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Cellular Metabolism of 3′-Azido-2′,3′-Dideoxyuridine with Formation of 5′-*O*-Diphosphohexose Derivatives by Previously Unrecognized Metabolic Pathways for 2′-Deoxyuridine Analogs

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SUMMARY

3′-Azido-2′,3′-dideoxyuridine (AzdU, CS-87) is a potent inhibitor of human immunodeficiency virus replication in human peripheral blood mononuclear cells (PBMC) with limited toxicity for human bone marrow cells (BMC). In the present study, metabolism of AzdU was investigated in human PBMC and BMC after exposure of cells to 2 or 10 μM [^{3}H]AzdU. 3′-Azido-2′,3′-dideoxyuridine-5′-monophosphate (AzdU-MP) was the predominant metabolite, representing approximately 55 to 65% of intracellular radioactivity in both PBMC and BMC at all times. The AzdU-5′-diphosphate and -5′-triphosphate intracellular levels were 10- to 100-fold lower than the AzdU-MP levels and, of note, AzdU-5′-triphosphate was not detected in human BMC. Using anion exchange chromatography, a new peak of radioactivity, distinct from any known anabolites, was detected. This chromatographic peak was found to be resistant to alkaline phosphatase but was hydrolyzed by 5′-phosphodiesterase, yielding AzdU-MP. Incubation of [^{3}H]AzdU and D-[1-^{14}C]glucose in PBMC and BMC produced a double-labeled peak with the same retention time as the anabolite, suggesting formation of a hexose derivative of AzdU. A novel high performance liquid chromatography method was developed that allowed for the separation of nucleosides, nucleotides, and carbohydrate derivatives thereof. Using this highly specific method, the putative AzdU-hexose actually was separated into two chromatographic peaks. These novel metabolites were identified as 3′-azido-2′,3′-dideoxyuridine-5′-*O*-diphosphoglucose and 3′-azido-2′,3′-dideoxyuridine-5′-*O*-diphospho-*N*-acetylglucosamine. Following 48 hr of incubation with [^{3}H]AzdU, as much as 20 and 30% of these AzdU metabolites accumulated in PBMC and BMC, respectively. When AzdU was removed from the cell cultures, intracellular AzdU diphosphohexose concentrations decayed in a monophasic manner, with an elimination half-life of 14.3 hr. By 48 hr, levels of 0.3 pmol/10^6 cells were still detected, reflecting a gradual anabolism of these metabolites. Elimination of AzdU-MP and AzdU-5′-diphosphate was characterized by a two-phase process, with a short initial half-life of 0.83 and 0.24 hr and a long terminal half-life of 14.10 and 8.24 hr, respectively. Similar diphosphohexoses of deoxyuridine (dUrd) were also detected in human PBMC and BMC after exposure to [^{3}H]dUrd, suggesting that dUrd derivatives are metabolized in a similar manner. In summary, the discovery of novel metabolic pathways for dUrd analogs demonstrates that AzdU has unique metabolic features that may contribute to the low toxicity of this anti-HIV agent in human BMC and also affect its mechanism of action. Furthermore, these findings may provide insight into the development of novel human immunodeficiency virus antiviral agents.

HIV has been recognized as the cause of AIDS (1, 2). Antiviral chemotherapy represents a major approach to preventing and/or treating AIDS. Several chemotherapeutic agents are being investigated for the treatment of AIDS, and 2′,3′-dideoxynucleosides are one of the most potent classes of inhibitors of HIV replication (3, 4). The antiretroviral effects of these compounds are thought to be due to their conversion, through cellular kinases, to their corresponding triphosphate metabolites, which competitively inhibit the HIV RT, terminate the

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ABBREVIATIONS: HIV, human immunodeficiency virus; AzdU, 3′-azido-2′,3′-dideoxyuridine; AzdU-MP, 3′-azido-2′,3′-dideoxyuridine-5′-monophosphate; AzdU-DP, 3′-azido-2′,3′-dideoxyuridine-5′-diphosphate; AzdU-TP, 3′-azido-2′,3′-dideoxyuridine-5′-triphosphate; AzdU-DP-Glc, 3′-azido-2′,3′-dideoxyuridine-5′-diphosphoglucose; AzdU-DP-GlcNAc, 3′-azido-2′,3′-dideoxyuridine-5′-diphospho-*N*-acetylglucosamine; AZT, 3′-azido-3′-deoxythymidine; AZT-MP, 3′-azido-3′-deoxythymidine-5′-monophosphate; AZT-TP, 3′-azido-3′-deoxythymidine-5′-triphosphate; PBMC, peripheral blood mononuclear cells; BMC, bone marrow cells; AIDS, acquired immunodeficiency syndrome; RT, reverse transcriptase; HPLC, high performance liquid chromatography; HBSS, Hanks' balanced salt solution; dUrd, deoxyuridine; Glc-1-P, glucose-1-phosphate; UDP-Glc, UDP-glucose; UDP-Gal, UDP-galactose; UDP-GalNAc, UDP-*N*-acetylgalactosamine; UDP-GlcNAc, UDP-*N*-acetylglucosamine; FAB, fast atom bombardment.

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newly synthesized viral DNA chain, or both (5–8). The first example of this class of inhibitors, AZT, was first synthesized by Horwitz *et al.* (9) in 1964. AZT is the only clinically approved drug for the treatment of AIDS, but its use in patients has been limited by its severe hematological toxicity (10–12). Studies over the past years have demonstrated the importance of the intracellular metabolism of AZT and how this metabolism plays a major role in both the antiviral and the cytotoxic effects of AZT in host cells (13–18). In the search for new agents with increased selectivity, a great deal of interest has been focused on other 2′,3′-dideoxynucleosides, including 2′,3′-didehydro-3′-deoxythymidine (19, 20), 2′,3′-dideoxyinosine (21), 2′,3′-dideoxycytidine (22), and 2′,3′-dideoxyadenosine (22). AzdU is a novel nucleoside analog currently being evaluated in phase I clinical trials (23, 24). This drug inhibits HIV replication in a variety of HIV-infected cells at concentrations close to or below 0.1 μM (25–27), a value at least 200-fold less than that which inhibits colony formation of human BMC (28). A recent report from this group (29) demonstrated that AzdU-TP competitively inhibits virus particle-derived HIV RT, with a K_i value of 0.0059 μM, and cellular DNA polymerase α, with a value of 51.5 μM, values that are similar to those observed with AZT-TP (8). In contrast, the affinities of AzdU and AZT for thymidine kinase were found to be different, with catalytic efficiency values of 4.6 and 162, respectively. These data suggest that the monophosphorylation step may be of importance in determining the different patterns of anti-HIV activity and cytotoxicity observed for these two anti-HIV agents.

The present study evaluates the detailed intracellular disposition of AzdU in human PBMC and BMC. These cells have been shown to be representative of the target site (30) and potential toxicity site for 2′,3′-dideoxynucleosides (31).

Analysis of AzdU metabolism in these primary human lymphocytes showed the expected formation of the 5′-mono-, 5′-di-, and 5′-triphosphate derivatives. Most notable was the detection of two previously unrecognized AzdU metabolites in primary lymphocytes and BMC. These intracellular products were enzymatically characterized as 5′-*O*-diphosphohexose derivatives of AzdU. Final identification using chemically synthesized metabolites demonstrated the formation of AzdU-DP-Glc and AzdU-DP-GlcNAc. Comparison with dUrd also revealed the formation of previously unrecognized dUDP-hexose derivatives in mammalian cells. The identification of novel and unique metabolic pathways for dUrd analogs in mammalian cells should lead to a better understanding of the cellular pharmacology of the anti-HIV drug AzdU.

Experimental Procedures

Chemicals. AzdU, AZT, and their phosphorylated derivatives were synthesized in our laboratory as previously published (20, 27). Purity was established by reverse phase and anion exchange HPLC methods and spectrophotometric analysis. [*methyl*-^{3}H]AZT (3 Ci/mmol), [5-^{3}H]AzdU (22 Ci/mmol), [2-^{14}C]AzdU (56 mCi/mmol), and [5-^{3}H]dUrd (20 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). D-[1-^{14}C]-Glucose (55 mCi/mmol) and [1-^{14}C]Glc-1-P were obtained from ICN Biochemicals Inc. (Irvine, CA). The purity of all radiolabeled compounds used was >99%, as ascertained by HPLC techniques described below. Glc-1-P, UDP-Glc, UDP-Gal, UDP-GalNAc, UTP, UDP-GlcNAc, venom phosphodiesterase I type VI, 5′-nucleotidase, UDP-Glc pyrophosphorylase (EC 2.7.7.9), and inorganic pyrophosphatase were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents were of the highest analytical grade available.

Preparation of human primary cells. Human rib specimens, obtained during thoracic surgery according to a protocol approved by the Institutional Review Board Committee at the University of Alabama at Birmingham, were the source for bone marrow cells. Cells were flushed from the ribs, under sterile conditions, with HBSS containing phenol red, 10% fetal calf serum, and 1% penicillin-streptomycin. The cell suspension was centrifuged at 1200 rpm in a Beckman GPR centrifuge for 10 min and the supernatant was discarded. After resuspension in HBSS, cells were gently layered onto 10 ml of Ficoll-Hypaque (Sigma) and were centrifuged at 1500 rpm for 35 min. The mononuclear cell layer was removed and washed twice with HBSS. Cells were counted using a hemacytometer and viability was >98%, as assessed by trypan blue exclusion. Under these conditions, approximately 4×10^8 cells/rib were collected. PBMC were obtained from the whole blood of healthy HIV- and hepatitis B virus-seronegative volunteers and collected by single-step Ficoll-Hypaque discontinuous gradient centrifugation, as described above. Cells were then stimulated for 3 days by 6 μg/ml phytohemagglutinin.

Incubation conditions and extraction of intracellular ^{3}H. Human PBMC and BMC (2×10^6 cells/ml) were suspended at 37° in a 5% CO_2 incubator in RPMI 1640 and McCoy's 5A media, respectively. Media were supplemented with nutrients and 15% dialyzed heat-inactivated fetal bovine serum. The experiments were initiated with addition of 2 or 10 μM [5-^{3}H]AzdU (specific activity, 400 or 200 mCi/mmol, respectively) and cells were exposed to drug for varying time periods. Cell viability was 98% or greater, as assessed by trypan blue exclusion, and cell number was constant for over 48 hr. At specified time periods, 2×10^7 cells were removed and washed three times with cold phosphate-buffered saline. Extraction of cell pellets was performed overnight at −20° with 1 ml of cold 60% methanol. After centrifugation at 15,000 × *g* in an Eppendorf model 5414 microcentrifuge for 1 min, the supernatant was concentrated to dryness at room temperature under a gentle stream of nitrogen. The residue was dissolved in 250 μl of distilled water and 200-μl aliquots were analyzed by liquid chromatography.

Analysis of [^{3}H]AzdU metabolites by HPLC. A high performance liquid chromatograph (Hewlett-Packard 1090) equipped with automatic injector, filter spectrophotometric detector, and chromatographic terminal (Hewlett-Packard 3393A) was used for analysis of AzdU metabolites. Absorbance was recorded at 254 nm. Analyses were performed by three distinct methods. System A was composed of anion exchange chromatography using a Partisil 10 SAX column, 4 × 250 mm (Whatman, Inc., Clifton, NJ), as stationary phase and a potassium phosphate buffer linear gradient as mobile phase, as we recently described (13), except that the gradient started at 10 min. Under these conditions, the retention times of the unlabeled markers, AzdU, AzdU-MP, AzdU-DP, and AzdU-TP, were 5.2, 10.8, 27.2, and 46.6 min, respectively. System B was developed to separate nucleosides, nucleotides, and carbohydrate derivatives. The extracts from cells were examined using a 5-μm Apex I carbohydrate reverse phase column, 4 × 250 mm (Jones Chromatography, Littleton, CO). Elution was carried out isocratically at 1 ml/min with 43 mM potassium phosphate buffer (pH 2.8)/acetonitrile (77:23, v/v) and 0.09% (v/v) triethylamine. Under these conditions, the retention times of the unlabeled markers, AzdU, AzdU-MP, *N*-acetylglucosamine, *N*-acetylglucosamine-1-phosphate, UDP-GlcNAc, UDP-GalNAc, UDP-Glc, and UDP-Gal, were 3.5, 5.5, 7.6, 11.1, 23.9, 26.6, 29.5, and 31.9 min, respectively. The di- and triphosphate derivatives of AzdU were strongly retained using this carbohydrate column. AzdU-DP and AzdU-TP were eluted with retention times of 53 and 62 min, respectively, using a 10-min linear gradient of 1 M potassium phosphate buffer (pH 2.8), 0.09% triethylamine, from 0 to 20% starting at 35 min, followed by a 20-min linear gradient of the same buffer from 20 to 80%.

System C was developed to evaluate whether AzdU-MP could be potentially converted to AZT-MP within cells. The stationary phase was identical to that of system B and elution was performed at 0.5 ml/min isocratically, with a mixture of potassium phosphate buffer (pH 2.8)/acetonitrile/triethylamine (77:23:0.09, v/v/v) and 1 M potassium

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phosphate buffer (pH 2.8), with 0.09% triethylamine (10:90, v/v). The retention times of AzdU, AZT-MP, and AzdU-MP were 4, 54, and 58 min, respectively.

For all analyses, timed fractions of 0.5 or 1 ml were collected into miniscintillation vials and radioactivity was measured using a Beckman LS-5801 liquid scintillation counter equipped with an automatic quench compensation program.

Identification of novel AzdU metabolites. Initial studies in which human primary cells in suspension were exposed to [^{3}H]AzdU at various concentrations were analyzed using HPLC system A. A peak of radioactivity at 18 min (referred to here as AzdU-X) on the HPLC radiochromatogram was found, which did not correspond to the retention time of any known phosphorylated metabolite. The following methods were used for identification of AzdU-X.

Incubation with alkaline phosphatase. A total of approximately 5000 dpm of AzdU-X was isolated by HPLC and incubated with 0.31 units of alkaline phosphatase in 50 mM potassium phosphate buffer, 1 mM $ZnSO_4$ (pH 6.5), for 4 hr at 37° (total volume, 150 μl). The reaction was terminated by the addition of 30 μl of cold 50% trichloroacetic acid. After 30 min at 4°, samples were centrifuged for 1 min at 15,000 × *g* in an Eppendorf model 5414 microcentrifuge. The supernatant was neutralized with 60 μl of 5 M potassium bicarbonate and an aliquot was analyzed by HPLC system A. Control incubations were performed with heat-inactivated enzyme.

Incubation with 5′-phosphodiesterase. Similar incubation conditions as described above were used, except that 50 μl of 0.048 units/ml (2 mg/ml) venom phosphodiesterase was used.

Exposure of human primary cells to [^{3}H]AzdU and D-[1-^{14}C] glucose. Cells were incubated simultaneously with 2 μM [^{3}H]AzdU (specific activity, 400 mCi/mmol) and 2.5 μCi/ml D-[1-^{14}C]glucose for 24 hr under identical conditions as described above. As a control, cells were incubated with 2.5 μCi/ml D-[1-^{14}C]glucose alone. Extraction procedures and HPLC conditions (system A) were similar to those described above.

Enzymatic synthesis of [^{14}C]AzdU-DP-Glc. To 150 μl of AzdU-TP (final concentration, 0.1 mM) were added 25 μl of 50 mM Glc-1-P (final concentration, 2.5 mM), 3.1 μCi of D-[1-^{14}C]Glc-1-P, 15 μl of 0.2 M $MgCl_2$ (final concentration, 6 mM), 50 μl of 0.67 M Tris·HCl, pH 7.6 (final concentration, 67 mM), 5 units of bacterial UDP-Glc pyrophosphorylase, and 2 units of inorganic pyrophosphatase, in a total volume of 0.5 ml. The reaction was started by addition of UDP-Glc pyrophosphorylase. After 4 hr at room temperature, the reaction was stopped with 36 μl of 7 N perchloric acid. After 30 min on ice, samples were centrifuged and supernatant was neutralized with 240 μl of 5 M potassium bicarbonate. After 30 min in ice, samples were recentrifuged and an aliquot was analyzed by HPLC, using system A. A negative control was performed with heat-inactivated enzyme. A positive control reaction, containing 0.1 mM UTP in place of AzdU-TP, yielded UDP-Glc, as demonstrated previously (32).

Chemical synthesis of AzdU-DP-Glc and AzdU-DP-GlcNAc. A general procedure for the preparation of AzdU-DP-hexoses is outlined as follows. The pyridinium salt of cyanoethyl phosphate (33) was reacted with AzdU in the presence of 1,3-dicyclohexylcarbodiimide (34) to give AzdU-5′-β-cyanoethyl phosphate, which was hydrolyzed by 1 N KOH at 100° for 15 min to give AzdU-MP. A more soluble and highly reactive AzdU-5′-phosphomorpholidiate was obtained by condensation of AzdU-MP with morpholine in the presence of 1,3-dicyclohexylcarbodiimide (35), which was reacted with freshly prepared α-D-hexose-1-phosphate-trioctyl amine salt (36) to form AzdU-DP-hexoses. AzdU-DP-GlcNAc was synthesized from AzdU-DP-Glc by a method similar to that described above and then acetylated (37). The crude AzdU-DP-Glc and AzdU-DP-GlcNAc obtained from the above procedure were purified by a DEAE-Sephadex column (38), using low performance liquid chromatography in combination with a Milton Roy UV detector monitoring the absorbance at 262 nm, and eluted with a linear gradient between 0 and 400 mM (pH 7.5) triethylammonium bicarbonate buffer (39). The quantitation of both compounds was accomplished by the determination of peak areas and peak heights using a Waters 740 integrator. A single spot of AzdU-DP-Glc and AzdU-DP-GlcNAc was observed by thin layer chromatography performed on a silica gel GF 250 plate with elution in 2-propanol/NH_3/H_2O (7:1:2) (38) or ethanol/NH_4OH/H_2O (15:10:1) (40). The structures of AzdU-DP-Glc and AzdU-DP-GlcNAc were confirmed by enzymatic hydrolysis and chemical identification. Both compounds were resistant to alkaline phosphatase but were converted by 5′-phosphodiesterase to only one product, which was identical to an AzdU-MP authentic standard. For enzyme digestion, similar conditions as described above were used.

^{1}H NMR spectra were recorded on a JEOL FX 90 Q Fourier transform spectrometer (90 MHz), with dimethylsulfoxide-d_6 as solvent. ^{31}P spectra were recorded on a Bruker WH 500 spectrometer (operating at 177 MHz), using 85% H_3PO_4 as a reference; chemical shifts are reported in ppm (δ) and signals are quoted as s (singlet), d (doublet), t (triplet), q (quartet), and b (broad). UV spectra were obtained on a Beckman DU-7 spectrophotomer. IR spectra were recorded on a Perkin-Elmer 21 spectrophotometer. Mass spectra were measured on a Varian MAT 311 A mass spectrometer equipped with an Ion Tech FAB gun. Glycerol was used as a matrix. AzdU-DP-Glc: IR:ν_{max}^{neat}, 2100 cm^{-1} (N_3); UV [$(CH_3CH_2)_3NHCO_3$]:λ_{max} (pH 7.5), 262.0 nm; ^{1}H NMR: δ 1.07 [t, J = 7.2 Hz, 18 H, 2 × $(CH_3CH_2)_3N$], 2.29 (t, J = 3 Hz, 2 H, H-2′), 2.85 [q, J = 7.3 Hz, 12 H, 2 × $(CH_3CH_2)_3N$], 4.2–4.5 (b s, 4 OH exchangeable), 5.39 (dd, J = 3.24 and 7.15 Hz, 1 H, H-1″), 5.70 (d, J = 8.2 Hz, 1 H, H-5), 6.05 (t, J = 7.15 Hz, 1 H, H-1′), 7.9 (d, J = 8.2 Hz, 1 H, H-6); ^{31}P NMR: δ −10.744 (d, βP); FAB mass spectrum: m/z 574 $(M - H)^-$, 111 $(uracil)^-$, 395 $(AzdU\text{-}DP\text{-}O)^-$, 412 $(AzdU\text{-}DP + H)^-$. AzdU-DP-GlcNAc: IR:ν_{max}^{neat}, 2100 cm^{-1} (N_3); UV [$(Ch_3CH_2)_3NHCO_3$]:λ_{max} (pH 7.5), 262.0 nm; ^{1}H NMR: δ 1.09 [t, J = 7.2 Hz, 18 H, 2 × $(CH_3CH_2)_3N$], 1.77 (s, 3 H, $-COCH_3$), 2.63 (t, J = 3.1 Hz, 2 H, H-2′), 2.91 [q, J = 7.2 Hz, 12 H, 2 × $(CH_3CH_2)_3N$], 4.2–4.4 (br s, 3 OH, exchangeable), 5.18 dd, J = 3.2 and 7.91 Hz, 1 H, H-1″), 5.73 (d, J = 8 Hz, 1 H, H-5), 6.05 (t, J = 6.93 Hz, H-1′), 7.9 (d, J = 8.1 Hz, 1 H, H-6); FAB mass spectrum: m/z 615 $(M - H)^-$, 111 $(uracil)^-$, 332 $(AzdU\text{-}MP)^-$.

Determination of intracellular elimination half-lives of AzdU metabolites. Mean intracellular concentration versus time data sets for each AzdU metabolite obtained in the wash-out experiments were evaluated using SIPHAR/Base (SIMED, Creteil, France). The $t_{1/2}$ values were determined using a peeling algorithm (41), which assumes zero-order input and first-order elimination, and according to previously described methods (42).

Results

HPLC analysis of intracellular ^{3}H following exposure of human PBMC and BMC to [^{3}H]AzdU. Fig. 1 illustrates

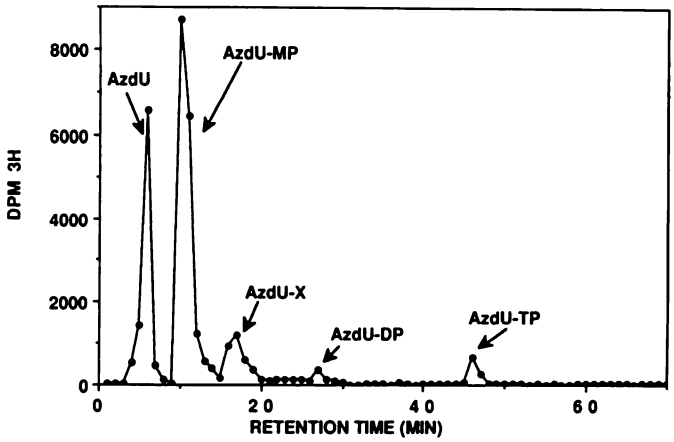

Fig. 1. HPLC radiochromatogram of intracellular ^{3}H 24 hr after incubation of phytohemagglutinin-stimulated human PBMC with 10 μM [^{3}H]AzdU. Cell extract was separated by HPLC system A and timed fractions of 1 ml were collected over 70 min. Retention times are indicated on the *abscissa*.

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the HPLC analysis of intracellular ^3H 24 hr after incubation of phytohemagglutinin-stimulated PBMC with 10 μM [^3H]AzdU. Unchanged drug was phosphorylated within cells to its mono-, di-, and triphosphates, with retention times identical to those of authentic, chemically synthesized, AzdU nucleotide derivatives. However, an additional radioactive chromatographic peak (to be referred to as AzdU-X) appeared, with a retention time of approximately 18 min using HPLC system A. This peak did not coelute with any of the known metabolites of AzdU. Similar AzdU metabolism with formation of this unknown metabolite, which eluted between AzdU-MP and AzdU-DP, was observed in human BMC (data not shown).

Characterization of AzdU-X. In order to identify the unknown AzdU metabolite, approximately 5000 dpm of [^3H] AzdU-X were isolated by HPLC and aliquots were treated with either alkaline phosphatase or venom phosphodiesterase. This metabolite was found to be resistant to alkaline phosphatase (Fig. 2B) under conditions where 5′-phosphorylated derivatives of AzdU were readily hydrolyzed to the nucleoside. In contrast, after incubation with 5′-phosphodiesterase, essentially all the radioactivity present in the metabolite fraction was converted to AzdU-MP (Fig. 2C), indicating that a phosphodiester linkage was present in the AzdU-X structure. Because sugar pyrimidine nucleotides, endogenously found in mammalian cells (43), are key intermediates in the formation of glycogen, glycoproteins, and glycolipids and because a similar metabolic pattern has been described for 5-fluorouracil and 5-fluorouridine, with fluorouridine nucleotide sugars being identified metabolites (44–46), we postulated that AzdU, although being a dUrd analog, may also form nucleotide sugars. Therefore, further efforts were directed towards determining whether AzdU-X was a nucleoside diphospho-sugar(s). Human PBMC were radiolabeled for 24 hr with 2 μM [5-^3H]AzdU (400 mCi/mmol) and D-[1-^{14}C] glucose (2.5 μCi/ml), in order to determine whether incorporation of both labels into a sugar nucleotide was possible. Using HPLC system A, cell extracts were found to contain a double-labeled chromatographic peak with the same retention time as AzdU-X (Fig. 3B), suggesting that this unknown metabolite was a sugar nucleotide derivative. In contrast, this peak was not detected when D-[1-^{14}C]glucose was incubated alone (Fig. 3A).

Based on these data and the susceptibility of the metabolite to venom phosphodiesterase but not alkaline phosphatase, the metabolite was postulated to be diphosphohexose derivative of AzdU. Endogenous sugar nucleotides include various derivatives with different sugar moieties, including glucose, galactose, and *N*-acetylhexosamines (47). To ascertain whether the AzdU-DP-hexose derivative detected at 18 min using the HPLC system A was a sole metabolite or the sum of several metabolites that were unseparated using these conditions, a new and highly specific HPLC methodology (system B) was developed to resolve nucleoside, nucleotide, and carbohydrate derivatives. Using this technique, the resolution factor *R* between UDP-Glc and UDP-GlcNAc and that between UDP-Gal and UDP-GalNAc were identical (0.8). These products were used to develop this novel HPLC method, because their chromatographic behavior was closest to that of AzdU-X.

Using HPLC system B, a portion of the cell extract analyzed above (see Fig. 3B) was injected, and timed fractions of 0.5 ml were collected over 65 min. Under these conditions, the AzdU-X eluted as two chromatographic peaks (to be referred to as AzdU-X_1 and AzdU-X_2), with retention times of 23 and 26 min

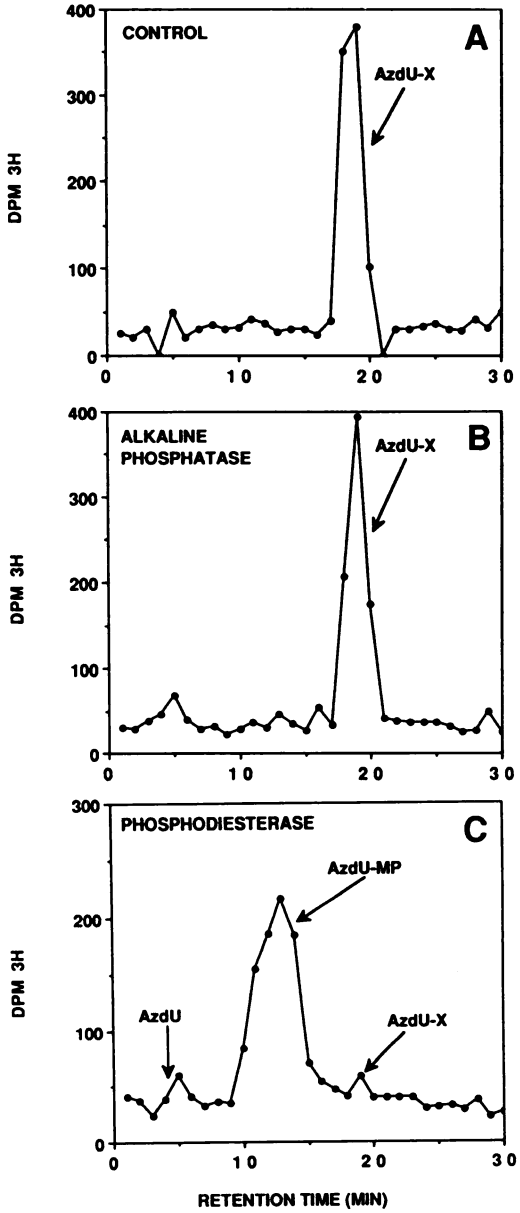

Fig. 2. HPLC profiles of peak AzdU-X after incubation in the presence of heat-inactivated enzyme (A), alkaline phosphatase (B), and 5′-phosphodiesterase (C). AzdU-X was treated for 4 hr at 37° with hydrolyzing enzymes and aliquots were analyzed using HPLC system A.

(Fig. 4). The *R* value between AzdU-X_1 and AzdU-X_2 was also approximately 0.8, suggesting that these metabolites could represent the diphosphohexose and the diphospho-*N*-acetylhexosamine derivatives of AzdU. The cellular synthesis of UDP-Glc is a cytoplasmic process, with UDP-Glc pyrophosphorylase catalyzing the synthesis of UDP-Glc from UTP and Glc-1-P. The formation of UDP-Gal, however, is mediated from UDP-Glc by either galactose-1-phosphate uridyl transferase (EC 2.7.7.12), or UDP-Gal 4′-epimerase (EC 5.1.3.2) (48). In order to elucidate the cellular synthetic pathway of AzdU-DP-hexoses, the *in vitro* formation of these AzdU metabolites was assessed using pure UDP-Glc pyrophosphorylase. This enzyme was incubated with 2.5 mM D-[1-^{14}C]Glc-1-P (2.8 mCi/mmol) in the presence of chemically synthesized AzdU-TP (0.1 mM). Inorganic pyrophosphate (2 units) was added to the reaction mixture in order to cleave the pyrophosphate formed and keep

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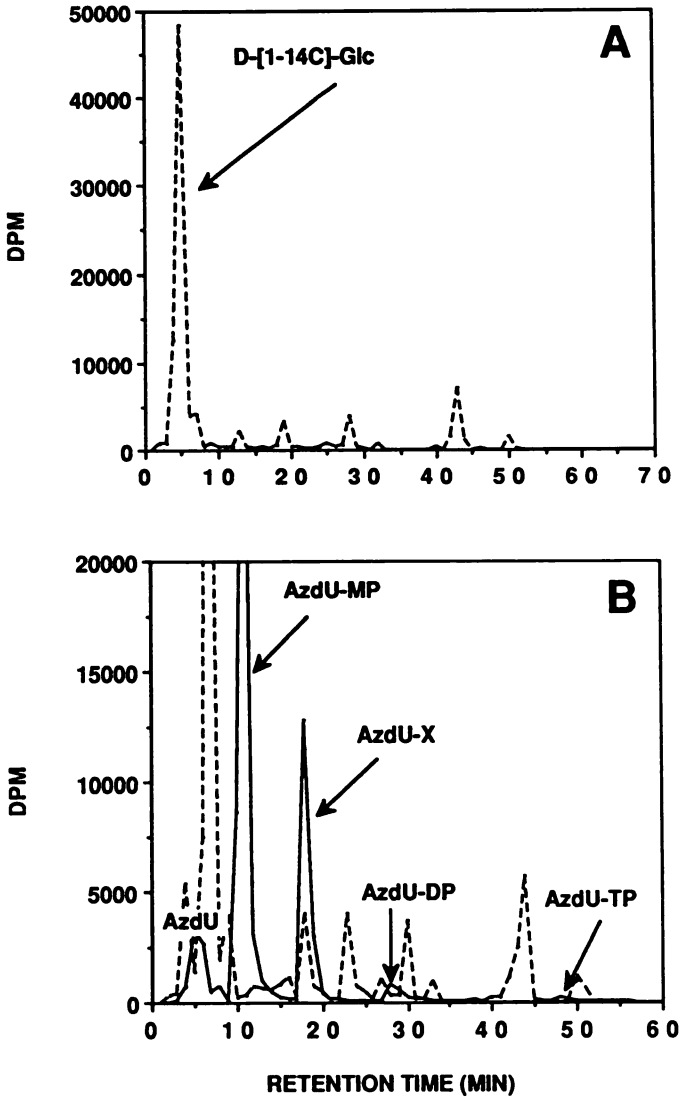

Fig. 3. HPLC radioactivity elution profile of a cell extract of human PBMC. Cells (2×10^7) were exposed for 24 hr to D-[1-^{14}C]glucose alone (A) or in the presence of 2 μM [^{3}H]AzdU (B). Extracts were analyzed by HPLC system A. Radioactivity derived from ^{14}C and ^{3}H is represented by a *dashed line* and a *solid line*, respectively.

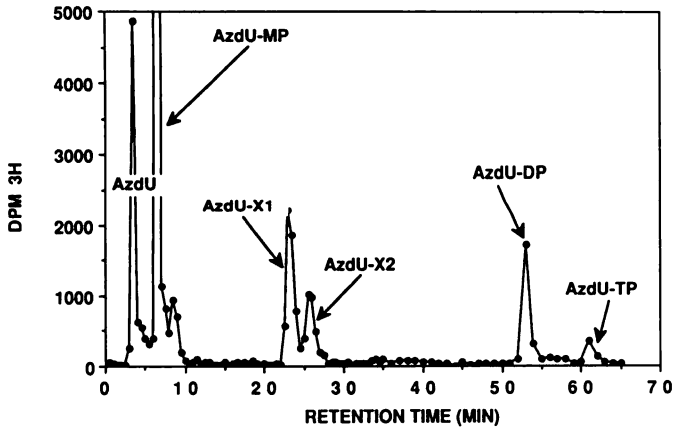

Fig. 4. HPLC analysis of formation of AzdU metabolites in human PBMC, using system B. Cell extracts were prepared under same conditions as described for Fig. 1. Timed fractions of 0.5 ml were collected after 65 min. Retention times are indicated on the *abscissa*.

the reaction unidirectional. The only enzymatically synthesized radiolabeled product formed under these conditions co-eluted with AzdU-X_2 detected "*in vivo*" on the HPLC/carbohydrate system, as shown in Fig. 5B. In the presence of heat-inactivated enzyme, no radioactivity was found in the AzdU-X_2 region and all radioactivity eluted at the same retention time as an authentic Glc-1-P standard (Fig. 5A). Under similar conditions, a positive control experiment was performed using D-[1-^{14}C] Glc-1-P and UTP. The radiolabeled product formed had the same retention time as a nonradiolabeled UDP-Glc standard (Fig. 5C). These data suggest that AzdU-X_2 represents AzdU-DP-Glc and that this metabolite is formed within cells from

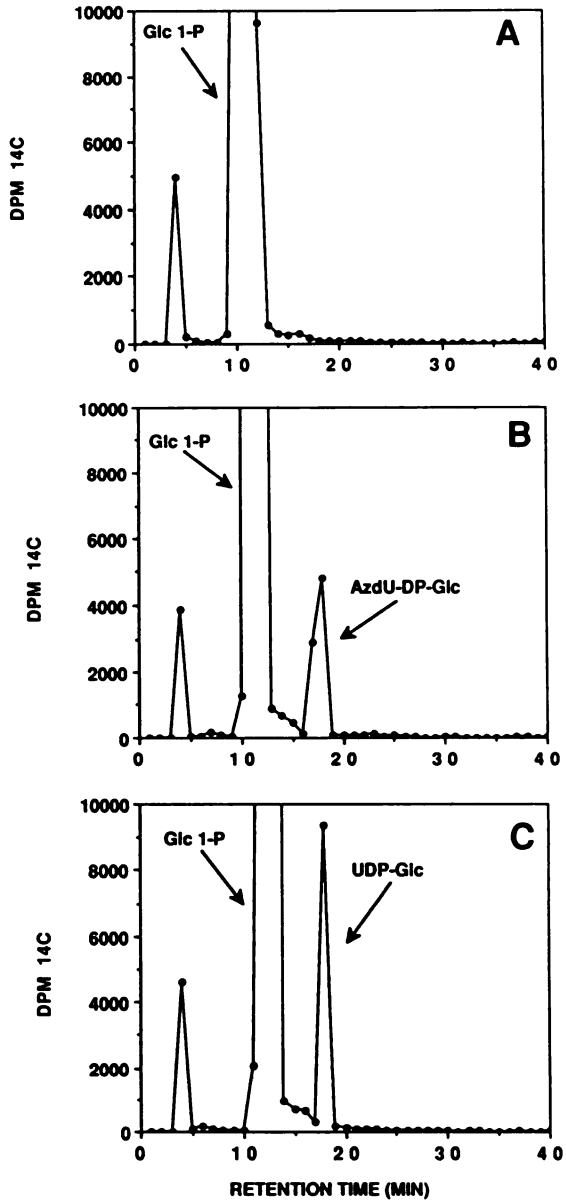

Fig. 5. *In vitro* formation of diphospho[^{14}C]glucose derivatives with UDP-Glc pyrophosphorylase. D-[1-^{14}C]Glc-1-P (2.5 mM, 2.8 mCi/mmol) was incubated for 4 hr at room temperature in the presence of inorganic pyrophosphatase (2 units), 0.1 mM AzdU-TP, and either heat-inactivated UDP-Glc pyrophosphorylase (A) or active UDP-Glc pyrophosphorylase (B). Incubation of UDP-Glc pyrophosphorylase with UTP (C) was performed with similar conditions. Acid-soluble fractions were analyzed by HPLC system A, as described in Experimental Procedures.

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AzdU-TP and Glc-1-P by UDP-Glc pyrophosphorylase. Because UDP-GlcNAc pyrophosphorylase was not available, enzymatic "*in vitro*" experiments could not be carried out to evaluate whether AzdU-X_1 was indeed AzdU-DP-GlcNAc, as postulated above. Identification of these two AzdU sugar nucleotides was obtained by comparing the HPLC retention times of the metabolites in cell extracts with those of chemically synthesized AzdU metabolites. Using the HPLC/carbohydrate system (system B), the chemically synthesized AzdU-DP-GlcNAc and AzdU-DP-Glc also had retention times of 23 and 26 min, respectively, thus comigrating with AzdU-X_1 and AzdU-X_2 detected within cells. Of note, AzdU-DP-Glc and AzdU-DP-GlcNAc had a disposition towards specific hydrolyzing enzymes similar to that of AzdU-X_1 and AzdU-X_2. In addition, with HPLC system A AzdU-DP-GlcNAc and AzdU-DP-Glc eluted together with a retention time of 18 min, similar to that observed initially for AzdU-X.

HPLC analysis of intracellular metabolism of [^{3}H] dUrd in human PBMC and BMC. Metabolism of dUrd in human primary cells was examined to evaluate whether the previously unrecognized metabolic pathway for AzdU in these cells may be unique to this drug or common to dUrd analogs in general. Therefore, phytohemagglutinin-stimulated PBMC were incubated for 24 hr with 2 μM [^{3}H]dUrd (specific activity, 450 mCi/mmol). Analysis of cell extracts using HPLC system A revealed that the unchanged compound was phosphorylated to its mono-, di-, and triphosphate derivatives, as expected; however, an additional chromatographic peak (similar to AzdU) was also observed at 18 min. The predominant metabolite was dUMP, with a concentration[1] of 0.11 ± 0.01 pmol/10^6 cells, whereas dUDP and dUTP achieved concentrations[1] of 0.05 ± 0.006 and 0.06 ± 0.002 pmol/10^6 cells, respectively. A concentration[1] of 0.076 ± 0.01 pmol/10^6 cells was calculated for the chromatographic peak detected at 18 min. Similar qualitative and quantitative results were obtained in human BMC. The chromatographic peak that eluted at 18 min was resistant to alkaline phosphatase but was a substrate for venom phosphodiesterase, yielding a single peak of ^{3}H-labeled dUMP. Incubation of phytohemagglutinin-stimulated PBMC with 2 μM [^{3}H]dUrd (specific activity, 300 mCi/mmol) and D-[1-^{14}C] glucose (2.2 μCi/ml) led to a doubly labeled dUrd sugar nucleotide, as ascertained by HPLC analysis of cell extracts. These data demonstrated that not only uridine and/or uridine analogs can be converted to sugar nucleotides (43–46) but endogenous dUrd and its analogs may also follow these metabolic pathways, with formation of dUDP-hexose derivatives.

Assessment of the putative conversion of AzdU-MP to AZT-MP in human primary cells. Because AzdU-MP is a major metabolite of AzdU in human primary cells and also an analog of dUMP, we examined whether AzdU-MP is a potential substrate for thymidylate synthase, an enzyme that catalyzes the conversion of dUMP to TMP within cells. It was also important to evaluate this potential metabolic pathway of AzdU-MP so as to ascertain whether the anti-HIV activity and/or toxicity of AzdU may be mediated through the formation of AZT 5′-phosphorylated derivatives. When 2 μM [2-^{14}C] AzdU (specific activity, 300 mCi/mmol) was added to phytohemagglutinin-stimulated PBMC for 24 hr, AZT-MP was not detected within cells, whereas more than 10,000 dpm were found in the AzdU-MP region. These data unambiguously demonstrate that AzdU-MP is not a substrate for thymidylate synthase in these cells. Similar data were obtained with human BMC, which are known to exhibit high thymidylate synthase activity (49). When purified thymidylate synthase derived from *Lactobacillus casei* was used, AzdU-MP was not found to be a substrate for that enzyme.[2]

Time course of appearance and decay of intracellular AzdU metabolites in human PBMC and BMC. In order to assess the intracellular kinetics of the diphosphohexose derivative pool of AzdU, as compared with its other 5′-phosphorylated metabolites, human PBMC or BMC were exposed to 10 μM [^{3}H]AzdU for specified time periods of up to 48 hr. The intracellular concentrations of AzdU metabolites after 2-, 6-, 24-, and 48-hr incubation periods are shown in Table 1. AzdU-MP was the predominant intracellular metabolite formed. However, AzdU-MP levels were 8- to 10-fold lower than those of AZT-MP formed in phytohemagglutinin-stimulated human PBMC[3] and BMC (13) after exposure to similar molar concentrations of the parent nucleoside. These data are consistent with previous studies from this group, showing a higher catalytic efficiency value for AZT, compared with AzdU, toward thymidine kinase (29). Formation of the 5′-phosphorylated nucleotides in both cell types was rapid, reaching maximum intracellular levels between 2 and 6 hr after initiation of the experiment. AzdU nucleotides subsequently declined or plateaued for the remainder of the experiment. Of interest, AzdU-TP was not detected in human BMC after exposure to 10 μM [^{3}H]AzdU having a specific activity of 200 mCi/mmol. In contrast, the pool of AzdU sugar nucleotides (representing the sum of AzdU-DP-Glc and AzdU-DP-GlcNAc) continuously increased over time, with a 3- and 15-fold increase in PBMC and BMC, respectively, between 2 and 48 hr. Most notably, these AzdU sugar nucleotides accounted for as much as 20 and 30% of the intracellular radioactivity in human PBMC and BMC, respectively. At 24 and 48 hr, the AzdU-DP-GlcNAc concentration was higher than that of AzdU-DP-Glc and was between 60 and 65% of the total AzdU sugar nucleotide pool.

With a lower concentration of 2 μM AzdU, intracellular levels of AzdU-MP substantially decreased, 2- to 3-fold, in both cell populations. Although substantial variations in the degree of phosphorylation by PBMC isolated from different individuals, as evidenced by the large standard deviations shown in Table 1, were observed, AzdU-DP and AzdU-TP also exhibited a dose-dependence behavior following incubation with either 2 or 10 μM AzdU. The concentration of the AzdU-sugar nucleotide pool remained unchanged in both PBMC and BMC after exposure of cells to either 10 or 2 μM AzdU for 24 hr. These previously unrecognized AzdU metabolites represented as much as 50% of the AzdU-MP concentration measured after a 24-hr incubation for both PBMC and BMC.

To determine the intracellular half-life of AzdU metabolites, human PBMC were incubated with 10 μM [^{3}H]AzdU for 24 hr, after which cells were washed and incubated in drug-free medium. At specified times, cell extracts were analyzed using HPLC system A. Elimination of AzdU-MP was initially quite rapid, with a half-life of 0.83 hr. This is similar to the kinetics of AZT-MP previously reported (5). However, this initial elimination phase of AzdU-MP was followed by a second phase,

[1] Mean ± standard deviation of at least three experiments with different lymphocytes donors.

[2] R. F. Schinazi *et al.*, unpublished data.

[3] J.-P. Sommadossi *et al.*, unpublished data.

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TABLE 1

Incorporation and metabolism of [^{3}H]AzdU in human primary cells

Values are mean ± standard deviation of at least three experiments with cells from different donors. Assuming a mean cellular volume of 1 pl (14), these values are similar when converted to μM.

Time of exposure	AzdU	Metabolite formed			
		AzdU-MP	AzdU-hexoses	AzdU-DP	AzdU-TP
hr	*μM*	*pmol/10^6 cells*			
Human PBMC					
2	2	5.22 ± 2.53	0.28 ± 0.04	0.15 ± 0.03	0.03 ± 0.02
	10	10.9 ± 15.1	0.38 ± 0.47	0.49 ± 0.69	0.18 ± 0.14
6	2	5.33 ± 0.31	0.86 ± 0.40	0.25 ± 0.01	0.03 ± 0.01
	10	11.1 ± 15.1	0.70 ± 0.92	0.53 ± 0.47	0.05 ± 0.04
24	2	4.18 ± 1.38	1.72 ± 1.06	0.27 ± 0.11	0.03 ± 0.01
	10	6.50 ± 4.70	1.20 ± 0.86	0.30 ± 0.23	0.15 ± 0.02
48	2	1.54 ± 1.48	0.59 ± 0.36	0.07 ± 0.05	ND[a]
	10	6.00 ± 3.80	1.23 ± 0.78	0.15 ± 0.11	0.04 ± 0.03
Human BMC					
2	2	1.11 ± 0.83	0.04 ± 0.05	0.04 ± 0.06	ND
	10	3.60 ± 4.30	0.16 ± 0.22	0.21 ± 0.17	ND
6	2	2.32 ± 1.58	0.24 ± 0.20	0.06 ± 0.04	ND
	10	4.80 ± 5.40	0.41 ± 0.55	0.07 ± 0.09	ND
24	2	3.32 ± 0.64	1.05 ± 0.62	0.09 ± 0.03	ND
	10	7.30 ± 3.10	1.36 ± 1.62	0.14 ± 0.07	ND
48	2	3.11 ± 1.34	1.03 ± 0.26	0.09 ± 0.04	ND
	10	6.20 ± 3.00	2.51 ± 1.31	0.22 ± 0.11	ND

[a] ND, not detected.

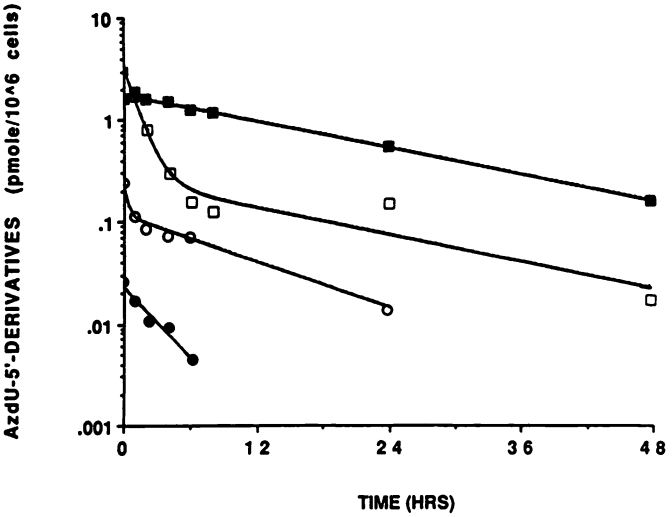

Fig. 6. Decay of AzdU 5′-derivatives in drug-free medium. PBMC were exposed for 24 hr to 10 μM [^{3}H]AzdU. At zero time, cells were washed and suspended in fresh medium. At specified times, cells were processed and AzdU-MP (□), AzdU-DP (○), AzdU-TP (●), and AzdU-DP-hexoses (▦) were quantitated by HPLC analysis. *Symbols*, mean values of at least three experiments with cells from different donors; *solid lines*, computer-fitted lines, as described in Experimental Procedures.

which began at 6 hr and had a half-life of 14.10 hr (Fig. 6). The AzdU-DP followed essentially the same two-phase process of elimination as the monophosphate derivative, with an initial half-life of 0.24 hr (for the first 2 hr) followed by a longer half-life of 8.24 hr. AzdU-TP had a half-life of 2.64 hr and was not detected after an 8-hr wash-out period under these conditions. In contrast, the AzdU-DP sugar nucleotides decayed by a one-phase process with a rather long half-life of 14.25 hr, and levels of 0.3 pmol/10^6 cells were still detected 48 hr after removal of drug. Essentially the same data were obtained in human BMC or when initial concentrations of 2 μM AzdU were studied (data not shown).

Discussion

AzdU is a potent inhibitor of HIV replication in infected human PBMC and yet is 20-fold less toxic than AZT in human bone marrow clonogenic assays (25–28). We have shown in the present study that AzdU can be phosphorylated to its corresponding mono-, di-, and triphosphate forms in human lymphocytes. The mechanism of the anti-HIV activity of AzdU can be explained by its intracellular sequential phosphorylation by cellular kinases to AzdU-TP, which, in turn, competes with the natural substrate TTP and/or terminates newly synthesized viral DNA chain. In that context, AzdU-TP has been shown to inhibit HIV RT activity (29).

Previous studies have also shown that AzdU was less potent than AZT in inhibiting HIV replication in infected human PBMC, when equimolar concentrations of the two compounds were compared (25–27). This difference cannot be explained by the affinities of these two compounds for HIV RT, because affinities of their 5′-triphosphate derivatives towards that enzyme are almost the same (29). Differences in *in vitro* anti-HIV activity might be explained by different affinities of AzdU and AZT for thymidine kinase, and a 40-fold higher catalytic efficiency has been observed with AZT, as compared with AzdU (28). However, results of phosphorylation experiments in the present study demonstrate that the primary intracellular metabolite of AzdU is AzdU-MP, indicating that thymidine kinase was not a rate-limited enzyme in the entire phosphorylation process. Like AZT-MP (5), AzdU-MP is a poor substrate for thymidylate kinase, with a K_m value of 378 μM as compared with a K_m value of 4.4 μM for dTMP.[2] Consequently, low levels of AzdU-DP and AzdU-TP might be formed intracellularly. Indeed, low concentrations of AzdU-TP were detected in human PBMC but, in contrast to AZT-TP (17), AzdU-TP levels were quite dependent on the initial AzdU concentration. After an exposure of human PBMC to only 2 μM AzdU, AzdU-TP levels were, at all time points, at least 10-fold higher than the K_i value for inhibition of HIV RT (29). In contrast, AzdU-TP

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was not detected in human BMC after exposure to 2 or 10 μM AzdU, whereas concentrations of 10 μM AZT resulted in the formation of approximately 0.1 pmol/10^6 cells AZT-TP (13). Although AZT and AzdU are structurally similar analogs, a major difference in their cellular pharmacology was demonstrated in human primary cells. Unknown chromatographic peaks migrating between AzdU-MP and AzdU-DP were detected and identified as AzdU-DP-Glc and AzdU-DP-GlcNAc.

These two novel AzdU metabolites contributed as much as 20 to 30% of all the AzdU metabolites in both human BMC and PBMC. The relatively high level of AzdU-DP-hexoses in PBMC and BMC may be explained in part by the conversion of AzdU-TP into those hexose derivatives when there is a sufficient intracellular AzdU supply. This would also explain the low AzdU-TP concentrations found in PBMC and the undetectable levels in BMC. The formation of AzdU-DP-Glc and AzdU-DP-GlcNAc by pyrophosphorylases is reversible; thus, a lack of intracellular AzdU would drive this reaction in reverse, leading to the formation of AzdU-TP from AzdU-DP-hexoses. In addition, AzdU-DP-Glc and AzdU-DP-GlcNAc may gradually be converted to AzdU-MP by 5′-phosphodiesterase hydrolysis or AzdU-DP by the action of glycogen synthase (EC 2.4.1.11) and subsequently converted to AzdU-TP. This is consistent with the data from the metabolite decay experiment and suggests that AzdU is not the only precursor of its 5′-phosphate derivatives within cells. After removal of AzdU from the media, in contrast to AZT (5), AzdU-MP and AzdU-DP were characterized as having long intracellular elimination terminal half-lives, which may reflect conversion of AzdU-DP-hexoses to these 5′-phosphorylated derivatives. Although AzdU-TP was not detected in PBMC after 6 hr of incubation, a possible explanation for this observation is that AzdU-TP levels were below the limit of detection of 0.015 pmol/10^6 cells, using a specific activity of 200 mCi/mmol.

The long elimination half-life of approximately 14 hr for these AzdU-DP-hexoses probably reflects the poor permeation of these compounds. However, transport of other diphosphosugar derivatives into cells has been previously demonstrated (50) and, therefore, the detailed metabolic fate of these AzdU-DP-hexose metabolites should be possible to evaluate in intact cells using radiolabeled standards. Nucleoside diphosphate sugars are endogenous donors of glycosyl residues essential in the biosynthesis of polysaccharides, glycolipids, and glycoproteins (43). Although several different monosaccharides can be found in naturally occurring sugar nucleotides, only ribonucleosides diphosphate analogs, including UDP and GDP, have been described as nucleotide-sugar complexes (47). Pogolotti *et al.* (44) and others (45, 46) have demonstrated that 5-fluorouracil and 5-fluorouridine can be converted to fluorouridine nucleotide sugars in mammalian cells, with a similar metabolic pattern analogous to that of UDP-sugars. Conversion of thymidine to thymidine diphosphohexose derivatives has been shown in plants, bacteria, and microorganism(s), with the demonstration that these 2′-deoxyribonucleotide sugars are major constitutents of the bacteria cell wall (51). However, to our knowledge, this report is the first to demonstrate that 2′-deoxyribonucleosides can also be metabolized to sugar nucleotides in mammalian cells. The formation of similar sugar nucleotides in human primary cells after exposure to endogenous dUrd further confirms that 2′-deoxyribonucleosides also serve as substrates for enzymes in UDP-sugar metabolism. Of note, AzdU and dUrd, at similar concentrations of 2 μM in medium for 24 hr, led to the formation of similar levels of AzdU-TP and dUTP, respectively, whereas the levels of AzdU-DP-sugars were 10-fold

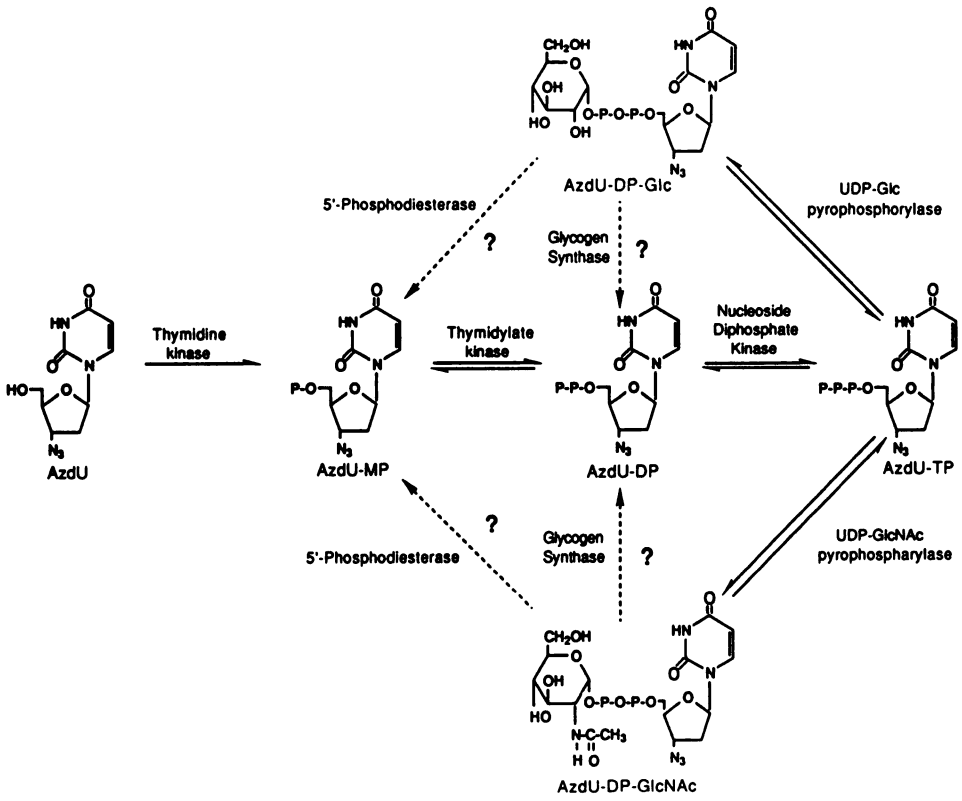

Fig. 7. Proposed metabolic pathways of AzdU.

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higher than those of dUDP-sugars. This suggests that AzdU-TP acts as a very favorable substrate in reactions catalyzed by UDP-Glc and UDP-GlcNAc pyrophosphorylases.

The discovery of these novel pathways for dUrd analogs and the finding that AzdU is extensively converted to AzdU-DP-Glc and AzdU-DP-GlcNAc may point to a second mechanism of action of this anti-HIV agent. Although inhibition of HIV replication is probably due primarily to inhibition of viral DNA synthesis by AzdU-TP, it cannot be excluded that AzdU sugar nucleotides may interfere with enzymes involved in the formation of HIV glycoproteins. Previous studies have demonstrated that such analogs may inhibit glycosylation processes of viral glycoproteins (52–54), and studies are in progress in our laboratory to evaluate whether AzdU-DP-hexoses may have similar effects.

The cellular pharmacokinetic properties of AzdU and the abilities of various cell types to form AzdU-DP-hexoses may have an impact on the efficacy and toxicity of AzdU. The formation of these metabolites may contribute to the observed low toxicity of this anti-HIV agent in human BMC, probably resulting from the major conversion of AzdU-TP to these derivatives, thereby decreasing the incorporation of AzdU into host cell DNA as compared with AZT (13). Furthermore, intracellular formation of these compounds may also serve as a depot for slow release of AzdU-TP after administration of AzdU, thus minimizing its toxicity while maintaining adequate AzdU-TP levels necessary for its anti-HIV activity.

These detailed cellular metabolic studies of AzdU also demonstrated that AzdU-MP is not converted to AZT-MP in human PBMC and BMC, indicating that AzdU is not a prodrug of AZT but, rather, a unique anti-HIV agent with cellular and kinetic properties different from those of AZT.

In conclusion, this is the first report on the cellular pharmacology of AzdU, an anti-HIV agent currently in phase I clinical trials in HIV-infected individuals (23, 24). Our data demonstrate novel metabolic pathways for dUrd analogs and indicate that AzdU has unique metabolic features (Fig. 7), as compared with other 2′,3′-dideoxynucleoside analogs. The discovery of the novel 2′-deoxynucleotide hexoses may provide insight into the synthesis of other antiviral agents with unique intracellular metabolism and selectivity.

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