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Modulation of the Metabolism and Cytotoxicity of Iododeoxyuridine by 5′-Amino-5′-Deoxythymidine

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SUMMARY

In HeLa and Vero cells the antiproliferative effects of iododeoxyuridine (IdUrd) were modulated in a biphasic manner by 5′-amino-5′-deoxythymidine (5′-AdThd). Low concentrations of 5′-AdThd increased the cytotoxicity of IdUrd whereas high concentrations of 5′-AdThd were antagonistic. Opposing effects on two enzymes, thymidine kinase (EC 2.7.1.21) and thymidylate kinase (EC 2.7.4.9), account for this unusual biphasic interaction. In the case of thymidine kinase, 5′-AdThd was found to antagonize the feedback inhibition which is normally exerted by the 5′-triphosphates of thymidine and IdUrd. Consequently, 5′-AdThd increased the rate of IdUrd phosphorylation. This stimulation (deinhibition) of enzyme activity was demonstrable in cell-free extracts and with a purified preparation of thymidine kinase provided that the 5′-triphosphates of IdUrd or thymidine were present. In their absence only enzyme inhibition was detected. In intact cells this stimulatory effect of 5′-AdThd was seen as a rapidly apparent, sustained increase in the steady-state levels of the phosphorylated IdUrd metabolites. As a result, IdUrd cytotoxicity was increased. Under these conditions, 5′-AdThd did not alter the relative abundance of the mono-, di-, and triphosphates of IdUrd. However, as the concentration of 5′-AdThd was raised, the percentage of IdUrd nucleotides present as iododeoxyuridylate increased dramatically. Corresponding reductions in the incorporation of IdUrd into cellular DNA and the associated cytotoxic effects were seen. These data suggested a second site of interaction, thymidylate kinase, the enzyme responsible for the conversion of iododeoxyuridylate to the diphosphate. In experiments measuring thymidylate kinase activity in cell-free extracts, 5′-AdThd effectively inhibited the phosphorylation of iododeoxyuridylate but not that of thymidylate. Additionally, 5′-AdThd did not produce an accumulation of thymidylate in intact cells. Thus, the ability of high concentrations of 5′-AdThd to antagonize the cytotoxicity produced by IdUrd without concomitantly inhibiting the phosphorylation of thymidylate and, thereby, reducing DNA synthesis was explained. Although the modulation of IdUrd metabolism produced by 5′-AdThd was qualitatively similar in Vero and HeLa cells, key quantitative differences were evident. Thus, 100 μM 5′-AdThd stimulated the uptake of 3 μM IdUrd in Vero cells but it was inhibitory in HeLa cells. Perturbation of nucleoside metabolism by agents such as 5′-AdThd may provide an important new way to achieve selective toxicity in cancer chemotherapy.

INTRODUCTION

The thymidine analogue, IdUrd,[3] is useful in the treatment of herpes simplex keratitis (1). However, the drug can be metabolically activated in uninfected tissues and toxicity may result, particularly if it is given systemically (2, 3). In this regard, IdUrd is typical of many antiviral agents. They are potent inhibitors of viral replication but are excessively toxic to uninfected tissues. In fact, the cytotoxic effects of IdUrd are sufficient to have warranted its evaluation as an antitumor drug (4, 5). Theoretically, the clinical utility of IdUrd could be improved both as an antiviral and an anticancer agent. For exam-

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[3] The abbreviations used are: IdUrd, 5-iodo-2′-deoxyuridine; IdUMP, IdUDP, IdUTP, the 5′-mono-, di-, and triphosphates of IdUrd; 5′-AdThd, 5′-amino-5′-deoxythymidine; PBS, phosphate-buffered saline.

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ple, preferential inhibition of the activation of IdUrd in uninfected cells is one strategy that could yield enhanced antiviral selectivity. This approach, which utilizes specific inhibitors, has been described (6, 7). Conversely, preferential activation of IdUrd in tumor tissues, as opposed to sensitive normal cells, would improve its anticancer selectivity.

We initially followed the first approach in an attempt to inhibit selectively the phosphorylation of IdUrd in uninfected cells with 5′-AdThd (7). 5′-AdThd was used because it is a potent inhibitor of mammalian thymidine kinase (8, 9), the enzyme responsible for the phosphorylation of IdUrd (10). In adition, 5′-AdThd is phosphorylated only by the herpes simplex virus-encoded thymidine kinase (11) and is a selective, but not particularly potent, anti-herpes agent (12, 13). We found that 5′-AdThd greatly enhanced the therapeutic index of IdUrd against herpes simplex virus infections *in vitro* (7). Although the drugs displayed additive antiviral effects, high concentrations of 5′-AdThd effectively antagonized the toxicity of IdUrd in uninfected cells. One of the aims of the present study was to elucidate the mechanism by which 5′-AdThd protects against the cytotoxicity of IdUrd.

In a recent study we found that 5′-AdThd could antagonize the inhibition of dThd kinase that is normally exerted by the feedback inhibitor dTTP (14). As a consequence of this deinhibition, the uptake of dThd was strongly stimulated by 5′-AdThd in both HeLa and Vero cells. The possibility that 5′-AdThd might alter IdUrd metabolism in a similar fashion thus became an important consideration. Indeed, we have found that low concentrations of 5′-AdThd increase the phosphorylation of IdUrd and thereby exacerbate its cytotoxicity. Such an effect may provide a means for achieving selective drug activation; therefore, the biochemical basis of this stimulatory action of 5′-AdThd has also been examined in this investigation.

EXPERIMENTAL PROCEDURES

Materials. IdUrd, IdUMP, and IdUTP were purchased from Calbiochem (San Diego, Calif.), and dThd, dTMP, dTDP and dTTP were obtained from Sigma Chemical Company (St. Louis, MO.). New England Nuclear Corporation (Boston, Mass.) supplied the [^{125}I]dUrd and [*methyl*-^{3}H]dThd, and Moravek Biochemicals Inc. furnished the [6-^{3}H] IdUrd, [6-^{3}H]IdUMP, and [^{14}C]dTMP. Drs. T.-S. Lin and M. S. Chen of the Yale University School of Medicine (New Haven, Conn.) kindly provided th 5′-AdThd and dThd kinase affinity gel, respectively. Thin-layer chromatographic sheets, MN PEI Cel 300 UV, were purchased from Brinkmann Instruments (Westbury, N. Y.).

Cells. Vero and HeLa cells were obtained from Flow Laboratories (Rockville, Md.). The cells were grown as monolayers in Dulbecco's modified minimal essential medium (Grand Island Biological Company, Grand Island, N. Y.) supplemented with 10% newborn calf serum (K. C. Biologicals, Kansas City, Mo.) at 37° in a humidified 5% CO_2 atmosphere. The cells were removed from plastic tissue cultureware using 0.1% porcine trypsin (K. C. Biologicals) and subcultured twice weekly. The cells were found to be free of mycoplasma contamination using the DNA staining technique described by Chen (15).

Cytotoxicity. Effects on cellular replication rates and the efficiency of colony formation were used as measures of cytotoxicity. The drug exposure period was 72 h in the growth rate experiments and 24 hr in the viability assays. In both cases, 1.5×10^5 cells were plated in 35-mm dishes and incubated for 24 hr prior to the addition of drugs. In the cell growth experiments the cells were dislodged from the dishes with PBS [137 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, and 1.1 mM KH_2PO_4 (pH 7.4)] containing 2.5 mM EDTA and then enumerated using an electronic particle counter (Coulter Electronics). These experiments were done in duplicate or triplicate and were repeated several times. The data are presented as the percentage of control cell number (mean ± standard error).

In the viability experiments, after the 24-hr period of drug treatment, the HeLa cells were washed with PBS and then removed from the dishes with 0.1% trypsin. The cells were diluted with medium and then plated in 35-mm culture dishes and incubated for 7–8 days. The plates were then stained with 1% methylene blue in methanol. Those cells forming colonies containing greater than 50 cells (read with a Bellco Plaque Viewer) were considered viable.

Uptake of IdUrd. The uptake of IdUrd into the acid-soluble and acid-insoluble pools of Vero and HeLa cells was determined in the following manner. Exponentially growing cells, plated in the manner already described, were used in all experiments. The cells were incubated for 4 hr, or as indicated, with the appropriate concentrations of 5′-AdThd and/or [^{125}I]dUrd (0.2 μCi/ml, or as indicated). The medium was removed and the cells were washed four times with ice-cold PBS prior to extraction with 0.2 N $HClO_4$. The acid-soluble material was collected by centrifugation, and a portion of the supernatant was saved for determination of radioactivity in a Beckman 4000 γ counter. The pellet was then resuspended in 0.2 N $HClO_4$ and collected by centrifugation; the supernatant was removed prior to measuring the amount of incorporated [^{125}I]dUrd into DNA. These experiments were repeated several times and the data are expressed as the mean ± standard error.

Distribution of metabolites. The distribution of the various metabolites of IdUrd was determined using thin-layer chromatography. Exponentially growing cells, plated in 60-mm dishes, were used in these experiments. The cells were exposed to various concentrations of 5′-AdThd and [^{125}I]dUrd (1 μCi/ml) for a 4-hr period. The medium was removed and the cells were washed four times with ice-cold PBS, extracted with 1 ml of cold 60% methanol for 30 min, and then scraped from the dish with a rubber policeman. The dish was washed with 1 ml of 60% methanol and the contents were pooled and centrifuged at 1000 × *g* for 5 min. The supernatant was transferred to another test tube and evaporated to dryness under vacuum. The residue was taken up in 50 μl of water, and 15-μl portions were applied to PEI Cel 300 strips previously spotted with IdUrd, IdUMP, and IdUTP markers. The chromatograms were developed in 1 M LiCl and the migration of the compounds was visualized under UV light. The strips were then cut into 1-cm segments and the distribution of radioactivity was determined. Several experiments were conducted to confirm that the amounts of radioactivity extracted with 60% methanol or into the 0.2 N $HClO_4$-soluble pool were the same. Similarly, the amounts of [^{125}I] dUrd associated with the 60% methanol-preciptable fraction and the 0.2 N $HClO_4$-insoluble pools were the same.

A similar procedure was used to analyze the metabolism of exogenously added [*methyl*-^{3}H]dThd. In these experiments, the extracts from cells labeled with [*methyl*-^{3}H]dThd (3 μCi/ml) were analyzed using a solvent system of 0.25 M LiCl and 0.06 M NH_4SO_4 with PEI Cel 300 thin-layer chromatograms (16). The strips were spotted with dThd, dTMP, dTDP, and dTTP and visualized with UV light. The developed chromatograms were cut into 1-cm strips, and the radioactivity was eluted with 1 ml of 0.7 M $MgCl_2$/0.02 M Tris-CCl (pH 7.5). The samples were counted in a Beckman LS 100 liquid scintillation spectrometer.

Thymidine kinase. Thymidine kinase, obtained from exponentially growing HeLa cells, was assayed in crude cellular extracts and in preparations purified by affinity column chromatography in a manner similar to procedures previously described (14, 17). For experiments using the purified enzyme preparation, a G-50 column was employed to remove the dThd required to elute the enzyme from the affinity column. The cells were suspended in an extraction buffer containing 25 mM Tris (pH 7.5), 5 mM ATP, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 10 mM KCl, and 10% glycerol; disrupted in a Dounce homogenizer; and then centrifuged at 12,000 rpm for 15 min in a JA-20 rotor, using a Beckman J21C centrifuge. Enzyme activity in the supernatant was assayed in a

reaction mixture that contained 2.5 mM ATP, 2.5 mM $MgCl_2$, 2.5 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 [6^{-3}H] IdUrd (30 μCi/ml) in a final volume of 90 μl. The reaction was carried out at 37° and was always checked for linearity with respect to the time of incubation. Portions (30 μl) were typically removed at 30 and 60 min and spotted on Whatman DE81 paper discs which were washed four times in 95% ethanol, dried, and counted in HFP-20 liquid scintillation fluid (Research Products International) with a Tracor Mark III scintillation counter.

Thymidine monophosphate kinase. dTMP Kinase was assayed in a manner similar to that of Cheng and Prusoff (18) in a reaction mixture containing 50 mM Tris (pH 7.5), 5mM ATP, 5 mM $MgCl_2$, 0.2% bovine serum albumin, 1 mM β-mercaptoethanol, 20 mM NaF, 4.5 mM phosphocreatine, 6 creatine kinase (units/ml), and either [6-^{3}H]IdUMP or [^{14}C]dTMP. The reaction was run at 37° for 30 min and stopped by spotting a portion of the reaction mixture on a PEI Cel 300 thin-layer chromatogram. The chromatograms, previously spotted with authentic nucleotide markers, were developed in 1.0 M LiCl or 0.4 M LiCl when IdUMP or TMP was used as the substrate, respectively. The areas corresponding to the mono-, di-, and triphosphates were identified under UV light, cut out, eluted with 0.7 M $MgCl_2$/0.02 M Tris (pH 7.5), and counted using ACS (Amersham).

RESULTS

Modulation of cytotoxicity. 5'-AdThd modulated the cytotoxicity of IdUrd in a biphasic manner. A characteristic pattern of exacerbation, followed by protection of IdUrd toxicity, was seen as the concentration of 5'-AdThd was increased progressively. These alterations in the cytotoxicity produced by IdUrd were evident in both antiproliferative and viability experiments. In the absence of 5'-AdThd the replication of Vero cells was inhibited by 42% after a 72-hr exposure to IdUrd (30 μM). The addition of 5'-AdThd (30 μM) increased the inhibition of growth to 70%, whereas it was reduced to 10% by 1000 μM 5'-AdThd (Fig. 1). Similarly, the inhibition of HeLa cell growth produced by IdUrd (30 μM) was enhanced by 5'-AdThd at lower concentrations (30 μM) and decreased by higher levels of 5'-AdThd (300 μm) (Fig. 1). Clonogenic assays were carried out in order to assess whether 5'-AdThd also influenced the survival of HeLa cells treated with IdUrd (Fig. 2). The viability of HeLa cells exposed to IdUrd (30 μM) for 24 hr was reduced from 11% to 4% by the addition of 5'-AdThd (30 μM). Survival was increased to 55% by a higher concentration of 5'-AdThd (300 μM). A similar modulation of cell killing was seen in experiments in which HeLa cells were exposed to 3 μM IdUrd (Fig. 2). The cloning efficiency of HeLa cells was not reduced by exposure to 5'-AdThd only.

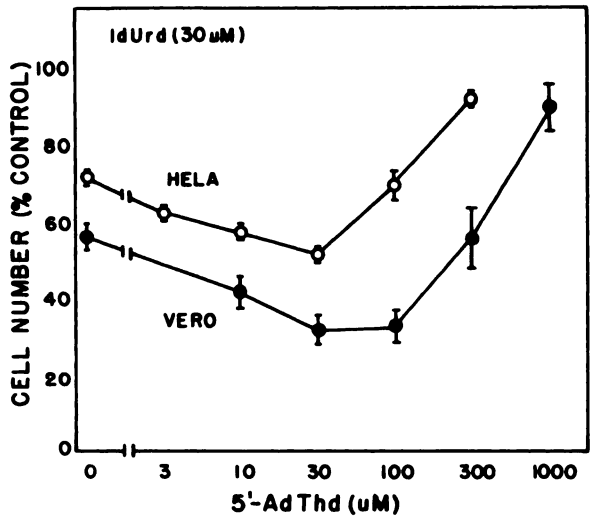

FIG. 1. *Effect of 5'-AdThd on the inhibition of cellular replication caused by IdUrd*

Exponentially growing HeLa cells (○) or Vero cells (●) were exposed for 72 hr to 30 μM IdUrd and the indicated concentrations of 5'-AdThd. The data are expressed as the percentage of control cell number (mean ± standard error; $n = 3$ for HeLa, $n = 4$ for Vero). Procedural details are presented under Experimental Procedures.

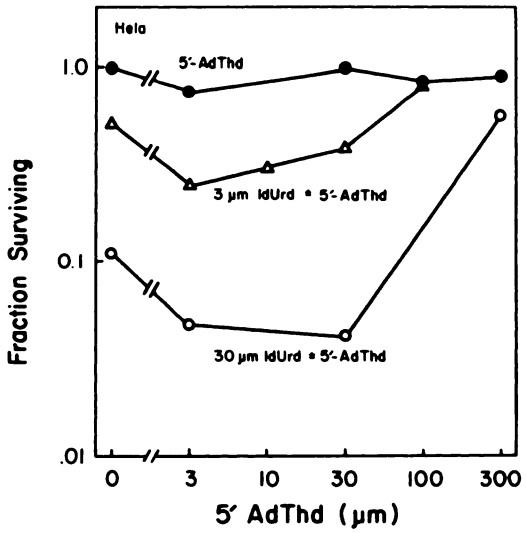

FIG. 2. *Effect of 5'-AdThd on the viability of HeLa cells exposed to IdUrd*

HeLa cells were exposed for 24 hr to 5'-AdThd (●) or to a combination of the indicated concentrations of 5'-AdThd and 30 μM IdUrd (○) or 3 μM IdUrd (△). The surviving fraction represents the percentage of cells in the treated population as compared with the control group that are able to form colonies of ≥50 cells. In these experiments the plating efficiency of the control cells was approximately 50%. These data are the averages from two separate experiments. Details for the cloning procedure are given under Experimental Procedures.

Effects of 5'-AdThd on the metabolism of IdUrd. The mechanisms underlying the unusual biphasic modulation of IdUrd cytotoxicity produced by 5'-AdThd were initially investigated by determining the effects of the aminonucleoside on the uptake and metabolism of IdUrd in both HeLa and Vero cells. Two important effects were seen. Under conditions in which the cytotoxicity of IdUrd was enhanced, 5'-AdThd also increased the intracellular levels of IdUMP, IdUDP, and IdUTP. Concentrations of 5'-AdThd which antagonized IdUrd toxicity produced a marked increase in the percentage of nucleotides present as IdUMP. These changes are shown in Figs. 3 and 4 for HeLa and Vero cells, respectively. These data were obtained 4 hr after the drugs were added, and they reflect new intracellular steady-state levels. In HeLa cells the intracellular levels of IdUrd nucleotides were increased 3-fold by 30 μM 5'-AdThd (Fig. 3A), whereas optimal stimulation in Vero cells required 30–100 μM 5'-AdThd (Fig. 4A). These increases were pronounced within minutes and were maintained for at least 24 hr. Changes in

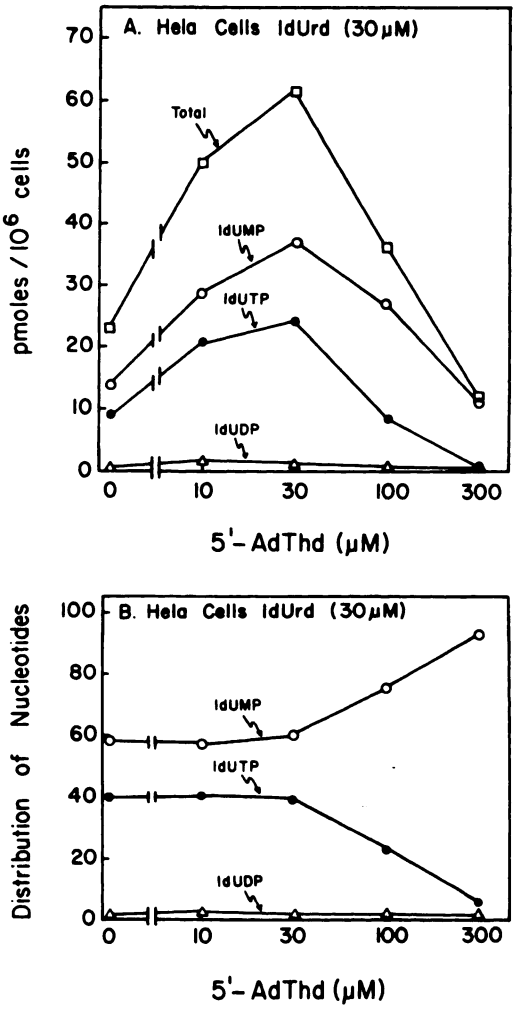

FIG. 3. *Effect of 5'-AdThd on the uptake and metabolism of 30 μM IdUrd in HeLa cells*

The influence of 5'-AdThd on the pattern of incorporation of [^{125}I] dUrd into IdUMP, IdUDP, IdUTP, and all three nucleotides is shown in A. The relative distribution of the nucleotides is shown in B as a function of the 5'-AdThd concentration. Experimental details for data presented here and in Fig. 4 are given under Experimental Procedures.

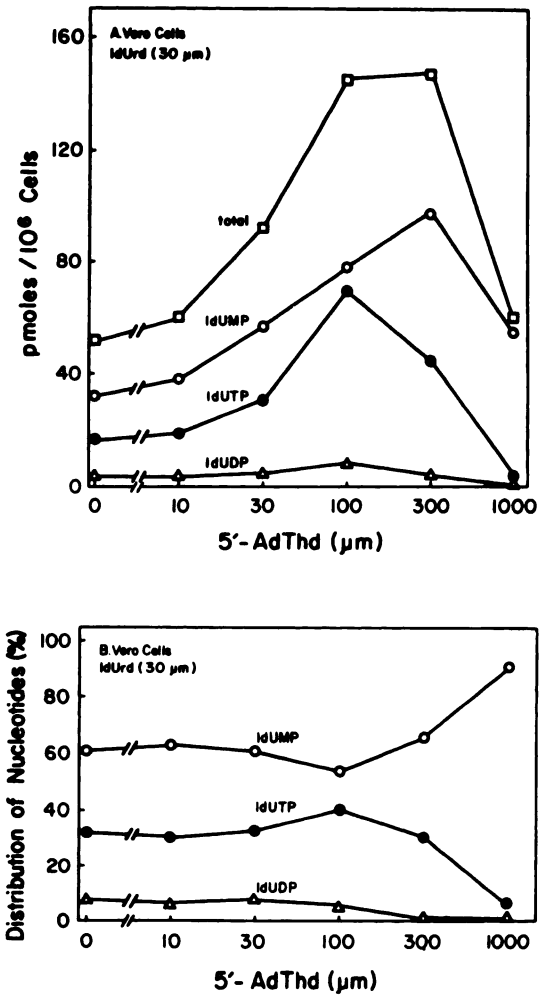

FIG. 4. *Effect of 5'-AdThd on the uptake and metabolism of 30 μM IdUrd in Vero cells*

The influence of 5'-AdThd on the pattern of incorporation of [^{125}I] dUrd into IdUMP, IdUDP, IdUTP, and all three nucleotides is shown in A. The relative distribution of the nucleotides is shown in B as a function of the 5'-AdThd concentration.

the relative distribution of the IdUrd nucleotides were not noted over the stimulatory portion of the dose-response curve (Figs. 3B and 4B). However, as the cells were exposed to higher concentrations of 5'-AdThd, the degree of stimulation decreased and the percentage of nucleotides present as IdUMP rose. Note the marked depletion of intracellular IdUTP levels produced by concentrations of 5'-AdThd (300 μM in HeLa, 1000 μM in Vero) that were sufficient to block IdUrd cytotoxicity.

The increased intracellular levels of IdUTP produced by 5'-AdThd were associated with increased rates of incorporation of IdUrd into DNA. Conversely, less IdUrd was incorporated into DNA under conditions in which 5'-AdThd reduced cytotoxicity. These data support the idea that the incorporation of IdUrd into DNA is responsible for its cytotoxicity (19). Figure 5 illustrates the effects of stimulatory (100 μM) inhibitory (1000 μM) concentrations of 5'-AdThd on the kinetics of incorporation of IdUrd into Vero cell DNA. In general, the effects of 5'-AdThd on incorporation of IdUrd into DNA were similar in HeLa and Vero cells; however, quantitative differences were seen. The data in Fig. 6, which compare the influence of 5'-AdThd on the incorporation of IdUrd into DNA and into the acid-soluble fraction of the two cell types, illustrate these points. The biphasic shape of the curves are similar, but at 100 μM 5'-AdThd the uptake of 3 μM IdUrd was strongly stimulated in Vero cells whereas it was inhibited in HeLa cells.

Inhibition of IdUrd catabolism to iodouracil could not account for the stimulation of IdUrd uptake produced by 5'-AdThd. Neither HeLa nor Vero cells produced levels of iodouracil detectable by thin-layer chromatography in these experiments. Approximately 95% of the added IdUrd was routinely recovered as IdUrd, IdUMP, IdUDP, and IdUTP. In addition, essentially identical results were obtained using either [^{125}I]dUrd or [^{3}H] IdUrd in numerous uptake and distribution experiments, indicating that thymidylate synthetase-catalyzed dehalogenation (20) was not a major factor in these studies.

Effects of 5'-AdThd on dThd metabolism. The accu-

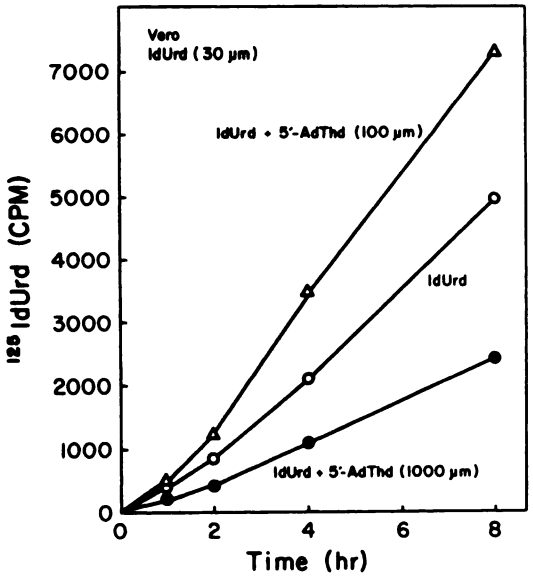

FIG. 5. *Effect of 5'-AdThd on the incorporation of [^{125}I]dUrd into Vero cell DNA*

Exponentially growing Vero cells were exposed to 30 μM [^{125}]IdUrd (0.4 μCi/ml) alone (○) or in the presence of 5'-AdThd at 100 μM (△) or at 1000 μM (●). At the indicated time the amounts of radioactivity incorporated into the acid-insoluble fractions were determined.

mulation of IdUMP and the depletion of IdUTP by concentrations of 5'-AdThd which antagonized IdUrd cytotoxicity suggested that inhibition of IdUMP phosphorylation was a critical effect. However, this finding raised an important question. Why is 5'-AdThd not a cytotoxic compound, since TMP kinase (the enzyme responsible for the phosphorylation IdUMP to IdUDP) also mediates the phosphorylation of TMP, an important step in TTP biosynthesis (21)? This is true whether TMP is generated from the salvage pathway (dThd kinase) or from *de novo* synthesis (TMP synthetase). Our previous work (14) had indicated that the phosphorylation of dThd was stimulated by low concentrations of 5'-AdThd. However, in contrast to our findings with IdUrd, the distribution of the dThd nucleotides was not altered, even by high concentrations of 5'-AdThd (Fig. 7). 5'-AdThd appeared to inhibit effectively the phosphorylation of IdUMP but not that of TMP and was, therefore, not cytotoxic.

Effects of 5'-AdThd on Thd kinase activity. 5'-AdThd can either stimulate or inhibit the phosphorylation of dThd by dThd kinase, depending on the presence or absence of the feedback inhibitor dTTP, respectively (14). That is, 5'-AdThd can act as a regulatory antagonist and reverse the inhibitory effects of dTTP on dThd kinase. It seemed that a similar mechanism was a plausible explanation for the effects of 5'-AdThd on IdUrd uptake described in this study. Our initial studies, with thymidine kinase activity measured in cellular extracts or in preparations purified from HeLa cells, support this contention. As shown in Fig. 8A, 5'-AdThd antagonized the inhibition of IdUrd phosphorylation exerted by IdUTP, a known feedback inhibitor of dThd kinase (22). These experiments were performed using dThd kinase from a crude cellular extract, and similar results have been obtained using dTTP as the feedback inhibitor. In an enzyme preparation purified by affinity column chromatogrpahy, the effects of 5'-AdThd on the phosphorylation of IdUrd were also shown to be critically dependent on the presence of dTTP (Fig. 8B). Clearly depending on the presence or absence of feedback inhibitors, marked stimulation or inhibition of IdUrd phosphorylation can be mediated by 5'-AdThd.

Effects of 5'-AdThd on TMP kinase activity. We com-

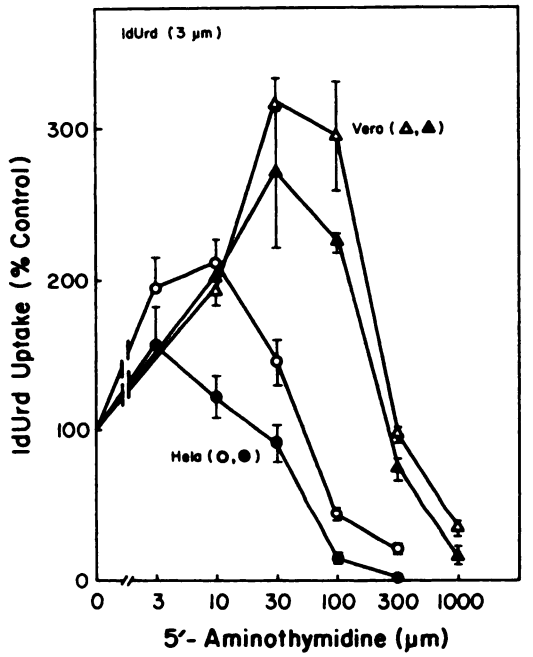

FIG. 6. *Comparison of the effect of 5'-AdThd on the uptake of IdUrd into Vero and HeLa cells*

The influence of various concentrations of 5'-AdThd on the uptake of [^{125}I]dUrd into the acid-soluble (△, ○) and acid-insoluble (▲, ●) fractions of Vero and HeLa cells. The data are expressed as the percentage of uptake obtained in the absence of 5'-AdThd (mean ± standard error; $n = 4$).

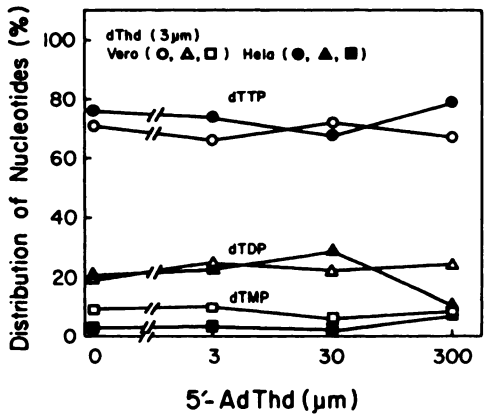

FIG. 7. *Effect of 5'-AdThd on the pattern of incorporation of [^{3}H] dThd into nucleotides in Vero and HeLa cells*

The relative distribution of the incorporation of [^{3}H]dThd into dTMP, dTDP, and dTTP is shown as a function of the concentration of 5'-AdThd. In HeLa cells, 300 μM 5'-AdThd reduced the total amount of labeled nucleotides from 10 pmoles/10^6 cells to 2.5 pmoles/10^6 cells. In Vero cells, the reduction was from about 12 pmoles/10^6 cells to 5 pmoles/10^6 cells.

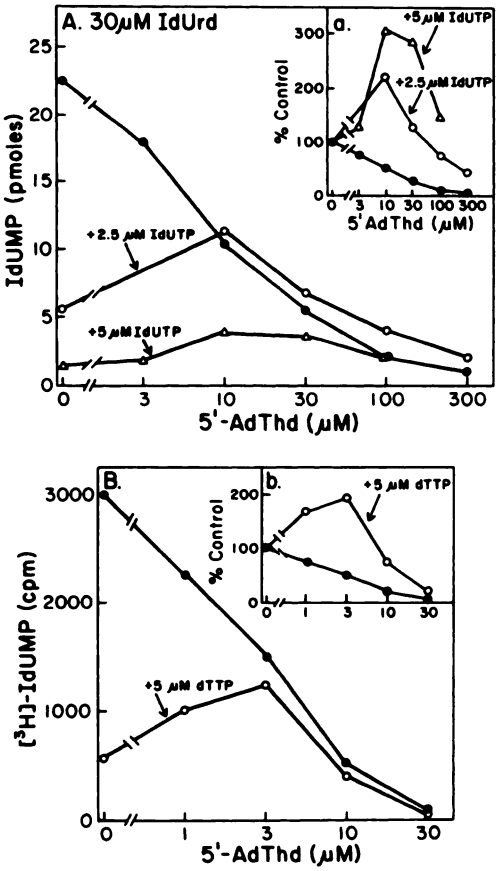

FIG. 8. *Effect of 5'-AdThd on the phosphorylation of IdUrd by dThd kinase*

Enzyme activity was measured in cellular extracts (A) or in a purified preparation (B) obtained from HeLa cells. The data were obtained in the presence or absence of IdUTP (A) or dTTP (B). The results are also expressed as the percentage of control (values in the absence of 5'-AdThd) in *insets* a and b. The concentration of IdUrd was 30 μM in A and 10.5 μM in B.

pared the effects of 5'-AdThd on the phosphorylation of [^{14}C]TMP and [^{3}H]IdUMP by TMP kinase extracted from HeLa cells (Fig. 9). The enzyme preparation and reaction conditions were identical except for the substrates. The amount of product formed was quite different for the two substrates. In the control reactions, 9 pmoles and 1.2 pmoles of substrate were phosphorylated using TMP and IdUMP, respectively. Second, under these conditions IdUMP phosphorylation was inhibited to a much greater extent than was TMP phosphorylation. These preliminary studies confirm the work of Cheng and Prusoff (18) in which 5'-AdThd was shown to be a weak inhibitor of TMP kinase when utilizing TMP as the substrate. These data are consistent with the ability of 5'-AdThd at nontoxic concentrations to inhibit the cytotoxicity of IdUrd by preferentially blocking the phosphorylation of IdUMP.

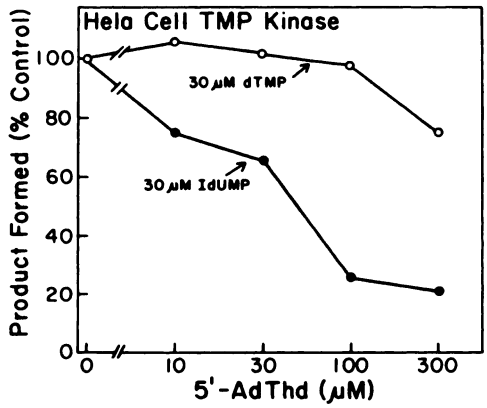

FIG. 9. *Effect of 5'-AdThd on dTMP kinase activity*

The inhibition of dTMP kinase activity by 5'-AdThd was compared using 30 μM [^{14}C]dTMP (○) and 30 μM [^{3}H]IdUMP (●) as the substrates. In the control reactions (no 5'-AdThd) 9 pmoles of dTMP and 1.2 pmoles of IdUMP were phosphorylated, respectively.

DISCUSSION

This study was designed to investigate the mechanisms by which 5'-AdThd modulates, in a biphasic manner, the cytotoxicity induced by IdUrd in HeLa and Vero cells. The data indicate two important sites of interaction, dThd kinase and dTMP kinase. Several lines of evidence suggest that 5'-AdThd antagonizes the inhibition of dThd kinase caused by dTTP and IdUTP and, thereby, stimulates (deinhibits) IdUrd phosphorylation. Studies on the cellular metabolism of IdUrd showed that 5'-AdThd increased the steady-state levels of IdUMP, IdUDP, and IdUTP at concentrations that enhanced IdUrd cytotoxicity. The stimulation was rapid and sustained, and resulted in higher levels of IdUrd incorporation into DNA. Inhibition of IdUrd catabolism was not a significant factor in these studies, and the production of iodouracil was not detectable. Most important, in experiments measuring dThd kinase activity, 5'-AdThd enhanced the rate of IdUrd phosphorylation in the presence of IdUTP or dTTP. These data, taken together, strongly suggest that 5'-AdThd acts as a regulatory antagonist of the feedback inhibition of dThd kinase in intact cells and greatly enhances the phosphorylation of a cytotoxic nucleoside.

In addition, 5'-AdThd is known to be a good inhibitor of dThd kinase (7, 8) and, as the concentration of 5'-AdThd is increased, interactions at the active site rather than the regulatory site predominate (Fig. 8). As a result, stimulation of IdUrd phosphorylation is not optimal, and the formation of IdUMP falls. We previously found that the phosphorylation of dThd could be perturbed in a similar manner (14).

Inhibition of IdUMP phosphorylation appears to account for the ability of 5'-AdThd to antagonize the cytotoxicity of IdUrd. Strong evidence was provided by the metabolic studies. Concentrations of 5'-AdThd sufficient to reduce IdUrd toxicity depleted intracellular IdUTP but not IdUMP (Figs. 3 and 4). For example, the inhibition of Vero cell growth caused by 30 μM IdUrd was abated by the addition of 1000 μM 5'-AdThd (Fig. 1). Under these conditions IdUTP pools were decreased from 18 to 3 pmoles/10^6 cells but IdUMP pools actually increased from 32 to 55 pmoles/10^6 cells (Fig. 4). As mentioned above, sufficiently high concentrations of 5'-AdThd can produce inhibition of both IdUrd and IdUMP phosphorylation. This is an interesting example of sequential enzyme inhibition (23, 24) in which two enzymes are inhibited by a single drug. Preliminary enzymological

experiments also indicate that 5′-AdThd can inhibit the phosphorylation of IdUMP by TMP kinase (Fig. 9).

Since Cheng and Prusoff (18) had found 5′-AdThd to be a weak inhibitor of TMP kinase, it was surprising to learn of the critical nature of this interaction in these studies. The differences in IdUMP and TMP as substrates for TMP kinase appear to be crucial. Our metabolic studies with dThd (Fig. 7) and our enzymatic studies with TMP (Fig. 9) support their data and emphasize this point. In contrast to the situation with dTMP and dTTP, IdUMP rather than IdUTP is the predominate nucleotide present intracellularly, suggesting that IdUMP is a relatively poor substrate of TMP kinase. This occurs in a variety of tissues, and Baugnet-Mauhieu and Goutier (25) attributed the relatively poor incorporation of IdUrd into DNA to the inefficient conversion of IdUMP to IdUTP. Kaufman and Davidson (26) have isolated a cell line resistant to IdUrd that possesses a TMP kinase with an altered substrate specificity. The enzyme effectively catalyzes the phosphorylation of TMP but not that of IdUMP. 5′-AdThd appears to produce a similar discrimination between the two substrates. The fact that TMP is a good substrate for TMP kinase accounts for the lack of cytotoxicity of 5′-AdThd, whereas interference with the phosphorylation of a poor substrate (IdUMP) accounts for the reduction in IdUrd toxicity. We are now investigating the basis for these differences in substrate behavior using a purified preparation of dTMP kinase.

The potential usefulness of developing enzyme regulatory site-directed drugs for application in chemotherapy has been discussed (14). However, the point is further illustrated in this study. We have shown that 5′-AdThd, a regulatory antagonist of dTTP- and IdUTP-mediated inhibition of dThd kinase, can markedly enhance the cytotoxicity of IdUrd. Of special importance is the finding that, under appropriate conditions, 5′-AdThd can exert selective, differential effects on two cell types (Fig. 6). The uptake of IdUrd was effectively enhanced in Vero cells by 100 μM 5′-AdThd, whereas it was strongly inhibited under the same conditions in HeLa cells. We are currently investigating the possibility that similar differences can be achieved between target and host tissues such that chemotherapeutic selectivity is greatly enhanced. The potential applicability of this approach in cancer and viral chemotherapy appears considerable.

Numerous interesting questions regarding the action and development of new regulatory-site antagonists require answers, which the following approaches may provide: a detailed kinetic investigation of their mechanism of action, structure-activity studies regarding the binding of compounds to regulatory as compared to active sites, and a determination of the applicability of this approach to other nucleoside kinases and to regulated enzymes in general. Of critical importance, as well, is an examination of the cellular and enzymatic parameters that control the quantitative responses of cells to regulatory antagonists such that exploitive chemotherapeutic regimens can be developed.

A second action of 5′-AdThd, the preferential inhibition of IdUMP phosphorylation, indicates another approach which could be used to increase selectivity in antiviral chemotherapy. The rationale for initially combining IdUrd and 5′-AdThd was, in fact, based on the concept of specific inhibitors (6, 7). This combination represented an attempt to reduce selectively the phosphorylation of IdUrd in uninfected cells. Our data indicate that TMP kinase, rather than dThd kinase, is the target enzyme, but the strategy involved is similar. Another approach, selective protection, was recently described by Cheng *et al.* (27). These investigators were able to protect against the cytotoxicity of *E*-5-propenyl-2′-deoxyuridine with thymidine without losing the antiviral activity of the deoxyuridine analogue.

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