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Molecular Basis of the Antineoplastic Activity of 3′-Amino-3′-deoxythymidine

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SUMMARY

3′-Amino-3′-deoxythymidine decreased the incorporation of [2-^{14}C]thymidine into DNA of L1210 cells *in vitro*, and produced an accumulation of [2-^{14}C]thymidine di- and triphosphate. The extent of these effects varied with the amount of recovery time after removal of 3′-amino-3′-deoxythymidine prior to addition of labeled thymidine. The distribution of radioactivity in the acid-soluble fraction derived from [^{3}H]3′-amino-3′-deoxythymidine was as follows: 50% as 3′-amino-3′-deoxythymidine, 20% as the monophosphate, 10% as the diphosphate, and 20% as the triphosphate derivatives. No incorporation of [^{3}H]3′-amino-3′-deoxythymidine into L1210 DNA could be detected. 3′-Amino-3′-deoxythymidine-5′-triphosphate is a competitive inhibitor against dTTP with a K_i of 3.3 μM, whereas the K_m for dTTP was 8 μM using activated calf thymus DNA as the template and DNA polymerase-α. These data indicate that a major site of inhibition by 3′-amino-3′-deoxythymidine is inhibition of the DNA polymerase reaction.

INTRODUCTION

Nucleoside analogues are not only useful chemotherapeutic agents but also tools in the study of biochemical mechanisms. Slight structural modifications of thymidine have produced dramatic changes in biological activities (1–3). AdThd[2] was synthesized by Miller and Fox (4) and Horwitz *et al.* (5), and was shown later by Lin and Prusoff (1) and Fischer *et al.* (6) to have potent cytotoxicity against L1210 cells in culture (ID_{50} = 1 μM) which could be prevented or even reversed by pyrimidine deoxyribonucleosides, but not by pyrimidine or purine ribonucleosides (6). AdThd was also found to have no effect on RNA or protein synthesis, but to be a selective inhibitor of DNA synthesis in L1210 cells. This analogue produced "cures" (>60-day survivors) in CDF_1 mice bearing the L1210 leukemia (7). In contrast, the 5′-amino analogues of thymidine and of 5-iodo-2′-deoxyuridine have essentially no cytotoxicity but have good but not potent antiviral activity (1, 2).

A related compound, 3′-fluoro-3′-deoxythymidine, was shown to be a good inhibitor of DNA synthesis *in vivo*, and also to be incorporated into DNA (8, 9). 2′,3′-Dideoxythymidine-5′-triphosphate was also shown to be incorporated into DNA and to act as a chain terminator (10). This report describes the metabolic studies of AdThd, its effect on DNA synthesis *in vitro*, and the inhibition by its triphosphate derivative of purified L1210 DNA polymerase-α.

EXPERIMENTAL PROCEDURES

Materials

AdThd was synthesized as described previously (1). Tritiated AdThd was obtained from New England Nuclear Corporation (Boston, Mass.) and purified as follows: Tritiated AdThd was purified by HPLC (column, HPAN-90 cation exchanger; buffer, 0.3 M NH_4ClO_4/0.1 M citric acid; temperature, 56°; flow rate, 1 ml/min). AdThd had a retention time of 58 min under this condition. The HPLC-purified tritiated AdThd was adsorbed onto a Dowex 50 H^+ column and eluted with a 100-ml 1 N NH_4OH/100-ml water gradient. The specific activity of this purified [^{3}H]AdThd was 1080 Ci/mole. [2-^{14}C]Thymidine (50 mCi/mmole) and [8-^{3}H]deoxyadenosine-5′-triphosphate were from Moravek Biochemicals (Brea, Calif.). [*methyl*-^{3}H]Thymidine triphosphate was from New England Nuclear Corporation. Poly(dA)·$d(pT)_{10}$ was from P-L Biochemicals, Inc. (Milwaukee, Wisc.), and nucleosides were from Sigma Chemical Company (St. Louis, Mo.). All other chemicals were reagent-grade.

AdThd-5′-monophosphate was synthesized by the procedure described by Yoshikawa *et al.* (11) and purified by chromatography on a DE-52 cellulose column (27.5 × 3 cm, equilibrated with 1 M sodium bicarbonate and washed thoroughly with water). The compound was eluted with a gradient of 300 ml of 0.7 M triethylamine bicarbonate and 300 ml of water. AdThd-5′-monophosphate was further purified on an activated carbon column and eluted with a solution of 1:1 ratio of 1 N NH_4OH/95% ethanol. AdThd-5′-triphosphate was synthesized and purified by the procedure of Hoard and Ott (12) and Hurlbert *et al.* (13).

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[2] The abbreviations used are: AdThd, 3′-amino-3′-deoxythymidine; HPLC, high-pressure liquid chromatography; BSA, bovine serum albumin; TLC, thin-layer chromatography; PBS, phosphate-buffered saline.

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Methods

Cell culture. Suspension cultures of murine leukemia L1210 cells were grown in Fisher's medium at 37° supplemented with 10% horse serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Grand Island Biological Company, Grand Island, N. Y.) in a 5% carbon dioxide incubator. Asynchronous, exponentially growing cells were used in all experiments. A Model ZBI Coulter counter was used for counting cells.

DNA polymerase assay. DNA polymerase from L1210 cells was purified by the procedure of Fisher and Korn (14), up to and including phosphocellulose chromatography, and the purified enzyme was kept at −70° until used. Activated calf thymus DNA was prepared by the procedure of Schlabach *et al.* (15). DNA polymerase activities were assayed as follows: 100 μl of incubation mixture contained 100 μM each of dATP, dCTP, dGTP, and dTTP; 47 mM $MgCl_2$; 1.25 mg of BSA; 8% glycerol; 6 μCi of [^{3}H]dATP; and 0.24 absorbance unit of activated DNA. After incubating at 37° for various periods of time, 15 μl were removed from the incubation mixture and spotted onto a Whatman 3-mm disc and dipped into a 5% trichloroacetic acid and 1% pyrophosphate solution. The discs were washed in the trichloroacetic acid/pyrophosphate solution three times for 5 min each, and finally washed in 95% ethanol. The amount of radioactivity was detected in a Beckman 7500 liquid scintillation counter.

Metabolic Studies

Thymidine. TLC separation of [2-^{14}C]thymidine and its phosphorylated derivatives in cell extracts used cellulose Polygram Cel 300 PEI/UV with 0.5 M LiCl or 2 N acetic acid/0.5 M LiCl as the solvent, as described previously (16). Cell extracts were prepared as follows: L1210 cells (0.5 to 1 × 10^7 cells) were extracted with 200 μl of 0.7 N $HClO_4$ for 20 min in a sonicated ice water bath. The sample was then centrifuged to pellet the cells, and 200 μl of the supernatant were removed and neutralized with 2 N KOH. Ten microliters of this neutralized extract were applied to TLC strips. A second extract was prepared and neutralized with KOH in the same manner. Portions of the first and second extracts were counted for their content of radioactivity. This represented the total extractable radioactivity.

The cell pellet was further extracted with 500 μl of 0.7 N $HClO_4$. The cell pellet was then transferred to a Whatman 3 MM paper disc (on a filter assembly) and washed two times with 5 ml of 5% trichloroacetic acid and finally with 95% ethanol. This represents the radioactivity associated with the acid-insoluble fraction.

AdThd. L1210 cells were incubated with 1.75 μM [^{3}H]AdThd (1.08 Ci/mmole) for 24 hr. After harvesting the cells by centrifugation, the cell pellet was washed once with PBS. The extraction of the acid-soluble AdThd metabolites was as described for thymidine. The neutralized extract containing the metabolites was subjected to analysis by HPLC using a Whatman SCX column. The HPLC conditions used for the analysis were as follows: I. Solvent: 0.4 M phosphate buffer/5% acetonitrile, pH 3.3; flow rate, 0.6 ml/min; temperature, 30°; retention time of AdThd-5′-triphosphate was 22 min, for dTTP was 35 min, and for dTDP 9 min. II. Solvent: 0.1 M phosphate buffer, pH 3.3; flow rate, 0.9 ml/min; temperature, 30°; retention time for AdThd-5′-diphosphate was 12 min, and for dTDP 32 min. III. Solvent: 10 mM phosphate buffer, pH 6.4; flow rate, 0.5 ml/min; temperature, 30°; the retention time for AdThd was 10 min and for the 5′-monophosphate of AdThd was 15 min, whereas dTMP had a retention time of 23 min.

DNA analysis. Twenty milliliters of a culture of L1210 cells (cell density 1.5 × 10^5 cells/ml) were incubated with 0.34 μM [^{3}H]AdThd (1.08 Ci/mmole) for 48 hr at 37°. During this incubation period, the cell density increased to 3.2 × 10^5 cells/ml. The cells were harvested by centrifugation and washed once with PBS. The cell pellet was digested with 0.5 ml 0.015 M NaCl/1.5 mM sodium citrate/20 mM EDTA (pH 7.3) containing 2 mg of pronase and 1% Sarkosyl at 37° overnight. The DNA was then isolated by CsCl density gradient centrifugation as described previously (17). The CsCl gradient-purified DNA was passed through a Sephadex G-50 column to remove the CsCl. The DNA was then digested with DNAse 1, followed by digestion with venom phosphodiesterase, and finally with *Escherichia coli* alkaline phosphatase. The incubation mixture was subjected to HPLC (Whatman SCX column, 10 mM phosphate buffer (pH 5.9), flow rate 1 ml/min). AdThd had a retention time of 15 min under this condition.

RESULTS

Effect of AdThd on the metabolism of [2-^{14}C]-thymidine in L1210 cells in vitro. The effect of a 90-min preincubation of L1210 cells with AdThd on the metabolism of thymidine when in the continued presence or absence of AdThd is shown in Table 1. Preincubation of L1210 cells with AdThd increased the formation of phosphorylated thymidine derivatives in the acid-soluble fraction, but decreased the incorporation of [2-^{14}C]thymidine into the acid-insoluble fraction. Washing the cells prior to addition of labeled thymidine increased the formation of dTDP, dTTP, and uptake into DNA.

The effect of preincubation of L1210 cells with AdThd and its subsequent removal on the metabolism of labeled thymidine is shown in Table 2. After AdThd had been removed from the medium for 3 hr, the amount of radioactive thymidine associated with the acid-insoluble fraction had recovered to that of the control, even though the concentrations of radioactive thymidine phosphorylated derivatives in the acid-soluble fraction were still higher than that in the control.

Identification of metabolically formed phosphorylated derivatives of [^{3}H]AdThd. The distribution of the radioactive metabolites of AdThd in the acid-soluble fraction after 24 hr of incubation was 20% as AdThd-5′-triphosphate, 10% as AdThd-5′-diphosphate, 20% as AdThd-5′-monophosphate, and 50% as AdThd.

Absence of AdThd in L1210-DNA. Attempts were made to determine whether AdThd was incorporated into the DNA of L1210 cells after 48 hr of incubation, during which time the cell number doubled. The conditions for growth, DNA isolation, digestion, and analysis by HPLC are presented under Methods. No radioactivity could be detected in the DNA fraction. Subsequent enzymatic

TABLE 1

Effect of a 90-min preincubation or continuous exposure to AdThd on the metabolism of [2-^{14}C]thymidine by L1210 cells

The procedure for pulse labeling of L1210 cells with [2-^{14}C]thymidine and isolation of metabolites is described under Methods.

Condition	Acid-soluble	TTP[a]	TDP[a]	Acid-insoluble
Control[b]	1.00	1.00	1.00	1.00
Preincubation with 10 μM AdThd for 90 min prior to addition of 0.18 μM [2-^{14}C]dThd for 30 min	1.55	1.35	1.21	0.43
Preincubation with 10 μM AdThd for 90 min, then washed to remove AdThd prior to addition of 0.18 μM [2-^{14}C]dThd for 30 min	1.82	1.69	1.67	0.57

[a] The percentage of total acid-soluble counts in the control cells represented by TTP is 74% and by TDP is 10%.

[b] The control data have been normalized to 1.00.

TABLE 2

Effect of a 60-min preincubation of L1210 cells with AdThd and its subsequent removal on the metabolism of [2-^{14}C]thymidine

Cells were treated with 7.5 μM AdThd (samples 2–7) for 1 hr, then all samples were centrifuged, resuspended in fresh media, and incubated for the amount of time indicated before time 0. [2-^{14}C]dThd (18 μM) was added for 30 min. The procedure for the isolation of the metabolites is described under Methods.

Sample	Condition	Time between removal of AdThd and addition of thymidine	Acid-soluble	dTTP[a]	dTDP[a]	Acid-insoluble
		hr				
1	Control[b]	0	1.00	1.00	1.00	1.00
2	AdThd, 7.5 μM	0	1.73	1.88	0.91	0.57
3	AdThd, 7.5 μM	1	1.56	1.50	1.73	0.69
4	AdThd, 7.5 μM	2	1.55	1.12	1.36	0.79
5	AdThd, 7.5 μM	3	1.49	1.39	1.55	0.92
6	AdThd, 7.5 μM	4	1.44	1.21	2.82	1.05
7	AdThd, 7.5 μM	5	1.35	1.24	2.91	0.99

[a] The radioactivity in the acid-soluble fraction of the control cells was 89% as dTTP and 9% as dTDP.
[b] The control data have been normalized to 1.00.

digestion of the DNA to the nucleosides and analysis by HPLC did not reveal the presence of either labeled or nonlabeled AdThd. This step was performed to eliminate the remote possibility that tritium exchange resulted in loss of label from AdThd if it had been incorporated.

Effect of AdThd-5'-triphosphate on L1210 DNA polymerase. Figure 1 shows the inhibition pattern of AdThd-5'-triphosphate against dTTP in the presence of 100 μM dGTP, dCTP, and dATP. With activated calf thymus DNA as the template, the K_m for dTTP was 8 μM, whereas the K_i for AdThd-5'-triphosphate against dTTP was 3.3 μM with competitive kinetics.

Figure 2 shows that the rate of the DNA polymerase reaction is linear in the presence of dGTP, dCTP, dATP, and [^{3}H]TTP with activated calf thymus DNA as template. AdThd-5'-triphosphate decreased markedly the rate of the enzyme reaction. When dTTP was the only nucleoside triphosphate in the enzyme assay mixture, the reaction rate was decreased by a factor of 8 relative to the rate when the other three deoxynucleoside-5'-triphosphates (dCTP, dGTP, and dATP) were present. This residual enzyme activity was not significantly inhibited by AdThd-5'-triphosphate. The K_m for dTTP for this residual enzyme activity was 2.2 μM.

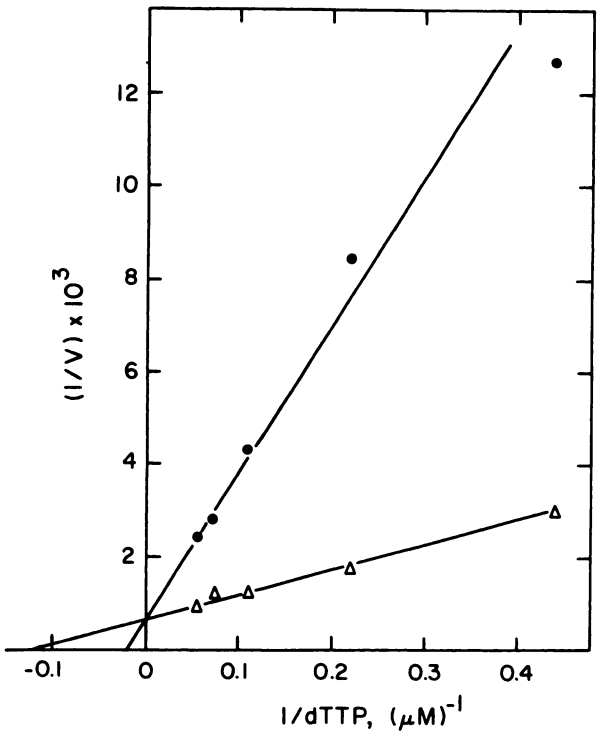

FIG. 1. *Inhibition by AdThd-5'-triphosphate of L1210 DNA polymerase-α*

AdThd-5'-triphosphate (18 μM) was either present (●) or absent (△) in the reaction mixture. The reaction mixture (100 μl) contained in addition 100 μM each of dATP, dCTP, and dGTP; BSA (1.25 mg/ml); 10% glycerol; 60 mM Tris (pH 8.0); 16 mM $MgCl_2$; 0.24 absorbance unit of activated DNA; the indicated amount of [^{3}H]dTTP; and L1210 DNA polymerase-α. The measurement of [^{3}H]dTMP incorporation into DNA is described under Methods.

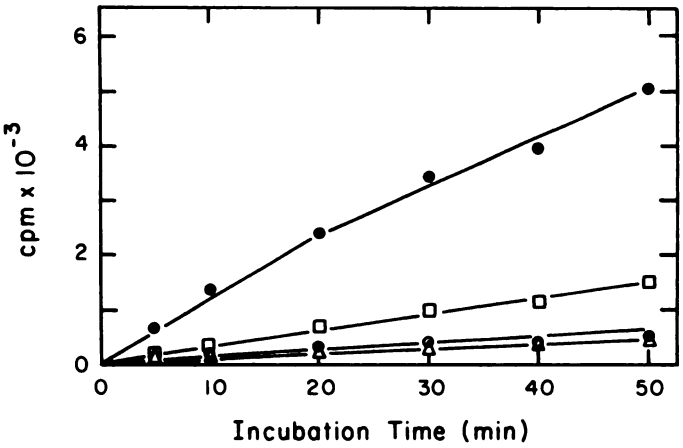

FIG. 2. *Effect of AdThd-5'-triphosphate on L1210 DNA polymerase-α in the presence and absence of 100 μM dCTP, dATP, and dGTP.*

The basic enzyme reaction mixture (200 μl) contained 11 μM [^{3}H]TTP, BSA (1.25 mg/ml), 10% glycerol, 60 mM Tris (pH 8.0), 16 mM $MgCl_2$, 0.24 absorbance unit of activated calf thymus DNA, and L1210 DNA polymerase-α. The following components were added: 1. 100 μM each of dCTP, dATP, and dGTP (●); 2. 100 μM each of dCTP, dATP, and dGTP, and 58 μM AdThd-5'-triphosphate (□); 3. no dCTP, dATP, or dGTP (○); 4. no dCTP, dATP, or dGTP, but 58 μM AdThd-5'-triphosphate (△). The measurement of the incorporation of [^{3}H]TMP into DNA is as described under Methods.

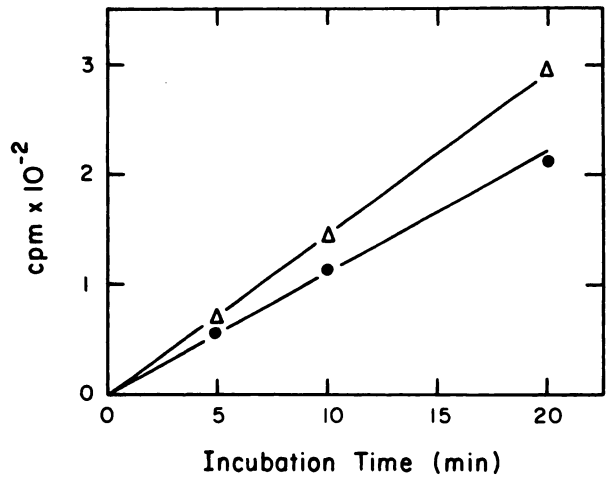

FIG. 3. *Effect of AdThd-5′-triphosphate on L1210 DNA polymerase-α when poly(dA)·d(pT)$_{10}$ was used as the template*

The enzyme reaction mixture (100 μl) contained BSA (1.25 mg/ml), 10% glycerol, 60 mM Tris (pH 8.0), 10 μM [^{3}H]dTTP, 16 mM $MgCl_2$, 0.25 absorbance unit of poly(dA)·d(pT)$_{10}$, and L1210 DNA polmerase-α; 10 μM AdThd-5′-triphosphate was either present (●) or absent (Δ). Measurement of the incorporation of [^{3}H]dTMP into DNA is as described under Methods.

The effect of AdThd-5′-triphosphate on the rate of enzyme reaction when poly(dA)·d(pT)$_{10}$ was used as the template is shown in Fig. 3. The reaction rates were linear in both the presence and absence of AdThd-5′-triphosphate. The K_m for dTTP with poly(dA)·d(pT)$_{10}$ as template was 33 μM.

The effect of AdThd-5′-triphosphate on the L1210 DNA polymerase-α reaction is shown in Table 3. The addition of AdThd-5′-triphosphate to the reaction mixture inhibited the incorporation of [^{3}H]dAMP into DNA. The incorporation of the labeled precursor into DNA was 24% below that found in reaction mixture 2, which contained dCTP, dGTP, and dATP but no dTTP.

Effect of AdThd on the growth of L1210 cells. The effect of pretreatment or the continuous presence of AdThd on the growth of L1210 cells is shown in Fig. 4. The cell culture that was treated with AdThd for 90 min prior to drug removal by washing showed the same rate of growth as the untreated control. The continued presence of AdThd in the media inhibited markedly the growth of the L1210 cells.

TABLE 3

Effect of AdThd-5′-triphosphate (AdTTP) on the L1210 DNA polymerase-α reaction

The enzyme reaction mixture (100 μl) contained BSA (1.25 mg/ml), 10% glycerol, 60 mM Tris (pH 8.0), 24 mM $MgCl_2$, 100 μM dCTP, 100 μM dGTP, 100 μM [^{3}H]dATP (6 μCi), 0.24 absorbance unit of activated calf thymus DNA, L1210 DNA polymerase-α, and the indicated amounts of dTTP and AdThd-5′-triphosphate.

Incubation mixture	dTTP	3′-AdTTP	[^{3}H]dAMP incorporated
	μM	μM	%
1	10	0	100
2	0	0	65
3	10	104	41

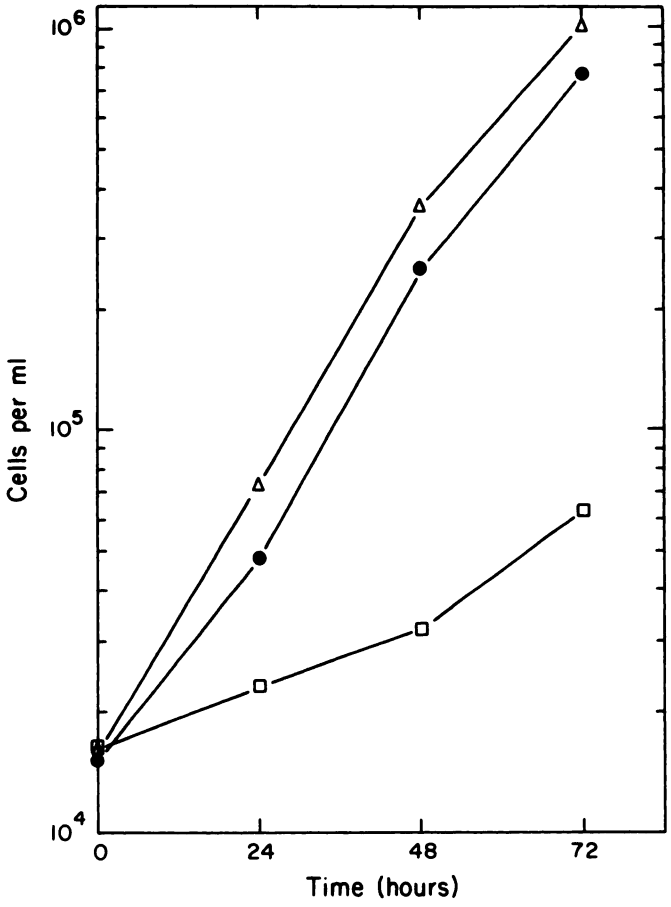

FIG. 4. *Effect of 3′-amino-3′-deoxythymidine on the replication of L1210 cells*

Two of three batches of exponentially growing L1210 cells were treated with 6.6 μM AdThd for 90 min (●, □), of which one batch (●) was then centrifuged, washed once with drug-free medium, and resuspended into drug-free medium. The second batch was continuously exposed (72 hr) to AdThd (□). The third batch was not exposed to AdThd (Δ). Cell counts were followed for 72 hr.

DISCUSSION

Exposure of L1210 cells to AdThd followed by a pulse label with [2-^{14}C]thymidine caused the accumulation of radioactive metabolites, primarily as dTTP in the acid-soluble fraction, and an inhibition of the incorporation of thymidine into DNA which returned to normal within 3 hr after AdThd was removed. [^{3}H]AdThd was not incorporated into DNA, but was phosphorylated to its triphosphate derivative, and was shown to be a competitive inhibitor of the utilization of dTTP by L1210 DNA polymerase-α with a K_i of 3.3 μM. A consequence of this inhibition was a decrease in the incorporation of [^{3}H]dAMP into DNA. Thus AdThd-5′-triphosphate not only inhibited the biosynthesis of DNA, but also was not utilized as a substrate since the rate of [^{3}H]dAMP incorporation into DNA was not above the base level.

The inhibitory effect of AdThd appears to be dependent on its continued presence, since the rate of replication of the inhibited L1210 cells resumed to that of the control cells when the analogue was removed.

This reversal is probably due to either a decrease in the concentration of the triphosphate analogue by cata-

bolic dephosphorylation inside the cell, or to a relative increase in the pool of dTTP. These data indicate that the inhibitory effect of AdThd occurs after conversion to the triphosphate derivative, and that the site of inhibition is at the DNA polymerase stage.

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