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ORIGINAL ARTICLE

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Long intracellular retention of 4′-thio-arabinofuranosylcytosine 5′-triphosphate as a critical factor for the anti-solid tumor activity of 4′-thio-arabinofuranosylcytosine

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Abstract 4′-Thio-arabinofuranosylcytosine (T-araC) is a new cytosine analog, which exhibits excellent antitumor activity against various solid tumor xenografts in mice. T-araC is a structural analog of arabinofuranosylcytosine (araC), which is known to be marginally active against solid tumors. We have continued to study the biochemical pharmacology of T-araC in solid tumor cells to further characterize the mechanism of action of this new agent and to elucidate why these compounds show a profound difference in antitumor activity against solid tumors. AraC was a slightly more potent inhibitor of cell growth than T-araC when cells were continuously exposed to the drugs. However, T-araC was markedly more cytotoxic than araC when high concentrations of the compounds were given for short periods of time. Despite the fact that T-araC is a much poorer substrate, as compared to araC, for deoxycytidine kinase (the rate-limiting step in the formation of the triphosphates), similar intracellular concentrations of T-araC-5′-triphosphate (T-araCTP) and araCTP were formed in cells at these high, pharmacologically relevant concentrations due to similar V_{max}'s. The major difference in the metabolism of araC and T-araC was that the half-life of T-araCTP was tenfold longer than that of araCTP and much higher levels of T-araCTP were sustained in cells for long durations after exposure to T-araC. Inhibition of cytidine deaminase, deoxycytidylate deaminase, or DNA replication did not affect the half-life of either araCTP or T-araCTP. In addition, the rates of disappearance of the mono- and tri-phosphates of araC and T-araC in crude cell extracts were similar. These results indicated that these enzymes were not rate-limiting in the degradation of the respective triphosphates. However, the rate of phosphorylation of T-araC-5′-monophosphate (T-araCMP) in crude cell extracts was about tenfold greater than that of araCMP. The results of this work suggested that the longer intracellular retention of T-araCTP was responsible for the superior activity of T-araC against solid tumors in vivo, and that the greater activity of T-araCMP as a substrate of UMP/CMP kinase was responsible for the long intracellular half-life of T-araCTP.

Keywords 4′-Thio-arabinofuranosylcytosine · Arabinofuranosylcytosine · Half-life of nucleoside triphosphate · Human UMP/CMP kinase · Solid tumors

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Introduction

As a part of our ongoing program to design nucleoside analogs with increased activity for the treatment of cancer, a series of 4′-thionucleosides have been synthesized and screened for their biological activities [21]. One of these compounds, 4′-thio-arabinofuranosylcytosine (T-araC, Fig. 1) exhibits excellent in vivo activity against a variety of human solid tumor xenograft models [22, 24]. It has better in vivo activity against most human solid tumor xenografts than 2′,2′-difluoro-2′-deoxycytidine (dFdC or gemcitabine) [22], which is widely used for the treatment of pancreatic and non-small cell lung cancers [2, 7]. T-araC is a structural analog of arabinofuranosylcytosine (araC or cytarabine, Fig. 1), which is used for the treatment of many hematological malignancies [10, 14, 19] but is poorly active against solid tumors in vivo [3, 4]. The only structural difference between araC and T-araC is replacement of the 4′-oxygen atom with sulfur (Fig. 1). Our research goal is to understand why this small structural change results in dramatically improved activity against solid tumors.

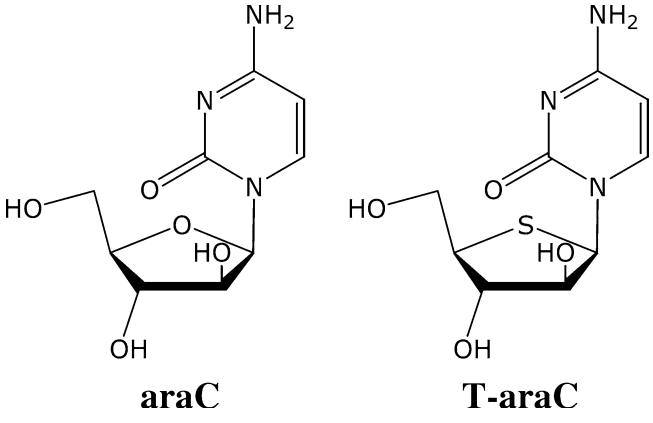

Fig. 1 Structures of araC and T-araC

Our previous studies using CCRF-CEM (human lymphocytic leukemia) cells showed that the basic mechanism of action of T-araC was similar to that of araC [12]. These compounds were phosphorylated to their respective triphosphates, which inhibited DNA replication. However, there were several quantitative differences in the metabolism and activity between these two compounds. First, the intracellular concentration of T-araC-5′-triphosphate (T-araCTP) was much lower than araC-5′-triphosphate (araCTP) at equitoxic concentrations of compound. In addition, T-araC and T-araC-5′-monophosphate (T-araCMP) were relatively poor substrates (with respect to araC and araCMP) for cytidine (Cyd) deaminase and deoxycytidylate (dCMP) deaminase, respectively. Finally, the intracellular half-life of T-araCTP was twofold longer than that of araCTP. However, none of these differences provided a clear reason to explain why T-araC is more active against solid tumors than araC. Therefore, in the current studies, we studied the biochemical pharmacology of T-araC in solid tumor cell lines to further define the mechanism of action of this new agent and to determine the basis of its activity against solid tumors.

Materials and methods

Materials

Arabinofuranosylcytosine, araCMP, araCTP, and aphidicolin were purchased from Sigma Chemicals Inc. (St. Louis, MO, USA). T-araC, T-araCMP, and T-araCTP were chemically synthesized in our laboratories as described previously [16, 18, 21]. 3,4,5,6-tetrahydro-2′-deoxyuridine (dTHU) was generously provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, NCI (Bethesda, MD, USA). [5−^{3}H]T-araC (7.7 Ci/mmol) was labeled with ^{3}H at the 5-position by Moravek Biochemicals Inc. (Brea, CA, USA). [5−^{3}H]araC (26 Ci/mmol) and [methyl−^{3}H]dThd (60 Ci/mmol) were purchased from Moravek Biochemicals Inc. Other compounds were of standard analytical grade.

Cell culture

HCT-116 (human colon cancer), MDA-MB-435 (human breast cancer, has been reported to be of melanoma origin) [5], SW-620 (human colon cancer), and NCI-H460 (human non-small cell lung cancer) cells were obtained from the Developmental Therapeutics Program Tumor Repository (Frederick, MD, USA). All of these cell lines were grown in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (Gibco-BRL), 10 U/ml penicillin, 10 μg/ml streptomycin, and 50 μg/ml gentamycin. Proliferation and viability of solid tumor cells were assessed with alamarBlue (Biosource, Camarillo, CA, USA) assay [17, 20]. Briefly, cells were incubated with fresh medium containing 10% alamarBlue at 37°C for 1 h. The percent of reduction of alamarBlue was calculated from OD values of the medium at 570 and 600 nm and was compared to that of control (no cells). After each alamarBlue measurement, medium containing alamarBlue was replaced by fresh medium.

Measurement of triphosphates in intact cells

Acid-soluble extracts were obtained from cells treated with radioactive nucleosides and were analyzed for their respective triphosphates using Partisil strong anion exchange (SAX, Keystone Scientific Inc., Bellefonte, PA, USA) HPLC [11]. ATP levels in these extracts were also determined by measuring its UV absorbance at 260 nm.

Measurement of dThd incorporation into DNA

To determine the relative rate of DNA synthesis in intact cells, the amount of dThd incorporated into DNA was measured. After incubating with 16 nM [methyl−^{3}H]dThd (1 μCi/ml) for 15 min, the cells were collected and treated with 0.5 M perchloric acid. The acid-insoluble material was captured by glass microfibre filters (Whatman International Ltd., Maidstone, England) and the radioactivity of each disc was determined as described [11]. A preliminary study showed that the incorporation of [methyl−^{3}H]dThd into DNA was linear for 1 h.

Measurement of nucleosides in culture medium

One milliliter of culture medium from cells treated with radioactive compounds was taken and acid-extracted. For the separation of araC from araU, the extract was applied to BetaMax reverse-phase column (Keystone Scientific Inc.) HPLC. The mobile phase was 0.5% acetonitrile in a 5 mM ADHP buffer (pH 4.5) at a flow rate of 1 ml/min. The column was then regenerated between each run by washing with 50% methanol for 30 min. The retention times of

araC and araU were 4.2 and 6.9 min, respectively. For the separation of T-araC from T-araU, the extract was applied to Hypersil BDS reverse-phase column (Keystone Scientific Inc.) HPLC as described previously [18].

Nucleotide phosphatase assay

A crude extract from HCT-116 cells was obtained as previously described [18]. Phosphatase activities of tri- and mono-phosphates of araC and T-araC were assayed in solutions containing 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 20 mM dithiothreitol, 30 mM KCl, 0.2 mg/ml bovine serum albumin, 50 μM nucleotide, and the crude extract (1 mg/ml protein concentration). After incubation at 37°C, the reaction was stopped by acid extraction. The rates of disappearance of araCTP and T-araCTP were determined using a Partisil SAX column HPLC as described above. The rates of disappearance of araCMP and T-araCMP were determined by HPLC using BioBasic anion exchange (AX, Keystone Scientific Inc.). The mobile phase was a 30 min linear gradient from 5 mM (pH 2.8) to 750 mM ADHP buffer (pH 6.0) at a flow rate of 1 ml/min. The retention times of araCMP and T-araCMP were 9.7 and 10 min, respectively. Nucleotides were detected by UV absorbance at 275 nm.

UMP/CMP kinase assay

UMP/CMP kinase activities of CMP, araCMP, and T-araCMP in crude extracts from cells were assayed in solutions containing 50 mM Tris (pH 8.0), 5 mM $MgCl_2$, 50 mM KCl, 1 mM ATP, various concentrations of nucleoside monophosphates, and sufficient enzyme (0.01 mg/ml protein concentration) to give a linear reaction. After incubation at 37°C, the reaction was stopped by acid extraction, and the neutralized extract was applied to BioBasic AX column HPLC analysis as described above. Nucleotides were detected by UV absorbance at 275 nm.

Table 1 Lack of correlation between in vitro cytotoxicity (continuous exposure) and in vivo efficacy of araC and T-araC

Cell type	In vitro cytotoxicity IC_{50} (μM)		In vivo antitumor activity T-C (days)[b]	
	AraC	T-araC	AraC	T-araC
HCT-116	9 ± 1[a]	34 ± 18[a]	8.5	> 56
MDA-MB-435	2 ± 1[a]	15 ± 7[a]	4.5	23

[a]Cells were incubated with various concentrations of araC or T-araC for 72 h, and the concentration of drug that was required to inhibit cell growth by 50% (IC_{50}) was determined using alamarBlue assay. The data presented are the mean ± SD of three separate experiments

[b]NCr-nu athymic mice were implanted subcutaneously with tumor cells. When tumors were approximately 100 mg, araC (13.3 mg/kg, 3 treatments each day for 9 consecutive days) and T-araC (90 mg/kg, daily treatment for 9 consecutive days) were administered via intraperitoneal injection as described [24]. Antitumor activity was assessed on the basis of delay in tumor growth. The days' delay in tumor growth (T-C days) was the difference between treated and control groups in the median times to double in mass two (MDA-MB-435) or three (HCT-116) times. The values shown are the mean of two separate experiments

Results

Correlation of in vitro cytotoxicity with in vivo antitumor efficacy

When the IC_{50}s of araC and T-araC were determined against various solid tumor cell lines with 72-h continuous incubation, the results did not correlate with the in vivo antitumor activities of araC and T-araC (Table 1). Even though T-araC was a less potent inhibitor of HCT-116 and MDA-MB-435 cell growth, treatment with T-araC resulted in good antitumor activity against these cells implanted in mice, whereas araC did not. According to our previous pharmacokinetic studies, the optimal in vivo dose of T-araC (100 mg/kg) resulted in peak plasma concentration of 100 μM and plasma half-life of 1 h [12]. Since there were clear differences in the pattern of drug exposure between the in vitro (low concentration + long-term exposure) and in vivo (high concentration + short-term exposure) studies, we hypothesized that cells in culture treated with high concentrations of drugs for short periods would correlate with in vivo antitumor activity. To test this hypothesis, HCT-116 cells were incubated with araC (10 μM) or T-araC (100 μM) for 2 h, and the growth of the cells was observed following removal of drug (Fig. 2). T-araC inhibited the growth of the cells for 9 days compared to control, whereas araC did not inhibit subsequent cell growth. When cells treated with T-araC were examined under a microscope, the cells were morphologically abnormal indicating cell death by oncosis and apoptosis [23]. These observations suggested that the subsequent growth of cells after treatment with T-araC was due to a small percentage of cells that survived the treatment. MDA-MB-435, SW-620, and NCI-H460 cells were also incubated with these compounds, and underwent 6-, 3-, and 4-day delay in their growth by T-araC, respectively (Table 2). Like HCT-116 cells, all of these cell lines were not sensitive to araC.

A lower concentration of araC (10 μM) was used in these studies because we determined that treatment of HCT-116 or MDA-MB-435 cells with either 10 or 100 μM araC for two hours did not affect subsequent cell growth ($N = 5$ in each cell type). In addition, we determined that incubation with either 10 or 100 μM araC for 2 h resulted in similar intracellular concentrations of araCTP. In HCT-116 cells treated with either 10 or 100 μM araC for 2 h the intracellular araCTP levels were 128 ± 75 ($N = 5$) or 88 ± 59 ($N = 3$) pmoles/mg protein, respectively (P value = 0.47, Student's t-test), and in MDA-MB-435, cells treated with either 10 or

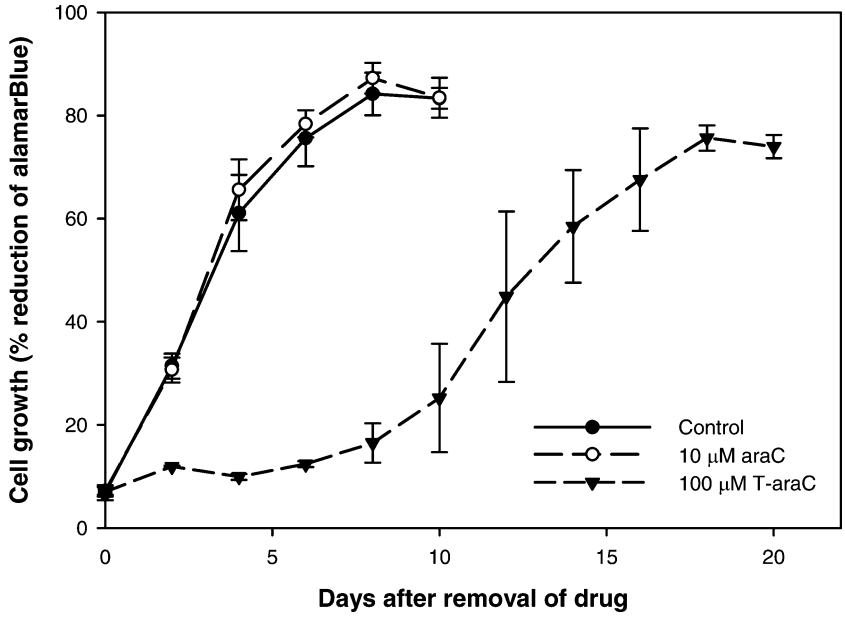

Fig. 2 Effect of 2 h treatment with araC or T-araC on the subsequent growth of HCT-116 cells. Near confluent HCT-116 cells were incubated with 10 μM araC or 100 μM T-araC for 2 h. After removing the medium, the cells were washed thrice with Earle's balanced salt solution (EBSS), harvested with 0.5% trypsin, resuspended in fresh medium, diluted with fresh medium to 10% of original concentration, and then reincubated at 37°C. The data presented are mean ± SD of three separate experiments

Table 2 The effect of araC or T-araC on in vitro (short-term exposure) and in vivo tumor growth

Cell type	AraC T-C (days)		T-araC T-C (days)	
	In vitro[a]	In vivo[b]	In vitro[a]	In vivo[b]
HCT-116	0.2	8.5	9	> 56
MDA-MB-435	0.5	4.5	6	23
SW-620	0.1	0	3	33
NCI-H460	0.3	2.5	4	25

[a]Solid tumor cells were treated with araC (10 μM) or T-araC (100 μM) for 2 h and their subsequent growth was determined using alamarBlue assay as described in the legend of Fig. 2. T-C days is the difference between treated and control groups in days for tumor cells to double, which was calculated from the growth curves (See Fig. 2). The values shown are the mean of two separate experiments

[b]T-C days of in vivo studies performed as described (32) was the difference between treated and control groups in the median times to double in mass two (MDA-MB-435), three (HCT-116, SW-620), or four (NCI-H460) times. The values shown are the mean of two separate experiments

100 μM araC for 2 h the intracellular araCTP levels were 396 ± 129 ($N = 5$) or 254 ± 55 ($N = 3$) pmoles/mg protein, respectively (P value = 0.13, Student's t-test). These results were consistent with those of others [25] and can be explained by the kinetic parameters of araC with dCK when UTP is used as the phosphate donor [18]. The results of these studies indicated that in vitro cytotoxicity after short-term exposure with high concentrations of araC or T-araC correlated with the in vivo antitumor activity of these two compounds against solid tumors. Furthermore, these results also indicated that biochemical analysis of the metabolism of araC and T-araC at high concentrations for short periods may lead to an understanding of the reason for the excellent solid tumor activity of T-araC.

Triphosphate accumulation and retention in solid tumor cells

According to our previous studies in CCRF-CEM cells [18], the rates of activation of araC and T-araC should be similar at pharmacologically relevant high concentrations. However, a substantial difference in cytotoxicity of araC and T-araC against the various solid tumor cell lines was observed (Table 2). To confirm that the rates of phosphorylation of these compounds were similar in these solid tumor cell lines under the conditions used in Fig. 2, the intracellular levels of araCTP and T-araCTP at the end of drug exposure were determined (Table 3). There were statistically significant differences between araCTP and T-araCTP levels in some of the cell lines, but the level of T-araCTP was significantly greater only in one cell line (SW-620). Therefore, the similar levels of araCTP and T-araCTP in these solid tumor cell lines cannot explain the marked differences in cytotoxicity between these two agents.

The intracellular retention of araCTP and T-araCTP was determined in HCT-116 cells. As shown in Fig. 3, T-araCTP was much more slowly degraded than araCTP. The intracellular half-lives of araCTP and T-araCTP were also determined in MDA-MB-435, SW-620, and NCI-H460 cells (Table 4). Like HCT-116 cells, the half-life of T-araCTP was much longer than that of araCTP in all of these solid tumors. These results strongly suggested that much longer intracellular retention of T-araCTP in solid tumors was responsible for the superior anti-solid tumor activity of T-araC over araC.

Prolonged inhibition of DNA synthesis by T-araCTP

dThd incorporated into DNA of HCT-116 cells treated with either araC or T-araC was determined to investi-

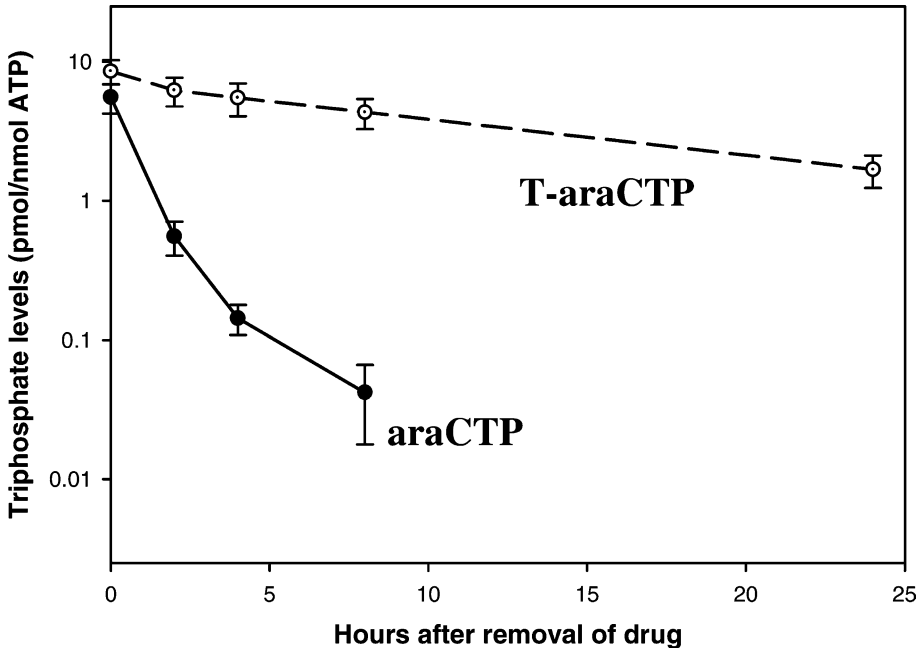

Fig. 3 Retention time of araCTP and T-araCTP in HCT-116 cells. Near confluent HCT-116 cells were incubated with 10 μM [5−^{3}H]araC or 100 μM [5−^{3}H]T-araC. After removing the medium, the cells were washed thrice with EBSS and then re-incubated in fresh medium. The medium was replaced with fresh medium 2, 4, and 8 h after removal of drugs. Cells were collected at various times after removal of drugs, and the triphosphate levels were determined. The data presented are mean ± SD of three separate experiments

gate whether longer intracellular retention of T-araCTP results in prolonged inhibition of DNA synthesis in these cells (Fig. 4). DNA synthesis of HCT-116 cells gradually recovered and returned to 25% of control 8 h after the removal of araC, whereas a 2-h treatment with T-araC completely inhibited DNA synthesis during the 8-h period after the removal of drug. DNA synthesis had not recovered from T-araC treatment 72 h after the drug removal (data not shown). These data indicated that longer intracellular retention of T-araCTP correlated with prolonged inhibition of DNA synthesis, which leads to cell death.

Effect of inhibition of Cyd deaminase and dCMP deaminase on araCTP retention time

It was known from our previous studies that T-araC was a tenfold poorer substrate for Cyd deaminase than araC [12] and that T-araCMP was a 20-fold poorer substrate for dCMP deaminase than araCMP (unpublished observation). In addition, when we analyzed the culture medium in the retention studies above the major end product of araCTP was araU while that of T-araCTP was T-araC (data not shown), which suggested that the rate-limiting step of araCTP degradation could be deamination. Since the inhibition of dCMP deaminase activity has been shown to be involved in the degradation of gemcitabine triphosphate [6], we hypothesized that the differences in these deaminases between araC/araCMP and T-araC/T-araCMP could explain why T-araCTP has a longer retention time than araCTP. Because treatment with dTHU is known to inhibit both of these deaminases, the retention time of araCTP and T-araCTP in intact HCT-116 cells in the presence of this compound was determined (Fig. 5a). However, inhibition of these deaminases did not affect the intracellular half-life of either araCTP or T-araCTP, which indicated that neither of these deaminases was responsible for faster degradation of araCTP in these cells. In the

Table 3 Intracellular levels of araCTP and T-araCTP

Cell type	AraC (10 μM) (pmol/nmol ATP)	T-araC (100 μM) (pmol/nmol ATP)
HCT-116	6 ± 2	8 ± 2
MDA-MB-435	14 ± 5*	7 ± 3*
SW-620	2 ± 0.05*	4 ± 0.4*
NCI-H460	3 ± 0.6	3 ± 1

Cells were incubated with [5−^{3}H]araC (10 μM) or [5−^{3}H]T-araC (100 μM) for 2 h. After washing with fresh medium, the cells were collected and the triphosphate levels were determined as described in Materials and methods. The presented data are the mean ± SD for at least three experiments

*The data are significantly different between araC and T-araC (Student t-test $P < 0.05$)

Table 4 Intracellular half-lives of araCTP and T-araCTP

Cell type	AraC (hours)	T-araC (hours)
HCT-116	1.1 ± 0.02*	11 ± 0.8*
MDA-MB-435	1.9 ± 0.5*	10 ± 0.4*
SW-620	0.8 ± 0.2*	9.0 ± 1.0*
NCI-H460	1.3 ± 0.01*	6.9 ± 0.3*

After incubation for 2 h with 10 μM [5−^{3}H]araC or 100 μM [5−^{3}H]T-araC, the cells were washed thrice with EBSS and reincubated in fresh medium. The medium was replaced with fresh medium 2, 4, and 8 h after removal of araC or T-araC. Samples were collected at various times after washing the cells, and the amount of radioactivity in the triphosphate region was determined as described in Materials and methods. Intracellular half-life of each triphosphate was calculated from the best line (time vs. log of triphosphate level) that was determined by linear regression ($R > 0.88$). The presented data are the mean ± SD for at least three experiments

*The data are significantly different between araC and T-araC (Student t-test $P < 0.05$)

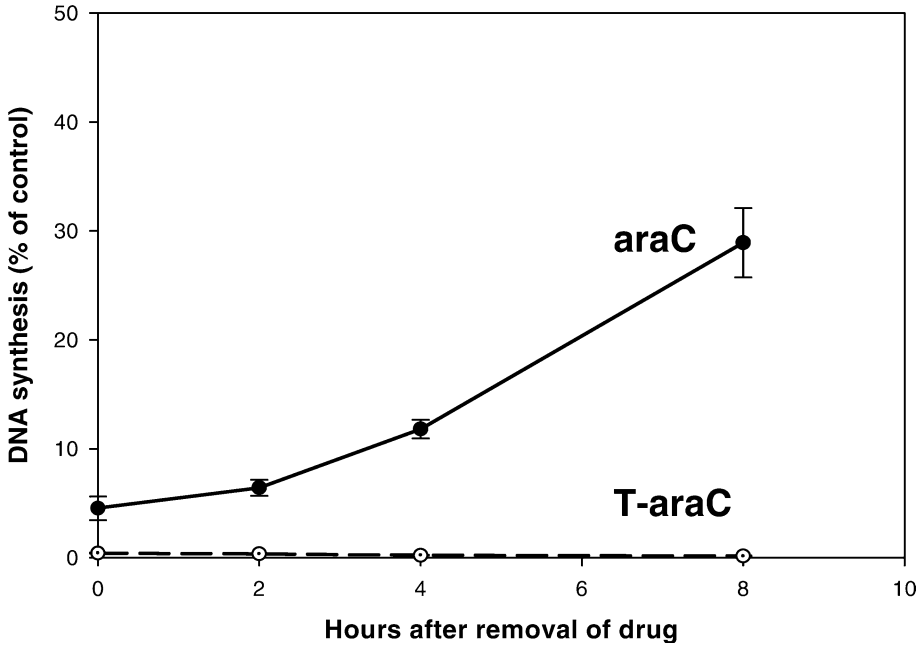

Fig. 4 Inhibition of DNA synthesis by araC and T-araC Near confluent HCT-116 cells were incubated with 10 μM araC or 100 μM T-araC. After removing the medium, the cells were washed thrice with EBSS and then reincubated in fresh medium. The medium was replaced with fresh medium 2, 4, and 8 h after removal of drugs. At the indicated times, the incorporation of [methyl−^{3}H]dThd into DNA was determined as described in Materials and methods. Each point is mean ± SD of three values and the experiment was repeated twice with similar results

presence of dTHU the major end product of araCTP was araC (data not shown).

Effect of inhibition of araC incorporation into DNA on araCTP retention time

Our second hypothesis to explain why T-araCTP had a longer retention than araCTP in solid tumor cells was that araCTP could be incorporated into DNA and excised by DNA repair enzymes more quickly than T-araCTP. Aphidicolin is a potent inhibitor of the major replicative DNA polymerases, such as DNA polymerase α and δ [13] and effectively inhibits the incorporation of dThd and araC [13, 14] into DNA. Thus, the intracellular retention time of araCTP and T-araCTP in intact HCT-116 cells in the presence of this compound was determined (Fig. 5b). Even though the levels of dThd incorporated into DNA were inhibited by >99% under these conditions (data not shown), inhibition of these polymerases did not affect retention time of either araCTP or T-araCTP.

Rates of disappearance of araC and T-araC nucleotides in crude extracts from HCT-116 cells

Because neither the deaminase pathway nor the DNA pathway was responsible for the difference in intracellular retention time between araCTP and T-araCTP in solid tumor cells, we hypothesized that the enzymes that dephosphorylated the triphosphates could differentiate between araCTP and T-araCTP. Since it was not clear what enzyme was responsible for the dephosphorylation of the nucleoside triphosphates, we simply determined the rates of degradation of triphosphates in crude HCT-116 cell extracts (data not shown). The rates of dephosphorylation of araCTP (0.28 pmole/μg protein-min; $N=2$) and T-araCTP (0.25 pmole/μg protein-min; $N=3$) were similar at concentrations of the triphosphates (50–100 μM) that were achieved after the 2-h incubation with 10 μM araC or 100 μM T-araC. The addition of ADP (as the phosphate acceptor) did not change the rate of disappearance of either triphosphate. These results indicated that the enzymes involved in the degradation of the triphosphates were not responsible for the difference in intracellular half-lives of these two triphosphates.

Since the enzyme that dephosphorylates the nucleoside monophosphates could be rate-limiting for the degradation of araCTP and T-araCTP, we determined the rates of degradation of the monophosphates in crude HCT-116 cell extracts. However, there was also no difference in the rate of dephosphorylation of araCMP and T-araCMP (0.058 pmole/μg protein-min; $N=2$: 0.074 pmole/μg protein-min; $N=2$, respectively).

AraCMP and T-araCMP as a substrate with UMP/CMP kinase

Because one of the 5′-nucleotidases, cytosolic nucleotidase II, requires ATP as an activator for its activity [1], we tried to determine the activities of araCMP and T-araCMP with this enzyme in the presence of ATP using crude extracts from HCT-116 cells. Contrary to our expectations, T-araCMP was completely converted to T-araCTP within 1 min of incubation, whereas araCMP

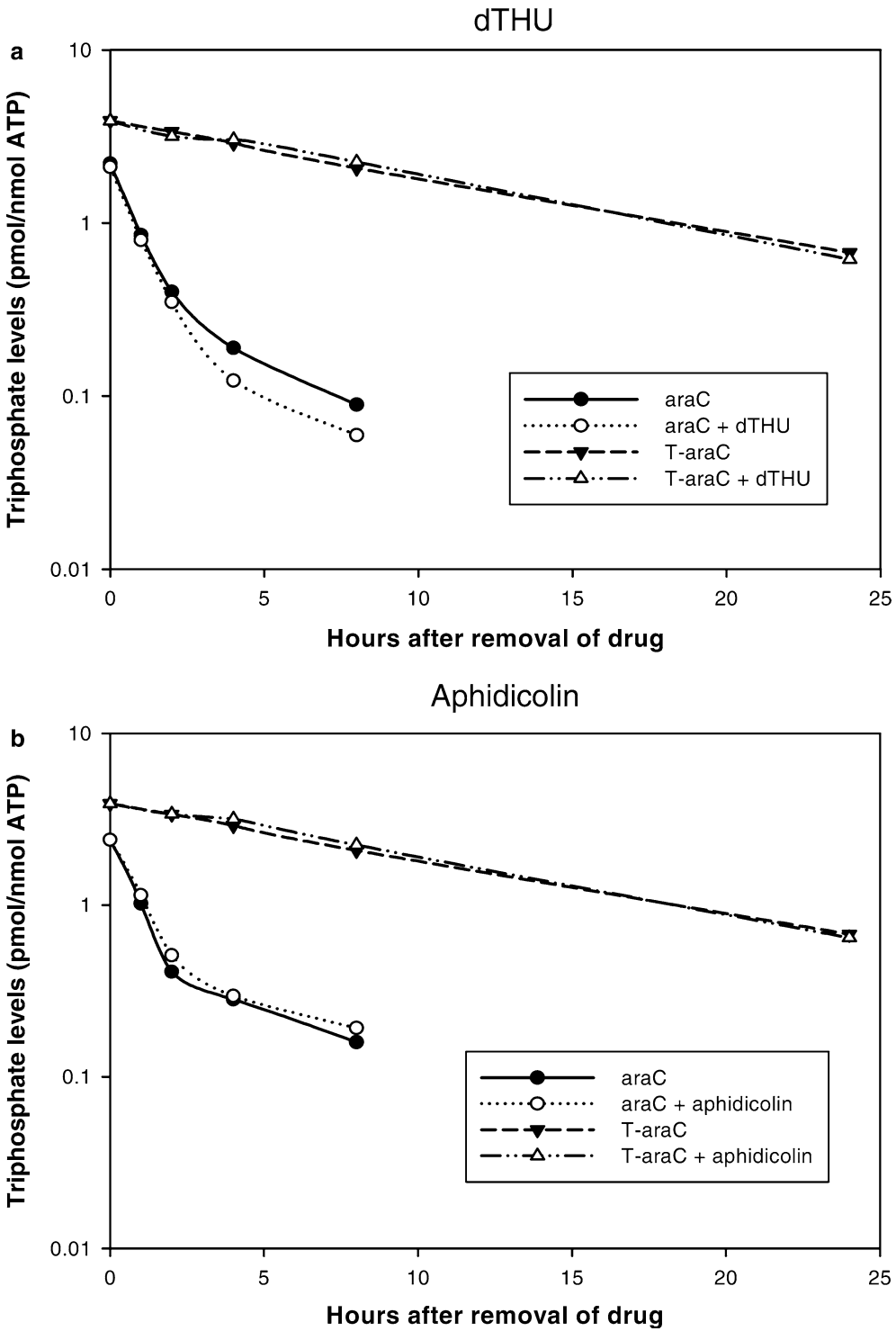

Fig. 5 Effects of dTHU or aphidicolin on intracellular half-lives of araCTP and T-araCTP. Near confluent HCT-116 cells were incubated with 10 μM araC or 100 μM T-araC as described in the legend of Fig. 2. After removing the medium, the cells were washed thrice with EBSS and then reincubated in fresh medium containing 100 μM dTHU (**a**), 30 μM (10 μg/ml) aphidicolin (**b**) or no addition. Samples were collected at various times after addition of fresh medium, and the levels of each triphosphate were determined as described in Materials and methods. *Each number* represents a single measurement, and the entire experiment was repeated once with identical results

still remained, which indicated that the rate of phosphorylation by UMP/CMP kinase of T-araCMP was much greater than that of araCMP. Therefore, the rates of phosphorylation of araCMP and T-araCMP using crude extracts from HCT-116 cells were determined and compared to that of CMP, which is the natural substrate of UMP/CMP kinase. The rate of phosphorylation of 20 μM CMP was 51 ± 22 pmol/μg protein-min ($N = 4$). The rates of phosphorylation of araCMP and T-araCMP at 20 μM were 15 and 120% of CMP, respectively.

Discussion

The intracellular retention times of cytotoxic nucleoside analog triphosphates are believed to be a critical aspect

of their mechanism of action that leads to antitumor activity [8, 15]. The observation that the intracellular half-life of T-araCTP was much longer than that of araCTP in solid tumor cells suggested that this parameter was responsible for the enhanced anti-solid tumor activity of T-araC over araC. Our results indicated that the rate of disappearance of the triphosphate in solid tumor cells was determined by the rate of the removal of the monophosphate. As illustrated in Fig. 6, once the triphosphate of either araC or T-araC is converted to their respective monophosphate, the monophosphates could be deaminated by dCMP deaminase to their respective uridylate analogs, they could be dephosphorylated to their respective nucleosides, or they could be re-phosphorylated by UMP/CMP kinase to their respective diphosphate metabolites. Once a deoxycytidine nucleotide has been deaminated, there are no metabolic pathways to recreate the nucleotides of the deoxycytidine analog. Furthermore, since the uracil derivatives are not toxic to cells, deamination of the deoxycytidine analog is a detoxifying metabolic step. Although nucleosides formed from the dephosphorylation of araCMP or T-araCMP could be re-phosphorylated by dCK, our data indicated that this was not an important component of the degradation of the triphosphates in solid tumors. Nucleosides formed from the nucleotide could either diffuse out of the cell or they could be deaminated by Cyd deaminase. If it diffused out of the cell, its concentration would be very low and subsequent phosphorylation by dCK would proceed at a very low rate. We determined that deamination of either the nucleoside or nucleotide was not an important component of the degradation of either araCTP or T-araCTP, and that the rate of dephosphorylation of araCMP and T-araCMP in crude cell extracts from HCT-116 cells were similar, which was consistent with our previous report in CCRF-CEM cells [18]. Since the rate of phosphorylation of T-araCMP by UMP/CMP kinase was tenfold greater than that of araCMP, there should be a tenfold greater chance that T-araCMP would be re-phosphorylated than araCMP, which suggested that the good activity of T-araCMP with UMP/CMP kinase was responsible for the longer intracellular retention time of T-araCTP. To our knowledge, this is the first report that has correlated UMP/CMP kinase activity with intracellular retention of nucleoside triphosphates.

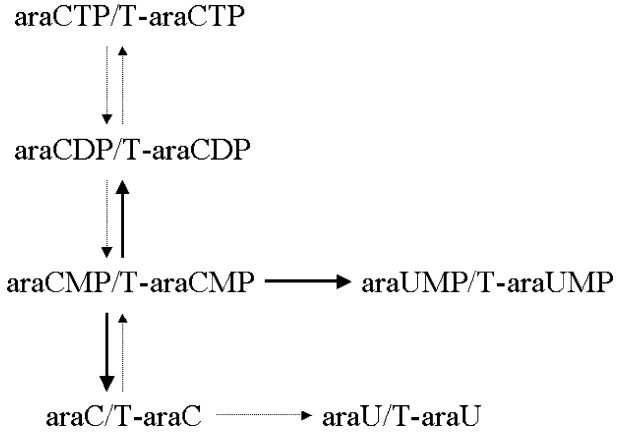

Fig. 6 Catabolic pathways of araCTP and T-araCTP

The rate of phosphorylation of T-araCMP in crude extracts was tenfold than that of araCMP. However, in an intact cell situation in the presence of extracellular nucleosides, the rate-limiting step in the intracellular production of triphosphate with both compounds is dCK. Therefore, the better activity of T-araCMP with UMP/CMP kinase does not lead a higher steady-state concentration of T-araCTP than that of araCTP in the presence of the extracellular nucleosides, and the tenfold preference of T-araCMP over araCMP is important in the triphosphate concentration only when there are no extracellular nucleosides.

Our data indicated that the rates of phosphorylation of both araCMP and T-araCMP were much greater than the rates of the other two reactions. For example, the rate of phosphorylation of araCMP was approximately 100-fold greater than the rate of dephosphorylation of araCMP in cell-free extracts (data not shown). If this marked difference between the ratio of the rate of phosphorylation versus dephosphorylation of the monophosphates existed in living cells, then the half-lives of T-araCTP and araCTP should be much longer than that observed (10 and 1 h, respectively). Therefore, these results suggest that there could be functional compartmentation of nucleotides in cells, in which the deoxycytidine monophosphate analogs have greater access to the dCMP deaminase and the 5′-nucleotidases than they do to UMP/CMP kinase.

In vitro assays using a long-time exposure of drug (72 h) are often used to screen new cytotoxic compounds for subsequent in vivo antitumor studies. We determined that the primary difference in the metabolism of araC and T-araC was the difference in the intracellular half-lives of their respective triphosphates. In long-term exposures the importance of the intracellular half-life of the active metabolite is diminished, because the cells are continually exposed to the nucleoside analog, which results in the maintenance of the triphosphate pool. Good antitumor activity is observed with T-araC in mice when it is given once per day [24]. Since the plasma half-life of T-araC is approximately 1 h [12], subcutaneous tumors are only exposed to T-araC for a short period of time. Under these in vivo conditions the retention time of the active metabolite is very important. Because our in vitro model using short-time exposure with high concentrations of drug approximates the pattern of drug exposure that actually occurs in mice, this model could be a better in vitro predictor of in vivo activity for other cytotoxic nucleosides.

4′-Thio-arabinofuranosylcytosine is a new agent with significant activity against solid tumor xenografts in mice. Our results indicate that the longer intracellular retention of T-araCTP is a critical factor for anti-solid

tumor activity of T-araC, and that the enhanced retention is due to the greater activity of T-araCMP for UMP/CMP kinase. Further studies are planned to characterize the interaction of araCMP and T-araCMP with purified UMP/CMP kinase and to examine how T-araCTP causes cell death. Although these studies were able to clarify the reasons for the difference in solid tumor activity between araC and T-araC, they did not reveal why some tumor types were more sensitive to T-araC than others, because the metabolism of T-araC and the half-life of T-araCTP were similar in all solid tumors tested. These studies indicate that factors unrelated to the metabolism of T-araC can impact the activity of T-araC. A complete understanding of the mechanism of action of T-araC should lead to an understanding of the actions of T-araC that are required for its activity against solid tumors. It is hoped that such understanding will aid in the design of new nucleoside analogs that have even better activity against solid tumors.

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