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Enzyme Regulatory Site-Directed Drugs: Study of the Interactions of 5′-Amino-2′,5′-dideoxythymidine (5′-AdThd) and Thymidine Triphosphate with Thymidine Kinase and the Relationship to the Stimulation of Thymidine Uptake by 5′-AdThd in 647V Cells

MIGUEL A. VAZQUEZ-PADUA,[1] KEITH KUNUGI, and PAUL H. FISCHER

Department of Human Oncology, University of Wisconsin Clinical Cancer Center, University of Wisconsin School of Medicine, Madison, Wisconsin 53792

Received June 27, 1988; Accepted October 21, 1988

SUMMARY

5′-Amino-2′,5′-dideoxythymidine (5′-AdThd) is a nontoxic thymidine (dThd) analogue capable of antagonizing the feedback inhibition exerted by thymidine triphosphate (dTTP) on thymidine kinase (EC 2.7.1.21). In intact cells, this results in stimulation of thymidine uptake by 5′-AdThd. We have studied the interaction between 5′-AdThd and thymidine kinase purified from 647V cells. We found that 5′-AdThd inhibited competitively thymidine kinase activity (K_i of 0.5 μM) in the absence of dTTP whereas dTTP inhibited thymidine kinase activity in a noncompetitive manner. However, in the presence of dTTP, 5′-AdThd was able to stimulate enzyme activity in a mode that suggests competition with dTTP for the regulatory site. Altered interactions were observed at high substrate (dThd) concentrations, with dThd showing competitive kinetics with dTTP. In intact cells, we evaluated the hypothesis that antagonism of feedback inhibition could account for stimulation of dThd uptake by 5′-AdThd. If inhibition of thymidine kinase activity by dTTP is critical, then depletion of cellular dTTP by methotrexate should reduce the ability of 5′-AdThd to stimulate dThd uptake. Indeed, this was the case. If the dTTP pools were repleted by the addition of higher concentrations of dThd, the ability of 5′-AdThd to stimulate dThd uptake was restored. Furthermore, effects of 5′-AdThd on nucleoside phosphorylase or cytoplasmic 5′-nucleotidase activity (dTMP breakdown) could not account for the stimulation of dThd uptake in 647V cells. In summary, our results indicate that 5′-AdThd interacts with thymidine kinase at the dTTP-binding site, resulting in stimulation of enzyme activity and stimulation of dThd uptake in intact cells.

Thymidine kinase catalyzes the phosphorylation of dThd, the rate-limiting step in the salvage pathway that results in the formation of dTTP. This enzyme is responsible also for the activation of various anticancer and antiviral drugs such as IdUrd, 5-fluorodeoxyuridine, 5-trifluorothymidine, and azidothymidine (1–4). Similar to other rate-limiting enzymes, thymidine kinase is regulated by end-product (i.e., dTTP or IdUTP) feedback inhibition (5–7). This could limit the uptake and activation of cytotoxic nucleoside analogues.[2] We have approached this problem by a direct intervention at the enzyme level, antagonizing the inhibition of the enzyme by using nucleoside analogues.

The 5′-amino derivatives of dThd, 5′-AdThd, and of IdUrd, 5-iodo-5′-amino-2′,5′-dideoxyuridine, are capable of stimulating thymidine kinase activity (8–10). The effects of 5′-AdThd on enzyme activity are dependent on the feedback inhibitor dTTP. In the absence of dTTP, 5′-AdThd inhibits thymidine kinase activity. However, in the presence of dTTP, 5′-AdThd partially antagonizes the dTTP-induced inhibition of thymidine kinase activity, thus resulting in a net increase in enzyme activity. In intact cells, 5′-AdThd enhances the cellular uptake and the cytotoxicity of dThd and IdUrd (8–9). This effect occurs preferentially in human bladder cancer cell lines (e.g., 647V) as compared with normal human urothelium propagated *in vitro* (11, 12). 5′-AdThd is not a substrate of the mammalian thymidine kinase and is a very nontoxic compound (13, 14). Thus, this and other analogues (15) provide a potentially useful new class of modulators of drug metabolism and action.

In this study we have addressed two questions. 1) What are

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[1] Present address: Department of Pharmacology, CB 7365, 915 FLOB, University of North Carolina, Chapel Hill, NC 27514.

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ABBREVIATIONS: dThd, 2′-deoxythymidine; 5′-AdThd, 5′-amino-2′,5′-dideoxythymidine; dTMP, dTTP, the 5′-mono- and triphosphate of dThd, respectively; IdUrd, 5-iodo-2′-deoxyuridine; MTX, methotrexate; PBS, phosphate-buffered saline.

the molecular interactions between thymidine kinase and 5′-AdThd that result in opposing effects (i.e., inhibition versus stimulation) on enzyme activity? 2) What is the mechanism of action of 5′-AdThd at the cellular level? Although studies using purified preparations of thymidine kinase indicate antagonism of feedback inhibition as the mechanism of action of 5′-AdThd, in the intracellular environment interactions with other metabolic enzymes could contribute as well. For instance, inhibition of the catabolic enzymes nucleoside phosphorylase (16, 17) or cytoplasmic 5′-nucleotidase (18) could potentially result in an increased accumulation of dThd metabolites. This could occur by either preventing the breakdown of dThd to thymine, thus maintaining substrate levels for thymidine kinase, and/or by preventing the conversion of dTMP to dThd, which would result in the intracellular retention of dThd metabolites.

Our data indicate that 5′-AdThd interacts with thymidine kinase at both the active and the regulatory sites. Also, its effects in intact cells are consistent with antagonism of feedback inhibition of thymidine kinase *in situ*.

Experimental Procedures

Materials. 5′-AdThd, dThd, and MTX were purchased from Sigma Chemical Co. (St. Louis, MO). dTTP was obtained from P.L. Biochemicals (Milwaukee, WI). [*methyl*-^{3}H]-dThd (60 or 70 Ci/mmol), [8-^{3}H] dATP (20 Ci/mmol), and [*methyl*-^{14}C]dTMP (56 mCi/mmol) were purchased from Moravek Biochemicals (Brea, CA). Alamine (tri-*n*-octylamine) was obtained from Aldrich Chemical Co. (Milwaukee, WI) and Freon (1,1,2-trichloro-1,2,2-trifluoroethane) from J. T. Baker Chemical Co. (Phillipsburg, NJ). All other reagents were purchased from Sigma Chemical Co., unless otherwise specified.

Cell culture. 647V cells, a human bladder cancer cell line (19), were subcultured twice a week and maintained as previously described (11). These cells tested negative for mycoplasma by the DNA fluorochrome staining technique (20). A 0.1% trypsin solution was used to detach cells from culture dishes. Determination of cell number was done using a model ZBI Coulter counter (Coulter Electronics, Hialeah, FL). Cell volumes were calculated from cell sizing analysis with a Canberra Series 35 Multichannel Analyzer coupled to the Coulter counter.

Thymidine kinase purification and assay. As previously described (11), we purified thymidine kinase from 647V by affinity column chromatography using a slight modification of the method of Lee and Cheng (21). Before the experiments were performed, thymidine kinase was separated from dThd with a G-50 column equilibrated with a buffer containing 5 mM Tris (pH 7.5), 3 mM β-mercaptoethanol, 30% glycerol, 2 mM $MgCl_2$, and 2 mM ATP. The enzyme reaction mixture contained 2.5 mM ATP, 2.5 mM $MgCl_2$, 2.5 mM dithiothreitol, 50 mM Tris (pH 7.8), 1% bovine serum albumin, [*methyl*-^{3}H]dThd (15 μCi/ml), and the test compounds, in a final volume of 80 μl. At two time points (usually 30 and 60 min), 30 μl of the reaction mixture were spotted on Whatman DE 81 filter strips and dropped into 95% ethanol. The filters were washed once in 95% ethanol, once in 1 mM ammonium formate, and three times in 95% ethanol. After drying they were counted in HFP-20 liquid scintillation fluid (Research Products International, Mount Prospect, IL).

Thymidine uptake. Cells in the midlog phase of cell growth were used. The cells were exposed for 1 hr to Eagle's minimum essential medium supplemented with 2 mM glutamine (GIBCO, Grand Island, NY), nonessential amino acids (GIBCO), and 10% dialyzed fetal bovine serum (K.C. Biologicals, Lenexa, KS) with or without 1 μM methotrexate at 37°. After this time period, the medium was aspirated and washed once with PBS and fresh medium was added (supplemented with 10% dialyzed serum). Then [*methyl*-^{3}H]dThd and 5′-AdThd were added as indicated in the figure legends. At the appropriate time points, the medium was aspirated and the cells were washed three times with ice-cold PBS and extracted with 0.5 N perchloric acid for 30 min. The cells were scraped with a rubber policeman and centrifuged. A portion of the supernatant was counted in aqueous counting solution (Amersham, Arlington Heights, IL).

Nucleoside phosphorylase activity. The breakdown of dThd to thymine was examined under conditions similar to those for the nucleoside uptake experiments. After exposing the cells to [*methyl*-^{3}H] thymidine (3 μM, 1 μCi/ml), aliquots were taken from the extracellular medium at different time points, ultrafiltered using Centrifree devices (Amicon Division, W.R. Grace & Co., Danvers, MA) and immediately frozen at −70°. Analysis of the appearance of thymine was accomplished by thin layer chromatography of the samples on silica-coated plates (EM Science, Cherry Hill, NJ). These were developed using a 6:1 mixture of chloroform/isopropanol (Mallinckrodt, Paris KY). The R_f values obtained for dThd and thymine were 0.12 and 0.33, respectively; for IdUrd and IUra, 0.17 and 0.34, respectively. The amount of radioactivity that comigrated with the standards was counted in aqueous counting solution after elution with a solution of 50 mM Tris (pH 7.4), 0.7 M $MgCl_2$.

Determination of dTTP pools. The cells were treated similarly to those in the uptake experiments, except no labeled material was added. After the appropriate treatment, the cells were washed with PBS and extracted with 0.5 N perchloric acid as indicated above. However, after centrifugation, the supernatant fraction was neutralized with 2 volumes of Freon-alamine (3.4:1). The samples were lyophilized and then reconstituted with 100 μl of distilled water. The assay for dTTP is a modification of the enzymatic method of Solter and Handschumacher (22) as described by Hunting and Henderson (23). The procedure has been described previously (11).

Cytosolic 5′-nucleotidase activity. A crude preparation from 647V cells was obtained using a modification of the procedure described by Madrid-Marina and Fox (24). After homogenization in 10 mM imidazole, 0.25 M sucrose, 20 mM $MgCl_2$ (pH 7.4) with 30 strokes in a Dounce homogenizer, the homogenate was centrifuged at 100,000 × *g* for 1 hr and the supernatant was stored at −70°. The reaction mixture contained 62.5 mM imidazole·HCl (pH 7.5), 1% bovine serum albumin, 20 mM $MgCl_2$, 2 mM [*methyl*-^{14}C]dTMP (2 μCi/ml), and the test compound, in a final volume of 80 μl. The assay was followed for two time points (usually 30 and 60 min) at 37°, and the reaction was stopped by heating the test tube at 85° for 2 min. A portion of the reaction mixture was spotted onto cellulose-coated thin layer chromatography plates (Brinkman Instruments, Inc., Westbury, NY). The chromatogram was developed using water as the mobile phase (25). Distribution of radioactivity as dTMP or dThd was quantified by determining the amount of radioactivity that comigrated with standards. Elution and counting were done as described above for nucleoside phosphorylase assays.

Results

Purified thymidine kinase studies. We investigated the interaction of 5′-AdThd with purified preparations of thymidine kinase derived from 647V cells. A double-reciprocal plot of enzyme activity using dThd as the substrate (Fig. 1) shows that 5′-AdThd behaves as a competitive inhibitor. A replot of the slope versus concentration of 5′-AdThd extrapolates to a K_i of 0.5 μM (data not shown).

We found that dTTP inhibition of thymidine kinase activity was characteristic of a noncompetitive type, as seen from the double-reciprocal plot of enzyme activity (Fig. 2). Analysis of Dixon, slopes or intercepts replots as a function of dTTP concentration resulted in parabolic curves (data not shown). Thus, no appropriate estimate of K_i was possible. On the other hand, using 3 μM dThd as the substrate, dTTP showed an IC_{50} value of 1.8 μM.[2]

As we have shown before (8–10), in the presence of dTTP, 5′-AdThd stimulates thymidine kinase activity, resulting in

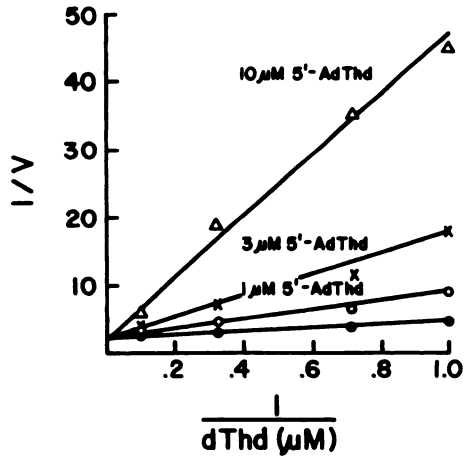

Fig. 1. Effect of 5′-AdThd on thymidine kinase activity. Enzyme activity using [^{3}H]dThd as substrate was followed in the presence of 0, 1, 3, and 10 μM 5′-AdThd as described in Experimental Procedures. The data are presented as the double-reciprocal plots of the calculated values (1/*v* given in min/pmol of dTMP formed). The data shown are the mean values from one experiment done in duplicates. Similar results were obtained in other experiments.

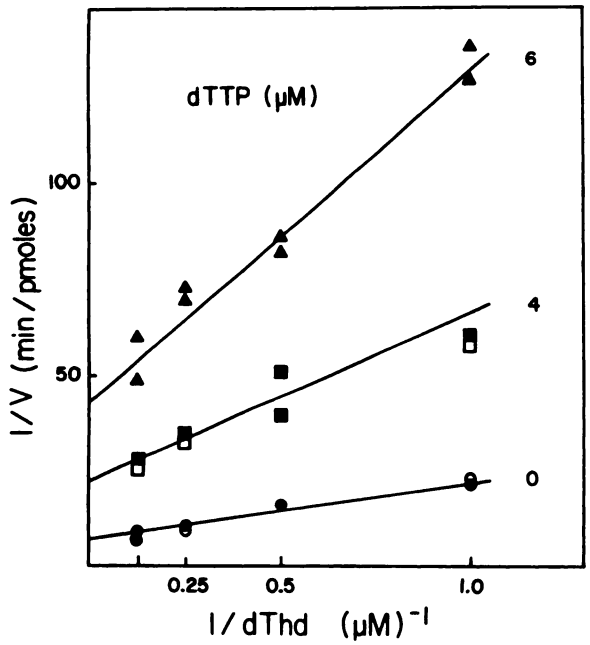

Fig. 2. Effect of dTTP on thymidine kinase activity. This was done as indicated in Fig. 1 but in the presence of 0, 4, and 6 μM dTTP. The data shown are from one experiment done in duplicates. Each data *point* has been plotted (the *open symbols* were used to distinguish data points that are too close). Similar results were obtained in other experiments.

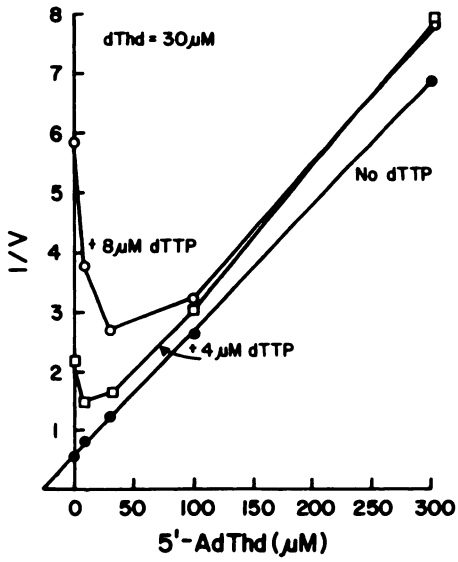

Fig. 3. Dixon plot of effect of 5′-AdThd on the inhibition of thymidine kinase activity by dTTP. Enzyme activity (1/*v*, min/pmol of dTMP formed) was determined at various concentrations of 5′-AdThd and 0, 4, or 8 μM dTTP using 30 μM dThd as the substrate. The procedure is described in Experimental Procedures. The data shown are the mean values from one experiment done in duplicates. Similar results were obtained in other experiments.

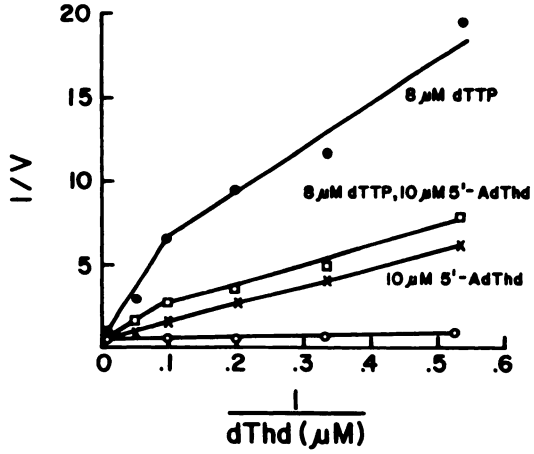

Fig. 4. Double-reciprocal plots of thymidine kinase activity in the presence of 8 μM dTTP and/or 10 μM 5′-AdThd using a wide range of concentrations of dThd. The procedure is described in Experimental Procedures. The data are plotted as 1/*v* (min/pmol of dTMP formed) and represent the mean values from one experiment done in duplicates. The results were reproduced in two other determinations.

enhanced phosphorylation of dThd (Fig. 3). Interestingly, the effect of 5′-AdThd was biphasic. In the presence of 4 or 8 μM dTTP, as the concentration of 5′-AdThd is increased, stimulation of enzyme activity augments until a maximum is achieved; however, as greater concentrations of 5′-AdThd are used, increased inhibition is observed (Fig. 3). Thus, antagonism of dTTP inhibition is obtained at lower concentrations of 5′-AdThd but the competitive nature of 5′-AdThd predominates at higher concentrations. We also found that the greater the inhibition of thymidine kinase by dTTP, the higher the concentration of 5′-AdThd required to produce maximal antagonism and the greater the degree of stimulation of enzyme activity. For example, 4 and 8 μM dTTP produced 75% and 88% of inhibition, respectively. Under these conditions, 3 and 10 μM 5′-AdThd were required to obtain maximal stimulation (175% and 325% of control, respectively).

In an attempt to further investigate the interactions of dThd, dTTP, and 5′-AdThd with thymidine kinase, we studied the enzyme activity over a wide range of substrate concentrations (2–100 μM). As expected, 5′-AdThd (10 μM) showed competitive type of inhibition over the range of substrate concentrations (K_m of dThd = 2 μM, data not shown) studied (Fig. 4). However, the dTTP-induced inhibition of enzyme activity showed two components, depending on the concentration of substrate used. At dThd concentrations below 10 μM, the dTTP inhibition curve extrapolates to a noncompetitive type of inhibition sim-

ilar to that shown in Fig. 2. At concentrations of dThd greater than 10 μM, the dTTP inhibition curve intercepts the control line at the vertical axis, showing competitive-type inhibition (Fig. 4). The presence of 5′-AdThd did not alter the two components of the dTTP inhibition curve but diminished the dTTP inhibitory properties. These results are consistent with an interaction of 5′-AdThd at the dTTP-binding site of the enzyme. Therefore, 5′-AdThd can interact at both the active and the regulatory sites of thymidine kinase.

Studies in intact cells. In intact 647V cells, 5′-AdThd significantly stimulates IdUrd and dThd uptake (11). Based on the enzyme studies, antagonism of the feedback inhibition produced by dTTP seems the most likely mechanism of action at the cellular level. However, inhibition of nucleoside phosphorylase or 5′-nucleotidase activities could also result in an enhanced retention or phosphorylation of dThd. Thus, we tested whether the stimulation of dThd uptake by 5′-AdThd is due to 1) antagonism of feedback inhibition of thymidine kinase, 2) inhibition of nucleoside phosphorylase activity, or 3) inhibition of cytoplasmic 5′-nucleotidase activity.

If 5′-AdThd works by antagonizing feedback inhibition of thymidine kinase, then perturbation of intracellular dTTP pool sizes (and thus the degree of inhibition of the enzyme *in situ*) should significantly affect the ability of 5′-AdThd to stimulate dThd uptake. We tested this hypothesis by taking advantage of the ability of MTX to inhibit dihydrofolate reductase and thus deplete intracellular dTTP pools (26–27). We would expect 5′-AdThd to inhibit or only slightly perturb dThd uptake in MTX-treated cells as compared with controls. In fact this was seen. Exposure of 647V cells to 1 μM methotrexate for 1 hr reduced intracellular dTTP pool sizes from 24 ± 1 μM to 12 ± 1 μM (mean ± standard error, seven determinations; data obtained from at least three experiments). In order to minimally perturb dTTP pools, we followed the uptake of a low concentration of dThd (0.3 μM) for relatively short time periods (2.5, 5, and 10 min). In cells not exposed to MTX, 5′-AdThd enhanced the uptake of dThd over the entire time period (Fig. 5A); however, 5′-AdThd became inhibitory or much less stimulatory in cells pretreated with MTX for 1 hr (Fig. 5B). These observations can be better appreciated in the replot of the data obtained at 10 min (see Fig. 7A). For example, by 10 min, 30 μM 5′-AdThd resulted in a stimulation of dThd uptake of 150% over control in nontreated cells, whereas it inhibited uptake to about 75% of control in MTX-pretreated cells.

To corroborate that the observations are due to the perturbation of dTTP pools by MTX and not to another effect as a result of exposure to MTX, we tested the effect of 5′-AdThd on the uptake of a higher concentration of dThd (3 μM) so that partial replenishment of dTTP pools was ensured. Under these conditions, the ability of 5′-AdThd to stimulate dThd uptake should be recovered in MTX-treated cells. The uptake of 3 μM dThd was enhanced by 5′-AdThd at 2.5, 5, and 10 min in the MTX-treated cells (Fig. 6). By 10 min, almost all concentrations of 5′-AdThd tested were stimulatory in both sets of cells (Fig. 7B). We found that, after 10 min, 3 μM dThd expanded dTTP pools to 38 ± 2 μM or 36 ± 1 μM (mean ± standard error, six determinations from three experiments) in controls or MTX-treated cells, respectively.

Nucleoside phosphorylase. We assayed for the presence of nucleoside phosphorylase activity in intact 647V cells under the same conditions under which we performed our dThd

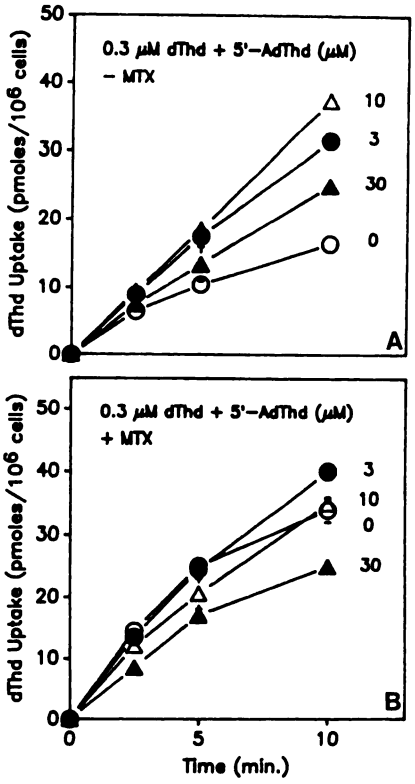

Fig. 5. Time course of the uptake of 0.3 μM [^{3}H]dThd in the presence of 5′-AdThd in 647V cells pretreated with or without 1 μM MTX for 1 hr. The procedure is detailed in Experimental Procedures. The data presented are the mean ± standard error (four determinations) for two experiments done in duplicates (in some cases, symbols are larger than error bars).

uptake experiments. After exposure of the cells to 3 μM [^{3}H] dThd (1 μCi/ml), we took aliquots from the extracellular media at various time points and then analyzed for the appearance of [^{3}H]thymine. We found no evidence of phosphorylase activity in mycoplasma-free cultures of 647V cells. No radioactivity corresponding to thymine appeared after up to 2 hr of incubation (Table 1).

Cytosolic 5′-nucleotidase. We studied the effects of 5′-AdThd on the dTMP phosphatase activity obtained from crude preparations from 647V cells. Due to the relatively high K_m values reported for this enzyme (18, 24, 28) we used 2 mM [^{14}C] dTMP as substrate in our experiments. The reaction, in the presence of 5′-AdThd, was followed for 20 and 40 min and the product, [^{14}C]dThd, was separated from the substrate by thin layer chromatography as detailed in Experimental Procedures. 5′-AdThd did not affect the breakdown of dTMP in three different crude preparations (Table 1). In other studies, 5′-AdThd also did not affect the breakdown of fluorodeoxyuridinemonophosphate to fluorodeoxyuridine by crude preparations of this cell line.[2] Thus, this catabolic step does not contribute to the intracellular mode of action of 5′-AdThd.

Discussion

The aims of this study were to investigate the molecular interactions between thymidine kinase and 5′-AdThd and to determine the mechanism of action of 5′-AdThd in intact 647V cells. The data from the purified enzyme studies strongly suggest that 5′-AdThd, which is not a substrate for the mammalian

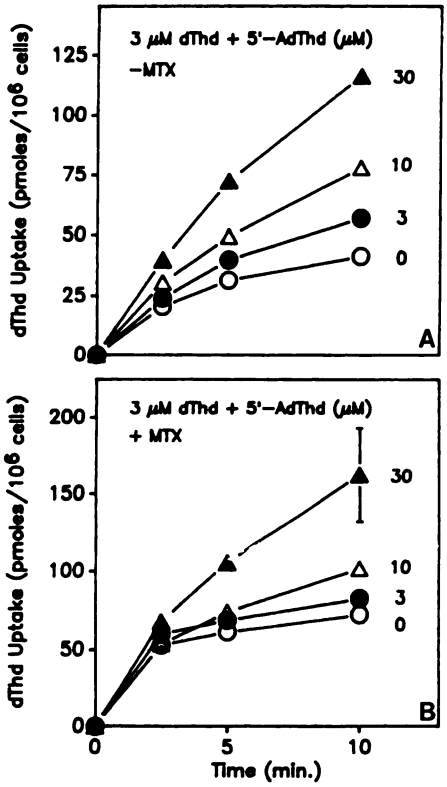

Fig. 6. Time course of the perturbation of the uptake of 3 μM [^{3}H]dThd by 5′-AdThd in 647V cells pretreated with or without 1 μM MTX for 1 hr. Conditions are similar to those indicated in Fig. 5.

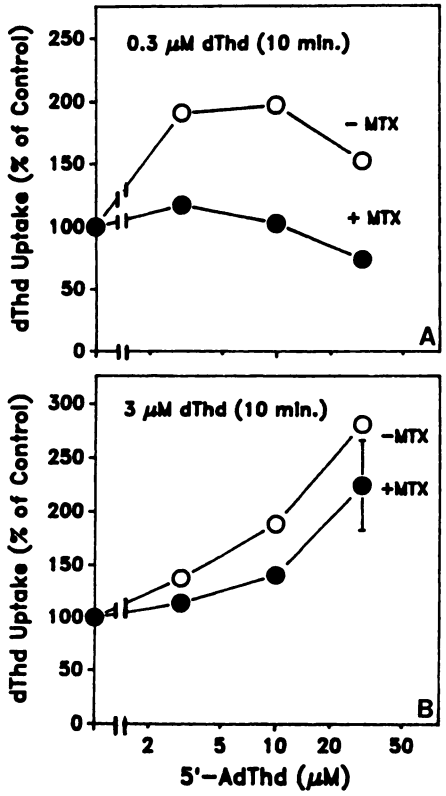

Fig. 7. Replot of the effect of 5′-AdThd on the uptake of 0.3 or 3 μM dThd at 10 min in 647V cells pretreated with or without 1 μM MTX. The data presented are derived from the values presented in Figs. 5 and 6. The data are presented as mean ± standard error for four determinations (in some cases, error bars are smaller than symbols).

thymidine kinase (13), interacts at both the active (Fig. 1) and the dTTP-binding sites (Figs. 3 and 4). Competitive inhibition of 5′-AdThd has been previously shown in thymidine kinase derived from mouse ascites sarcoma 180 (29). The stimulation of thymidine kinase activity by 5′-AdThd in the presence of dTTP seems to occur as a result of interactions of both compounds with the regulatory site. Binding to a third site, however, cannot be ruled out from our studies.

dTTP interacts with thymidine kinase in a noncompetitive manner (Fig. 2), similar to what others have previously observed (1, 29). However, the replots of the data for slopes or intercepts as a function of the concentration of dTTP were parabolic, indicating more complex interactions. Others have previously reported different types of behavior for the dTTP inhibition of thymidine kinase. For example, Prusoff and Chang (30) observed competitive kinetics whereas Munch-Petersen (31) observed parabolic curves in Lineweaver-Burk plots.

The interactions between 5′-AdThd and thymidine kinase are interesting because 1) it is not a substrate for the mammalian thymidine kinase (13), 2) at physiological pH it has the opposite charge as dTTP (e.g., p K_a of 5′-AdThd = 8.5, p K_a of dTTP = 7.5), and 3) it is an example of a nucleoside analogue affecting a nucleotide binding site. The third point is of particular interest because this approach could provide a new insight to studying other enzymes with nucleotide binding sites. Some of the possible candidates are deoxycytidine kinase, uridine-cytidine kinase, ribonucleotide reductase, GTP proteins, reverse transcriptases, and the polymerases. Appropriate structure-activity studies could help exploit this new approach to design new drugs with different mechanisms of action.

In this study we have shown that the mechanism of action of 5′-AdThd in intact cells is consistent with antagonism of the feedback inhibition that regulates thymidine kinase. This was evidenced by the sensitivity of the 5′-AdThd effects on dThd uptake to the perturbation of the intracellular dTTP pools by MTX (Figs. 5–7). Although inhibition of catabolism of dThd could result in increased uptake, these reactions were not involved (Table 1). Furthermore, it should be noted that the dose-response curve for the stimulation of dThd uptake by 5′-AdThd (Fig. 7) is biphasic. This parallels the results obtained in the experiments using purified enzyme; at higher concentrations of 5′-AdThd, interactions at the active site (competitive inhibition) predominate over those at the allosteric site. At the cellular level, interactions with the nucleoside transporter should not be of importance because of the high

TABLE 1

Catabolism in 647V cells

Phosphorylase activity was determined as indicated in Experimental Procedures. The data are presented as mean ± standard error (five determinations) from two experiments. Usually [^{3}H]thymine counts were less than 10% of total radioactivity (dThd plus thymine). dTMP phosphatase activity from 647V cells was assayed in the presence of various concentrations of 5′-AdThd as indicated in Experimental Procedures. The data presented are the mean ± standard error (three determinations) from one experiment done in triplicates.

Phosphorylase		Phosphatase	
Time	Thymine formed	5′-AdThd	dThd formed
min	*cpm*	*μM*	*nmol/min/mg of protein*
0	41 ± 4	0	10.86 ± 0.29
60	34 ± 2	3	11.02 ± 0.58
120	38 ± 1	30	10.41 ± 0.56
		300	11.40 ± 0.96

capacity of the transporter (32), the relatively low concentrations used compared with the reported K_m values (32), and the relatively long time course.

Antagonism of feedback inhibition is an innovative approach to anticancer and antiviral chemotherapy. We have previously shown that 5′-AdThd enhances the uptake and the cytotoxicity of IdUrd in HeLa and Vero cells (9). Interestingly, these effects occur preferentially in various human bladder cancer cell lines but not in the normal human urothelium propagated *in vitro* (11). An interesting application to antiviral chemotherapy is the combination of trifluorothymidine and 5′-AdThd. 5′-AdThd selectively reduces the phosphorylation and cytotoxicity of trifluorothymidine in uninfected versus *Herpes simplex*-infected cells (33). Furthermore, 5′-AdThd enhances the uptake and antiviral activity of trifluorothymidine in cytomegalovirus-infected cells.[3]

Feedback inhibition is a regulatory mechanism characteristic of several metabolic pathways. Other enzymes of nucleotide metabolism involved in the activation of anticancer drugs are also regulated by such a mechanism. For example, cytosine arabinoside, a potent antileukemic agent, is activated by deoxycytidine kinase (34), which is regulated by deoxycytidine triphosphate (35). UTP and CTP are feedback inhibitors of uridine-cytidine kinase (36), which mediates the activation of 5-fluorouridine and 5-azacytidine (37). One approach used by other groups to enhance drug activation by these enzymes has been to inhibit the synthesis of the feedback inhibitor of another drug. This has resulted in an enhanced activation of cytosine arabinoside (38) and 5-fluorouridine (39). In contrast, our approach deals with an interaction directly at the enzyme level.

In general, 5′-AdThd is an interesting compound with a unique property of interacting at the active and the regulatory sites of thymidine kinase. Its action as an antagonist of the feedback regulation that modulates the activity of the enzyme in intact cells provides a new approach to drug design (15). It opens the possibility of exploiting differences in the regulation of enzyme activity among different cells, and, it is hoped, between normal, neoplastic, and virally infected cells.

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References

1. Bresnick, E., and U. B. Thompson. Properties of deoxythymidine kinase partially purified from animal tumors. *J. Biol. Chem.* **240**:3967–3974 (1965).
2. Harbers, E., N. K. Chandhur, and C. Heidelberger. Studies on fluorinated pyrimidines. VIII. Further biochemical and metabolic investigations. *J. Biol. Chem.* **234**:1255–1262 (1959).
3. Bresnick, E., and S. S. Williams. Effects of 5-trifluoromethyldeoxythymidine upon deoxythymidine kinase. *Biochem. Pharmacol.* **16**:503–507 (1967).
4. Furman, P. A., J. A. Fyfe, M. H. St. Clair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. N. Lehrman, D. P. Bolognesi, S. Broder, H. Mitsuya, and D. W. Barry. Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **83**:8333–8337 (1986).
5. Maley, F., and G. F. Maley. On the nature of a sparing effect by thymidine on the utilization of deoxycytidine. *Biochemistry* **1**:847–851 (1962).
6. Breitman, T. R. The feedback inhibition of thymidine kinase. *Biochim. Biophys. Acta* **67**:153–158 (1963).
7. Ives, D. H., P. A. Morse, and V. R. Potter. Feedback inhibition of thymidine kinase by thymidine triphosphate. *J. Biol. Chem.* **238**:1467–1474 (1963).
8. Fischer, P. H., and D. Baxter. Enzyme regulatory site-directed drugs: modulation of thymidine triphosphate inhibition of thymidine kinase by 5′-amino-5′-deoxythymidine. *Mol. Pharmacol.* **22**:231–234 (1982).
9. Fischer, P. H., M. A. Weddle, and R. D. Mossie. Modulation of the metabolism and cytotoxicity of iododeoxyuridine by 5′-amino-5′-deoxythymidine. *Mol. Pharmacol.* **23**:709–716 (1983).
10. Fischer, P. H., and A. W. Phillips. Antagonism of feedback inhibition: stimulation of the phosphorylation of thymidine and 5-iodo-2′-deoxyuridine by 5-iodo-5′-amino-2′,5′-dideoxyuridine. *Mol. Pharmacol.* **25**:446–451 (1984).
11. Fischer, P. H., M. A. Vazquez-Padua, C. A. Reznikoff, and W. J. Ratschan. Preferential stimulation of iododeoxyuridine phosphorylation in human bladder cancer cells *in vitro*. *Cancer Res.* **46**:4522–4526 (1986).
12. Fischer, P. H., M. A. Vazquez-Padua, and C. A. Reznikoff. Perturbation of thymidine kinase regulation: a novel chemotherapeutic approach. *Adv. Enzyme Regul.* **25**:21–34 (1986).
13. Chen, M. S., G. T. Shiau, and W. H. Prusoff. 5′-Amino-5′-deoxythymidine: synthesis, specific phosphorylation by herpes virus thymidine kinase, and stability to pH of the enzymically formed diphosphate derivative. *Antimicrob. Agents Chemother.* **18**:433–436 (1980).
14. Pavan-Langston, D., N. H. Park, J. Lass, J. Papale, D. M. Albert, T. Lin, W. H. Prusoff, and D. H. Percy. 5′-Amino-5′-deoxythymidine: topical therapeutic efficacy in ocular herpes and systemic teratogenic and toxicity studies (41386). *Proc. Soc. Exp. Biol. Med.* **170**:1–7 (1987).
15. Fischer, P. H., T.-T. Fang, T.-S. Lin, A. Hampton, and J. Bruggink. Structure activity analysis of antagonism of the feedback inhibition of thymidine kinase. *Biochem. Pharmacol.* **37**:1293–1298 (1988).
16. Woodman, P. W., A. M. Sarrif, and C. Heidelberger. Specificity of pyrimidine nucleoside phosphorylases and the phosphorolysis of 5-fluoro-2′-deoxyuridine. *Cancer Res.* **40**:507–511 (1980).
17. Nakayama, C., Y. Wataya, R. B. Meyer, D. V. Santi, M. Saneyoshi, and T. Ueda. Thymidine phosphorylase: substrate specificity for 5-substituted 2′-deoxyuridines. *J. Med. Chem.* **23**:962–964 (1980).
18. Fritzon, P. Regulation of nucleotidase activities in animal tissues. *Adv. Enzyme Regul.* **16**:43–61 (1978).
19. Elliot, A. Y., D. L. Bronson, N. Stein, and E. E. Fraley. *In vitro* cultivation of epithelial cells derived from tumors of the human urinary tract. *Cancer Res.* **36**:365–369 (1976).
20. Chen, T. R. *In situ* detection of mycoplasma contamination in cell cultures by fluorescent Hoescht 33258 stain. *Exp. Cell Res.* **104**:255–262 (1977).
21. Lee, L. S., and Y.-C. Cheng. Human deoxythymidine kinase. I. Purification and general properties of the cytoplasmic and mitochondrial isozymes derived from blast cells of acute myelocytic leukemia. *J. Biol. Chem.* **251**:2600–2604 (1976).
22. Solter, A. W., and R. E. Handschumacher. A rapid quantitative determination of deoxyribonucleoside triphosphate based on the enzymatic synthesis of DNA. *Biochim. Biophys. Acta* **174**:585–590 (1969).
23. Hunting, D., and J. F. Henderson. Methods for the determination of deoxyribonucleotide triphosphate concentrations. *Methods Cancer Res.* **20**:245–283 (1982).
24. Madrid-Marina, V., and I. H. Fox. Human placental 5′-nucleotidase: kinetic properties and inhibition. *J. Biol. Chem.* **261**:444–452 (1986).
25. Reyes, P. PEI-Cellulose thin-layer chromatography: a highly versatile system for separating purine and pyrimidine nucleotides from nucleosides and free bases. *Anal. Biochem.* **50**:35–39 (1972).
26. Tattersall, M. H. N., and K. R. Harrap. Changes in the deoxyribonucleoside triphosphate pools of mouse 5178Y lymphoma cells following exposure to methotrexate or 5-fluorouracil. *Cancer Res.* **33**:3086–3090 (1973).
27. Kyburz, S., J.-C. Schaer, and R. Schindler. Effects of methotrexate on thymidine triphosphate levels in chinese hamster cell cultures. *Biochem. Pharmacol.* **28**:1885–1891 (1979).
28. Itoh, R. Purification and some properties of cytosol nucleotidase from rat liver. *Biochim. Biophys. Acta.* **657**:402–410 (1981).
29. Cheng, Y.-C., and W. H. Prusoff. Mouse ascites sarcoma 180 deoxythymidine kinase: general properties and inhibition studies. *Biochemistry* **13**:1179–1185 (1974).
30. Prusoff, W. H., and P. K. Chang. Regulation of thymidine kinase activity by 5-iodo-2′-deoxyuridine 5′-triphosphate and deoxythymidine 5′-triphosphate. *Chem.-Biol. Interact.* **1**:285–299 (1969/70).
31. Munch-Petersen, B. Differences in the kinetic properties of thymidine kinase isoenzymes in unstimulated and phytohemagglutinin-stimulated human lymphocytes. *Mol. Cell. Biochem.* **64**:173–185 (1984).
32. Plagemann, P. G., and R. M. Wohlhueter. Permeation of nucleoside, nucleic acids bases, and nucleotides in animal cells. *Curr. Top. Membr. Transp.* **14**:225–330 (1980).
33. Fischer, P. H., D.G. Murphy, and R. Kawahara. Preferential inhibition of 5-trifluoromethyl-2′-deoxyuridine phosphorylation by 5′-amino-5′-deoxythymidine in uninfected versus *Herpes simplex*-infected cells. *Mol. Pharmacol.* **24**:90–96 (1983).
34. Kessel, D. Properties of deoxycytidine kinase partially purified from L1210 cells. *J. Biol. Chem.* **243**:4739–4744 (1968).
35. Ives, D. H., and J. P. Durham. Deoxycytidine kinase. III. Kinetics and allosteric regulation of the calf thymus enzyme. *J. Biol. Chem.* **245**: 2285–2294 (1970).
36. Anderson, E. P., and R. W. Brockman. Feedback inhibition of uridine kinase by cytidine triphosphate and uridine triphosphate. *Biochim. Biophys. Acta.* **91**:380–386 (1964).

[3] P. H. Fischer and K. Fredericks, unpublished observations.

37. Lee, T., M. Karon, and R. L. Momparler. Kinetic studies on phosphorylation of 5-azacytidine with the purified uridine-cytidine kinase from calf thymus. *Cancer Res.* **34**:2482–2488 (1974).
38. Kinahan, J. J., E. J. Kowal, and G. B. Grindey. Biochemical and antitumor effects of the combination of thymidine and 1-β-D-arabinofuranosylcytosine against leukemia L1210. *Cancer Res.* **41**:445–451 (1981).
39. Holstege, S., B. Herrmann, and D. Keppler. Increased formation of nucleotide derivatives of 5-fluorouridine in hepatoma cells treated with inhibitors of pyrimidine synthesis and d-galactosamine. *FEBS Lett.* **95**:361–365 (1978).

Send reprint requests to: Paul Fischer, Pfizer Central Research, Eastern Point Road, Groton, CT 06340.