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Antagonism of Feedback Inhibition

Stimulation of the Phosphorylation of Thymidine and 5-Iodo-2′-deoxyuridine by 5-Iodo-5′-amino-2′,5′-dideoxyuridine

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SUMMARY

The phosphorylation of thymidine and iododeoxyuridine by thymidine kinase was stimulated by 5-iodo-5′-amino-2′,5′-dideoxyuridine (AIdUrd). Antagonism of the feedback inhibition that is normally exerted by the 5′-triphosphates of thymidine and iododeoxyuridine appears to account for the stimulation. The effect of AIdUrd on thymidine kinase purified from HeLa cells by affinity column chromatography was critically dependent on the presence of these feedback inhibitors. In the presence of thymidine triphosphate or iododeoxyuridine triphosphate, AIdUrd could markedly stimulate (deinhibit) enzyme activity, whereas, in their absence, AIdUrd inhibited thymidine kinase with an apparent K_i of 0.7 μM. Stimulation was evident over a wide range of concentrations of both iododeoxyuridine and adenosine triphosphate. In intact HeLa and Vero cells, phosphorylation of thymidine and iododeoxyuridine was strongly enhanced by AIdUrd. Large increases in the intracellular levels of nucleotides derived from exogenous thymidine and iododeoxyuridine were apparent. As a consequence, the cytotoxicity of both nucleosides was exacerbated by AIdUrd. The reductions in cellular replication rates and colony formation produced by iododeoxyuridine were enhanced by AIdUrd. Although the replication of HeLa cells was not inhibited by either thymidine (30 μM) or AIdUrd (300 μM), in combination they were strongly synergistic and produced a 60% inhibition of cellular growth. Under these conditions, the uptake of thymidine was increased over 300% by AIdUrd. AIdUrd represents a new regulatory antagonist of thymidine kinase which may be useful in novel chemotherapeutic strategies.

INTRODUCTION

Thymidine kinase can play an important role in cellular replication by expediting the utilization of exogenous thymidine for DNA biosynthesis and thereby decreasing dependence on the *de novo* synthetic pathway (1). Enzyme activity increases as cells shift from a quiescent to a replicative state (2), and its presence and ioszymic pattern have been used as markers for neoplastic disease (3, 4). In addition, thymidine kinase catalyzes the phosphorylation of several nucleoside analogs used in cancer and viral chemotherapy (5–7). dTTP regulates dThd kinase activity by feedback inhibition (8–10), and this mechanism normally limits the overproduction of dTTP. However, in a chemotherapeutic setting, feedback inhibition may be detrimental and could limit the activation of nucleoside analogues. We recently suggested that compounds that antagonize the feedback inhibition of critical enzymes could be used to increase drug activation (11). For example, we found that 5′-aminothymidine could relieve the inhibition of thymidine kinase produced by dTTP (11) and IdUTP,[1] (12) and thereby stimulate nucleoside phosphorylation. Increased levels of intracellular dThd and IdUrd nucleotides were produced by 5′-aminothymidine, and, as a consequence, their cytotoxic effects were enhanced. However, higher concentrations of 5′-aminothymidine inhibited the phosphorylation of IdUMP and reduced intracellular IdUTP levels (12). This interaction limited the concentration range over which 5′-aminothymidine exacerbated IdUrd toxicity. The present study was undertaken to answer several questions. Could AIdUrd, the 5-iodo derivative of 5′aminothymidine, enhance the cytotoxicity of IdUrd and dThd? If so, does the mechanism involve antagonism

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[1] The abbreviations used are: AIdUrd, 5-iodo-5′-amino-2′,5′-dideoxyuridine; IdUrd, 5-iodo-2′-deoxyuridine; IdUMP, IdUDP, and IdUTP, the 5′-mono-, di-, and triphosphates of IdUrd.

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of the feedback inhibition of thymidine kinase? Does AIdUrd block IdUMP phosphorylation? AIdUrd, originally synthesized by Lin *et al.* (13), is a nontoxic nucleoside possessing selective antiherpes virus activity (14, 15).

EXPERIMENTAL PROCEDURES

Materials. Calbiochem supplied the AIdUrd, IdUrd, IdUMP, and IdUTP. New England Nuclear Corporation furnished the [*methyl*-^{3}H]dThd, Amersham the [^{125}I]dUrd, and Moravek Biochemicals Inc., the [6-^{3}H]IdUrd. Thin-layer chromatographic sheets, MN PEI cel 300 UV, and MN Sil N-HR/UV were purchased from Brinkmann Instruments.

Cells. Vero and HeLa cells, obtained from Flow Laboratories, were maintained as previously described (12, 16). The cells were found to be free of mycoplasma contamination using the DNA staining technique described by Chen (17). The growth inhibition and clonogenic assay procedures have been described (12, 16).

Uptake of nucleosides. The distribution of the various metabolites of IdUrd was determined essentially as described (12). Briefly stated, exponentially growing HeLa or Vero cells were exposed to various concentrations of AIdUrd and [^{125}I]dUrd (0.6–1.0 μCi/ml) for a 4-hr period. The cells were extracted for 30 min with ice-cold 60% methanol following four washes with cold phosphate-buffered saline. The precipitable material was collected by centrifugation, and the supernatant was then transferred to another test tube and evaporated under vacuum. The residue was dissolved in water, and portions were applied to PEI Cell 300 strips previously spotted with IdUrd, IdUMP, and IdUTP markers. The compounds were visualized under UV light after development of the chromatogram in 1 M LiCl. The strips were cut into 0.8-cm segments, and the radioactivity was quantified in a Searle Model 1285 gamma counter.

The uptake of [*methyl*-^{3}H]dThd (1 μCi/ml) into the 60% methanol soluble fraction was assessed similarly. In these experiments, a portion of 60% methanol extract was counted in a Tracor Mark III scintillation spectrometer using ACS scintillation fluid (Amersham). The conversion of thymidine to thymine and IdUrd to iodouracil was assessed by separation of the nucleosides and bases with silica gel thin-layer chromatography and $CHCl_3$:isopropanol (3.5:1) as the solvent.

Thymidine kinase. Procedures for the purification of thymidine kinase from HeLa cells by affinity column chromatography using a slight modification of the method of Lee and Cheng (18) have already been described (12, 16). In these experiments, the enzyme was separated from the dThd used for elution of the enzyme from the affinity gel with a G-50 column equilibrated with the following buffer: 5 mM Tris (pH 7.5), 3 mM β-mercaptoethanol, 30% glycerol, 2 mM $MgCl_2$, and 2 mM ATP. Unless otherwise indicated, 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, and [6-^{3}H]IdUrd (6.25 μCi/ml) in a final volume of 80 μl. The reaction was carried out at 37° and was always checked for linearity with respect to the time of incubation. After 30 and 60 min of incubation, portions (30 μl) of the reaction mixture were spotted on Whatman DE 81 paper discs which were washed once in 95% ethanol, once in 1 mM ammonium formate, and thrice more in 95% ethanol. The filters were dried and counted in HFP-20 liquid scintillation fluid (Research Products International).

RESULTS

AIdUrd is a nontoxic analogue of IdUrd (14) and, as such, did not inhibit the growth or viability of Vero or HeLa cells (Figs. 1 and 2). However, it greatly exacerbated the cytotoxicity produced by IdUrd. The inhibition of HeLa or Vero cell replication caused by a 72-hr exposure to 30 μM IdUrd was increased in a dose-dependent manner by AIdUrd (Fig. 1). The cell killing produced by IdUrd, as measured by colony formation, could also be enhanced by AIdUrd (Fig. 2). In these experiments, the

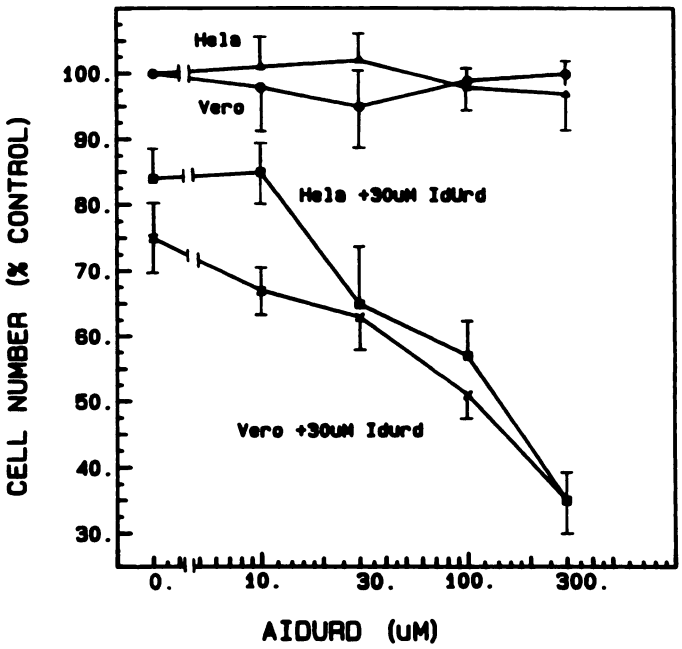

FIG. 1. *Effect of AIdUrd on the inhibition of cell growth by IdUrd*
Exponentially growing HeLa cells (▲, ■) or Vero cells (●, ×) were exposed for 72 hr to the indicated concentrations of AIdUrd in the absence (▲, ●) or presence of 30 μM IdUrd (■, ×). The data are expressed as the percentage of control cell number (mean ± standard error, $n \geq 3$).

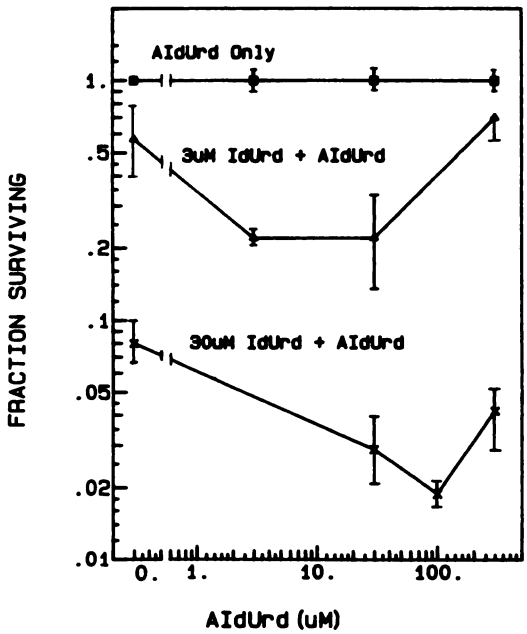

FIG. 2. *Effect of AIdUrd on the viability of HeLa cells exposed to IdUrd*
Exponentially growing HeLa cells were exposed for 24 hr to AIdUrd (■) or to a combination of the indicated concentrations of AIdUrd and 3 μM IdUrd (▲) or 30 μM IdUrd (×). The fraction surviving (mean ± standard error, $n \geq 3$) represents the percentage of cells in the treated population as compared with the control group that are able to form colonies of 50 cells or more. In these experiments, the plating efficiency of the control cells was approximately 49%.

percentage of HeLa cells surviving a 24-hr exposure to 3 μM IdUrd was reduced from 57% to 22% by the addition of 3 μM AIdUrd. A very high concentration of AIdUrd (300 μM) antagonized the IdUrd cytotoxicity. When the HeLa cells were exposed to 30 μM IdUrd, cell survival was reduced from 8% to 2% by 100 μM AIdUrd. Thus, the inhibition of cell growth and colony formation produced by IdUrd was exacerbated by AIdUrd unless quite high concentrations of AIdUrd were used.

The data in Fig. 3 illustrate an interesting example of a synergistic drug interaction. In this experiment, HeLa cells were exposed for 72 hr to 30 μM dThd in the absence or presence of various concentrations of AIdUrd. The combination of dThd (30 μM) and AIdUrd (300 μM) inhibited cell growth by 60%, whereas neither agent alone was toxic. The addition of increasing amounts of AIdUrd inhibited HeLa cell growth in a dose-dependent fashion.

Modulation of nucleoside metabolism. Since the cytotoxicity of dThd and IdUrd is dependent on conversion to their respective triphosphates, the effects of AIdUrd on their metabolism was determined. The enhancement of dThd cytotoxicity produced by AIdUrd (Fig. 3) was associated with a parallel increase in the uptake of exogenous [*methyl*-^{3}H]dThd (Fig. 4). Significant catabolism of dThd to thymine did not occur, and dTTP was the primary thymine-nucleotide present. Radioactive thymine was not detected by thin-layer chromatographic analysis (silica gel) in either the cellular extracts or the tissue culture media following incubation with [*methyl*-^{3}H]dThd. The phosphorylated derivatives and the free nucleoside accounted for more than 95% of the radioactive derivatives in the cell extracts. The increases in thymidine uptake produced by AIdUrd at concentrations

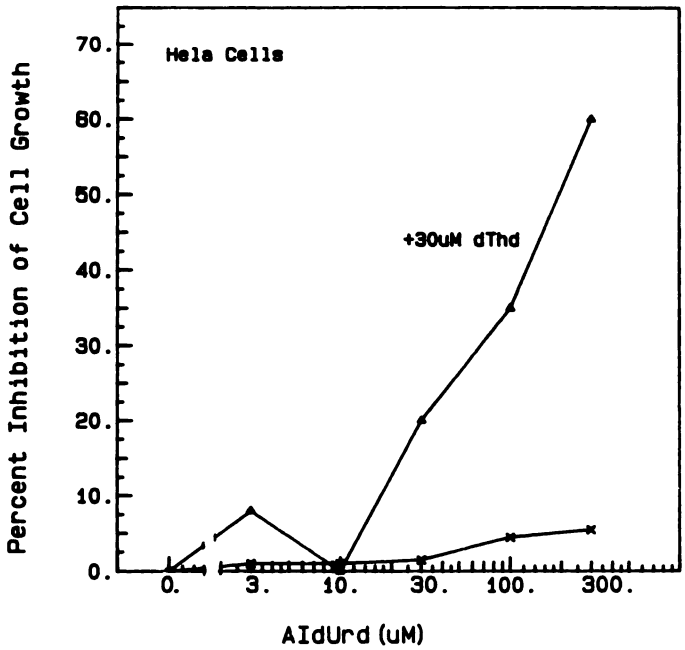

FIG. 3. *Effect of AIdUrd on the cytotoxicity of dThd in HeLa cells*
Exponentially growing HeLa cells were exposed for 72 hr to the indicated concentrations of AIdUrd in the absence (×) or presence of 30 μM dThd (▲). The data are expressed as the percentage inhibition of cell growth; the mean coefficient of variation was 0.09.

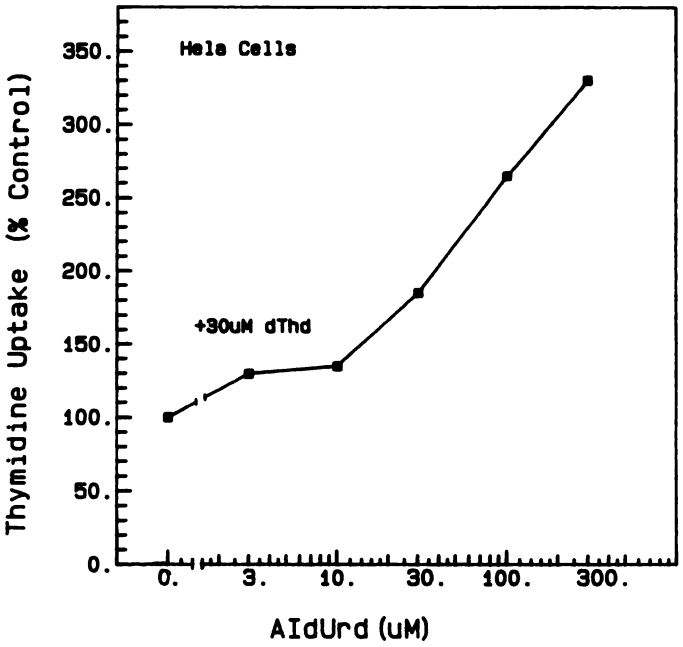

FIG. 4. *Modulation of dThd uptake by AIdUrd*
The influence of AIdUrd on the uptake of 30 μM [*methyl*-^{3}H]dThd into the 60% methanol-soluble portion of exponentially growing HeLa cells was determined. The cells were exposed to [*methyl*-^{3}H]dThd (specific activity = 0.036 Ci/mmole) and the indicated concentrations of AIdUrd for 60 min. The data are expressed as the percentage of uptake obtained in the absence of AIdUrd.

of 30 μM–300 μM correlated well with inhibition of cell growth.

Alterations in IdUrd uptake were also produced by AIdUrd. The accumulation of IdUMP, IdUDP, and IdUTP in HeLa cells exposed to either 3 μM (Fig. 5) or 30 μM (Fig. 6) IdUrd for 4 hr was augmented by AIdUrd. For example, IdUTP pools were elevated 300% by 100 μM AIdUrd in cells treated with 30 μM IdUrd (Fig. 6). AIdUrd produced little change in the relative abundance of the three nucleotides (Figs. 4 and 5). Thus, in contrast to the effects of 5′-aminothymidine (12), AIdUrd did not inhibit the phosphorylation of IdUMP, and the IdUTP pools were maintained. The uptake of IdUrd (30 μM) was also strongly stimulated by AIdUrd in Vero cells (Fig. 7). IdUTP levels were increased over 400% by 300 μM AIdUrd with no evidence of inhibition of IdUMP phosphorylation. In fact, the relative abundance of IdUMP decreased from 70% to 50% in the presence of 300 μM AIdUrd. Iodouracil, as measured by silica gel thin-layer chromatography, was not detected in either the cell extracts or the tissue culture media. These studies indicate that the effects of AIdUrd on IdUrd cytotoxicity in both HeLa and Vero cells can be accounted for, both qualitatively and quantitatively, by modulation of IdUrd metabolism.

Effects on thymidine kinase. AIdUrd inhibited the activity of thymidine kinase in the absence of feedback inhibitors, whereas in their presence enzyme activity was stimulated. This was illustrated by using an enzyme preparation purified from HeLa cells by affinity chromatography. In the absence of IdUTP, the phosphorylation of IdUrd (3, 10, and 30 μM) was inhibited progres-

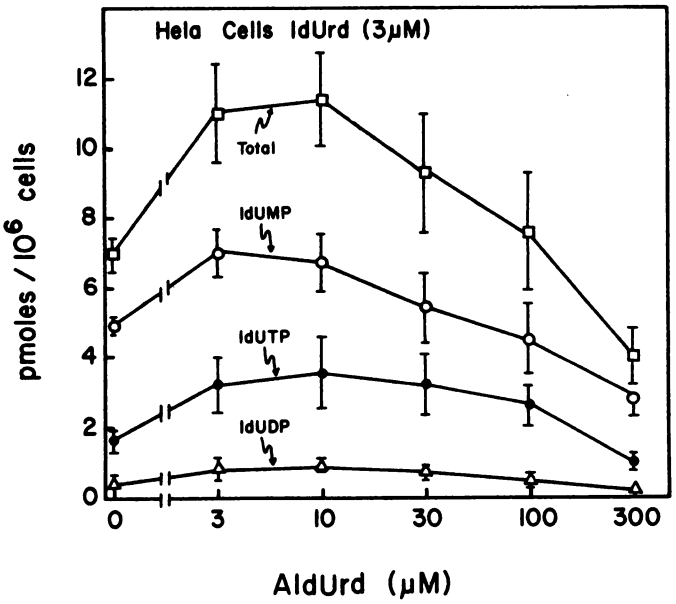

FIG. 5. *Effect of AIdUrd on the uptake and metabolism of 3 μM IdUrd in HeLa cells*

The influence of AIdUrd on the pattern of incorporation of [^{125}I] dUrd into IdUMP, IdUDP, IdUTP, and all three nucleotides is shown. Experimental details for the data presented here and in Figs. 6 and 7 are given under Experimental Procedures. These data are presented as picomoles of nucleotide per 10^6 cells (mean ± standard error, $n = 3$).

sively by increasing concentrations of AIdUrd (Fig. 8). In other experiments, the pattern of inhibition was shown to be competitive and the apparent K_i of AIdUrd was estimated to be 0.7 μM (data not shown). The addition of IdUTP (5 μM) to the reaction mixture dramatically altered the effects of AIdUrd (Fig. 9). Under these conditions, enzyme activity could be markedly stimulated. The feedback inhibition exerted by IdUTP (19) was reduced over a wide concentration range of both the substrate, IdUrd, and the antagonist, AIdUrd. At the highest concentrations of AIdUrd, the stimulatory effects

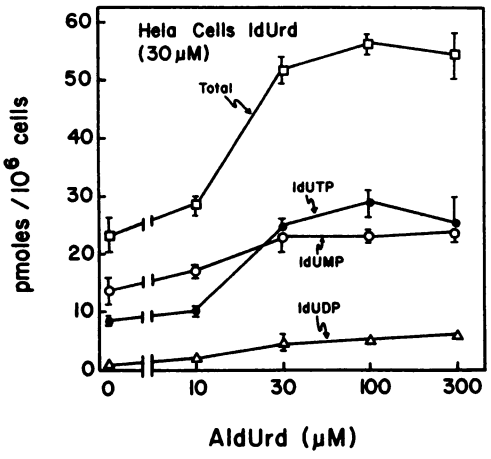

FIG. 6. *Effect of AIdUrd on the uptake and metabolism of 30 μM IdUrd in HeLa cells*

The influence of AIdUrd on the pattern of incorporation of [^{125}I] dUrd into IdUMP, IdUTP, IdUTP, and all three nucleotides is shown. The data are expressed as the mean ± range of two separate experiments.

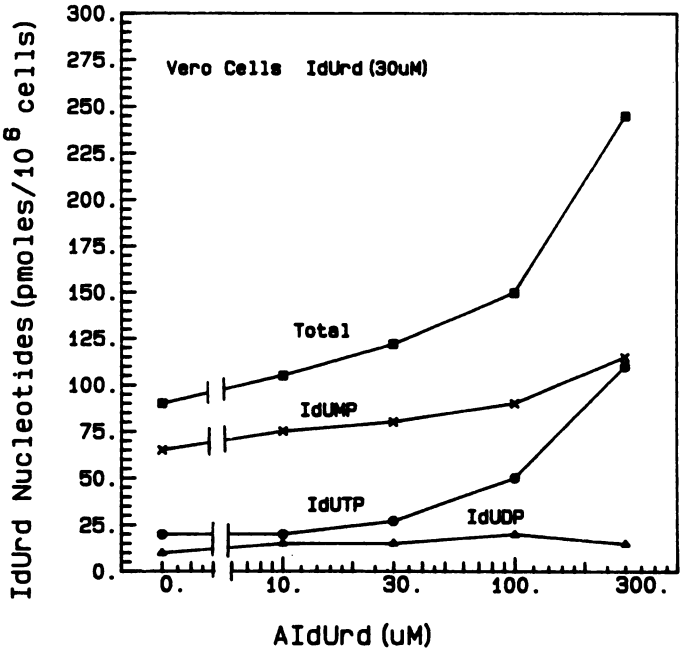

FIG. 7. *Effect of AIdUrd on the uptake and metabolism of 30 μM IdUrd in Vero cells*

The influence of AIdUrd on the pattern of incorporation of [^{125}I] dUrd into IdUMP, IdUDP, IdUTP, and all three nucleotides is shown.

were reversed. Varying the ATP concentration alters the degree of inhibition produced by IdUTP but not that produced by AIdUrd. Thus, the ability of AIdUrd to antagonize IdUTP inhibition was compared at different ATP-Mg^{2+} concentrations (Fig. 10). In the absence of IdUTP, inhibition of thymidine kinase by AIdUrd was approximately 35% at all three concentrations of ATP-

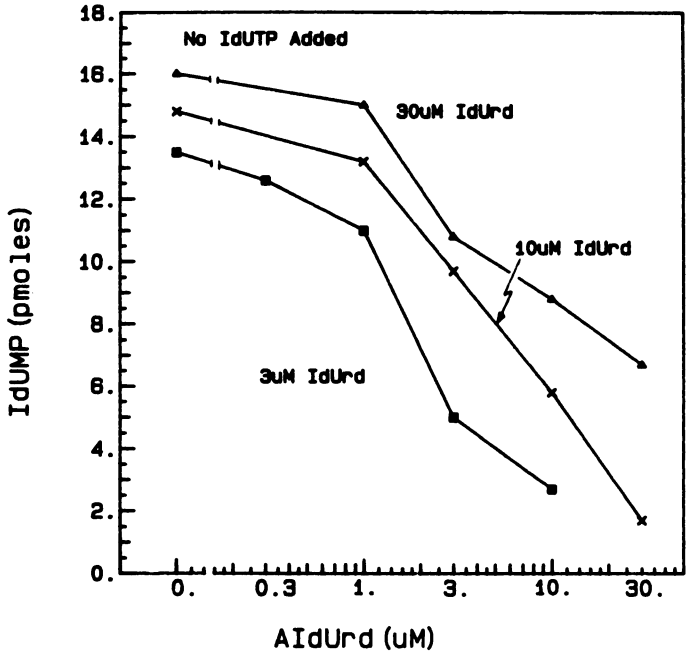

FIG. 8. *Inhibition of IdUrd phosphorylation by AIdUrd*

Thymidine kinase was assayed in a preparation purified from HeLa cells by affinity column chromatography. The influence of AIdUrd on enzyme activity was determined at 3 μM (■), 10 μM (×), and 30 μM (▲) IdUrd.

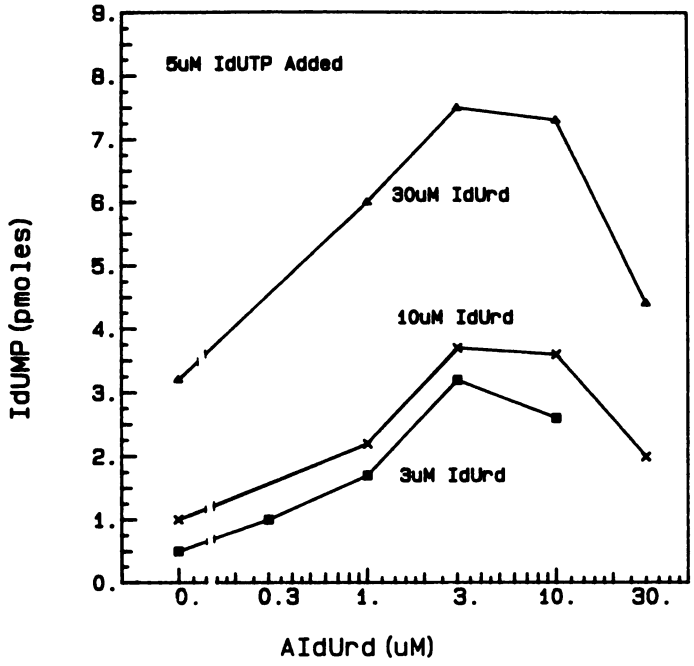

FIG. 9. *Antagonism of feedback inhibition by AIdUrd*
The influence of AIdUrd on thymidine kinase activity was determined at 3 μM (■), 10 μM (×), and 30 μM (▲) IdUrd in the presence of 5 μM IdUTP.

Mg^{2+}, whereas IdUTP inhibition decreased from 95% at 1 mM ATP-Mg^{2+} to 53% at 5 mM ATP-Mg^{2+}. Antagonism of feedback inhibition by AIdUrd was particularly evident at 2.5 mM and 5.0 mM ATP-Mg^{2+}.

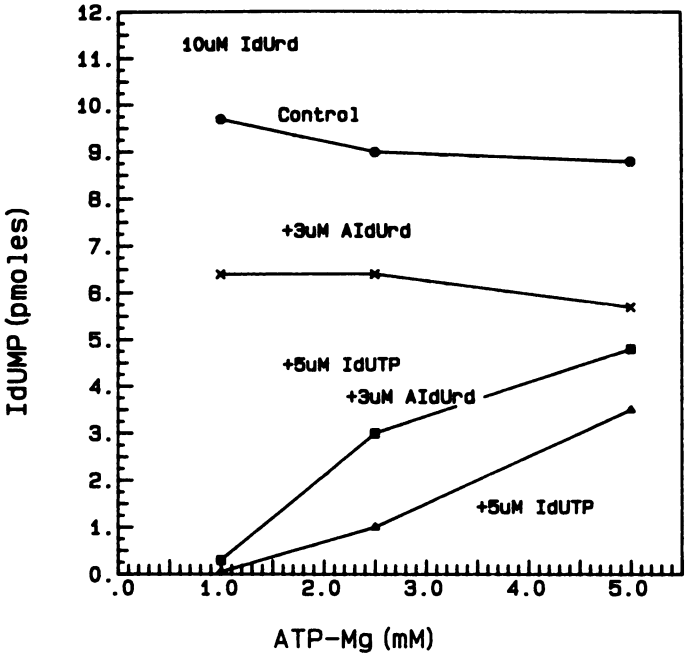

FIG. 10. *Effect of ATP on the antagonism of IdUTP inhibition of thymidine kinase by AIdUrd*
The influence of ATP on the phosphorylation of 10 μM IdUrd by thymidine kinase purified from HeLa cells was determined in the presence of 3 μM AIdUrd (×), 5 μM IdUTP (▲), or both 3 μM AIdUrd and 5 μM IdUTP (■).

DISCUSSION

Our recent findings indicated that 5′-aminothymidine can stimulate the activity of thymidine kinase by antagonizing feedback inhibition (11, 12). As a consequence, the phosphorylation and cytotoxicity of dThd and IdUrd are potentiated. Since the use of antagonists of feedback inhibition represents a new approach to increase the activation of numerous chemotherapeutic agents (11), studies of the structural constraints of such compounds were initiated. This investigation dealt with the effects of AIdUrd, the 5-iodo analogue of 5′-aminothymidine, on the metabolism of dThd and IdUrd. Our results indicated that AIdUrd effectively antagonized the feedback inhibition of thymidine kinase and thereby stimulated the phosphorylation of IdUrd and dThd and elevated the intracellular levels of IdUTP and dTTP. These metabolic effects accounted for the ability of AIdUrd to enhance the cytotoxicity of dThd and IdUrd.

Several lines of evidence support these conclusions. Experiments utilizing a preparation of thymidine kinase purified from HeLa cells illustrated how IdUTP, a known feedback inhibitor (19), critically influences the effect of AIdUrd on the enzyme's activity (Figs. 8–10). In the absence of IdUTP, AIdUrd potently inhibited thymidine kinase activity; however, stimulation of enzyme activity was produced if the reaction was allowed to proceed in the presence of a feedback inhibitor. The de-inhibition of thymidine kinase seen in these experiments was sufficient to account for the increase in IdUrd uptake produced by AIdUrd in the HeLa and Vero cells (Figs. 5–7). In addition, the concentration-response curves for the modulation of IdUrd uptake in the intact cells and for the effect of AIdUrd on thymidine kinase activity in the presence of IdUTP were quite similar.

The effects of AIdUrd on the uptake and metabolism of IdUrd and dThd were consistent with perturbations of thymidine kinase activity. Little or no change in the relative distribution of the various phosphorylated derivatives was produced by AIdUrd. Thus, in contrast to the inhibition of IdUMP phosphorylation produced by 5′-aminothymidine (12), elevated intracellular levels of IdUTP were produced by a wide range of AIdUrd concentrations (Figs. 6 and 7). In addition, Vero and HeLa cells did not significantly catabolize IdUrd or dThd, and so inhibition of nucleoside phosphorylase activity by AIdUrd does not appear to be necessary for the stimulation of nucleoside uptake. Additionally, under conditions of the thymidine kinase assay, conversion of thymidine to thymine was not detected. Thymidine phosphorylase is not retarded by the affinity column used for the purification of thymidine kinase (18). The fact that thymidine kinase activity was stimulated by AIdUrd only in the presence of dTTP also argues strongly against an important interaction with nucleoside phosphorylase. The uptake of IdUrd (3 μM) was modulated in a biphasic manner by AIdUrd (Fig. 5). This pattern of interaction corresponded closely, both qualitatively and quantitatively, with the results obtained using a purified preparation of thymidine kinase in which IdUTP had been added to the reaction mixture (Fig. 9). At higher concentrations of AIdUrd, both the stimulation of IdUrd (3 μM)

uptake in intact cells (Fig. 5) and the antagonism of IdUTP inhibition of thymidine kinase (Fig. 9) were decreased. Presumably, binding of AIdUrd at the active site of thymidine kinase ($K_i = 0.7\ \mu M$) accounts for both effects. However, it is important to note that AIdUrd is not a substrate of the mammalian thymidine kinase. Chen *et al.* (20) demonstrated that AIdUrd was phosphorylated only in herpes simplex-infected cells. Thus, in uninfected cells, only AIdUrd—but not any of its phosphorylated derivatives—would perturb IdUrd metabolism.

The cytotoxic effects produced by IdUrd or dThd in combination with AIdUrd were also consistent with the alterations in nucleoside metabolism. Incorporation of IdUrd into DNA appears to be a critical event in the inhibition of cellular replication (21), and IdUTP is the key fraudulent substrate for DNA polymerase. The levels of this metabolite correlated well with the lethality produced by IdUrd in these experiments. In particular, note the similarities in the modulation of IdUTP levels in Figs. 5 and 6 with the changes in cell viability shown in Fig. 2. Similarly, a good correlation between increases in dThd uptake (Fig. 4) and inhibition of HeLa cell replication (Fig. 3) was illustrated. Thus, the data presented in this paper support the hypothesis that AIdUrd exacerbates the toxicity of IdUrd and dThd by increasing the phosphorylation of these nucleosides by thymidine kinase. Furthermore, the mechanism appears to involve antagonism of the feedback inhibition normally exerted by dTTP and IdUTP. The results indicate that, although modification of 5′-aminothymidine with a 5-iodo group does not alter its ability to antagonize feedback inhibition, inhibition of IdUMP phosphorylation is eliminated. This is not surprising since IdUTP is an excellent inhibitor of thymidine kinase (19) and IdUrd is an avid substrate of the enzyme (6), whereas IdUMP is a poor substrate for TMP kinase (12, 22, 23). Thus, it appears that AIdUrd shares some of the characteristics of other 5-iodo-substituted analogues.

The use of 5′-aminothymidine, AIdUrd or other regulatory antagonists may be useful additions in viral and cancer chemotherapy and provide a means of selectively increasing drug activation. Our preliminary findings indicate that responses to regulatory antagonists vary markedly among different types of cells. Of particular interest is our observation that several normal cell types are only minimally affected by these antagonists. We are currently investigating the mechanisms which account for these differences and exploring their potential for chemotherapeutic exploitation.

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