Mechanism of Inhibition of HIV Reverse Transcriptase by Toxiusol, a Novel General Inhibitor of Retroviral and Cellular DNA Polymerases†

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ABSTRACT: Toxiusol, a natural product isolated from the Red Sea sponge *Toxiclona toxius*, has been shown to be a potent inhibitor of various viral reverse transcriptases (RT) [i.e., of human immunodeficiency virus (HIV-1), equine infectious anemia virus, and murine leukemia virus] and cellular DNA polymerases (i.e., of DNA polymerases α and β and *Escherichia coli* DNA polymerase I). A thorough investigation of the mode of inhibition was conducted with HIV-1 RT-associated DNA polymerase activity. The inhibition is unaffected by the nature of template-primer used. The inhibitory active site of toxiusol is attributable to the polar moieties at the benzene ring. The presence of either sulfate groups in the natural lead compound or hydroxyl groups in the corresponding hydroquinone is critical, because both compounds are equally effective at low micromolar concentrations. Conversely, the presence of acetyl groups in the same position in the derivative toxiusol diacetate lowers significantly or abolishes the inhibitory activity. Toxiusol binds the HIV-1 RT irreversibly and in a noncompetitive way with high affinity ($K_i = 1.2$ μM), probably through polar groups. The replacement with acetyl moieties in the analog toxiusol diacetate hampers the binding of the inhibitor to the enzyme (K_i increases to about 26 μM). Still, the compound binds irreversibly, probably through its hydrophobic structure skeleton. Toxiusol diacetate loses its ability to inhibit the first step in the DNA polymerization process (that is, the formation of the DNA−enzyme complex as measured by a gel retardation assay), which contributes to its poor inhibitory capacity. On the other hand, toxiusol has been demonstrated to effectively block the binding of HIV-1 RT to its template-primer. This general mechanism of inhibition is likely to be typical of a universal inhibitor of DNA polymerases such as toxiusol.

Reverse transcriptase (RT)[1] plays an essential role in the early steps of the replication cycle of retroviruses. The enzyme is responsible for the conversion of the viral genomic RNA into proviral DNA (De Clercq, 1992). The viral encoded RT is considered an ideal target for the specific chemotherapeutic treatment of retroviral infections, especially those of human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS). In fact, the clinical potential of compounds that interfere with the function of HIV-1 RT enzyme has been demonstrated by the use of nucleoside analogs such as 3′-azido-2′,3′-dideoxythymidine (AZT) and other dideoxynucleoside analogs. These compounds need to be phosphorylated intracellulary to their triphosphate forms before they can compete effectively with the natural nucleotide substrates and act as chain terminators in the synthesis of DNA (Mitsuya & Broder, 1986; Reardon & Miller, 1990). Yet, their structural resemblance to authentic substrates leads to recognition by DNA polymerases other than RTs, which eventually may be the cause for the observed toxic side effects in treated patients (Richman et al., 1987; Fischl et al., 1989). On the other hand, the recently described nonnucleoside inhibitors of HIV-1 RT (NNIRT), such as TIBO and HEPT, all of which fall into chemically distinct classes of compounds, are highly selective against HIV-1 RT. These compounds exhibit no inhibitory activity against the RTs of HIV-2, avian myeloblastosis virus, murine leukemia virus (MuLV), simian immunodeficiency virus, and feline leukemia virus, nor is there activity against human DNA polymerases α, β, γ, and δ (Debyser et al., 1992; De Clercq, 1993).

In contrast to the HIV-1 RT-specific NNIRT, there are many other RT-targeted nonnucleoside inhibitors. Our own screening program for HIV-associated RT inhibitors from marine organisms have identified structurally diverse compounds, all novel nonnucleoside inhibitors, such as avarol analogs, illimaquinone, halocynthiaxanthine, 3,5,8-hydroxy-4-quinolone (Loya & Hizi, 1990; Loya et al., 1990, 1992, 1994). Recently, we have isolated from the Red Sea sponge *Toxiclona toxius* a novel sulfated hexaprenoid hydroquinone, toxiusol. This substance preferentially inhibits the HIV-1 RT-associated DNA polymerase activity (Loya et al., 1993). The inhibitory effects of toxiusol on other DNA polymerases is unknown. Furthermore, the mechanism underlying this inhibition has not been clarified. Thus, we have examined the effect of the lead natural compound, toxiusol, and two

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[1] Abbreviations: RT, reverse transcriptase; HIV-1 and HIV-2, human immunodeficiency virus type 1 and type 2, respectively; AIDS, acquired immunodeficiency syndrome; AZT, 3′-azido-2′,3′-dideoxythymidine; NNIRT, nonnucleoside inhibitors of HIV-1 RT; TIBO, tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-one and -thione; HEPT, 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine; MuLV, murine leukemia virus; EIAV, equine infectious anemia virus; KF, Klenow fragment of *E. coli* DNA polymerase I; TDA, toxiusol diacetate; PAGE, polyacrylamide gel electrophoresis.

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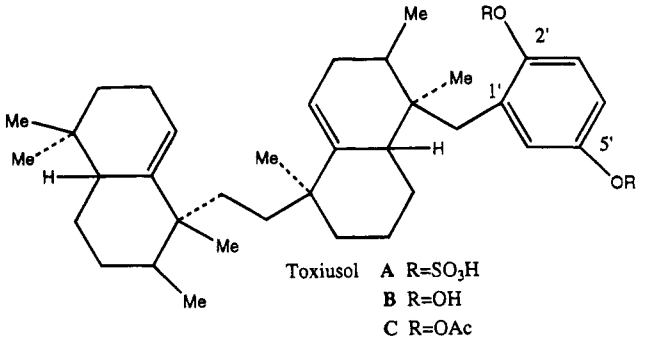

FIGURE 1: Structural formulas of toxiusol (A) and its analogs, the hydroquinone (B), and toxiusol diacetate (C).

analogs on HIV-1, equine infectious anemia virus (EIAV), and MuLV-encoded RTs and on cellular DNA polymerases, eukaryotic DNA polymerase α and β, and the prokaryotic Klenow fragment of *E. coli* DNA polymerase I (KF).

In the current study, we show that toxiusol effectively inhibits all DNA polymerases tested. The mode of inhibition of HIV-1 RT-associated DNA polymerase activity is studied in detail. Toxiusol is a noncompetitive inhibitor and binds the enzyme irreversibly. We speculate that toxiusol blocks the first essential step of DNA polymerization, i.e., the formation of the HIV-1 RT−DNA complex. The structure−activity relationship studies suggest that the polar sulfate moieties on the benzene ring of toxiusol play an essential role in obstructing the formation of the RT−DNA complex, and hence interfering with the overall DNA polymerization process.

MATERIALS AND METHODS

Chemicals. The natural compound toxiusol, designated as compound A (Figure 1), was isolated from the Red Sea sponge *T. toxius* and was identified as described previously (Issacs et al., 1993). Compound B (Figure 1) is the corresponding *p*-hydroquinone derivative of the natural sulfated compound A. The hydrolysis of the two sulfates at positions 2′ and 5′ in the benzene ring of toxiusol was performed with 1% (v/v) trifluoroacetic acid, and the structure was identified as described (Issacs et al., 1993). The analog of both compounds, toxiusol diacetate (TDA) (designated as compound C; Figure 1), was prepared by acetylation of compound B with a mixture of acetic anhydride/pyridine (1:1). Synthetic template-primers poly(rA)$_n$•oligo(dT)$_{12-18}$ and poly(dA)$_n$•oligo(dT)$_n$ were the products of Pharmacia. Activated gapped DNA was prepared by a limited digestion of herring sperm DNA with bovine pancreatic DNase-I as described elsewhere (Spanos et al., 1981).

Enzymes. The RTs used were all recombinant enzymes expressed in *Escherichia coli* and purified from bacterial extracts to homogeneity as described previously (Clark et al., 1990). The HIV-1 RT expression plasmid was derived from the BH-10 proviral isolate (Hizi et al., 1988), EIAV expression plasmid was constructed as described (Shaharabany et al., 1993), and MuLV expression plasmid was derived from the *K* proviral isolate (Hizi & Hughes, 1988). The purified RTs of HIV-1 and EIAV were p66/p51 heterodimers, and the MuLV was the p70 monomer. Prolonged storage of enzymes was performed in 50% glycerol (v/v), 2 mM dithiothreitol, 25 mM Tris-HCl, pH 8.0, at −80 °C. DNA polymerase α was purified from calf thymus by immunoaffinity column chromatography according to Perrino and Loeb (1989) and was a generous gift from Dr. M. Fry. Recombinant human DNA polymerase-β was prepared as described (Abbots et al., 1988) and was a generous gift from Dr. S. H. Wilson.

Enzymatic Assays. Enzymatic assay of HIV-1, EIAV, and MuLV RTs were carried out as described in detail previously (Hizi et al., 1991). DNA polymerase activity was assayed by following the poly(rA)$_n$•oligo(dT)$_{12-18}$-directed incorporation of [^{3}H]dTTP into DNA. In all inhibition experiments, the enzymes were preincubated for 5 min at 30 °C in the absence or in the presence of inhibitor at various concentrations. The enzymatic reactions were initiated by adding the appropriate substrate followed by an incubation for 30 min at 37 °C (or for 10 min in the case of kinetic studies). The residual enzymatic activities were calculated relative to the initial linear reaction rates observed when no drug was added. The inhibitor concentrations leading to 50% inhibition of the enzymatic activities (IC_{50} values) were calculated from the inhibition curves as a function of inhibitor concentrations. The kinetic constants were calculated by plotting reciprocal velocity of polymerization against reciprocal substrate concentration. The kinetic constants from the Lineweaver−Burk and Dixon plots were determined by computer-generated linear regression analyses. Enzymatic activities were defined as follows: 1 unit of DNA polymerase activity is the amount of enzyme that catalyzes the incorporation of 1 pmol of dNTP into DNA product after 30 min at 37 °C under the standard assay conditions. For calf-thymus DNA polymerase α, the assay was carried out as described with activated DNA as the template-primer (45 μg/mL) (Abbots et al., 1988). The assay of human recombinant DNA polymerase-β was carried out in a final volume of 0.1 mL containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM $MnCl_2$, 3 mM dithiothreitol, 5 μg/mL poly(dA)$_n$•oligo(dT)$_{12-18}$, and [^{3}H]-dTTP at 5 μM (specific activity 1300−1500 cpm/pmol).

Binding Assays. The binding of HIV-1 RT to a double-stranded oligonucleotide DNA was performed by PAGE-band mobility shift assay (Bakhanashvili & Hizi, 1994). Complex formation between the ^{32}P end-labeled oligonucleotide DNA and highly purified HIV-1 RT was detected by the electrophoretic retardation of DNA as a result of its association with the enzyme. The binding reaction assays were conducted in a final volume 12.5 μL containing 10 mM HEPES−KOH, pH 8.0, 50 mM ammonium sulfate, 0.25 mM dithiothreitol, 100 μg/mL bovine serum albumin, 10 mM KCl, 1.2 pmol of HIV-1 RT, and 0.12 pmol of the labeled oligonucleotide template-primer. The reaction mixtures were incubated for 10 min at 32 °C in the absence or in the presence of each inhibitor at various concentrations. The protein−oligonucleotide mixtures were electrophoresed at 4 °C under 10 V/cm for 2−3 h through a 5% polyacrylamide gel in 1 × TBE. After electrophoresis, gels were dried and subjected to autoradiography. Quantification of the RT−DNA complex formation was calculated employing phosphoimaging scanning procedures.

RESULTS

Effect of Toxiusol and Its Hydroquinone Derivative on Various Reverse Transcriptases. In our continuous search for marine metabolites with potent anti-HIV RT activity, we have isolated from a Red Sea sponge *T. toxius* a new sulfated hexaprenoid hydroquinone, toxiusol (Figure 1). The natural

Table 1: Effect of Toxiusol (A) and Its Hydroquinone Derivative (B) on DNA Polymerase Activity of Various Retroviral RTs[a]

	reverse transcriptases					
	HIV-1		EIAV		MuLV	
inhibitor	IC_{50}	IC_{95}	IC_{50}	IC_{95}	IC_{50}	IC_{95}
toxiusol (A)	1.5 ± 0.2	4.6 ± 0.1	1.80 ± 0.3	4.55 ± 0.7	0.76 ± 0.0	3.98 ± 0.87
hydroquinone derivative (B)	2.8 ± 0.09	11.1 ± 2.5	3.67 ± 0.07	6.37 ± 0.83	1.04 ± 0.14	3.29 ± 0.17

[a] The IC_{50} and IC_{95} values (the inhibitor concentrations which lead to the inhibition of the initial enzymatic activity by 50% or 95%, respectively) are expressed in μM. All data represent mean values (± range) of at least three separate experiments.

product has been reported to inhibit preferentially the DNA polymerase functions of HIV-1 RT (Loya et al., 1993). We have also reported that a derivative of the natural sulfated compound toxiusol, the corresponding *p*-hydroquinone (designated as compound B) (Figure 1), behaves similarly. Both compounds are potent inhibitors of the RNA-directed DNA synthesis of HIV-1 RT with IC_{50} values achieved at about 1.5 and 2.8 μM toxiusol and its hydroquinone derivative (B), respectively (Table 1). To assess whether there is a general inhibitory effect of toxiusol on retroviral RTs, we have tested the effect of toxiusol on RTs of the closely related lentivirus—EIAV—and of the more distant retrovirus, MuLV. As can be seen in Table 1, toxiusol strongly inhibits the poly(rA)$_n$•oligo(dT)$_{12-18}$-directed DNA synthesis of both enzymes (IC_{50} values of about 2.3 and 0.76 μM for EIAV and MuLV RTs, respectively). As a matter of fact, there is no substantial difference in the sensitivity of the DNA polymerase activity of HIV-1, EIAV, and MuLV RTs to toxiusol. Nor there is a pronounced difference in the response of the three retroviral RTs to the derivative of toxiusol, compound B (Table 1). Furthermore, as shown in Table 1, the IC_{50} values calculated for the natural inhibitor and its derivative with any given enzyme are almost inseparable. In general, it seems that the hydrolysis of the two sulfates of toxiusol to the corresponding hydroquinone does not affect its inhibitory potential.

Effect of Toxiusol and Its Analog Toxiusol Diacetate on Various Retroviral RTs. In an attempt to find the active site of the native toxiusol (A) and its hydrolyzed hydroquinone compound (B), we have prepared an analog, TDA, designated as compound C (Figure 1). The potential reactive groups at positions 2′ and 5′ in the benzene ring have been substituted with acetyl groups. The analog TDA was tested for its ability to inhibit the DNA polymerase function associated with three retroviral RTs (i.e., of HIV-1, EIAV, and MuLV). As can be seen in Figure 2, TDA is substantially less effective in inhibiting the DNA polymerase activity. It is apparent from dose–response inhibition curves of HIV-1 RT-associated DNA polymerase activity with both toxiusol and TDA (Figure 2A) that the analog is about 40-fold less effective than its natural parent compound (IC_{50} values of about 1.4 and 41 μM for toxiusol and TDA, respectively). The same pattern of reduced inhibition by TDA relative to toxiusol has been detected with the DNA polymerase of either EIAV or MuLV RTs. Thus, the analog is about 14-fold less potent in inhibiting the EIAV RT-associated DNA polymerase activity than is toxiusol (Figure 2B; IC_{50} values of about 1.8 μM with toxiusol versus 25 μM with TDA). A more profound difference of about 50-fold in the inhibition of MuLV-associated DNA polymerase functions can be detected between the natural inhibitor and its analog (Figure 2C; IC_{50} values of about 0.75 μM for toxiusol and 35 μM for TDA). Taken together, the importance of the sulfate or the hydroxyl groups at position 2′ and 5′ in the benzene ring for the

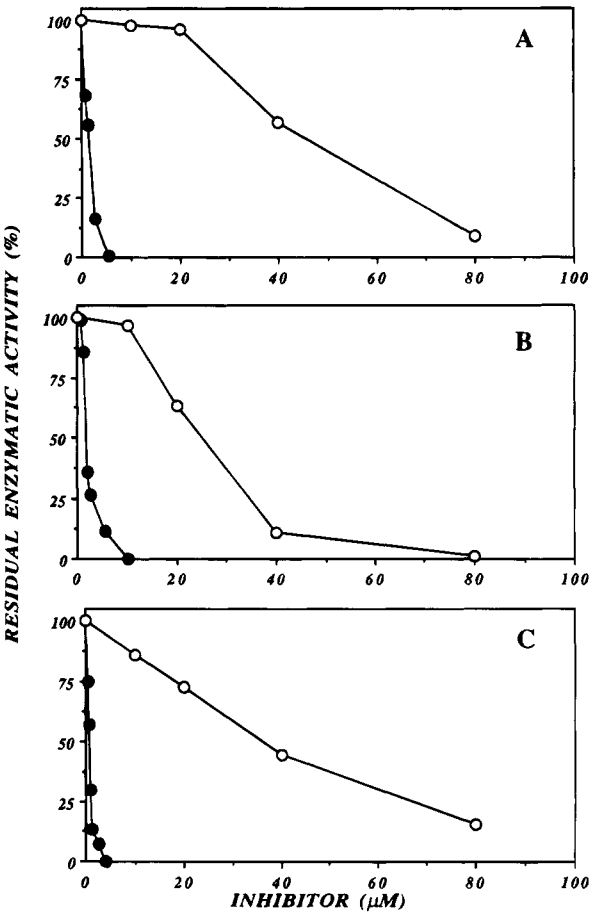

FIGURE 2: Effect of toxiusol and its analog TDA on various retroviral RTs. The dose–response experiments of HIV-1 RT (A), EIAV RT (B), and MuLV RT (C) were carried out as described in Materials and Methods. The DNA polymerase activity was assayed by following the poly(rA)$_n$•oligo(dT)$_{12-18}$-directed incorporation of [^{3}H]dTTP into DNA in the presence of various concentration of either toxiusol (●) or TDA (○).

inhibitory activity of the DNA polymerase function is established by the relatively ineffective acetylated analog.

Effect of Toxiusol and Its Analog TDA on Other Eukaryotic DNA Polymerases. To assess whether toxiusol possesses a general inhibitory activity against DNA polymerases, we have further analyzed its effects on cellular DNA polymerases. We have measured the effects of toxiusol and its analog TDA on the prokaryotic KF and on two other eukaryotic DNA polymerases, α and β. The resulting inhibition curves are shown in Figure 3. DNA polymerases β and α are very sensitive to toxiusol. Fifty percent inhibition of calf-thymus DNA polymerase-α and recombinant human DNA polymerase-β are achieved approximately at 1.2 and 0.9 μM, respectively (Figure 3A,B). As seen in Figure 3C, toxiusol also exhibits comparable inhibitory activity toward KF (IC_{50} equals about 0.93 μM). Interest-

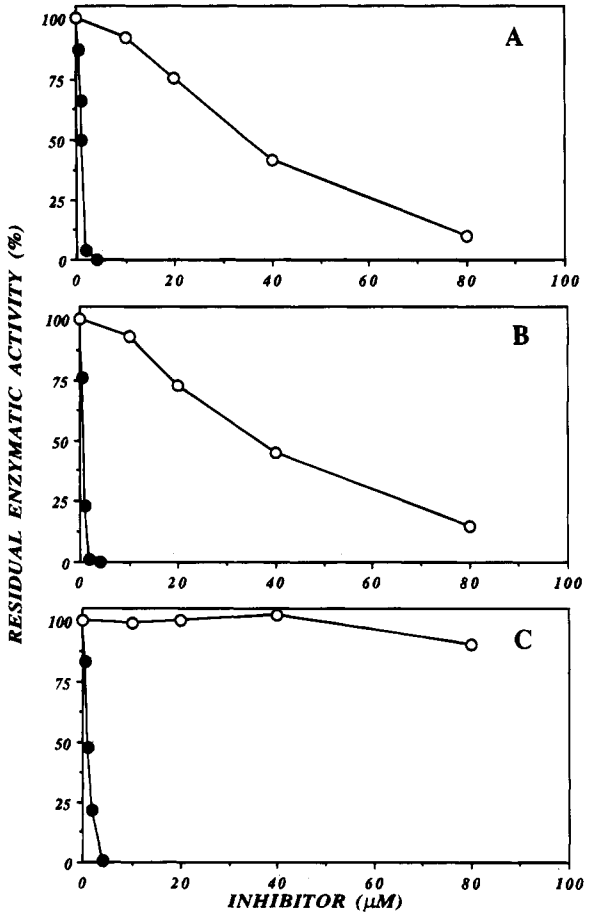

FIGURE 3: Effect of toxiusol and its analog TDA on cellular DNA polymerases. The dose–response inhibition experiments of calf-thymus DNA polymerase-α (A), human DNA polymerase-β (B), and KF (C) were carried out as described under Materials and Methods. The enzymatic activity of KF and DNA polymerase-α were followed with activated DNA as template-primer and DNA polymerase-β with poly(dA)$_n$•oligo(dT)$_{12-18}$ as template-primer in the presence of either toxiusol (●) or TDA (○).

ingly, as in the case of the various RTs studied, the analog TDA exhibits significantly lower inhibitory activity compared to that of the natural compound. The IC_{50} values for both DNA polymerase-α and DNA polymerase-β are about 36 μM, i.e., differences of about 30- and 40-fold in the IC_{50} values between toxiusol and TDA for polymerase-α and polymerase-β, respectively. The insensitivity of KF to the analog is even more profound. KF retains most of its DNA-polymerizing activity even in the presence of 80 μM TDA (Figure 3C). Since the pattern of the inhibition of all DNA polymerases tested seems to be similar, we have elected HIV-1 RT for a thorough study of the mechanism of inhibition by toxiusol and its analog TDA.

Mode of Inhibition of the DNA Polymerase Activity of HIV-1 RT by Toxiusol and TDA. Steady-state kinetics were performed by increasing the concentrations of either dTTP or the template-primer, poly(rA)$_n$•oligo(dT)$_{12-18}$, in the presence of increasing inhibitor concentrations. The linear initial rates of [^{3}H]dTTP incorporation into DNA product were calculated, and the kinetic constants K_m and V_{max} were determined from the double-reciprocal plot of the initial velocity versus substrate concentrations (Figure 4). It is clear from Figure 4A that toxiusol is a noncompetitive inhibitor with respect to dTTP. The apparent K_m is 4.4 ± 0.3 μM, irrespective of the presence of toxiusol. Conversely, the V_{max} values are suppressed as a function of increasing inhibitor

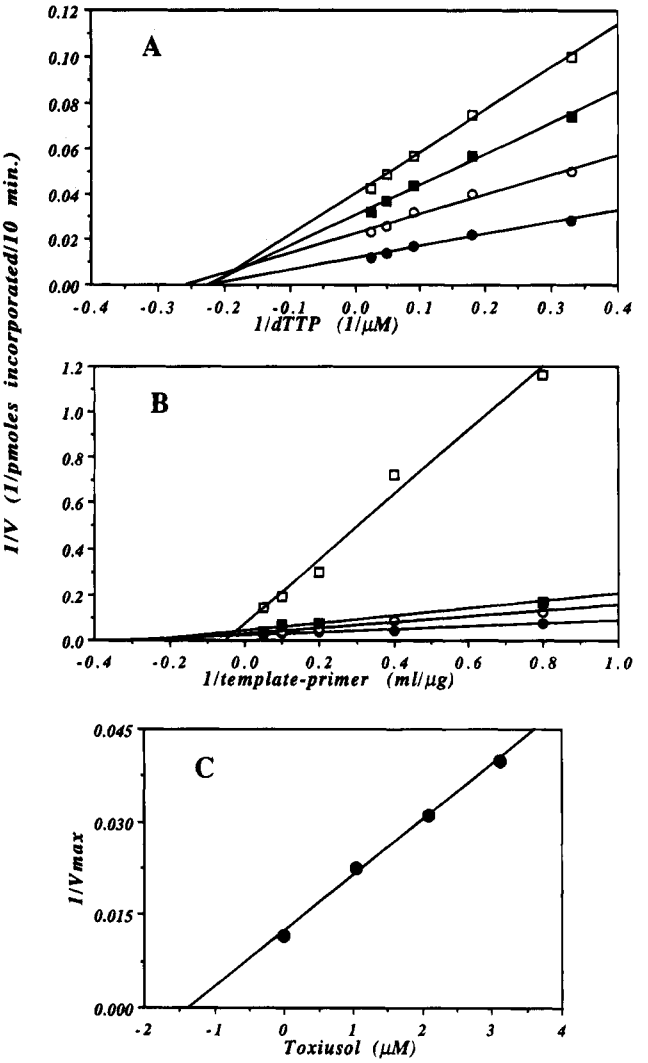

FIGURE 4: Kinetic analysis of the inhibition of HIV-1 RT-associated DNA polymerase activity by toxiusol. The double-reciprocal plots of increasing concentrations of either dTTP (panel A) or poly(rA)$_n$•oligo(dT)$_{12-18}$ (panel B) as a function of the initial velocity of [^{3}H]dTTP incorporation by HIV-1 RT. (Panel A) The enzymatic reaction in the absence (●) or in the presence of 1.04 (○), 2.08 (■), or 3.12 μM (□) toxiusol. (Panel B) The enzymatic reaction in the absence (●) or the presence of 0.69 (○), 1.04 (■), or 1.38 μM (□) toxiusol. (Panel C) Secondary replot (Dixon plot) of the reciprocal maximal velocity (calculated from panel A) versus various toxiusol concentrations. The kinetic constants K_m and K_i were computer-generated by linear regression analysis. The regression coefficient values (r) were as high as 0.99 for each curve, indicating a high degree of linear relationship between the reciprocal velocity of the enzymatic reaction and the reciprocal concentration of the substrate.

concentrations. The inhibition constant (K_i) for toxiusol is derived from replots of intercepts from Figure 4A. The plot is linear ($r = 0.99$) and yields a K_i value of 1.2 μM. Toxiusol binds the enzyme with greater affinity than dTTP ($K_i/K_m < 1$). When the template-primer is the variable substrate, the mode of inhibition varies from noncompetitive at lower toxiusol concentrations to mixed linear at higher concentrations (Figure 4B). It seems that the affinity of the template-primer for the enzyme decreases at higher concentrations of the inhibitor. Thus, the apparent K_m value increases by 5-fold (from 4 to 20 μg/mL).

We have shown that TDA, the analog of toxiusol, lost significantly its inhibitory activity. It was of interest to explore its mode of inhibition at concentrations in the range of its IC_{50} value. Will the mode of inhibition be changed as

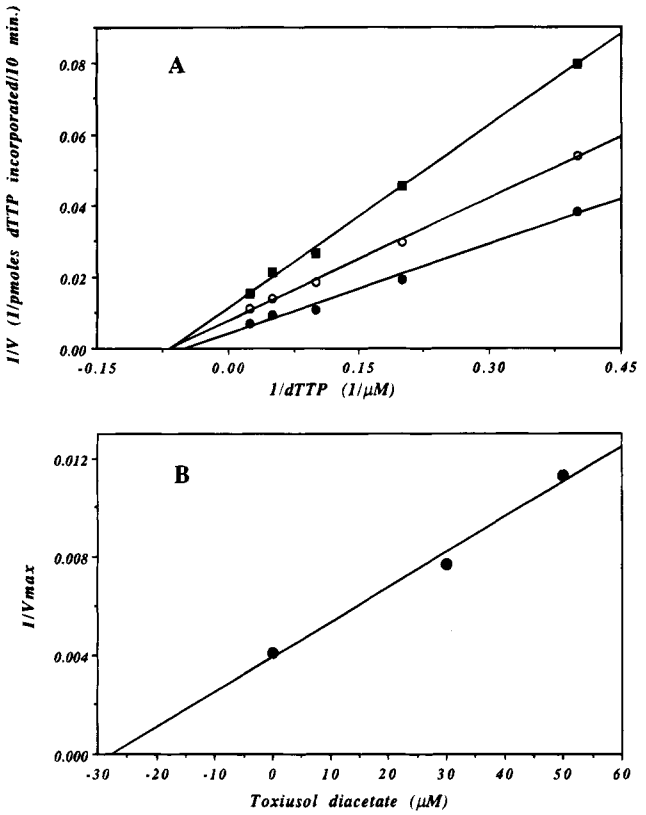

FIGURE 5: Kinetic analysis of the inhibition of HIV-1 RT-associated DNA polymerase activity by TDA. The double-reciprocal plots of the effect of increasing concentration of dTTP (panel A) on the incorporation of [^{3}H]dTTP by HIV-1 RT in the absence (●) and in the presence of 30 (○) and 50 μM (■) TDA. Panel B represents secondary replots of the intercepts (from panel A) versus TDA concentrations. The derived K_m and K_i values were computer-generated by linear regression analysis with a high value of 0.99.

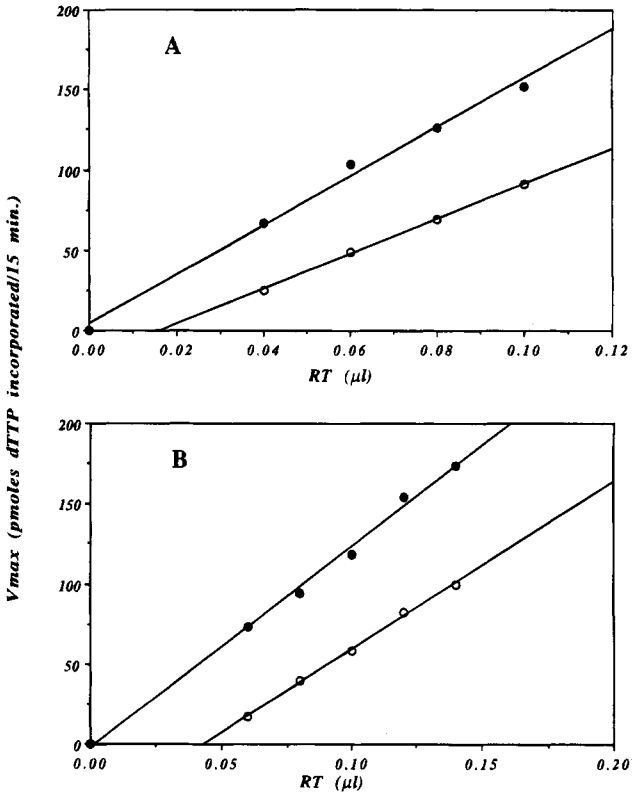

FIGURE 6: Maximal rate of HIV-1 RT-associated DNA polymerase activity as a function of enzyme concentration in the presence of either toxiusol (A) or TDA (B). The DNA polymerase activity associated with HIV-1 RT was assayed by following the poly(rA)$_n$·oligo(dT)$_{12-18}$-directed incorporation of [^{3}H]dTTP into DNA in the absence (●) (panels A and B) and in the presence of 0.69 μM toxiusol (○) (panel A) or 50 μM TDA (○) (panel B). The reactions were performed under saturation concentrations of the template-primer (9 μg/mL) and of the radiolabeled substrated dTTP (15 μM) for 15 min at 37 °C. The plots were computer-generated by regression analysis with regression coefficients of 0.99, indicating a high degree of linear relationship between the rate of reaction and enzyme concentrations.

a result of the substitution of sulfates by acetyl groups? To answer this question, we have conducted steady-state kinetic studies by increasing concentrations of dTTP in the presence of the inhibitor TDA. It is clear from the double-reciprocal plots (Figure 5A) that, like toxiusol, the analog TDA is a noncompetitive inhibitor with respect to dTTP. The substrate binds the enzyme equally well in the absence or presence of the inhibitor. However, the inhibition constant (K_i), derived from the replots of the intercepts from Figure 5A, reflects the change in the affinity of TDA to the enzyme. TDA interacts with the enzyme with low affinity (K_i = 26 μM); a decrease of about 22-fold in the affinity of the analog compared to its parent natural inhibitor. TDA binds the enzyme with lower affinity than dTTP (K_i/K_m > 1).

Any inhibitor that interacts irreversibly with an enzyme may resemble a noncompetitive inhibitor since V_{max} decreases but K_m remains unchanged. Thus, we have asked whether the inhibition of the DNA polymerase activity associated with HIV-1 RT by either toxiusol or it analog TDA is reversible. The velocity of the reaction is measured at serial dilutions of the enzyme in the presence of saturating concentrations of the template-primer poly(rA)$_n$·oligo(dT)$_{12-18}$ (9 μg/mL) and the substrate [^{3}H]dTTP (15 μM) (Figure 6). When the enzyme concentration is decreased, the maximum velocities of the control reactions and of the reactions with either 0.69 μM toxiusol (Figure 6A) or 50 μM TDA (Figure 6B) are not reduced proportionally. An increase in the inhibitory activity of both toxiusol and TDA is noted as a function of lowering the enzyme concentrations. When V_{max} = 0, the enzyme−axis intercept represents the amount of enzyme which is completely removed from the recation, thus resulting in a decrease in V_{max}. Toxiusol and its analogs TDA inhibit the DNA polymerase activity of HIV-1 RT irreversibly.

Effect of Toxiusol and TDA on the Binding of HIV-1 RT to Template-Primer. To further investigate the possible mechanism by which the HIV-1 RT-associated DNA polymerase activity is inhibited by toxiusol, we have examined whether this inhibitor interferes with the interaction of the enzyme with the DNA template-primer. It is known that in the process of DNA synthesis the binding of the DNA polymerase to template-primer precedes the binding of the enzyme to dNTP (Majumadar et al., 1988). Therefore, the interaction of the RT with its DNA template-primer may constitute another facet for the interference of anti-HIV RT drugs. We have recently developed a gel retardation assay which has been used to study the effects of different inhibitors on the binding of HIV-1 RT to DNA oligomer (Bakhanashvili & Hizi, 1994). As can be seen in Figure 7A, a complex formation between the ^{32}P end-labeled oligonucleotide DNA and HIV-1 RT is detected (Figure 7A, lane 1) by the electrophoretic retardation of the DNA as a result of its association with the enzyme. When the reaction is performed in the presence of increasing toxiusol concentrations, a marked decrease in the intensity of the bands is observed (Figure 7A, lanes 2−5). The quantitation of the complexes as a function increasing toxiusol concentrations

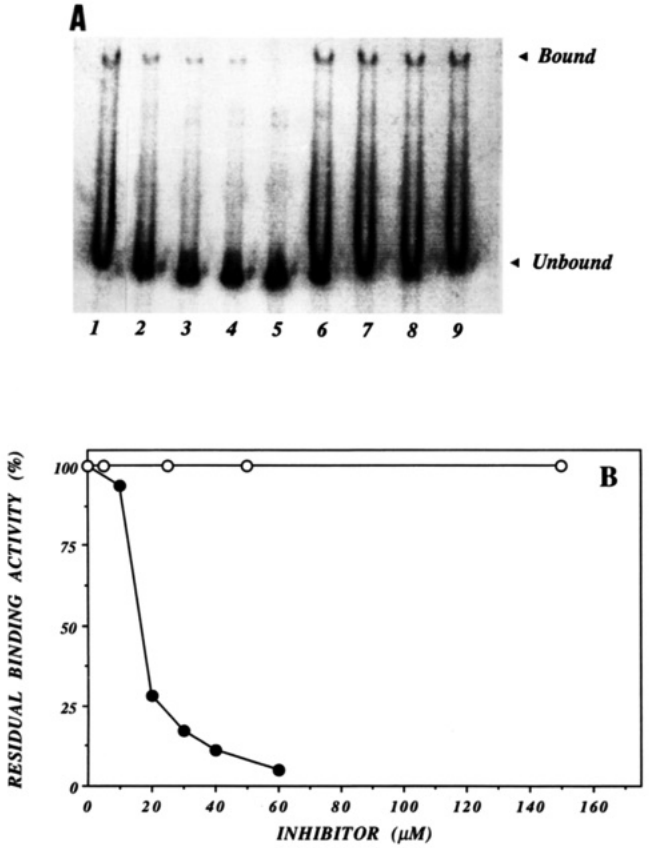

FIGURE 7: Effect of toxiusol and its analog TDA on the formations of HIV-1 RT−DNA complex. The binding of HIV-1 RT to ^{32}P end-labeled double-stranded oligonucleotide DNA was preformed as described in detail in Materials and Methods. (Panel A) Autoradiogram of a gel mobility shift assay. Lane 1, control binding with no competitor. Lanes 2−5, binding with increasing concentrations of toxiusol [at final concentrations of 10 (lane 2), 20 (lane 3), 40 (lane 4), or 60 μM (lane 5)]. Lanes 6−9, binding with increasing concentrations of TDA [at final concentrations of 5 (lane 6), 25 (lane 7), 50 (lane 8), or 150 μM (lane 9)]. The gel represents one out of two independent experiments done (both gave identical results). (Panel B) Quantification of the inhibition of the binding of the enzyme to DNA by toxiusol and TDA. The extent of inhibition is derived from an experiment similar to that presented in panel A. The binding of HIV-1 RT to DNA was measured as a function of increasing concentrations of either toxiusol (●) or TDA (○). The inhibition is calculated relative to control with no inhibitor.

(Figure 7B) clearly demonstrates that toxiusol inhibits the formation of DNA−RT complexes (IC_{50} = about 16 μM). In the presence of the analog toxiusol diacetate, on the other hand, there is no effect on the DNA−RT complex formation even at a concentration as high as 150 μM (Figure 7A, lanes 6−9; Figure 7B). Substitution of the sulfate group by acetyl group results in a loss in the ability to interfere with the formation of the DNA template-primer enzyme complex. This result is fully compatible with the relative insensitivity of RT to TDA observed earlier (Figure 2A).

DISCUSSION

Toxiusol, a natural product isolated from the Red Sea sponge *T. toxius*, has been shown to be a general potent inhibitor *in vitro* of both viral and cellular DNA polymerases. This compound inhibits equally well the DNA polymerase activity associated with RTs of HIV-1, EIAV, and MuLV. It should be noted that MuLV RT represents a more distant retroviral RT species, which sets it apart from two closely-related lentiviral RTs (i.e., EIAV and HIV-1). For example, the fidelity of DNA synthesis of lentiviral RTs studied so far is substantially lower than that of MuLV RT (Bakhanashvili & Hizi, 1993a,b). In addition, toxiusol is capable of blocking the DNA polymerase function of the RTs of two other distant retroviruses, i.e., of mouse mammary tumor virus and bovine leukemia virus (unpublished results). Toxiusol also inhibits effectively various cellular DNA polymerases. These enzymes represent three main subfamilies: the first is referred to as the DNA polymerase I family (as represented in this study by KF); the second is homologous to the human DNA polymerase-α (represented by calf-thymus DNA polymerase-α); the third subfamily includes the DNA polymerase-β (Delarue et al., 1990). The inhibitory effect of toxiusol is achieved irrespective of the template-primer used. For example, toxiusol inhibits both the poly(rA)$_n$·oligo(dT)$_{12-18}$-directed or activated DNA-directed DNA synthesis of HIV-1 RT (Loya et al., 1994).

To locate the potential inhibitory active site of the lead compound, toxiusol, we have conducted structure−activity relationship studies focusing primarily on substitutions at positions 2′ and 5′ in the benzene ring. Compound B, in which the two sulfate moieties have been hydrolyzed, exhibits high potency in blocking the DNA polymerase activity associated with the three retroviral RTs. Thus, substitution of the sulfate with hydroxyl groups appears to have no dramatic effect on activity. On the other hand, acetylation at the same positions has a determinant effect on potency. Modification of both polar moieties by acetyl groups in TDA resulted in a very weak or inactive inhibitor. This poor inhibitory activity was exhibited against all DNA polymerases tested. We speculate, therefore, that hydrophilic polar moieties are required for optimum inhibition. Similarly, we have previously shown that the hydroxyl group in the pyridinone ring of the natural product 3,5,8-trihydroxy-4-quinolone and in the quininone ring of avarol analogs and of illimaquinone is a key element for their anti-HIV-1 RT inhibitory activity (Loya & Hizi, 1990; Loya et al., 1990, 1994).

The mode of inhibition of the RT-associated DNA polymerase activity by toxiusol was found to be irreversible and noncompetitive with respect to dTTP and to poly(rA)$_n$·oligo(dT)$_{12-18}$ at only low toxiusol concentrations. However, at higher inhibitor concentrations, the mode of inhibition with respect to template-primer varies to mixed linear. Consequently, toxiusol binds the enzymes with high affinity (K_i = 1.2 μM) at sites distinct from those occupied by either substrate. Likewise, TDA, devoid of polar moieties in the benzene ring, is capable of binding the enzyme irreversibly and in a noncompetitive manner but at a much lower affinity (K_i = 26 μM). The efficacy of inhibition decreased by 22-fold due to the loss of the polar groups. Therefore, toxiusol and TDA probably interact with the enzyme with their hydrophobic structure skeleton. In addition to the hydrophobic interactions, toxiusol and the hydroquinone derivative (compound B) are capable of interacting with the enzyme, probably through a hydrogen bond. This, in turn, contributes to the high potency of toxiusol. The importance of the polar moieties was further established by the fact that toxiusol inhibits the physical binding of RT to the double-stranded oligonucleotide used, whereas the acytelated analog TDA was devoid of any inhibitory activity (Figure 7).

The binding of toxiusol to RT with an affinity higher than that of the substrate destabilizes RT−DNA complex, thereby inhibiting the overall polymerization reaction. Indeed, kinetic

studies of RT have already demonstrated that polymerization is an ordered mechanism in which the template-primer binds first, followed by the addition of nucleoside triphosphates (Majumadar et al., 1988). Thus, other RT nonnucleoside inhibitors, such as peyssonol A and peyssonol B (Loya et al., 1995) and 3,5,8-trihydroxy-4-quinolone (Bakhanashvili & Hizi, 1994), inhibit the binding of the template-primer to the enzyme. Consequently, the subsequent addition of the incoming dNTP during the polymerization process would be impaired. In contrast to these natural inhibitors, TIBO, representing the NNRTI (De Clercq, 1993), was recently found by us not to affect the stability of the RT−DNA complex (Bakhanashvili & Hizi, 1994). The NNRTI binding site lies in a hydrophobic pocket near the DNA polymerase active site (Kohlstaedt et al., 1992; Jacobo-Molina et al., 1993) and probably interferes with the binding to dNTP.

We have shown that toxiusol is a general inhibitor of various DNA polymerases. Is there any common denominator between all DNA polymerases? For one, all DNA polymerases are responsible for the DNA synthesis. The chemical reaction is the same, i.e., the formation of phosphodiester linkage with the 3′-hydroxyl group of the deoxyribonucleotide as substrate. Sequence alignment studies have shown that in spite of wide apparent sequence variability there are three conserved sequence motif domains common to all DNA-dependent DNA polymerases (Delarue et al., 1990). From the comparison of the backbone structure of the p66 polymerase domain of HIV-1 RT with that of KF, it is clear that both enzymes exhibit an overall right-hand-like shape, with subdomains designated as palm, thumb, and fingers (Kohlstaedt et al., 1992; Beese et al., 1993; Jacobo-Molina et al., 1993; Moras, 1993). A large cleft is created in the palm between the thumb and the fingers that is wide enough to accommodate the template-primer. The crystal structure of the catalytic domain of the DNA polymerase-β has been recently determined (Davies et al., 1994; Sawaya et al., 1994). Here again, the protein has three subdomains arranged in an overall U-shaped molecule, creating a groove for the template-primer. The palm regions of the three enzymes (HIV-1 RT, KF, and DNA polymerase-β), the only DNA polymerases whose three-dimensional structures are already known, superimposed fairly well. It is conceivable, therefore, that in all DNA polymerases, in analogy with the known structure of HIV-1 RT, the enzyme is assumed to surround the template during the polymerization process. The binding of the inhibitor to the enzyme is likely to result in conformational changes, thereby the template-primer cannot occupy its respective binding site, which is probably common to all DNA polymerases. Indeed, inhibition of the formation of enzyme−DNA complex is one of the major mechanisms expected from a general inhibitor of DNA polymerases as is toxiusol. We plan to continue and study further the molecular mechanisms of inhibition both biochemically and structurally including by X-ray crystalography.

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