MOLECULAR PHARMACOLOGY, 22:231-234



SHORT COMMUNICATIONS

# Enzyme Regulatory Site-Directed Drugs

## Modulation of Thymidine Triphosphate Inhibition of Thymidine Kinase by 5′-Amino-5′-deoxythymidine

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Received March 22, 1982; Accepted May 18, 1982

SUMMARY

5′-Amino-5′-deoxythymidine (5′-AdThd) was found to antagonize the feedback inhibition exerted by dTTP on dThd kinase (EC 2.7.1.21). This effect was demonstrable in intact cells, cellular extracts, and purified enzyme preparations. Thus, 5′-AdThd markedly stimulated the uptake of dThd in Hela and Vero cells and reduced the inhibitory effects of dTTP on the dThd kinase activity measured in extracts from both cell types. dThd kinase was purified by affinity column chromatography from Hela and Vero cells, and 5′-AdThd was again shown to reduce the inhibition caused by dTTP. The ability of 5′-AdThd to disrupt the homeostatic mechanisms normally regulating the uptake of dThd was sufficient to convert a noncytotoxic concentration of dThd (30 μM) to one that inhibited Hela cell growth by 40%. Since the activity of regulatory enzymes critically influences the pharmacological response produced by many agents, we propose the design of compounds specifically targeted at enzyme regulatory sites as an approach to drug development.

The biochemical responses produced by many chemotherapeutic agents are frequently influenced by the activity of regulatory or allosteric enzymes. For example, the reactions catalyzed by these enzymes may be target sites (1–3) or can play important roles in drug activation (4–7). Since the patterns of sensitivity and resistance of cells to various agents can be markedly influenced by the activity of key regulatory enzymes (8–10), we propose the design of compounds specifically targeted at enzyme regulatory sites as an approach to drug development. The use of such agents, particularly in combination chemotherapy, could lead to enhanced therapeutic efficacy or selectivity for new and existing agents. Compounds that mimic normal regulatory metabolites would be regulatory agonists, whereas regulatory antagonists would prevent the normal biochemical response. In this report we describe a regulatory antagonist, 5′-AdThd.[3] This nucleoside, an analogue of dThd, reduces the feedback inhibition normally exerted by dTTP on dThd kinase. As a result, 5′-AdThd can cause marked increase in the intracellular accumulation of dThd nucleotides derived from exogenously added dThd and thereby enhance the cytotoxicity of dThd. Our data clearly indicate the feasibility of using regulatory site-directed agents to modulate the metabolism and action of nucleosides.

5′-AdThd, first synthesized by Horwitz *et al.* (11), was shown by Lin *et al.* (12) to be an effective inhibitor of herpes simplex virus replication without causing host cell toxicity. The fact that the virally encoded dThd kinase, but not the host cytosolic enzyme, can phosphorylate this aminonucleoside (13) appears to account for its high degree of selectivity. We have been investigating the effects of 5′-AdThd on nucleoside metabolism in an attempt to maximize the potential usefulness of 5′-AdThd and other nucleosides in combination chemotherapy regimens (14). We found that 5′-AdThd produced an unusual biphasic effect on the uptake of dThd in both Hela (Fig. 1A) and Vero (Fig. 1B) cells. As shown, low concentrations of 5′-AdThd (3 μM, 30 μM) stimulated [$^3$H]dThd uptake, whereas a high concentration of 5′-AdThd (300 μM) was inhibitory. In other experiments maximal stimulation of dThd (3 μM) uptake was also produced by 30 μM 5′-AdThd; both 10 μM and 100 μM 5′-AdThd were less effective. 5′-AdThd (3 μM, 100 μM) was also capable of

This work was supported by the Pharmaceutical Manufacturers Association Foundation Inc. and by National Institutes of Health Grant AI 19043-01.

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[3] The abbreviation used is: 5′-AdThd, 5′-amino-5′-deoxythymidine.

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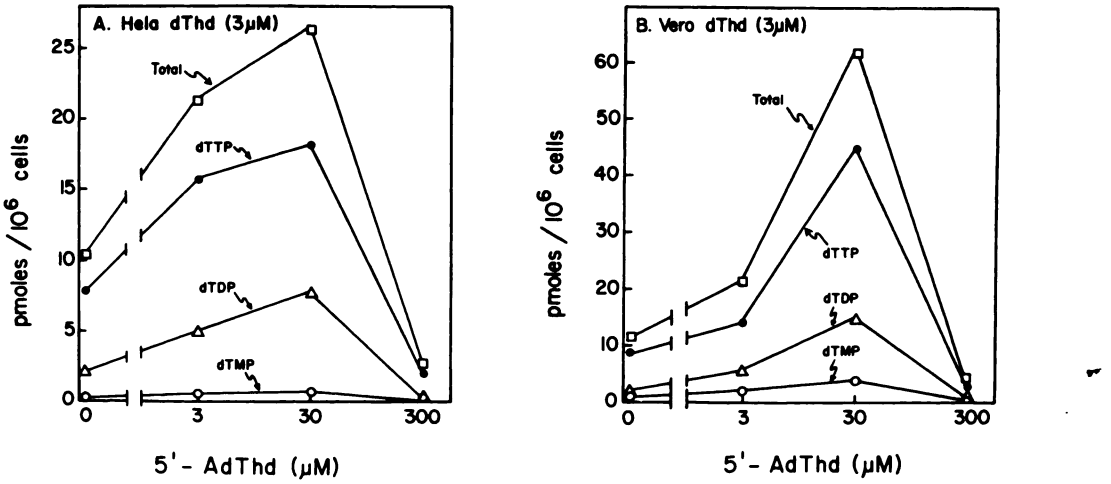


FIG. 1. *Effect of 5'-AdThd on the uptake and metabolism of dThd in HeLa and Vero (African green monkey kidney) cells*

The influence of 5'-AdThd (kindly provided by Dr. T.-S. Lin, Yale University) on the pattern of incorporation of tritiated dThd into dTMP, dTDP, dTTP, and all three nucleotides is shown for Hela cells (A) and Vero cells (B). These cells (obtained from Flow Laboratories, Rockville, Md.) were grown as monolayers in Dulbecco's modified essential medium supplemented with 10% newborn calf serum (K. C. Biologicals, Kansas City, Mo.) in a humidified 5% $CO_2$ atmosphere. Exponentially growing cells (~1 × $10^6$ cells/25 $cm^2$ dish) were exposed for 4 hr to [*methyl*-$^3$H] dThd (3 μM; 1 Ci/mmole; New England Nuclear Corporation, Boston, Mass.) in the absence or presence of 5'-AdThd at concentrations of 3 μM, 30 μM, or 300 μM. The cells were washed four times with ice-cold phosphate-buffered saline (137 mM NaCl, 2.6 mM KCl, 8.1 mM $Na_2HPO_4$, and 1.1 mM $KH_2PO_4$, pH 7.4) and then extracted with ice-cold 60% methanol. The cells were scraped from the dishes and the precipitate was collected by centrifugation at 1000 × *g* for 5 min. A portion of the supernatant was counted to determine the accumulation of methanol-soluble [$^3$H]dThd metabolites and the remainder was evaporated to dryness, resuspended in water, and spotted on Polygram CEL 300 polyethyleneimine (Macherey-Nagel) thin-layer chromatograms to determine the pattern of incorporation. The strips, previously spotted with authentic dThd, dTMP, dTDP, and dTTP (Sigma Chemical Company, St. Louis, Mo.), were developed with a solvent system of 0.25 M LiCl and 0.06 M $NH_4SO_4$ (15), and the markers were visualized with UV light. The chromatograms were cut into 1-cm strips and radioactivity was eluted with 1 ml of buffer (0.7 M $MgCl_2$ and 0.02 M Tris-HCl, pH 7.5) and quantified in a Beckman LS100 liquid scintillation spectrometer.

enhancing (3-fold) the uptake of lower (0.3 μM) and higher (30 μM) concentrations of dThd. In Vero cells the increases in uptake were also considerable; the dTTP levels derived from exogenous [$^3$H]dThd were elevated 5-fold by 5'-AdThd. Despite the marked changes in the degree of [$^3$H]dThd uptake, the pattern of incorporation into the various dThd derivatives was not significantly changed. The enhancement, but not the inhibition, of dThd uptake was quite unexpected since 5'-AdThd is known to be a good inhibitor of the mammalian dThd kinase (16, 17). It was of interest to understand the mechanism of this unusual stimulatory effect, since dThd and its analogues are important in viral and cancer chemotherapy.

Analysis of the effects of 5'-AdThd on dThd kinase activity revealed that the increase as well as the decrease in dThd uptake was mediated by interactions with this enzyme. The experiments described in Fig. 2 clarify the manner by which 5'-AdThd exerts both a stimulatory (de-inhibition) and an inhibitory effect on dThd phosphorylation. We found that dTTP, a potent allosteric inhibitor of dThd kinase (20, 21), plays a crucial role in determining whether 5'-AdThd stimulates or inhibits dThd kinase activity. Thus, in the absence of dTTP, the dThd kinase activity present in crude cellular extracts of HeLa cells was progressively inhibited by 5'-AdThd (Fig. 2A). However, if dTTP (5 μM) was included in the reaction mixture, a stimulatory effect of 5'-AdThd on enzyme activity was apparent. This stimulation (de-inhibition) of enzyme activity is more clearly indicated in a replot of the data (Fig. 2A). At low concentrations, 5'-AdThd behaved as a regulatory antagonist and counteracted the inhibitory actions of dTTP and thus increased enzyme activity. However, 5'-AdThd is also a competitive inhibitor of dThd kinase, and at higher concentrations of 5'-AdThd this effect prevailed and a biphasic dose-response curve was generated. The experiment using dThd kinase purified from HeLa cells by affinity chromatography (Fig. 2B) demonstrates that this effect was, indeed, a result of interactions at the level of dThd kinase, and was not mediated by effects on other enzymes.

A similar pattern of interaction between these compounds was seen using Vero cells. The biphasic effect of 5'-AdThd on dThd kinase activity was evident only if dTTP was present. This pattern was seen if the enzyme activity was measured in cellular extracts (Fig. 2C) or with a purified preparation (Fig. 2D). Thus, in two cell types, the stimulatory and inhibitory effects of 5'-AdThd on dThd uptake in intake cells appear to be mediated at the level of dThd kinase. At low concentrations, 5'-AdThd preferentially reduced the feedback inhibition normally exerted by dTTP, enzyme activity was increased, and greater amounts of dThd metabolites accumulated. At high concentrations the interactions of 5'-AdThd at the active site predominated (16, 17), dThd kinase activity was inhibited, and the accumulation of dThd metabolites was reduced.

The ability of 5'-AdThd to disrupt the feedback loop which normally regulates the phosphorylation of dThd is considerable. This is evident in the experiment shown in Fig. 3 in which the presence of 5'-AdThd converted a noncytotoxic concentration of dThd (30 μM) to one that

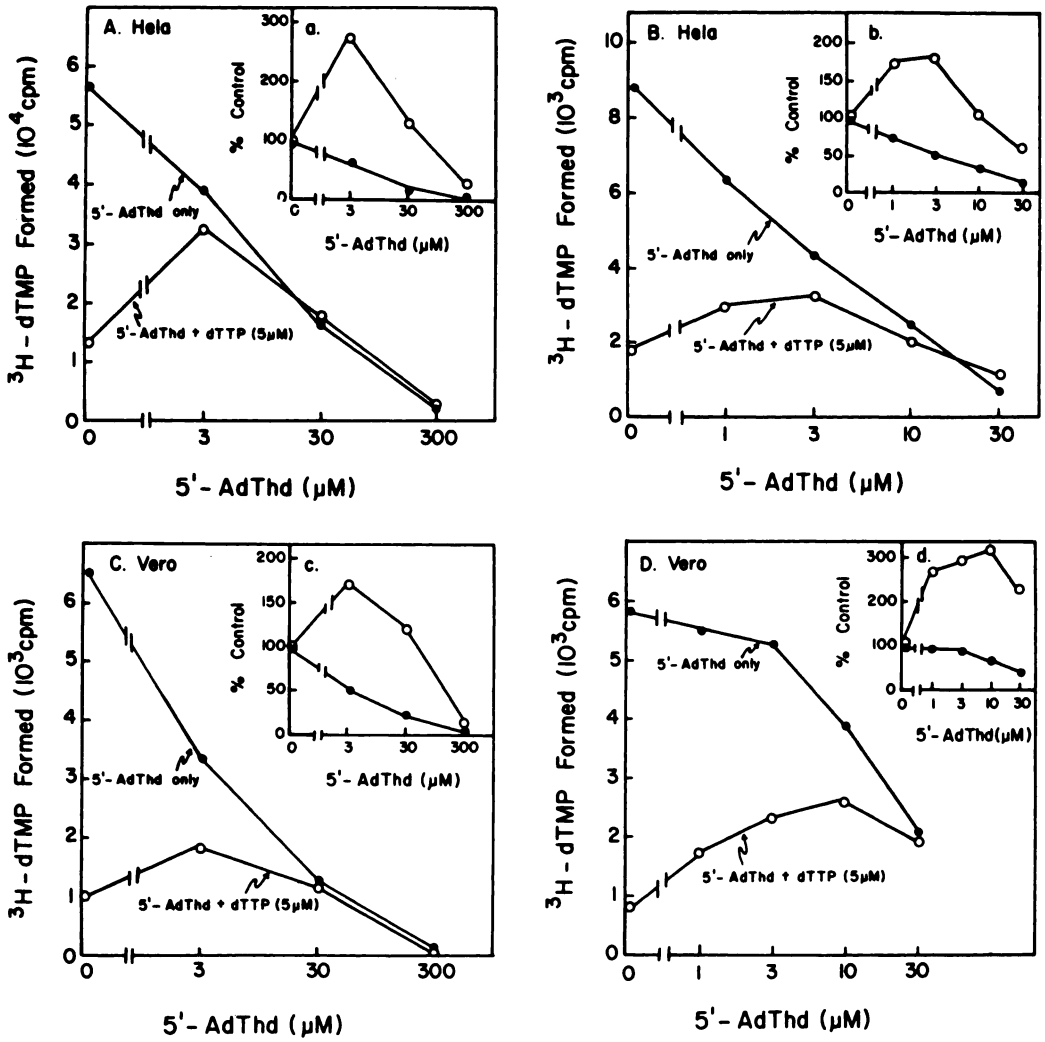


FIG. 2. *Modulation of dTTP inhibition of dThd kinase by 5'-AdThd*

Enzyme activity was determined in either crude cellular extracts or highly purified preparations obtained from HeLa cells (A and B) or Vero cells (C and D), respectively. The data were obtained in the presence and absence of 5 μM dTTP. If the enzyme activities are normalized to the values obtained in the absence of 5'-AdThd, the data can be expressed as the percentage of control (a, b, c, and d). To obtain a crude cellular extract, exponentially growing cells were scraped from the flasks, collected by centrifugation, and disrupted by sonication in a dThd kinase extraction buffer [20 mM Tris-HCl (pH 7.8), 50 μM dThd, 5 mM mercaptoethanol, and 10% glycerol]. The dThd kinase reaction mixture [2.5 mM ATP·$MgCl_2$, 2 mM dithiothreitol, 4.5 mM phosphocreatine, creatine kinase (6 units/ml), 20 mM NaF, 50 mM Tris (pH 7.8), 1% bovine serum albumin, and [*methyl*-$^3$H]dThd (50 μCi/ml)] and assay procedure were similar to described methods (18).

dThd kinase was purified from HeLa and Vero cells by affinity column chromatography (18, 19), using a column (Sepharose 4B connected to dThd at position 3' utilizing an amino substituent) kindly provided by Dr. Ming Chen (Department of Pharmacology, Yale University). The enzyme was eluted from the column with a solution of 300 mM Tris-HCl (pH 7.5), 10% glycerol, 3 mM mercaptoethanol, and 200 μM dThd, and assayed as previously described. The following concentrations of dThd were used: A, 3.6 μM; B, 20 μM; C, 10 μM; and D, 20 μM.

inhibited HeLa cell growth by 40%. Although neither dThd nor 5'-AdThd alone was cytotoxic, the modulation of dThd metabolism by 5'-AdThd produced a biphasic cytotoxic response. The inhibition of cellular replication produced by this drug combination was similar to that produced by 300 μM dThd alone and, as would be expected, the increased accumulation of dThd metabolites produced by 5'-AdThd was approximately equal to that obtained with 300 μM dThd alone. As the concentration of 5'-AdThd was increased, dThd phosphorylation was enhanced and cytotoxicity became evident. With higher amounts of 5'-AdThd, the stimulation of dThd uptake was reversed and cytotoxicity decreased. Thus, 5'-AdThd represents an effective, but not optimal, regulatory antagonist of dTTP since the ability of 5'-AdThd to bind at the active site of dThd kinase rapidly becomes limiting. We are currently investigating compounds in which interactions with regulatory metabolites rather than with substrates predominate over a wide range of concentrations in an attempt to obtain highly specific probes for a number of different enzyme systems.

Modulation of the activity of regulatory enzymes has been used to improve the chemotherapeutic response to various agents (7, 9, 22–27). The general approach has been to inhibit the synthesis of a feedback inhibitor with one drug so that the metabolic activation of a second compound will be increased. Cytosine arabinoside, for example, is phosphorylated by dCyd kinase (28, 29), an

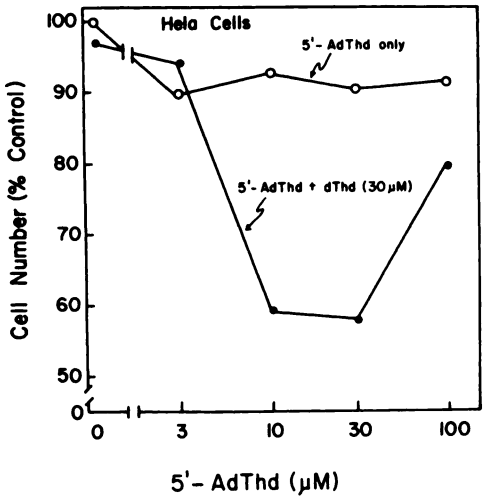


FIG. 3. *Effect of dThd and 5'-AdThd on the replication of HeLa cells.*

HeLa cells were plated at a density of $1.5 \times 10^5$ cells/35-mm well and incubated as previously described (Fig. 1) for 24 hr prior to the addition of the indicated concentration of 5'-AdThd in the absence and presence of 30 μM dThd. After a 72-hr exposure period the cells were removed from the dishes [with phosphate-buffered saline (see legend to Fig. 1) and 2.5 mM EDTA] and enumerated (Coulter counter Model ZBI). The data are expressed as the percentage of control cell number and represent the average of two experiments, each conducted in triplicate.

enzyme that is tightly regulated by dCTP (7, 30). Treatments that reduce the levels of this feedback inhibitor clearly enhance the phosphorylation and cytotoxicity of cytosine arabinoside (7, 23, 24, 26, 27). We propose a more direct approach to this general problem: the design of drugs specifically targeted at the regulatory sites of key enzymes but which do not require metabolic activation. This is an important consideration, since certain compounds such as 6-mercaptopurine ribonucleotide, which are known to act as pseudofeedback inhibitors (2), are also metabolized to other active species. The result is a wide range of biochemical effects and a loss of target-site specificity. Agents analogous to the regulatory antagonist described in this report, 5'-AdThd, could be used selectively to disrupt normal homeostatic mechanisms and provide a means of altering the metabolism of many substances in a variety of biological systems.

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