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CHARACTERIZATION OF A NOVEL INHIBITOR OF HUMAN DNA POLYMERASES: 3,4,5-TRI-*O*-GALLOYLQUINIC ACID*

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Abstract—Various galloyl derivatives of quinic acid were found to be inhibitors of human DNA polymerases. Among them, 3,4,5-tri-*O*-galloylquinic acid (TGQA) was the most potent inhibitor of DNA polymerase α. Under identical conditions, this compound was 60-fold more potent than aphidicolin as an inhibitor of DNA polymerase α. The inhibition of DNA polymerase α by this compound was not competitive with either the template or any of the deoxynucleoside triphosphates with a K_i of 0.28 μM. Under similar reaction conditions, DNA polymerases β and γ were much less sensitive to the effects of these compounds and, in contrast to the effect seen with DNA polymerase α, the inhibition of DNA polymerases β and γ by TGQA was competitive with respect to the template with K_i values of 44.4 and 7.5 μM respectively. The potency of these compounds against DNA polymerase γ varied according to the assay conditions used. The inhibition of DNA polymerase γ by TGQA could be increased substantially by using $MnCl_2$ in place of $MgCl_2$ and by including 50 mM potassium phosphate, pH 7.5, in the assay mixture. DNA polymerase β was also more sensitive to TGQA when measured with $MnCl_2$. However, potassium phosphate had little, if any, effect on the inhibition by TGQA of either DNA polymerase α or β. DNA polymerase α was less sensitive to TGQA when assayed with $MnCl_2$. TGQA was not a potent inhibitor of human KB cell growth in culture, which could be due to its degradation or poor uptake. Nevertheless, this compound could serve as a model for developing antitumor drugs targeted at DNA polymerases.

DNA polymerases have received much consideration as potential targets of antiviral and antitumor drugs. A number of compounds [i.e. aphidicolin, Refs. 1 and 2] have been isolated from natural sources which are specific inhibitors of DNA polymerases. Recently, galloyl derivatives of glucose (gallotannins) were reported to be selective inhibitors of reverse transcriptase from avian myeloblastosis virus [3]. We have isolated a number of galloyl derivatives of quinic acid from tannic acid and have found that they are also potent inhibitors of HIV reverse transcriptase and human DNA polymerases [4]. The structures of these compounds can be seen in Fig. 1 and consist of a quinic acid moity which is linked to one or more gallic acid moities through an ester linkage with the hydroxyl groups at C-3, C-4, and C-5 of quinic acid. In this work we have examined the effects of the galloyl derivatives of quinic acid on DNA polymerases α, β, and γ. Because of the potency of these compounds against DNA polymerase α, this work suggested that these structures should be explored as potential antitumor agents.

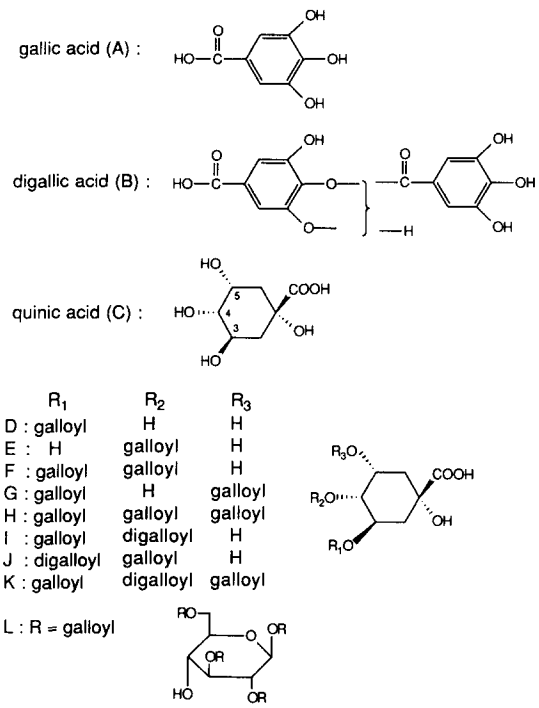

Fig. 1. Structures of galloyl derivatives of quinic acid.

METHODS

Chemicals and supplies. The nucleoside triphosphates used in this study (TTP, dATP, dCTP, and dGTP) were obtained from the Sigma Chemical Co. (St Louis, MO). [*Methyl*-^{3}H]TTP (20 Ci/mmol), [5-^{3}H]dCTP (26 Ci/mmol), [8-^{3}H]dATP (20 Ci/mmol),

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and [8-^{3}H]dGTP (20 Ci/mmol) were obtained from ICN Radiochemicals (Irvine, CA). Poly(rA) oligo(dT)$_{10}$ and poly(dA) oligo(dT)$_{10}$ were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Aphidicolin was obtained from the Drug Synthesis and Chemistry Branch at the National Cancer Institute (Bethesda, MD). Compounds A, B, and C were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Galloylquinic acids were isolated from tannic acid, which was obtained from the Aldrich Chemical Co. Compounds D, E, F, G, H [see Refs 5–7], and L [see Ref. 8] were known compounds and were characterized by comparing their physical data with those reported in the literature [5–8]. Compounds I, J, and K are new compounds and will be reported elsewhere [4]. Compound M [9] and compound N [6] were derived from compound H using methods in the literature. All other chemicals were of standard analytical grade.

Purification of DNA polymerases α, β, and γ. DNA polymerase α and DNA primase were purified from chronic myelogenous leukemia cells (K 562 cells) grown in culture as described previously [10]. The specific activity of DNA polymerase α was 94 units per mg protein. DNA polymerases β and γ were purified from chronic lymphocytic leukemia cells obtained from patients by leukophoresis as described [11]. The specific activity of DNA polymerase β was 1000 units per mg of protein, and for DNA polymerase γ it was 61 units per mg of protein. One unit of DNA polymerase activity is defined as the amount that catalyzes the incorporation of 1 nmol of TMP into the acid-insoluble fraction per hr at 37°.

Enzyme assays. Unless otherwise indicated, DNA polymerase α activity was assayed in 50-μl reactions containing 25 mM Tris, pH 8.0, 1 mg/ml bovine serum albumin, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg/ml of activated DNA [12], 10 μM [^{3}H]TTP (1 Ci/mmol), 50 μM each of dATP, dCTP, and dGTP, and approximately 0.01 units of DNA polymerase activity. After incubation at 37° for the desired time, the DNA was precipitated onto glass fiber filters with a 5% trichloroacetic acid, 10 mM pyrophosphate solution and counted for radioactivity as previously described [10]. DNA polymerases β and γ were assayed in the same manner except that 100 mM KCl was included in the reaction. RNA-dependent DNA polymerase activity was measured in 50-μl reactions containing 25 mM Tris, pH 8.0, 1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 0.5 mM $MnCl_2$, 25 μg/ml poly(rA) oligo(dT)$_{10}$, 100 mM KCl, 5 μM [^{3}H]TTP (1 Ci/mmol), and 0.01 units of DNA polymerase γ. After incubation at 37° for the desired time, the DNA was precipitated onto glass fiber filters as described above.

RESULTS

Structure–activity relationships of galloyl derivatives of quinic acid. Various galloyl derivatives of quinic acid were isolated from tannic acid, and their abilities to inhibit DNA polymerases α, β, and γ were determined (Table 1). Note that the concentration of each compound used in the DNA polymerase α assay was 0.33 μg/ml, in the DNA polymerase β assay it was 10 μg/ml, and in the DNA polymerase γ assay it was 33 μg/ml. Gallic acid, quinic acid, digallic acid, 3-*O*-galloylquinic acid, and 4-*O*-galloylquinic acid (compounds A through E) did not inhibit DNA polymerase α at the concentration tested. The addition of galloyl groups to the C-3 and C-4 hydroxyl of quinic acid (compound F) did result in a compound which was active against DNA polymerase α. However, the addition of galloyl groups at the C-3 and C-5 hydroxyls of quinic acid (compound G) did not produce an active compound. The substitution of either the 3 or 4 gallic acid moity of 3,4-di-*O*-galloylquinic acid (compound F) with digallic acid (compounds I and J) modestly increased the potency of this compound. The most potent inhibitor of DNA polymerase α was 3,4,5-tri-*O*-galloylquinic acid (TGQA, compound H). The addition of a galloyl group to the C-4 galloyl of TGQA (Compound K) did not result in a compound with increased ability to inhibit DNA polymerase α. When these galloylquinic acids were tested against DNA polymerase β at 10 μg/ml, only 3,5-di-*O*-galloyl-4-*O*-digalloylquinic acid (compound K) was active. DNA polymerase γ activity was inhibited 80% by 33 μg/ml 3,4,5-tri-*O*-galloylguinic acid (compound H) and was totally inhibited by 33 μg/ml of 3,5-di-*O*-galloyl-4-*O*-digalloylquinic acid (compound K). Tetra-*O*-galloyl glucose (compound L, a gallotannin) also inhibited all of these DNA polymerases, which indicated the importance of the galloyl groups to the inhibition seen with these compounds. Methylation of the carboxylic acid of the quinic acid reduced the inhibitory potential of these compounds against DNA polymerases α and γ, as did the methylation of all of the hydroxyls (compare the action of compounds M and N with that of compound H).

Characterization of TGQA. Because TGQA (compound H) was the most sensitive inhibitor of DNA polymerase α, we chose it to further characterize this class of compounds. Under similar assay conditions, DNA polymerase α was 230- and 500-fold more sensitive to this compound than was DNA polymerase γ or β respectively (Fig. 2). Furthermore, TGQA was approximately 60-fold more potent than aphidicolin as an inhibitor of DNA polymerase α. To determine whether or not these compounds inhibited these polymerases in competition with any of the deoxynucleoside triphosphates, the inhibition of DNA polymerase activity by TGQA was determined using two concentrations of each radiolabeled deoxynucleoside triphosphate (Table 2). These data indicated that TGQA did not compete with any of the deoxynucleoside triphosphates. For DNA polymerase α, the inhibition seen with TGQA was noncompetitive with respect to the template (the K_i was 0.28 μM, Fig. 3). In contrast, for DNA polymerases β and γ the inhibition of TGQA was competitive with respect to the template. The inhibition of DNA polymerase β by TGQA involved more than one binding site as indicated by a hyperbolic concave upward slope versus inhibitor concentration plot (data not shown; [13]). However, a plot of the slope versus (the drug concentration)$^{3.4}$ was linear. From this replot the apparent K_i of TGQA for DNA polymerase β was 44.4 μM. The evaluation of the effect of TGQA on DNA polymerase γ using activated DNA as template was difficult, because the inter-

Table 1. Inhibition of DNA polymerases by galloylated derivatives of quinic acid

Compound	Percent of control polymerase activity		
	α (0.33 μg/ml)	β (10 μg/ml)	γ (33 μg/ml)
(A) Gallic acid (170)	102 ± 6	99 ± 8	103 ± 4
(B) Digallic acid (322)	104 ± 4	102 ± 5	99 ± 2
(C) Quinic acid (QA) (192)	99 ± 8	104 ± 2	98 ± 10
(D) 3-*O*-galloyl-QA (344)	107 ± 10	101 ± 10	98 ± 7
(E) 4-*O*-galloyl-QA (344)	105 ± 5	96 ± 8	97 ± 6
(F) 3,4-di-*O*-galloyl-QA (496)	45 ± 4	103 ± 6	95 ± 12
(G) 3,5-di-*O*-galloyl-QA (496)	105 ± 10	101 ± 6	99 ± 4
(H) 3,4,5-tri-*O*-galloyl-QA (648)	17 ± 3	100 ± 6	22 ± 6
(I) 4-*O*-digalloyl-3-*O*-galloyl-QA (648)	37 ± 6	102 ± 5	88 ± 17
(J) 3-*O*-digalloyl-4-*O*-galloyl-QA (648)	30 ± 5	103 ± 8	85 ± 17
(K) 3,5-di-*O*-galloyl-4-*O*-digalloyl-QA (817)	32 ± 8	38 ± 5	0
(L) 1,2,3,6-tetra-*O*-galloyl glucose (788)	55 ± 9	8 ± 4	1 ± 2
(M) Methyl ester of Compound H* (660)	95 ± 8	101 ± 7	80 ± 20
(N) Permethylate of Compound H* (788)	103 ± 8	100 ± 3	99 ± 11

DNA polymerase activity was determined as described in Methods, using gapped duplex DNA as template. The number in parentheses following the name of each compound is the molecular weight of the compound. For DNA polymerase α, control reactions incorporated approximately 12.6 pmol of [^{3}H]TTP into DNA per hr; for DNA polymerase β, control [^{3}H]TTP incorporation was 28.6 pmol per hr; and for DNA polymerase γ, control [^{3}H]TTP incorporation was 7.02 pmol per hr. Each value is the mean ± the standard deviation from three experiments.

* The structure of compound M is methyl 3,4,5-tri-*O*-galloylquinate and compound N is methyl 3,4,5-tri-*O*-(tri-*O*-methylgalloyl)quinate.

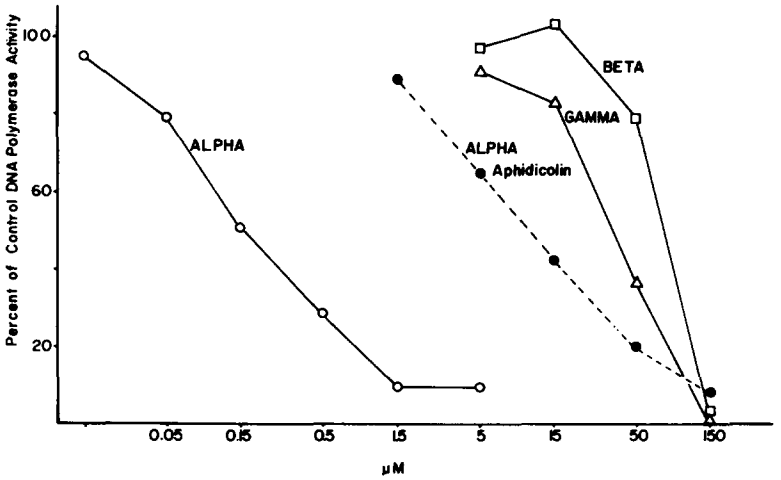

Fig. 2. Inhibition of DNA polymerases α, β, and γ by TGQA. DNA polymerase activity was measured as described in Methods except that the concentration of dCTP was 10 μM instead of 50 μM. The effect of aphidicolin (●) on DNA polymerase α activity was also determined. For DNA polymerase α, control reactions incorporated 3.14 pmol of [^{3}H]TTP into DNA per hr; for DNA polymerase β, control [^{3}H]TTP incorporation was 15.6 pmol per hr; and for DNA polymerase γ, control [^{3}H]TTP incorporation was 6.7 pmol per hr.

action of DNA polymerase γ with activated DNA did not follow classic Michaelis–Menten kinetics (substrate inhibition as well as cooperative interaction of the template with the enzyme was observed). Because DNA polymerase γ did not exhibit substrate inhibition using poly(dA) oligo(dT)$_{10}$ as template, the kinetics of inhibition of TGQA against DNA polymerase γ was done with this template. Because linear Lineweaver–Burk plots were obtained by plotting $1/V$ vs $1/[\text{template}]^2$, these data suggested that DNA polymerase γ had at least two binding sites for poly(dA) oligo(dT)$_{10}$ that acted in a cooperative manner [13]. From a replot of the slope versus the inhibitor concentration, the apparent K_i of TGQA for DNA polymerase γ was 7.5 μM. TGQA up to a concentration of 30 μM did not inhibit the poly(dC) DNA primase activity [measured as in Ref. 10] which was associated with DNA polymerase α.

During the course of this study it was found that the inhibition of DNA polymerase γ by these quinic acid derivatives could be increased approximately

Table 2. Effect of deoxynucleoside triphosphate concentration on the inhibition of DNA polymerase α, β, and γ by TGQA

	Percent of control DNA polymerase activity					
	α		β		γ	
Label	Concn of label (μM)	%	Concn of label (μM)	%	Concn of label (μM)	%
[^{3}H]TTP	2.5	80.7	2.5	70.8	0.25	13.2
	10.0	81.3	10.0	72.5	1.0	14.2
[^{3}H]dATP	2.5	74.8	2.5	75.9	0.25	15.3
	10.0	71.7	10.0	74.4	1.0	12.5
[^{3}H]dCTP	2.5	72.8	2.5	79.6	0.25	12.2
	10.0	75.8	10.0	80.2	1.0	12.5
[^{3}H]dGTP	2.5	74.0	2.5	77.7	0.25	12.3
	10.0	76.3	10.0	81.9	1.0	12.0

DNA polymerase activity was measured as described in Methods except that the concentration of the radiolabeled nucleoside triphosphate was as shown in the table, and the remaining deoxynucleoside triphosphates were at 50 μM. The concentration of TGQA was 0.15 μM in the DNA polymerase α assay and was 50 μM in both the DNA polymerase β and γ assays.

DNA polymerase α control reactions using either 2.5 or 10 μM [^{3}H]dNTP incorporated approximately 10.6 or 18.5 pmol of label into the DNA per hr respectively. DNA polymerase β control reactions using either 2.5 or 10 μM [^{3}H]dNTP incorporated approximately 9.5 or 20 pmol of label into the DNA per hr respectively. DNA polymerase γ control reactions using either 0.25 or 1 μM [^{3}H]dNTP incorporated approximately 5.2 or 6.95 pmol of label into the DNA per hr respectively.

35-fold by the addition of 50 mM potassium phosphate, pH 7.5 (data not shown). Potassium phosphate at this concentration and pH had no effect on the inhibition of DNA polymerase β and only slightly increased the inhibition by TGQA of DNA polymerase α (approximately 2-fold). Therefore, the selectivity of TGQA for DNA polymerase α over DNA polymerase γ was reduced significantly when the assay included 50 mM potassium phosphate, pH 7.5. When 50 mM potassium phosphate, pH 7.5, was included in the assay, 3,4-di-*O*-galloylquinic acid (compound F) was a more selective inhibitor of DNA polymerase α versus DNA polymerase γ than was TGQA (40-fold difference in the IC_{50}). Potassium phosphate has been shown to affect DNA polymerase γ activity [14], and this could be related to the effect seen with TGQA.

TGQA also inhibited the RNA-dependent DNA polymerase activity which was associated with DNA polymerase γ (IC_{50} of 0.7 μM, Fig. 4). DNA polymerase γ using this particular assay was much more sensitive to the action of TGQA than was the assay using activated DNA. It was possible that this difference reflected some characteristic of the particular templates and not a difference in the sensitivity of these two activities to TGQA. Therefore, the effect of TGQA on DNA-dependent DNA polymerase activity using poly(dA) oligo(dT)$_{10}$ as template with either $MnCl_2$ or $MgCl_2$ as the divalent cation was determined. Interestingly, TGQA was as potent an inhibitor of DNA-dependent DNA polymerase activity as it was of RNA-dependent DNA polymerase activity when the divalent cation was Mn^{2+}. However, when the same reaction was done in the presence of $MgCl_2$ instead of $MnCl_2$, DNA-dependent DNA polymerase was much less sensitive to TGQA (IC_{50} of 17 μM), which was similar to the inhibition seen with activated DNA (IC_{50} of 35 μM). When the divalent cation was changed from $MnCl_2$ to $MgCl_2$ in the RNA-dependent DNA synthesis assay, a biphasic pattern of inhibition was observed. The underlying mechanism is unclear. Nevertheless, these results showed that DNA-dependent DNA synthesis by DNA polymerase γ was as sensitive to TGQA as was the RNA-dependent DNA synthesis of DNA polymerase γ, and that the choice of divalent cation can have an important effect on the ability of TGQA to inhibit DNA synthesis by DNA polymerase γ. Replacement of 10 mM $MgCl_2$ by 0.5 mM $MnCl_2$ in the assay of DNA polymerase β also increased the sensitivity of DNA polymerase β to TGQA by more than 2-fold, and the addition of $MnCl_2$ to the assay of DNA polymerase α decreased the sensitivity of DNA polymerase α to TGQA (data not shown). Using activated DNA as template, the relative activity with 0.5 mM $MnCl_2$ was 0, 61, or 8% of that seen with 10 mM $MgCl_2$ using DNA polymerases α, β, and γ respectively. However, these results with $MnCl_2$ are not likely to be relevant to *in vivo* DNA synthesis, since Mg^{2+} is the predominant metal activator within cells [15].

The inhibition of DNA polymerase α, β, or γ was independent of the concentration of bovine serum albumin in the reaction mix, indicating that the different sensitivities to TGQA were not due to nonspecific protein binding of TGQA.

Cytotoxicity of 3,4,5-*tri*-O-*galloylquinic acid.* In preliminary experiments it was found that TGQA was not a good inhibitor of KB cell growth. After 72 hr of incubation the IC_{50} for TGQA was 21 μM (data not shown), which was much higher than the concentration required to inhibit DNA polymerase

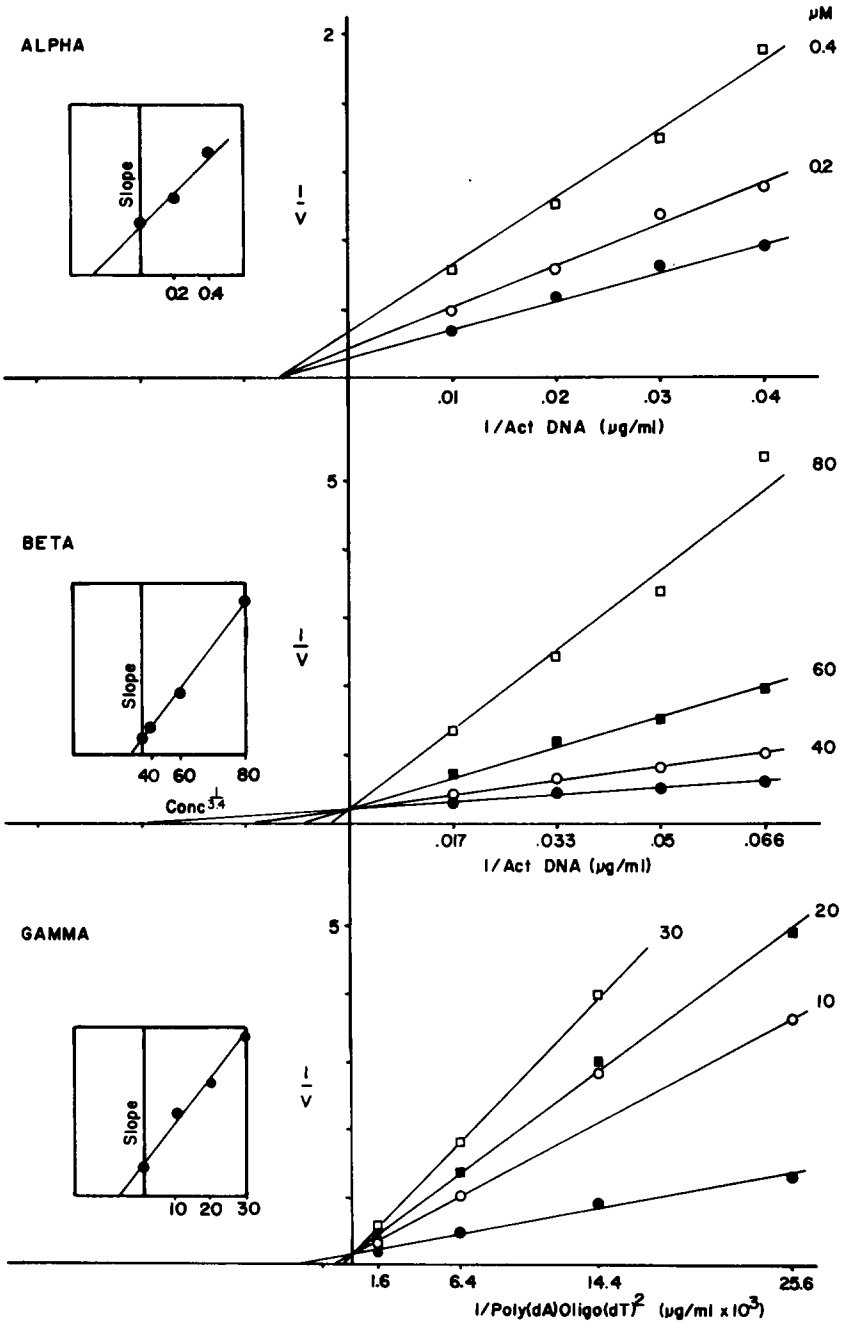

Fig. 3. Lineweaver–Burke analysis of the inhibition of DNA polymerases α, β, and γ by TGQA in relation to the concentration of template. For DNA polymerases α and β, DNA polymerase activity was measured using activated DNA as described in Methods, except that the concentration of DNA was varied as shown in the figure. For DNA polymerase γ, DNA polymerase activity was measured using poly(dA) oligo(dT)$_{10}$ as template and 10 mM $MgCl_2$ as described in the legend to Fig. 4.

α in vitro (IC_{50} of 0.15 μM). It is possible that the reason for this discrepancy was due to either the inability of this compound to enter the cell or that the compound was degraded in the medium or the cell. A preliminary experiment was done to determine whether or not this compound was degraded during incubation with KB cells. We found that TGQA (31 μM) was degraded in culture with KB cells in a biphasic manner with half-lives of 4.73 and 42.4 hr. In contrast, TGQA (31 μM) was stable in H_2O for 24 hr at 37°. These data indicated that TGQA was degraded in cell culture, but it could only partially explain the high concentration of TGQA necessary to inhibit cell growth. Because the IC_{50} of TGQA against DNA polymerase *α in vitro* was 100-fold less than the concentration required to inhibit KB cell growth by 50% and because there was only a 5-fold loss of TGQA during 24 hr of incubation,

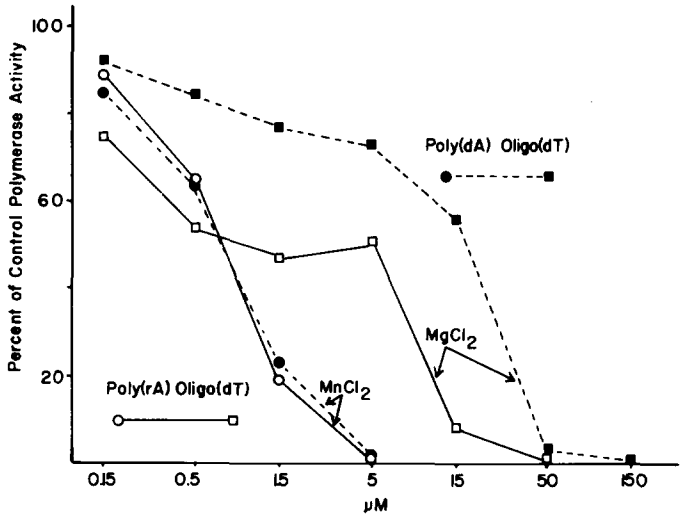

Fig. 4. Effect of TGQA on the RNA-dependent DNA polymerase which is associated with DNA polymerase γ. RNA-dependent DNA polymerase activity using $MnCl_2$ (○) was measured as described in Methods (control activity was 7.3 pmol of TMP incorporated in 30 min). This assay was also done using 10 mM $MgCl_2$ (□, control activity was 1.9 pmol of TMP incorporated in 30 min) in place of 0.5 mM $MnCl_2$. DNA-dependent DNA polymerase activity using either 0.5 mM $MnCl_2$ (●, control activity was 3.9 pmol of TMP incorporated in 30 min) or 10 mM $MgCl_2$ (■, control activity was 36.1 pmol incorporated in 30 min) was measured as described for RNA-dependent DNA polymerase except that poly(dA) oligo$(dT)_{10}$ replaced poly(rA) oligo$(dT)_{10}$ in the assay.

these data suggested that there were additional explanations responsible for its low cytotoxicity (i.e. poor uptake and/or degradation within the cells).

DISCUSSION

In summary, we have isolated a novel and potent inhibitior of human DNA polymerases. Against DNA polymerase α this compound was much more potent than aphidicolin, but was similar to aphidicolin in that its structure does not suggest a mechanism of inhibition. The inhibition of DNA polymerase α by TGQA was not competitive with respect to the template or any of the deoxynucleoside triphosphates and, therefore, the mechanism is different from aphidicolin. TGQA inhibited both DNA polymerases β and γ by competing with the template. TGQA was not a potent inhibitor of KB cell growth, presumably due to the degradation of TGQA and its poor uptake. These studies suggested that the development of a useful antitumor drug from this compound would require the production of an analog which can penetrate the cell membrane and is resistant to degradation.

There are many similarities between TGQA and gossypol, a polyphenolic compound that is being used as a male antifertility agent in China. The structures [16] of these two compounds are similar in that both compounds contain hydroxylated phenol groups and, like TGQA, gossypol inhibits DNA polymerase α in a noncompetitive manner with respect to the four deoxynucleoside triphosphates and the DNA template [16]. This suggests that these two compounds may inhibit DNA polymerase α by a similar mechanism. The K_i of gossypol against DNA polymerase α was approximately 14 μM [16], whereas for TGQA it was 0.28 μM. Gossypol has been tested for antitumor activity against a few murine tumors [17], and was found to be active against the mouse mammary adenocarcinoma 755 (Ca 755). However, the therapeutic range for gossypol was very narrow. It would be interesting to determine the antitumor effect of TGQA or an analog and compare it to that seen with gossypol.

The inhibition of DNA polymerases β and γ by TGQA was similar to the inhibition of avian myeloblastosis virus reverse transcriptase by gallotannins [3] in that the inhibition in all cases was competitive in respect to the template. Furthermore, those investigators showed that increasing the number of galloyl groups on glucose increased the ability of these compounds to inhibit the reverse transcriptase activity. This was also true using galloyl derivatives of quinic acid for DNA polymerases β and γ, but not the case for DNA polymerase α. They also reported that hexa-galloyl glucose was not a sensitive inhibitor of calf thymus DNA polymerase α (IC_{50} greater than 10 μM), whereas we showed that tetra-galloyl glucose (compound L) was a sensitive inhibitor of human DNA polymerase α (IC_{50} of approximately 0.4 μM). Taken together, these results suggested that the addition of more galloyl groups to glucose (greater than 4) may have a negative impact on the ability of these compounds to inhibit DNA polymerase α.

These compounds may also prove to be useful as an aid in the study of human DNA polymerases. Along with aphidicolin, dideoxynucleosides, and salt these compounds can be used to differentiate DNA polymerases α, β, and γ from one another. Furthermore, the understanding of the mechanism of inhibition of DNA polymerase α by TGQA may lead

to a greater understanding of the function of DNA polymerase α or the function of proteins which are associated with it.

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