Phosphorylation of 4’-thio-arabinofuranosylcytosine and its analogs by human deoxycytidine kinase

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d) Abbreviations used: araC, β-D-arabinofuranosylcytosine; araCMP, 5’-monophosphate of araC; araCTP, 5’-triphosphate of araC; Br-dCyd, 5-bromo-2’-deoxycytidine; Br-T-araC, 5-bromo-4’-thio-β-D-arabinofuranosylcytosine; CH3-dCyd, 5-methyl-2’-deoxycytidine; CH3-T-araC, 5-methyl-4’-thio-β-D-arabinofuranosylcytosine; Cl-dCyd, 5-chloro-2’-deoxycytidine; Cl-T-araC, 5-chloro-4’-thio-β-D-arabinofuranosylcytosine; dCK, deoxycytidine kinase; dCyd, 2’-deoxycytidine; dThd, thymidine; F-araC, 5-fluoro-β-D-arabinofuranosylcytosine; F-dCyd, 5-fluoro-2’-deoxycytidine; F-T-araC, 5-fluoro-4’-thio-β-D-arabinofuranosylcytosine; HPLC, high pressure liquid chromatography; IC50, concentration of compound that inhibits cell growth by 50%; MS, mass spectrometry; NMR, nuclear magnetic resonance; NTP, 5’-triphosphate of nucleoside; SAX, strong anion exchange; t1/2, half-life; T-araC, 4’-thio-β-D-arabinofuranosylcytosine; T-araCMP, 5’-monophosphate of T-araC; T-araCTP, 5’-triphosphate of T-araC; T-dCyd, 4’-thio-2’-deoxycytidine.

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ABSTRACT

4'-Thio-β-D-arabinofuranosylcytosine (T-araC) exhibits excellent \textit{in vivo} antitumor activity against a variety of solid tumors despite its structural similarity to β-D-arabinofuranosylcytosine (araC), an agent which is poorly active against solid tumors \textit{in vivo}. It is of great interest to elucidate why these compounds show profound difference in antitumor activity. Because deoxycytidine kinase (dCK) is the critical enzyme in the activation of both compounds, here we report the differences in the substrate characteristics with human dCK between these compounds. The catalytic efficiency ($V_{\text{max}}/K_m$) of araC was 100-fold higher than that of T-araC using either ATP or UTP as the phosphate donor. However, $V_{\text{max}}$ values of araC and T-araC were similar when UTP was the phosphate donor. Since UTP is believed to be the true phosphate donor for dCK in intact cells, these data indicated that the rates of phosphorylation of these two compounds at high pharmacologically relevant concentrations would be similar. This prediction was confirmed in intact cell experiments, which supported the hypothesis that UTP is the physiological phosphate donor for dCK phosphorylation in cells. The relative lack of importance of phosphate donor to the phosphorylation of T-araC by dCK revealed important insights into the activation of this compound in human cells at pharmacological doses. These studies indicated that the replacement of 4’-oxygen with sulfur significantly reduced the substrate activity of nucleoside analogs with dCK and that the superior activity of T-araC with respect to araC against solid tumors was not due to superior activity with dCK.
T-araC (Figure 1) is the most promising antitumor agent among the 4'-thionucleoside analogs that have been synthesized in our drug development program (Tiwari et al., 2000). It exhibits excellent *in vivo* antitumor activity against a variety of human solid tumor xenografts, such as CAK-1 (renal), NCI-H23 (non small cell lung), HCT-116 (colon), LOX (melanomas), PANC-1 (pancreas), and DU-145 (prostate) (Tomkinson et al., 2002; Waud et al., 1999). Because of these results, it is being evaluated for effectiveness in clinical trials by OSI Pharmaceuticals as OSI-7836. T-araC is a structural analog of araC (Figure 1), which is clinically used in the treatment for acute myelogenous leukemia and other hematological malignancies (Mastrianni et al., 1992; Peters et al., 1987; Stryckmans et al., 1987) but is poorly active against solid tumors *in vivo* (Cheng and Capizzi, 1982; Davis et al., 1974). It is of great interest to elucidate how the minor structural difference between araC and T-araC (the 4'-oxygen atom in the arabinofuranosyl ring is replaced by a sulfur atom, Figure 1) results in the profound difference in antitumor activity that is observed with these two agents.

Our previous studies indicated that the basic mechanisms of action of these two agents were similar. They were phosphorylated to their respective triphosphates, which inhibited DNA replication. (Parker et al., 2000). Blajeski et al. (2002) have shown that araCTP and T-araCTP similarly inhibit DNA synthesis (i.e. they are both alternative substrates for DNA polymerase α and δ, which results in chain termination). However, there were many quantitative differences in the metabolism and activity between araC and T-araC; 1) T-araC was phosphorylated to active metabolites at 1% the rate of araC, 2) T-araCTP was 20-fold more potent as an inhibitor of DNA synthesis than was araCTP, 3) the t½ of T-araCTP was twice that of araCTP, 4) the catalytic efficiency of T-araC with cytidine deaminase was 10% that of araC, and 5) araCMP was a better substrate for dCMP deaminase than was T-araCMP. Although it is possible that some of these differences could contribute to the activity of T-araC against solid tumors, it is still uncertain why T-araC is a superior agent against solid tumors.

As a part of a series of investigations to define the mechanism of action of T-araC, we initiated this study to determine the difference in the substrate characteristics between araC and T-araC with deoxycytidine kinase (dCK), the
enzyme that mediates the rate-limiting step in the activation of both compounds. In addition, the substrate properties of 
dCyd, T-dCyd, and 5-substituted analogs of T-araC, such as F-T-araC, Cl-T-araC, Br-T-araC and CH$_3$-T-araC with dCK 
were also determined in order to obtain insight into the structure-activity relationship of 4'-thionucleoside analogs with 
regard to their utilization by dCK.
Materials and Methods

**Materials.** dCyd, araC and araCMP were purchased from Sigma Chemicals Inc. (St. Louis, MO). T-dCyd, T-araC, F-T-araC, Cl-T-araC, Br-T-araC, and CH₃-T-araC were chemically synthesized in our laboratories (Secrist *et al.*, 1991; Tiwari *et al.*, 2000; Tiwari *et al.*, 2000). T-araCMP was synthesized from T-araCTP using phosphodiesterase I that was obtained from Sigma Chemicals Inc. (95% yield, 95% purity). Its structure and purity were confirmed by ¹H-NMR, ³¹P-NMR, MS, and HPLC. The structures of the other compounds were verified by MS and NMR. [5-³H]dCyd and [5-³H]araC (5.0 and 9.6 Ci/mmol, respectively) were labeled with ³H at the 5-position by Moravek Biochemicals Inc. (Brea, CA). [5-³H]dCyd (26.5 Ci/mmol) and [5-³H]araC (26 Ci/mmol) were also purchased from Moravek Biochemicals Inc. Other compounds were of standard analytical grade.

**Cell culture.** Wild-type CEM cells (human leukemia cell line, American Type Culture Collection, Rockville, MD), dCK deficient CEM cells, which were generously provided by Dr. Jan Balzarini (Rega Institute for Medical Research, Leuven, Belgium), and HCT-116 cells (human colon cancer cell line, NCI tumor repository) were grown in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD) containing 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 1 mg/ml sodium bicarbonate, 10 U/ml penicillin, 10 µg/ml streptomycin, and 50 µg/ml gentamycin. Cell numbers of wild-type and dCK deficient CEM cells were determined with a Coulter Counter, and the concentration of compound that resulted in inhibition of cell growth by 50% over a 72-hr incubation period was determined (IC₅₀).

**Purification of deoxycytidine kinase.** dCK was partially purified from both CEM and HCT-116 cells as described by Datta *et al.* (1989) with some modifications. Briefly, CEM or HCT-116 cell pellets were homogenized in 3-5 volumes of 50 mM Tris (pH=7.5), 0.5 mM EDTA, 5 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride. The suspension was centrifuged at 40,000 rpm for 1 hr and the supernatant fluid was dialyzed against the homogenization buffer containing 30% glycerol. The crude extract was applied onto a 1 ml HiTrap Q Sepharose HP anion exchange
column (Amersham Pharmacia Biotech Inc., Piscataway, NJ) that had been equilibrated with the homogenization buffer containing 5% glycerol (running buffer), and the protein was eluted using a step gradient of 200, 250, 300, and 1000 mM KCl in the running buffer (5 ml each). dCK eluted with the 250 mM KCl wash. Active fractions were pooled and dialyzed against the running buffer. Glycerol sufficient to yield 30% (v/v) was added, and the sample was stored at -85°C. This procedure removed 97% of cellular protein and resulted in an increase in specific activity of approximately 10-fold. dThd kinase (Parker et al., 1995), UMP/CMP kinase, and nucleotide phosphatase activities were not detected in the enzyme preparation. In order to measure UMP/CMP kinase activity, [3H]dCyd and its phosphorylated metabolites generated in dCK assay were separated from each other by chromatography on a Partisil SAX column (Keystone Scientific Inc., Bellefonte, PA). The column was washed with a 50 min linear gradient from 5 (pH=2.8) to 750 mM NH₄H₂PO₄ (pH=3.7) buffer with a flow rate of 2 ml/min. One-minute fractions were collected from the column and counted for radioactivity.

Deoxycytidine kinase assay. In the assays with radiolabeled nucleosides, the reaction was carried out as described by Parker et al. (1999). Briefly, solutions containing 50 mM Tris (pH=8.0), phosphate donor (either ATP or UTP), 7.5 mM MgCl₂, 20 mM NaF, various concentrations of radiolabeled nucleosides, and sufficient enzyme to give linear reaction were incubated at 37°C. The reactions were terminated by putting 40 µl of each assay onto Whatman DE-81 anion exchange filter discs (Whatman International Ltd., Maidstone, England). The radioactivity of each disc was determined after washing three times with 1 mM ammonium formate solution and twice with 95% ethanol. With non-radiolabeled nucleosides, the reaction was carried out in the same way as above, except that the reaction was stopped by boiling at 100°C or by addition of 0.5 mM HClO₄ followed by neutralizing with 8 N KOH. The particulate matter was removed by filtration or centrifugation, and the extract was applied to Nucleosil SB silica SAX column (Interchim, Montlucon Cedex, France) as described by Aussenac et al. (2001) with some modifications. The samples were eluted with a 30 min linear gradient from 7 to 500 mM KH₂PO₄ (pH=4.0) and followed by 20 minutes of 500 mM KH₂PO₄ (pH=4.0) at a flow rate of 2 ml/min. When ATP was used as the phosphate donor in the assays, the ATP was eluted with
20 minutes of 2 M KCl in place of 500 mM KH$_2$PO$_4$. Nucleoside monophosphates were detected by UV absorbance at 260 nm. dCK kinase activity with non-radiolabeled compounds was also determined by observing the disappearance of substrate from the reaction mixture. The reactions were carried out and an acid-soluble extract was obtained as described above. The extract was applied to Hypersil BDS reverse phase column (Keystone Scientific Inc., Bellefonte, PA). The mobile phase was 2.5% acetonitrile in a 25 mM NH$_4$H$_2$PO$_4$ buffer (pH=4.5) at a flow rate of 1 ml/min. The nucleosides were detected by UV absorbance at 260 nm.

**Nucleotide phosphatase assay.** Phosphatase activity of araCMP or T-araCMP was assayed in solutions containing 50 mM Tris (pH=8.0), 7.5 mM MgCl$_2$, 50 or 100 µM nucleoside monophosphate and various concentration of enzyme. After an 8-hr incubation at 37°C, the reaction was stopped by acid extraction as described above. The extract was applied to the reverse phase HPLC analysis as described above to observe the formation of nucleoside from respective monophosphate.
Results

Substrate characteristics of dCyd, T-dCyd, araC, and T-araC with dCK using ATP as the phosphate donor. The substrate characteristics of dCyd, T-dCyd, araC, and T-araC with dCK from CEM cells using 5 mM ATP as the phosphate donor were determined (Table 1). T-dCyd had the lowest $V_{\text{max}}$ value among the four compounds, indicating that the 4'-sulfur atom made the reaction velocity lower. AraC had a 20-fold greater $K_{m}$ value than that of dCyd, indicating that the arabino configuration caused the lower affinity for the enzyme. T-araC had the highest $K_{m}$ value among the four compounds and a much lower $V_{\text{max}}$ value than that of dCyd, indicating that the two substitutions of 4'-oxygen to 4'-sulfur and deoxyribose to arabinofuranose caused both the affinity to the enzyme and the reaction velocity to be much lower. The catalytic efficiency of T-araC using ATP as the phosphate donor was 1% that of araC. Similar results were obtained with dCK isolated from HCT-116 cells (data not shown).

Substrate characteristics of dCyd, araC, and T-araC with dCK using UTP as the phosphate donor. Since UTP is considered to be the physiological phosphate donor for dCK phosphorylation (Hughes et al., 1997; Shewach et al., 1992; White and Capizzi, 1991), the substrate characteristics of dCyd, araC, and T-araC with the dCK from CEM cells using 5 mM UTP were also determined (Table 1). The change of the phosphate donor from 5 mM ATP to 5 mM UTP decreased the $K_{m}$ value of araC by 40-fold while decreasing those of dCyd and T-araC by only 4-fold. The phosphate donor substitution also resulted in a decrease in the $V_{\text{max}}$ value of araC by 15-fold, while there was only a moderate decrease in $V_{\text{max}}$ for dCyd and almost no decrease in $V_{\text{max}}$ for T-araC. Because the intracellular concentration of UTP is known to be lower than that of ATP (Shewach et al., 1992; White and Capizzi, 1991), the kinetic values of araC and T-araC using 0.1 mM UTP were also determined (data not shown). The results were similar to those obtained at 5 mM UTP, which indicated that the kinetic parameters for araC and T-araC were independent of UTP concentration. Even though the kinetic parameters of araC were affected much more than those of T-araC by changing the phosphate donor,
the catalytic efficiency of araC was still 100-fold greater than that of T-araC using UTP as the phosphate donor.

**Metabolism of araC and T-araC at micro molar concentrations in intact cells.** The theoretical rates of phosphorylation of araC and T-araC were calculated at 0.1 and 100 µM using the kinetic parameters obtained with ATP or UTP (Table 2). The kinetic parameters indicated that if ATP is the physiological phosphate donor, then phosphorylation of araC would be much greater than T-araC at both high and low concentrations. However, if UTP is the physiological phosphate donor, then the phosphorylation of T-araC would be similar to araC at high concentrations. The concentration is important because peak plasma levels in mice treated with a therapeutic dose of T-araC (100 mg/kg) are approximately 100 µM (Waud et al., 1999; Parker et al., 2000). In order to confirm this prediction, the difference in the rates of phosphorylation of araC and T-araC at 0.1 and 100 µM in intact CEM cells was determined (Figure 2). The difference in metabolism of these agents at 100 µM was only 2- to 3-fold, while the difference at 0.1 µM was approximately 60-fold. Similar results were obtained with intact HCT-116 cells (data not shown). The differences in rates of phosphorylation of araC and T-araC in intact cells at both high and low concentrations were consistent with the differences in the theoretical rate of phosphorylation using UTP as the phosphate donor. The theoretical difference in the rate of phosphorylation of araC and T-araC at 100 µM using ATP as the phosphate donor (33-fold) was more than 10-fold greater than the results obtained in the intact cell experiment (2- to 3-fold). Therefore, these results strongly supported the idea that UTP is the physiological phosphate donor for dCK.

**Phosphatase activities of araCMP or T-araCMP in crude extract from CEM cells.** In our previous work, there was a discrepancy between the results obtained with purified dCK and CEM cell extract (Parker et al., 2000). Results with purified enzyme indicated that the catalytic efficiency was similar for araC and T-araC, which indicated that metabolism in CEM cells of the two compounds would be similar. However, the difference in rate of phosphorylation of araC and T-araC at low concentrations by crude extracts from CEM cells was 100-fold, which was consistent with the kinetic data reported in the current work. A possible explanation of the previous results was that T-araCMP was degraded
more quickly than araCMP in the crude CEM cell extracts. In order to test this hypothesis, the difference in phosphatase activities in crude extracts from CEM cells between araCMP and T-araCMP was examined (data not shown). At either 50 or 100 µM, the phosphatase activity of araCMP was 2-fold higher than that of T-araCMP. In addition, araCMP and T-araCMP phosphatase activities were not detected in the purified preparation of dCK used in the current work. Therefore, these data indicated that the discrepancy did not come from the difference in the phosphatase activities in the crude extracts or the backward reaction of the dCK. Although the reason for the discrepancy is still unclear, all the results in the current work favored the observation obtained using crude extracts from CEM cells.

**Effects of araC, T-araC, and 5-substituted analogs of T-araC on wild-type or dCK deficient CEM cell growth.** The IC₅₀s of araC and T-araC against dCK deficient CEM cell growth were determined (Table 3). Wild-type CEM cells were slightly less sensitive to T-araC than they were to araC. However, T-araC was more potent against the dCK deficient CEM cell growth than was araC. dCK activity in this cell line was approximately 1% of that found in wild-type cells. As a part of our ongoing program to design and synthesize new antitumor agents, several 5-substituted analogs of T-araC (F-T-araC, Cl-T-araC, Br-T-araC, and CH₃-T-araC) were synthesized and evaluated for biological activity. The Cl, Br, and CH₃ analogs were much less toxic (100- to 5000-fold) to wild-type CEM cells than T-araC (Table 3). However, F-T-araC was about 10-fold more potent than T-araC.

**Substrate activities of 5-substituted analogs of T-araC for dCK phosphorylation.** In order to understand the structure-activity relationship of 5-substituted analogs of T-araC for dCK activity, substrate activities of T-araC, F-, Cl-, Br-, and CH₃-T-araC for dCK phosphorylation were examined using UTP as the phosphate donor. T-araCMP and F-T-araCMP were detected in dCK assays using Nucleosil SAX HPLC to separate nucleosides from nucleotides (Figure 3). As an estimation of the Vₘₐₓ value, the rate of phosphorylation of F-T-araC at a saturating concentration (1 mM) using UTP as the phosphate donor was determined by Nucleosil SAX HPLC and compared to that of T-araC (Table 3). At this concentration, the amount of product formed was similar for both compounds. Similar results were obtained using ATP
as the phosphate donor. In order to obtain an estimate of the $K_m$ of F-T-araC, the inhibition constant ($K_i$) of F-T-araC against dCyd phosphorylation was determined (Figure 4). The $K_i$s of T-araC and F-T-araC were 551 ± 50 and 50 ± 9.4 using ATP as the phosphate donor, and 180 ± 25 and 27 ± 3.1 µM using UTP as the phosphate donor (mean ± standard deviation, N=3), respectively. Based on these results, it was estimated that the $K_m$ value of F-T-araC for dCK phosphorylation using ATP or UTP as the phosphate donor was approximately 8 or 3 µM, respectively. These results indicated that the catalytic efficiency of dCK with F-T-araC was about 10-fold better than that of T-araC.

The monophosphates of Cl-, Br-, or CH$_3$-T-araC were not detected in dCK assays when using Nucleosil SAX HPLC (Table 3). Similar results were obtained using ATP as the phosphate donor. Substrate activity was also not detected with the three compounds for dCK when the disappearance of substrate was followed as a measurement of enzyme activity (Table 3), which indicated that these compounds were at least 5-fold poorer as substrates for dCK phosphorylation than T-araC at 100 µM. Furthermore, the concentration of Cl-, CH$_3$-, and Br-T-araC required to inhibit dCK phosphorylation of 1 µM T-araC by 50% was 500-1000 µM (data not shown), which suggested that the three compounds were 500- to 1000-fold poorer substrates than T-araC. The results from these three experiments indicated that Cl-, Br-, and CH$_3$-T-araC were very poor substrates for dCK.
Discussion

We have previously discovered a structural analog of araC (T-araC) that has demonstrated excellent anti-tumor activity against a wide variety of human solid tumor xenografts (Tomkinson et al., 2002; Waud et al., 1999). As with many other nucleoside analogs used in the treatment of cancer and viral infections, dCK is the rate-limiting step in its activation to cytotoxic nucleotides (Parker et al., 2000). Therefore, we studied the phosphorylation of T-araC by dCK to increase our understanding of the biochemical pharmacology of this new agent.

One of the more important findings of this work was the discovery of the relative lack of importance of the phosphate donor to the phosphorylation of T-araC by dCK, which revealed important insights into the activation of this compound in human cells. The substitution of ATP with UTP increased the catalytic efficiencies of both araC and T-araC by about 3-fold and thus there was no change in the difference in the catalytic efficiency between the two compounds. However, there were significant differences in the degree of change in the $K_m$ and $V_{max}$ values between the two compounds (for araC there was a 40-fold decrease in the $K_m$ and 15-fold decrease in the $V_{max}$ value when UTP was used in place of ATP, whereas there was only a 4-fold decrease in the $K_m$ and little, if any, decrease in $V_{max}$ value for T-araC). These differences indicated that at low concentrations there is a 100-fold difference in the rate of phosphorylation between the two compounds, irrespective of whether ATP or UTP is used as the phosphate donor. However, at high concentrations only the kinetic parameters with UTP can adequately predict the difference in the rate of phosphorylation between the two compounds in intact cells (Figure 2). Since the peak plasma concentration of T-araC at the optimal therapeutic dose of T-araC (100 mg/kg) is 100 µM (Parker et al., 2000; Waud et al., 1999), it is important to realize that the difference in the rate of phosphorylation between the two compounds in tumor cells in animals treated with these agents should be similar, not 100-fold as indicated in our previous publication (Parker et al., 2000).

The fact that the rate of metabolism of 100 µM T-araC was similar to that of araC in intact cells and that this
result was predicted using the kinetic parameters for araC and T-araC with UTP as the phosphate donor is further evidence that UTP is the primary phosphate donor in intact cells when both UTP and ATP were present for this purpose. The importance of UTP to dCK in intact cells was first suggested by White and Capizzi (1991), who showed that the kinetics of araC phosphorylation by intact cells was quantitatively similar to that seen with purified dCK using UTP. In addition, only the deletion of UTP from NTP mixtures in dCK assays significantly reduced the rate of phosphorylation of dCyd and araC (Shewach et al., 1992; White and Capizzi, 1991). Other results with purified enzymes have indicated that the catalytic efficiencies of dCyd and several other nucleoside analogs were greater using UTP as the phosphate donor rather than ATP, and that UTP is preferred over ATP as a phosphate donor (Cheng et al., 1977; Hughes et al., 1997; Krawiec et al., 1995; Shewach et al., 1992).

T-araC was more potent against dCK deficient CEM cells than araC, which is different from the result observed in wild-type CEM cells (Table 3). It is clear from our results (Parker et al., 2000) that dCK is primarily responsible for the activation of both compounds in wild-type cells. Therefore, the change in the relative potency of the two agents in these two cell lines initially suggested to us that T-araC was phosphorylated by another nucleoside kinase that did not recognize araC as a substrate. However, this change in the relative potency of these two agents can be explained by the differences observed in the kinetic parameters of T-araC and araC using UTP as the phosphate donor. If one assumed that ATP was the physiological phosphate donor, then araC would always be more potent than T-araC because the relative rates of phosphorylation would be similar at both low (nM) and high (µM) concentrations of compounds. However, if UTP is the phosphate donor, then the rate of activation of T-araC would be similar to that of araC at high concentrations, but not at low concentrations. Theoretically, there is only a 4-fold difference in the phosphorylation of araC and T-araC at 15 µM when UTP is the phosphate donor. Our previous results (Parker et al., 2000) indicated that T-araCTP was about 20-fold more potent than araCTP to inhibit CEM cell growth. Therefore, at concentrations leading to a similar rate of activation, T-araC would be more cytotoxic than araC.
The characterization of the kinetic parameters with dCK of many nucleoside analogs has been done with ATP as the phosphate donor. For comparison of catalytic efficiencies of nucleoside analogs, it may not matter whether ATP or UTP was used as the phosphate donor because the substitution of ATP with UTP usually decreases both $K_m$ and $V_{max}$ for dCK phosphorylation (Hughes et al., 1997; Johansson and Karlsson, 1995; Shewach et al., 1992; White and Capizzi, 1991) and thus the difference in their catalytic efficiencies is only slightly changed. Our data, however, argue that one could be misled by results obtained with ATP and that utilization of UTP to determine kinetic parameters of nucleoside analogs activated by dCK is crucial for the comparison of their rates of phosphorylation and prediction of their cytotoxicity in an in vivo setting.

Unlike dCyd and araC, which exhibit substrate inhibition when UTP is used as the phosphate donor at concentrations above 1-3 and 10-20 µM, respectively (Hughes et al., 1997; Shewach et al., 1992; White and Capizzi, 1991), no substrate inhibition was observed with T-araC at concentrations up to 1 mM (data not shown). In addition to the relative lack of effect of phosphate donor on the kinetic parameters, this observation also suggests that T-araC may be a unique cytosine analog in terms of dCK phosphorylation. Substrate inhibition could cause a problem for nucleosides with low $K_m$ values with dCK. If a nucleoside analog has a low $K_m$ value, one would want to dose high enough to overcome the rate-limiting step of the uptake process (Mackey et al., 1998) but too much compound may result in substrate inhibition. Therefore, in order to obtain the maximum triphosphate formation, an ideal nucleoside analog should have a moderate $K_m$ and large $V_{max}$ value for dCK phosphorylation.

In this work we have also evaluated the cytotoxicity and phosphorylation of numerous 5-substituted analogs of T-arac. Equivalent differences in cytotoxicity (10-fold) and catalytic efficiency for dCK (10-fold) between T-arac and F-T-arac suggest that the enhanced cytotoxicity of F-T-arac to cells in culture was related to its enhanced substrate activity for dCK. However, it is possible that other factors could also be involved in the enhanced potency of F-T-arac. Our results with F-T-arac are consistent with previous studies with murine dCK, which indicated that F-dCyd and F-arac
were good substrates of this enzyme (Balzarini and De Clercq, 1982; Cooper and Greer, 1973). The Cl, Br, and CH$_3$ substituted analogs of T-araC were poorer substrates for human dCK, and likewise, did not have significant effects on the growth of CEM cells. These results are different from those of Br-, Cl-, and CH$_3$-dCyd obtained with calf thymus and mouse dCK (Balzarini and De Clercq, 1982; Cooper and Greer, 1973; Krenitsky et al., 1976). However, since these data were obtained using ATP or TTP as the phosphate donor, the results might be different if UTP was used as the phosphate donor.

Unlike araC, T-araC demonstrates excellent activity against solid tumor xenografts, which indicates that there must be actions of T-araC which are different from those of araC and result in activity against solid tumors. In the current work we have fully characterized the interaction of T-araC with dCK, the enzyme responsible for the activation of both compounds. Although our previous results (Parker et al., 2000) indicated that there was 100-fold difference in the rate of phosphorylation between araC and T-araC, the current study has revealed that the actual difference is 2 to 3-fold at pharmacologically relevant concentrations. However, since araC is still a better substrate for dCK at any concentration, the difference in substrate activity between these agents with dCK cannot explain why T-araC is a superior agent against solid tumors. In our previous studies (Parker et al., 2000) we identified two biochemical differences between araC and T-araC (greater potency of T-araCTP and longer intracellular t$_{1/2}$ of T-araCTP), which could help compensate for the poor activation of T-araC by dCK. Because we now know that at pharmacological doses T-araC is phosphorylated at a rate that is similar to that of araC, these biochemical differences do not just compensate for the poor activation of T-araC, but they enhance the activity of T-araC beyond that of araC. It is hoped that increasing our knowledge of the biochemical pharmacology of T-araC will lead to identification of the activity or activities of this compound that results in its excellent activity against solid tumors and that this information would be useful in the design of new and better nucleoside analogs.

In order to further characterize the mechanism of action of T-araC, studies are planned to examine the differences in the interaction of the metabolites of araC and T-araC with enzymes involved in DNA replication.
Acknowledgment

None
References


Footnotes

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   A preliminary report of this work has been presented in 2002 Annual Meeting of American Association for Cancer Research (Someya et al., 2002).

b) Send reprint requests to: Dr. William B. Parker, Southern Research Institute, 2000 Ninth Avenue South, Birmingham, AL 35205.

c) None
Legends for figures

Figure 1  
Structures of dCyd, T-dCyd, araC, and T araC

Figure 2  
Metabolism of araC and T araC in intact CEM cells

Wild-type CEM cells were incubated with either (A) 0.1 or (B) 100 µM [5-³H]araC (●) or [5-³H]T-araC (V). The cells were collected by centrifugation after 30, 60, and 120 min of incubation, and an acid-soluble extract was prepared, and analyzed by Partisil SAX HPLC as described in Materials and Methods. Each plot represents the amount of triphosphate, which was calculated from the summation of radioactivity in triphosphate region and the subsequent conversion to pmol/10⁶ cells. The experiment has been repeated with similar results.

Figure 3  
Phosphorylation of T-araC and F-T-araC by dCK

dCK was incubated with 5 mM UTP plus 500 µM T-araC (A), 500 µM F-T-araC (B), or No drug (C) for 24 hours. Enzyme activity was stopped by acid extraction and samples were injected into Nucleosil SAX HPLC. The products of the reaction were detected by UV at 260 nm as they eluted from the column.

Figure 4  
Kᵢ values of T-araC and F-T-araC

Inhibition constants (Kᵢ) of T-araC (A) and F-T-araC (B) against ³H-dCyd phosphorylation by dCK from CEM cells using 5 mM UTP as the phosphate donor were determined from linear replots of inhibitor concentration vs. slope of the double-reciprocal plots of substrate concentration vs. velocity. The best lines were drawn by linear regression from at least five points of substrate concentration vs. velocity or from at least four points of inhibitor concentration vs. slope.
Table 1

**Substrate characteristics of dCyd, T-dCyd, araC, and T-araC with human dCK**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Phosphate donor</th>
<th>N</th>
<th>K_m (µM)</th>
<th>V_max (nmol/mg/min)</th>
<th>V_max/K_m</th>
</tr>
</thead>
<tbody>
<tr>
<td>dCyd</td>
<td>ATP</td>
<td>9</td>
<td>0.57 ± 0.42</td>
<td>4.0 ± 2.0</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>UTP</td>
<td>4</td>
<td>0.13 ± 0.030</td>
<td>1.2 ± 0.22</td>
<td>9.4</td>
</tr>
<tr>
<td>T-dCyd</td>
<td>ATP</td>
<td>3</td>
<td>0.32 ± 0.035</td>
<td>0.12 ± 0.010</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>UTP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>araC</td>
<td>ATP</td>
<td>7</td>
<td>15 ± 4.1</td>
<td>9.3 ± 1.0</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>UTP</td>
<td>4</td>
<td>0.41 ± 0.19</td>
<td>0.69 ± 0.33</td>
<td>1.7</td>
</tr>
<tr>
<td>T-araC</td>
<td>ATP</td>
<td>6</td>
<td>88 ± 16</td>
<td>0.46 ± 0.052</td>
<td>0.0052</td>
</tr>
<tr>
<td></td>
<td>UTP</td>
<td>3</td>
<td>20 ± 0.90</td>
<td>0.40 ± 0.055</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Kinetic parameters were determined from linear double-reciprocal plots of substrate concentration vs. velocity. The nucleoside substrates were labeled with ^3^H, and product formation was determined using the filter disc assay described in Materials and Methods section. The concentrations of ATP and UTP were 5 mM. The best line was drawn by linear regression from at least five points. Each experiment was done in triplicate and the experiments were repeated at least three times (N=number of experiments).
Table 2

Theoretical rates of phosphorylation of araC and T-arac with dCK

<table>
<thead>
<tr>
<th></th>
<th>Rate of phosphorylation (nmol/mg/min)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low concentration (0.1 µM)</td>
<td>High concentration (100 µM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>UTP</td>
<td>ATP</td>
</tr>
<tr>
<td>araC</td>
<td>0.062</td>
<td>0.13</td>
<td>8.1</td>
</tr>
<tr>
<td>T-arac</td>
<td>0.00052</td>
<td>0.0020</td>
<td>0.24</td>
</tr>
<tr>
<td>araC/T-arac</td>
<td>120</td>
<td>66</td>
<td>33</td>
</tr>
</tbody>
</table>

The values were calculated from Michaelis-Menten equation based on the kinetic parameters shown in Table 1.
### Table 3

Cytotoxicities and dCK activities of araC, T-araC, and 5-substituted analogs of T-araC

<table>
<thead>
<tr>
<th></th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>Monophosphate detected by Nucleosil SAX chromatography</th>
<th>Rate of disappearance of substrate (nmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type CEM cells</td>
<td>dCK deficient CEM cells</td>
<td>(nmol/mg/min)</td>
</tr>
<tr>
<td>araC</td>
<td>0.0055 ± 0.0028&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19 ± 8.1</td>
<td>-</td>
</tr>
<tr>
<td>T-araC</td>
<td>0.024 ± 0.0092&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.7 ± 3.9</td>
<td>0.87</td>
</tr>
<tr>
<td>F-T-araC</td>
<td>0.0020 ± 0.00086</td>
<td>-</td>
<td>0.77</td>
</tr>
<tr>
<td>Cl-T-araC</td>
<td>51</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Br-T-araC</td>
<td>3.0</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>CH₃-T-araC</td>
<td>&gt;150</td>
<td>-</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup>: (-) = Not determined, ND=Not detected

<sup>b</sup>: IC<sub>50</sub>s against wild-type or dCK deficient CEM cells were determined over a 72-hr incubation period. The data presented are the average ± standard deviation of at least 3 measurements, or the average of 2 values.

<sup>c</sup>: The data have been published (Parker et al., 2000).

<sup>d</sup>: The assays were performed with the compounds at 1 mM over a 24-hr incubation period using UTP as the phosphate donor. dCK activities were determined by measuring the product formation using Nucleosil SAX HPLC to separate substrate from product.

<sup>e</sup>: The assays were performed with the compounds at 100 µM over a 24-hr incubation period using UTP as the phosphate donor. dCK activities were determined by observing the disappearance of substrate using reverse phase HPLC. The data presented are mean ± standard deviation of three experiments.

<sup>f</sup>: Although the disappearance of these nucleosides was not detected, these compounds could be substrates for dCK with a maximum activity of 0.13 nmol/mg/min. The detection limit of the system is due to the technical difficulty of measuring less than 10% difference in substrate.
Figure 1

dCyd

T-dCyd

araC

T-araC
Figure 2

(A) 0.1 μM

(B) 100 μM

Triphosphate Concentrations (pmol/10^6 cells)

Incubation time (Minutes)
Figure 3

(A) T-araC (500 µM)

(B) F-T-araC (500 µM)

(C) No drug
Figure 4

(A) T-araC

(B) F-T-araC