization experiments with TIBO are more likely to be successful if carried out in the presence of an adequate template—i.e., poly(C).

Whereas all experimental data indicate an RT-mediated mechanism of action of the TIBO compounds, it should be recognized that the concentration required to inhibit the HIV-1 induced cytopathicity is significantly lower than that required to inhibit the HIV-1 RT. This may suggest that some other as-yet-unknown factors which are present in the cell-based systems but absent from the cell-free enzyme assays play a part in the inhibition of viral growth. In the enzyme assays used to determine RT inhibition, to which exogenous template/primer has been added, certain cofactors, which at the cellular level contribute to the RT process, may not be functional.

In conclusion, our studies indicate that the TIBO compounds which are highly potent and selective inhibitors of HIV-1 replication in cell culture represent a class of anti-HIV-1 drugs that interact with the HIV-1 RT in a unique fashion, different from that of other known RT inhibitors. The study of the mechanism of RT inhibition by TIBO compounds may contribute to a better understanding of the mechanism of reverse transcription.

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