Polycitone A, a novel and potent general inhibitor of retroviral reverse transcriptases and cellular DNA polymerases

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Polycitone A, an aromatic alkaloid isolated from the ascidian *Polycitor* sp. exhibits potent inhibitory capacity of both RNA- and DNA-directed DNA polymerases. The drug inhibits retroviral reverse transcriptase (RT) [i.e. of human immunodeficiency virus type 1 (HIV), murine leukaemia virus (MLV) and mouse mammary tumour virus (MMTV)] as efficiently as cellular DNA polymerases (i.e. of both DNA polymerases α and β and *Escherichia coli* DNA polymerase I). The mode and mechanism of inhibition of the DNA-polymerase activity associated with HIV-1 RT by polycitone A have been studied. The results suggest that the inhibitory capacity of the DNA polymerase activity is independent of the template-primer used. The RNase H function, on the other hand, is hardly affected by this inhibitor. Polycitone A has been shown to interfere with DNA primer extension as well as with the formation of the RT–DNA complex. Steady-state kinetic studies demonstrate that this inhibitor can be considered as an allosteric inhibitor of HIV-1 RT. The target

site on the enzyme may be also spatially related to the substrate binding site, since this inhibitor behaves competitively with respect to dTTP with poly(rA)·oligo(dT) as template primer. Chemical transformations of the five phenol groups of polycitone A by methoxy groups have a determinant effect on the inhibitory potency. Thus, the pentamethoxy derivative which is devoid of all hydroxy moieties, loses significantly, by 40-fold, the ability to inhibit the DNA polymerase function. Furthermore, this analogue lacks the ability to inhibit DNA primer extension as well as the formation of the RT–DNA complex. Indeed, inhibition of the first step in DNA polymerization, the formation of the RT–DNA complex, and hence, of the overall process, could serve as a model for a universal inhibitor of the superfamily of DNA polymerases.

Keywords: DNA binding, polycitrin A, reverse transcriptase inhibitor, toxiusol.

INTRODUCTION

Reverse transcriptase (RT) plays a critical role in the early steps of the life cycle of the human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS) [1,2], and for over a decade has been one of the major targets of AIDS therapy. This enzyme possesses three catalytic activities, RNA- and DNA-directed DNA polymerases and RNase H, all of which are responsible for the conversion of the HIV-1 single-stranded RNA genome into double stranded DNA [3]. The present anti-RT drugs used to control HIV infection interfere with the DNA polymerase functions of the enzyme. The compounds can be divided into two classes, nucleoside analogues and non-nucleoside RT inhibitors (NNRTI). The nucleoside analogues in their active form lack the 3'-hydroxyl group and thus compete with the natural nucleoside substrate, leading to premature termination of DNA polymerization [4,5]. Unfortunately, their effectiveness in therapy is hampered by toxic side effects [6,7], which may result from the inhibition of cellular DNA polymerases and the rapid emergence of resistant viral strains [8,9].

The NNRTI, on the other hand, which belong to unrelated classes of aromatic compounds, are highly selective against HIV-1 RT and therefore, are low in toxicity and high in their potency of inhibition. As a rule, all NNRTI are allosteric inhibitors of HIV-1, RT, i.e. they interact with the enzyme at a non-substrate binding site. However, these inhibitors, by selecting RT mutations, lead to a very rapid development of drug-resistant

viral strains [10]. The NNRTI are still used in combination therapy with nucleoside analogues, since their separate mechanisms of inhibition lead to synergism [11]. Although combination therapy has now become the standard care against AIDS, no single combination has proved completely effective in all patients and, thus, there is still a need to develop new drugs with disparate inhibition mechanisms.

During our survey of novel marine natural products for their anti-HIV RT activity, we have isolated structurally-diverse non-nucleoside compounds, none of which belonged to the anti-HIV-1 specific NNRTI. These compounds inhibited both HIV-1 and HIV-2 RTs and exhibited either poor or no activity against cellular DNA polymerases [12–16]. In addition, we have isolated two compounds, toxiusol and 2-hexaprenyl-hydroquinone, that exhibit a broad spectrum of inhibitory capacity of the DNA polymerase function [17,18]. Similarly, we report herein, on a new general inhibitor of DNA polymerases. Polycitone A, an alkaloid isolated from a marine ascidian *Polycitor* sp. [19], is a potent inhibitor of retroviral RTs of HIV-1, murine leukaemia virus (MLV) and mouse mammary tumour virus (MMTV) as well as of cellular DNA polymerases, the eukaryotic DNA polymerases α and β and the prokaryotic Klenow fragment of *E. coli* DNA polymerase I (KF). The extent, mode and mechanism of inhibition are described below. We demonstrate that, like toxiusol and unlike 2-hexaprenyl hydroquinone, polycitone A interferes with the first step of DNA polymerization, i.e. the formation of the RT–DNA complex. Structure–activity studies point to the crucial role of the polar hydroxyl moieties of

Abbreviations used: DD, DNA dependent; DDDP, DNA-dependent DNA polymerase; KF, Klenow fragment of *E. coli* DNA polymerase I; MLV, murine leukaemia virus; MMTV, mouse mammary tumour virus; NNRTI, non-nucleoside RT inhibitors; pol β , DNA polymerase β ; RD, RNA dependent; RDDP, RNA-dependent DNA polymerase; RT, reverse transcriptase; TBE, Tris-borate/EDTA.

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polycitone A in obstructing the formation or stability of the RT-DNA complex and hence with the overall process of DNA polymerization.

MATERIALS AND METHODS

Chemicals

The natural compound, Polycitone A (Figure 1) an alkaloid, isolated from the Indo Pacific ascidian *Polycitor* sp. was isolated and identified as described [19]. The pentamethoxy derivative (compound 2) was prepared by methylation of polycitone A with CH_3I in the presence of K_2CO_3 at 60°C for 5 h as described [19] and the tetramethoxy derivative (compound 3) was prepared using a similar procedure except for the methylation time of only 2 h. Polycitrin A (compound 4) was isolated from the same marine organism as described [19]. The trimethoxy derivative of polycitrin A was prepared by the same methylation procedure as described for the pentamethoxy derivative of polycitone A. Synthetic template-primers poly(rA)·oligo(dT) and poly(rC)·oligo(dG) 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 [20].

Enzymes

The RTs of HIV-1 and of MMTV are recombinant enzymes expressed in *E. coli* DH5 α and purified from the bacterial extracts. The HIV-RT, a p66/p51 heterodimer, was derived from the BH-10 clone [21] and was purified to homogeneity as described [22]. 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 six-histidine extension at the N-terminus) as described [23]. The MMTV RT, a p66 monomer, was purified by Ni^{+2} -nitriloacetic acid chromatography followed by carboxymethyl-Sepharose ion exchange chromatography [23]. Recombinant MLV RT, a p70 monomer, was purchased from Amersham. Pol α was purified from calf thymus by immunoaffinity column chromatography and was generously donated by Dr. M. Fry (Technicon, Haifa, Israel). Recombinant pol β was a generous gift from Dr. Z. Hostomsky (Agouron, San Diego, CA, U.S.A.). KF was purchased from New England Biolabs (Beverly, MA, U.S.A.).

Enzyme assays

Enzymatic reactions were performed basically as described previously [24]. The RNA-dependent DNA polymerase (RDDP) activity was assayed by monitoring either the poly(rA)·oligo(dT)-directed incorporation of [^3H]dTTP or the poly(rC)·oligo(dG)-directed incorporation of [^3H]dGTP into DNA. The DNA-directed DNA polymerase (DDDP) activity associated with all retroviral RTs, pol α , pol β and KF was assayed using activated gapped DNA and four deoxynucleotides (of which only one, dTTP was labelled). The assays were carried out at 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 $\mu\text{g/ml}$ activated DNA, 25 μM of each unlabelled dATP, dGTP, dCTP and [^3H]dTTP at a final concentration of 5 μM (1800–3500 cpm/pmol). RNase H activity of HIV-1 RT was assayed by measuring the release of trichloroacetic acid-soluble material from the synthetic substrate [^3H]poly(rA)·poly(dT) as described previously [24]. One unit of DNA polymerase activity is defined as the amount of enzyme that catalyses the incorporation of 1 pmol dNTP into DNA product for 30 min at 37°C under standard assay conditions. Inhibition of enzyme activity was calculated relative to the initial linear rates observed under identical conditions when no drug was added.

In all inhibition experiments, the enzymes were preincubated with increasing concentrations of the inhibitor for 3 min at 30°C . The reaction was initiated by adding the relevant [^3H]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 concentrations using computer-generated linear regression analyses.

DNA primer extension

Single-stranded circular $\phi\text{X174am3}$ DNA (New England Biolabs) was primed with a synthetic 15mer oligonucleotide that hybridizes with the DNA employed at positions 588–602 [25]. 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 per-

- 1 polycitone A, R=R'=H
2 pentamethoxy derivative R=R'=Me
3 tetramethoxy derivative R=Me, R'=H

- 4 polycitrin A, R=H
5 trimethoxy derivative R=Me

Figure 1 The structural formulae of polycitone A and its derivatives and of polycitrin A and its derivative

formed in 20 mM Tris/HCl at pH 7.5, 2 mM DTT, 10 mM MgCl₂ and 0.1 mg/ml bovine serum albumin. The reactions were divided into two sets of experiments. In one set of experiments the enzyme was preincubated with increasing amounts of inhibitor for 3 min on ice prior to initiating the polymerization reaction with the end-labelled template-primer and all four dNTPs (each at a final concentration of 10 μ M). In the second set of reactions, the polymerization was allowed to initiate for 2 min on ice before adding increasing amounts of inhibitor. All reactions, each at a final volume of 12.5 μ l, were incubated at 37 °C for 20 min. The reactions were stopped by adding an equal volume of formamide dye mix, denatured at 100 °C for 3 min, quickly chilled on ice and analysed by electrophoresis on 8% polyacrylamide-urea gels as described earlier [25]. The gel autoradiographs were analysed by a densitometry scanning procedure.

Binding assays

The binding of HIV-1 RT to a double-stranded oligonucleotide was measured by using PAGE-band mobility shift assays [26]. Complex formation between [³²P]-5'-end labelled oligonucleotide and HIV-1 RT is detected by the electrophoretic retardation of the DNA as a result of its association with the enzyme. HIV-1 RT was incubated for 10 min at 32 °C with an oligonucleotide duplex (oligo 1/oligo 2; nucleotide sequence as described in [27]) of 54 bp with recessed 2nt 3'-end, in which only one strand (oligo 1) was radioactively end-labelled at 5'-end with ³²P. The binding reaction assays were conducted in a final volume of 12.5 μ l containing 10 mM Hepes-KOH, pH 8.0, 30 mM ammonium sulphate, 0.25 mM dithiothreitol, 20 μ g/ml bovine serum albumin, 10 mM KCl, 1.2 pmol HIV-1 RT and 0.12 pmol of the labelled oligonucleotide template primer. The reaction mixtures were electrophoresed through 6.5% polyacrylamide gel in TBE at 4 °C under 15 V/cm for about 3 h. The gel autoradiographs were analysed by densitometry scanning procedure.

RESULTS

The effect of polycitone A on HIV-1 RT

The natural alkaloid Polycitone A was isolated from the Indo Pacific ascidian *Polycitor* sp. and identified as described [20]. This compound has been found to be a very potent inhibitor of HIV-1 RT-associated DNA polymerase activities. The IC₅₀ values were calculated from the dose-response curves and were as low as 245 $\pm$ 15 nM and 470 $\pm$ 22 nM for the RNA- and DNA-directed DNA polymerase functions, respectively (Figure 2 and Table 1). Contrary to this high inhibitory potency, polycitone A displays a relatively weak capacity to inhibit the RNase H activity of HIV-1 RT, with an IC₅₀ value of 30 $\pm$ 5 μ M (a 120-fold increase in the IC₅₀ value compared with that of RDDP activity, Figure 2). It is clear that polycitone A preferentially inhibits the DNA polymerase function. Consequently, we have continued the study with polycitone A only on its effects on the RT-associated DNA polymerase activities. It was reported that the efficacy of inhibition may vary according to the template-primer used. For example, tetrahydroimidazo[4,5,1-jk][1,4]-benzodizepine-2(1H)one and thione (TIBO) exhibit a marked template-primer preference, i.e. with poly(rC)·oligo(dG) the IC₅₀ is 17-fold lower than with poly(rA)·oligo(dT) as the template-primer [27]. When we measured the capacity of polycitone A to inhibit HIV-1 RT with different synthetic template-primers, no substantial difference was detected. Thus, the IC₅₀ values derived from inhibition curves (not shown) are 245 $\pm$ 5 nM and 295 $\pm$ 15 nM for poly(rA)·oligo(dT) and poly(rC)·oligo(dG)-

Figure 2 The effect of polycitone A on HIV-1 RT-associated RDDP, DDDP and RNase H activities

The dose-response experiments were carried out as described in the Materials and methods section. The DNA polymerase activity was assayed by following either the poly(rA)·oligo(dT)-directed (●) or the activated DNA-directed DNA synthesis (○). The RNase H activity (▲) was assayed by following the release of trichloroacetic acid-soluble material from the labelled substrate in the presence of increasing concentrations of polycitone A. The residual activities were calculated as percentage of control samples with no inhibition.

Table 1 Effects of polycitone A on the DNA polymerase activities of retroviral RTs and cellular DNA polymerases

The IC₅₀ and IC₉₅ values are derived from dose-response curves and assayed with either poly(rA)·oligo(dT)-direct or activated-DNA-directed DNA synthesis in the presence of increasing concentrations of polycitone A. The data represent mean values $\pm$ S.D. of at least three independent experiments with duplicate determinations in each experiment. RD, RNA dependent; DD, DNA dependent.

Enzyme	DNA polymerase function	IC ₅₀ (nM)	IC ₉₅ (nM)
HIV-1 RT	RD	245 $\pm$ 15	477.5 $\pm$ 22.5
	DD	470 $\pm$ 21.6	1750 $\pm$ 70
MuLV RT	RD	73 $\pm$ 7	230 $\pm$ 20
	DD	410 $\pm$ 10	985 $\pm$ 35
MMTV RT	RD	225 $\pm$ 35	845 $\pm$ 35
	DD	587.5 $\pm$ 62.5	2213 $\pm$ 63
Calf-thymus pol α	DD	285 $\pm$ 5	815 $\pm$ 25
Human pol β	DD	600 $\pm$ 16	1670 $\pm$ 70
KF	DD	92.5 $\pm$ 7.5	380 $\pm$ 20

directed DNA synthesis, respectively. These IC₅₀ values are also quite similar to that obtained with the activated DNA. Thus, the efficiency of polycitone inhibition seems to be independent of the template-primer used.

The effect of polycitone A on other retroviral RTs and cellular DNA polymerases

To assess whether polycitone A affects also other retroviral RTs, we have tested its inhibitory effect on RTs of both type C and B retroviruses, i.e. of MLV and MMTV, respectively. These two retroviruses are distantly related to HIV-1, a prototype of the lentivirus subfamily of retroviruses. As can be seen in Table 1, the response to the inhibitor of these RTs is similar to the one displayed by the RT of HIV-1. Polycitone A, effectively inhibited the RNA- and DNA-dependent DNA polymerase activities

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

The double reciprocal plots of increasing concentrations of either dTTP (panel A), dGTP (panel B) and poly(rA)·oligo(dT) (panel C) as a function of the initial velocity of [3 H]dNTP incorporation by HIV-1 RT. **A**, The enzymatic reaction in the absence (●) or in the presence of 100 nM (○), 200 nM (▲) and 400 nM (■) polycitron A. **B**, The enzymatic reaction in the absence (●) or in the presence of 150 nM (○), 300 nM (▲) and 450 nM (■) polycitron A. **C**, Reactions in the absence (●) or in the presence of 100 nM (○), 200 nM (▲) and 300 nM (■) polycitron A. The plots were computer-generated by linear regression analyses, and the regression coefficient values (r) ranged between 0.98 and 0.99, indicating a high degree of linear relationships between the reciprocal velocity of the enzymatic reaction and the reciprocal concentration of the substrate.

Table 2 Effects of polycitron A and its methylated derivatives (2 and 3), polycitrin A (4) and its methylated derivative (5) on the HIV-1 RT associated DNA polymerase and RNase H activities

The IC_{50} values are derived from dose-response curves as described in the Materials and methods section. All data represent the mean $\pm$ S.D. of 2–4 independent experiments with duplicate determinations in each experiment. The figures in parentheses are the ratio between the IC_{50} values calculated for the indicated compound and that of polycitron A.

Compound	DNA polymerase IC_{50} (μ M)	RNase H IC_{50} (μ M)
1. Polycitron A	0.25 ± 0.01 (1)	30 ± 5
2. Pentamethoxy derivative	9.3 ± 2 (38)	≥ 100
3. Tetramethoxy derivative	3.7 ± 0.1 (15)	≥ 100
4. Polycitrin A	4.75 ± 0.75 (19.5)	≥ 100
5. Trimethoxy derivative	14.9 ± 2.3 (61)	≥ 100

(RDDP, DDDP) of both enzymes. The IC_{50} values for MLV are 73 ± 7 nM and 410 ± 10 nM, for the RDDP and DDDP functions respectively, and for MMTV RT, 225 ± 35 nM for the RDDP activity and 588 ± 63 nM for the DDDP function. The general inhibitory pattern exhibited by all RTs studied is similar. Nor could we detect a significant dichotomy between the sensitivities to the inhibitor of RDDP and DDDP activities associated with the retroviral RTs studied. Thus, we have extended the study and analysed the capacity of polycitron A to inhibit other cellular DNA polymerases from prokaryotic and eukaryotic sources. As summarized in Table 1, the natural inhibitor effectively inhibited the DNA-directed DNA synthesis of KF, human pol β and calf-thymus pol α , with IC_{50} values of 93 ± 8 nM, 600 ± 16 nM and 285 ± 5 nM, respectively. In short, polycitron A is a general inhibitor of the DNA polymerase activity, regardless of the template-primer used and the enzyme studied. The general pattern of the response to the inhibitor of all enzymes studied is almost identical. As a result, we have chosen to extend the study on the mode and mechanism of polycitron A inhibition only with HIV-1 RT, representing the DNA polymerases tested.

Mode of inhibition of DNA polymerase activity of HIV-1 RT by polycitron A

The mode of inhibition of HIV-1 RT by polycitron A was analysed under various conditions. Double reciprocal plots of initial velocity vs. substrate concentrations reveal that the inhibition of the enzyme by polycitron A is competitive with respect to dTTP as the variable substrate [with poly(rC)·oligo(dT) as template-primer; Figure 3A]. The apparent V_{max} is unaffected by the inhibitor and is 92 ± 8 pmol dTTP/10 min under the experimental conditions employed. Conversely, the apparent K_m values for dTTP continually increases as a function of increasing concentrations of the inhibitor. The K_i value of 132 nM was derived from a replot of the apparent K_m values vs. the corresponding inhibitor concentrations at which they were obtained (not shown). Polycitron A binds the enzyme with greater affinity than the substrate dTTP ($K_i/K_m = 9.6 \times 10^{-3}$). Interestingly, this mode of inhibition is not general, since in experiments where dGTP is the variable substrate [with poly(rC)·oligo(dG) as template-primer], a noncompetitive inhibition is observed (Figure 3B). The apparent K_m value is 7.65 ± 1.2 μ M, irrespective of the presence of polycitron A. Conversely, the V_{max} values are suppressed as a function of increasing inhibitor concentrations. The inhibition constant (K_i)

Figure 4 DNA primer extension in the presence of increasing concentrations of polycitone A (A) and its pentamethoxy derivative (B)

Reactions were performed with single stranded ϕ X174am3 DNA annealed to 15mer [32 P]-end labelled primer and the DNA products were analysed by polyacrylamide-urea gel electrophoresis as described in detail in the Materials and methods section. Molecular size markers were *Hinf*I-cleaved double-stranded dephosphorylated ϕ X174 DNA (Promega). The markers were 5'-end labelled with [γ - 32 P]ATP as described previously [51]. The arrows indicate the nucleotide lengths of single-stranded DNA fragments. **A**, lane m, marker; lane 1, control with no enzyme; lanes 2–6 preincubation of enzyme with increasing amounts of polycitone A prior to initiation of reaction at 37 °C for 20 min [at a final concentration of 0, 2.5, 5, 10 and 20 μ M, respectively]; lanes 7–12, the reaction was allowed to initiate for 2 min on ice before adding increasing amounts of polycitone A [0, 2.5, 5, 10 and 20 μ M inhibitor in lanes 8–12, respectively], and further incubation for 20 min at 37 °C; lane 7, the reaction was stopped with equal volume of formamide–dye mix after 2 min incubation on ice. **B**, lane m, marker; lane 1, control with no enzyme; preincubation of enzyme without inhibitor (lane 2) and with 20 μ M pentamethoxy derivative of polycitone A (lane 3); primer extension allowed to initiate for 2 min on ice after which the reaction was either stopped (lane 4), or further incubated at 37 °C for 20 min without (lane 5) or with 20 μ M pentamethoxy derivative of polycitone A (lane 6).

is derived from a replot of the intercepts from Figure 3B (not shown). The plot is linear ($r = 0.98$) and yields a K_i value of 216 nM. The kinetics of inhibition were also studied with various concentrations of poly(rA)·oligo(dT) and found to be of a mixed-type, which is a form of non-competitive inhibition (Figure 3C). Polycitone A and the template-primer bind the enzyme independently of each other. A K_i value of 72 nM was derived from the replot of either the intercepts or the slopes obtained from Figure 3C (not shown). The fact that both replots were linear ($r = 0.99$) indicates that the polycitone A affects the affinity of the enzyme for the substrate. Thus, the apparent K_m value increases by 3-fold from 0.44 μ g/ml to 1.3 μ g/ml.

The effects of polycitone A derivatives on HIV-1 RT

To analyse the active moieties in polycitone A that are required to potentiate the inhibition of the DNA polymerase activity, we have prepared the pentamethoxy (compound 2) and the tetramethoxy (compound 3) derivatives. The effect of these compounds on the polymerase activity of HIV-1 RT has been examined with poly(rA)·oligo(dT) as template primer. Compound 2, in which the phenol functionality in each of the aromatic rings is methylated, substantially loses its ability to inhibit the enzyme. A 40-fold increase in the IC_{50} value is observed compared with that of the parental aromatic alkaloid

(Table 2). Methylation of four hydroxyl moieties, leaving only one hydroxyl group as in derivative 3, reduces significantly the capacity to inhibit the HIV-1 RT DNA polymerase activity (by 15-fold), but improves by about 3-fold this activity compared with the pentamethoxy derivative. The natural triphenyl polycitrin A (compound 4, Figure 1), displays an IC_{50} value of about $4 \mu M$, which is at least 15-fold higher compared with that of polycitron A. That is to say, the absence of the two phenolic rings affects the inhibitory potential. As can be seen in Table 2, the trimethoxy derivative of polycitrin A (compound 5), in which all three hydroxyl groups are blocked by methyl groups, exhibits an IC_{50} value of about $15 \mu M$. That means at least a 3-fold reduction in the inhibitory capacity compared with parental compound 4. In all, compound 5 is at least 60-fold less effective than polycitron A.

As can be seen in Table 2, the weak capacity to inhibit the HIV-1 RT-associated RNase H activity exhibited by the natural inhibitor is completely lost by derivatives 2 and 3, polycitrin A (compound 4) and its derivative, compound 5. Even at high inhibitor concentrations ($100 \mu M$) no inhibitory activity is detected.

DNA synthesis in the presence of polycitron A and its pentamethoxy derivative

To attain a better understanding of the mechanism of inhibition of the HIV-1 RT DNA polymerase activity, we have followed the capacity of the enzyme to extend a 5'-end-labelled 15mer primer, (using $\phi X174$ am3 DNA as template) in the presence of polycitron A and its pentamethoxy derivative (Figure 4). Increasing concentrations of inhibitor were preincubated with RT for 3 min prior to addition of the template-primer and all four dNTPs (Figure 4A, lanes 2–6; Figure 4B, lanes 2, 3). In parallel experiments, DNA extension was initiated and allowed to continue for 2 min on ice before adding the inhibitor at increasing concentrations (Figure 4A, lanes 7–12; Figure 4B, lanes 4–6). As can be seen, HIV-1 RT is capable of extending the primer up to about 500mer. There are several strong pausing sites with the most apparent ones obtained under the assay conditions employed, at the approximate positions of 90mer, 120mer and 300mer. Interestingly, the 90mer pause is enhanced in the presence of the inhibitor. It was already reported by us that there are no obvious patterns in the sequence of the $\phi X174$ am3 DNA template that are responsible for these pausing positions [24,26]. It was, therefore, suggested that secondary structures of the single stranded DNA may contribute to these pausing patterns. Thus, we speculate that conformational changes induced by the drug may have an effect on the 90mer pause position. Polycitron A interferes with primer extensions, i.e. at $2.5 \mu M$ (Figure 4A, lane 3). Here, the longest product extends only up to 140mer. At higher inhibitor concentrations, i.e. 5, 10 and $20 \mu M$, the DNA synthesis is completely blocked, i.e. no DNA extension is detected (Figure 4A, lanes 3–6). When polycitron A is added after extension has been allowed to continue for 2 min on ice (Figure 4A, lane 7), it is apparent that the inhibitor affects extension, although to a significantly lesser extent than that obtained in the former set of experiments. While in the absence of drug, the primer is extended up to 500mer in length, in the presence of increasing inhibitor concentrations (i.e. 2.5, 5, 10 and $20 \mu M$) the primer is extended only to 140, 90, 90 and 66mer, respectively (Figure 4A, lanes 9–12). It seems that the polycitron A inhibits the HIV-1 RT polymerase by preventing its reassociation with the DNA primer after it dissociates from the template-primer during extension. This idea is further supported by the results obtained with the pentamethoxy derivative, com-

Figure 5 Effect of polycitron A and its pentamethoxy derivative on the formation of HIV-1 RT-DNA complex

PAGE band mobility shift assays were performed as described under the Materials and methods section. The lanes in the autoradiograms are as follows: lane 1, no enzyme present; lane 2, binding in the absence of inhibitor; lanes 3–7, binding in the presence of increasing concentrations of polycitron A (2.5 , 5 , 10 , 20 and $50 \mu M$ inhibitor, respectively); binding in the presence of $10 \mu M$ (lane 8) and $50 \mu M$ (lane 9) of the pentamethoxy derivative of polycitron A; binding in the presence of $50 \mu M$ toxiusol (lane 10).

ound 2. As can be seen in Figure 4B, the extension patterns with and without inhibitor are indistinguishable (lanes 2, 3, 5, 6). Moreover, no difference can be detected in the overall DNA extension if the inhibitor was added to the reaction before initiation of DNA synthesis (lanes 2, 3) or, alternatively, after it was initiated (Figure 4B, lanes 5, 6).

Effects of polycitron A and its pentamethoxy derivative on the binding of HIV-1 RT to template-primer

The above results have led us to examine the possibility that polycitron A interferes with the interaction of the enzyme with DNA template-primer. It is well accepted that DNA polymerization is an ordered reaction in which the physical association of the enzyme with its template-primer precedes the binding of the enzyme to dNTP [28]. Therefore, disturbing this step constitutes another drug target for anti-HIV RT therapy. We have used a gel shift assay to analyse the effect of polycitron A on the binding of RT to double-stranded DNA oligonucleotide (Figure 5). As expected, a complex formation between the [^{32}P]-5'-end-labelled oligonucleotide (54mer) and HIV-1 RT is detected (Figure 5, lane 2) due to the retardation of the electrophoretic mobility of the DNA as a result of its association with the enzyme. When the binding reaction is performed in the presence of increasing polycitron A concentrations (lanes 3–7), a marked drop in the intensity of the RT-bound DNA band is observed. Polycitron A inhibits either the formation or the stability of DNA-RT complexes and the IC_{50} value is somewhere between 5 and $10 \mu M$ inhibitor. The pentamethoxy derivative, on the other hand, does not affect the level of the RT-DNA complex even at concentrations as high as $50 \mu M$ (Figure 5, lanes 8, 9). In contrast, at this high concentration, polycitron A and toxiusol completely abolish the DNA-enzyme complex (Figure 5, lanes 7, 10, respectively), since no band is detected, similar to the control with no enzyme present (lane 1). These results are fully compatible with the relative insensitivity of RT to the polycitron A pentamethoxy derivative (compound 2) observed above (Table 2) and

to the lack of the effects on the DNA primer extension patterns obtained in the presence or absence of this compound (see Figure 4B).

DISCUSSION

We have shown in the present study that polycitone A is a general inhibitor of DNA polymerases. This compound inhibits preferentially and with high potency the RNA- and the DNA-directed DNA synthesis of HIV-1 RT. The RNase H function associated with this enzyme, on the other hand, is hardly affected by polycitone A. Unlike the conventional NNRTIs, polycitone A was found to be also a potent inhibitor of HIV-2 RT (unpublished data). Moreover, this compound inhibits as efficiently as the lentiviral RTs other more distantly-related RTs of both C and B retroviruses, i.e. of MLV and MMTV prototypes, respectively. These results support the notion that reverse transcriptases from different genera share similar conserved motifs, that is, about one- to two-thirds of the approx. 175 acid residues that constitute the most conserved part of RT [29]. In contrast to the NNRTIs, which exhibit marked differences in their template-primer preferences [30], the inhibitory capacity of polycitone A does not depend on the template-primer used. Furthermore, the enzymatic activity of both the prokaryotic DNA polymerase KF and the eukaryotic DNA polymerases α and β are also efficiently impaired in the presence of this natural inhibitor.

Polycitone A is a non-competitive inhibitor of HIV-1 RT with respect to both dGTP and the template-primer poly(rA)·oligo(dT) and hence, can be considered as an allosteric inhibitor of HIV-1 RT. However, despite the fact that polycitone A bears no structural relationship to dTTP, it is a competitive inhibitor with respect to dTTP. This indicates that the inhibitor binding site on the enzyme may be functionally or spatially related to the substrate binding site. Similar results have already been obtained by others with some NNRTIs [10,31,32].

Initial structure-activity relationship studies with polycitone A derivatives, DNA primer extension and gel-shift binding studies point to the importance of the free polar moieties in each of the five aromatic rings present in the natural intact molecule. It is possible that the lipophilic structure of polycitone A interacts with the hydrophobic region of the enzyme, whereas the polar negatively-charged groups may contribute to the formation of the enzyme-inhibitor complex through hydrogen bonding. These results are in line with our previous findings that phenol groups are key elements in anti-HIV-1 RT activity [12,13,15,17,18]. In short, optimal anti-HIV-1 RT inhibitory effects are achieved by an intact molecule containing both the N-acylated tyramine unit and the two types of dibromophenol moieties. Polycitone A like other reported inhibitors [15,14,17] interferes with the physical binding of the RT to the cognate double-stranded oligonucleotide substrate. The drug binds to RT with an affinity higher than that of the substrate ($K_i < K_m$), destabilizing the RT-DNA complex, and thereby inhibiting the overall polymerization function. On the other hand, TIBO, representing a conventional NNRTI, was found not to affect the stability of the RT-DNA complex [26]. These NNRTIs appear to inhibit specifically HIV-1 RT by interacting with an hydrophobic pocket near the active site of the enzyme, and as a result, a switch to a more stable but inactive conformation of key residues occurs, mimicking the inactive polymerase site in p51 [33]. The fact that polycitone A destabilizes the RT-DNA complex has led us to test whether mutations of HIV-1 RT in residues 74 and 89 would be resistant to this drug. These residues are located in a position shown to interact directly with template-primer [34,35]. The resistance to nucleoside analogues due to mutations in these residues is a result of

repositioning of the template-primer [35–37]. We have found that Leu74Val and Glu89Gly mutants of HIV-1 RT are as sensitive to polycitone A as the wild-type HIV-1 RT (data not shown).

A general inhibitor should exert its inhibitory activity through a common mechanism shared by all DNA polymerases. Indeed, the fact that polycitone A perturbs the RT-DNA complex complies with such an idea. Rapid quench pre-steady-state kinetic analyses supports the notion of a common stepwise polymerization mechanism where the template-primer binds first to the enzyme [38–40]. Crystallographic studies further support the notion that all DNA polymerases have remarkable structural similarities [41–46], even though they share only limited sequence homologies. The polymerase 'superfamily' reveals a characteristic common protein folding format an overall right-hand-like shape with three subdomains designated as 'palm', 'thumb' and 'fingers' [42–43]. The two motifs A and C within the core of the palm subdomain are the only universally conserved motifs present in all polymerases [45,47]. Both motifs contain carboxylic acid residues thought to participate directly in catalysis through a two-metal phosphoryl transfer mechanism as proposed for HIV-1 RT [48], MLV RT [46] and pol β [49] or three-metal mechanism suggested for KF [50]. In HIV-1 RT the conserved active site, Tyr183-Met184-Asp185-Asp186 sequence, may have a direct effect on the incoming dNTP [35]. Thus, the mutant Met184Val of HIV-1 RT leads to resistance to both nucleoside analogues ddI (didanosine) and 3TC (3'-thiacytidine). When the two single mutants of HIV-1 RT Tyr183Phe and Met184Leu were tested by us for their response to polycitone A in comparison with the parental wild-type RT, no difference was detected (data not shown). This implies that residue 184 and the hydroxyl group of residue 183 are probably not the target sites for this inhibitor. We assume, therefore, that polycitone A like toxiusol, probably binds the enzyme in a highly conserved motif commonly shared by all DNA polymerases.

Taken together, the binding of polycitone A to DNA polymerase leads probably to subtle conformational changes, thereby preventing the template-primer from occupying its respective site in a manner appropriate for catalysis. Inhibition of the formation of the initial enzyme nucleic acid complex prevents the further binding to dNTP to form a catalytically-competent ternary complex. This is one of the major mechanisms of inhibition expected from a general inhibitor of DNA polymerases. The results presented here indicate that polycitone A, by being a general inhibitor of DNA polymerases cannot serve as an anti-HIV drug. However, we believe that structural modification, together with the identification of the active inhibitory sites of the natural inhibitor, can be an important step towards the rational design of new derivatives with selective anti-HIV RT and possibly anti-AIDS drug.

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