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Metabolism of 4′-Thio-β-D-arabinofuranosylcytosine in CEM Cells

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ABSTRACT. Because of the excellent *in vivo* activity of 4′-thio-β-D-arabinofuranosylcytosine (T-araC) against a variety of human solid tumors, we have studied its metabolism in CEM cells to determine how the biochemical pharmacology of this compound differs from that of β-D-arabinofuranosylcytosine (araC). Although there were many quantitative differences in the metabolism of T-araC and araC, the basic mechanism of action of T-araC was similar to that of araC: it was phosphorylated to T-araC-5′-triphosphate (T-araCTP) and inhibited DNA synthesis. The major differences between these two compounds were: (i) T-araC was phosphorylated to active metabolites at 1% the rate of araC; (ii) T-araCTP was 10- to 20-fold more potent as an inhibitor of DNA synthesis than was the 5′-triphosphate of araC (araCTP); (iii) the half-life of T-araCTP was twice that of araCTP; (iv) the catalytic efficiency of T-araC with cytidine deaminase was 10% that of araC; and (v) the 5′-monophosphate of araC was a better substrate for deoxycytidine 5′-monophosphate deaminase than was the 5′-monophosphate of T-araC. Of these differences in the metabolism of these two compounds, we propose that the prolonged retention of T-araCTP is a major factor contributing to the activity of T-araC against solid tumors. The data in this study represent another example of how relatively small structural changes in nucleoside analogs can profoundly affect the biochemical activity. BIOCHEM PHARMACOL **60**;12:1925–1932, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. arabinofuranosyl nucleosides; 4′-thionucleosides; anti-cancer agents; phosphorylation; metabolism; deoxycytidine kinase; cytidine deaminase

In the last few years, we have designed and synthesized many 4′-thionucleoside analogs in our drug development program. The most promising antitumor compound that we have discovered in this series is T-araC§ [1, 2], which is structurally related to araC (Fig. 1), an agent currently used in the treatment of acute myelogenous leukemia. T-araC was first reported by Whistler *et al.* in 1971 [3], and although it was determined to be cytotoxic to KB cells (IC_{50} of 0.42 μM), no further biological studies were reported, presumably due to the lack of compound and the difficulty of the synthetic route used to make it. Our synthetic procedures have allowed us to generate large amounts of T-araC and test it in various animal tumor models. Of particular interest is the broad spectrum of activity that was observed with T-araC. Unlike araC, T-araC has demonstrated excellent *in vivo* antitumor activity against many solid tumors, including human colon, non-small cell lung, prostate, and renal tumor xenografts, and it is highly effective when administered once daily during the treatment period, whereas multiple daily doses of araC are necessary to obtain marginal antitumor activity [2].

The only structural difference between T-araC and araC is the replacement of the oxygen atom in the arabinose ring by a sulfur atom. The reasons why this relatively minor structural difference resulted in profound differences in antitumor activity are of great interest, and a complete understanding of the differences in the biochemical pharmacology of these two agents could give insight into the characteristics of nucleoside analogs that are required for activity against solid tumors. Therefore, we have initiated studies to characterize the biochemical pharmacology of T-araC to determine how it and its metabolites interact with the various enzymes associated with dCyd metabolism. For comparison purposes, we also have evaluated the metabolism of araC, T-dCyd, and dCyd. These studies, which are the first to characterize the metabolism of

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§ *Abbreviations*: araC, β-D-arabinofuranosylcytosine; araCMP, 5′-monophosphate of araC; araCTP, 5′-triphosphate of araC; araU, β-D-arabinofuranosyluracil; araUMP, 5′-monophosphate of araU; Cyd, cytidine; Cyt, cytosine; dCyd, 2′-deoxycytidine; dThd, thymidine; dUrd, 2′-deoxyuridine; F-dUrd, 5-fluoro-2′-deoxyuridine; IC_{50}, concentration of compound that inhibits cell growth by 50%; T-araC, 4′-thio-β-D-arabinofuranosylcytosine; T-araCMP, 5′-monophosphate of T-araC; T-araCTP, 5′-triphosphate of T-araC; T-araU, 4′-thio-β-D-arabinofuranosyluracil; T-araUMP, 5′-monophosphate of T-araU; T-dCyd, 4′-thio-2′-deoxycytidine; T-dCMP, 5′-monophosphate of T-dCyd; T-dCTP, 5′-triphosphate of T-dCyd; T-dThd, 4′-thiothymidine; T-dUrd, 4′-thio-2′-deoxyuridine; T-dUMP, 5′-monophosphate of T-dUrd; and Urd, uridine.

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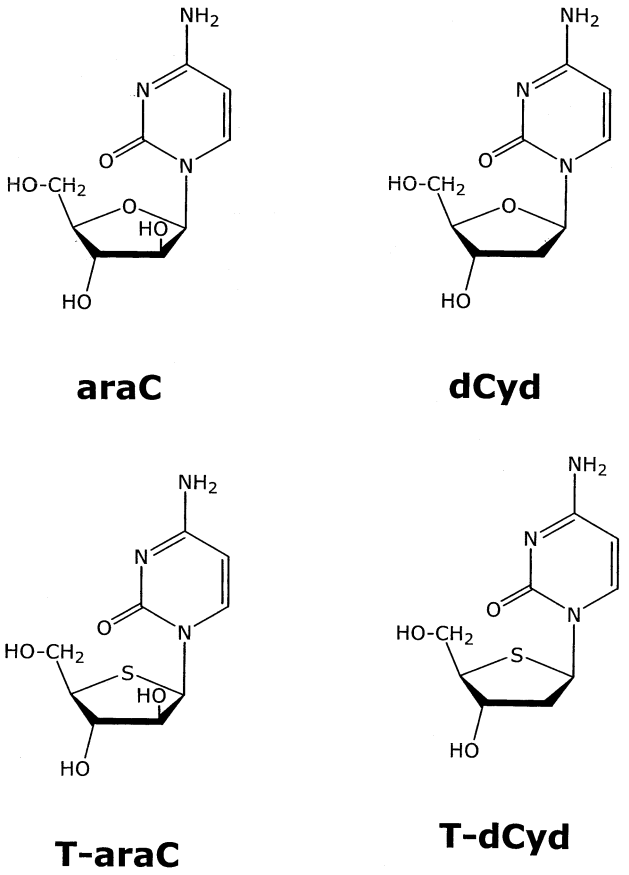

FIG. 1. Structures of araC, T-araC, dCyd, and T-dCyd.

4′-thio-deoxycytidine analogs, have identified many quantitative differences in the biochemical pharmacology of T-araC and araC. A preliminary report of this work has been presented [4].

MATERIALS AND METHODS

Materials

T-dCyd and T-araC were synthesized as described [1, 5] and radiolabeled with ^3H at the 5-position (5.0 and 9.6 Ci/mmol, respectively) by Moravek Biochemicals Inc. [*methyl*-^{3}H]dThd (60 Ci/mmol), [5-^{3}H]Urd (20 Ci/mmol), [4,5-^{3}H]l-leucine (120 Ci/mmol), [5-^{3}H]araC (26 Ci/mmol), and [5-^{3}H]dCyd (22.2 Ci/mmol) were also purchased from Moravek Biochemicals Inc. CEM cells (American Type Culture Collection) were grown in RPMI 1640 medium (Gibco-BRL) containing 10% fetal bovine serum (Atlanta Biologicals), 1 mg/mL of sodium bicarbonate, 10 U/mL of penicillin, 10 μg/mL of streptomycin, and 50 μg/mL of gentamycin. Cell numbers were determined with a Coulter Counter, and the concentration of compound that resulted in 50% inhibition of cell growth over a 72-hr incubation period was determined (IC_{50}) and used as a measure of cytotoxicity. Male athymic NCr-*nu* mice were obtained from various NCI-approved commercial suppliers. All procedures were approved by the Southern Research Institute's Institutional Animal Care and Use Committee, which conforms to the current Public Health Service *Policy on Humane Care and Use of Laboratory Animals* and the *Guide for the Care and Use of Laboratory Animals*.

Biochemical Methodology

Cyd deaminase was purified to the ammonium sulfate step from human placenta as described by Laliberte *et al.* [6]. Reactions were carried out in solutions containing 20 mM potassium phosphate (pH 7.4), 100 mM KCl, various concentrations of radiolabeled nucleoside, and sufficient enzyme to give linear reaction. The reactions were stopped with acid, and the substrate was separated from the product by reverse phase HPLC as described below. Recombinant human dCyd kinase was obtained, and the kinetic constants were measured as described by Shewach *et al.* [7]. dCyd kinase activity in CEM cell extracts and the effect of each compound on DNA, RNA, and protein synthesis were measured as described [8]. DNA from cells incubated with radiolabeled compounds was isolated using CsCl gradients [9]. The DNA was dialyzed extensively and was then degraded to nucleosides with alkaline phosphatase and phosphodiesterase. The nucleosides were separated by reverse phase HPLC. To determine if the analogs were at the 3′-terminus of DNA chains, the DNA was also degraded with micrococcal nuclease and spleen phosphodiesterase as described by Rubsam and Shewach [10]. The products of the reaction, 3′-monophosphates and nucleosides, were separated by reverse phase HPLC. Uptake of [^{3}H]T-araC and [^{3}H]araC was measured using the oil-stop method described by Paterson *et al.* [11].

HPLC

The Cyt nucleosides were separated from their deaminated products using a Hypersil BDS reverse phase column (Keystone Scientific Inc.). The mobile phase was 1% acetonitrile in a 10 mM ammonium phosphate buffer (pH 4.5) at a flow rate of 1 mL/min. One-minute fractions were collected from the column and counted for radioactivity. This HPLC system resulted in clear resolution of dCyd/dUrd, T-dCyd/T-dUrd, araC/araU, and T-araC/T-araU. In addition, these nucleosides were resolved from H_2O, which eluted in the void volume, and from the natural deoxynucleosides (dAdo, dThd, and dGuo). The acid-soluble extracts were obtained from CEM cells incubated with radiolabeled nucleosides and were analyzed using strong anion exchange HPLC as described [8].

RESULTS

Inhibition of CEM Cell Growth

CEM cells were slightly less sensitive to T-araC than they were to araC. The IC_{50} for T-araC was 24 ± 9 nM (N = 3), whereas for araC it was 6 ± 3 nM (N = 3). T-dCyd, IC_{50} of 2200 ± 1400 nM (N = 3), was much less toxic to CEM cells than were either of the arabinose analogs. The toxicity

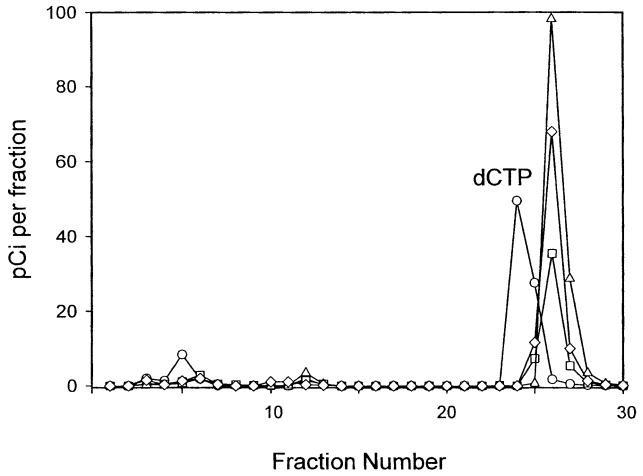

FIG. 2. Metabolism of dCyd, T-dCyd, araC, and T-araC in CEM cells. CEM cells were incubated with 100 nM [5-^{3}H]dCyd (○), [5-^{3}H]T-dCyd (□), [5-^{3}H]araC (△), or [5-^{3}H]T-araC (◇). Cells were collected by centrifugation after 15 min (dCyd, T-dCyd, araC) or 8 hr (T-araC) of incubation, and an acid-soluble extract was prepared, which was injected onto a SAX HPLC system [8]. One-minute fractions were collected as they eluted from the column, and the radioactivity in each was determined. The results shown in this figure are representative of many chromatograms.

of each of these compounds was prevented by incubation with dCyd, which suggested that these compounds were metabolized to active nucleotides by dCyd kinase.

Metabolism of T-araC and T-dCyd by CEM Cells

CEM cells were treated with 100 nM [^{3}H]dCyd, [^{3}H]T-dCyd, [^{3}H]araC, or [^{3}H]T-araC. A representative profile of the radioactivity in the acid-soluble pool for each compound is shown in Fig. 2. Most of the radioactivity in the acid-soluble fraction from each of the compounds eluted from the SAX HPLC column between 24 and 28 min, which was similar to the elution time of dCTP. The radioactive peak eluting at approximately 26 min for each of these compounds was confirmed to be phosphorylated forms of the parent compounds by degradation with phosphodiesterase and alkaline phosphatase followed by reverse phase HPLC of the resulting nucleosides. These data indicated that all four compounds were phosphorylated to their respective 5′-triphosphates. The phosphorylation of both T-araC and araC was inhibited by addition of dCyd to the culture medium (data not shown).

The level of triphosphate formed from each nucleoside as a function of incubation time is shown in Fig. 3A. Within 15 min, similar amounts of araCTP, dCTP, and T-dCTP were formed in CEM cells, but with dCTP and T-dCTP the levels of triphosphate did not continue to increase over the next 45 min. Both dCTP and T-dCTP were incorporated efficiently in the acid-insoluble fraction (Fig. 3B), which suggested that the plateau in the accumulation of these triphosphates was due to the equilibrium between their synthesis and utilization. Much less T-araCTP was formed

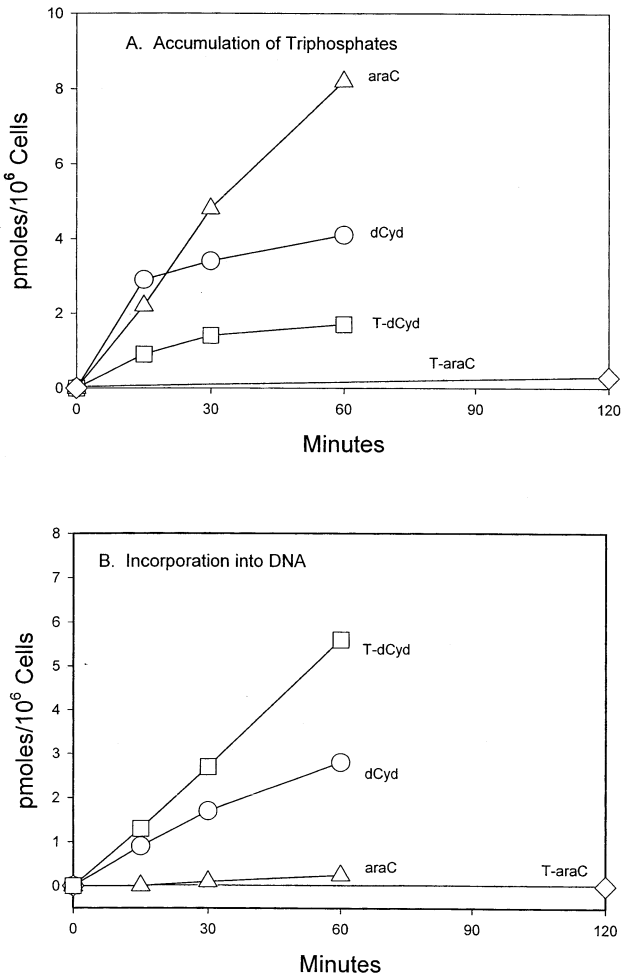

FIG. 3. Accumulation of nucleoside triphosphates in the acid-soluble pool and incorporation of nucleosides into DNA. CEM cells were incubated with 100 nM [5-^{3}H]dCyd (○), [5-^{3}H]T-dCyd (□), [5-^{3}H]araC (△), or [5-^{3}H]T-araC (◇). At the indicated times the amount of radioactivity in the triphosphate region (panel A) and the incorporation of radiolabel into the acid-insoluble pool (panel B) was determined. This precise experiment has been done once, but is representative of many others (eight) that measured relative metabolism of these four agents.

in CEM cells. The amount of T-araCTP in CEM cells after 2 hr was 0.28 pmol/10^6 cells, whereas 8.2 pmol of araCTP/10^6 cells were formed in a 1-hr period. T-araCTP continued to accumulate in CEM cells over a 24-hr period to 1.9 pmol/10^6 cells (data not shown). T-dCyd and dCyd were incorporated into DNA at a similar rate (approximately 4 pmol/10^6 cells/hr based on the 15-min result, Fig. 3B). The incorporation of T-dCyd into DNA was linear with respect to time over the 1-hr period. The lack of linear incorporation of dCyd over this time period was likely due to the utilization of the dCyd. After a 1-hr incubation, only 38% of the dCyd remained in the culture medium, whereas more than 90% of the T-dCyd was still present in the medium. Very little araC (0.25 pmol/10^6 cells/hr) or T-araC (0.0025 pmol/10^6 cells/hr) was incorporated into the DNA. Although not shown in this figure, the incorporation of

TABLE 1. Metabolism of dCyd, T-dCyd, araC, and T-araC in CEM cells

Nucleoside	Time of incubation (hr)	Original compound	Deaminated compound	H_2O	Nucleoside triphosphate	DNA
		(percent of total radioactivity)				
dCyd	1	38	0	55	4	3
T-dCyd	1	90	0	0	2	8
araC	1	87	0	0	13	0.4
T-araC	24	95	0	0	4	0.3

CEM cells were incubated with 100 nM [5-^{3}H]T-dCyd, [5-^{3}H]dCyd, ^{3}H]araC, or [5-^{3}H]T-araC for the times indicated, and the complete metabolism of each compound was determined. The medium was analyzed for the original compound, its deaminated form, and H_2O using reverse phase HPLC. The acid-soluble extract was analyzed by SAX HPLC for phosphorylated metabolites, and the incorporation of radioactivity into acid-precipitable material (DNA) was determined. All of the original radioactivity was accounted for in these fractions. This experiment is representative of a total of eight experiments that were done to characterize the metabolism of these four agents.

T-araC into DNA increased over the 24-hr period. The difference in the incorporation of araC and T-araC into DNA (100-fold) was similar to the difference in the triphosphate levels of these two nucleotides (60-fold).

dCyd analogs can be deaminated by Cyd deaminase. However, as noted by Fridland and Verhoef [12], we were not able to detect this activity in CEM cell extracts. Furthermore, tetrahydrouridine (at 10 μM), a specific inhibitor of Cyd deaminase activity [13], did not affect the metabolism of any of the four compounds when present at a concentration of 100 nM. These results indicated that Cyd deaminase was not involved in the metabolism of these compounds in CEM cells.

dCyd and its analogs can also be deaminated at the monophosphate level by dCMP deaminase to dUMP analogs, which can be converted to dThd nucleotides by thymidylate synthetase. However, because the tritium label of each of these nucleosides was at the 5-position of the cytosine ring, no thymidine nucleotides formed from any of these agents would be detected in the previous experiments. Instead, the tritium label would be removed as the dUMP was converted to TMP by thymidylate synthetase and would be present in the cell culture as H_2O. [^{3}H]H_2O generated from these compounds rapidly equilibrates across the cell membrane and elutes in the void volume of the reverse phase HPLC column. In cells treated with [^{3}H]dCyd, a considerable amount of radioactivity in the culture medium eluted in the void fraction (data not shown). This radioactivity was volatile, and its appearance in the culture medium was inhibited by treatment with F-dUrd, which indicated that it was [^{3}H]H_2O that was generated by the action of thymidylate synthetase on [^{3}H]dUMP. Approximately 90% of the dCMP formed in CEM cells was converted to H_2O rather than being phosphorylated further to dCyd nucleotides. In contrast to these results, no H_2O or deaminated products were detected in experiments using T-dCyd, araC, or T-araC, which indicated that these compounds were relatively poor substrates for dCMP deaminase activity. A representative sample of the complete metabolism of these four compounds in CEM cells is shown in Table 1. The rate of utilization of dCyd, T-dCyd, araC, and T-araC by CEM cells was 53 ± 9, 8 ± 1.5, 7.5 ± 3.3, and 0.16 ± 0.07 pmol/hr/10^6 cells, respectively (means ± SD of 3 or 4 experiments).

dUrd, T-dUrd, and araU were detected in the medium of cells treated with dCyd, T-dCyd, or araC in the presence of 10 μM F-dUrd, respectively, and there were increases in the accumulation of monophosphate nucleosides in the acid-soluble fraction. These metabolites were likely dUMP, T-dUMP, or araUMP (respectively) that accumulated due to the inhibition of thymidylate synthetase. These results indicated that both araCMP and T-dCMP could be deaminated by dCMP deaminase in CEM cells under conditions of thymidine nucleotide starvation. In contrast to these results, no T-araU or T-araUMP was detected in the medium or CEM cell extracts treated with T-araC plus F-dUrd (data not shown), which indicated that T-araCMP was a much poorer substrate for dCMP deaminase than was araCMP.

Retention of Triphosphates

The $T_{1/2}$ for the disappearance of each triphosphate was determined (Table 2). The $T_{1/2}$ of T-araCTP was approximately 2-fold greater than that for araCTP and was 10-fold greater than that for both dCTP and T-dCTP. Upon longer incubation, it was apparent that T-araCTP levels decreased less than araCTP (Fig. 4). In 72 hr, T-araCTP had decreased to 1% of its initial concentration, whereas, the

TABLE 2. Half-life of dCTP, T-dCTP, araCTP, and T-araCTP in CEM cells

Nucleotide	$T_{½}$ (hr)
dCTP	0.94 ± 0.16
T-dCTP	1.10 ± 0.35
araCTP	5.31 ± 0.31
T-araCTP	10.8 ± 1.80

After incubation of CEM cells for 1 hr with 100 nM [5-^{3}H]dCyd, [5-^{3}H]T-dCyd, [5-^{3}H]araC, or [5-^{3}H]T-araC, the cells were collected, washed with fresh medium, and resuspended in fresh medium that did not contain radiolabeled nucleosides. Samples were collected at various times after the cells were resuspended in fresh medium, and the amount of radioactivity in the 5′-triphosphate peak was determined using SAX HPLC. The data presented are the means ±SD from three separate experiments.

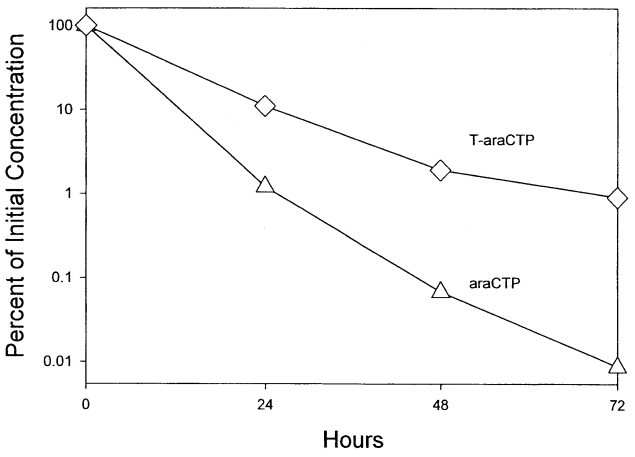

FIG. 4. Rate of disappearance of T-araCTP and araCTP. After incubation of CEM cells for 1 hr with either 5 nM [5-^{3}H]araC (△) or 200 nM [5-^{3}H]T-araC (◇), the cells were collected, washed with fresh medium, and resuspended in fresh medium that did not contain radiolabeled nucleosides. Samples were collected at various times after the cells were resuspended in fresh medium, and the amount of radioactivity in the 5′-triphosphate peak was determined using SAX HPLC. In this experiment, there was 0.639 pmol araCTP/10^6 cells and 0.246 pmol of T-araCTP after the 1-hr incubation with radiolabeled compound. This experiment has been repeated twice with similar results.

araCTP concentration had decreased to 0.01% of its original value. Both dCTP and T-dCTP rapidly declined to undetectable levels during the 72-hr incubation period (data not shown). A 1-hr treatment with these agents at the concentration used in this experiment was not toxic to the CEM cells. Therefore, the cells continued to proliferate during the 72-hr period, and the amount of radioactivity in the acid-insoluble fraction (DNA) per cell decreased during the wash-out period with a rate that was similar to the doubling time of the CEM cells. These results indicated that neither araC nor T-araC was being excised from the DNA under these nontoxic conditions.

Incorporation into DNA

In the above experiments, the incorporation of radioactivity into the acid-insoluble fraction was interpreted to be a measure of the incorporation of compound into DNA. To verify that each of the compounds was indeed incorporated into DNA, the DNA from cells treated with the radiolabeled compounds was obtained from CsCl gradients [9]. After dialysis, it was degraded to nucleosides by phosphodiesterase and alkaline phosphatase, and the nucleosides were identified using reverse phase HPLC. In each case, the original compound was isolated from the DNA. The DNA from cells treated with these compounds was also degraded using micrococcal nuclease and spleen phosphodiesterase to determine the percent of compound at the 3′-terminus. In each case, the 3′-monophosphate of each nucleoside accounted for most of the radioactivity that was incorporated into DNA, which indicated that all four nucleosides were incorporated into internal linkages in the DNA. In these experiments, very little acid-precipitable radioactivity was detected in the RNA portion of the CsCl gradients (the pellet).

TABLE 3. Substrate characteristics of dCyd, T-dCyd, araC, and T-araC with dCyd deaminase activity isolated from human placenta

Compound	K_m (μM)	V_{max} (pmol/mg/min)	V_{max}/K_m
dCyd	23 ± 2.7	13 ± 1.5	0.55
T-dCyd	110 ± 77	37 ± 12	0.33
araC	240 ± 130	14 ± 7	0.058
T-araC	2900 ± 1000	21 ± 6	0.0072

Kinetic constants were determined from linear plots of 1/velocity versus 1/concentration of the substrate. The best line was determined by linear regression from at least 5 datum points, and the K_m and V_{max} were determined from the x- and y-intercepts. The data presented are the means ± SD from three separate experiments.

Cytidine Deaminase Activity and Deamination in Mice

Although Cyd deaminase activity was not involved in the metabolism of these compounds in CEM cells, this enzyme is important in the metabolism of dCyd and its analogs in intact animals [14]. Therefore, Cyd deaminase activity was purified from human placenta, and its ability to deaminate these nucleosides was determined (Table 3). T-dCyd was a good substrate for this enzyme with a catalytic efficiency similar to that of dCyd. However, T-araC was a very poor substrate. Its K_m was approximately 3000 μM, 10-fold greater than that of araC and 100-fold greater than that of dCyd.

Unlike araC, T-araC is effective against tumors in animals when administered once daily during the treatment period [2]. It is possible that the decreased rate of deamination of T-araC by Cyd deaminase could explain the schedule differences between these two compounds. Therefore, mice were injected i.p. with 100 mg/kg of either araC or T-araC, and the amount of parent nucleoside and deaminated nucleoside in the plasma was determined at various times after injection (Fig. 5). After injection of T-araC or araC, there was much less T-araU in the plasma than araU (respectively), which supported the kinetic studies of these compounds with Cyd deaminase. However, the plasma levels of T-araC and its rate of disappearance were similar to those seen with araC, which suggested that the decreased activity with Cyd deaminase had little, if any, effect on the pharmacokinetics of T-araC.

Uptake of T-araC and araC

It is possible that the large difference seen in the metabolism of T-araC and araC in CEM cells could be due to differences in the uptake of these compounds rather than

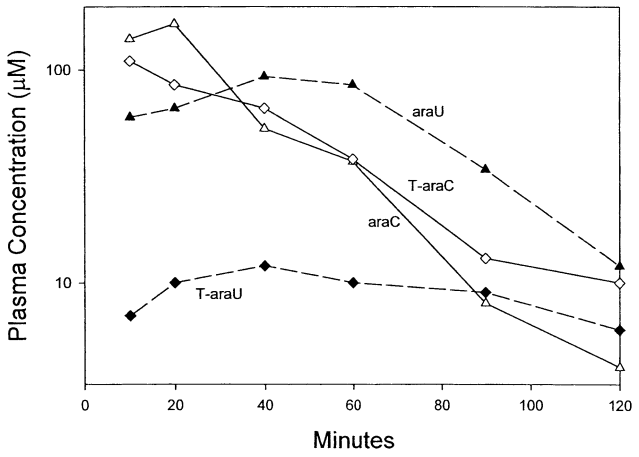

FIG. 5. Pharmacokinetics of T-araC and araC after i.p. injection. Male athymic NCr-*nu* mice were injected i.p. with 100 mg/kg of either [5-^{3}H]T-araC (◇,◆) or [5-^{3}H]araC (△,▲). Mice were killed, and plasma samples were obtained at the times indicated. The amounts of parent compound (T-araC or araC) and deaminated product (T-araU or araU) were determined using reverse phase HPLC. Four mice were used for each data point shown. The experiment has been repeated three times with similar results.

their phosphorylation. However, as seen in Fig. 6, the initial uptake of T-araC in CEM cells was similar to that for araC. Therefore, differences in uptake cannot explain the differences in the accumulation of the respective triphosphates. In this experiment, the total intracellular accumulation of radiolabeled compound was measured, which included any metabolites that were formed. The rate-limiting step in the utilization of araC at low concentrations is uptake [15]. Therefore, an equilibrium between the intracellular and extracellular concentrations of araC is not reached in these early times because as araC enters the cell

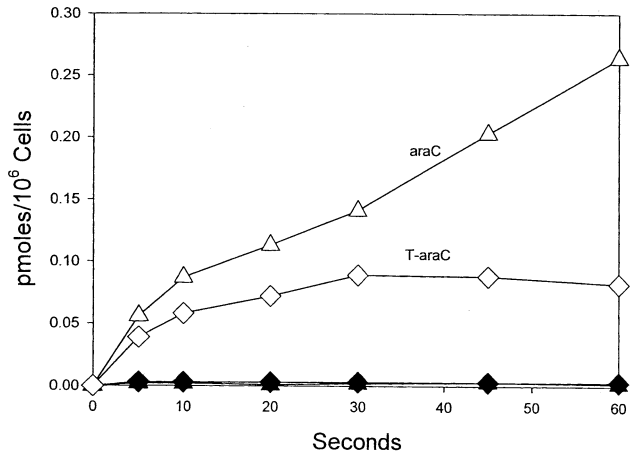

FIG. 6. Uptake of T-araC and araC. CEM cells were incubated with 100 nM [5-^{3}H]araC (△) or [5-^{3}H]T-araC (◇). Samples were taken at the times shown, the cells was separated from the medium by centrifugation through oil as described [11], and the radioactivity associated with the cells was determined. The filled symbols are experiments with no cells. This experiment has been repeated with similar results.

TABLE 4. Substrate characteristics of dCyd, araC, and T-araC with human dCyd kinase

Compound	K_m (μM)	Relative V_{max}	V_{max}/K_m
dCyd	1.2	1	0.8
araC	15	0.1	0.006
T-araC	93	0.46	0.005

Human dCyd kinase was expressed in *Escherichia coli* and purified as described [7]. Each assay was done in duplicate, and the kinetic constants were determined from linear plots of 1/velocity versus 1/concentration of the substrate. The best line was determined by linear regression from at least five datum points, and the K_m *and* V_{max} were determined from the x- and y-intercepts. Each result is the average of two separate determinations.

it is rapidly phosphorylated to araCTP. In contrast, because of the low rate of phosphorylation of T-araC, the intracellular concentration of T-araC quickly reaches equilibrium with the extracellular concentration. For these reasons the observed differences in the radioactivity associated with CEM cells that was detected at the later time points reflect differences in phosphorylation of the two compounds, not their uptake.

Activity with Purified dCyd Kinase

The phosphorylation of T-araC and araC was compared with purified recombinant human dCyd kinase (Table 4). The catalytic efficiency of this enzyme with T-araC was similar to that of araC. Although the K_m for T-araC was 6-fold that of araC, its V_{max} was also increased by 5-fold, so that the V_{max}/K_m ratio for araC and T-araC was very similar. These results are contrary to the results obtained in crude CEM cell extracts, where the rate of phosphorylation of 1 μM T-araC (0.76 ± 0.3 pmol/mg/min, N = 3) was 100-fold less than the rate of phosphorylation of 1 μM araC (74 ± 20 pmol/mg/min, N = 3). The reason for the discrepancy in these results is not known, but could be due to phosphatase activity in the crude cell extracts that could selectively cleave thionucleosides, resulting in less net phosphorylation of T-araC.

Effect of araC, T-araC, and T-dCyd on Macromolecular Synthesis

Each of these compounds at a concentration that was 30 times the IC_{50} inhibited the incorporation of [^{3}H]dThd into DNA during a 4-hr incubation period, but had a minimal effect on the incorporation of either [^{3}H]Urd or [^{3}H]leucine into RNA or protein, respectively (data not shown). This result indicated that inhibition of an enzyme involved in DNA synthesis was responsible for the cytotoxicity of these agents. With T-araC and araC, the effect on dThd incorporation was concentration-related (5–150 nM for araC; 25–750 nM for T-araC). However, dThd incorporation into DNA was inhibited the same amount by 2, 6, 20, or 60 μM T-dCyd (inhibition of approximately 75%).

DISCUSSION

These studies indicated that the basic mechanism of cell kill for T-araC was similar to that of araC. It is phosphorylated via dCyd kinase to T-araCTP, which inhibits DNA replication. Prior studies indicate that araCTP is an alternative substrate for the DNA polymerases that are associated with DNA replication, but that its incorporation into the 3′-terminus inhibits further elongation of the DNA strand [15]. Our studies in intact cells suggest that T-araCTP would have a similar effect on the DNA polymerases. However, the fact that the level of accumulation of araCTP was 60- to 100-fold greater than that of T-araCTP, and that araC was only 5-fold more potent than T-araC, indicated that much less (~20-fold) phosphorylation of T-araC and its subsequent incorporation into DNA was needed to cause toxicity to CEM cells. These results suggest that T-araCTP is a more potent inhibitor of DNA synthesis than is araCTP.

Although the basic mechanism of action of T-araC was similar to that of araC, there were many quantitative differences in the metabolism of these two agents that could have an important effect on the antitumor activity of T-araC. It is possible that the longer half-life of T-araCTP could explain the effectiveness of T-araC against solid tumors. Although its half-life was only 2-fold greater than that of araCTP, the amount of T-araCTP 72 hr after the removal of compound was approximately 100-fold greater than that of araCTP. AraC is primarily active against cells in S-phase, which is likely to be the case with T-araC, and it is believed that the small percentage of cells in S phase is the primary reason that solid tumors are not sensitive to araC. Because dCyd kinase is expressed in all phases of the cell cycle [15], both araCTP and T-araCTP would be formed in phases of the cell cycle, such as G_0 or G_1, and for agents such as T-araC, that have a prolonged retention of the analog triphosphate, the active metabolite would still be at sufficient concentrations in the tumor cells to inhibit the DNA polymerases when the cells entered S phase.

The relatively high levels of Cyd deaminase activity with respect to dCyd kinase activity have been suggested as one of the reasons that araC is not effective against solid tumors [16]. It is proposed that most of the araC that enters a tumor cell would be inactivated instead of being phosphorylated to active metabolites. In addition, araCMP can be deaminated (inactivated) by dCMP deaminase [17], which could also contribute to the resistance of solid tumors to treatment with araC. Therefore, it is possible that the relatively poor deamination of T-araC and T-araCMP by these two enzymes could also contribute to its effectiveness against solid tumors.

Cyd deaminase is a major enzyme in the metabolism of araC in whole animals, and most of the administered drug is deaminated and inactivated by this enzyme before it has a chance to interact with a tumor cell [14]. The catalytic efficiency of human Cyd deaminase with T-araC as substrate was 10-fold less than it was with araC as substrate, which indicated that this enzyme would have less importance in the metabolism of T-araC than it does with araC. Consistent with the kinetic parameters of T-araC and araC with Cyd deaminase, much less deamination of T-araC was detected in mice than araC after i.p. injection of each agent. However, this decreased deamination of T-araC did not result in greatly different plasma levels of T-araC and araC. These results indicated that the difference in the deamination of araC and T-araC by Cyd deaminase was not responsible for the differences in the optimal treatment schedules of these two agents. It is likely that the longer half-life of T-araCTP is responsible for the differences in the optimal treatment schedule between T-araC and araC.

The results with T-dCyd indicated that the mechanism of action of T-dCyd was fundamentally different from that of T-araC and araC. T-dCyd was converted to T-dCTP and was incorporated into DNA as readily as dCyd, which indicated that T-dCTP was a very good substrate for the DNA polymerases involved in DNA replication and that these DNA polymerases readily extended the DNA chain after the incorporation of T-dCyd. We have shown previously that T-dThd is also readily used as a substrate for DNA synthesis in L1210 cells [18], which indicated that, in general, pyrimidine nucleoside kinases, nucleotide kinases, and DNA polymerases utilized 4′-thio-deoxynucleosides and nucleotides with an efficiency that was similar to the natural nucleosides and nucleotides. Although DNA synthesis was selectively inhibited by T-dCyd, our results indicated that direct inhibition of DNA polymerase activity by T-dCTP was not responsible for the antitumor activity of T-dCyd. The basis for the toxicity of these agents has not been determined precisely. In our previous work [18] with T-dThd, its incorporation into DNA was sufficient to explain the toxicity of T-dThd to L1210 cells. Inhibition of ribonucleotide reductase by the 5′-triphosphate of T-dThd was not involved in the toxicity of this agent. Therefore, we believe that it is likely that the incorporation of T-dCyd into DNA and the disruption of some unknown function are also responsible for its toxicity to CEM cells. Unfortunately, neither T-dCyd nor T-dThd has demonstrated much antitumor activity in animals.

In summary, these studies have identified many differences between the metabolism of T-araC and araC that could explain the difference observed in the antitumor activity of these two agents. This research again demonstrates how relatively minor structural changes in nucleoside analogs can result in profound effects on antitumor activity and metabolism. Studies are planned to compare the metabolism of these compounds in solid tumors to identify which of these characteristics is responsible for the good activity of T-araC against solid tumors.

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