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STRUCTURE–ACTIVITY ANALYSIS OF ANTAGONISM OF THE FEEDBACK INHIBITION OF THYMIDINE KINASE*

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Abstract—The effects of a variety of 5-, 5'-, and 3'-substituted deoxyuridine derivatives on the cytoplasmic thymidine kinase (EC 2.7.1.21) purified from a human colon carcinoma cell line, HCT 116, were determined. Of particular interest was elucidation of the structural features important for antagonism of the feedback inhibition of thymidine kinase exerted by thymidine triphosphate. Substitutions at the 5-position altered the potency of the 5'-modified compounds. The replacement of the 5-hydrogen with a methyl group or an iodine greatly increased the affinity of compounds for the thymidine kinase. This was evident for enzyme substrates with 5'-hydroxyl groups [2'-deoxyuridine (dUrd), 2'-deoxythymidine (dThd) and 5-iodo-2'-deoxyuridine (IdUrd)], feedback inhibitors with 5'-triphosphate substitutions (dUTP, dTTP and IdUTP), and for 5'-amino derivatives [5'-amino-2',5'-dideoxyuridine (5'-AdUrd), 5'-amino-2'-5'-dideoxythymidine (5'-AdThd) and 5-iodo-5'-amino-2',5'-dideoxyuridine (5'-AIdUrd)]. Qualitatively, however, the 5-substitutions did not affect the nature of the interactions with dThd kinase. For example, in the presence of dTTP, 5'-AdUrd stimulated dThd kinase activity as much as 5'-AdThd, but approximately a 100-fold greater concentration of 5'-AdUrd was required. Similar results were obtained using intact cells in which substitutions at the 5-position affected the potency, but not the efficacy, of the 5'-amino derivatives to stimulate dThd phosphorylation. In contrast, substitutions at the 5'-position did alter the nature of the interaction with dThd kinase. Thus, the 5'-hydroxyl compounds, dUrd, dThd and IdUrd, did not reverse the enzyme inhibition produced by dTTP nor did they stimulate dThd uptake in intact cells. 5'-Deoxy-5'-(ethylthio)thymidine, 5'-deoxy-5'-[(2-hydroxyethyl)thio]thymidine, and dTMP, but not dTDP, also antagonized the inhibition of dThd kinase produced by dTTP. In comparison to 5'-AdThd, the 3'-amino derivatives, 3'-AdThd and 3'-5'-diAdThd, were much less potent, but still efficacious, antagonists of feedback inhibition. These results indicate that a wide range of dUrd derivatives can disrupt the regulation of dThd kinase and provide leads for the development of new nucleotide analogues.

The rate-limiting step in the dThd¶ salvage pathway, the conversion of dThd to dTMP, is catalyzed by dThd kinase. This enzyme is also required for the activation of several antiviral and anticancer nucleosides [1–3]. Feedback inhibition, exerted by the end product thymidine triphosphate [4–6], can limit the rate at which chemotherapeutic nucleosides are activated. The 5'-amino derivatives of dThd and IdUrd can reduce the feedback inhibition produced by dTTP and thereby increase the phosphorylation and toxicity of dThd and IdUrd [7–9]. The effects of 5'-AdThd on dThd kinase are critically dependent on dTTP. In the absence of feedback inhibition, 5'-AdThd competitively inhibits enzyme activity by binding to the active site. In the presence of dTTP, however, 5'-AdThd can stimulate dThd kinase activity by reducing the degree of feedback inhibition. Furthermore, 5'-AdThd can preferentially stimulate the phosphorylation of IdUrd in human bladder cancer cells as compared to normal human urothelial cells *in vitro* [10, 11]. Thus, compounds which can disrupt enzyme regulation appear to be an interesting new class of agents.

The present study was undertaken to elucidate some of the key structural features necessary for the antagonism of the feedback inhibition of cytosolic dThd kinase. A variety of compounds substituted at the 5-, 5'- and 3'-positions of dUrd were examined. Our results indicate that compounds modified at the 5-position primarily affect the potency, but not the type, of effect produced. Several analogs, in addition

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¶ Abbreviations: dThd, 2'-deoxythymidine; dUrd, 2'-deoxyuridine; IdUrd, 5-iodo-2'-deoxyuridine; dTMP, dTDP, and dTTP, the 5'-mono-,di- and triphosphates of dThd; dUTP, the 5'-triphosphate of dUrd; IdUTP, the 5'-triphosphate of IdUrd; 5'-AdUrd, 5'-amino-2',5'-dideoxyuridine; 5'-AdThd, 5'-amino-2',5'-dideoxythymidine; 5'-AIdUrd, 5-iodo-5'-amino-2',5'-dideoxyuridine; 3'-AdThd, 3'-amino-2',3'-dideoxythymidine; 3',5'-diAdThd 3,5'-diamino-2',3',5'-trideoxythymidine; 3'-dTMP, the 3'-monophosphate of dThd; 5'-NPE-dTMP, the *p*-nitrophenyl ester of dTMP; 5'-Morph-dTMP, the morpholidate of dTMP; 5'-SCH_2CH_3-dThd, 5'-(ethylthio)-2',5'-dideoxythymidine; 5'-$S(CH_2)_2OH$-dThd, 5'-[(2-hydroxyethyl)thio]-2',5'-dideoxythymidine; and 3'-OCH_2CH_3-dThd, 3'-*O*-ethyl-2'-deoxythymidine.

to the 5'-amino derivatives, were able to disrupt the regulation of dThd kinase produced by dTTP.

MATERIALS AND METHODS

Materials. 5'-AdUrd, 3'-AdUrd and 3'-,5'-diAdThd were synthesized as previously described [12–14]. 5'-AdThd and 5'-AIdUrd were obtained from the Sigma Chemical Co. (St. Louis, MO) and CalBiochem (San Diego, CA) respectively. The syntheses of 5'-deoxy-5'-(ethylthio)thymidine [15], 5'-deoxy-5'-[(2-hydroxyethyl)thio]thymidine [15] and 3'-ethoxythymidine [16] have been described. [*Methyl*-^{3}H]Thymidine (25 Ci/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA).

Cells. HCT 116 cells, a human colorectal carcinoma cell line, were obtained from Dr. M. Brattain at the Bristol-Baylor Laboratory (Houston, TX) and have been characterized previously [17]. The cells were grown in Eagle's Minimal Essential Medium (K. C. Biologicals, Lenexa, KS) supplemented with 8% fetal bovine serum, 0.1 mM nonessential amino acids, and 2 mM 1-glutamine. The cells were subcultured weekly and tested negatively for mycoplasma [18].

Thymidine uptake. Approximately 2×10^4 cells were plated into 26 mm multiwell culture dishes and maintained at 37° in a humidified 5% CO_2 atmosphere. [^{3}H]Thymidine (3 μM; 5 μCi/ml) and the test compounds were added to the cells 24 hr later and incubated for an additional 60 min. The medium was then aspirated, and the cells were washed three times with ice-cold phosphate-buffered saline. The acid-soluble nucleotides were extracted with cold 0.5 N $HClO_4$ for 15 min. After removing the cells from the dish with a rubber policeman, the precipitate was collected by centrifugation and a portion of the supernatant fraction was used for liquid scintillation counting.

Thymidine kinase. Procedures for the purification of dThd kinase from HCT 116 cells by affinity column chromatography using the method of Lee and Cheng [19] have been described [7–9]. The enzyme was separated from the dThd used for elution of the enzyme from the affinity gel with a G-50 column equilibrated with 5 mM Tris (pH 7.5), 3 mM β-mercaptoethanol, 2 mM ATP, 2 mM $MgCl_2$, and 30% glycerol. The enzyme reaction mixture contained 50 mM Tris, pH 7.8, 2.5 mM ATP, 2.5 mM $MgCl_2$, 2.5 mM dithiothreitol, 1% bovine serum albumin, 20 mM NaF, and 3 μM [^{3}H]dThd (6.7 μCi/nmol) in a final volume of 80 μl. The reaction was carried out at 37° and was linear for at least 60 min. Portions (20 μl) were spotted on Whatman DE 81 paper strips that were washed once in 95% ethanol, once in 1 mM ammonium formate, and three more times in 95% ethanol. The filters were dried and counted using liquid scintillation spectrometry.

Chromatography. To verify that the effects of 3'-AdThd and 3',5'-diAdThd were not produced by small contaminating quantities of 5'-AdThd, an HPLC procedure for separating these derivatives was developed. An Altex Ultrasil-ODS 250 × 4.6 mm column was used with a Spectraphysics 8700 solvent delivery system, a Kratos Spectroflow 773 UV detector and an LDC/Milton Roy CI-10 integrator. The mobile phase, run at 2 ml/min, consisted of 90% filtered, double-distilled water, 10% methanol, 0.1% acetic acid, and 5 mM 1-heptanesulfonic acid (Sigma Chemical Co.). Under these conditions, the retention time was 20 min for 5'-AdThd and 25 min for 3'-AdThd. No 5'-AdThd could be detected in the 3'-AdThd sample under conditions in which a 0.05% contaminant would have been picked up. The 3',5'-dAdThd was eluted from the column at 37.5 min if the concentration of methanol was increased to 50% between 30 and 35 min. 5'-AdThd was not detectable in the 3',5'-diAdThd sample. Thus, the effects of the 3'-AdThd and 3',5'-diAdThd on dThd kinase activity and on dThd uptake cannot be ascribed to the presence of small amounts of 5'-AdThd.

RESULTS

The effects of 5'-AdUrd, 5'-AIdUrd, and 5'-AdThd on dThd kinase purified from HCT 116 cells are shown in Fig. 1. The critical effect of dTTP on the nature of the interaction between these analogs and dThd kinase is demonstrated. In the absence of dTTP, the 5'-amino derivatives inhibited enzyme activity in a dose-dependent fashion. Previously this interaction was shown to be competitive in nature [20, 21]. In the presence of dTTP, however, a biphasic dose–response curve was generated. At lower concentrations, the amino compounds antagonized the dTTP inhibition and stimulated enzyme activity. At higher concentrations inhibition became

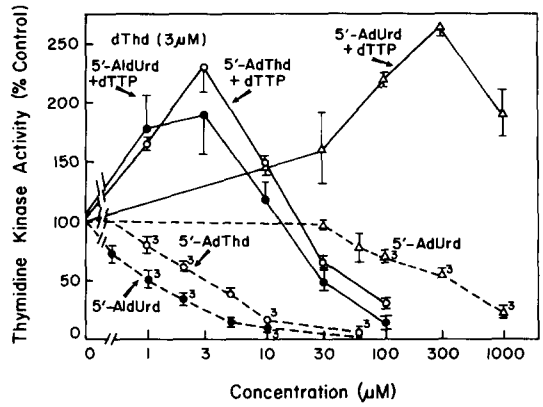

Fig. 1. Modulation of thymidine kinase activity by derivatives of 5'-aminodeoxyuridine. The effects of 5'-AIdUrd (●), 5'-AdThd (○) and 5'-AdUrd (△) on thymidine kinase purified from HCT 116 cells were determined in the presence (solid lines) or absence (dashed lines) of 4 μM dTTP. The concentration of dThd (labeled with [*methyl*-^{3}H]thymidine) was 3 μM. Under these conditions, 4 μM dTTP inhibited enzyme activity by an average of 82%. In a typical experiment, the activity of the control averaged 9660 ± 187 dpm/15 min and that of the reaction inhibited by 4 μM dTTP 1870 ± 244 dpm/15 min (mean ± SE; N = 3). The data are expressed as the percent of thymidine kinase activity seen in the absence of the indicated amino nucleoside. At least two separate experiments, each done in duplicate or triplicate, were done for each point. The data are presented as the mean ± SE (N = 3) for those points from three separate experiments or as the mean and range for the data from two separate experiments.

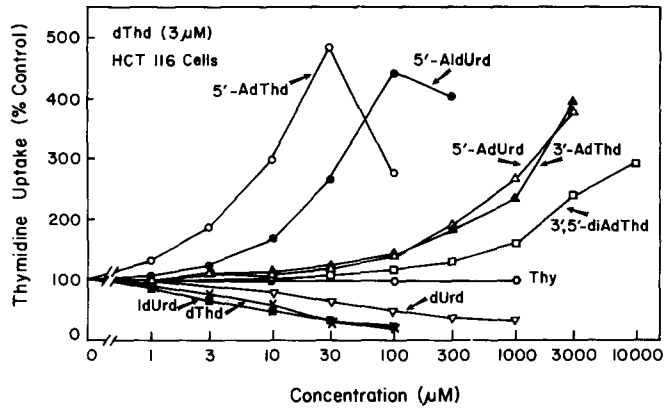

Fig. 2. Modulation of thymidine uptake in HCT 116 cells. The effects of 5'-AdThd (○), 5'-AIdUrd (●), 5'-AdUrd (△), 3'-AdThd (▲), 3',5'-diAdThd (□), thymine (○), IdUrd (■), dThd (×), and dUrd (▽) on the uptake of 3 μM dThd (labeled with [*methyl*-^{3}H]thymidine) in HCT 116 cells were determined. The data are expressed as the percent of dThd uptake seen in the absence of the added test compound. The uptake experiments were performed in triplicate and done at least twice.

evident and eventually predominated. These modifications at the 5-position altered the potency, but not the intrinsic activity, of these compounds either as inhibitors of dThd kinase or as antagonists of the dTTP inhibition.

Similarly, in experiments using intact HCT 116 cells, modification of the 5'-amino derivatives at the 5-position changed the potency, but not the ability of these compounds to stimulate the uptake of thymidine into the acid soluble fraction. As shown in Fig. 2, the uptake of dThd was increased by concentrations of 5'-AdUrd as high as 3 mM. Limited availability of compound precluded testing at higher concentrations to determine the amount of 5'-AdUrd needed for optimal stimulation of dThd uptake. 5'-AdThd was much more potent than 5'-AdUrd and produced a similar effect at 30 μM. It was also found that dUrd was much less potent than IdUrd as an inhibitor of dThd kinase (Table 1) and, in contrast to the findings with the 5'-amino derivatives, dUrd, dThd and IdUrd did not stimulate the uptake of dThd in intact cells (Fig. 2). It should be noted that dUrd and IdUrd decreased the phosphorylation of dThd by dThd kinase by acting as alternate substrates. The percent inhibition of dThd kinase activity produced by these 5'-hydroxyl nucleosides was somewhat reduced in the presence of dTTP, but no stimulation of enzyme activity was produced (Table 1).

These results show that changes at the 5-position

Table 1. Modulation of thymidine kinase activity

Compound	Concn (μM)	Thymidine kinase activity (% control)	
		No dTTP	+dTTP
Thymine	1000	98	109
Thymidine	30	19	45
Iododeoxyuridine	30	13	60
Deoxyuridine	300	26	60
3'-dTMP	2500	58	140
5'-NPE-dTMP	1000	90	142
5'-Morph-dTMP	2500	49	55
5'-SCH_2CH_3-dThd	3000	81	172
5'-$S(CH_2)_2OH$-dThd	3000	96	150
3'-OCH_2CH_3-dThd	100	73	173

The effects of a variety of compounds on thymidine kinase purified from HCT 116 cells were determined. The assays were run in the presence or absence of dTTP (4–5 μM) using 3 μM dThd (labeled with [*methyl*-^{3}]thymidine) as the substrate. These data are selected from dose–response curves generated for each of the derivatives. In a typical experiment, the control reaction averaged 3460 ± 205 dpm/15 min and that of the reaction inhibited by 5 μM dTTP 339 ± 20 dpm/15 min (mean ± SE; N = 4). The compounds were generally tested over a 300-fold concentration range. The data are expressed as the percent of enzyme activity obtained in the absence of the test compound.

have similar effects on substrates, such as dThd and IdUrd, and on the antagonists of feedback inhibition, such as 5′-AdThd and 5′-AIdUrd. The 5-iodo compounds were the most potent, whereas the 5-H derivatives were the least potent. Enzyme inhibition studies were done with the 5-triphosphates to further examine this structure–activity relationship. As shown in Fig. 3, approximately 100-fold less IdUTP than dUTP was needed to inhibit dThd kinase activity by 50%. dTTP was about three times less potent than IdUTP as an inhibitor of dThd kinase. The dose–response curves produced by the three triphosphates were essentially parallel, and complete inhibition of enzyme activity could be obtained.

The results obtained with the 5′-amino derivatives are consistent with the idea that certain nucleoside analogs can bind to the dTTP site of dThd kinase and reduce the effects of feedback inhibitors. Thus, it seemed possible that dTMP might, in an analogous fashion, be able to antagonize the inhibition produced by dTTP. The results shown in Fig. 4 indicate that dTMP, but not dTDP, can significantly reduce dTTP inhibition. Thus, the beta phosphate conferred a major increase in the potency of dTDP as an inhibitor of dThd kinase.

The influence of the position of the amino group on interactions with dThd kinase was assessed by comparing 5′-AdThd with 3′-AdThd and 3′,5′-diAdThd. The 3′-amino derivatives produced the same qualitative results as 5′-AdThd, but they were much less potent. Their effects on dThd kinase were critically dependent on dTTP (Fig. 5). In the absence of dTTP, millimolar concentrations of 3′-AdThd and 3′,5′-diAdThd were necessary to inhibit enzyme activity. In the presence of dTTP, their ability to stimulate dThd kinase activity was considerable. The effects seen with the purified enzyme were also evident in intact HCT 116 cells. High concentrations of the 3′-amino derivatives produced significant stimu-

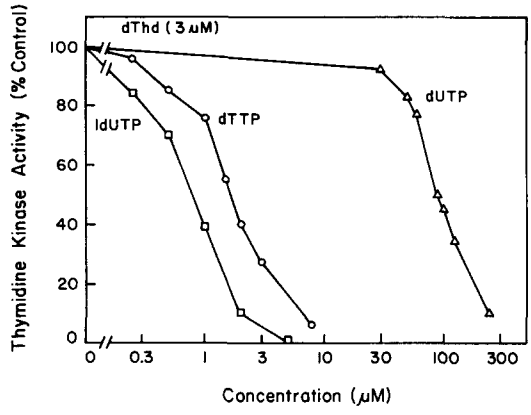

Fig. 3. Inhibition of thymidine kinase by nucleoside triphosphates. The effects of IdUTP (□), dTTP (○), and dUTP (△) on the activity of thymidine kinase purified from HCT 116 cells were determined. The concentration of dThd (labeled with [*methyl*-^{3}H]thymidine) was 3 μM, and the data are expressed as the percent of enzyme activity present in the absence of the added test compound. These dose–response curves are compiled from three separate experiments done in duplicate. The control reactions averaged 13,300 ± 716 dpm/15 min (mean ± SE; N = 4).

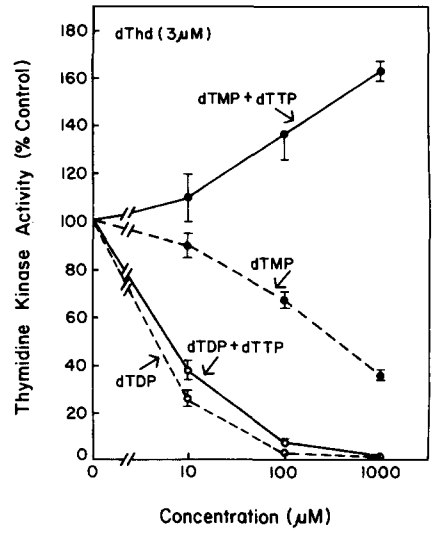

Fig. 4. Perturbation of thymidine kinase activity by dTMP and dTDP. The effects of dTMP (●) and dTDP (○) on thymidine kinase activity were determined in the presence or absence of 5 μM dTTP using 3 μM dThd (labeled with [*methyl*-^{3}H]thymidine) as the substrate. These experiments were done three times in triplicate. The control reaction averaged 5200 ± 74 dpm/15 min and that of the reaction inhibited by 5 μM dTTP 850 ± 38 dpm/15 min (mean ± SE; N = 4) in a typical experiment. The data are expressed as the percent of enzyme activity present in the absence of added dTMP or dTDP and are presented as the mean ± SE (N = 3).

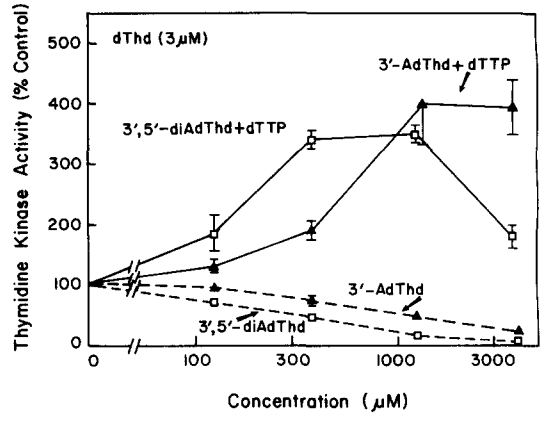

Fig. 5. Modulation of thymidine kinase activity by 3′-AdThd and 3′,5′-diAdThd. The effects of 3′-AdThd (▲) and 3′,5′-diAdThd (□) on the activity of thymidine kinase purified from HCT 116 cells were determined in the presence (solid lines) or absence (dashed lines) of 10 μM dTTP using 3 μM dThd (labeled with [*methyl*-^{3}H]thymidine) as the substrate. These experiments were done twice in triplicate. The control reactions averaged 3970 ± 271 dpm/15 min and that of the reaction inhibited by 5 μM dTTP 150 ± 21 dpm/15 min (mean ± SE; N = 4) in these experiments. The results are expressed as the percent of thymidine kinase activity obtained in the absence of the amino derivatives and are presented as the mean and range of the two experiments.

lation of dThd uptake in these cells (Fig. 2). As was seen with 5′-AdThd, these compounds modulated dThd uptake in a biphasic manner and at very high concentrations there was a decrease in dThd uptake.

Several other compounds were examined for their ability to antagonize the feedback inhibition of dThd kinase produced by dTTP (Table 1). The findings with dTMP prompted us to test the 5′-*p*-nitrophenyl ester and morpholidate of dTMP. The phenyl ester modestly antagonized dTTP inhibition of dThd kinase but concentrations of 1 mM were required. The morpholidate was a weak inhibitor of the enzyme and did not reverse the effects of dTTP. Since the 3′-amino derivative of dThd could antagonize feedback inhibition, we also examined the 3′-monophosphate of dThd. It was similar to dTMP, but less potent, and concentrations of 2.5 mM were required. The 3′-OCH_2CH_3 derivative of dThd was much more potent, producing significant antagonism of dTTP inhibition at 100 μM. Finally, two 5′-thio derivatives of dThd were compared. Both the 5′-SCH_2CH_3 and the 5′-$S(CH_2)_2OH$ compounds could antagonize feedback inhibition, but concentrations of 3 mM were necessary.

DISCUSSION

The ability of a variety of nucleoside analogs to antagonize the inhibition of dThd kinase exerted by dTTP is the key finding reported in this study. Thus, the interaction between the 5′-amino group of 5′-AdThd and dThd kinase was not uniquely required to antagonize the effects of dTTP. Although 3′-AdThd was much less potent than 5′-AdThd, it was still quite efficacious in reducing feedback inhibition of dThd kinase and in stimulating the uptake of dThd in intact cells. Similarly, the ability of dTMP, its *p*-nitrophenyl ester, and the 5′-thio derivatives of dThd to stimulate dThd kinase activity in the presence of dTTP suggests considerable structural tolerance at the 5′-position. In addition to 3′-AdThd, the 3′-monophosphate and 3′-ethoxy derivatives of dThd could antagonize the effects of dTTP. These findings have importance in regard to the design of new antagonists of feedback inhibition, in particular, and of nucleotide analogs, in general. Thus, in certain cases nucleosides may be effective nucleotide analogs. Since nucleotides are poorly transported into cells, this could be a major advantage in drug penetration and delivery. The recently reported 5′-haloacetamido derivatives of dThd, which are effective thymidylate synthetase inhibitors, provide another example of nucleosides which can function as nucleotide analogs [22].

Modifications at the 5-position primarily altered the potency of the compounds evaluated. The relative potencies of 5′-AdUrd, 5′-AdThd and 5′-AIdUrd, both as inhibitors of dThd kinase and as antagonists of dTTP inhibition, were similar to those seen for the 5′-hydroxyl (dUrd, dThd and IdUrd) and 5′-triphosphate derivatives (dUTP, dTTP and IdUTP). The relatively poor affinity of dUrd for dThd kinase was demonstrated previously in studies using dThd kinase obtained from other cell types [2, 23]. dUTP inhibits dThd kinase from acute myelocytic leukemia cells less well than dTTP [23]. In contrast, experiments with other derivatives have shown that 5-ethylaminothymidine has virtually no binding to the active site, whereas its 5′-triphosphate does retain inhibitory activity [15]. Taken together these findings suggest that there is considerable similarity, but not identity between the active and the regulatory sites of dThd kinase with regard to binding of the pyrimidine ring. Substitutions on the deoxyribose were necessary to produce enzyme stimulation. This was evidenced by the inability of dThd, IdUrd or dUrd to antagonize dTTP inhibition. The nucleobase, thymine, neither inhibited enzyme activity nor reversed the effects of dTTP.

The ability of the various amino derivatives to antagonize the feedback inhibition produced by dTTP was highly correlated with their stimulation of dThd uptake in HCT 116 cells. This relationship was evident for all of these compounds even though their potencies varied by a factor of 1000. In addition, dUrd, IdUrd and dThd, which inhibited dThd kinase activity in the presence of dTTP, also inhibited dThd uptake in the intact cells. Thymine, which did not affect dThd kinase activity, also did not perturb dThd uptake. These findings provide further support for disruption of feedback inhibition as the mechanism by which 5′-AdThd stimulates the uptake of dThd and IdUrd in a variety of cell types [7–10].

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