Mode of inhibition of HIV-1 reverse transcriptase by polyacetylenetriol, a novel inhibitor of RNA- and DNA-directed DNA polymerases

Shoshana LOYA*, Amira RUDI†, Yoel KASHMAN† and Amnon HIZI*[1]

*Department of Cell Biology and Histology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv 69978, Israel, and †School of Chemistry, Tel-Aviv University, Tel-Aviv 69978, Israel

Polyacetylenetriol (PAT), a natural marine product from the Mediterranean sea sponge *Petrosia* sp., was found to be a novel general potent inhibitor of DNA polymerases. It inhibits equally well the RNA- and DNA-dependent DNA polymerase activities of retroviral reverse transcriptases (RTs) (i.e. of HIV, murine leukaemia virus and mouse mammary tumour virus) as well as cellular DNA polymerases (i.e. DNA polymerases α and β and *Escherichia coli* polymerase I). A study of the mode and mechanism of the polymerase inhibition by PAT has been conducted with HIV-1 RT. PAT was shown to be a reversible non-competitive inhibitor. PAT binds RT independently and at a site different from that of the primer-template and dNTP substrates with high affinity ($K_i = 0.51\ \mu$M and $K_i = 0.53\ \mu$M with dTTP and with dGTP as the variable substrates respectively). Blocking the polar hydroxy groups of PAT has only a marginal effect on the inhibitory capacity, thus hydrophobic interactions are likely to play a major role in inhibiting RT. Preincubation of RT with the primer-template substrate prior to the interaction with PAT reduces substantially the inhibition capacity, probably by preventing these contacts. PAT does not interfere with the first step of polymerization, the binding of RT to DNA, nor does the inhibitor interfere with the binding of dNTP to RT/DNA complex, as evident from the steady-state kinetic study, whereby K_m remains unchanged. We assume, therefore, that PAT interferes with subsequent catalytic steps of DNA polymerization. The inhibitor may alter the optimal stereochemistry of the polymerase active site relative to the primer terminus, bound dNTP and the metal ions that are crucial for efficient catalysis or, alternatively, may interfere with the thumb sub-domain movement and, thus, with the translocation of the primer-template following nucleotide incorporation.

Key words: enzyme kinetics, natural products, retroviruses.

INTRODUCTION

The enzyme reverse transcriptase (RT) is crucial to the replicative cycle of retroviruses, HIV-1 and HIV-2. Therefore it is one of the major targets for anti-AIDS chemotherapy. RT is a multifunctional enzyme displaying an RNA-dependent DNA polymerase (RDDP), a DNA-dependent DNA polymerase (DDDP) and an RNase H function. This virus-specific enzyme converts the genomic single-stranded RNA into double-stranded DNA [1]. To date, about ten anti-RT compounds are approved for the treatment of HIV-infected individuals [2]. These drugs fall into two categories: nucleoside analogues and non-nucleoside inhibitors (NNRTI). The nucleoside analogues lack the 3′-hydroxy groups and thus compete with the normal substrate, leading to premature termination of DNA polymerization [3,4]. Although these drugs appear to provide some clinical benefit for AIDS patients, their use is limited by serious toxic side effects [5,6] and the emergence of resistant viral strains [7,8]. The NNRTI are chemically diverse, but all are highly specific for HIV-1 RT with no effect on HIV-2 RT. They inhibit the enzyme by binding to a common allosteric site near to, but distinct from, the polymerase active site [9]. In contrast with the nucleoside analogues, the NNRTI exhibit considerably lower cellular toxicity, which probably results from the lack of activity against cellular DNA polymerases. However, the efficacy of NNRTI is hampered by the very rapid development of drug-resistant mutants. In some cases, even a single mutation in the viral RT permits the virus to escape from the effect of the drug [2,10]. The NNRTI are used in combination therapy with nucleoside analogues, since their separate mechanisms of inhibition lead to synergism [11]. However, the highly active anti-retroviral therapy currently used, with a combination of three or more drugs (nucleoside analogues, NNRTI and protease inhibitors) is not potent enough to completely suppress virus replication or drug-resistance development and hence eradicate HIV. Therefore, there is still an urgent need to develop new drugs with disparate and novel mechanisms of inhibition.

Another category of anti-RT drug includes compounds which can be structurally characterized as non-nucleoside inhibitors originating from natural sources, as opposed to both the synthetically produced nucleoside analogues and the conventional NNRTI [12]. In our search for such compounds we have isolated two classes of RT inhibitors: (1) compounds with equal capacity to inhibit effectively both HIV-1 and HIV-2 RTs, but with poor or no ability to inhibit cellular DNA polymerases, and (2) compounds with a wide spectrum of inhibitory capacity against DNA polymerases with no specificity against RT. Toxiusol [13] and polycitone A [14] belong to the group of general inhibitors of DNA polymerases. They are capable of inhibiting DNA polymerization by blocking RT–DNA complex formation. On the other hand, 2-hexaprenylhydroquinone, another general inhibitor of the RNA- and DNA-directed DNA polymerases,

Abbreviations used: RT, reverse transcriptase; RDDP, RNA-dependent DNA polymerase; DDDP, DNA-dependent DNA polymerase; MuLV, murine leukaemia virus; MMTV, mouse mammary tumour virus; KF, Klenow fragment of *Escherichia coli* DNA polymerase I; PAT, polyacetylenetriol; pol α, DNA polymerase α; pol β, DNA polymerase β; NNRTI, non-nucleoside RT inhibitors; TIBO, tetrahydroimidazo[4,5,1-jk][1,4]benzodiazepine-2(1*H*)-one and thione; TBE, Tris·borate/EDTA.

[1] To whom correspondence should be addressed (e-mail ahizy@post.tau.ac.il).

inhibits the nucleotidyl-transfer catalytic reaction [15]. Similarly, we describe in the present paper a novel natural marine product, polyacetylenetriol (PAT) from the Mediterranean sea sponge *Petrosia* sp., which exhibits a general inhibition of retroviral DNA polymerases [HIV-1, HIV-2, murine leukaemia virus (MuLV) and mouse mammary tumour virus (MMTV)] and cellular DNA polymerases [calf thymus DNA polymerase α (pol α), human DNA polymerase β (pol β) and Klenow fragment of *E. coli* pol I (KF)]. PAT is a reversible non-competitive inhibitor capable of interacting with RT at a non-substrate binding allosteric site through hydrophobic interactions. PAT does not block both the formation of the RT–DNA complex (the first step of DNA polymerization) and dNTP binding step. We speculate that the presence of PAT distorts the precise stereochemistry of the RT active site relative to the primer terminus, bound dNTP and the associated metal ions consequently blocking the catalytic step of the overall DNA polymerization reaction.

MATERIALS AND METHODS

Chemicals

The natural compound, a polyacetylenetriol (Figure 1), was isolated from *Petrosia* sp. using the following procedure. The freeze-dried sponge (10 g) was extracted with methanol/ethyl acetate (1:1, v/v) to produce a brown 'gum' (100 mg). Solvent partition between water/methanol (2:8, v/v) and tetrachloromethane gave the crude active material in the tetrachloromethane phase. Sephadex LH-20 chromatography [eluted with light petroleum (b.p. 60–70 °C)/chloroform/methanol (2:1:1, by vol.)] yielded 15 mg of the pure compound. Elucidation of the structural formula by spectroscopic means, one- and two-dimensional NMR and mass spectra revealed a compound which is identical in all respects with petroformyne-2 reported previously by Cimino et al. [16]. The acetylation of the natural compound PAT was performed in acetic anhydride/pyridine (1:1, v/v) for 24 h at room temperature. The mixture was evaporated and the residue obtained was chromatographed on a Sephadex LH-20 column to yield a triacetyl derivative (Figure 1). Synthetic template-primers poly(rA)$_n$·oligo(dT)$_{12-18}$ and poly(rC)$_n$·oligo(dG)$_{12-18}$ were purchased from Pharmacia. Activated gapped DNA was prepared by limited digestion of herring sperm DNA by bovine pancreatic DNase I as described in detail in [17].

Enzymes

The RTs of HIV-1 and of MMTV are recombinant enzymes expressed in *E. coli* DH5α and purified from the bacterial extracts. The HIV-1 RT, a heterodimer of a large 66 kDa subunit and a smaller 51 kDa subunit (p66/p51), was derived from the BH-10 clone [18] and was purified to homogeneity as described in [19]. The MMTV RT was derived from the pUC1002 proviral plasmid generated from the BR6 strain. The MMTV RT coding DNA insert was then transferred into pUC12N6H plasmid (with a hexahistidine extension at the N-terminus) as described in [20]. The MMTV RT, a p66 monomer, was purified by Ni^{2+}-nitrilotriacetate chromatography followed by CM-Sepharose ion-exchange chromatography [20]. Recombinant MuLV RT, a p70 monomer, was purchased from Amersham Biosciences (Little Chalfont, Bucks., U.K.), pol α was purified from calf thymus by immunoaffinity column chromatography and was generously donated by Dr M. Fry (Technion, Haifa, Israel). Recombinant pol β was generously given by Dr Z. Hostomsky (Agouron, San Diego, CA, U.S.A.). KF was purchased from New England Biolabs (Beverley, MA, U.S.A.).

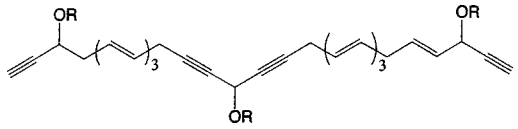

Figure 1 Structural formulae of polyacetylenetriol and its triacetylated derivative

Where 'R' is H, this denotes polyacetylenetriol; where 'R' is Ac, this represents the triacetylated derivative.

Enzyme assays

Enzymic reactions were performed as described previously [21]. The RDDP activity was assayed by monitoring either the poly(rA)$_n$·oligo(dT)$_{12-18}$-directed incorporation of [^{3}H]dTTP or the poly(rC)$_n$·oligo(dG)$_{12-18}$-directed incorporation of [^{3}H]-dGTP into DNA. The DDDP activity associated with all retroviral RTs, as well as pol α, pol β and KF was assayed using activated gapped DNA and four deoxynucleotides (of which only dTTP was labelled). The assays were carried out in a final volume of 0.1 ml containing 50 mM Tris/HCl, pH 7.5, 40 mM KCl, 3 mM dithiothreitol, 8 mM $MgCl_2$, 60 μg/ml activated DNA, 25 μM of each unlabelled dATP, dGTP, dCTP, and [^{3}H]dTTP at a final concentration of 5 μM (1800–3500 c.p.m./pmol). One unit of DNA polymerase activity is defined as the amount of enzyme that catalyses the incorporation of 1 pmol of dNTP into DNA product in 30 min at 37 °C under standard assay conditions. The RNase H activity of HIV-1 RT was assayed by measuring the release of trichloracetic-soluble material from the synthetic substrate [^{3}H]poly(rA)·poly(dT) as described previously [21]. Inhibition of enzyme activities was calculated relative to the initial linear reaction rates observed under identical conditions with the drug omitted. In all dose–response experiments of the inhibition of DNA polymerase, the enzymes were first preincubated with increasing concentrations of the inhibitor for 3 min at 30 °C. The reaction was initiated by adding the relevant [^{3}H]dNTP and substrates followed by incubation at 37 °C for 30 min (or for 10 min in the steady-state kinetic studies). Kinetic constants were derived from the double-reciprocal plots of velocity versus substrate concentration using computer-generated linear regression analyses.

DNA primer extension

Single-stranded circular ϕX174am3 DNA (New England Biolabs, Beverly, MA, U.S.A.) was primed with a synthetic 15-mer oligonucleotide (which hybridizes to the DNA at positions 588–602 [22]). The sequence of this synthetic primer is 5′-AAAGCGAGGG TATCC-3′. The primer was labelled with T4 polynucleotide kinase at its 5′-end with [γ-^{32}P]ATP and was annealed to a 2-fold molar excess of unlabelled template. The reactions were performed in 20 mM Tris/HCl, pH 7.5, 2 mM dithiothreitol (DTT), 10 mM $MgCl_2$ and 0.1 mg/ml BSA. The reactions were divided into two sets. In the first set of reactions, the enzyme was preincubated with increasing amounts of inhibitor for 5 min at room temperature prior to initiating the polymerization reaction with the template-primer and all four dNTPs (each at a final concentration of 10 μM). In the second set of reactions, the enzyme was preincubated with the template-primer for 5 min at room temperature before adding increasing amounts of inhibitor and all four dNTPs to initiate the reaction. All reactions, each at a final volume of 12.5 μl, were incubated at

37 °C for 20 min. The reactions were stopped by adding an equal volume of formamide dye mix, denaturing at 100 °C for 3 min and immediately chilling on ice before being analysed by electrophoresis on 8 %-(w/v)-polyacrylamide-urea gels as described previously [22]. The gel autoradiographs were analysed by densitometry scanning procedures.

Binding assay

The binding of HIV-1 RT to a double-stranded oligonucleotide was measured using electrophoretic-mobility shift assays [23]. A single-stranded 54-mer oligonucleotide (oligo 1) was labelled with T4 polynucleotide kinase at its 5′-end with [γ-^{32}P] ATP and was annealed to another single-stranded 54-mer nucleotide (oligo 2) to produce a duplex oligonucleotide of 54 bp with recessed 2 nt at 3′-ends. The sequences of these two oligonucleotides were as follows: oligo 1: 5′-AAT GAA AGA CCC CAC CTG TAG GTT GGA TCC TTA CCC GTC AGC GGG GGT CTT TCA-3′; oligo 2: 3′-A CTT TCT GGG GTG GAC ATC CAA CCT AGG AAG GGG CAG TCG CCC CCA GAA AGT AA-5′.The formation of a complex between ^{32}P-5′-end-labelled oligonucleotide and HIV-1 RT was detected by the electrophoretic retardation of the DNA as a result of its association with the enzyme. HIV-1 RT was first incubated with the inhibitor at room temperature for 5 min before the onset of the binding reaction by the addition of the labelled oligonucleotide duplex (oligo1–oligo2). The binding reaction assays were incubated for 10 min at 32 °C in a final volume of 12.5 μl containing 10 mM Hepes/KOH, pH 8.0, 30 mM ammonium sulphate, 0.25 mM dithiothreitol, 20 μg/ml BSA, 10 mM KCl, 1.2 pmol HIV-1 RT and 0.12 pmol of the double-stranded labelled oligonucleotide. The reaction mixtures were electrophoresed through 6.5 %-polyacrylamide gel in 0.5 × TBE (45 mM Tris/borate/1 mM EDTA, pH 8) at 4 °C under 15 V/cm for about 3 h. The gel autoradiographs were analysed by densitometry scanning.

RESULTS

Effects of PAT and its acetylated derivative on HIV-1-RT-associated enzymic functions

A screening of many extracts from marine organisms for their anti-(HIV-1 RT) activities has yielded a new natural compound identified as polyacetylenetriol (PAT) (Figure 1). This compound was isolated from *Petrosia* sp. and was found to be identical in all respects with petroformyne-2, described by Cimino et al. [16]. PAT is shown to be a potent inhibitor of the HIV-1 RT-associated DNA polymerase functions. The poly(rA)$_n$ · oligo(dT)$_{12–18}$, as well as the gapped activated DNA-directed DNA synthesis, are strongly inhibited by PAT with IC_{50} values (inhibitor concentrations which lead to the inhibition of the initial enzymic activity by 50 %) achieved at 0.95 ± 0.05 μM and 2.60 ± 0.5 μM PAT respectively. The RNase H activity of HIV-1 RT, on the other hand, is hardly affected by the inhibitor; even at a concentration as high as 100 μM there was only a marginal effect of 10 % inhibition (Table 1). In all, PAT selectively inhibits only the DNA polymerase activities of HIV-1 RT. A chemical derivative of the natural product PAT was prepared by substituting three hydroxy groups with acetyl moieties. The acetylated derivative, in which the three potential active sites are blocked, has been tested for its capacity to inhibit the HIV-1-associated RT functions. As can be seen in Table 1, the IC_{50} values for the derivative, calculated from dose–response curves for both DNA polymerase and RNase H activities, are quite similar to the corresponding values for the natural inhibitor. In other words, the derivative exhibits a potent inhibitory capacity against both HIV-1-associated DNA activities and insignificant weak activity against RNase H function (with IC_{50} values of 1.55 ± 0.55 μM and 9.0 ± 0.8 μM for poly(rA)$_n$ · oligo(dT)$_{12–18}$-directed and activated DNA-directed DNA synthesis respectively).

It is known that the efficacy of inhibition may vary according to the template-primer used. For example, TIBO {tetrahydroimidazo[4,5,1-jk][1,4]benzodiazepine-2(1*H*)-one and thione} exhibits a marked template-primer preference, i.e. with the poly(rC)$_n$ · oligo(dG)$_{12–18}$ the IC_{50} value is 17-fold lower than with poly(rA)$_n$ · oligo(dT)$_{12–18}$ [24]. Therefore we have tested the dependence of inhibition by PAT on the template-primer used. The IC_{50} values calculated from the dose–response curves in Figure 2 were 1.4 ± 0.4 μM, 0.53 ± 0.08 μM and 2.6 ± 0.6 μM of PAT for poly(rA)$_n$ · oligo(dT)$_{12–18}$-, poly(rC)$_n$ · oligo(dG)$_{12–18}$- and activated DNA-directed DNA synthesis respectively. The capacity of PAT to inhibit the DNA polymerase function only improves slightly (by 2–3-fold) and with the poly(rC)$_n$ · oligo(dG)$_{12–18}$ as the template-primer; namely the

Table 1 Effects of PAT and its acetylated derivative on the RNA- and DNA-dependent DNA polymerases and RNase H activities of HIV-1 RT

The IC_{50} values were derived from dose–response curves as described in the Materials and methods section. The RDDP function was tested with poly(rA)$_n$ · oligo(dT)$_{12–18}$ as template-primer. Results are means ± S.D. for two to four independent experiments with duplicate determinations in each experiment.

	IC_{50} (μM)		
	DNA polymerase function		
Compound	RDDP	DDDP	RNase H
PAT	0.95 ± 0.05	2.6 ± 0.6	> 100
Acetylated PAT	1.55 ± 0.55	9.0 ± 0.8	⩾ 100

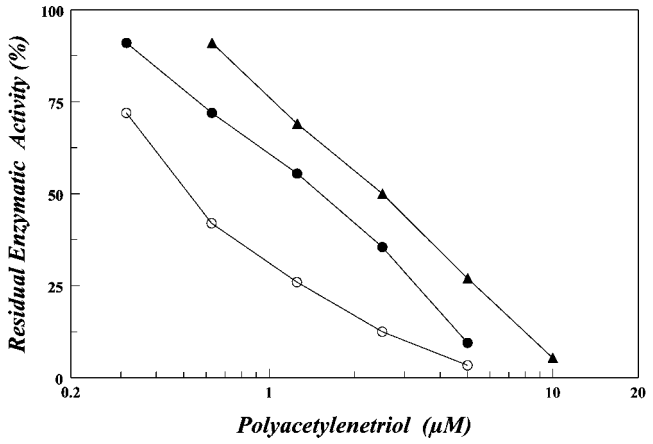

Figure 2 Dose–response curves for inhibition of the HIV-1 RT associated DNA polymerase activity by PAT using different template-primers

Inhibition experiments were carried out as described in the Materials and methods section. The DNA polymerase activity of HIV-1 RT was assayed by monitoring poly(rA)$_n$ · oligo(dT)$_{12–18}$ (●), poly(rC)$_n$ · oligo(dG)$_{12–18}$ (○) or gapped activated DNA (▲) -directed DNA synthesis in the presence of increasing concentrations of PAT. The IC_{50} values calculated from the curves are 1.4 ± 0.4, 0.53 ± 0.08 and 2.6 ± 0.6 μM respectively. Activities of 100 % correspond to 77, 31, and 54 units for the poly(rA)$_n$ · oligo(dT)$_{12–18}$, poly(rC)$_n$ · oligo(dG)$_{12–18}$ and activated DNA-directed DNA polymerase activities respectively.

Table 2 Effects of polyacetylenetriol on the DNA polymerase activities of retroviral RTs and cellular DNA polymerases

The IC_{50} values and IC_{95} values are derived from dose–response curves and assayed with either poly(rA) · oligo(dT) or activated-DNA-directed DNA synthesis in the presence of increasing concentrations of PAT. The IC_{50} values for HIV-1 RT are taken from Table 1. Results are means ± S.D. for at least three independent experiments with duplicate determinations in each experiment.

Enzyme	DNA polymerase function	IC_{50} (μM)	IC_{95} (μM)
HIV-1 RT	RDDP	0.95 ± 0.05	4.0 ± 0.6
	DDDP	2.6 ± 0.6	9.5 ± 0.3
MuLV RT	RDDP	0.40 ± 0.1	3.9 ± 0.3
	DDDP	0.38 ± 0.03	4.2 ± 0.5
MMTV RT	RDDP	1.4 ± 0.3	6.7 ± 1.7
	DDDP	6.9 ± 0.96	≫10
Calf-thymus pol α	DDDP	1.1 ± 0.3	2.6 ± 0.3
Human pol β	DDDP	1.25 ± 0.15	7.1 ± 1.1
		(2.8 ± 0.8)*	(9.0 ± 1.0)*
KF	DDDP	0.78 ± 0.23	5.0 ± 0.3
		(4.0 ± 0.5)*	(20 ± 2)*

* These values are calculated from dose–response inhibition curves with the acetylated derivative.

inhibition is practically unaffected by the nature of the template-primer used.

Effect of PAT on other retroviral RTs and cellular DNA polymerases

The specificity of PAT as an anti-(HIV-1 RT)-associated DNA polymerase drug has been evaluated by testing its effect on RTs of both C-type and B-type retroviruses, i.e. of MuLV and MMTV respectively. These two viruses are distantly related to HIV-1 RT, a prototype of the lentiviruses subfamily of retroviruses. As seen in Table 2, PAT inhibits DNA polymerase activity of both MuLV and MMTV RTs as well as HIV-1 RT. The RNA- and the DNA-dependent DNA polymerase activities were blocked effectively, exhibiting IC_{50} values of 0.4 ± 0.1 μM and 0.38 ± 0.03 μM PAT respectively, for the MuLV RT and 1.4 ± 0.3 μM and 6.9 ± 0.96 of PAT respectively, for the MMTV RT.

Since no substantial differences in the response of the various retroviral RTs to PAT were apparent, we have extended the study to DNA polymerases from both prokaryotic and eukaryotic sources. As shown in Table 2, all DNA polymerases tested are equally sensitive to PAT. The IC_{50} values calculated for calf thymus pol α, human pol β and KF were 1.1 ± 0.3 μM, 1.25 ± 0.15 μM and 0.78 ± 0.23 μM PAT respectively. The acetylated derivative, likewise, exhibited a potent inhibitory capacity against bacterial and cellular DNA polymerases. As shown in Table 2, the IC_{50} values were 4.0 ± 0.5 μM for KF and 2.8 ± 0.8 μM for pol β. As in the case of HIV-1 RT, it seems that blocking the hydroxy groups by acetylation has only a minor effect on the inhibition capacity of PAT. In all, PAT inhibits indiscriminately all polymerases tested and, therefore, can be considered as a general inhibitor of the DNA polymerases. To further study the mode of PAT inhibition, we have used HIV-1 RT as a representative of the DNA polymerases tested.

The mode of HIV-1 RT inhibition by PAT

Steady-state kinetic studies were performed by increasing concentrations of either dTTP or the template-primer

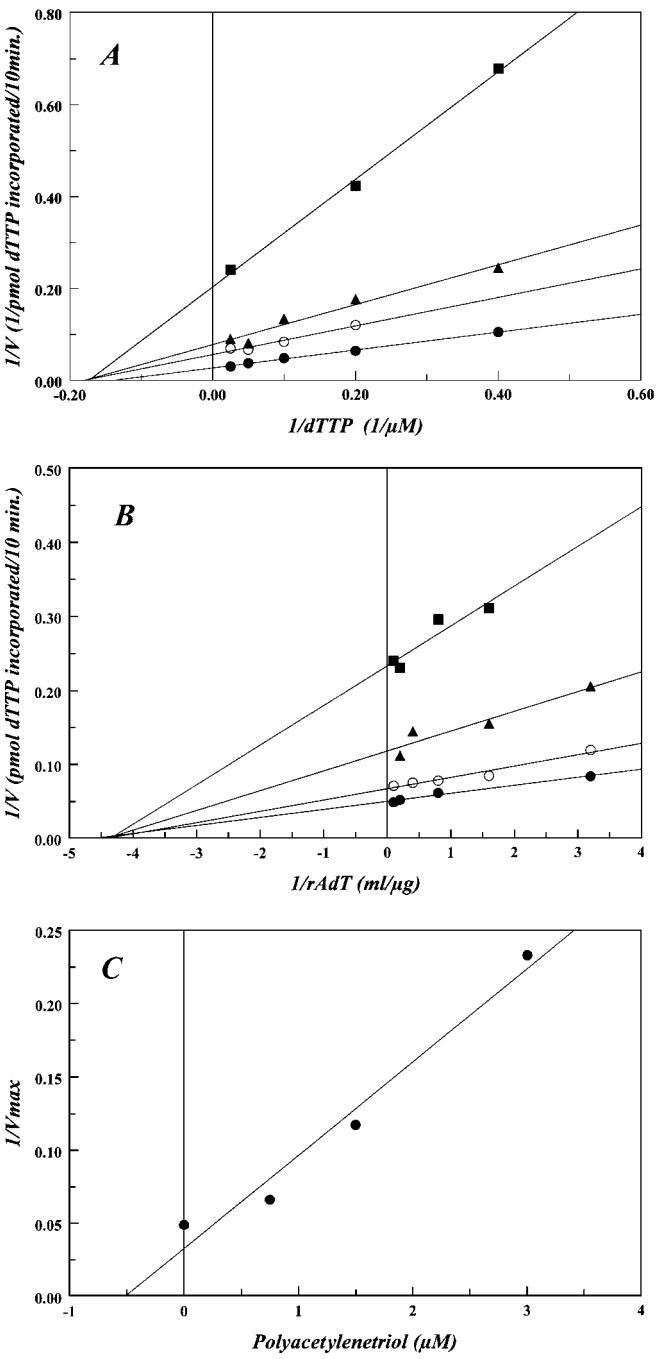

Figure 3 Kinetic analysis of the inhibition of HIV-1 RT-associated DNA polymerase activity by PAT

Double-reciprocal plots of the initial velocity of RDDP activity of HIV-1 RT as a function of substrate concentration. (**A**) Increasing concentrations of dTTP in the absence of (●) or in the presence of 1 (○), 2 (▲) or 4 μM (■) PAT. (**B**) Increasing concentrations of poly $(rA)_n$·oligo$(dT)_{12–18}$ in the absence of (●) or in the presence of 0.75 (○), 1.5 (▲) or 3 μM (■) PAT. (**C**) Secondary replot (Dixon) of the reciprocal maximal velocity (calculated from **B**) versus various PAT concentrations. The kinetic constants K_m and K_i were computer-generated by a linear regression analysis.

poly$(rA)_n$·oligo$(dT)_{12–18}$ in the presence of various concentrations of PAT. As shown in Figure 3, the type of inhibition is non-competitive with respect to both dTTP and the template-primer. Accordingly, the K_m values (derived from Lineweaver–Burk plots) for dTTP (6.0 ± 0.7 μM) and for

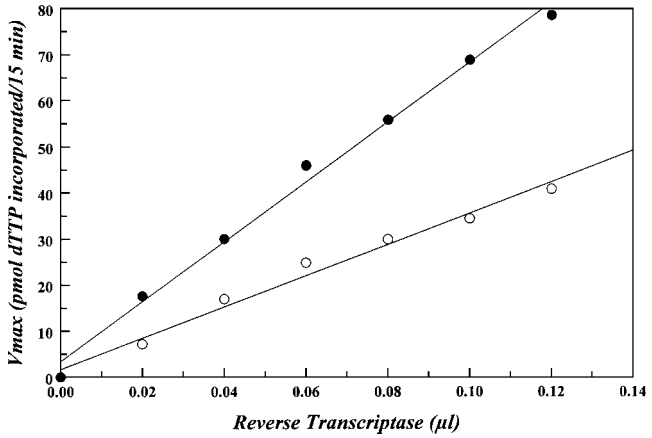

Figure 4 Maximal velocity of HIV-1 RT-associated DNA polymerase activity as a function of enzyme concentration

The DNA polymerase activity was monitored by measuring the poly(rA)$_n$ · oligo(dT)$_{12–18}$-directed incorporation of [^{3}H]dTTP into DNA in the absence (●) or presence of 1 μM PAT (○). The reactions were performed with saturating concentrations of dTTP (15 μM) and template-primer (8.5 μg/ml) for 15 min at 37 °C. The curves were computer-generated by a linear-regression analysis. The values were as high as 0.99, indicating a high linear relationship between V_{max} values and enzyme concentrations (1 μl = 0.24 μg of enzyme).

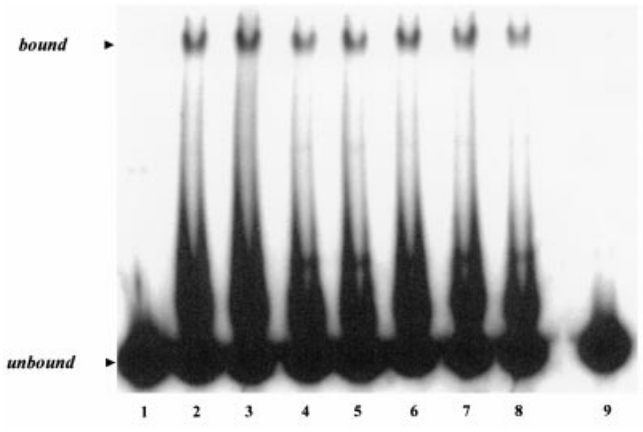

Figure 5 A gel-shift assay to determine the effects of PAT and its triacetylated derivative on the formation of HIV-1 RT–DNA complex

The binding of HIV-1 RT to ^{32}P-end-labelled double-stranded oligonucleotide DNA (54-mer) was carried out in the absence or in the presence of increasing concentrations of inhibitor as described in the Materials and methods section. The lanes in the scanned autoradiogram of the electrophoretic-mobility-shift assays are as follows: lane 1, control with no enzyme present; lane 2, binding of RT DNA to DNA with no inhibitor; lanes 3–6, binding of RT to DNA in the presence of PAT [at final concentration of 6.25 (lane 3), 12.5 (lane 4), 25 (lane 5) and 50 μM (lane 6)]; lanes 7 and 8, binding of RT to DNA in the presence of 12.5 and 50 μM triacetylated PAT derivative respectively; lane 9, binding of RT to DNA in the presence of 50 μM toxiusol.

poly(rA)$_n$ · oligo(dT)$_{12–18}$ (0.226 ± 0.005 μg/ml) were not significantly affected by the presence of increasing concentrations of PAT (Figure 3A and 3B). The V_{max} values, on the other hand, were suppressed as a function of increasing inhibitor concentrations, i.e. the rate of the catalytic process (K_{cat}) decreases by 8-fold, from 0.28 s^{-1} to 0.038 s^{-1}. It is clear that the inhibitor and the substrate are not mutually exclusive and both can bind the enzyme independently. A replot of $1/V_{max}$ values (intercept) against the corresponding inhibitor concentrations at which they were obtained (Dixon plot; Figure 3C) is linear ($r = 0.98$) and yields a K_i value of 0.513 μM. Preincubation of RT with increasing concentrations of poly(rA)$_n$ · oligo(dT)$_{12–18}$ before adding the inhibitor affects both K_{cat} and K_m (results not shown). That is, a mixed-type inhibition, which is actually a form of a non-competitive inhibition, has been obtained. PAT was found also to be a non-competitive inhibitor with respect to dGTP and the template-primer poly(rC)$_n$ · oligo(dG)$_{12–18}$ (results not shown). K_m values were calculated to be 1.10 ± 0.03 μM for the variable dGTP substrate and 1.38 ± 0.22 μg/ml for the variable template-primer. In the presence of 2 μM PAT, the apparent K_{cat} value for the incorporation of dGTP decreases by 4-fold, from 0.016 s^{-1} without the inhibitor to 0.004 s^{-1}. The inhibition constant (K_i) of 0.53 ± 0.04 μM PAT was derived from a linear Dixon plot ($r = 0.99$). This information implies that PAT interacts with RT at a non-substrate binding allosteric site.

In a non-competitive inhibition, the substrate and inhibitor bind reversibly, and independently at different sites. However, a compound that combines irreversibly with an enzyme may resemble a classical non-competitive inhibitor, because K_m remains unchanged and V_{max} decreases as a result of complete removal of some enzyme from the reaction. To distinguish between these two possibilities, we have measured the V_{max} of RT-associated DNA synthesis (with saturating amounts of dNTP and template-primer) with increasing amounts of RT in the presence of 1 μM inhibitor. As can be seen in Figure 4, the V_{max} values in both curves, with and without PAT, are proportionately reduced as a function of the decrease in enzyme concentration. The reaction velocity in the presence of inhibitor is diminished at all enzyme concentrations tested compared with the control reaction. In other words, the inhibitor capacity of PAT remains unchanged (53 ± 6 %) at all enzyme dilutions. It is likely, from these results, that PAT is a reversible non-competitive inhibitor of the RT-associated DNA polymerase function.

Effect of PAT on the binding of HIV-1 RT to its cognate template-primer DNA

DNA polymerization by HIV-1 RT demonstrates an ordered sequential mechanism with the template-primer bound first to the enzyme [25]. Interference with this initial step may inhibit the overall DNA synthesis reaction pathway. The formation of the RT–DNA complex can be monitored by a gel-shift assay, in which an electrophoretic retardation of labelled DNA is detected as a result of its association with the enzyme. The effects of increasing concentrations of PAT and its acetylated derivative on the formation of complexes between the RT and ^{32}P-5′-end-labelled double-stranded oligonucleotide (54-mer) are shown in Figure 5. A band of RT bound to DNA is shown in lane 2 with no inhibitor present, as well as in lanes 3–6 in the presence of increasing concentrations of PAT (6.25 up to 50 μM) or in the presence of its triacetylated derivative in lanes 7 and 8 (12.5 μM and 50 μM, respectively). PAT, as well as its triacetylated analogue, does not affect the formation of RT/DNA complexes. This is in contrast with toxiusol which, as has already been shown previously [15], blocks completely the formation of RT/DNA complex at a concentration of 50 μM (no band can be detected in lane 9, or in the control in lane 1 with no enzyme present).

Primer extension by HIV-1 RT in the presence of PAT

To obtain a better insight into the mechanism of inhibition of the HIV-1 RT-associated DNA polymerase activity by PAT, we

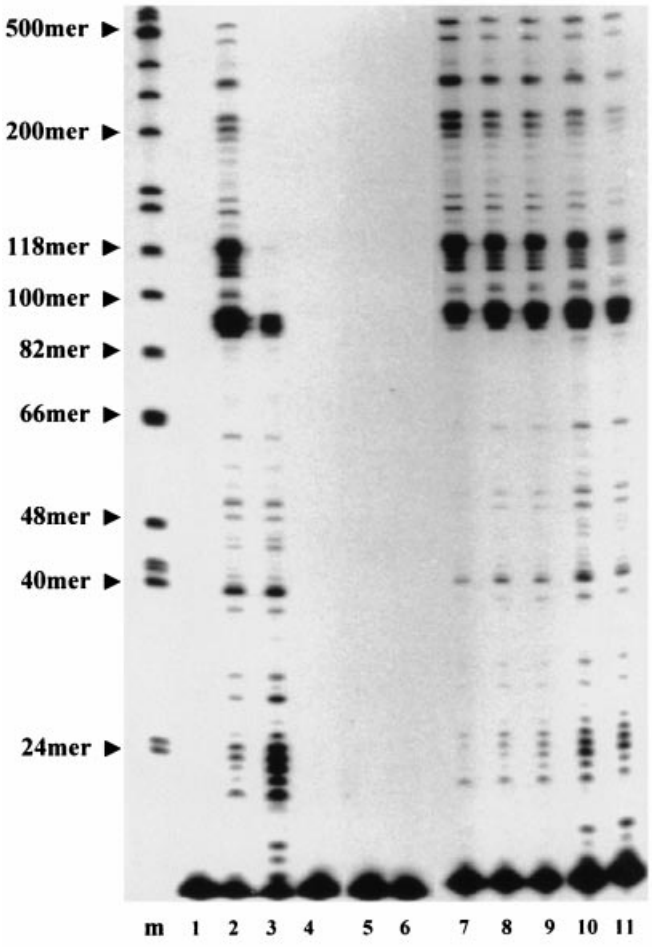

Figure 6 Effects of PAT on DNA primer extension with HIV-1 RT

Primer-extension reactions were carried out as described in the Materials and methods section. The DNA products of the extended ^{32}P-end-labelled 15-mer oligonucleotide annealed to single-stranded ϕX174am3 DNA were analysed by urea/PAGE. Molecular size markers were *Hin*f I-cleaved double-stranded dephosphorylated ϕX174 DNA (Promega). The markers were 5′-end-labelled with [γ-^{32}P]ATP as described in [51]. The arrows indicate the lengths of single-stranded DNA fragments. The lanes of the scanned autoradiogram are as follows: m, marker; lane 1, control with no enzyme; lanes 2–6, pre-incubation of RT with increasing amounts of PAT for 5 min at room temperature prior to initiation of reaction (0, 6.25, 12.5, 25 and 50 μM respectively); lanes 7–11, pre-incubation of enzyme with the template-primer ϕX174 DNA-oligonucleotide for 5 min at room temperature prior to addition of increasing amounts of PAT (0, 6.25, 12.5, 25 and 50 μM PAT respectively). The reactions were initiated by adding all four dNTPs and were carried out for 20 min at 37 °C.

have followed the extension products of a 5′-end-labelled 15-mer primer annealed to ϕX174 single-stranded DNA in the presence of the natural inhibitor. HIV-1 RT was incubated with increasing concentrations of PAT for 5 min at room temperature prior to addition of the labelled primer-template and the four unlabelled dNTPs. The overall primer-extension of the ϕX174am3 DNA is presented in Figure 6. HIV-1 RT is able to extend the primer in the absence of PAT (lane 2) to product DNA up to about 500-mer in length with several strong pausing sites; the strongest ones lead to the accumulation of DNA species of about 90-mer and 120-mer in length. Since we were not able to detect any unique sequences in the template that may contribute to these pausings [23], it may be that secondary structures of single-stranded DNA are responsible for them. The extent of primer elongation in the presence of 6.25 μM PAT is substantially impaired. As can be seen in lane 3, PAT prevents the extension of DNA beyond products of 90-mer in length. There is also an increase in the formation of shorter DNA species (of about 20-mer) relative to the control with no PAT present (lane 2). Moreover, at higher concentrations of PAT, i.e. 12.5, 25 and 50 μM, the capacity of RT to extend the primer is completely blocked (lanes 4–6). Interestingly, preincubation of the RT with template-primer for 5 min at room temperature prior to addition of PAT and dNTPs profoundly affects the results (lanes 7–11). The extension patterns of the products irrespective of the presence or absence of PAT are quite similar, with a modest increase in the pausing frequencies at approximate positions of 20–24-mer and a decrease in the longest products of 100–150-mer in length (lanes 10 and 11). It seems that the pre-formation of RT–DNA complexes prevent the natural inhibitor from exerting its activity on the polymerization function of RT.

DISCUSSION

Polyacetylenetriol was found to be a general potent inhibitor of retroviral RTs and DNA polymerases from both prokaryotic and eukaryotic sources. This compound inhibits efficiently both the RNA- and DNA-directed DNA polymerase associated with the RTs of HIV-1 and HIV-2 (results not shown), MuLV and MMTV. MuLV, a prototype of mammalian C-type viruses, MMTV a prototype of B-type retroviruses and the lentviral HIV-1 and HIV-2 possess RTs that differ in both size and subunit organization and in several of their catalytic properties [20,21]. Nevertheless, retroviral RTs are similar proteins with highly conserved motifs and overall sequence identity of 25% [26,27]. The RNase H function of RT, on the other hand, is hardly affected by this inhibitor. In this respect PAT resembles other natural compounds that were already found to be general inhibitors, namely toxiusol [13], 2-hexaprenylhydroquinone [15] and polycitone A [14]. PAT displays a similar paradigm of inhibition with cellular DNA polymerases from three different families, that is, calf thymus pol α from the α family (B family), pol β from the terminal transferase family and KF from the pol I family [28]. DNA polymerases constitute a 'superfamily' of enzymes, which use a similar machinery for DNA synthesis. All polymerases studied so far have a common overall shape that resembles a right hand with subdomains designated palm, thumb and fingers. These subdomains play similar functional roles by using analogous secondary structural elements. The palm region is the most conserved subdomain among all DNA polymerases with a common folding unit of three β-strands flanked by two α-helices [29]. Motifs A and C, which are situated on β-sheets, are the only universally conserved motifs present in all RNA- and DNA-directed DNA polymerases studied [30,31].

To be able to inhibit all DNA polymerase, we postulate that PAT exerts its inhibitory capacity through a common feature shared by these enzymes. Consequently, the mode and mechanism of inhibition have been further evaluated with HIV-1 RT. Owing to its critical role in AIDS, HIV-1 RT has become one of the most studied enzymes. To date, many crystal structures of HIV-1 RT are available, namely unligated [33,34], in complex with double-stranded primer-template [35] and together with the Fab fragment of monoclonal antibody [36], in complex with NNRTI [37–40] and in complex with a polypurine tract RNA·DNA [41] and a ternary complex of RT–DNA–dNTP [42]. The DNA polymerization by HIV-1 RT is an ordered stepwise mechanism with the template-primer binding first to the enzyme [25]. Subsequently, the appropriate dNTP binds to the template-primer RT complex, whereby a conformational change takes place. Through a nucleophilic attack of two-metal-ion catalytic

mechanism [43], a single nucleotide is incorporated into a growing DNA strand, yielding a phosphodiester bond and release of a pyrophosphate. After dNTP incorporation, the enzyme either dissociates from the template-primer or translocates along the DNA product to the next newly formed 3′ end (i.e. a processive mode of polymerization). Like TIBO [23] and 2-hexaprenylhydroxyquinone [15], PAT does not interfere with the binding of RT to DNA. In this respect, it differs from the other general inhibitors, toxiusol and polycitone A [13,14] and behaves similarly to the other natural inhibitors peyssonols A and B and 3,5,8-trihydroxyquinolone, [23,44]. PAT also does not affect the subsequent binding of dNTP to the RT–DNA complex. An incoming dNTP could still bind to the template-primer–RT complex with the same affinity irrespective of increasing concentrations of PAT. We assume, therefore, that interference with the dNTP binding step does not account for the inhibition detected. It is possible that PAT blocks the catalytic step, i.e. the chemical formation of the phosphodiester bond, and thereby the overall DNA polymerization process. In addition, at low concentrations PAT reduces substantially the extent of DNA elongation (Figure 6, lane 3). Similarly, pre-steady-state kinetic data have shown that NNRTI binding inhibits the catalytic step of HIV-1 RT polymerization [45].

PAT is a non-competitive inhibitor with respect to dNTPs and the template-primers. It binds RT at an allosteric site with high affinity and mainly through hydrophobic interactions. The removal of the three polar hydroxy groups of PAT, as in the triacetylated derivative, does not affect substantially the extent of inhibition. This emphasizes the potential significance of the hydrophobic backbone of PAT to the formation of RT/inhibitor complexes, as opposed to the minor contribution of the electrostatic interactions via hydrogen bonds. It is possible that PAT molecules are inserted between two hydrophobic surfaces which have complementary shapes in the enzyme, thus interfering with these putative contacts formed in its absence. Similarly, hydrophobic interactions have been suggested for the formation of RT/NNRTI complexes. Kinetic and structural studies reveal that various NNRTIs bind HIV-1 RT at an allosteric site, located some 10 Å (1 nm) away from the catalytic site in a hydrophobic pocket, which is created through a conformational switch of key residues to mimic the inactive polymerase site in the p51 RT subunit [40].

Preincubation of RT with the template-primer substantially affects the inhibitory capacity of PAT. It seems that, by binding to template-primer, the RT is largely protected from the effects of PAT; hence DNA synthesis continues even in its presence. This phenomenon is compatible with the reversible mode of inhibition displayed by PAT. It could well be that the RT molecules, complexed to double-stranded DNA, are not accessible to PAT. Alternatively, the transition from a close conformation of unligated RT [33,34] to an open state of RT complexed double-stranded DNA restricts the ability of PAT to complement and mimic the shape of the hydrophobic surfaces within the enzyme. Indeed, the binding of RT to DNA involves a major conformational rotation of the thumb subdomain of p66. The bound DNA is in the B form, except for the nucleotides close to the active site, which have an A-like structure [35,46]. A similar conformation has been reported for DNA bound to other DNA polymerases [30,47].

RT interaction with the template-primer involves primarily amino acid residues of the fingers, palm and thumb subdomains of p66. These residues constitute the primer grip and the template-grip that play a role in positioning the template-primer in the optimal orientation for nucleophilic attack on an incoming dNTP [36,42]. Binding dNTP to RT/DNA induces substantial conformational changes in the HIV-1 RT [45]. Parts of the finger subdomain are closing on the polymerase active site. Similar changes have been reported for the analogous structures in human DNA polymerase β [48] and in *E. coli* pol I [49]. Upon dNTP and metal binding, the primer 3′-terminus is repositioned with respect to the polymerase site, and one carboxylate residue (Asp_{110} in HIV-1 RT, Asp_{610} in *E. coli* pol I and Asp_{192} in polymerase β) comes close to, and chelates, one of the two metal ions, which is bound to the incoming dNTP. In short, binding the inhibitor may displace the primer, which disturbs the position of the 3′-terminus of the primer strand at the polymerase active site, thus changing the stereochemical relationship between the primer terminus, a bound dNTP and the bivalent cations. Alternatively, the binding of PAT may restrict the mobility of the thumb subdomain from an open form to a close complex, leading to suppression of the RT translocation along the nascent DNA towards a new 3′-primer terminus. These models allow the binding of incoming dNTP to the RT–PAT complex without affinity alteration as evident from the steady-state kinetic data.

General inhibition by a single molecule such as PAT can be understood only in terms of a common mechanism of DNA polymerization shared by the commonly folded structures of all DNA polymerases. Unfortunately, the lack of specificity excludes PAT from use as an anti-HIV drug. Nevertheless, the fact that other polyacetylenes, petrosynol and petrosolic acid also inhibit effectively HIV-1 RT [50] calls for further work. It is possible that structural modification of the side chains of the lead polyacetylenic molecule may produce new potent and selective anti-AIDS drugs.

We thank the High Throughput Screening (HTS) group, Dr Ram Alststock, Ruth Gottlieb and Professor Yossi Loya for the involvement in the screening procedure of the natural products. We also thank Dr Z. Hostomsky and Dr M. Fry for generously providing pol β and calf thymus pol α, respectively. This work was supported by a grant from the Ministry of Science, Culture and Sports of the State of Israel.

REFERENCES

1 Le Grice, S. F. J. (1993) HIV reverse transcriptase. In Reverse Transcriptase (Skalka, A. M. and Goff, S. H., eds.), pp. 163–191, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
2 Jonckheere, H., Anné, J. and De Clercq, E. (2000) The HIV-1 reverse transcription (RT) process as target for RT inhibitors. Med. Res. Rev. **20**, 129–154
3 Mitsuya, H. and Broder, S. (1986) Inhibition of the *in vitro* infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2′-, 3′-dideoxynucleosides. Proc. Natl. Acad. Sci. U.S.A. **83**, 1911–1915
4 De Clercq, E. (1997) In search of a selective antiviral chemotherapy. Clin. Microbiol. Rev. **10**, 647–693
5 Richmann, D. D., Fisch, M. A., Grieco, M. H., Gotlieb, M. S., Volberding, P. A., Laskin, O. L., Leedom, J. M., Grooman, J. E., Mildvan, D., Hirsch, M. S. et al. (1987) The toxicity of azidothimidine (AZT) in the treatment of patients with AIDS and AIDS-related complex; a double blind placebo-controlled trial. N. Engl. J. Med. **317**, 192–197
6 Richman, D. D. (1994) Resistance, drug failure and disease progression. AIDS Res. Human Retrovir. **10**, 901–905
7 Larder, B. A. and Kemp, S. D. (1989) Multiple mutations in HIV-1 RT confer high-level resistance to Ziodovudine (AZT). Science **246**, 1155–1158
8 Larder, B. A., Darby, G. and Richman, D. D. (1989) HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science **243**, 1731–1734
9 De Clercq, E. (1996) Non-nucleoside RT inhibitors (NNRTIs) for the treatment of HIV-1 infections: strategies to overcome drug resistance development. Med. Res. Rev. **16**, 125–157
10 Arnold, E., Das, K., Ding, J., Yadav, P. N. S., Hsiou, Y., Boyer, P. L. and Hughes, S. H. (1996) Targeting HIV RT for anti-AIDS drug design: structural and biological considerations for chemotherapeutic strategies. Drug Des. Discov. **13**, 29–47
11 Yuasa, S., Sadakata, Y., Takashima, H., Sekiya, K., Inouya, N., Ubasawa, M. and Baba, M. (1993) Selective and synergistic inhibition of HIV-1 RT by a non-nucleoside inhibitor MKC-442. Mol. Pharmacol. **44**, 895–900

12 De Clercq, E. (2000) Current lead natural products for the chemotherapy of HIV infection. Med. Res. Rev. **20**, 323–349
13 Loya, S., Bakhanashvili, M., Kashman, Y. and Hizi, A. (1995) Mechanism of inhibition of HIV reverse transcriptase by toxiusol, a novel general inhibitor of retroviral and cellular DNA polymerases. Biochemistry **34**, 2260–2266
14 Loya, S., Rudi, A., Kashman, Y. and Hizi, A. (1999) Polycitone A. A novel and potent general inhibitor of retroviral reverse transcriptases and cellular DNA polymerases. Biochem. J. **344**, 85–92
15 Loya, S., Rudi, A., Kashman, Y. and Hizi, A. (1997) Mode of inhibition of HIV reverse transcriptase by 2-hexaprenylhydroquinone, a novel general inhibitor of RNA- and DNA-directed polymerases. Biochem. J. **324**, 721–727
16 Cimino, G., De Giulio, A., De Rosa, S. and Di Marzo, V. (1989) High molecular weight polyacetylene from *Petrosia ficiformis*: further structural analysis and biological activity. Tetrahedron Lett. **30**, 3563–3566
17 Shaharabany, M. and Hizi, A. (1991) The DNA-dependent and RNA-dependent DNA polymerase activities of the reverse transcriptases of HIV types 1 and 2. AIDS Res. Hum. Retrovir. **7**, 883–888
18 Hizi, A., McGill, C. and Hughes, S. H. (1988) Expression of soluble, enzymatically active, HIV reverse-transcriptase in *Escherichia coli* and analysis of mutants. Proc. Natl. Acad. Sci. U.S.A. **85**, 1212–1218
19 Clark, P. K., Ferris, A. L., Miller, D. A., Hizi, A., Kim, K. W., Dringer-Boyer, S. M., Mellini, M. L., Clark, Jr., A. D., Arnold, G. F., Lebherz, W. B. et al. (1990) HIV-1 reverse transcriptase purified from a recombinant strain of *Escherichia coli*. AIDS Res. Hum. Retrovir. **6**, 753–764
20 Taube, R., Loya, S., Avidan, O., Perach, M. and Hizi, A. (1998) Reverse transcriptase of mouse mammary tumor virus; expression in bacteria, purification and biochemical characterization. Biochem. J. **329**, 578–579
21 Hizi, A., Tal, R., Shaharabany, M. and Loya, S. (1991) Catalytic properties of the reverse transcriptase of HIV type 1 and type 2. J. Biol. Chem. **266**, 6230–6239
22 Avidan, O. and Hizi, A. (1998) The processivity of DNA synthesis exhibited by drug-resistant variants of HIV-1 reverse transcriptase. Nucleic Acids Res. **26**, 1713–1717
23 Bakhanashvili, M. and Hizi, A. (1994) Interaction of the reverse transcriptase of HIV type 1 with DNA. Biochemistry **33**, 12222–12228
24 Debyser, Z., Pauwels, R., Andries, K., Desmyter, J., Kukla, M., Janssen, P. A. J. and De Clercq, E. V. (1991) An antiviral target on reverse transcriptase of HIV-1 revealed by tetrahydroimidazo-[4,5,1-jk][1,4]benzoadiazepine-2(1*H*)one and -thione derivatives. Proc. Natl. Acad. Sci. U.S.A. **88**, 1451–1455
25 Majumadar, C., Abbots, J., Broder, S. and Wilson, S. H. (1988) Studies on the mechanism of HIV reverse transcriptase. Steady-state kinetics, processivity and polynucleotide inhibition. J. Biol. Chem. **263**, 15657–15665
26 Johnson, M. S., McClure, M. A., Feng, D. F., Gray, J. and Doolittle, R. F. (1986) Computer analysis of retroviral pol genes: assignment of enzymatic functions to specific sequences and homologies with nonviral enzymes. Proc. Natl. Acad. Sci. U.S.A. **83**, 7648–7652
27 Georgiadis, M. M., Jenssen, S. M., Ogata, C. M., Telesnitsky, A., Goff, S. P. and Hendrickson, W. A. (1995) Mechanistic implications from the structure of a catalytic fragment of Moloney murine leukemia virus reverse transcriptase. Structure **3**, 879–892
28 Steitz, T. A. (1999) DNA polymerases: structural diversity and common mechanisms. J. Biol. Chem. **274**, 17395–17398
29 Brautigam, C. A. and Steitz, T. A. (1998) Structural and functional insights provided by crystal structures of DNA polymerases and their substrate complexes. Curr. Opin. Struct. Biol. **8**, 54–63
30 Sawaya, M. R., Pelletier, H., Kumar, A., Wilson, S. H. and Kraut, J. (1994) Crystal structure of rat DNA polymerase β: evidence for a common polymerase mechanism. Science **264**, 1930–1935
31 Sousa, R. (1996) Structural and mechanistic relationships between nucleic acid polymerases. Trends Biochem. Sci. **21**, 186–190
32 Esnouf, R., Ren, J., Ross, R., Jones, Y., Stammers, D. and Stuart, D. (1995) Mechanism of inhibition of HIV-1 reverse transcriptase by non-nucleoside inhibitors. Nat. Struct. Biol. **2**, 303–308
33 Rodgers, D. W., Gamblin, S. J., Harris, B. A., Ray, S., Culp, J. S., Hellmig, B., Woolf, D. J., Debuck, C. and Harrison, S. C. (1995) The structure of unliganded reverse transcriptase from the HIV type 1. Proc. Natl. Acad. Sci. U.S.A. **92**, 1222–1226
34 Hsiou, Y., Ding, J., Das, K., Clark, A. D. Jr., Hughes, S. H. and Arnold, E. (1996) Structure of unliganded HIV-1 reverse transcriptase at 2.7 Å resolution: implications of conformational changes for polymerization and inhibition mechanisms. Structure **4**, 853–860
35 Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, A. D. Jr., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., Hizi, A., Hughes, S. H. and Arnold, E. (1993) Crystal structure of HIV type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 Å resolution shows bent DNA. Proc. Natl. Acad. Sci. U.S.A. **90**, 6320–6324
36 Ding, J., Das, K., Hsiou, Y., Sarafianos, S. G., Clark, Jr., A. D., Jacobo-Molina, A., Tantillo, C., Hughes, S. H. and Arnold, E. (1998) Structure and functional implications of the polymerase active site region in a complex of HIV-1 RT with a double-stranded DNA template-primer and an antibody Fab fragment at 2.8 Å resolution. J. Mol. Biol. **284**, 1095–1111
37 Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A. and Steitz, T. A. (1992) Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science **256**, 1783–1790
38 Ren, J., Esnouf, R., Garman, E., Somers, D., Ross, C., Kirby, I., Keeling, J., Darby, G., Jones, Y., Stuart, D. and Stammers, D. (1995) High resolution structures of HIV-1 RT from four RT-inhibitors. Nat. Struct. Biol. **2**, 293–302
39 Ding, J., Das, K., Moereels, H., Koymans, L., Andries, K., Janssen, P. A. J., Hughes, S. H. and Arnold, E. (1995) Structure of HIV-1 RT/TIBO R 86183 complex reveals similarity in the binding of diverse non nucleoside inhibitors. Nat. Struct. Biol. **2**, 407–415
40 Hopkins, A. L., Ren., J., Esnouf, R. M., Wilcox, B. E., Jones, E. Y., Ross, C., Miyasaka, T., Walker, R. T., Tamaka, H., Stammers, D. K. and Stuart, D. I. (1996) Complexes of HIV-1 reverse transcriptase with inhibitors of the HEPT series reveal conformational changes relevant to the design of potent non-nucleoside inhibitors. J. Med. Chem. **39**, 1589–1600
41 Sarafianos, S. G., Das, K., Tantillo, C., Clark, Jr., A. D., Ding, J., Whitcomb, J. M., Boyer, P. L., Hughes, S. H. and Arnold, E. (2001) Crystal structure of HIV-1 reverse transcriptase in complex with a polypurine tract RNA:DNA. EMBO J. **20**, 1449–1461
42 Haung, H., Chopra, R., Verdine, G. L. and Harrison, S. (1998) Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. Science **282**, 1669–1675
43 Patel, P. H., Jacobo-Molina, A., Ding, J., Tantillo, C., Clark, Jr., A. D., Raag, R., Nanni, R. G., Hughes, S. H. and Arnold, E. (1995) Insights into DNA polymerization mechanisms from structure and function analysis of HIV-1 reverse transcriptase. Biochemistry **34**, 5351–5363
44 Loya, S., Bakhanashvili, M., Kashman, Y. and Hizi, A. (1995) Peyssonols A and B, two novel inhibitors of the reverse transcriptases of HIV types 1 and 2. Arch. Biochem. Biophys. **316**, 789–796
45 Rittinger, K., Divita, G. and Goody, R. S. (1995) HIV reverse transcriptase substrate-induced conformational changes and the mechanism of inhibition by nonnucleoside inhibitors. Proc. Natl. Acad. Sci. U.S.A. **92**, 8046–8049
46 Sarafianos, S. G., Das, K., Ding, J., Boyer, P. L., Hughes, S. H. and Arnold, E. (1999) Touching the heart of HIV-1 drug resistance; the figures close down on the dNTP at the polymerase active site. Chem. Biol. **6**, R137–R146
47 Li, Y., Korolev, S. and Waksman, G. (1998) Crystal structures of open and closed forms of binary and ternary complexes of the large fragment of *Thermus aquaticus* DNA polymerase I: structural basis for nucleotide incorporation. EMBO J. **17**, 7514–7525
48 Sawaya, M. R., Prasad, R., Wilson, S., Krant, J. and Pelletier, H. (1997) Crystal structures of human DNA polymerase β complexed with gapped and nicked DNA: evidence for an induced fit mechanism. Biochemistry **36**, 11205–11215
49 Beese, L. S., Derbyshire, V. and Steitz, T. A. (1993) Structure of DNA polymerase I Klenow fragment bound to duplex DNA. Science **260**, 352–355
50 Issac, S., Kashman, Y., Loya, S. and Hizi, A. (1993) Petrosynol and petrosolic acid, two novel natural inhibitors of the reverse transcriptase of HIV from *Petrosia sp.* Tetrahedron **49**, 10435–10438
51 Bakhanashvili, M. and Hizi, A. (1993) Fidelity of DNA synthesis exhibited *in vitro* by the reverse transcriptase of the lentivirus equine infectious anemia virus. Biochemistry **32**, 7559–7567

Received 18 July 2001/23 October 2001; accepted 20 December 2001