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Interaction of 2-Halogenated dATP Analogs (F, Cl, and Br) with Human DNA Polymerases, DNA Primase, and Ribonucleotide Reductase

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SUMMARY

Recently, 2-halogenated deoxyadenosine analogs (F, Cl, and Br) have been shown to have antitumor activity. These analogs are phosphorylated by cells and are believed to exert their cytotoxic action at the nucleoside triphosphate level. In this work the interaction of these nucleoside triphosphate analogs with potential targets, such as DNA polymerase α, β, and γ, DNA primase, and ribonucleotide reductase was examined in detail. All of these compounds competitively inhibited the incorporation of dAMP into DNA by DNA polymerase α, β, or γ. F-dATP was able to completely substitute for dATP using DNA polymerase α and γ, but not with DNA polymerase β. Cl-dATP and Br-dATP substituted poorly for dATP using DNA polymerase α and β. Extension of a ^{32}P-labeled primer by DNA polymerase α, β, or γ on a single-stranded M13 template showed that these compounds were incorporated into the 3′ end of the growing DNA chain and that elongation beyond the incorporated analogs was significantly retarded for Cl-dATP and Br-dATP using either DNA polymerase α or β. DNA primase using poly(dC) as template was inhibited by these compounds at a concentration 4 to 5 times greater than that required for 2-F-araATP. The 2-halogenated dATP analogs were potent inhibitors of ADP reduction by ribonucleotide reductase. In conclusion, the cytotoxic action of 2-Cl-deoxyadenosine and 2-Br-deoxyadenosine may partially be mediated through the mechanism of "self-potentiation," by depression of the deoxynucleoside triphosphate pools due to inhibition of ribonucleotide reductase, which would facilitate their incorporation into DNA and result in the inhibition of DNA synthesis.

dAdo has been considered for development as a possible antileukemic and antilymphocytic agent because the elevation of dATP is believed to be responsible for the lymphopenia observed in children who have a genetic deficiency in adenosine deaminase (1). This suggests that there may be some selectivity of the toxic actions of dAdo against lymphocytes when compared with other normal tissues. dAdo is also cytotoxic in a wide variety of other biological systems (2). One problem with the use of dAdo as an antitumor agent in otherwise normal patients is that it is rapidly deaminated, and thereby inactivated, by adenosine deaminase. However, the 2-halogenated analogs of dAdo (F, Cl, and Br) are resistant to deamination and are potent inhibitors of cell growth (3–8) and cell viability (7, 8). Cl-dAdo has shown some promise as an antitumor agent against 1) the L1210 mouse leukemia *in vivo* (4, 6, 8, 9), 2) bone marrow specimens grown in culture obtained from patients with acute leukemia (3), and 3) various hematological malignancies that were refractory to conventional chemotherapy (10). Of further interest is the ability of Cl-dAdo to kill resting and nonproliferating human lymphocytes (3), due to the reduction of intracellular NAD levels (11) because of its ability to inhibit DNA repair (12).

The inhibition of ribonucleotide reductase by dATP is thought to play an important role in the cytotoxicity of dAdo (2). However, the mechanism of cytotoxicity of the 2-halogenated dAdo analogs is not clear. Unlike dAdo, these compounds are metabolized primarily by dCyd kinase to their respective nucleoside monophosphates (4), which are then phosphorylated further to the triphosphate level (3, 4). It is believed that the nucleoside triphosphate of each analog is responsible for the cytotoxicity observed with these compounds (4). *In vivo* studies have determined that these compounds are potent inhibitors of DNA synthesis [determined by the incorporation of [^{3}H]deoxythymidine into the acid-insoluble fraction of cells treated with nucleoside analogs (4, 7)] and cause cells to accumulate in S phase (7, 8). Sato *et al.* (13) have shown that F-dAdo is a potent

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ABBREVIATIONS: dAdo, deoxyadenosine; F-dAdo, 2-fluoro-2′-deoxyadenosine; Cl-dAdo, 2-chloro-2′-deoxyadenosine; Br-dAdo, 2-bromo-2′-deoxyadenosine; F-araA, 9-β-D-arabinofuranosyl-2-fluoroadenine; araA, 9-β-D-arabinofuranosyladenine; F-dATP, 2-fluoro-2′-deoxyadenosine 5′-triphosphate; Cl-dATP, 2-chloro-2′-deoxyadenosine 5′-triphosphate; Br-dATP, 2-bromo-2′-deoxyadenosine 5′-triphosphate; F-araATP, 9-β-D-arabinofuransyl-2-fluoroadenine 5′-triphosphate; araATP, 9-β-D-arabinofuranoyladenine 5′-triphosphate; ddATP, 2′,3′-dideoxyadenosine 5′-triphosphate; IC_{50}, concentration of drug required to inhibit the activity in question by 50%; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

inhibitor of ribonucleotide reductase *in vivo*, and Cl-dAdo has been shown to decrease intracellular deoxynucleoside triphosphate concentrations (14). Furthermore, Carson *et al.* (3) have shown that Cl-dAdo is incorporated into the DNA of proliferating CCRF-CEM cells. These studies suggest that two likely targets for this class of compounds are ribonucleotide reductase and DNA polymerase α.

In order to better understand the mechanism of action of the 2-halogenated dAdo analogs, we have studied the interaction between the triphosphate analogs of these compounds and some probable intracellular targets, such as DNA polymerase α, DNA polymerase β, DNA polymerase γ, DNA primase, and ribonucleotide reductase. Because of the clinical interest in developing F-araA as an antitumor agent (15–17), we have also studied the effect of araATP and F-araATP on these same targets so that comparisons can be made between these similar classes of drugs. Our results suggested a "self potentiation" mechanism of action for the Cl- and Br-dAdo analogs similar to that observed for F-araA (18), which involves the depletion of dATP by inhibition of ribonucleotide reductase followed by the incorporation of the analog into DNA and the inhibition of further DNA elongation. A preliminary report of this research has already appeared (19).

Materials and Methods

Chemicals and supplies. K652 cells (American Type Culture Collection CCL 243, human chronic myelogenous leukemia) were grown in RPMI 1640 media (GIBCO, Grand Island, NY) supplemented with 5% fetal bovine serum (GIBCO). Some of the nucleoside triphosphates used in this study (TTP, dATP, dGTP, ATP, GTP, and araATP) were obtained from Sigma Chemical Co. (St. Louis, MO). [*methyl*-^{3}H]TTP (20 Ci/mmol), [8-^{3}H]dATP (20 Ci/mmol), [8-^{3}H]dGTP (20 Ci/mmol), and [α-^{32}P]ATP (3000 Ci/mmol) were obtained from ICN Radiochemicals (Irvine, CA). [U-^{14}C]ADP (520 mCi/mmol) was obtained from Amersham Corporation (Arlington Heights, IL). Poly(dC), poly(dT), T4 polynucleotide kinase, and ddATP were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). F-araA, F-dAdo, Cl-dAdo, Br-dAdo, F-araATP, F-dATP, Cl-dATP, and Br-dATP were obtained from Southern Research Institute (Birmingham, AL). Single strand (+) M13 mp9 DNA was prepared from *Escherichia coli.* cells (JM 103) transfected with M13 phage RF DNA (Bethesda Research Laboratory, Gaithersburg, MD) as described (20). The 17-base oligodeoxynucleotide primer (5′-GTAAAACGACGGCCAGT-3′) was synthesized with an American Bionuclear (Emeryville, CA) automated DNA synthesizer by Dr. Dana Fowlkes, Pathology Department, University of North Carolina School of Medicine (Chapel Hill), according to the manufacturer's specification. The primer sequence is complementary to the 3′- end of the distinct multiple cloning site of the M13 template. All other chemicals were of standard analytical grade.

Purification of enzymes. DNA polymerase α and DNA primase were purified from chronic myelogenous leukemia cells (K562 cells) grown in culture as described previously (21). The specific activity of DNA polymerase α was 640 units/mg of protein. DNA polymerase β was purified from human acute myeloblastic leukemia cells obtained by leukophoresis as described previously (22) to a specific activity of 1000 units/mg of protein. DNA polymerase γ was purified from chronic lymphocytic leukemia cells obtained by leukophoresis as described (23). The specific activity was approximately 50 units/mg of protein. One unit of polymerase activity is defined as the amount that catalyzes the incorporation of 1 nmol of TMP (or dGMP for DNA primase) into the acid-insoluble fraction per hr at 37°. DNA polymerase α, β, and γ were differentiated from each other by their sensitivities to dideoxynucleotides, aphidicolin, aranucleotides, and salt. Furthermore, DNA polymerase γ was identified by its ability to use poly(rA) oligo(dT) as template and by its low K_m for the deoxynucleoside triphosphates.

The cell extract used to measure ribonucleotide reductase activity was prepared from exponentially growing K562 cells. Approximately 1×10^9 cells were collected by centrifugation and washed twice with ice-cold phosphate-buffered saline. The cell pellet was frozen and thawed three times, mixed with 2 volumes of extraction buffer, and then homogenized with a ground glass tissue grinder (50 strokes). The homogenate was centrifuged at 25,000 × *g* for 30 min at 4°, and the supernatant fluid was collected and dialyzed against the extraction buffer for 3 hr at 4°. The extraction buffer was 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, and 20% glycerol.

Enzyme assays. DNA polymerase α was measured in 50- μl reactions containing 25 mM Tris·HCl, pH 8.0, 1 mg/ml bovine serum albumin, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μm/ml activated DNA (24), 10 μM [^{3}H]TTP (1 Ci/mmol), a 50 μM concentration each of dCTP, dGTP, and dATP (unless otherwise noted), and 0.01 units of enzyme. After incubation for the desired time at 37°, the DNA was precipitated onto glass fiber filters with a 5% trichloroacetic acid/10 mM pyrophosphate solution and counted for radioactivity as previously described (21). DNA polymerase β activity was measured in the same manner, except that 125 mM KCl was included in the reaction. DNA polymerase γ was measured as described for DNA polymerase α except that Tris·HCl was replaced with 50 mM HEPES, pH 7.5, and 100 mM KCl and 50 mM KPO_4, pH 7.5, were included in the reaction. For determination of the K_i of the dATP analogs, varying concentrations of [^{3}H]dATP (1 Ci/mmol) were used in place of [^{3}H]TTP. TTP (50 μM) replaced dATP in these reactions. DNA primase, using poly(dC) as template, was measured in 50-μl reactions containing 25 mM Tris·HCl, pH 8.0, 0.2 mg/ml bovine serum albumin, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.4 units/ml poly(dC), 10 μM [^{3}H]dGTP (1 Ci/mmol), 50 μM GTP, and 0.01 units of DNA primase as previously described (21). RNA primer formation by DNA primase was measured in 75-μl reactions containing 25 mM Tris·HCl, pH 8.0, 1 mg/ml bovine serum albumin, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 unit/ml poly(dT), 2.5 mM [^{32}P]ATP (0.1 Ci/mmol), 50 μM dATP, and 0.06 units of DNA primase. After incubation at 37° for 1 hr, the RNA was extracted and analyzed as previously described for RNA primer formation with [^{32}P]GTP and poly(dC) (21). ADP reduction was measured in 100-μl reactions containing 30 mM HEPES, pH 7.5, 5 mM dGTP, 10 mM $MgCl_2$, 1.25 mM NaF, 15 μM [^{14}C]ADP (3.66 Ci/mmol), 5 mM dithiothreitol, and 25 μl of K562 extract as described (25, 26).

The ability of DNA polymerase α, β, or γ to extend a ^{32}P-labeled primer that was annealed to single strand M13 template was examined as previously described (22). Briefly, 0.005 units of DNA polymerase α, β, or γ was incubated in 10-μl reactions that contained the ingredients listed above for each specific polymerase, except that the activated DNA was replaced with 25 μg/ml of single strand (+) M13 DNA mp 9 that was annealed to an equimolar concentration of a 17-base oligomer, which had been labeled on the 5′ end with [γ-^{32}P]ATP by T4 polynucleotide kinase. After incubation for 1 hr at 37° the reactions were terminated by adding 10 μl of 80% formamide, 40 mM EDTA, and 0.3% bromophenol blue, boiled, loaded onto a 10% polyacrylamide/7 M urea gel, and electrophoresed for 3 hr at 30 W constant power. The radioactivity in the gel was visualized by autoradiography.

Results

Effects on cell growth. 2-Halogenated dAdo analogs were potent inhibitors of K562 cell growth (Table 1). Cl-dAdo was the most potent inhibitor of the three analogs, with an IC_{50} of 24 nM. For comparison, the effect of F-araA on K562 cell growth was also determined. All of the halogenated dAdo analogs were much more potent inhibitors of cell growth than was F-araA (IC_{50} of 4.7 μM).

Inhibition of DNA polymerases. The 2-halogenated dATP analogs competitively inhibited the incorporation of dAMP into DNA using DNA polymerase α, β, or γ (Table 1).

TABLE 1

Effect of 2-halogenated dAdo nucleoside analogs on cell growth and their triphosphates on DNA polymerase α, β, and γ

The Michaelis-Menten kinetic constants for dATP and its analogs were obtained from linear double reciprocal plots of 1/*V* *versus* 1/dATP using gapped duplex DNA as substrate. The IC_{50} is the concentration of analog required to inhibit cell growth by 50% after 72 hr of incubation. Each value represents the mean ± standard deviation of at least two determinations. The value in parentheses is the ratio of K_i/K_m

Nucleotide	IC_{50} of nucleoside	K_i (K_m)		
		α	β	γ
	nM		μM	
dATP		1.9 ± 0.5	3.0 ± 0.8	0.3 ± 0.03
F-dATP	270 ± 141	3.8 ± 1.8 (2.0)	5.2 ± 1.1 (1.7)	2.5 ± 0.0 (8.3)
Cl-dATP	24 ± 8	3.5 ± 1.4 (1.8)	4.2 ± 0.4 (1.4)	21 ± 3.2 (70)
Br-dATP	94 ± 44	9.2 ± 5.3 (4.8)	12.2 ± 2.5 (4.0)	24 ± 12 (80)
F-araATP	4700 ± 140	0.7 ± 0.09 (0.4)	5.8 ± 0.4 (1.9)	9.5 ± 0.8 (32)
araATP		0.8 ± 0.1 (0.4)	7.5 ± 0.7 (2.5)	2.5 ± 0.6 (8.3)

For comparison, the K_i of both araATP and F-araATP was also determined using DNA polymerase α, β, and γ. The K_i obtained for both araATP and F-araATP agreed well with previously published results for DNA polymerase α (18, 27, 28) and β (18, 28). These results showed competition between these analogs and dATP for the DNA polymerases, but they did not indicate whether or not these analogs were alternative substrates of the DNA polymerases in place of dATP. Therefore, the ability of each analog to substitute for dATP with each DNA polymerase was determined using [^{3}H]TTP to label the newly synthesized DNA. As can be seen in Fig. 1, DNA synthesis using F-dATP was 85 and 72% of that obtained with dATP using either DNA polymerase α or γ, respectively, which showed that F-dATP was almost as good as substrate for these enzymes as was dATP. DNA synthesis with DNA polymerase β using F-dATP was 80% of that obtained with dATP. However, these results are hard to interpret because of the high activity of DNA polymerase β in the absence of dATP (almost 70% of the activity seen in the presence of all four deoxynucleotide triphosphates). In contrast, DNA synthesis by DNA polymerase α and γ in the absence of dATP was only 20 and 9%, respectively, of that in the presence of all four deoxynucleoside triphosphates. We believe that this phenomenon observed with DNA polymerase β, which has been observed with other preparations of DNA polymerase β (22, 29, 30), was due to the distributive nature of the enzyme. When the primer is extended to a position that would require the insertion of dAMP, DNA polymerase β would be free to diffuse to another primed site that did not require the insertion of dAMP (unlike DNA polymerases α and γ, which are processive enzymes). DNA synthesis with all polymerases using the Cl and Br analogs of dATP was much less than that observed with dATP (Fig. 1). However, when 10 μM dATP was included in the reaction mixture, high concentrations of either Cl-dATP or Br-dATP (>100 μM) were needed to inhibit these polymerases (data not shown).

A primer extension assay (22) was used to determine whether each analog was incorporated into the DNA and what effect this incorporation may have on the further elongation of DNA by each DNA polymerase. In this assay, DNA polymerase α, β, or γ was incubated with the desired analog, TTP, dCTP, dGTP, and single strand M13 template primed with a 17-base oligomer labeled with ^{32}P on the 5′ end. After incubation, the size of the DNA that has been synthesized from the 3′ terminus of the ^{32}P-labeled primer can be determined by electrophoresis of the products through a 10% polyacrylamide/7 M urea gel followed by autoradiography, in a manner similar to that described for dideoxy sequencing techniques. The termination of DNA synthesis by a nucleotide analog at a specific site should result in the accumulation of products of distinct length, which can be detected by autoradiography of the gel. The sequence of DNA following the 17-base primer was determined using the dideoxy sequencing technique of Sanger *et al.* (31). It was clear from Fig. 2 that each polymerase showed a distinct pattern under control conditions (50 μM dATP; Fig. 2, *lanes A*). DNA polymerase α paused at three discrete sites along the M13 template,

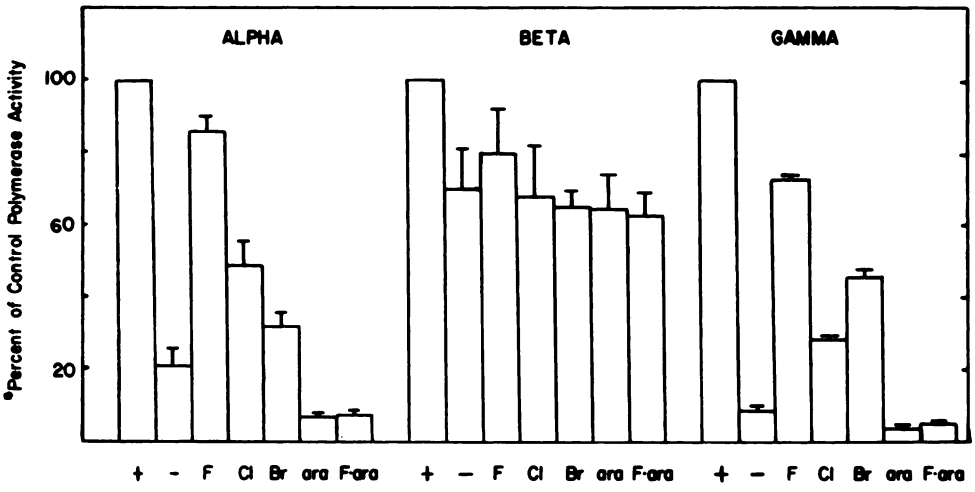

Fig. 1. Ability of DNA polymerases to utilize dATP analogs for DNA synthesis. DNA synthesis was measured using DNA polymerase α, β, or γ with gapped duplex DNA, 50 μM dCTP, 50 μM dGTP, 10 μM [^{3}H]TTP with (+) or without (−) 50 μM dATP or one of the following analogs at 50 μM each; F-dATP (*F*), Cl-dATP (*Cl*), Br-dATP (*Br*), araATP (*ara*), or F-araATP (*F-ara*). Control activity was defined as that amount seen in the reaction with 50 μM dATP. Each *bar* represents the mean ± standard deviation of three separate experiments.

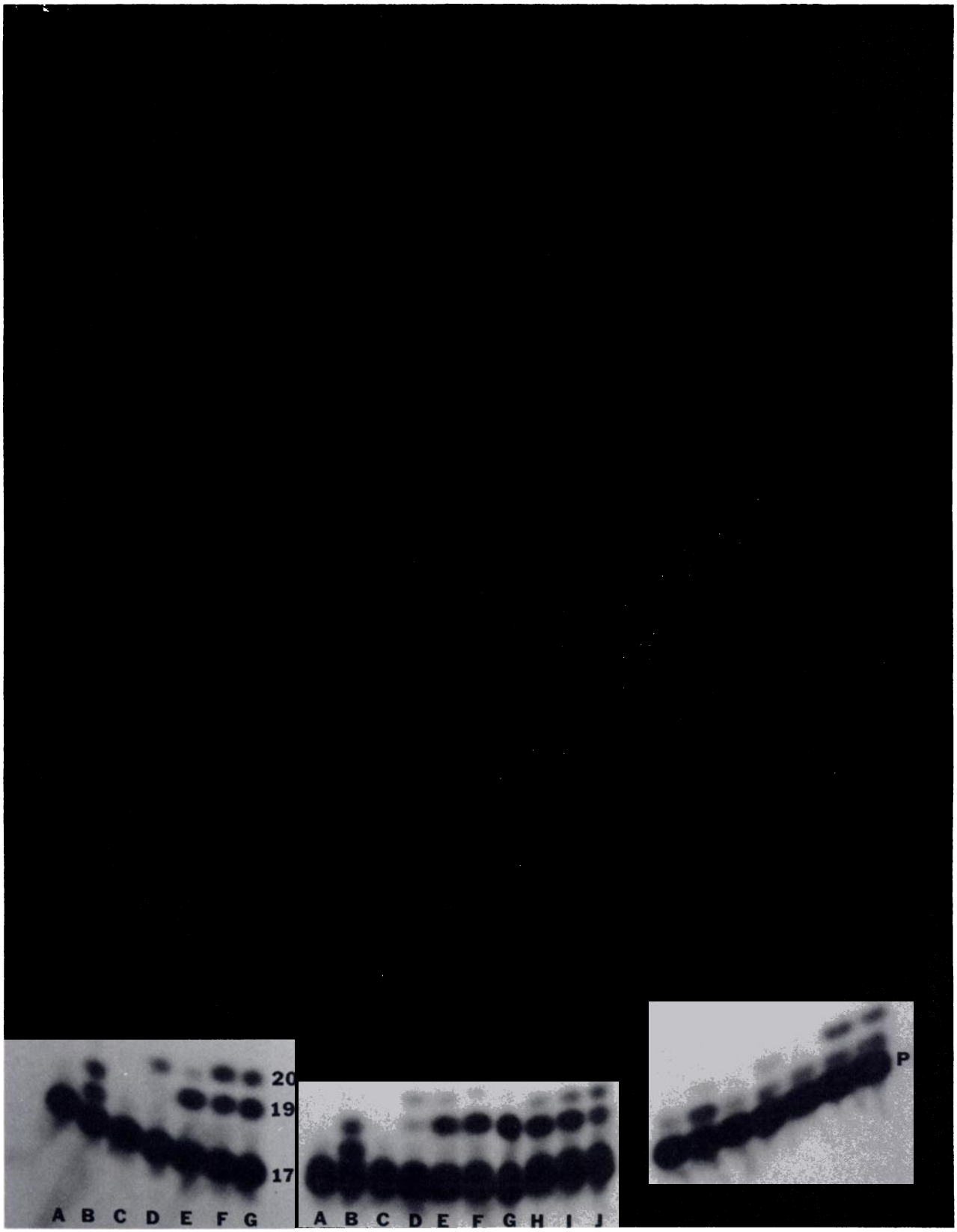

Fig. 2. Effect of dATP analogs on primer extension assay. DNA polymerase α, β, or γ was incubated with single-strand M13 DNA primed with a 17-base oligomer that was labeled with ^{32}P at the 5′ end and 50 μM each of TTP, dCTP, and dGTP. The reactions also included 50 μM of the following: dATP (*A*), F-dATP (*C*), Cl-dATP (*D*), Br-dATP (*E*), araATP (*F*), or F-araATP (*G*). In *lane B* there were no extra additions. With DNA polymerase β, ddATP (5, 20, or 50 μM, *lanes H*, *I*, and *J*, respectively) was incubated with 10 μM to identify the TMP sites in the template DNA. After incubation for 1 hr, the DNA was extracted and loaded onto a 10% polyacrylamide/7 M urea gel. After electrophoresis the bands were visualized by autoradiography. The positions where dAMP was incorporated into the growing DNA chain are marked by an *A* in the lane to the *right* of *lane J*. The sequence of incorporation of deoxynucleotides by the polymerases into the growing DNA chain is as follows; 5′-primer-GAATTCCCGGGGATCCGACCTGCAGCC . . . 3′. The length of the DNA products is shown in the lane to the *right* of *lane G* in the DNA polymerase α experiment.

presumably due to secondary structure of the template, and very little high molecular weight product was formed. DNA polymerases β and γ were also slowed at certain sites on the template, but a greater percentage of the label was incorporated into high molecular weight DNA, which does not enter the gel. It was also clear from the patterns using F-dATP, Cl-dATP, and Br-dATP in place of dATP that all three analogs of dATP were incorporated into the DNA by all three enzymes and that the incorporation of each analog into the growing DNA chain has varying effects on further DNA polymerization, depending on the analog and the enzyme that was used. For polymerase α, F-dATP had little, if any, effect on the elongation of DNA. Incorporation of Cl-dAMP and Br-dAMP strongly inhibited DNA elongation. These results suggested that the incorporation of two consecutive Cl-dAMP into the DNA by DNA polymerase α was required for the inhibition of DNA chain elongation, because there was little accumulation of product at the first site at which Cl-dAMP could be incorporated (19-base product), and there was significant accumulation of product at the next site (20-base product). However, incorporation of only one Cl-dAMP into a normal pause site was also sufficient to retard DNA elongation. Very little elongation of DNA occurred after the incorporation of just one Br-dAMP. For DNA polymerase β, F-dATP, Cl-dATP, and Br-dATP all inhibited DNA elongation with the same order of effectiveness as seen with DNA polymerase α (i.e., F-dATP < Cl-dATP < Br-dATP). Furthermore, all of these compounds retard DNA chain elongation after only one analog had been incorporated into the DNA chain. The pattern for these compounds was very similar to that seen with ddATP (Fig. 2, *lanes H, I,* and *J*), which indicated the positions in the sequence where dAMP should be incorporated.

The action of these compounds on DNA polymerase γ was quite different from that seen with either DNA polymerase α or β. For instance, the elongation of DNA by DNA polymerase γ was inhibited most by Cl-dATP and was only slightly inhibited by Br-dATP. Furthermore, the pause pattern seen with Cl-dATP and Br-dATP was not easily understood. The termination of DNA elongation with Cl-dATP and Br-dATP was not necessarily at positions across from TMP in the template strand (note that there was very little accumulation of product at the first five dAMP sites). These compounds appeared to impede DNA elongation when they were incorporated at a position that correlated with a pause site for DNA polymerase α.

The incorporation of araAMP or F-araAMP into the DNA by any of the polymerases inhibited the further elongation of the DNA by these polymerases more than was observed with any of the 2-halogenated dATP analogs. However, the effect observed with Br-dATP using DNA polymerase α or β was similar to that of either araATP or F-araATP. The data obtained with the primer extension assay (Fig. 2) were consistent with those obtained using the routine filter disk assay with gapped duplex DNA as template (Fig. 1).

DNA primase activity. The 2-halogenated dATP analogs were less potent inhibitors of DNA primase activity than were either araATP or F-araATP. F-, Cl-, and Br-dATP inhibited the poly(dC) DNA primase assay at high concentrations (IC_{50} values were as follows: Br-dATP, 80 μM; Cl-dATP, 88 μM; F-dATP, 220 μM; data not shown). Under identical assay conditions, only 50 μM araATP and 16 μM F-araATP were required to inhibit the poly(dC) DNA primase activity by 50% (20). The effect of these dATP analogs on DNA primase activity was also studied by measuring the effect of these compounds on the incorporation of [α-^{32}P]ATP into the RNA primer produced by DNA primase using poly(dT) as template. F-, Cl-, and Br-dATP did not inhibit DNA primase in this assay at the highest concentration tested (300 μM), whereas araATP and F-araATP were potent inhibitors in this assay (IC_{50} values of approximately 10 to 30 μM). These results suggested that at low concentrations DNA primase was not an important target of the 2-halogenated dAdo analogs.

Inhibition of ribonucleotide reductase activity. The 2-halogenated dATP analogs were very potent inhibitors of ADP reduction by ribonucleotide reductase (Fig. 3). The IC_{50} for Cl-dATP and Br-dATP was approximately 130 nM and for F-dATP the IC_{50} was 480 nM. These values were at least 10 times lower than that seen for dATP (6 μM). These compounds also inhibited CDP reduction, but at higher concentrations (data not shown). F-dATP was 2 to 3 times more potent as an inhibitor of ADP reduction than was F-araATP (IC_{50} of 1.2 μM). The inhibition of ADP reduction by F-araATP, dATP, and araATP was similar to that seen with HeLa cells (18).

Discussion

The 2-halogenated dAdo analogs are an interesting class of compounds, which may have some clinical utility. Previous work has suggested that ribonucleotide reductase and DNA polymerase α may be important intracellular targets for these drugs. In this work we studied the interaction of the nucleoside triphosphate analogs with their potential targets *in vitro* and have found that 1) all of these analogs were potent inhibitors of ADP reduction by ribonucleotide reductase; 2) all analogs competively inhibited the incorporation of dAMP into DNA using DNA polymerases α, β, and γ; 3) all analogs were incorporated into the growing DNA chain by all three enzymes and the incorporation of each analog into the DNA had varying effects on further polymerization depending on the analog and the enzyme which were used; and 4) none of these compounds were sensitive inhibitors of DNA primase.

Mechanism of action. These results suggested that the mechanism of action of F-dAdo involved primarily the inhibition of ribonucleotide reductase, with some possible contribution from the inhibition of DNA polymerase β. Another possible consequence of F-dAdo treatment, that has not been addressed, is the possible effects of the incorporation of F-dAMP into the DNA on the ability of polymerases to faithfully utilize such a template for either RNA or DNA synthesis. Furthermore, the effect of the incorporation of F-dAMP into the DNA on the integrity of the DNA is also not known. Our results show that F-dAMP was readily incorporated into the DNA.

The intracellular concentrations of Cl-dATP or Br-dATP that are achieved after treatment with either of these nucleoside analogs is very important to the mechanism of cytotoxicity for these two compounds. If only low concentrations of the nucleotide triphosphates are achieved, then these two compounds should initially stop cell growth primarily by the inhibition of ribonucleotide reductase. However, if micromolar concentrations of the nucleoside triphosphates are obtained, then their action initially on both ribonucleotide reductase and DNA polymerases could be considered. In either case, a "self-potentiation" mechanism of action could be proposed that is similar

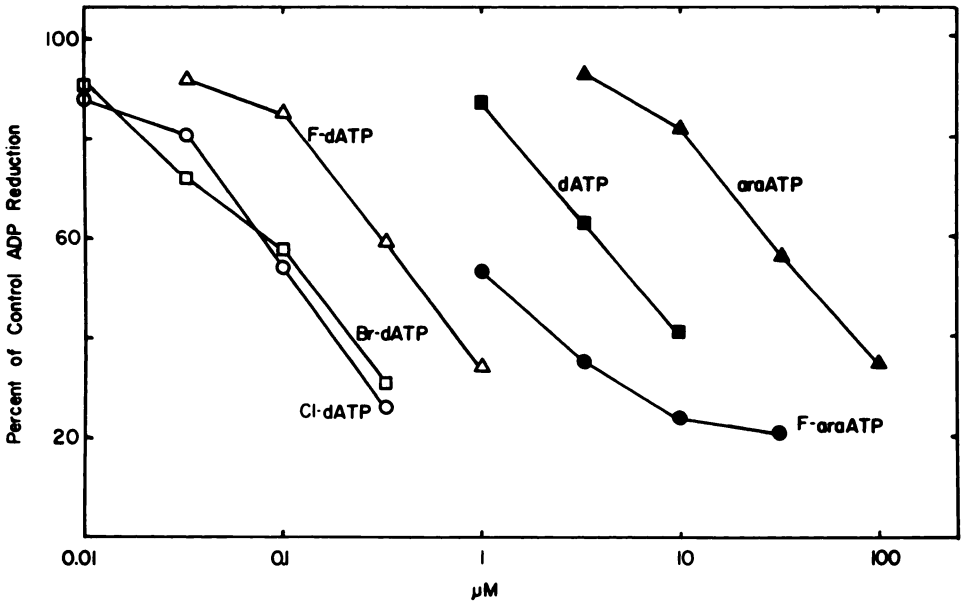

Fig. 3. Effect of dATP analogs on ADP reduction by ribonucleoside reductase. Crude extracts from K562 cells were incubated with [^{14}C]ADP and the dATP analogs shown in the figure. After incubation the substrate and products were degraded to the nucleoside and separated using Dowex-1-borate columns.

to that proposed for F-araATP (18). The inhibition of ribonucleotide reductase would decrease the concentration of dATP, and Cl-dATP and Br-dATP would be more readily used for DNA synthesis by both DNA polymerase α and β. This incorporation of Cl-dAMP or Br-dAMP would then impede the ability of either polymerase to elongate the DNA. This self-potentiation mechanism would also apply to F-dATP and DNA polymerase β. Some work has been done to evaluate the metabolism of these compounds to the nucleoside triphosphate. Carson *et al.* (3) showed that the incubation of CCRF-CEM cells with 200 nM Cl-dAdo for 24 hr results in the accumulation of 100 pmol of Cl-dATP/10^8 cells (concentration of approximately 1 μM, assuming that 10^9 cells is equal to 1 ml). In addition, this group (4) showed that incubation of WI-L2 cells for 4 hr with 20 μM F-dAdo results in 30 pmol of F-dATP/10^6 cells (approximately 30 μM) and that incubation for 2 hr with 400 nM Cl-dAdo results in 40 pmol/10^8 cells (approximately 0.4 μM). These results suggest that for some treatment regimens, enough halogenated nucleoside triphosphate can be produced to interfere directly with DNA polymerase α and β. However, more studies are needed to clarify this important point.

Comparison of F-araATP with F-dATP. Recently, there has been much interest in F-araA in the treatment of leukemias and lymphomas (15–17). Because of this interest, we have compared the effects of F-araATP and F-dATP on the various intracellular targets. F-dAdo was shown in this work and in other cell culture systems (4, 6, 13) to be approximately 10 times more potent as an inhibitor of cell growth than was F-araA. We showed that F-dATP was also 2 to 3 times more potent as an inhibitor of ADP reduction by ribonucleotide reductase than was F-araATP, and Sato *et al.* (13) showed that F-dAdo is 5-fold more active than F-araA in inhibiting the formation of deoxycytidine nucleotides *in vivo*. On the other hand, the K_i for F-araATP against DNA polymerase α was approximately 5-fold less than that seen with F-dATP. Furthermore, the incorporation of F-dATP into the DNA did not impede DNA elongation by DNA polymerase α, whereas the incorporation of F-araATP into the DNA strongly inhibited the further enlongation of the DNA by DNA polymerase α (this was also true for DNA polymerase γ). The K_i against DNA polymerase β was similar for both compounds, and they both inhibited DNA elongation by DNA polymerase β after they were incorporated. Finally, F-araATP was a more potent inhibitor of DNA primase than was F-dATP. These results suggested that the mechanism of action of these two compounds is quite different. The primary target for F-dATP appeared to be ribonucleotide reductase, with the possible inhibition of DNA polymerase β, whereas the primary target for F-araATP appears to be DNA polymerase α, with the capability to potentiate its effect on DNA polymerase α by inhibition of ribonucleotide reductase (18). The actions of Br-dATP and F-araATP against the various cellular targets appeared to be more similar. However, there were some significant differences between these two compounds as well (potency against ribonucleotide reductase and the K_i against DNA polymerase α).

Mechanism of toxicity toward resting cells. In resting human lymphocytes Cl-dAdo has been shown to 1) cause a reduction in NAD, ATP, and RNA synthesis (11), 2) cause DNA strand breaks (11), and 3) inhibit DNA repair and unscheduled DNA synthesis after exposure to γ radiation (12). It was suggested that the damage of the DNA by treatment with Cl-dAdo results in the synthesis of poly(ADP-ribose), which causes the rapid consumption of cellular NAD. The depletion of NAD is the principle cause of death in normal resting human lymphocytes (11). These results suggest that Cl-dATP may have an effect on DNA polymerase β, the polymerase primarily responsible for DNA repair synthesis (32), but this was not studied directly. In our work we showed that Cl-dATP was a sensitive inhibitor of DNA polymerase β, with a K_i of 5 μM, and that the ability of DNA polymerase β to elongate the DNA past the incorporation of Cl-dAMP was severely impaired. This was also true (to differing degrees) for F-dATP and Br-dATP. Our data support the work of Seto *et al.* (11, 12) and suggested that the inhibition of DNA polymerase β by Cl-dATP was partially responsible for the cytotoxicity against resting human lymphocytes seen with Cl-dAdo.

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