Mode of inhibition of HIV reverse transcriptase by 2-hexaprenylhydroquinone, a novel general inhibitor of RNA-and DNA-directed DNA polymerases

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A natural compound from the Red Sea sponge *Ircinia* sp., 2-hexaprenylhydroquinone (HPH), has been shown to be a general inhibitor of retroviral reverse transcriptases (from HIV-1, HIV-2 and murine leukaemia virus) as well as of cellular DNA polymerases (*Escherichia coli* DNA polymerase I, and DNA polymerases α and β). The pattern of inhibition was found to be similar for all DNA polymerases tested. Thus the mode of inhibition was studied in detail for HIV-1 reverse transcriptase. HPH is a non-competitive inhibitor and binds the enzyme irreversibly with high affinity ($K_i = 0.62\ \mu$M). The polar hydroxy groups have been shown to be of key importance. A methylated derivative, mHPH, which is devoid of these polar moieties, showed a significantly decreased capacity to inhibit all DNA polymerases tested. Like the natural product, mHPH binds the enzyme independently at an allosteric site, but with reduced affinity ($K_i = 7.4\ \mu$M). We show that HPH does not interfere with the first step of the polymerization process, i.e. the physical formation of the reverse-transcriptase–DNA complex. Consequently, we suggest that the natural inhibitor interferes with the subsequent steps of the overall reaction. Since HPH seems not to affect the affinity of dNTP for the enzyme (the K_m is unchanged under conditions where the HPH concentration is increased), we speculate that its inhibitory capacity is derived from its effect on the nucleotidyl-transfer catalytic reaction. We suggest that such a mechanism of inhibition is typical of an inhibitor whose mode of inhibition should be common to all RNA- and DNA-directed polymerases.

INTRODUCTION

Since the discovery of HIV as the causative agent of AIDS [1,2], enormous efforts have been invested worldwide in the search for effective drugs against this devastating disease. Studies on the molecular biology of HIV have identified its reverse transcriptase (RT) as one of the main targets for AIDS therapy. RT plays an essential role in the early steps of the replication cycle of retroviruses. RT catalyses the transcription of the HIV-encoded single-stranded RNA into double-stranded DNA [3]. Many of the currently approved anti-AIDS agents are potent inhibitors of retroviral RT. These compounds include both nucleoside analogues and non-nucleoside RT inhibitors (NNRTI). The nucleoside analogues need to be phosphorylated first by cellular kinases to their triphosphate forms before competing with the natural nucleosides, leading to premature termination of DNA polymerization [4,5]. However, their structural resemblance to authentic substrates leads to recognition by cellular DNA polymerases other than RT, and thus to the toxic side effects observed in treated patients [6,7].

The NNRTI, as opposed to the nucleoside analogues, constitue a number of different, structurally unrelated, classes of compounds that are highly selective against HIV-1 RT and are targeted at a non-substrate binding site of this enzyme. The NNRTI exhibit no inhibitory activity against other retroviral RTs [e.g. those of HIV-2, avian myeloblastosis virus, murine leukaemia virus (MuLV), simian immunodeficiency virus and feline leukaemia virus] or against human DNA polymerases α (pol α), β (pol β) and δ [8,9]. As opposed to the HIV-1-specific NNRTI, there are a wide range of various non-nucleoside substances with anti-RT activity [10]. During a survey of marine natural products for activity against HIV RT, we have isolated various structurally diverse compounds, such as avarol analogues, illimaquinone, halocynthiaxanthin, 3,5,8-hydroxy-4-quinolone, and peyssonols A and B [11–15]. All of these compounds have the capacity to inhibit HIV-1 and HIV-2 RTs, and exhibit poor or no activity against cellular DNA-dependent DNA polymerases. Toxiusol, on the other hand, has been shown to be a potent inhibitor not only of viral RTs (i.e. those of HIV-1, equine infectious anaemia virus and MuLV) but also of cellular DNA polymerases [pol α, pol β and the Klenow fragment of *Escherichia coli* DNA polymerase I (KF)], simply by blocking formation of the HIV-1 RT–DNA complex, the first step in the polymerization process [16].

In the present study we show that the compound 2-hexaprenylhydroquinone (HPH), a natural product of the Red Sea sponge *Ircinia* sp., is a potent general inhibitor of all RTs and cellular DNA polymerases tested. The mode of inhibition of HIV-1-RT-associated DNA polymerase activity is studied in detail. Similar to toxiusol, HPH was found to bind the enzyme irreversibly in a non-competitive fashion. However, unlike toxiusol, HPH does not interfere significantly with the first step of polymerization,

Abbreviations used: HPH, 2-hexaprenylhydroquinone; mHPH, 1,4-dimethyl derivative of HPH; RT, reverse transcriptase; NNRTI, non-nucleoside RT inhibitors; MuLV, murine leukaemia virus; KF, Klenow fragment of *Escherichia coli* DNA polymerase I; pol α, DNA polymerase β; pol β, DNA polymerase β; TIBO, tetrahydroimidazo[4,5,1-*jk*][1,4]-benzodizepine-2(1H)-one and -thione.

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i.e. the physical binding of the enzyme to its template primer. We speculate that HPH might interfere with subsequent steps of the DNA polymerization process, such as the nucleotidyl-transfer reaction, which leads to the incorporation of a single dNTP.

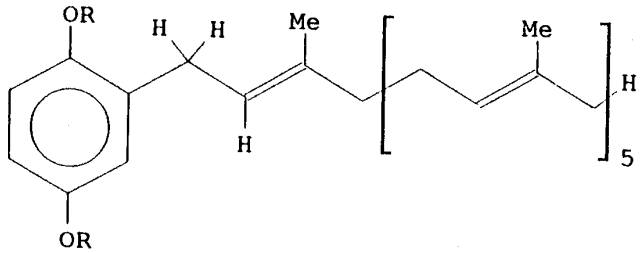
MATERIALS AND METHODS

Chemicals

The natural compound HPH (Figure 1) was isolated from the sponge *Ircinia* sp., which had been collected from the Gulf of Eilat in the Red Sea. The compound was isolated by the following procedure. The sponge (5 g dry weight) was extracted with a 1:1 (v/v) mixture of methanol/ethyl acetate to produce a brown 'gum' (100 mg). Solvent partition between aqueous methanol and tetrachloromethane gave the crude hydroquinone (HPH) in the tetrachloromethane phase. Sephadex LH-20 chromatography of the latter material (eluted with hexane/ethyl acetate/methanol, 2:1:1, by vol.) resulted in the pure compound HPH in high yield (50 mg). The structural formula of HPH was elucidated by spectroscopic means, one-dimensional and two-dimensional NMR and mass spectra. Electron-impact MS: m/z 518 (M^+, 20), 393 (10), 246 (84), 161 (74), 135 (55), 123 (91), 109 (45), 69 (100). NMR (500 MHz): δ_H 6.63 (1 H, m), 6.58 (1 H, m), 6.55 (1 H, m), 5.28 (1 H, t, J 7 Hz), 5.09 (5 H, m), 3.27 (2 H, d, J 7.5 Hz), 1.95–2.08 (20 H, m), 1.73 (3 H, s), 1.67 (3 H, s), 1.58 (15 H, s). The natural compound was found to be identical in all respects to 2-hexaprenylhydroquinone reported previously by Cimino et al. [17].

The compound mHPH is a 1,4-dimethyl derivative of the natural compound HPH, and was prepared as follows. HPH (5 mg) in acetone (2 ml) was treated with methyliodide (50 mg) in the presence of K_2CO_3 (20 mg). After 24 h at room temperature, the mixture was filtered and evaporated. The residue obtained was chromatographed on a short silica gel column to yield a pure mHPH compound. The structure was identified by using MS and one-dimensional and two-dimensional NMR. Electron-impact MS: m/z 546 (M^+,15), 69 (100). NMR (500 MHz): δ 6.74 (3 H, m), 5.32 (1 H, b, r, t), 5.11 (5 H, b, r, t), 3.78 (OMe, s); 3.74 (OMe, s), 3.30 (2H, d). The dried compounds were dissolved in 100% DMSO. The final DMSO concentration in the enzymic assays was always adjusted to 1%, a concentration that did not affect the various DNA polymerase activities.

The synthetic template primers poly(rA)$_n$·oligo(dT)$_{12-18}$ and poly(rC)$_n$·oligo(dG)$_{12-18}$ were products of Pharmacia. Activated gapped DNA was prepared by limited digestion of herring sperm DNA with bovine pancreatic DNase I [18].



-R=H; 2-hexaprenylhydroquinone (HPH)

-R=CH_3 methylated derivative (mHPH)

Figure 1 Structural formulae of HPH and mHPH

Enzymes

HIV-1 and HIV-2 RTs were recombinant enzymes expressed in *E. coli* and purified from bacterial extracts to homogeneity as described previously [19]. The HIV-1 RT expression plasmid was derived from the BH-10 proviral isolate [20], and the HIV-2 expression plasmid was derived from the pRod isolate [21,22]. The purified RTs from HIV-1 and HIV-2 were composed of p66/p51 and p68/p55 heterodimers respectively. MuLV RT, a p70 monomer, is a recombinant enzyme and was purchased from USB. Pol α was purified from calf thymus by immunoaffinity column chromatography as described by Perrino and Loeb [23], and was generously donated by Dr. M. Fry (Technion, Haifa, Israel). Recombinant pol β was a gift from Dr. Z. Hostomsky (Agouron, San Diego, CA, U.S.A.). KF was purchased from Advanced Biotechnologies Ltd. HIV-1 and HIV-2 RTs were kept in 25 mM Tris/HCl, pH 8.0, 50% (v/v) glycerol and 2 mM dithiothreitol. Long-term storage of all enzymes was at −80 °C.

Enzyme assays

Assays of HIV-1, HIV-2 and MuLV RTs were carried out as described in detail previously [24]. In brief, the RNA-dependent DNA polymerase 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 the DNA product. DNA-dependent DNA polymerase activity was assayed using activated gapped DNA as primer-template and with the four deoxynucleotides present (of which only one, dTTP, was labelled). In all inhibition experiments, the enzymes were preincubated for 5 min at 30 °C in the presence of inhibitor at various concentrations. The enzymic reaction was initiated by adding the relevant [^{3}H]dNTP followed by incubation at 37 °C for 30 min (or for 10 min in the kinetic studies). Inhibition of enzyme activity was calculated relative to the initial linear reaction rates observed under identical conditions when no drug was added. IC_{50} values were calculated from the inhibition dose-dependent curves. Steady-state kinetic constants were calculated from the double-reciprocal plots of velocity against substrate concentration. Kinetic constants were determined by computer-generated linear regression analyses.

Assays of calf thymus pol α, pol β and KF 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 $MgCl_2$, 45 μg/ml activated DNA, unlabelled dATP, dGTP and dCTP (each at 25 μM) and [^{3}H]dTTP at a final concentration of 5 μM (specific radioactivity 2000–4000 c.p.m./pmol). RNase H activity was assayed by measuring the release of trichloroacetic acid-soluble material from the synthetic substrate [^{3}H]poly(rA)$_n$·poly(dT)$_n$, as described in detail previously [24]. Enzymic activity was defined as follows: 1 unit of DNA polymerase activity is the amount of enzyme that catalyses the incorporation of 1 pmol dNTP into DNA product after 30 min at 37 °C, under the standard assay conditions.

Binding assays

The binding of HIV-1 RT to a double-stranded oligonucleotide DNA was measured using PAGE-band mobility shift assays [25]. Complex-formation between the ^{32}P-5′-end-labelled oligonucleotide DNA (54mer) and highly purified HIV-1 RT was detected by the electrophoretic retardation of the DNA as a result of its association with the enzyme. The binding reaction assays were conducted as described previously [16], except that the BSA concentration in the reaction mixture was reduced to 20 μg/ml.

The quantification of RT–DNA complex-formation was calculated by employing the densitometry scanning procedure.

RESULTS

Effects of HPH and mHPH on various retroviral RTs

In an attempt to identify a new marine natural product with potential activity against HIV RT, we have isolated from the Red Sea sponge *Ircinia* sp. the hexaprenoid hydroquinone HPH (Figure 1). The structure of HPH was found to be identical to that described previously by Cimino et al. [17]. The effects of HPH on the DNA polymerase activity of HIV-1 RT have been studied. The natural compound is a potent inhibitor of both poly(rA)$_n$·oligo(dT)$_{12-18}$- and DNA-directed DNA synthesis associated with HIV-1 RT (Figure 2 and Table 1) (IC_{50} $2.7 \pm 0.1\ \mu M$ and $4.5 \times \pm 0.5\ \mu M$ respectively). On the other hand, RT-associated RNase H activity was hardly affected by HPH, i.e. the IC_{50} was as high as $200\ \mu M$. This emphasizes the selective capacity of HPH to inhibit DNA polymerase function. The responses of RTs from the closely related lentivirus HIV-2 and from a more distant retrovirus, MuLV, to the inhibitor were very similar. The two polymerase activities associated with each enzyme, i.e. RNA-and DNA-dependent DNA polymerase, were equally sensitive to HPH. Thus IC_{50} values of around $1\ \mu M$ were calculated for both functions of the enzymes.

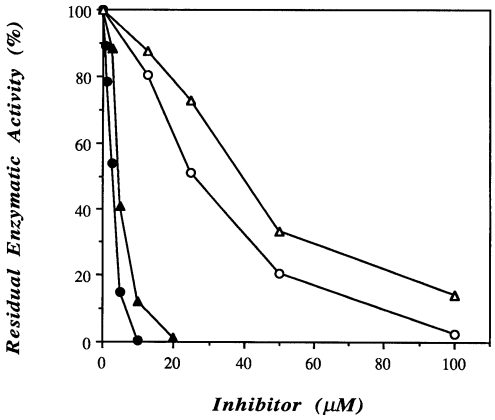


Figure 2 Dose–response curves for inhibition of the DNA polymerase activity of HIV-1 RT by HPH and mHPH

Inhibition experiments were carried out as described in the Materials and methods section. The DNA polymerase activity of HIV-1 RT was assayed either by following poly(rA)$_n$·oligo(dT)$_{12-18}$-directed DNA synthesis in the presence of increasing concentrations of HPH (●) and mHPH (○) or by following activated DNA-directed DNA synthesis in the presence of HPH (▲) and mHPH (△). An activity of 100% corresponds to 72 units and 34 units for RNA- and DNA-directed DNA synthesis respectively (see the Materials and methods section).

A derivative of the natural product HPH, designated mHPH (Figure 1), was prepared by substituting the two hydroxy groups of the hydroquinone ring with methoxy moieties. The methylated derivative, in which the two potential inhibitory active sites are blocked, was tested for its capacity to inhibit both DNA polymerase functions of HIV-1, HIV-2 and MuLV RTs. From the results obtained (Table 1 and Figure 2), it is clear that mHPH is about 10-fold less effective in inhibiting the RT catalytic functions than is the natural product. The IC_{50} and IC_{90} values were increased by 8–10-fold compared with the corresponding values obtained with HPH, regardless of the catalytic activity or the RT tested. In short, a general inhibitory effect of HPH and its derivative mHPH has been established. Moreover, there is no dichotomy in the response to the inhibitor of the two DNA polymerase functions associated with RT, i.e. the RNA-dependent and DNA-dependent DNA polymerase activities.

Effects of HPH and mHPH on other cellular DNA polymerases

To assess the possibility that HPH has a general inhibitory effect on DNA polymerases, we further analysed its capacity to inhibit cellular DNA polymerases. The natural compound HPH strongly inhibited the DNA polymerase activities of KF, pol α and pol β (IC_{50} values of $0.58 \pm 0.03\ \mu M$, $1.1 \pm 0.1\ \mu M$ and $1.9 \times \times \pm 0.1\ \mu M$ respectively; Table 1). Here again, as with the retroviral RTs, the sensitivity of the DNA polymerases to the analogue mHPH was significantly reduced (Table 1). Thus 20-, 15- and 17-fold increases in the IC_{50} values calculated for mHPH relative to the natural prodcut were observed for pol α, pol β and KF respectively.

The overall responses of the RNA- and DNA-directed activities of the retroviral RTs, as well as of the cellular DNA polymerases tested, to HPH and its methylated derivative were almost identical. Therefore we chose to continue to study the mode of inhibition using HIV-1 RT as a representative of all the enzymes

Table 1 Effects of HPH and mHPH on the DNA polymerase activities of various retroviral RTs and cellular DNA polymerases

IC_{50} and IC_{90} values were derived from dose–response experiments of the different enzymic activities by increasing the concentration of either HPH or mHPH. RNA-dependent DNA polymerase activity is associated with retroviral RTs, whereas the DNA-dependent DNA polymerase function is associated with all DNA polymerases. All data are means ± range of at least three separate experiments.

Enzyme	DNA polymerase function	HPH		mHPH	
		IC_{50} (μM)	IC_{90} (μM)	IC_{50} (μM)	IC_{90} (μM)
HIV-1 RT	RNA-dependent	2.7 ± 0.1	5.4 ± 0.6	23.5 ± 1.5	64.5 ± 5.5
	DNA-dependent	4.5 ± 0.5	11.0 ± 2	38.5 ± 3.5	105.5 ± 9.5
HIV-2 RT	RNA-dependent	1.2 ± 0.2	2.6 ± 0.3	10.0 ± 1.5	21.5 ± 1.0
	DNA-dependent	1.5 ± 0.5	4.3 ± 0.8	12.8 ± .28	49.8 ± 4.8
MuLV RT	RNA-dependent	0.8 ± 0.3	2.2 ± 0.2	7.1 ± 1.7	23.5 ± 0
	DNA-dependent	1.5 ± 0.1	5.0 ± 0.5	15.0 ± 4.5	54.5 ± 5.5
Calf-thymus pol α	DNA-dependent	1.1 ± 0.1	3.2 ± 0.6	22.5 ± 1.5	80 ± 0
Human pol β	DNA-dependent	1.9 ± 0.1	4.6 ± 0.1	28 ± 2	82 ± 1
KF	DNA-dependent	0.58 ± 0.03	2.15 ± 0.05	10 ± 0	43.8 ± 1.3

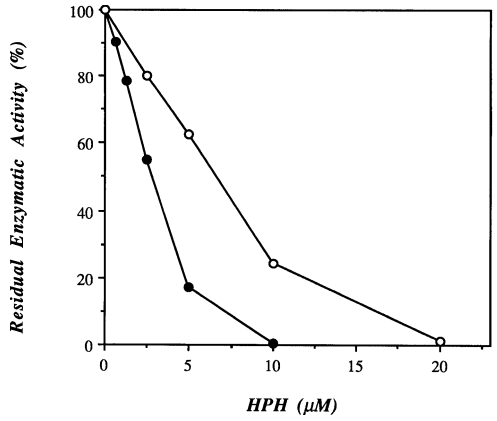


Figure 3 Effect of HPH on RNA-directed DNA synthesis using different template-primers

The experiments were carried out as described in the Materials and methods section. An activity of 100% with poly(rA)$_n$·oligo(dT)$_{12-18}$ (●) and with poly(rC)$_n$·oligo(dG)$_{12-18}$ (○) as template-primer corresponds to 74 and 34 units respectively.

tested. There are known cases in which the efficiency of inhibition varies according to the template-primer used, as reported, for example, for various NNRTI. Thus tetrahydroimidazo[4,5,1-*jk*][1,4]-benzodizepine-2(1H)-one and -thione (TIBO) and 2′5′-bis-*O*-(t-butyldimethylsilyl)-3′-spiro-5″-(4″-amino-1″2″-oxathiole-2″, 2″-dioxide) (TSAO-T) were shown to be more efficient in inhibiting poly(rC)$_n$·oligo(dG)$_{12-18}$-directed compared with poly(rA)$_n$·oligo(dT)$_{12-18}$-directed DNA synthesis [26,27]. Therefore we decided to test whether the inhibitory capacity of HPH is dependent on the synthetic template-primer used. The efficacy of HPH inhibition was increased only by 2-fold when using poly(rA)$_n$·oligo(dT)$_{12-18}$ as template-primer (Figure 3). The IC_{50} values calculated from the dose–response curve in Figure 3 were 2.8 ± 0 and 6.7 ± 0.1 μM for poly(rA)$_n$·oligo(dT)$_{12-18}$-directed and poly(rC)$_n$·oligo(dG)$_{12-18}$-directed synthesis respectively.

Mode of inhibition of the DNA polymerase activity of HIV-1 RT by HPH and mHPH

Steady-state kinetic studies of HIV-1 RT were carried out by increasing the concentration of either dTTP or poly(rA)$_n$·oligo(dT)$_{12-18}$ in the presence of HPH, according to Michaelis–Menten kinetics. It is apparent that HPH is a non-competitive inhibitor with respect to dTTP (Figure 4A) and varies from being non-competitive (at low HPH concentrations) to uncompetitive (at higher HPH concentrations) with respect to poly(rA)$_n$·oligo(dT)$_{12-18}$ (Figure 4B). The apparent K_m values of 7.5 ± 0.4 μM for dTTP and 0.65 ± 0.06 μg/ml for the template-primer were not affected by the presence of increasing concentrations of HPH. The inhibition constant (K_i) for HPH was derived from a secondary re-plot of $1/V_{max}$ (intercept) against inhibitor concentration (Dixon plot; not shown). The curve was linear and yielded a K_i value of 0.62 μM. It is clear that HPH binds the enzyme with greater affinity than does dTTP ($K_i/K_m < 1$).

We have shown that mHPH showed a significantly lower capacity than HPH to inhibit DNA polymerases. It was, therefore, of interest to test whether the substitution of the two hydroxy groups by methoxy groups had any effect on the mode of inhibition observed. To this end, we tested the effect of mHPH

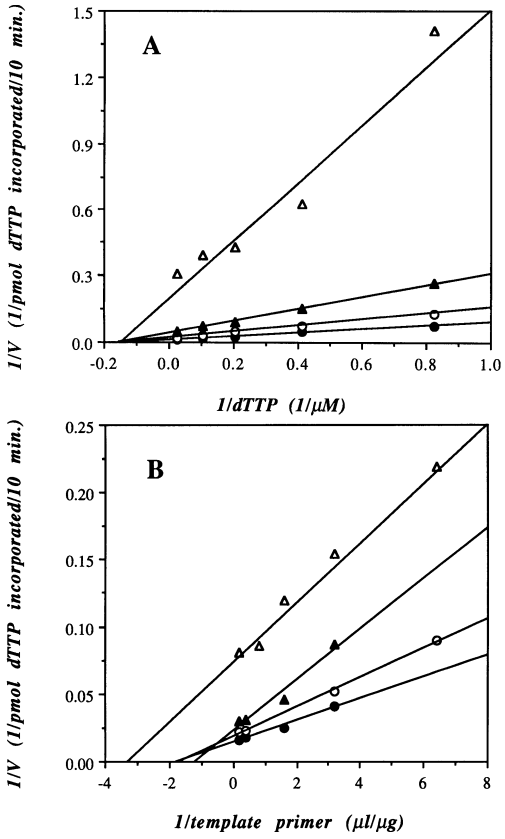


Figure 4 Kinetic analysis of the inhibition of HIV-1-RT-associated DNA polymerase activity by HPH

Shown are double-reciprocal plots obtained with increasing concentrations of dTTP (**A**) or poly(rA)$_n$·oligo(dT)$_{12-18}$ (**B**) as a function of the initial velocity of [^{3}H]dTTP incorporation by HIV-1 RT. (**A**) Reaction in the absence (●) or in the presence of 1 μM (○), 2 μM (▲) or 4 μM (▲) HPH; (**B**) reaction in the absence (●) or presence of 0.75 μM (○), 1.5 μM (▲) or 3 μM (▲) HPH. The plots were computer-generated by linear-regression analysis, and the regression coefficient values (r) were as high as 0.99 for each curve [except for one plot in (**A**) (4 μM HPH), for which $r = 0.98$], indicating a highly linear relationship between the parameters.

(at concentrations in the range of its IC_{50} value) on the initial-velocity pattern with either dTTP or poly(rA)$_n$·oligo(dT)$_{12-18}$ as the variable substrate. The results indicate that, like the natural product, mHPH is a non-competitive inhibitor with respect to both substrates (Figures 5A and 5B). However, the inhibition constant derived from Dixon plot (not shown) reflected a substantial decrease in the affinity of the inhibitor for the enzyme. This suggests that the derivative binds the enzyme independently at different sites than does either substrate, with a 10-fold decrease in the K_i value ($K_i = 7.4 \pm 1.6$ μM).

Kinetic analyses of non-competitive inhibitors that bear no structural resemblance to the substrate do not distinguish between true reversible inhibitors and irreversible ones. To differentiate between these two possibilities, we measured the velocity of DNA synthesis at serial dilutions of HIV-1 RT in the presence of saturating concentrations of poly(rA)$_n$·oligo(dT)$_{12-18}$ (10 μg/ml) and [^{3}H]dTTP (15 μM). As the enzyme concentration was decreased, the maximal velocities of the control and the corresponding inhibition reactions in the presence of either 2 μM HPH (Figure 6A) or 25 μM mHPH (Figure 6B) were not decreased proportionally. The inhibitory activities of HPH and its analogue mHPH were increased at low enzyme concentrations.

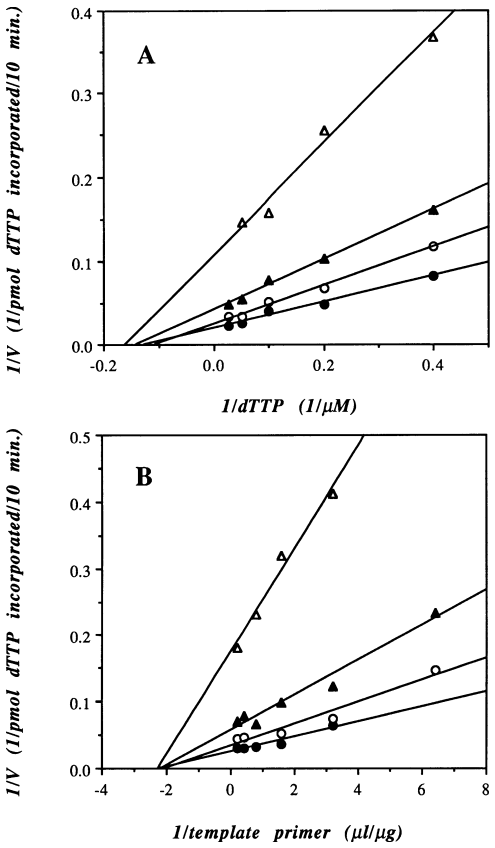


Figure 5 Kinetic analysis of the inhibition of HIV-1-RT-associated DNA polymerase activity by mHPH

Shown are double-reciprocal plots obtained with increasing concentrations of either dTTP (**A**) or poly(rA)$_n$·oligo(dT)$_{12–18}$ (**B**) as a function of the initial velocity of [^{3}H]dTTP incorporation by HIV-1 RT in the absence (●) or in the presence of 12.5 μM (○) 25 μM (▲) or 50 μM (△) mHPH. All curves were computer-generated by linear-regression analysis. The *r* values were as high as 0.99.

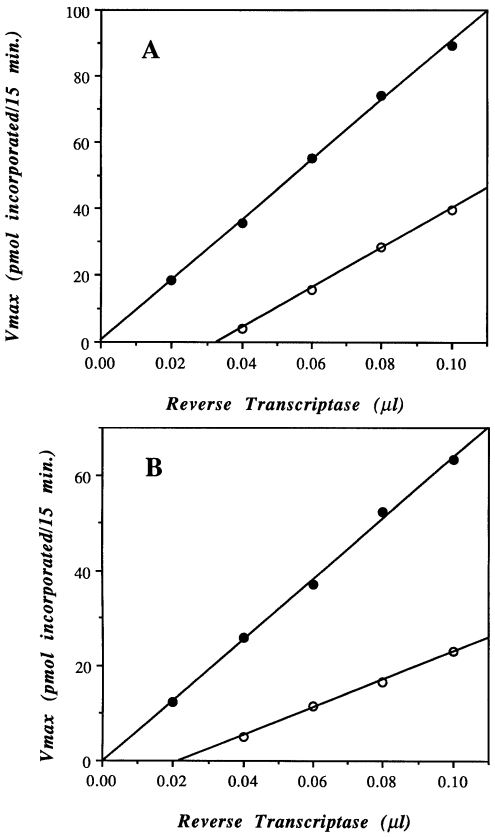


Figure 6 Maximal velocity of HIV-1-RT-associated DNA polymerase activity as a function of enzyme concentration

The experiment was carried out in the presence of HPH (**A**) or mHPH (**B**). DNA polymerase activity was followed by measuring poly(rA)$_n$·oligo(dT)$_{12–18}$-directed incorporation of [^{3}H]dTTP into DNA in the absence (●) or in the presence (○) of 2 μM HPH (**A**) or 25 μM mHPH (**B**). The reactions were performed with saturating concentrations of the radiolabelled substrate dTTP (15 μM) and the template-primer (10 μg/ml) for 15 min at 37 °C. The curves were computer-generated by regression analyses and the regression coefficients were as high as 0.99, indicating a highly linear relationship between the rate of reaction and the enzyme concentration.

When $V_{max} = 0$, the RT axis intercept represents the amount of enzyme that is completely removed from the reaction (0.033 μl and 0.022 μl of RT in the presence of HPH and mHPH respectively), resulting in a decrease in V_{max}. In conclusion, HPH and its derivative mHPH are both irreversible inhibitors.

Effects of HPH and mHPH on the binding of HIV-1 RT to template-primer DNA

The first step in DNA synthesis involves physical association of the enzyme with its nucleic acid substrate. Only then does a subsequent dNTP binding step occur [28]. Thus interference with template-primer binding to HIV-1 RT constitutes a possible target for drugs against HIV RT. We have analysed the effects of the natural inhibitor HPH and its methylated derivative on the stability of the RT complexed to a ^{32}P-5′-end-labelled double-stranded DNA oligonucleotide. To this end, we have used a gel shift assay [25]. Complex-formation between the ^{32}P-end-labelled double-stranded oligonucleotide DNA (54mer) and HIV-1 RT was detected (Figure 7, lanes 2–8) by the electrophoretic retardation of the DNA as a result of its association with the enzyme. When the reaction was performed in the presence of increasing concentrations of HPH and mHPH (lanes 3–5 and lanes 6,7 respectively), there was no significant change in the

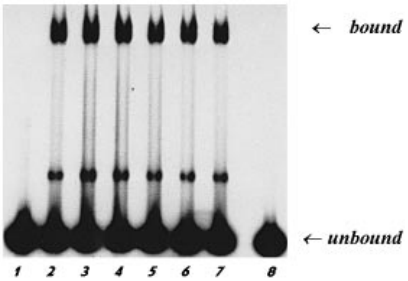


Figure 7 Effects of HPH and mHPH on formation of the HIV-1 RT–DNA complex

The binding of HIV-1 RT to ^{32}P-end-labelled double-stranded oligonucleotide DNA (54mer) was carried out as described in the Materials and methods section. The lanes in the autoradiogram of the gel mobility shift assays are as follows: lane 1, control (no enzyme present); lane 2, binding with no inhibitor; lanes 3–5, binding in the presence of increasing concentrations of HPH [final concentrations of 12.5 μM (lane 3), 25 μM (lane 4) and 50 μM (lane 5)]; lanes 6 and 7, binding in the presence of increasing concentrations of mHPH [final concentrations of 100 μM (lane 6) and 200 μM (lane 7)]; lane 8, binding in the presence of 50 μM toxiusol.

intensity of the bands. Furthermore, quantification of the RT–DNA complexes supports this conclusion, even at concentrations as high as 100 μM HPH and 400 μM mHPH (results not shown). In contrast, the presence of 50 μM toxiusol completely abolished the formation of the DNA–enzyme complex [Figure 7, lane 8; no band is detected, similar to the control with no enzyme present (lane 1)].

DISCUSSION

HPH, a natural product isolated from the Red Sea sponge *Ircinia* sp., is shown here to be a general *in vitro* inhibitor of DNA polymerases from retroviral, prokaryotic and eukaryotic sources. HPH exhibits equal potency in inhibiting the DNA polymerase activity associated with two closely related RTs from the lentivirus subfamily of retroviruses (i.e. those of HIV-1 and HIV-2) and with a more distantly related RT, from MuLV. MuLV RT differs from the two HIV RTs in several enzymic properties, such as its high sensitivity to thiol reagents [24] and its significantly higher fidelity of DNA synthesis [29,30]. Nevertheless, our results are compatible with the fact that retroviral RTs are related proteins with conserved motifs and an overall sequence identity of around 25% [31,32]. The inhibitory activity of HPH occurs irrespective of the primer-template used. Thus the natural inhibitor exhibits potent inhibition of DNA synthesis directed by $poly(rA)_n \cdot oligo(dT)_{12-18}$, $poly(rC)_n \cdot oligo(dG)_{12-18}$ or gapped DNA. It should also be noted that HPH fails to inhibit effectively the HIV-1-RT-associated RNase H function, emphasizing the specificity of the inhibition. In addition, we have shown that HPH is a potential inhibitor of cellular DNA polymerases from three different subfamilies: the first includes the pol I class of DNA polymerases, which is represented in this study by KF; the second subfamily is represented by mammalian pol α (in this study by calf thymus pol α); and the third class of DNA polymerases includes pol β [33].

The natural product, HPH, possesses two hydroxy groups on the hydroquinone ring (Figure 1). We have previously demonstrated the importance and the contribution of hydroxy groups to inhibitory activity against HIV RT, as in the case of the avarol analogues illimaquinone, 3,5,8-trihydroxy-4-quinolone and toxiusol [11,12,14,16]. Therefore we decided to replace the two hydroxy moieties on the quinone ring in order to block the potential inhibitory active sites of the lead compound. As expected, the inhibitory capacity of the methylated derivative was affected significantly. Modification of both hydroxy groups yielded a drug with an approx. 8–21-fold decreased inhibitory capacity with all DNA polymerases tested (Table 1). It is clear that these polar side-residues are important for optimal inhibition and probably contribute to the final formation of the drug–enzyme complex through hydrogen bonding. Indeed, the inhibitory potency of HPH reflects tight binding to the enzyme; a 12-fold greater K_i value is observed when the natural inhibitor, as compared with its methylated derivative, interacts with the RT. It should be noted that the hexaprenyl side chain of HPH is also essential for the formation of the drug–enzyme complex, probably through hydrophobic interactions. The hydroquinone ring by itself is devoid of any inhibitory activity towards HIV-1 RT (S. Loya, A. Rudi, Y. Kashman and A. Hizi, unpublished work). Moreover, the derivative mHPH, as opposed to the hydroquinone, retains in part the potential to inhibit RT, albeit at a significantly decreased level. In short, both the hydroxy groups and the hydrophobic hexaprenyl side chain contribute to the inhibitory capacity of HPH, and thus to combined hydrophilic and hydrophobic interactions with the protein.

The non-competitive inhibition by HPH observed with dTTP (Figure 4A) indicates the presence of an allosteric binding site on the RT that is independent of the incoming dNTP. The mechanism of inhibition by HPH, where the template-primer varies, is, however, dependent on the inhibitor concentration. At high concentrations the mode of HPH inhibition of RT changes from non-competitive to uncompetitive (Figure 4B). This suggests that HPH binding to RT occurs after the template-primer binds to the enzyme. Thus the K_m decreases by 2-fold (from 0.65 to 0.3 μg/ml).

Since HPH is a general inhibitor of all DNA polymerases tested in the present study, i.e. KF, pol α and pol β, as well as of both RNA- and DNA-directed DNA synthesis by various retroviral RTs, we assume that this inhibitory effect is exerted through a common mechanism. DNA polymerases constitute a superfamily of enzymes that use similar machinery for DNA polymerization. By using rapid-quench, pre-steady-state kinetic analysis of HIV-1 RT, the sequential steps and the rate of each step of polymerization have been studied [34–36]. The first step involves physical binding of the enzyme to the DNA, followed by binding of the appropriate dNTP to the RT–DNA complex and subsequent incorporation of the nucleotide by a nucleotidyl-transfer reaction. We have shown, by using the gel-retardation technique, that HPH and its methylated derivative have no substantial influence on the formation or stability of the RT–DNA complex, the first step in DNA polymerization. Therefore we speculate that HPH inhibits subsequent steps in DNA polymerization, such as dNTP binding or the chemical formation of the phosphodiester bond. In this respect HPH differs from toxiusol (another general inhibitor of DNA polymerases) and the HIV RT inhibitors peyssonols A and B and 3,5,8-trihydroxyquinolone, all of which inhibit the binding of the template-primer to the enzyme [15,16,25]. In contrast to these natural inhibitors, TIBO, representing the NNRTI [9], was found by us not to affect the stability of the RT–DNA complex [25].

The NNRTI binding site lies within a hydrophobic pocket near the DNA polymerase active site [37–41]. Rittenger et al. [42] reported recently that the major inhibitory effect of TIBO is on the nucleotidyl-transfer reaction rather than on the dNTP binding step. Since increasing concentrations of HPH do not significantly affect the affinity of the dNTP for the enzyme, we may assume that interference with the nucleotide binding step in the overall polymerization process probably does not account for the inhibition detected. Therefore the inhibitory capacity of HPH might be derived from its effect on the catalytic step leading to the final incorporation of a single nucleotide.

Sequence alignment studies have highlighted conserved sequence motif domains that are common to all DNA-dependent DNA polymerases analysed [33]. Three-dimensional analyses have revealed a similarity in the folding KF and of the comparable p66 DNA polymerase domain of HIV-1 RT. Both exhibit an overall hand-like shape with three subdomains designated as 'palm', 'thumb' and 'fingers' [37,38,43,44]. The most extensive structural similarity between the DNA polymerase domains of the two enzymes is confined to the palm subdomain. In fact, the palm regions of the three enzymes HIV-1 RT, KF and pol β (the only DNA polymerases for which the three-dimensional structures are known so far) superimpose fairly well [45,46]. Motifs A and C, which are situated on β-sheets within the core of the palm subdomain, are the only universally conserved motifs present in all DNA- and RNA-directed DNA polymerases, irrespective of template or substrate specificity [46,47]. Both motifs contain invariant aspartic acid residues that are directly involved in the formation of the phosphodiester bond. A two-metal phosphoryl-transfer mechanism has been proposed for HIV-1 RT [48],

MuLV RT [32] and pol β [49], as opposed to the three-metal mechanism proposed for KF [50]. A general inhibitor would be expected to exert its effect through a mechanism common to all DNA polymerases. Therefore we speculate that HPH interacts with highly conserved motifs common to all DNA polymerases, such as the palm subdomain of the protein.

A general inhibitor such as the natural compound HPH cannot serve as a candidate for an anti-HIV drug. We have found that HPH and, to a lesser extent, its methylated derivative are toxic to mammalian cells in culture (S. Loya, A. Rudi, Y. Kashman and A. Hizi, unpublished work). However, a better understanding of the interactions between the inhibitor and DNA polymerases might result in structural modifications of the lead compound HPH and contribute to the rational design of new effective and selective drugs against HIV RT.

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REFERENCES

1 Barre-Sinoussi, F., Cherman, J. C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., Daguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., et al. (1983) Science **220**, 868–871
2 Gallo, R. C., Salahuddin, S. Z., Popovic, M., Shearer, G. M., Kaplan, M., Haynes, B. F., Palker, T. J., Redfield, R., Oleske, J., Safai, B., et al. (1984) Science **224**, 500–503
3 De Clercq, X. (1992) AIDS Res. Hum. Retroviruses **8**, 119–134
4 Mitsuya, H. and Broder, S. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 1911–1915
5 Reardon, J. E. and Miller, W. H. (1990) J. Biol. Chem. **265**, 20302–20307
6 Richman, D. D., Fischl, 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) N. Engl. J. Med. **317**, 192–197
7 Fischl, M. A., Richman, D. D., Causey, D. M., Grieco, M. H., Bryson, Y., Mildvan, D., Laskin, O. L., Groopman, J. E., Volberding, P. A., Shooley, R. T., et al. (1989) J. Am. Med. Assoc. **262**, 2405–2410
8 Debyser, Z., Pauwls, R., Andries, K. and De Clercq, E. (1992) J. Enzyme Inhib. **6**, 47–53
9 De Clercq, E. (1993) Med. Res. Rev. **13**, 229–258
10 De Clercq, E. (1995) Clin. Microbiol. Rev. **8**, 200–239
11 Loya, S. and Hizi, A. (1990) FEBS Lett. **269**, 131–134
12 Loya, S., Tal, R., Kashman, Y. and Hizi, A. (1990) Antimicrob. Agents Chemother. **34**, 2009–2012
13 Loya, S., Kashman, Y. and Hizi, A. (1992) Arch. Biochem. Biophys. **293**, 208–212
14 Loya, S., Rudi, A., Tal, R., Kashman, Y., Loya, Y. and Hizi, A. (1994) Arch. Biochem. Biophys. **309**, 315–322
15 Loya, S., Bakhanashvili, M., Kashman, Y. and Hizi, A. (1995) Arch. Biochem. Biophys. **316**, 789–786
16 Loya, S., Bakhanashvili, M., Kashman, Y. and Hizi, A. (1995) Biochemistry **34**, 2260–2266
17 Cimino, G., De Stefano, S. and Minale, L. (1972) Tetrahedron **28**, 1315–1324
18 Shaharabany, M. and Hizi, A. (1991) AIDS Res. Hum. Retroviruses **7**, 883–888
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, C. F., Lebherz, W. B., et al. (1990) AIDS Res. Hum. Retroviruses **6**, 753–764
20 Hizi, A., McGill, C. and Hughes, S. H. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 1218–1222
21 Guyader, M., Emerman, M., Songio, P., Clavel, F., Montagnier, L. and Alizon, M. (1987) Nature (London) **326**, 662–669
22 Hizi, A., Tal, R. and Hughes, S. H. (1991) Virology **180**, 339–346
23 Perrino, F. W. and Loeb, L. A. (1989) J. Biol. Chem. **264**, 2898–2905
24 Hizi, A., Tal, R., Shaharabany, M. and Loya, S. (1991) J. Biol. Chem. **266**, 6230–6239
25 Bakhanashvili, M. and Hizi, A. (1994) Biochemistry **33**, 12222–12228
26 Debyser, Z., Pauwels, R., Andries, K., Desmyr, J., Kulkan, M. and Janssen, P. A. J. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 1451–1455
27 Balzarini, J., Perez-Perez, M. J., San Felix, A., Camarasa, M. J., Bathurst, I. C., Barr, P. J. and De Clercq, E. (1992) J. Biol. Chem. **267**, 11831–11836
28 Majumadar, C., Abbots, J., Broder, S. and Wilson, S. H. (1988) J. Biol. Chem. **263**, 15657–15665
29 Bakhanashvili, M. and Hizi, A. (1993) FEBS Lett. **319**, 201–205
30 Bakhanashvili, M. and Hizi, A. (1993) Biochemistry **32**, 7559–7567
31 Johnson, M. S., McClure, M. A., Feng, D.-F, Gray, J. and Doolittle, R. F. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 7648–7652
32 Georgiadis, M. M., Jenssen, S. M., Ogata, C. M., Telesnitsky, A., Goff, S. P. and Hendrickson, W. A. (1995) Structure **3**, 879–892
33 Delarue, M., Poch, O., Tordo, N., Moras, D. and Argos, P. (1990) Protein Eng. **3**, 461–467
34 Kati, W. M., Johnson, K. A., Jerva, L. F. and Anderson, K. S. (1992) J. Biol. Chem. **267**, 25988–25997
35 Reardon, J. E. (1992) Biochemistry **31**, 4473–4479
36 Hsieh, J., Zinner, S. and Modrich, P. (1993) J. Biol. Chem. **268**, 24607–24613
37 Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A. and Steitz, T. A. (1992) Science **256**, 1783–1790
38 Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, Jr., A. D., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., et al. (1993) Proc. Nat. Acad. Sci. U.S.A. **90**, 6320–6324
39 Smerdon, S. J., Jager, J., Wang, J., Kohlstaedt, L. A., Chirino, A. J., Friedman, J. M., Rice, P. A. and Steitz, T. A. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 3911–3915
40 Ren, J., Esnouf, R., Hopkins, A., Ross, C., Jones, Y., Stammers, D. and Stuart, D. (1995) Structure **3**, 915–925
41 Ding, J., Das, K., Moereels, H., Koymans, L., Andries, K., Janssen, P. A. J., Hughes, S. H. and Arnold, E. (1995) Struct. Biol. **2**, 407–415
42 Rittenger, K., Divita, G. and Goody, R. S. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 8046–8049
43 Beese, L. S., Friedman, J. M. and Steitz, T. A. (1993) Biochemistry **32**, 14095–14101
44 Moras, D. (1993) Nature (London) **364**, 572–573
45 Davies, J. F., Almassy, R. J., Hostomsky, Z., Ferre, R. A. and Hostomsky, Z. (1994) Cell **76**, 1123–1133
46 Sawaya, M. R., Pelletier, H., Kumar, A., Wilson, S. H. and Kraut, J. (1994) Science **264**, 1930–1935
47 Sousa, R. (1996) Trends Biochem. Sci. **21**, 186–190
48 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) Biochemistry **34**, 5351–5363
49 Pellitier, H., Sawaya, M. R., Kumar, A., Wilson, S. H. and Kraut, J. (1994) Science **264**, 1891–1903
50 Steitz, T. A. (1993) Curr. Opin. Struct. Biol. **3**, 31–38

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