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METABOLISM AND MODE OF SELECTIVE INHIBITION OF HUMAN IMMUNODEFICIENCY VIRUS REPLICATION BY 3′-AZIDO-2′,3′-DIDEOXY-5-IODOURIDINE AND 3′-AZIDO-2′,3′-DIDEOXY-5-BROMOURIDINE

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Abstract—3′-Azido-2′,3′-dideoxy-5-iodouridine (AzIdUrd) and 3′-azido-2′,3′-dideoxy-5-bromouridine (AzBdUrd), previously shown to be potent and selective inhibitors of human immunodeficiency virus replication *in vitro* were minimally toxic to the uninfected human lymphoid cell line H9 (IC_{50} = 197 and 590 μM, respectively). Both compounds strongly inhibited the incorporation of [^{3}H]thymidine but not [^{3}H]deoxyadenosine into DNA, and we observed no significant inhibition of [^{3}H]uridine incorporation into RNA or [^{3}H]amino acid incorporation into protein. Exposure of H9 cells to AzIdUrd or AzBdUrd (100 μM, 24 hr) and pulse-labeling with [^{3}H]thymidine resulted in approximately 80% reduction in levels of tritiated dTMP, dTDP, and dTTP relative to control. [^{125}I]AzIdUrd was phosphorylated rapidly in H9 cells with the monophosphate accounting for over 90% of total soluble radioactivity. A relatively low but stable level of AzIdUTP was maintained over a 12-hr period. [^{125}I]AzIdUrd was phosphorylated by a cell free extract of H9 cells at a rate approximately three times that of thymidine and its phosphorylation was inhibited by excess thymidine. AzIdUrd was found to be a competitive inhibitor of cytosolic thymidine kinase with a K_i of 2.63 μM and AzIdUMP a weak competitive inhibitor of thymidylate kinase with a K_i of 55.3 μM. Both AzIdUTP and AzBdUTP were potent competitive inhibitors of HIV-1 reverse transcriptase (K_i = 0.028 and 0.043 μM, respectively) and relatively poor inhibitors of H9 cell DNA polymerase α (K_i = 42.0 and 42.7 μM, respectively). Thus, the high therapeutic index of these compounds is due to the sensitivity of the viral reverse transcriptase, coupled with the relative insensitivity of the host cell DNA polymerase α.

The acquired immune deficiency syndrome (AIDS) is a disease characterized by extensive immunosuppression that predisposes patients to life-threatening opportunistic infections and unusual forms of neoplasms. The retrovirus human immunodeficiency virus (HIV-1)† is the etiologic agent responsible for AIDS [1, 2]. Various nucleoside analogs have been found to inhibit the replication and cytopathic effects of HIV-1 in cell culture including 3′-azido-3′-deoxythymidine (AZT, Zidovudine) [3], and 2′,3′-dideoxyinosine (ddI) [4], 2′,3′-dideoxycytidine [4, 5], 2′,3′-dideoxycytidin-2′-ene [6–9], ribavirin [10, 11], 3′-fluoro-3′-deoxythymidine [12–14], 3′-azido-2′,3′-dideoxyuridine [15, 16], 3′-deoxythymidin-2′-ene (d4T) [7, 9, 17, 18], and 2′,3′-dideoxyguanosin-2′-ene [19]. Of these, AZT and ddI have been approved for the therapy of AIDS in patients; however, their use is complicated by unfavorable toxicities such as AZT-induced bone marrow suppression [20] and ddI-induced peripheral neuropathy and pancreatitis [21]. Thus, there is a need for agents which possess improved therapeutic efficacy and decreased toxicities.

We previously reported that the thymidine analogues 3′-azido-2′,3′-dideoxy-5-iodouridine (AzIdUrd) and 3′-azido-2′,3′-dideoxy-5-bromouridine (AzBdUrd) are active against Moloney-murine leukemia virus (M-MuLV) with EC_{50} values of 1.5 and 3.0 μM, respectively, in SC-1 cells [22], and against HIV-1 with EC_{50} values of 1.1 and 1.0 μM, respectively, in human peripheral blood mononuclear cells [16]. AzBdUrd also has been tested *in vitro* against HIV-1 in HUT 78 cells with an EC_{50} of 2.3 μM [22]. The present report describes the cellular metabolism of AzIdUrd and a study of the biochemical events relating to the mechanism whereby these two compounds exert their biological actions.

MATERIALS AND METHODS

Chemicals. AzBdUrd [22] and AzIdUrd [22] were

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† Abbreviations: HIV-1, human immunodeficiency virus (type 1); AMV, avian myeloblastosis virus; RT, reverse transcriptase; AzIdUrd, 3′-azido-2′,3′-dideoxy-5-iodouridine; AzBdUrd, 3′-azido-2′,3′-dideoxy-5-bromouridine; AZT, 3′-azido-3′-deoxythymidine; ddI, 2′,3′-dideoxyinosine; d4T, 3′-deoxythymidin-2′-ene; dThd, thymidine; dAdo, 2′-deoxyadenosine; Urd, uridine; AzIdUMP, AzIdUDP and AzIdUTP, 3′-azido-2′, 3′-dideoxy-5-iodouridine mono-, di- and triphosphates; AzBdUMP, AzBdUDP and AzBdUTP, 3′-azido-2′,3′-dideoxy-5-bromouridine mono-, di- and triphosphates; EC_{50}, the concentration of a compound required to inhibit virus replication by 50%; IC_{50}, the concentration of a compound required to reduce the cell number in an uninfected cell culture by 50%; TCA, trichloroacetic acid; and PBS, phosphate-buffered saline.

prepared by published procedures, and their 5'-mono- and triphosphate derivatives were synthesized by P. Chang by the method of Yoshikawa *et al.* [23] and Hoard and Ott [24]. Optifluor and Instafluor scintillation fluids and Soluene tissue solubilizer were obtained from the Packard Instrument Co. (Downers Grove, IL) and the template-primer poly $(rA)_n$ oligo$(dT)_{12-18}$ was obtained from Pharmacia P-L Biochemicals (Piscataway, NJ). Thymidine-Sepharose affinity matrix was a gift of Y-C. Cheng.

Radiochemicals. [^{3}H]Amino acid mixture (33 Ci/mmol) was obtained from the Amersham Corp. (Arlington Heights, IL); [^{3}H-*methyl*]thymidine (dThd) (89 Ci/mmol), [2,8-^{3}H]deoxyadenosine (dAdo) (36 Ci/mmol), [6-^{3}H]uridine (Urd) (50 Ci/mmol) and [^{3}H-*methyl*]dTTP (26 Ci/mmol) were obtained from ICN Biomedicals Inc. (Irvine, CA); [5-^{125}I]AzIdUrd (4.5 mCi/mmol) was synthesized as described [25].

Cells. H9 cells were grown in RPMI 1640 medium containing 1% fetal bovine serum, 1% newborn calf serum, 2 mM L-glutamine, 1 mM HEPES buffer (pH 7.4), and 1% HL-1 Supplement (Ventrex Laboratories, Portland, ME), as previously described [26].

Cell growth assay. The effect of AzBdUrd or AzIdUrd on cell growth was assessed by measuring the inhibition of cell proliferation over a 72-hr period. H9 cells (5×10^4 cells/mL) were cultured in medium which contained various concentrations of test compounds, and the cell number was determined each day for 3 days on a model ZM Coulter Counter/model 256 Channelyzer (Coulter Electronics, Hialeah, FL). The IC_{50} values were determined graphically.

Incorporation of radiolabeled precursors into DNA, RNA, or protein. Into a flask containing H9 cells (2×10^5 cells/mL) and 100 μM AzIdUrd or AzBdUrd was introduced [^{3}H]dThd (1.5 μCi/mL), [^{3}H]dAdo (1.5 μCi/mL), [^{3}H]Urd (1.5 μCi/mL) or [^{3}H]amino acids (2.0 μCi/mL) at time 0. At various time points, 2 mL was removed and the cells were washed twice in ice-cold phosphate-buffered saline (PBS). The cell pellet was resuspended in 500 μL of 5 mM EDTA in PBS and 25 μL of the cell suspension was transferred in quadruplicate to Whatman No. 1 filter paper discs. The discs were dropped immediately into ice-cold 5% trichloroacetic acid (TCA) and were washed twice in cold 5% TCA (10 mL/disc for 10 min). The filters were dehydrated in 95% ethanol and air dried, and the radioactivity was determined in 5 mL Optifluor.

Enzymes. Extracts of H9 cells were prepared by three freeze–thaw cycles in 4 vol. of extraction buffer (10 mM Tris–HCl, pH 7.5, 10 mM KCl, 1 mM $MgCl_2$, 3 mM dithiothreitol) followed by centrifugation at 10,000 *g* as described by Furman *et al.* [27]. Cytosolic thymidine kinase (EC 2.7.1.21) and thymidylate kinase (EC 2.7.4.9) from H9 cells were prepared by affinity chromatography as described by Cheng and Ostrander [28]. DNA polymerase α was prepared as described [29, 30]. Avian myeloblastosis virus-reverse transcriptase (AMV-RT) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), and HIV-RT was a gift from the Bristol Myers-Squibb Co. (Wallingford, CT).

Enzyme assays. Phosphorylation of [^{3}H]dThd and [^{125}I]AzIdUrd by a cell-free extract was measured by a DEAE-filter disc method as described [27]. Standard reaction mixtures contained 50 mM Tris–HCl (pH 7.5), 5 mM ATP-Mg^{2+}, 10 μM [^{3}H]dThd or [^{125}I]AzIdUrd, the indicated concentration of unlabeled dThd or AzIdUrd, and cell extract in a total volume of 50 μL. Assays were incubated at 37° for 30 min, and terminated by spotting 35 μL onto a DE-81 filter disc. The discs were washed four times for 5 min each in H_2O and twice in 95% ethanol, and the dried discs were counted in 5 mL Instafluor + 3% Soluene tissue solubilizer. The activity of purified H9 thymidine kinase was measured as described above, except that the standard reaction mixture contained: 50 mM Tris–HCl (pH 7.5), 2 mM ATP-Mg^{2+}, various concentrations of labeled and unlabeled dThd and AzIdUrd, and enzyme in a total volume of 50 μL. The enzyme activity was proportional to enzyme concentration and time of reaction for the experiments described.

Measurements of H9 cell thymidylate kinase were carried out according to Lee and Cheng [31], and the standard reaction mixtures contained 50 mM HEPES (pH 7.7), 2.7 mg/mL phosphocreatine, 88.2 μg/mL phosphocreatine kinase, 6.7 U/mL nucleoside-5'-diphosphate kinase (Sigma Chemical Co., St. Louis, MO), 0.88 mg/mL bovine serum albumin fraction V, 2.2 mM dithiothreitol, 2 mM ATP, 2 mM $MgCl_2$, various concentrations of [^{14}C]-dTMP (5 cpm/pmol) and 3'-azido -2',3'-dideoxy-5-iodouridine monophosphate (AzIdUMP), and enzyme in a total volume of 50 μL.

HIV-1 reverse transcriptase activity was determined by the method of Cheng *et al.* [32]: 50 mM Tris–HCl (pH 8.5), 2 mM dithiothreitol, 8 mM $MgCl_2$, 100 mM KCl, various concentrations of [^{3}H]dTTP (1500 cpm/pmol) and AzIdUTP or 3'-azido-2',3'-dideoxy-5-bromouridine triphosphate (AzBdUTP), 0.5 O.D.$_{260}$ U/mL of template-primer poly $(rA)_n$ $(dT)_{12-18}$ and 0.05 U of enzyme (total volume = 50 μL). The assay conditions for AMV-RT were as described by Cheng *et al.* [32]: 50 mM Tris–HCl (pH 8.5), 10 mM $MgCl_2$, 40 mM KCl, various concentrations of [^{3}H]dTTP (1500 cpm/pmol) and AzIdUTP or AzBdUTP, and 0.5 O.D.$_{260}$ U/mL poly $(rA)_n$ $(dT)_{12-18}$. The assay for DNA polymerase α was performed as described by Elion *et al.* [33] and Furman *et al.* [34]: 50 mM Tris–HCl (pH 8.5), 12 mM $MgCl_2$, 1.2 mM dithiothreitol, 100 μM each dATP, dCTP, dGTP and [^{3}H]dTTP (30–60 cpm/pmol), plus 0.25 mg/mL activated calf thymus DNA, and various concentrations of AzIdUTP or AzBdUTP.

All three polymerase assays were incubated for 30 min at 37°, and terminated by spotting 35 μL of the reaction mixture on a DE-81 filter disc. The discs were washed six times for 5 min each in 5% Na_2HPO_4 (10 mL/disc), rinsed with H_2O, and dehydrated in 95% ethanol; the radioactivity on the dried filters was determined in 3% Soluene tissue solubilizer in Instafluor after standing overnight.

High pressure liquid chromatographic analysis. The 60% methanol extracts of H9 cells treated with [^{125}I]AzIdUrd or [^{3}H]dThd were analyzed by anion exchange HPLC with a Whatman Radial PAK

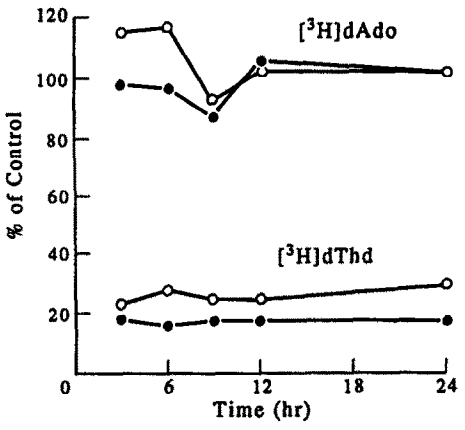

Fig. 1. Effects of AzIdUrd and AzBdUrd on cellular DNA synthesis. H9 cells in log phase (2×10^5 cells/mL) were incubated for 24 hr in the presence and absence of 100 μM AzBdUrd (○) or AzIdUrd (●) with the indicated radiolabeled precursor, and incorporation into acid-precipitable material was determined as described in Materials and Methods. Control values for incorporation were as follows (in cpm/10^5 cells): for dAdo incorporation 59,300, 82,000, 101,600, 121,000 and 158,000 at 3, 6, 9, 12, and 24 hr, respectively; and for dThd incorporation 238,000, 450,000, 585,000, 805,000 and 872,000 at 3, 6, 9, 12 and 24 hr, respectively. Data points are the averages of 3–5 determinations.

Cartridge 8P SAX10 column. Elution was with a linear gradient of $(NH_4)H_2PO_4$, 0.01 to 0.4 M at pH 4.0 to 5.0 over 90 min, at a flow rate of 2 mL/min. Fractions (4 mL) were collected every 2 min, and aliquots of each fraction were counted for radioactivity. The radioactive metabolites were identified by comparing their retention times with those of authentic AzIdUrd, AzIdUMP, AzIdUTP, dThd, dTMP, dTDP, and dTTP.

RESULTS

Effects of AzIdUrd and AzBdUrd on H9 cell growth and macromolecular synthesis. The effects of AzIdUrd and AzBdUrd on the replication of H9 cells were measured after 72 hr of incubation. The inhibition of cell proliferation was proportional to concentration over the range of 12.5 to 400 μM (data not shown). The IC_{50} values of AzIdUrd and AzBdUrd were 197 and 590 μM when H9 cells were seeded initially at 5×10^4 cells/mL.

The effects of AzIdUrd and AzBdUrd on the synthesis of RNA, DNA, and proteins in H9 cells were investigated. The incorporation of [^{3}H]dThd by H9 cells incubated with 100 μM AzIdUrd or AzBdUrd was inhibited markedly to approximately 20% of control (Fig. 1). This inhibitory effect appeared early (within the first 3 hr of incubation) and remained at this level for 24 hr. In contrast, no significant inhibition of [^{3}H]dAdo incorporation was observed (Fig. 1). No significant inhibition of [^{3}H]-Urd or [^{3}H]amino acid incorporation was observed (data not shown).

Effects of AzIdUrd and AzBdUrd on thymidine metabolism. H9 cells were treated with 100 μM AzIdUrd or AzBdUrd for 24 hr with a 1-hr pulse of [^{3}H]dThd in the last hour resulting in a decrease in the cell-associated levels of labeled dThd to 61.6 and 47.7% of control, respectively (Fig. 2). The levels of [^{3}H]dTMP, dTDP and dTTP were decreased markedly by AzIdUrd (to 7, 15, and 10% of control). AzBdUrd inhibited the phosphorylation of thymidine into mono-, di-, and triphosphates to a similar extent (5, 9, and 8% of control, respectively) (Fig. 2).

Metabolism of AzIdUrd. [^{125}I]AzIdUrd was phosphorylated rapidly in H9 cells. Within 1 hr, 282 pmol/10^6 cells of [^{125}I]AzIdUMP and 0.38 pmol/10^6 cells of [^{125}I]AzIdUTP were detected following incubation with 20 μM [^{125}I]AzIdUrd (Table 1). [^{125}I]AzIdUDP was not detected in our system, presumably due to its low concentration. The main metabolic product detected was AzIdUMP which accounted for over 90% of total soluble radioactivity. The level of the monophosphate decreased gradually from 282 pmol/10^6 cells to 154 pmol/10^6 cells during the 12-hr incubation. The level of triphosphate was quite stable and remained at 0.31 to 0.38 pmol/10^6 cells during the 12-hr incubation. Radioactivity from [^{125}I]AzIdUrd was also present in the methanol-insoluble fractions, suggesting incorporation into DNA; however, the low

Table 1. Anabolism of [^{125}I]AzIdUrd in H9 cells*

Time (hr)	pmol/10^6 cells			
	Monophosphate	Diphosphate	Triphosphate	MeOH-insoluble
1	282 (270)†	ND‡	0.38 (0.36)	0.43
3	267 (255)	ND	0.37 (0.35)	0.56
6	228 (217)	ND	0.33 (0.31)	0.36
12	154 (147)	ND	0.31 (0.30)	0.42

* H9 cells (10^6 cells/mL) were incubated with 20 μM [^{125}I]AzIdUrd (specific activity: 4.5 mCi/mmol), and a 60% methanol extract was prepared [26]. The methanol-soluble fraction was analyzed by anion exchange HPLC as described in Materials and Methods.

† The number in parentheses indicates the intracellular micromolar concentration of metabolite, calculated using an average cell volume of 10.5 μL/10^7 cells [26].

‡ Not detected.

Table 2. Phosphorylation of dThd and AzIdUrd by cell-free extracts of H9 cells*

[^{3}H]dThd (μM)	AzIdUrd (μM)	pmol/min/mg protein	% of Control
10	0	22.9	100.0
10	10	22.7	99.6
10	50	16.3	71.3
10	100	11.6	50.6
10	200	5.5	24.1
10	400	3.7	16.9
dThd (μM)	**[^{125}I]AzIdUrd (μM)**	**pmol/min/mg protein**	**% of Control**
0	10	68.2	100.0
10	10	63.4	93.0
50	10	30.3	44.4
100	10	20.2	29.6
200	10	11.6	17.1
400	10	7.5	10.9

* Assays were performed as described in Materials and Methods. Protein determinations were performed by the Coomassie Blue dye binding assay (Bio-Rad Laboratories, Richmond, CA). Results are averages of at least 3 experiments.

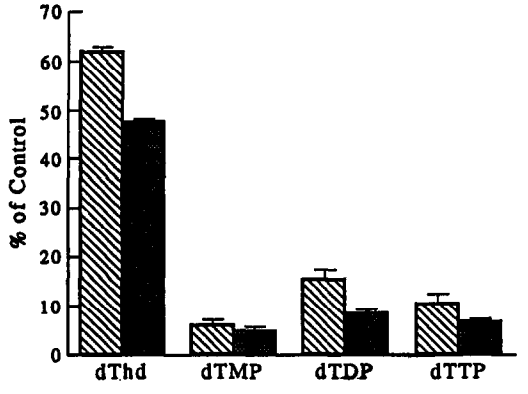

Fig. 2. Effects of AzIdUrd and AzBdUrd on thymidine metabolism. H9 cells in log phase were exposed to 100 μM AzIdUrd (▨), or 100 μM AzBdUrd (■) for 24 hr, and pulse-labeled during the last hour with 100 μCi [^{3}H]dThd (74 Ci/mmol). The 60% methanol extracts were resolved by anion exchange HPLC (see Materials and Methods). Control values were as follows (cpm/10^6 cells): dTMP, 76,200; dTDP, 30,400; and dTTP, 106,000. Data points are the means ± SD of 3–5 determinations.

level of radioactivity present precluded complete characterization of the extent of incorporation.

Phosphorylation of [^{125}I]AzIdUrd by H9 cell extracts and purified enzymes. [^{3}H]dThd and [^{125}I]-AzIdUrd (10 μM), when incubated with a cell-free extract of H9 cells, were phosphorylated at the rate of 22.9 and 68.2 pmol/min/mg protein, respectively (Table 2). The phosphorylation of [^{125}I]AzIdUrd was inhibited by thymidine, and [^{3}H]dThd phosphorylation was inhibited by AzIdUrd. Thymidine kinase and thymidylate kinase were purified from H9 cells as described in Materials and Methods. Kinetic studies gave the K_m values of 2.06 and 5.06 μM for thymidine and thymidylate, respectively (Table 3). These K_m values were comparable to those reported by Furman *et al.* [34]. Inhibition kinetic studies with thymidine kinase using AzIdUrd (5–15 μM) are shown in Fig. 3A, and it was found to be a competitive inhibitor of dThd phosphorylation with a K_i = 2.63 μM (Table 3). AzIdUMP was found to be a very weak competitive inhibitor of thymidylate phosphorylation by thymidylate kinase (Fig. 3B), with a K_i = 55.3 μM (Table 3).

Effects of AzBdUTP and AzIdUTP on retroviral RT and cellular DNA polymerase α. AzIdUTP and AzBdUTP were potent competitive inhibitors of dTTP incorporation by either HIV-1-RT or AMV-

Table 3. Kinetic values for the inhibition of thymidine and thymidylate kinase by AzIdUrd and AzIdUMP

Enzyme	Substrate	Inhibitor	K_m (μM)	K_i (μM)
Thymidine kinase	[^{3}H]dThd	AzIdUrd	2.06 ± 0.29	2.63 ± 0.51
Thymidylate kinase	[^{14}C]dTMP	AzIdUMP	5.06 ± 0.61	55.3 ± 7.1

Values are means ± SD of 3–7 determinations. K_i values were determined by replotting the Lineweaver–Burk data (Fig. 3) in the slope versus intercept form.

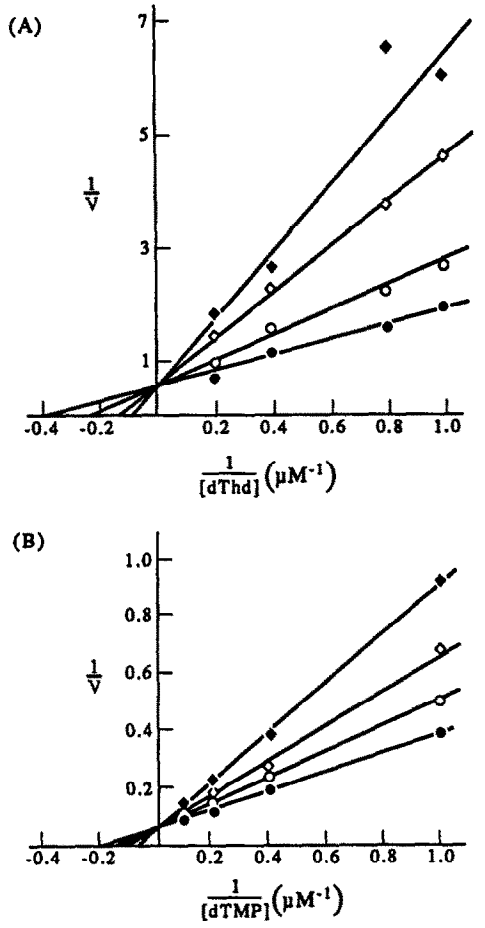

Fig. 3. Double-reciprocal plots of the effects of AzIdUrd and AzIdUMP on H9 cell thymidine kinase (A) and thymidylate kinase (B), respectively. (A): AzIdUrd at 0 (●), 5 (○), 10 (◊), and 15 (♦) μM; (B): AzIdUMP at 0 (●), 20 (○), 40 (◊), and 80 (♦) μM. Data points are the averages of 4–7 determinations.

RT with poly $(rA)_n$ $(dT)_{12-18}$ as the template (Fig. 4). The K_i values (Table 4) were: AzIdUTP, 0.028 μM for HIV-1 RT and 0.65 μM for AMV-RT; AzBdUTP, 0.043 μM for HIV-1 RT and 0.32 μM for AMV-RT. With purified H9 cell DNA polymerase α, activated calf thymus as the template, and [^{3}H]-dTTP as substrate, the K_i values were 42.0 and 42.7 μM for AzIdUTP and AzBdUTP, respectively (Table 4). Thus, the host cell DNA polymerase α is several orders of magnitude less sensitive than are the viral DNA polymerases to the inhibitory effects of these compounds.

Octanol/H_2O partition coefficients. Partition coefficients in *n*-octanol/10 mM potassium phosphate (pH 7.4) of AzIdUrd and AzBdUrd were determined by the procedure of Lin [36]. The coefficients of 3.43 ± 0.11 and 1.89 ± 0.03 (mean ± range) for AzIdUrd and AzBdUrd, respectively, indicate that these compounds were markedly more lipophilic than AZT (0.98 ± 0.06 [37]).

DISCUSSION

Previous studies from this laboratory have shown that AzIdUrd and AzBdUrd are potent inhibitors of HIV-1 and M-MuLV replication *in vitro* [16, 22]. Both compounds displayed low toxicity toward the uninfected human lymphocytic cell line H9, with IC_{50} values of 197 and 590 μM, respectively. The 3′-azido-5-halo compounds (100 μM) inhibited dThd incorporation into DNA by approximately 80% (Fig. 1), but had no significant effect on the incorporation of [^{3}H]dAdo into cellular DNA, suggesting that the primary effect of these compounds in uninfected H9 cells is an inhibition of the dThd salvage pathways and not an inhibition of DNA synthesis at the polymerase level. In addition, no significant inhibition of RNA or protein synthesis by either compound was observed.

A further examination of the effects of AzIdUrd and AzBdUrd on the metabolism of dThd (Fig. 2) revealed that both compounds strongly inhibited the phosphorylation of [^{3}H]dThd in intact H9 cells, with approximately a 90% reduction in the intracellular level of tritiated dTMP, dTDP, and dTTP relative to non-drug-treated cells. In a similar assay, we previously showed that AZT treatment of H9 cells resulted in an accumulation of [^{3}H]dTMP and a reduction in [^{3}H]dTDP and -dTTP levels [38]. Furman *et al.* [27] found AzdTMP to be an inhibitor of dTMP-kinase, which would account for the accumulation of dTMP in its presence. In the present experiments, we observed no accumulation of [^{3}H]-dTMP, suggesting that AzIdUrd and AzBdUrd, unlike AZT, exert a primary inhibitory effect at the level of dThd kinase.

Direct phosphorylation of [^{125}I]AzIdUrd was catalyzed by cell-free extracts of H9 cells (Table 2) at a rate approximately three times that of [^{3}H]dThd. Since unlabeled dThd inhibited the phosphorylation of [^{125}I]AzIdUrd and unlabeled AzIdUrd inhibited the phosphorylation of [^{3}H]dThd, the same enzyme is presumably responsible for both phosphorylations. Studies with a purified preparation of H9 cell thymidine kinase show AzIdUrd to be a potent competitive inhibitor of dThd phosphorylation. As predicted above, AzIdUMP was only a weak competitive inhibitor of dTMP phosphorylation by purified H9 cell dTMP-kinase.

Direct study of the metabolism of AzIdUrd was facilitated by the synthesis of the compound bearing an [^{125}I]-label [25]. As shown in Table 1, [^{125}I]-AzIdUrd was phosphorylated rapidly to the 5′-monophosphate by H9 cells. Using an average cell volume of 10.5 μL/10^7 cells [26], the calculated concentration of AzIdUMP was approximately 270 μM after 1 hr of incubation, which is over 10-fold greater than extracellular nucleoside. Subsequent phosphorylation to the 5′-triphosphate also occurred, and the intracellular triphosphate concentration remained low but stable over a 12-hr exposure (0.30 to 0.36 μM). We were unable to detect AzIdUDP in our cell extract, presumably due to its presence in amounts below our detection limits. Thus, the rate-limiting metabolic step appears to be the phosphorylation of AzIdUMP to the diphosphate.

Small amounts of radioactivity from [^{125}I]AzIdUrd

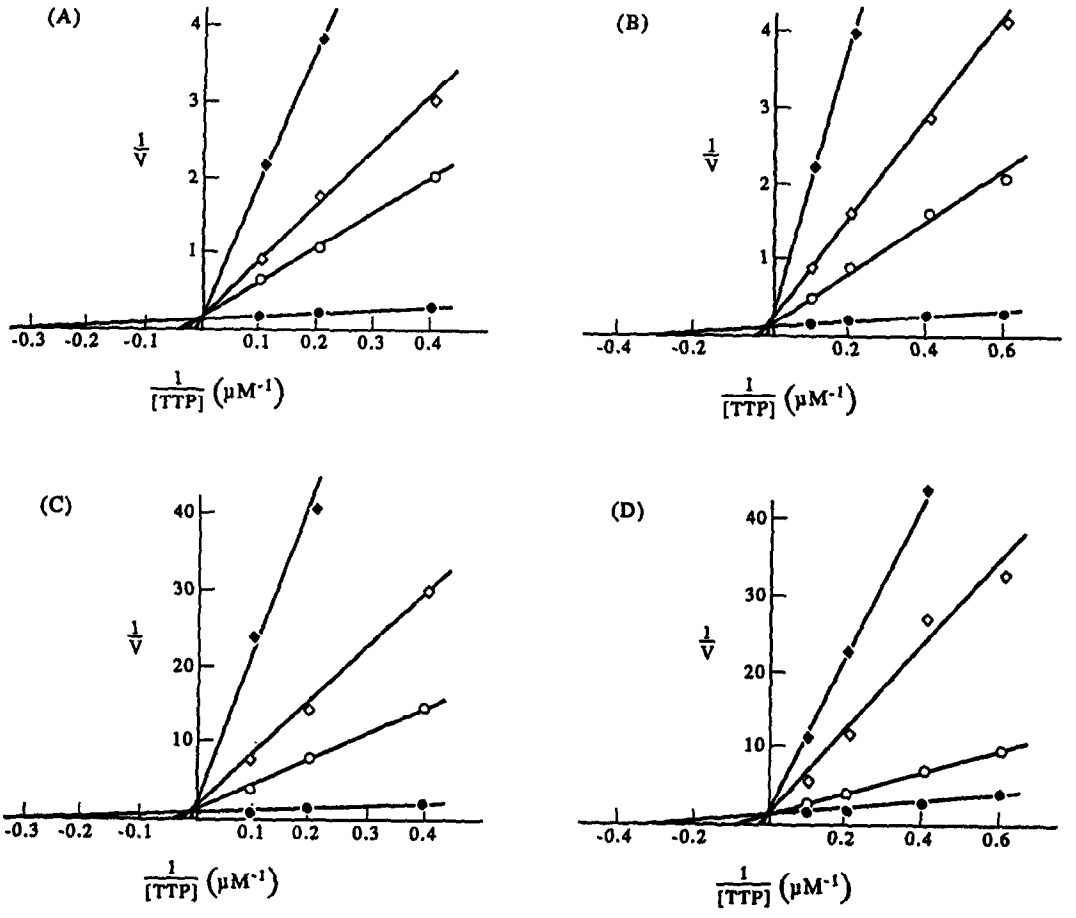

Fig. 4. Double-reciprocal plots of the effects of AzIdUTP and AzBdUTP on the activity of HIV-RT (A and B) and AMV-RT (C and D). Assays were performed as described in Materials and Methods with $(rA)_n$ $(dT)_{12-18}$ as the template and [^{3}H]*d*TTP concentrations as shown. (A): 0 (●), 0.25 (○), 0.5 (◊), and 2 (♦) μM AzIdUTP; (B): 0 (●), 0.2 (○), 0.5 (◊), and 2 (♦) μM AzBdUTP; (C): 0 (●), 5 (○), 10 (◊), and 30 (♦) μM AzIdUTP; and (D): 0 (●), 1 (○), 5 (◊), and 10 (♦) μM AzBdUTP.

Table 4. Inhibition of HIV-RT, AMV-RT, and DNA polymerase α by AzIdUTP and AzBdUTP

Enzyme	Inhibitor	K_i (μM)	Type
HIV-RT	AzIdUTP	0.028 ± 0.006	Competitive
	AzBdUTP	0.043 ± 0.002	Competitive
AMV-RT	AzIdUTP	0.65 ± 0.04	Competitive
	AzBdUTP	0.32 ± 0.10	Competitive
DNA polymerase α	AzIdUTP	42.0 ± 5.0	
	AzBdUTP	42.7 ± 5.1	

K_i values for HIV-RT and AMV-RT were determined by Dixon plot analysis, and are the means ± SD of 3–5 determinations. K_i values for DNA polymerase α (means ± SD, N = 3) were calculated by the method of Cheng and Prusoff [35].

were also present in the 60% methanol-insoluble fraction of H9 cells, suggesting that the compound may be incorporated into the DNA of growing cells. Since AzIdUrd lacks a 3′-hydroxyl moiety, addition to a nascent DNA strand would result in chain termination as has been shown for AZT [39] and d4T [26]. However, as shown above, the synthesis of DNA (as measured by [^{3}H]dAdo incorporation)

continues in the presence of AzIdUrd. Even though the utilization of exogenous dThd for DNA synthesis is significantly inhibited by AzIdUrd, dTMP derived from *de novo* synthesis may not be, and thus sufficient dTTP for DNA synthesis would be derived from the *de novo* formation of dTMP and subsequent phosphorylation. In addition, since AzIdUTP is such a poor inhibitor of cellular DNA polymerase α (K_i = 42.0 μM) the utilization of dATP or dTTP (once formed) should not be affected significantly.

The 5′-triphosphates of various nucleoside analogs which possess anti-retroviral activity are potent inhibitors of the viral-encoded RT. Both AzIdUTP and AzBdUTP are likewise potent competitive inhibitors of HIV-RT and AMV-RT with K_i values for HIV-RT (Table 4) comparable to those previously reported for the 5′-triphosphates of AZT [27] and d4T [37]. When H9 cells were incubated with 20 μM [^{125}I]AzIdUrd (Table 1), the intracellular triphosphate pools measured 0.30 to 0.36 μM, which is some 10-fold greater than the K_i for HIV-RT. However, both AzIdUTP and AzBdUTP were very poor inhibitors of the cellular DNA polymerase α with K_i values several orders of magnitude greater than that observed with the viral RT. Therefore, the high therapeutic index of these compounds is due to the sensitivity of the viral RT coupled with the relative insensitivity of the host cell DNA polymerase α.

The favorable pharmacokinetics and central nervous system penetration of AZT are consistent with diffusion of a lipophilic compound across cell membranes allowing access to every compartment of the body [40]. AzIUdR and AzBUdR are significantly more lipophilic than AZT, as shown by their octanol/water partition coefficients suggesting increased penetration through the blood–brain barrier.

Therefore, we have shown that AzIdUrd and AzBdUrd, potent inhibitors of HIV-1 replication *in vitro* [16], are relatively non-toxic to the uninfected human T-cell line H9. AzIdUrd is phosphorylated by cellular enzymes with accumulation of the 5′-monophosphate and, to a lesser extent, the triphosphate. Both compounds, as the 5′-triphosphates, were shown to be potent inhibitors of HIV-RT and AMV-RT and relatively poor inhibitors of the cellular DNA polymerase α. Although structurally similar to AZT, these 3′-azido-5-halo-analogs exhibit a fundamental difference in their interference with the anabolic phosphorylation of dThd. Whether this difference will confer any therapeutic advantage over AZT remains to be seen. However, on the basis of their low toxicity towards uninfected cells and high antiviral potency, AzIdUrd and AzBdUrd merit further investigation as agents for use in the treatment of AIDS.

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