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METABOLISM AND METABOLIC ACTIONS OF 4′-THIOTHYMIDINE IN L1210 CELLS

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Abstract—4′-Thiothymidine (S-dThd) is a potent inhibitor of L1210 cell growth and is active against P388 leukemia in mice. Because of these activities and its novel structure, we have begun studies of its metabolism and metabolic actions in L1210 cells in order to understand its mechanism of cytotoxicity. S-dThd inhibited the incorporation of radiolabeled precursors into DNA, but did not inhibit the incorporation of either uridine or leucine into RNA or protein, respectively, which indicated that the mechanism of its toxicity was due to its inhibition of DNA synthesis. S-dThd did not decrease the concentration of any of the natural deoxynucleoside triphosphates, which indicated that its cytotoxicity was not due to the inhibition of ribonucleotide reductase. S-dThd was readily phosphorylated and used as a substrate for DNA synthesis. Because the rate of incorporation of S-dThd into DNA was 20% that of thymidine, it is likely that the mechanism of action of S-dThd is not due to inhibition of DNA polymerases by the 5′-triphosphate of S-dThd, but instead to its incorporation into the DNA and its subsequent disruption of some function of DNA.

Key words: thiothymidine; metabolism; cytotoxicity; mechanism of action

Numerous nucleoside analogs have been synthesized that contain a sulfur atom in place of the 4′-oxygen in an effort to create new chemotherapeutic compounds [1–13]. 4′-Thionucleosides are poor substrates for phosphorylases and, therefore, are more stable in biological systems than are natural nucleosides [2, 9]. Some of these agents have been shown to have potent activity against certain herpes viruses [7] and HIV [10]. We recently synthesized a number of 2′-deoxy-4′-thionucleosides as potential antitumor agents [8, 11]. The most potent of these agents against murine and human cells in culture was S-dThd† (Fig. 1). In addition, S-dThd has been found to have modest antitumor activity against the P388 mouse leukemia model: a 45% increase in lifespan at the maximally tolerated dose‡. Because very little is known about the metabolism and biochemical actions of any of these compounds, we have evaluated the biochemical pharmacology of S-dThd in L1210 cells in an effort to understand the biochemical perturbations caused by S-dThd that lead to cytotoxicity. A preliminary report of this work has been presented [14].

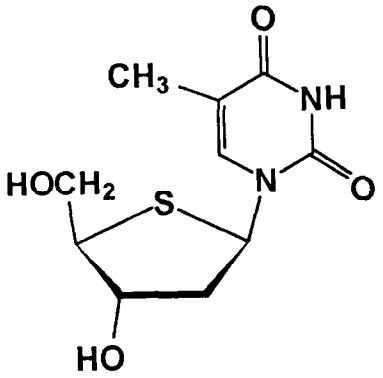

Fig. 1. Structure of 4′-thiothymidine.

MATERIALS AND METHODS

Materials. S-dThd and [^{3}H]S-dThd were synthesized in our laboratories as described [8]. [*Methyl*-^{3}H]S-dThd (19 Ci/mol) was synthesized using 25 mCi of [*Methyl*-^{3}H]thymine (50–70 Ci/mmol) that was obtained from Moravek Biochemicals, Brea, CA. During the course of these experiments, the purity of [^{3}H]S-dThd was 90–97% as judged by reverse-phase HPLC analysis. [*Methyl*-^{3}H]dThd (65 Ci/mmol), [5-^{3}H]uridine (23 Ci/mmol), [8-^{14}C]hypoxanthine (56 mCi/mmol), [8-^{14}C]deoxyguanosine (56 mCi/mmol), and [2-^{14}C]dCyd (56 mCi/mmol) also were obtained from Moravek Biochemicals. [4, 5-^{3}H]Leucine (69 Ci/mol) was obtained from the Amersham Corp. (Arlington Heights, IL).

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† Abbreviations: dThd, thymidine; S-dThd, 4′-thiothymidine; S-dTTP, 5′-triphosphate of S-dThd; dCyd, 2′-deoxycytidine; IC_{50}, concentration of drug required to inhibit cell growth by 50%; S-dGTP; 5′-triphosphate of 6-thio-2′-deoxyguanosine; and Br-dUTP, 5′-triphosphate of 5-bromo-2′-deoxyuridine.

‡ Waud WR, unpublished observation. Cited with permission.

Escherichia coli DNA polymerase I, dATP, dCTP, dGTP, dTTP, poly(dI-dC), and poly(dA-dT) were obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). 9-Benzyl-9-deazaguanine was prepared as described [15].

Cell culture. L1210 cells, obtained from the American Type Culture Collection (Rockville, MD), were grown in Fischer's medium (Life Technologies, Grand Island, NY) containing heat-inactivated 10% horse serum (Life Technologies), 500 U/mL penicillin (local distributer), 50 μg/mL streptomycin (ICN Biomedicals, Inc., Costa Mesa, CA), and 50 μg/mL gentamicin (Life Technologies). Cells were tested routinely for the presence of mycoplasma. All experiments were conducted with cells that were proliferating at maximal rates. For determination of drug effect on cell growth, the cell numbers were determined using a Coulter Counter 24 and 48 hr after addition of drug. To determine the effect of drug on the ability of L1210 cells to form colonies, cells were incubated with S-dThd for 2 hr. Drug was then washed from the cells, and the cells were resuspended in conditioned culture medium that contained 0.2% agar and 20% horse serum. Conditioned medium was obtained from 48-hr L1210 cell suspensions (approximately 3.5×10^5 cells/mL). Cells were removed by centrifugation and filtration through a 0.45-μm filter. After 14 days of incubation at 37° in a humidified 5% CO_2 atmosphere, the colonies were visible to the naked eye and could be counted. The cloning efficiency of L1210 cells was approximately 30%.

Measurement of RNA, DNA, and protein synthesis. The effect of S-dThd on the incorporation of radiolabeled precursors into RNA, DNA, and protein was determined as described [16, 17].

Measurement of acid-soluble metabolites of radiolabeled nucleosides. L1210 cells were incubated with labeled nucleosides for the indicated times at 37°. The cells were collected by centrifugation, and were resuspended in 110 μL of ice-cold 0.5 M perchloric acid. The acid-insoluble material was removed by centrifugation at 12,000 *g* for 20 min, and after extended washing it was counted for radioactivity. One hundred microliters of the supernatant fluid was removed and neutralized with 7.5 μL of 1 M potassium phosphate (pH 7.4) and 12.5 μL of 4 M KOH. $KClO_4$ was removed by centrifugation at 12,000 *g* for 20 min, and a portion of the supernatant fluid was injected onto a Partisil-10 SAX anion exchange column (Keystone Scientific Inc., State College, PA). Elution of the nucleotides was accomplished with a 50-min linear gradient from 5 mM $NH_4H_2PO_4$ (pH 2.8) to 750 mM $NH_4H_2PO_4$ (pH 3.7) buffer with a flow rate of 2 mL/min. The natural nucleotides were detected by measurement of the UV absorbance at 254 nm, and the radioactive acid-soluble metabolites were detected by counting 1-min fractions that eluted from the column.

Measurement of the incorporation of nucleosides into DNA. L1210 cells incubated with either [^{3}H]S-dThd or [^{3}H]dThd were collected by centrifugation and resuspended in 0.5 mL of 10 mM Tris (pH 8.0), 40 mM EDTA, 0.5% SDS, and 200 μg/mL of proteinase K. The mixture was incubated at 37° overnight, mixed with CsCl, and centrifuged to equilibration as described previously [18]. The gradients were fractionated, and the DNA in each sample was precipitated onto glass fiber filters with a 5% trichloroacetic acid solution containing 10 mM pyrophosphate. These filters were washed three times with this 5% trichloroacetic acid solution followed by two washes with 95% ethanol, dried, and counted for radioactivity. To verify that the radioactivity associated with the DNA fractions was due to the incorporation of [^{3}H]S-dThd or [^{3}H]dThd, the DNA samples were degraded to their constituent nucleosides, which were separated by reverse-phase HPLC as described [18, 19].

Determination of deoxynucleotide pools. The deoxyribonucleoside triphosphate pools were measured using the method of Solter and Handschumacher [20] with slight modifications [21]. In this assay, *E. coli* DNA polymerase I reaction mixtures that were deficient in only one of the deoxynucleoside triphosphates were supplemented with cell extract, and the reaction rate could be related to the amount of the missing deoxynucleotide in the extract.

Measurement of dThd kinase activity in L1210 *cell extracts.* Cell-free extracts were prepared as described [22]. Approximately 1 mL of packed L1210 cells was mixed with 5 mL of 10 mM Tris, pH 7.5, 10% glycerol, 0.15 M NaCl, 20 μM dThd, 2 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride and homogenized using a Dounce homogenizer with a tight-fitting pestle (pestle A). The sample was centrifuged at 100,000 *g* for 1 hr. Streptomycin sulfate was added to the supernatant solution to a final concentration of 2%, and the sample was centrifuged at 10,000 *g* for 10 min. This sample was dialyzed overnight against 10 mM Tris, pH 7.5, 30% glycerol, 2 mM dithiothreitol, 0.5 mM EDTA, and 100 mM KCl. The phosphorylation of dThd by this extract was not affected by 1000-fold excess of dCyd, which indicated that the activity being measured was cytosolic dThd kinase activity and not mitochondrial dThd kinase [23]. dThd kinase activity was measured in 100 μL volumes containing 200 mM Tris, pH 7.5, 2 mM ATP, 2 mM $MgCl_2$, 1 mg/mL BSA, 10 mM dithiothreitol, 10 mM NaF, L1210 extract, and either [^{3}H]dThd or [^{3}H]S-dThd. The reaction was stopped by spotting 50 μL of the reaction mixture onto DE-81 filters. The disks were washed three times with 95% ethanol/5% H_2O, and the radioactivity on each disk was determined.

RESULTS

Effect of S-dThd on cell growth. S-dThd was approximately 100-fold more potent as an inhibitor of L1210 cell growth than was dThd. The IC_{50} of S-dThd was dependent on the initial number of cells that were treated with S-dThd. The IC_{50} of S-dThd was 590 nM when the initial L1210 cell density was 167,000 cells/mL, and it was 41 ± 10 nM when the initial L1210 cell density was 22,000 cells/mL (data not shown). Treatment of L1210 cells with S-dThd for as little as 2 hr was sufficient to reduce the ability of L1210 cells to form colonies in soft agar: the concentration of S-dThd required to inhibit colony formation by 50% was 50 nM (data not shown). The

IC_{50} of S-dThd in CEM cells deficient in dThd kinase activity was 100-fold greater than its IC_{50} against wild-type CEM cells, which indicated that the cytotoxicity of S-dThd was dependent on its phosphorylation by dThd kinase (data not shown).

Effect of S-dThd on macromolecular synthesis. Treatment of L1210 cells with S-dThd did not affect the incorporation of leucine into protein or the incorporation of uridine, cytidine, or hypoxanthine into RNA (data not shown). Incubation of L1210 cells with S-dThd inhibited the incorporation of both hypoxanthine and deoxyguanosine into the DNA (Fig. 2). Incubation of cells with 1 μM S-dThd inhibited DNA synthesis by approximately 50%. A similar inhibition of the incorporation of deoxyguanosine into DNA was observed after either 0.5 or 20 hr of preincubation with S-dThd (0.1 to 1.0 μM). These results suggested that the toxicity of S-dThd to L1210 cells was related to its inhibition of DNA synthesis. However, the modest decrease in DNA synthesis was consistent with the possibility that neither S-dThd nor its metabolites directly inhibited enzymes involved in the synthesis of DNA, such as ribonucleotide reductase and DNA polymerases.

Metabolism of S-dThd and its incorporation into DNA. The primary metabolite of S-dThd in L1210 cells was S-dTTP (Fig. 3). As seen in this figure, the metabolism of S-dThd to its acid-soluble metabolites was very similar to the metabolism seen with dThd, except that the concentration of S-dTTP was approximately 20% of dTTP. Cells rapidly converted both S-dThd and dThd to their respective triphosphates. Peak levels of both nucleotides were achieved in the first hour of treatment (Fig. 4). The intracellular concentration of both triphosphates appeared to be stable until the medium was depleted of nucleoside (Fig. 4 and Table 1).

S-dThd was readily incorporated into the DNA of L1210 cells. Greater than 95% of the total acid-insoluble radioactivity in cells treated with S-dThd was found in the DNA band of the CsCl gradient. Degradation of the radiolabeled DNA to its component nucleosides followed by HPLC analysis confirmed that the radioactivity associated with the DNA in the CsCl gradient was S-dThd (Fig. 5). Incubation of L1210 cells with 0.2, 2, or 20 μM S-dThd resulted in similar amounts of S-dThd in DNA (Table 1). The S-dThd that was incorporated into the DNA was not removed from the DNA for up to 72 hr (data not shown), which indicated that the S-dThd in the DNA was not recognized by any DNA repair enzyme as a fraudulent nucleoside.

The results shown in Table 1 compare the conversion of dThd and S-dThd to their respective triphosphates and their incorporation into DNA. In this experiment, 78% of all of the dThd (0.2 μM) was incorporated into the DNA in the first hour, and within 2 hr all of the dThd had been taken up from the medium and incorporated into cellular DNA. Under similar conditions, 50% of the S-dThd (0.2 μM) was taken up by the cells and incorporated into the DNA in 6 hr of incubation. Most (>90%) of the S-dThd at 0.2 μM could be taken up by L1210 cells and incorporated into the DNA after longer incubation periods. These results indicated that the

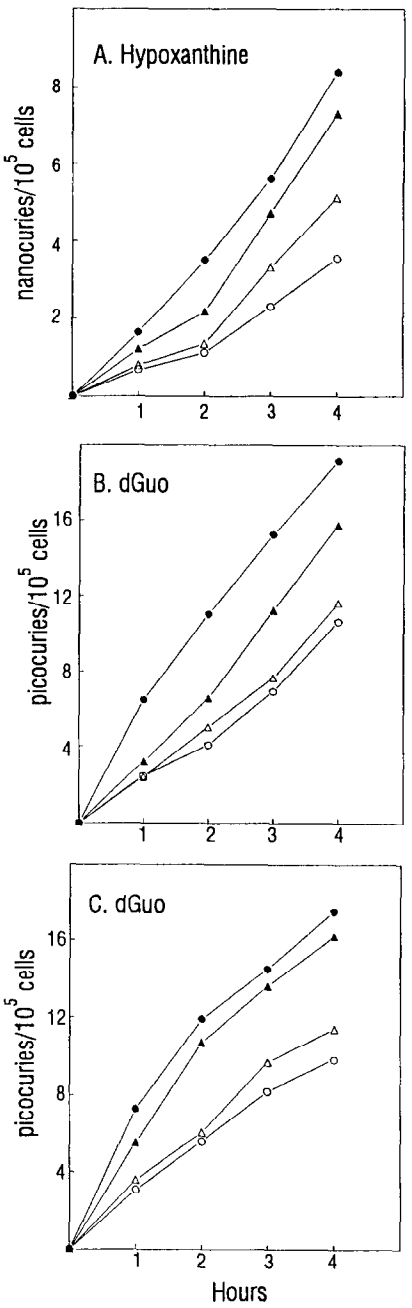

Fig. 2. Effect of S-dThd on the incorporation of hypoxanthine and deoxyguanosine into DNA. (A) [8-^{14}C]-hypoxanthine was added to L1210 cultures 30 min after the addition of 0 (●), 0.1 (▲), 0.3 (△), or 1 (○) μM S-dThd. Samples were removed at 1, 2, 3, and 4 hr after the addition of [8-^{14}C]hypoxanthine, and the incorporation of label into acid-insoluble/alkali stable fraction was determined. (B) the same as in panel A except that the cells were incubated with [^{14}C]deoxyguanosine plus 9-benzyl-9-deazaguanine (2 μg/mL) instead of [8-^{14}C]hypoxanthine. 9-Benzyl-9-deazaguanine is a potent inhibitor of purine nucleoside phosphorylase and was used to prevent the conversion of dGuo to guanine. (C) the same as panel B except that [^{14}C]deoxyguanosine plus 9-benzyl-9-deazaguanine were added 20 hr after S-dThd.

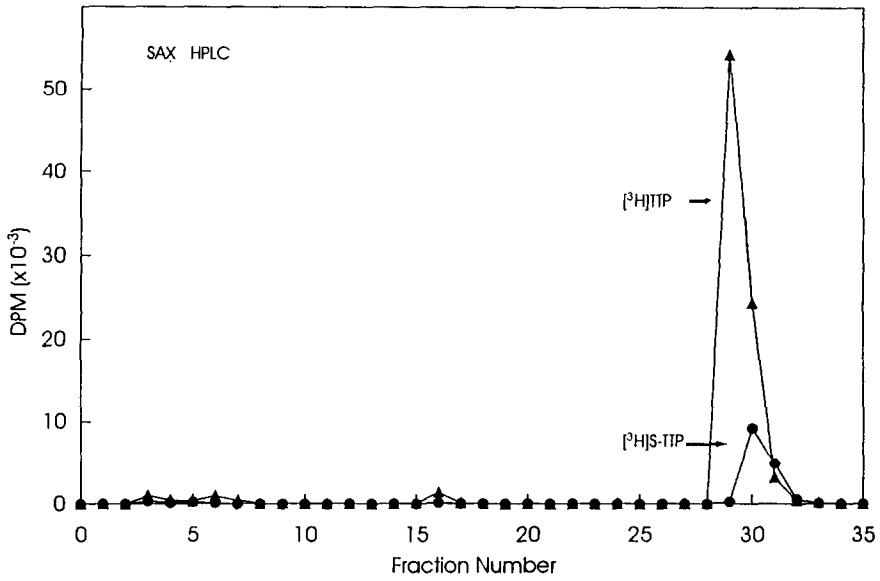

Fig. 3. Metabolism of S-dThd in L1210 cells. L1210 cells were incubated with a 20 μM concentration of either [^{3}H]dThd (19 Ci/mol) or [^{3}H]S-dThd (19 Ci/mol) for 1 hr at 37°. The cells were collected, and the acid-soluble nucleotides were separated using SAX HPLC. Each fraction eluting from the column was counted for radioactivity.

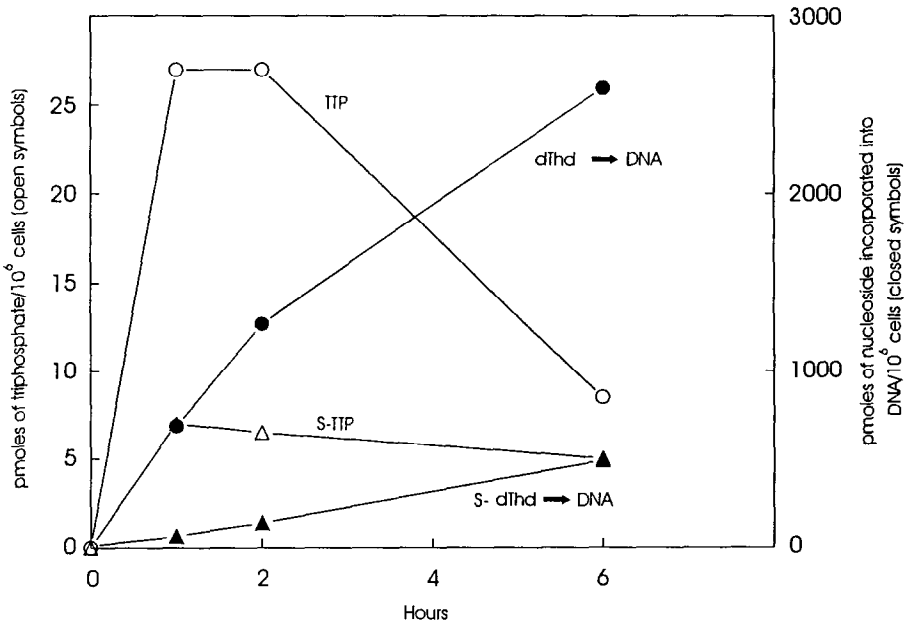

Fig. 4. Incorporation of dThd and S-dThd into the triphosphate pool and DNA of L1210 cells. L1210 cells were incubated at 37° with a 2 μM concentration of either [^{3}H]dThd (19 Ci/mol) or [^{3}H]S-dThd (19 Ci/mol). At 1, 2, and 6 hr after the beginning of the experiment, the nucleotides in the acid-soluble extract were separated using SAX HPLC, and the amount of triphosphate of each nucleoside was determined. The incorporation of radioactivity into the acid-insoluble fraction (DNA) of each sample was also determined at these times.

DNA polymerases responsible for the majority of DNA synthesis in L1210 cells were able to efficiently use S-dTTP as a substrate for DNA synthesis.

Treatment of L1210 cells with either 0.2 or 2 μM S-dThd resulted in a peak concentration of S-dTTP of 4 or 7 pmol/10^6 cells (Table 1), which was approximately 20% of the control dTTP concentration (see Table 3). Therefore, the cytotoxicity

Table 1. Incorporation of dThd and S-dThd into the triphosphate pool and DNA of L1210 cells

Time (hr)	Concn (μM)	Thymidine dTTP	Thymidine DNA	Thiothymidine S-dTTP	Thiothymidine DNA
		(pmol/10^6 cells)			
1	0.2	12.7	248	3.7 (29)	50 (20)
	2.0	26.9	687	7.0 (26)	64 (9)
	20	71.5	695	12.5 (17)	72 (10)
2	0.2		320	1.8 (—)	85 (26)
	2.0	27	1270	6.5 (24)	139 (11)
	20	45	1780	10.0 (22)	123 (7)
6	0.2	0.4	342	0.4 (100)	170 (50)
	2.0	8.5	2595	5.0 (59)	489 (19)
	20	37.3	3220	12.3 (33)	268 (8)

L1210 cells were incubated at 37° with a 0.2, 2, or 20 μM concentration of either [^{3}H]dThd (19 Ci/mol) or [^{3}H]S-dThd (19 Ci/mol). At 1, 2, and 6 hr after the beginning of the experiment, the nucleotides in the acid-soluble extract were separated using SAX HPLC, and the amount of triphosphate of each nucleoside was determined. The incorporation of radioactivity into the acid-insoluble fraction (DNA) of each sample was also determined at these times. The numbers in parentheses represent the percent of the value observed with dThd under the same conditions. The maximum amount of dThd or S-dThd that can be incorporated into DNA in these experiments is 0.32, 3.2, and 32 nmol/10^6 cells for the 0.2, 2, and 20 μM concentration of nucleoside, respectively. The results shown are those from a typical experiment.

of S-dThd occurred under conditions where only a small percentage of the dTTP pool was replaced by S-dTTP. It is possible that the procedure used to measure the dTTP pool did not distinguish between S-dTTP and dTTP. Therefore, in cells treated with S-dThd the value of the dTTP concentration could represent the intracellular concentration of both S-dTTP and dTTP.

Addition of dCyd to L1210 cells treated with S-dThd prevented the growth inhibition caused by S-dThd and inhibited the incorporation of S-dThd into DNA (Table 2). The coefficient of correlation for a plot of the percent of control growth versus incorporation of S-dThd into DNA during the first 24 hr was 0.9695, which suggested that the toxicity of S-dThd was related to its incorporation into DNA (Fig. 6). A similar r value was obtained when the percent of control growth was plotted against the incorporation of S-dThd into DNA during the first 6 hr of incubation. Addition of dCyd to S-dThd-treated cells also caused a decrease in the S-dTTP pool that was similar to the decrease in its incorporation into DNA.

Effect of S-dThd on deoxynucleotide pools. Addition of either S-dThd or dThd to cells treated with [5-^{3}H]dCyd resulted in an increase in the incorporation of dCyd into DNA (data not shown). The effect of dThd on dCyd incorporation into DNA is due to its feedback inhibition of ribonucleotide reductase, which causes a decline in intracellular dCTP concentration [24]. The apparent increase in anabolism of dCyd in dThd-treated cells occurs for two reasons. First, a decline in the intracellular concentrations of dCTP results in increased dCyd kinase activity (due to the removal of a negative feedback inhibitor), which results in greater phosphorylation of dCyd. Second, decreased *de novo* synthesis of dCTP results in an increase in the radiospecificity of the [^{3}H]dCTP that is used in the synthesis of DNA.

The increase in incorporation of [^{3}H]dCyd into

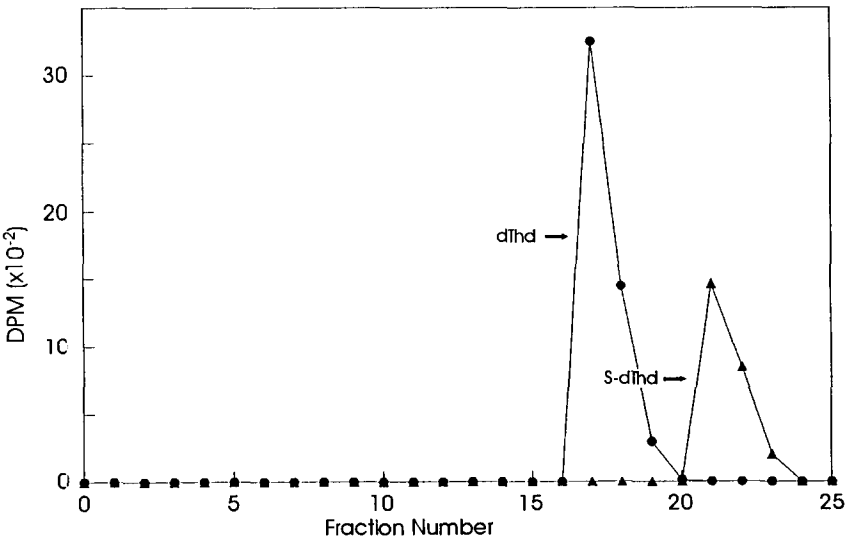

Fig. 5. Identification of the radioactivity in DNA of L1210 cells incubated with [^{3}H]S-dThd or [^{3}H]-dThd. L1210 cells were incubated at 37° with either[^{3}H]dThd or [^{3}H]S-dThd for 24 hr. DNA from CsCl gradients was degraded to deoxynucleosides by treatment with DNase I, phosphodiesterase I, and alkaline phosphatase, and the deoxynucleosides were separated from one another by reverse-phase HPLC as described in Materials and Methods. Each fraction was counted for radioactivity.

Table 2. Effect of dCyd on the inhibition of cell growth by S-dThd and the incorporation of S-dThd into DNA

	Concentration of dCyd					
	0 μM		10 μM		100 μM	
S-dThd (μM)	% Control growth	nmol incorp.	% Control growth	nmol incorp.	% Control growth	nmol incorp.
0.05	80	0.052	87	0.042	79	0.030
0.1	62	0.094	73	0.072	78	0.054
0.2	44	0.173	60	0.116	69	0.092
0.4	25	0.279	46	0.143	54	0.127

L1210 cells were treated with [^{3}H]S-dThd with or without dCyd as shown in the table. A sample of each treatment was collected after 24 hr of incubation, and the incorporation of [^{3}H]S-dThd into the acid-insoluble fraction (DNA) was determined. After 48 hr, the cell numbers in each treatment group were determined. The numbers shown are the means from two separate experiments.

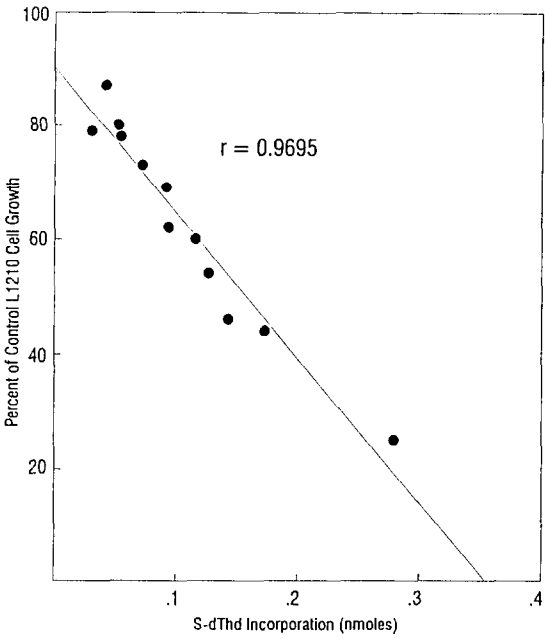

Fig. 6. Correlation between inhibition by S-dThd of cell growth and its incorporation into DNA. Plot of nanomoles of S-dThd incorporated into DNA in a 24-hr period versus the percent of control growth (see Table 2).

DNA due to treatment with S-dThd suggested that S-dThd may also inhibit ribonucleotide reductase. However, in contrast to the results with dThd, treatment of cells with S-dThd (1–100 μM for 4 or 24 hr) did not decrease dCTP or any other deoxynucleotide pool (Table 3). Indeed, treatment with 100 μM S-dThd resulted in a 5-fold increase in the dCTP pools. Treatment of L1210 cells with 100 μM dThd resulted in a 4-fold decline in the dCTP pool and a 3-fold increase in the dTTP pool (Table 3). Note that 100 μM dThd resulted in approximately the same inhibition of cell growth as 1 μM S-dThd. These results indicated that the cytotoxicity of S-dThd, unlike dThd, was not due to the inhibition of ribonucleotide reductase activity.

Effect of S-dThd on dCyd metabolism. As noted, treatment of L1210 cells with S-dThd resulted in an increase in the incorporation of radiolabeled [5-^{3}H]dCyd into DNA. As discussed above, this increased incorporation into DNA was not due to the inhibition of ribonucleotide reductase. Therefore, studies were done to characterize the effect of S-dThd on dCyd metabolism in an effort to better understand biochemical actions of S-dThd or its metabolites. S-dThd (1 μM) increased the metabolism of 0.05 μM [^{14}C]dCyd to both dCTP and dTTP, which suggested that S-dThd or one its metabolites increased the activity of dCyd kinase.

Substrate characteristics of S-dThd with dThd kinase. Because S-dThd was readily phosphorylated in L1210 cells and incorporated into DNA, it was of interest to determine the kinetic parameters of S-dThd with dThd kinase isolated from L1210 cells. S-dThd competitively inhibited the phosphorylation of dThd by L1210 dThd kinase with a K_i of approximately 12 μM. S-dThd was a good substrate for L1210 dThd kinase (Table 4). A comparison of the V_{max}/K_m ratio for S-dThd and dThd indicated that the efficiency of the reaction with S-dThd was 13% that of dThd.

DISCUSSION

Our results suggested that the cytotoxicity of S-dThd to L1210 cells is due to its incorporation into DNA and disruption of its function. Structural studies have shown that the conformation of S-dThd closely resembles that of dThd [25]. It is clear from our studies that the enzymes involved in the phosphorylation of dThd and its incorporation into DNA readily accept S-dThd and its metabolites as substrates.

Treatment of cells with S-dThd did not result in a decline in any of the natural deoxynucleotide pools, which indicated that ribonucleotide reductase was not a target of S-dThd or any of its metabolites.

Table 3. Effect of S-dThd and dThd on dNTP pools

	Incubation time (hr)	Concn (μM)	dATP	dTTP*	dCTP	dGTP
			Percent of control			
S-dThd	4	1	140 (4)	127 (4)	170 (3)	129 (4)
		10	140 (3)	105 (3)	150 (2)	107 (3)
		100	149 (3)	68 (3)	235 (2)	151 (3)
	24	1	123 (2)	101 (2)	168 (2)	157 (2)
		10	186 (2)	138 (2)	318 (2)	188 (2)
		100	161 (2)	133 (2)	583 (2)	260 (2)
dThd	4	1	125 (1)	120 (1)	81 (1)	114 (1)
		10	148 (1)	181 (1)	70 (1)	136 (1)
		100	106 (1)	357 (1)	28 (1)	193 (1)
	24	1	83 (1)	61 (1)	128 (1)	102 (1)
		10	62 (1)	95 (1)	36 (1)	92 (1)
		100	121 (1)	252 (1)	22 (1)	330 (1)

L1210 cells were incubated with a 1, 10, or 100 μM concentration of either S-dThd or dThd for either 4 or 24 hr. The deoxynucleotide pools were determined as described in Materials and Methods. In these experiments, control cells contained 29 ± 12 pmol dTTP/10^6 cells, 7.3 ± 2.8 pmol dATP/10^6 cells, 4.9 ± 0.8 pmol dGTP/10^6 cells, and 15 ± 7.5 pmol dCTP/10^6 cells. The numbers in parentheses represent the number of experiments.

* The assay used to measure dTTP levels may not distinguish between S-dTTP and dTTP. Therefore, the value for dTTP in this table may represent the concentration of both dTTP and S-dTTP.

Table 4. Kinetic parameters of S-dThd as a substrate for dThd kinase

Nucleoside	K_m (μM)	V_{max} (pmol/min/mg)	V_{max}/K_m
dThd	1.2 ± 0.1*	442 ± 192*	368
S-dThd	11 ± 4	524 ± 171	48

* Means ± SD, N = 3–5.

This is an interesting observation because the toxicity to cells of dThd is due to its inhibition of ribonucleotide reductase and the subsequent decline in dCTP pools [24]. It is possible that insufficient accumulation of S-dTTP even at very high extracellular concentrations of S-dThd was responsible for the lack of activity of S-dThd against ribonucleotide reductase. S-dTTP accumulated to only 12 pmol/10^6 cells in cells treated with 20 μM S-dThd, and our results indicated that increased extracellular concentrations of S-dThd would not result in greatly increased S-dTTP levels [an increase in the extracellular concentration of S-dThd from 2 to 20 μM resulted in only a 2-fold increase in S-dTTP levels (Table 1), and 100 μM S-dThd only modestly affected the combined dTTP/S-dTTP pool (Table 3)]. In contrast to these actions of S-dThd, treatment with 100 μM dThd resulted in a 3.5-fold increase in the dTTP pool from 29 to 100 pmol/10^6 cells. This concentration of dThd was required to depress dCTP levels by 70–80% (Table 3). However, it is also possible that S-dTTP would not inhibit ribonucleotide reductase activity even if it accumulated to concentrations similar to those of dTTP. This question will only be resolved when pure S-dTTP is available for study.

The mechanism responsible for the protection of L1210 cells from S-dThd by dCyd is likely to be due to its anabolism to dThd nucleotides, which would interfere with the phosphorylation of S-dThd monophosphate to S-dTTP and compete with S-dTTP for incorporation into DNA. A high percentage (80%) of the dCyd that is salvaged from the medium by L1210 cells is converted to dThd nucleotides and is incorporated into the DNA (data not shown; [26]). The deamination step occurs at dCMP rather than dCyd [26]. The stimulation of dCyd incorporation into DNA by S-dThd is similar to that observed with dThd, which initially suggested to us that inhibition of ribonucleotide reductase may have been responsible for the toxicity of S-dThd. However, the lack of effect of S-dThd on dCTP pools makes this possibility unlikely. S-dThd also caused an increase in the conversion of [^{14}C]dCyd to both the dCTP and dTTP pools. This indicated that S-dThd did not inhibit the deamination of dCyd or dCMP. If this had occurred, then the increase in [^{3}H]dCyd incorporation into DNA could have been due to the decreased deamination of [^{3}H]dCyd, which would allow for more of the [^{3}H]dCyd to be phosphorylated and incorporated into DNA (because the [5-^{3}H] is removed from dUMP by thymidylate synthetase, [5-^{3}H]dCyd does not label the dThd nucleotides). The increase in the conversion of dCyd to both dCTP and dTTP suggests that S-dThd or one of its metabolites increases dCyd kinase activity. High levels of dTTP are known to activate dCyd kinase activity [24].

The ability of L1210 cells to totally clear all of the S-dThd from the culture medium and incorporate it into their DNA indicated that S-dTTP was efficiently

used by DNA polymerases involved in the bulk of DNA synthesis, such as DNA polymerases α, δ, and ε. However, our studies do not rule out the possibility that S-dTTP does inhibit some cellular DNA polymerase that is responsible for only a small percentage of the total DNA synthesis in the cell, inhibition of which would not be detected in our experiments. Proliferating human tumor cells express at least six DNA polymerases. Studies of the effect of S-dTTP on all cellular polymerases are needed to determine if any are inhibited by this nucleotide analog.

The function of DNA that may be disrupted resulting from the incorporation of S-dThd into DNA is not known. DNA duplexes have been made which contain S-dThd in place of dThd [27]. The melting temperature and CD spectrum of these oligomers are similar to those of the dThd-containing oligomers. The CD spectrum of S-dThd-containing DNA was compatible with a B-DNA structure. However, these S-dThd-containing oligonucleotides did not interact with the *Eco*RV restriction endonuclease and its associated methylase as did natural DNA oligomers. In addition, oligoribonucleotides containing 4′-thiouridine are resistant to the action of four nucleases [28]. These results indicate that DNA and RNA oligomers containing 4′-thionucleosides are not recognized by some of the enzymes involved in their processing. It is possible that the inability of other enzymes involved in the metabolism of DNA to interact with DNA containing S-dThd would lead to cytotoxicity.

Our results indicated that S-dThd was a reasonably good substrate for dThd kinase. Other 4′-thionucleoside analogs also appear to be good substrates for other nucleoside kinases. For example, 4′-thioadenosine is readily used as a substrate for adenosine kinase [6, 29]. In addition, various 4′-thionucleosides are toxic to cells in culture or have antiviral activity, which suggests that these compounds are substrates for their respective activating kinases (examples are: 4′-thio-5-fluorouridine, uridine kinase [5, 9]; 4′-thioarabinofuranosyl cytosine [4], 4′-thio-2′-deoxycytidine [8], 4′-thio-2′, 3′-dideoxycytidine [10], 4′-thio-2-chloro-2′-deoxyadenosine [11], deoxycytidine kinase; 4′-thiotoyocamycin, adenosine kinase [3]; and 4′-thio-5-bromovinyl-2′-deoxyuridine, HSV dThd kinase [7]). These results indicate that nucleoside kinases, in general, recognize 4′-thionucleosides as substrates.

The mechanism of cytotoxicity of S-dThd appears to be similar to that of 6-mercaptopurine, 6-thioguanine, and 5-Br-2′-deoxyuridine in that the active nucleotides of these agents (S-dTTP, S-dGTP, and Br-dUTP) are good substrates for human DNA polymerases, and their use as a substrate does not inhibit the DNA synthesis reaction [30, 31]. With each of these compounds their incorporation into DNA followed by some subsequent disruption of its function appears to be responsible for their cytotoxicity. However, the DNA function that is disrupted by these agents does not appear to be the same. Cells treated with 6-thioguanine, 6-mercaptopurine, or 5-bromo-2′-deoxyuridine continue to progress through the cell cycle and divide to form two daughter cells that are not able to replicate [31, 32]. In contrast, incubation with S-dThd resulted in the immediate inhibition of cell growth, which suggests that the functions that are impaired by the incorporation of S-dThd into DNA occur relatively soon after its incorporation.

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