Biochemical Pharmacology, Vol. 44, No. 5, pp. 973–980, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

CATABOLIC DISPOSITION OF 3′-AZIDO-2′,3′-DIDEOXYURIDINE IN HEPATOCYTES WITH EVIDENCE OF AZIDO REDUCTION BEING A GENERAL CATABOLIC PATHWAY OF 3′-AZIDO-2′,3′-DIDEOXYNUCLEOSIDES

ERIKA M. CRETTON,* MENG-YU XIE,* NAGANNA M. GOUDGAON,† RAYMOND F. SCHINAZI,† CHUNG K. CHU‡ and JEAN-PIERRE SOMMADOSSI§

*Department of Pharmacology, Center for AIDS Research, Comprehensive Cancer Center, Division of Clinical Pharmacology, University of Alabama at Birmingham, Birmingham, AL 35294; and †Veterans Affairs Medical Center, Decatur, GA 30033, and Laboratory of Biochemical Pharmacology, Department of Pediatrics, Emory University School of Medicine, Atlanta, GA 30322; and ‡Department of Medicinal Chemistry and Pharmacology, College of Pharmacy, The University of Georgia, Athens, GA 30602, U.S.A.

(*Received* 10 *February* 1992; *accepted* 23 *April* 1992)

Abstract—3′-Azido-2′,3′-dideoxyuridine (AzddU, CS-87) is a potent inhibitor of human immunodeficiency virus replication *in vitro* with low bone marrow toxicity. Although AzddU is currently being evaluated in clinical trials, its catabolic disposition is unknown. Pharmacokinetic studies in rhesus monkeys have demonstrated that a 5′-*O*-glucuronide is excreted in urine. The present study examined the catabolic disposition of AzddU is isolated rat hepatocytes, a model for the study at the cellular level of biosynthetic, catabolic and transport phenomena in the liver. Following exposure of cells to 10 μM [^{3}H]AzddU, low intracellular levels of two catabolites, identified as 3′-azido-2′,3′-dideoxy-5′-β-D-glucopyranosyluridine (GAzddU) and 3′-amino-2′,3′-dideoxyuridine (AMddU), were detected. Studies using rat microsomes demonstrated that GAzddU formation was only detected in the presence of uridine 5′-diphosphoglucuronic acid, and that the rate of AMddU formation increased significantly in the presence of NADPH. Under similar conditions, reduction of the 3′-azido function was also demonstrated herein with 3′-azido-2′,3′-dideoxycytidine (AzddC), 3′-azido-2′,3′-dideoxy-5-methylcytidine (AzddMeC) and 3′-azido-2′,3′-dideoxyguanine (AzddG), suggesting that enzymatic reduction to a 3′-amino derivative is a general catabolic pathway of 3′-azido-2′,3′-dideoxynucleosides at the hepatic site.

Antiviral chemotherapy currently represents the major approach for the treatment of patients with acquired immunodeficiency syndrome. 3′-Azido-2′,3′-dideoxyuridine (AzddU‖, CS-87), a novel pyrimidine analog which is being evaluated in phase I/IIa clinical trials,¶** is the first nucleoside analogue with a uracil base to be a potent inhibitor of human immunodeficiency virus replication *in vitro* [1–3]. When compared to 3′-azido-3′-deoxythymidine (AZT), AzddU exhibits a lower toxicity towards human bone marrow cells *in vitro* [4] consistent with the absence of myelosuppression in humans after prolonged treatment with this drug.¶**

Metabolic studies in primary human cells have demonstrated that AzddU is sequentially phosphorylated to the active 5′-triphosphate derivative, AzddU-TP [5]. In addition, two previously unidentified AzddU metabolites were also detected and identified as 5′-*O*-diphosphohexose derivatives, i.e. 3′-azido-2′,3′-dideoxyuridine-5′-diphosphoglucose and 3′-azido- 2′,3′-dideoxyuridine- 5′-diphospho- *N*-acetylglucosamine [5]. Both derivatives are readily formed, reaching significant intracellular levels and may serve as "prodrugs" of AzddU-5′-monophosphate and -triphosphate [5].

Detailed knowledge of the catabolic pathway of AzddU is still limited. In preliminary pharmacokinetic studies using rhesus monkeys, AzddU demonstrated similar pharmacokinetic characteristics when compared to AZT including formation of a 5′-*O*-glucuronide conjugate which was identified

§ Corresponding author: Dr. Jean-Pierre Sommadossi, Department of Pharmacology, University of Alabama at Birmingham, Volker 600, Birmingham, AL 35294-0019. Tel. (205) 934-8226; FAX (205) 934-8240.

‖ Abbreviations: AzddU, 3′-azido-2′,3′-dideoxyuridine; GAzddU, 3′-azido-2′,3′-dideoxy-5′-β-D-glucopyranosyluridine; AMddU, 3′-amino-2′,3′-dideoxyuridine; AzddC, 3′-azido-2′,3′-dideoxycytidine; AMddC, 3′-amino-2′,3′-dideoxycytidine; AzddMeC, 3′-azido-2′,3′-dideoxy-5-methylcytidine; AMddMeC, 3′-amino-2′,3′-dideoxy-5-methylcytidine; AzddG, 3′-azido-2′,3′-dideoxyguanine; AZT, 3′-azido-3′-deoxythymidine; AzddU-TP, 3′-azido-2′,3′-dideoxyuridine-5′-triphosphate; AMT, 3′-amino-3′-deoxythymidine; GAMT, 3′-amino-3′-dideoxy-5′-β-D-glucopyranosylthymidine; BFU-E, burst-forming unit-erythroid; CFU-GM, colony-forming unit-granulocyte-macrophage; and UDPGA, uridine-5′-diphosphoglucuronicacid.

¶ Pollis M, Darvey R, Lee D, Fallon J, Kovacs J, Metcalf J, Arnannea M, Zurlo K, Rosenthal Y, Wallenberg J, Desai A, Lane H and Masur H, A dose escalation study to evaluate the safety, anti-viral and immunological effects of 3′-azido-2′,3′-dideoxyuridine (AzddU) in patients with HIV-1 infection. In: *The Sixth International Conference on AIDS*, San Francisco, p. 206, 1990.

** Mitsuyasu RT, Miles SA, Wallenberg J, Williams G and Marcus S, Phase 1 trial of 3′-azido-2′,3′-dideoxyuridine (AzddU) in patients with symptomic HIV infections. In: *The Sixth International Conference on AIDS*, San Francisco, p. 206, 1990.

as 3′-azido-2′,3′-dideoxy-5′-β-D-glucopyranosyluridine (GAzddU) through specific enzymatic hydrolysis by β-glucuronidase [6].

Recently our laboratory demonstrated the usefulness of an isolated rat hepatocyte system for evaluating the *in vitro* metabolism of AZT in intact liver cells [7]. These *in vitro* studies led to the discovery of novel metabolites of AZT, 3′-amino-3′-deoxythymidine (AMT) and its glucuronide, GAMT. The formation of these AZT metabolites was confirmed in rhesus monkeys [8] and, more importantly, recent clinical pharmacokinetic studies of AZT performed by our group have shown that the AUC of AMT represents as much as 20–30% of the AUC of AZT intravenous administration of AZT in naive patients [9]. The demonstration of an enhanced toxicity of AMT in human burst-forming unit-erythroid (BFU-E) and colony-forming unit-granulocyte-macrophage (CFU-GM) as compared to AZT [7], and its antagonistic effect on the antiretroviral activity of AZT suggest that this metabolite, present in patients treated with AZT, may affect the pharmacodynamic properties of AZT in relation to both its activity against HIV and cytotoxicity to host cells.

Using a similar *in vitro* approach, AzddU catabolism was assessed in liver systems. Formation of GAzddU and 3′-amino-2′,3′-dideoxyuridine (AMddU) was detected. Similar to the parent drug [4], AMddU had a limited *in vitro* bone marrow toxicity. The present study also examined the potential of other 3′-azido-2′,3′-dideoxynucleosides to be metabolized by liver microsomes to their corresponding 3′-amino derivatives. The results of these studies and those previously published [7] demonstrate that enzymatic reduction of the 3′-azido moiety is a general catabolic pathway of 3′-azido-2′,3′-dideoxynucleosides.

MATERIALS AND METHODS

Chemicals

[5-^{3}H]AzddU (22.0 Ci/mmol) and [5-^{3}H]3′-azido-2′,3′-dideoxy-5-methylcytidine (AzddMeC, CS-92) (16.0 Ci/mmol) were synthesized by Moravek Biochemicals, Inc. (Brea, CA) and were 99% pure as ascertained by the HPLC technique described below. [*carboxyl*-^{14}C]Inulin was purchased from Dupont Research Products (Wilmington, DE). Unlabeled AzddU, 3′-azido-2′,3′-dideoxycytidine (AzddC, CS-91), and AzddMeC (CS-92) were synthesized in our laboratories [2]. All compounds had a purity of >99%, as assessed by HPLC and NMR spectra. IR spectra were recorded on a Perkin–Elmer 684 infra-red spectrophotometer. UV spectra were obtained on a Beckman DU-7 spectrophotometer. ^{1}H NMR spectra were recorded on a JEOL FX 90Q fourier transformation spectrometer using Me_4Si as an internal standard: chemical shifts are reported in parts per million (δ) and signals are quoted as an s (singlet), d (doublet), br s (broad singlet), t (triplet) or m (multiplet). Saccharo-1,4-β-lactone, β-glucuronidase (from bovine liver type B-10), Triton X-100, β-NADPH (reduced form), uridine-5′-diphosphoglucuronic acid (UDPGA) and Ninhydrin spray were purchased from the Sigma Chemical Co. (St. Louis, MO).

Preparation of hepatocyte suspension and incubation conditions

Studies were performed utilizing rat hepatocytes in suspension freshly isolated from Sprague–Dawley rats (180–200 g, Harlan Laboratories, Indianapolis, IN) by modification of the technique of Berry and Friend [10] which increases cell yield and viability as described previously [11]. Cell viability, determined by trypan blue exclusion, was 90% or greater in all experiments. Hepatocytes were suspended to a final cytocrit of 10% and were incubated at 37° in Krebs–Henseleit buffer as previously described [7]. Experiments were initiated with the addition of [^{3}H]AzddU (sp. act. 200 dpm/pmol) to achieve a final concentration of 10 μM. Times of incubation in the text represent the length of time between introduction of radiolabel into the cell suspension and starting of the microfuge. Portions of the cell suspension (0.5 mL) were removed and extracellular and intracellular medium separated as previously described in detail [7].

Analysis of intracellular and extracellular [^{3}H]AzddU and its catabolites by HPLC

Aliquots (50–100 μL) of the intracellular or extracellular compartments were analyzed by HPLC using a Hewlett–Packard model 1090 liquid chromatograph equipped with automatic injector, fixed wavelength spectrophotometer and chromatographic terminal (Hewlett–Packard 85 B). Reversed-phase chromatography was carried out using a Hypersil ODS 5 μm column (Jones Chromatography, Littleton, CO). Elution was performed at 1 mL/min with 25 mM phosphoric acid, adjusted to pH 7.2 and a 35-min linear gradient of acetonitrile from 0 to 30% starting at the time of injection. Column temperature was maintained at 25° and absorbance was recorded at 254 nm. Eluent from the column was directed via a low dead volume connection line into an LKB 2112 Redirac fraction collector (LKB Instruments, Rockville, MD) and timed fractions of 0.5 mL were collected into mini-scintillation vials over 35 min. After adding 5 mL of Budget-Solve (Research Products International Corp., Mount Prospect, IL) scintillation fluor, radioactivity was measured using a Beckman 5801 Liquid Scintillation Counter equipped with an automatic quench correction program. Under the above conditions, retention times of the unlabeled markers, AMddU, GAzddU and AzddU, were 7.3, 10.8, and 18.0 min, respectively. The total radioactivity applied to the column was recovered for both extracellular and intracellular compartments in 35 min (95 ± 2% recovery based upon 96 runs).

Determination of intracellular water

Intracellular and extracellular water volumes were determined on unwashed pellets using [^{14}C]inulin as an extracellular marker and gravimetric determination of wet and dry cell weights, as previously described in detail [7, 11].

Preliminary identification of AzddU catabolites

Liver microsomal assays. Studies were performed

utilizing rat liver microsomes isolated from Sprague–Dawley rats as previously described [12]. Protein content was determined by the method of Lowry *et al.* [13]. Assay mixtures contained 0.04% (v/v) Triton X-100, 50 mM $MgCl_2$, 3 mg/mL microsomal protein, 5 mM saccaro-1,4-β-lactone and 1 mM [^{3}H]-AzddU in a final volume of 0.5 mL. Reactions were initiated by adding either: (i) no cofactors, (ii) 6 mM NADPH, (iii) 6 mM NADPH and 5 mM UDPGA, and (iv) 5 mM UDPGA. All samples were incubated at 37° for 60 min. Control incubations were carried out in the absence of cofactors, and experiments were done in triplicate under conditions leading to linear reaction rates with time and protein concentration. Incubations were terminated by boiling for 30 sec, and proteins were removed by centrifugation at 15,000 *g* for 4 min. Aliquots (50–100 μL) were analyzed by HPLC as described above.

Ninhydrin assay. Preliminary identification of X_1 was done using ninhydrin which reacts with free amino groups resulting in a colored product. X_1 aliquots were isolated by HPLC from microsomal incubations, pooled and lyophilized. The dry residue was re-dissolved in 1 mL of water and a 250-μL portion was chromatographed as described above to ensure complete separation of X_1 from X_2 and AzddU. Approximately 5000 dpm of X_1 was then spotted, together with AzddU and GAzddU authentic standards on a silica gel GF 500 μm thin-layer chromatography plate; aspartic acid and AMT were used as positive controls. The plate was developed in ethyl acetate/acetic acid (10:1) for about 2 hr, air dried and sprayed with 0.2% ninhydrin solution in ethanol. This technique has been previously described for the identification of AMT [7].

Identification of X_2. Samples of X_2 isolated from hepatocyte incubations were pooled and lyophilized. The dry residue was re-dissolved in 2 mL of water, passed through a 0.4 μm Acro LC filter (Gelman Sciences, Ann Arbor, MI) and then applied to a Sep-pak C18 cartridge (Waters Associates Inc., Milford, MA). The cartridge was then washed with 5 mL of water and 80–90% of the initial radioactivity placed on the Sep-pak was recovered. Portions were pooled and lyophilized. The dry residue was dissolved in 1 mL of water and 250-μL portions, containing approximately 2000 dpm, were lyophilized and resuspended in 500 μL of 0.2 M sodium acetate (pH 4.5). Each sample was incubated in an Eppendorf microfuge tube for 2 hr at 37° with either 5000 units of β-glucuronidase inactivated by boiling for 30 sec, to be used as control, 5000 units of β-glucuronidase or 5000 units of β-glucuronidase in the presence of 20 mM saccharo-1,4-β-lactone. Following incubation, the samples were lyophilized and then resuspended in 250 μL of water; 200 μL was analyzed by HPLC.

Chemical synthesis of AMddU

AzddU (470 mg, 1.8 mmol) and triphenylphosphine (700 mg, 2.7 mmol) were dissolved in pyridine (5 mL) and the reaction was performed under identical conditions as described previously [7]. Recrystallization of the solid residue from ethanol afforded 198 mg (48%) of AMddU (m.p. 159–161°). The chromatographic properties and the ^{1}H NMR spectrum were identical with those of an authentic sample [14].

Chemical synthesis of GAzddU

To a solution of methyl-1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranuronate (3.0 *g*, 8.0 mmol) and AzddU (1.34 *g*, 5.3 mmol) in anhydrous acetonitrile (40 mL) was added anhydrous stannic chloride (3.2 mL, 27.2 mmol) at 0–4°. The reaction mixture was stirred for 30 hr at 0–4°. A cold solution of saturated sodium bicarbonate and ethyl acetate (1:1, v/v) was poured into the reaction mixture. After 0.5 hr the organic layer was extracted with ethyl acetate (150 mL), washed with water, dried (Na_2SO_4) and concentrated. The residue was chromatographed on a silica-gel column (chloroform/methanol, 95/5 as an eluant) to afford AzddU-5′-(methyl-1,2,3-tri-*O*-acetyl)-β-D-glucopyranuronate. AzddU-5′-(methyl-1,2,3-tri-*O*-acetyl)-β-D glucopyranuronate (611 mg, 21%): m.p. 90°; UV (MeOH) λ_{max} 261.5 nm; IR (KBr) 2100 (N_3), 1750 (COO), 1690 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.03 (s, 9H, 3 × CH_3CO), 2.24–2.39 (m, 2H, 2′-H), 3.76 (s, 3H, CO_2CH_3) 4.03–4.29 (m, 3H, 5′-CH_2 and 5″-glucuronide), 4.58 (m, 1H, 4′-H), 4.89–5.33 (m, complex, 5H) 5.82–5.92 (m, 2H, 5-H, and 1″-glucuronide), 6.13–6.27 (t, 1H, J = 6.5 Hz, 1′-H), 7.72 (d, 1H, J = 8.1 Hz, 6-H).

A solution of this intermediate (220 mg, 0.386 mmol) in methanol (15 mL) was treated with 1 N NaOH (pH 9). The solution was stirred at room temperature for 1.5 hr and progress of the reaction was followed by thin-layer chromatography analysis (isopropanol/acetic acid/water, 90:2:8, by vol.). Solvent was evaporated and the residue was chromatographed on a sephadex-DEAE column, eluted with a linear gradient from 0 to 400 mmol of triethylammonium bicarbonate (pH 7.5). The fractions containing GAzddU, obtained in the triethylamine salt form, were concentrated and coevaporated with methanol. The residue was dissolved in water and the solution was then passed through an ion exchange column of Dowex-50X8-100 (hydrogen) to afford GAzddU.

GAzddU (125 mg, 75%): UV (MeOH) λ_{max} 262 nm; IR (KBr) 3380 (OH), 2100 (N_3), 1680 (C=O), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.29–2.36 (m, 2H, 2′-H), 2.90–4.17 (m, complex, 9H), 4.26–4.57 (br s, OH, glucuronide, exchangeable), 5.31 (d, 1H, J = 4.6 Hz, 1″-H glucuronide), 5.68 (d, 1H, J = 8.2 Hz, 5-H), 6.11–6.17 (t, 1H, J = 5.6 Hz, 1′-H), 7.89 (d, 1H, J = 7.7 Hz, 6-H).

Chemical synthesis of 3′-amino-2′,3′-dideoxy-5-methylcytidine (AMddMeC)

A solution of AzddMeC (133 mg, 0.5 mmol) in 20 mL of MeOH was hydrogenated under 40 psi of hydrogen pressure at room temperature for 4 hr in the presence of 10% palladium on charcoal (50 mg). The catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was passed through a short silica gel column using $CHCl_3$-MeOH (6:4) as eluent to yield the title compound as white crystalline solid (90 mg, 53%). The analytically pure sample was obtained by crystallization from EtOH m.p. 170° (dec). ^{1}H NMR

(Me_2SO-d_6) δ 1.78 (s, 3H, C-5-CH_3), 1.92–2.18 (m, 2H, C-2′-CH_2), 3.32–3.68 (m, 5H, C-5′-CH_2, C-3′-H, C-3′-NH_2, D_2O exchangeable), 4.17 (m, 1H, C-4′-H), 5.02 (br s, 1H, C-5′-OH, D_2O exchangeable), 6.11 (t, 1H, C-1′-CH), 7.21 (br d, 2H, C-4-NH_2, D_2O exchangeable), 7.80 (s, 1H, C-6-H).

Chemical synthesis of 3′-amino-2′,3′-dideoxycytidine (AMddC)

A solution of AzddC (30 mg) in 10 mL of MeOH was hydrogenated in the presence of 10% palladium on charcoal (15 mg) under identical conditions as described above for AMddMeC and afforded AMddC (12 mg), m.p. 201–205°. The chromatographic properties and the ^1H NMR spectrum were identical with those for the authentic sample [14].

Clonogenic assays for drug cytoxicity

Human bone marrow cells were collected by aspiration from the posterior iliac crest of normal healthy volunteers, according to a protocol approved by the Institutional Review Board Committee at the University of Alabama at Birmingham. The clonogenic assays were performed using either a bilayer soft agar (CFU-GM) or methyl cellulose (BFU-E). These procedures have been described previously by us in detail [15].

In vitro *reduction of 3′-azido-2′,3′-dideoxynucleosides*

Studies were performed using rat liver microsomal preparations as described above for AzddU. Microsomes were exposed to 1 mM AzddC, AzddMeC or 3′-azido-2′,3′-dideoxyguanine (AzddG) and incubated in either (i) the absence of 6 mM NADPH or (ii) the presence of 6 mM NADPH for 60 min at 37°. Proteins were removed by boiling and centrifugation. The supernatant was filtered through a 0.45 μm Acro LC 13 filter (Gelman Sciences, Ann Arbor, MI) and lyophilized. The dry residue was re-dissolved in 500 μL of ethanol, portions were spotted on a Whatman 3 mm filter and air dried, and 2–3 drops of 0.2% ninhydrin were added. The disk was then air dried at room temperature and color development was assessed 60 min later. In addition, microsomes were also exposed to 1 mM [^3H]AzddMeC and incubated under the above conditions.

RESULTS

HPLC analysis of AzddU and its catabolites in rat hepatocytes

The HPLC chromatogram of intracellular tritium, 30 min after incubating hepatocytes with 10 μM [^3H]-AzddU, is shown in Fig. 1. In addition to AzddU, two chromatographic peaks, labeled X_1, and X_2, were detected. These catabolites did not correspond to any previously reported metabolites of AzddU [5].

Identification of AzddU catabolites

Identification of X_1. The ninhydrin assay was positive for the unknown chromatographic peak X_1, indicating that this catabolite contains a free amino group. In addition, under the same chromatographic conditions to those described above, X_1 co-eluted with an authentic AMddU standard, further indicating that this catabolite is very likely the 3′-amino derivative of AzddU. As expected, the reaction was negative for AzddU and GAzddU standards since these compounds do not contain a primary amino group.

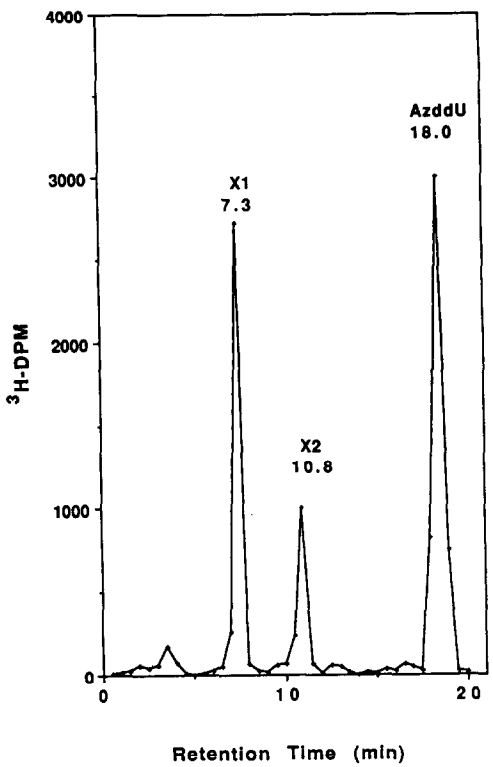

Fig. 1. HPLC profile of intracellular AzddU and its catabolites X_1 and X_2 in freshly isolated rat hepatocytes 30 min after the exposure of cells to 10 μM [^3H]AzddU. The parent drug and catabolites were resolved on a Hypersil ODS column with phosphoric acid (pH 7.2) and a 35-min linear acetonitrile gradient.

Identification of X_2. Figure 2 represents the HPLC chromatogram of intracellular extracts incubated with (A) boiled β-glucuronidase, (B) β-glucuronidase and (C) β-glucuronidase in the presence of saccharo-1,4-β-lactone as described in Materials and Methods. In the presence of β-glucuronidase (Fig. 2B), the peak labeled X_2 as completely converted to a peak which co-eluted with authentic AzddU. This conversion was inhibited by the addition of boiled enzyme (Fig. 2A) and in the presence of inhibitor (Fig. 2C), suggesting that X_2 is a glucuronide conjugate of AzddU. Furthermore, under identical chromatographic conditions, X_2 co-eluted with an authentic 5′-*O*-GAzddU standard.

Liver microsomal assays

Table 1 summarizes the data obtained when rat microsomes were incubated with 1 mM [^3H]AzddU and (i) no cofactors, (ii) NADPH, (iii) NADPH and UDPGA and (iv) UDPGA. In experiments that

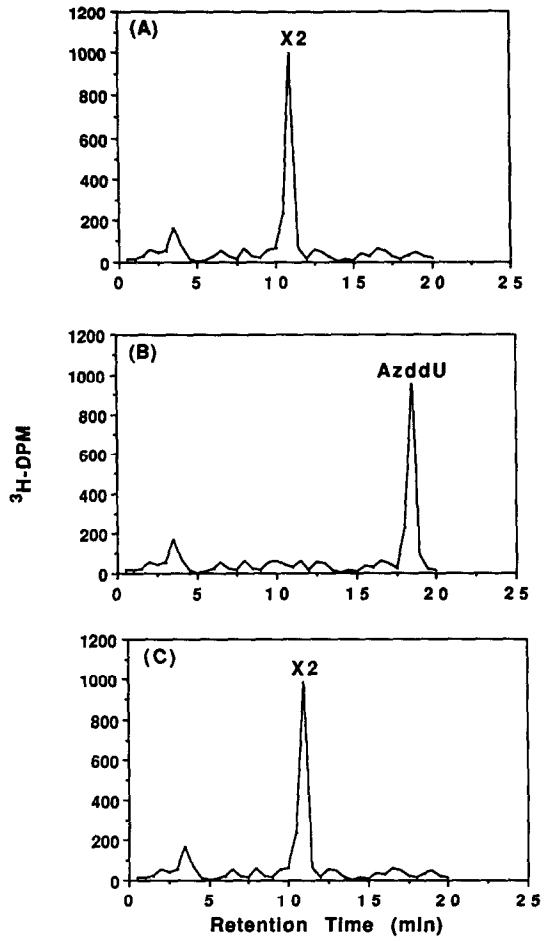

Fig. 2. HPLC radiochromatogram of the AzddU metabolite X_2 following incubation in the presence of (A) heat-inactivated β-glucuronidase, (B) β-glucuronidase, and (C) β-glucuronidase and 20 mM saccharo-1,4-β-lactone. Data represent three different experiments.

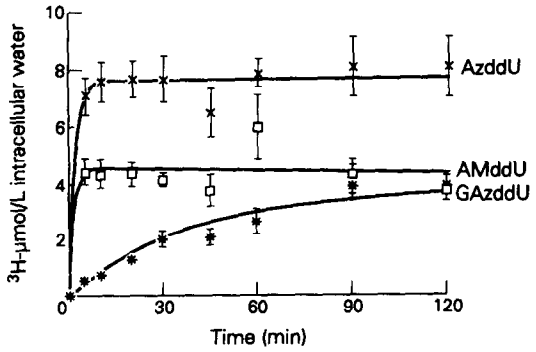

Fig. 3. Time course of appearance of intracellular AzddU and its catabolites AMddU and GAzddU following exposure of cells to 10 μM [^{3}H]AzddU. At the indicated times, a portion of the cell suspension was separated and extracted, and total intracellular [^{3}H] was assayed by HPLC. Data are the means ± SEM of five experiments.

Table 1. Rate of formation of AzddU catabolites in rat liver microsomes in the presence of NADPH, NADPH and UDPGA, and UDPGA as described in Materials and Methods

	Catabolite (pmol/min/mg protein)	
	AMddU	GAzddU
Control*	1490 ± 170†	ND‡
NADPH	5690 ± 190	ND
NADPH + UDPGA	5480 ± 340	290 ± 120
UDPGA	1560 ± 110	400 ± 330

* Additional controls were carried out containing cofactors but not microsomes. Neither catabolite was detected.

† Values are means ± SD of three experiments performed in duplicate.

‡ ND: not detected.

contained NADPH, AMddU was the major catabolite with a rate of formation of 5690 ± 190 pmol/min/mg protein, suggesting that the formation of AMddU involves an NADPH-dependent enzyme system. UDPGA had no effect on the AMddU formation. In contrast, GAzddU was only detected in incubations containing UDPGA (conditions iii and iv). When compared to AMddU, the rate of GAzddU formation was lower by approximately 15- to 20-fold. This suggests that AzddU is a poor substrate for glucuronidation in rat microsomes.

Time course of intracellular AMddU, GAzddU and AzddU

The time course of appearance of unchanged drug and its catabolites in the intracellular compartment following exposure to 10 μM [^{3}H]AzddU is depicted in Fig. 3. Unmetabolized AzddU was detected at all time points over the 2-hr exposure. Intracellular AzddU levels were approximately equal to extracellular levels, suggesting that this 2',3'-dideoxynucleoside, like AZT, crosses biological membranes via passive diffusion. Both AMddU and GAzddU were detected within the cell as early as 5 min following the addition of 10 μM [^{3}H]AzddU to the hepatocyte suspension. Surprisingly, AMddU, and not GAzddU, was the major intracellular catabolite, attaining a level of 4.9 ± 2.3 μM by 60 min and slowly decreasing to 3.6 ± 0.7 μM by 2 hr. In contrast, GAzddU levels were intially low, increasing to a maximum concentration of 3.8 ± 1.1 μM by 2 hr.

Analysis of the time course of the disappearance of extracellular AzddU and the appearance of catabolites

Extracellular AzddU decreased by only 10% over the 2 hr of the experiment, indicating that AzddU is not catabolized extensively in rat hepatocytes. Both AMddU and GAzddU levels were low, reaching maximum levels of 0.60 ± 0.30 and 0.23 ± 0.08 μM, respectively, by 60 min. Similar

results were obtained for both intracellular and extracellular compartments when hepatocytes were exposed to 10 μM [2-^{14}C]AzddU (data not shown).

Identification of the 3'-amino derivatives of AzddC, AzddMeC, and AzddG

Samples, isolated from microsomal extracts, were exposed to AzddC, AzddMeC, and AzddG, incubated with NADPH as described in Materials and Methods, and tested with ninhydrin to detect the presence of a primary amino group. The assay was positive for all three compound extracts, suggesting that their corresponding 3'-amino derivatives were formed and detected. As expected, the ninhydrin test was negative to chemically synthesized AzddG.

Analysis of the radiochromatogram of microsomes exposed to 1 mM [^{3}H]AzddMeC demonstrated a chromatographic peak eluting at 8.5 min which was only detected in samples which contained NADPH. In addition, this peak at 8.5 min co-eluted with an authentic chemically synthesized AMddMeC, further demonstrating that this nucleoside analogue is also reduced to its corresponding amino derivative. These data indicate that an NADPH-dependent system is involved in the reduction of 3'-azido-2',3'-dideoxynucleosides investigated in the present study, as recently demonstrated with AZT [7].

Toxicity of 3'-azido-2',3'-dideoxynucleosides and their 3'-amino catabolites in human hematopoietic progenitor cells in vitro

The effects of continuous exposure (14 days) to increasing concentrations of 3'-azido-2',3'-dideoxynucleosides and their corresponding 3'-amino catabolites on the growth of human CFU-GM and BFU-E is summarized in Table 2. The 50% inhibitory concentration (IC_{50}) of AzddMeC, AzddU, AZT and AMT for either CFU-GM or BFU-E was similar to that previously reported [4, 7, 16]. As previously demonstrated, BFU-E was more sensitive to these compounds [4]. The 3'-amino catabolites were either equally or more toxic to both CFU-GM and BFU-E than the parent compound.

Table 2. Effects of 3'-azido-2',3'-dideoxynucleosides and their 3'-amino catabolites on human CFU-GM and BFU-E clonal growth

Compound	Clonogenic assay	IC_{50}* (μM)
AZT	CFU-GM	2.0
	BFU-E	0.6
AMT	CFU-GM	0.4
	BFU-E	0.1
AzddU	CFU-GM	10.0
	BFU-E	100.0
AMddU	CFU-GM	40.0
	BFU-E	25.0
AzddC	CFU-GM	1.0
	BFU-E	ND†
AMddC	CFU-GM	2.0
	BFU-E	4.0
AzddMeC	CFU-GM	40.0
	BFU-E	100.0
AMddMeC	CFU-GM	5.0
	BFU-E	1.0

* Fifty per cent inhibitory concentration. Each point is the mean of at least six experiments with different marrow donors.

† ND: not determined.

DISCUSSION

The present study assessed the catabolism of AzddU in intact cells using isolated rat hepatocytes. Figure 4 demonstrates the catabolic pathways of AzddU in the hepatocyte. In a previous report, we demonstrated the usefulness of this system for the analysis of the formation of AZT catabolites and their disposition in hepatic cells [7].

In contrast to the findings reported for AZT, AzddU is not extensively glucuronidated in rat hepatocytes. Following incubation of hepatocytes with radiolabeled AzddU, substantial levels of AMddU were detected intracellularly, suggesting that reduction of the 3'-azido group to the 3'-amino group was the major catabolic reaction. In addition, intracellular levels were much higher than extracellular levels, suggesting that this catabolite is slowly effluxed from the cell, possibly due to the high polarity of the 3'-amino group as observed with AMT [7].

Handlon and Oppenheimer [17] recently reported that thiols are able to reduce AZT *in vitro*. Although AzddU reduction was not examined, one can assume that similar results would be observed since AZT and AzddU are structurally close analogs. With respect to the results reported herein, it should be emphasized that samples, either during incubation of cells or processing of samples, or HPLC analysis, did not come into contact with exogenous reducing agents at any time, thus eliminating the possibility that the formation of AMddU detected in this study occurs by a non-enzymatic process. Furthermore, when rat microsomes were exposed to AzddU in the presence of NADPH, the rate of formation of AMddU increased approximately 5-fold. This suggests that the formation of AMddU is mediated by an NADPH-dependent system, possibly a cytochrome P450 reductase, as recently suggested for the reduction of AZT [7]. Recent unpublished data by our group demonstrated that AMddU is formed in rhesus monkeys treated with radiolabeled AzddU, indicating that AzddU reduction can also occur *in vivo*. Currently, characterization of this enzymatic step at the hepatic site is being pursued for AzddU, AZT, and related compounds.

Since the amino derivative of AZT was shown to be highly toxic to bone marrow cells [7], the direct effects of AMddU on myeloid and erythroid human progenitor cells *in vitro* were examined. Interestingly, AMddU showed significantly lower toxicity when compared to AMT, suggesting that reduction of the 3'-azido moiety does not necessarily result in a toxic catabolite. The second catabolic pathway of AzddU

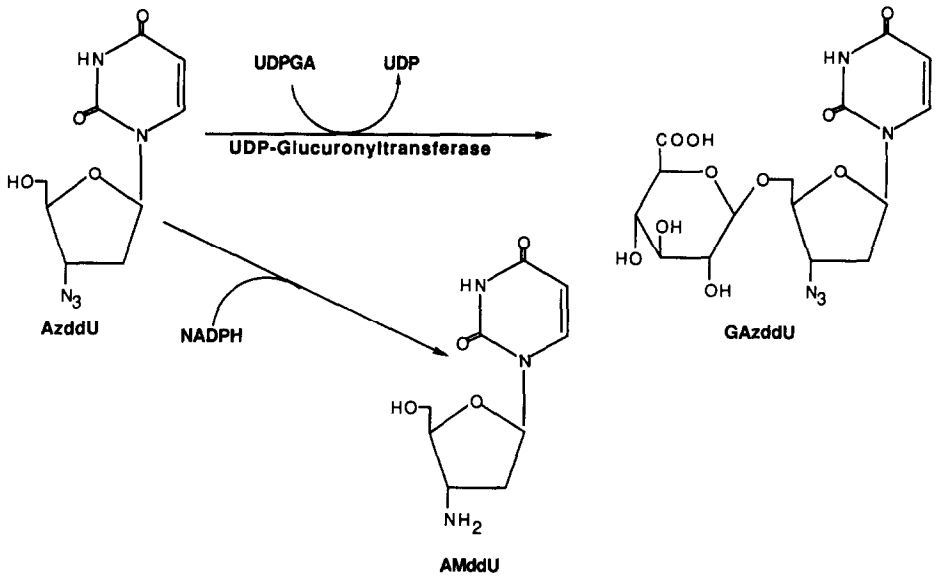

Fig. 4. Proposed catabolic pathway for AzddU.

involves conjugation with UDPGA resulting in GAzddU. Interestingly, intracellular GAzddU levels were significantly lower than intracellular AMddU in rat hepatocytes. The rate of formation of GAzddU in rat liver microsomes exposed to AzddU and UDPGA was approximately 20-fold lower than the rate of AMddU formation, suggesting that AzddU is a poor substrate for rat uridine 5′-diphosphoglucuronyltransferase (UDPGT) *in vitro*. These data are in good agreement with a recent report which demonstrated that AzddU is a less efficient substrate than AZT for UDPGT in human microsomes [18]. In that study, hydrophobicity of 2′,3′-dideoxynucleosides was suggested to correlate with their degree of glucuronidation.

Reduction of the 3′-azido moiety of AzddC, AzddMeC and AzddG was also detected, following incubation of microsomes with drug and NADPH. In view of the observations reported for AZT [8, 9] and AzddU (Schinazi RF *et al.*, unpublished data) in monkeys, these data indicate that the reduction of these compounds is very likely to occur *in vivo*, suggesting that formation of a 3′-amino catabolite is probably a general catabolic pathway for 3′-azido-2′,3′-dideoxynucleosides. As demonstrated in this study, the 3′-amino catabolites were very inhibitory to both CFU-GM and BFU-E, with the exception of AMddU. This may explain the lack of bone marrow toxicity observed in humans undergoing AzddU treatment.*† Potential interactions, in terms of toxicity and pharmacological action, between a 3′-azido-2′,3′-dideoxynucleoside and its 3′-amino catabolite should be investigated carefully. These results further emphasize the uniqueness of each 2′,3′-dideoxynucleoside and the need for caution when extrapolating conclusions on metabolism and pharmacological action of parent drug and potentially toxic metabolite based only on their chemical structure.

Acknowledgements—This work was supported by United States Public Health Service Grants HL-42125, AI-25784, and RR-00032 (J-P.S); AI-25889 (R.F.S. and C.K.C.), and the Department of Veterans Affairs (R.F.S.). J-P.S. is the recipient of a Faculty Research Award from the American Cancer Society. We would like to thank Janna Stockinger for preparing and editing this manuscript.

REFERENCES

1. Chu CK, Schinazi RF, Arnold BH, Cannon DL, Doboszewski B, Bhadti VB and Gu Z, Comparative activity of 2′,3′-saturated and unsaturated pyrimidine and purine nucleosides against human immunodeficiency virus type 1 in peripheral blood mononuclear cells. *Biochem Pharmacol* **37**: 3543–3548, 1988.
2. Chu CK, Schinazi RF, Ahn MK, Ullas GV and Gu ZP, Structure–activity relationships of pyrimidine nucleosides as antiviral agents for human immunodeficiency virus type 1 in peripheral blood mononuclear cells. *J Med Chem* **32**: 612–617, 1989.
3. Schinazi RF, Chu CK, Ahn MK and Sommadossi J-P, Selective *in vitro* inhibition of human immunodeficiency virus (HIV) by 3′-azido-2′,3′-dideoxyuridine (CS-87). *J Cell Biochem Suppl* **11D**: 74, 1987.
4. Sommadossi J-P, Zhou Z, Carlisle R, Xie MY, Weidner DA and el Kouni MH, Novel pharmacologic approaches for the treatment of AIDS and potential use of

* Pollis M, Darvey R, Lee D, Fallon J, Kovacs J, Metcalf J, Arnannea M, Zurlo K, Rosenthal Y, Wallenberg J, Desai A, Lane H and Masur H, A dose escalation study to evaluate the safety, anti-viral and immunological effects of 3′-azido-2′,3′-dideoxyuridine (AzddU) in patients with HIV-1 infection. In: *The Sixth International Conference on AIDS*, San Francisco, p. 206, 1990.

† Mitsuyasu RT, Miles SA, Wallenberg J, Williams G and Marcus S, Phase 1 trial of 3′-azido-2′,3′-dideoxyuridine (AzddU) in patients with symptomic HIV infections. In: *The Sixth International Conference on AIDS*, San Francisco, p. 206, 1990.

uridine phosphorylase inhibitors. In: *Advances in Chemotherapy of AIDS* (Eds. Diasio RB and Sommadossi J-P), pp. 63–73. Pergamon Press, New York, 1990.
5. Zhu Z, Schinazi RF, Chu CK, Williams GJ, Colby CB and Sommadossi J-P, Cellular metabolism of 3′-azido-2′,3′-dideoxyuridine with formation of 5′-*O*-diphosphohexose derivatives by previously unrecognized metabolic pathways for 2′-deoxyuridine analogs. *Mol Pharmacol* **38**: 929–938, 1990.
6. Boudinot FD, Schinazi RF, Gallo JM, McClure HM, Anderson DC, Doshi KJ, Kambhampathi PC and Chu CK, 3′-Azido-2′,3′-dideoxyuridine (AzddU): Comparative pharmacokinetics with 3′-azido-3′-deoxythymidine (AZT) in monkeys. *AIDS Res Hum Retroviruses* **6**: 219–228, 1990.
7. Cretton EM, Xie MY, Bevan RJ, Goudgaon NM, Schinazi RF and Sommadossi J-P, Catabolism of 3′-azido-3′-deoxythymidine in hepatocytes and liver microsomes with evidence of formation of 3′-amino-3′-deoxythymidine, a highly toxic catabolite for human bone marrow cells. *Mol Pharmacol* **39**: 258–266, 1991.
8. Cretton EM, Schinazi RF, McClure HM, Anderson DC and Sommadossi J-P, Pharmacokinetics of 3′-azido-3′-deoxythymidine and its catabolites and interactions with probenecid in rhesus monkeys. *Antimicrob Agents Chemother* **35**: 801–807, 1991.
9. Stagg MP, Cretton EM, Kidd L, Diasio RB and Sommadossi J-P, Clinical pharmacokinetics of 3′-azido-3′-deoxythymidine (AZT) and catabolites with formation of a toxic catabolite, 3′-amino-3′-deoxythymidine (AMT). *Clin Pharmacol Ther* **51**: 668–676, 1992.
10. Berry MN and Friend DS, High yield preparation of isolated rat liver parenchymal cells: A biochemical and fine structure study. *J Cell Biol* **43**: 506–520, 1969.
11. Sommadossi J-P, Gewirtz DA, Diasio RB, Aubert C, Cano J-P and Goldman ID, Rapid catabolism of 5-fluorouracil in freshly isolated rat hepatocytes as analyzed by high performance liquid chromatography. *J Biol Chem* **257**: 8171–8176, 1982.
12. Cretton EM, Waterhous DV, Bevan R and Sommadossi J-P, Glucuronidation of 3′-azido-3′-deoxythymidine by rat and human liver microsomes. *Drug Metab Dispos* **18**: 369–372, 1990.
13. Lowry OH, Rosebrough, NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
14. Lin TS and Mancini WR, Synthesis and antineoplastic activity of 3′-azido and 3′-azido analogues of pyrimidine deoxyribonucleoside. *J Med Chem* **26**: 544–548, 1983.
15. Sommadossi J-P and Carlisle R, Toxicity of 3′-azido-3′-deoxythymidine and 9-(1,3-dihydroxy-2-propoxymethyl)guanine for normal human hematopoietic progenitor cells *in vitro*. *Antimicrob Agents Chemother* **31**: 452–454, 1987.
16. Schinazi RF, Chu CK, Eriksson BF, Sommadossi J-P, Doshi KJ, Boudinot FD, Oswald B, and McClure HM, Antiretroviral activity, biochemistry, and pharmacokinetics of 3′-azido-2′,3′-dideoxy-5-methylcytidine. *Ann NY Acad Sci* **616**: 385–397, 1990.
17. Handlon AL and Oppenheimer NJ, Thiol reduction of 3′-azidothymidine to 3′-aminothymidine: Kinetics and biological implications. *Pharm Res* **5**: 297–299, 1988.
18. Resetar A, Minick D and Spector T, Glucuronidation of 3′-azido-3′-deoxythymidine catalyzed by human liver UDP-glucuronosyltransferase: Significance of nucleoside hydrophobicity and inhibition by xenobiotics. *Biochem Pharmacol* **42**: 559–568, 1991.