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GLUCURONIDATION OF 3′-AZIDO-3′-DEOXYTHYMIDINE CATALYZED BY HUMAN LIVER UDP-GLUCURONOSYLTRANSFERASE

SIGNIFICANCE OF NUCLEOSIDE HYDROPHOBICITY AND INHIBITION BY XENOBIOTICS

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Abstract—The enzymatic glucuronidation of 3′-azido-3′-deoxythymidine (AZT) catalyzed by human liver microsomal UDP-glucuronosyltransferase (EC 2.4.1.17, UDPGT) was inhibited by a number of nucleoside analogs. The inhibitory potency of these nucleoside analogs correlated with their hydrophobicity ($r^2 = 0.90$, N = 13). Since similar results were obtained with solubilized UDPGT ($r^2 = 0.87$, N = 7), the affinity of the nucleosides for UDPGT was probably being assessed rather than the ability of the compounds to access the membrane-bound enzyme. Three homologous inhibitors, 3′-azido-2′,3′-dideoxyuridine (AzddU), 5-ethyl-AzddU, and 5-propyl-AzddU, were also studied as substrates of UDPGT. The substrate efficiency (V_{max}/K_m) of these three compounds and AZT also correlated with their hydrophobicity ($r^2 = 0.94$). Sixteen drugs that are structurally unrelated to nucleosides also inhibited the glucuronidation of AZT. The mechanism of inhibition was competitive for seven compounds tested. K_i values were estimated from Dixon plots for nine other less soluble inhibitors; their mechanism of inhibition was assumed to be competitive. Since the peak physiological drug concentrations of the tested inhibitors are considerably less than their K_i values, none of these compounds are expected to strongly inhibit AZT glucuronidation in humans. However, the rank order of these drugs with respect to their inhibitory potential is probenecid > chrloramphenicol > naproxen > phenylbutazone ≫ other drugs tested.

3′-Azido-3′-deoxythymidine (AZT,‖ zidovudine, Retrovir®, BW A509U), a potent inhibitor of HIV replication *in vitro* [1, 2], is used in the treatment of AIDS [3–5]. Although preclinical studies indicated that AZT undergoes limited metabolism in various animal species [6–9], AZT is extensively converted to the inactive metabolite 3′-azido-3′-deoxy-5′-β-D-glucopyranuranosylthymidine (GAZT) in humans [9–11]. UDP-glucuronosyltransferase (EC 2.4.1.17) enzymatically catalyzes this biotransformation [11–13].

Although many endogenous and xenobiotic compounds are converted to more polar, water-soluble metabolites by enzymatic glucuronidation [14], the generation of a nucleoside 5′-ether-glucuronide is uncommon. Therefore, studies were initiated to determine what factors allow AZT to be converted to this unusual nucleoside metabolite by human liver UDPGT. Hydrophobicity may be an important factor since AZT is significantly more hydrophobic than either thymidine or dideoxythymidine [15], two nucleoside analogs that have not been reported to be glucuronidated either *in vivo* or *in vitro*. The results of the studies presented here indicate that both the binding affinity and the substrate efficiency of a series of nucleoside analogs correlated with nucleoside hydrophobicity.

The present study also examined the potential of other drugs to interfere with AZT glucuronidation. Multiple drugs are co-administered with AZT in the treatment of AIDS, associated opportunistic infections, and additional medical complications. Any co-administered xenobiotics that are substrates of UDPGT may also be alternative-substrate inhibitors of AZT glucuronidation. Inhibition of AZT glucuronidation may increase the efficacy of AZT against HIV replication [3–5] and/or toxicity to bone marrow progenitor cells [16, 17]. The effects of two UDPGT substrates, the uricosuric agent probenecid [18] and the analgesic acetaminophen [19], on the metabolism and pharmacokinetics of AZT have been reported. Probenecid increases the

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‖ Abbreviations: AIDS, acquired immunodeficiency syndrome; AzddA, 3′-azido-2′,3′-dideoxyadenosine; AzddC, 3′-azido-2′,3′-dideoxycytidine; AzddG, 3′-azido-2′,3′-dideoxyguanosine; AzddU, 3′-azido-2′,3′-dideoxyuridine; AZT, 3′-azido-3′-deoxythymidine; BSA, bovine serum albumin; ddT, 2′,3′-dideoxythymidine; d4T, 2′,3′-dideoxy-2′,3′-didehydrothymidine; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; GAZT, 3′-azido-3′-deoxy-5′-β-D-glucopyranuranosylthymidine; HIV, human immunodeficiency virus; K_i, inhibition constant; K_m, Michaelis–Menten constant; 4MUB, 4-methylumbelliferone; NOG, *N*-octyl-β-D-glucoside; TAPS, *N*-tris[hydroxy-methyl]methyl-3-amino-propanesulfonic acid; UDPGA, UDP-glucuronic acid; UDPGT, UDP-glucuronosyltransferase; v_i, reaction velocity in the presence of inhibitor; v_o, reaction velocity in the absence of inhibitor; V_{max}, maximal velocity; and V_{max}/K_m, substrate efficiency.

area under the plasma concentration–time curve for AZT by a factor of two [20–22], whereas acetaminophen does not alter AZT pharmacokinetics [23–25]. Therefore, not all compounds that are glucuronidated *in vivo* affect AZT metabolism and pharmacokinetics.

Sixteen drugs that are glucuronidated in humans [14] were tested as inhibitors of AZT glucuronidation *in vitro*. The results of these studies indicate that a number of structurally unrelated drugs can inhibit AZT glucuronidation. However, the low plasma levels of these drugs relative to their inhibition constants suggest that while none of the compounds tested would be expected to strongly inhibit AZT glucuronidation in humans, some small effects on AZT metabolism and pharmacokinetics may be observed.

MATERIALS AND METHODS

Chemicals. Acetaminophen, chloramphenicol, cimetidine, dapsone, ethinylestradiol, furosemide, lorazepam, morphine sulfate, 4-methylumbelliferone (4MUB), (+)-naproxen, oxazepam, phenylbutazone, probenecid, sulfinpyrazone, zomepirac, UDPGA (sodium salt), *N*-octyl-β-D-glucoside, *Escherichia coli* β-glucuronidase (Type X-A, EC 3.2.1.31), D-saccharic acid 1,4-lactone, dithiothreitol (DTT), and *N*-tris[hydroxy-methyl]methyl-3-amino-propanesulfonic acid (TAPS) were obtained from the Sigma Chemical Co. (St. Louis, MO); Coomassie Blue reagent was from Pierce Biochemicals (Rockford, IL); and bovine serum albumin (BSA) from Calbiochem (La Jolla, CA); HPLC grade ammonium phosphate (monobasic), acetonitrile, and water were from Mallinckrodt (St. Louis, MO); and Scintilene and Scintiverse BD from Fisher Scientific (Fair Lawn, NJ). AZT, [5′-^{3}H]AZT, GAZT and other nucleoside analogs used in this study were synthesized at the Wellcome Research Laboratories. All other chemicals were of reagent grade.

Purification of [^{3}H]AZT. Approximately 0.6 μmol [^{3}H]AZT (12.5 Ci/mmol) in 1 mL of 10 mM ammonium phosphate, pH 5.5 (Buffer A), was loaded onto a 300 mg PrepSep™ C_{18} column (Fisher Scientific) equilibrated with Buffer A. The column was washed with 6 mL of Buffer A and eluted with 5 mL of 25% MeOH in Buffer A. Fractions (1 mL) were collected and monitored for radioactivity. Purified [^{3}H]AZT eluted in 25% MeOH in Buffer A and was stored at −25°. This material was greater than 99% homogeneous as determined by HPLC analysis.

Liver samples. Human liver samples were obtained from organ donors or autopsy specimens. The livers were perfused with ice-cold phosphate-buffered saline, diced, and stored at −75°.

Microsome preparation. Microsomes were prepared by differential centrifugation as described [12, 26] and could be stored at −75° for 1 year with no apparent loss of UDPGT.

Solubilization of microsomal protein. Microsomes were resuspended in 25 mM TAPS, pH 8.2 (4°), 20% glycerol, and 1 mM DTT and were solubilized with 1.25 mg *N*-octyl-β-D-glucoside (NOG) mg protein. The final protein concentration was 10 mg/mL. The mixture was stirred for 60 min at 4° and then centrifuged at 100,000 *g* for 60 min. Approximately 70% of the microsomal protein and 75% of the glucuronidating activity (assayed with 4MUB as substrate) were recovered in the supernatant. 4MUB glucuronidation was assayed as described previously [12, 27]. Solubilized enzyme was dialyzed against 25 mM TAPS, pH 8.2, 20% glycerol, and 0.05% NOG for 12 h to remove DTT and reduce the NOG concentration prior to assays of AZT glucuronidation. Solubilized enzyme could be stored at −75° for 1 month with no apparent loss of UDPGT.

Protein assays. A refined Coomassie Blue assay was used to quantitate protein [28]. BSA was used as the protein standard.

Inhibition of AZT glucuronidation. A radiochemical TLC method described previously [12] was used to assay AZT glucuronidation. Nucleoside analogs (at concentrations that produced 30–80% inhibition) were tested as inhibitors of AZT glucuronidation in reaction mixtures (40 μL) containing 100 mM TAPS, pH 8.5, 10 mM $MgCl_2$, 20 mM UDPGA, 100–200 μg microsomal or solubilized protein, and 0.1 mM [^{3}H]AZT (150 Ci/mol). Samples (4 μL) were taken at 0, 20, 40, 60, 80 and 100 min to determine if product formation was linear with time. Since adenosine deaminase was detected in microsome preparations and AzddA was an excellent substrate for ADA (unpublished observation), deoxycoformycin (10 μM), a potent ADA inhibitor [29], was included in reaction mixtures containing AzddA. Deoxycoformycin had no effect on AZT glucuronidation. Pharmaceuticals were tested as inhibitors of 0.1 mM AZT glucuronidation at three to six concentrations. DMSO (5%) was present in reaction mixtures containing dapsone, lorazepam or oxazepam, and ethanol (2%) was present in reaction mixtures containing ethinylestradiol to increase the solubilities of the inhibitors. AZT glucuronidation was inhibited 20% by either 5% DMSO or 2% ethanol. Therefore, AZT glucuronidation rates obtained in the presence of dapsone, lorazepam, oxazepam, or ethinylestradiol were compared to rates obtained from control reactions that contained 5% DMSO or 2% ethanol. When the mechanism of inhibition of AZT glucuronidation was studied, reaction mixtures (20 μL) contained 0.5–10 mM [^{3}H]AZT (10 Ci/mol) and inhibitors at concentrations from zero to six times their K_i values. Samples (4 μL) were taken and analyzed after 60 min of incubation at 37°. Previous studies indicated that reactions were linear for at least 80 min.

Glucuronidation of AzddU analogs. AzddU analogs (1 mM) were assayed as substrates of UDPGT in reaction mixtures (500 μL) containing 100 mM TAPS, pH 8.5, 10 mM $MgCl_2$, 20 mM UDPGA, and 2–4 mg microsomal protein. Reactions were initiated with enzyme and incubated at 37°. Control reactions were incubated in the absence of protein or UDPGA. At various time intervals, 100-μL samples were taken, quenched with 0.5 mL acetonitrile plus 0.5 mL water, and centrifuged at 13,000 *g* for 5 min. Supernatants were evaporated to dryness in a Speed Vac (Savant Instruments, Farmingdale, NY), resuspended in 500 μL of 25 mM ammonium phosphate, pH 7.2, and stored at −25°. Product formation was analyzed by HPLC as described below.

To confirm that the enzymatic products generated from AzddU analogs were glucuronides, reactions (350 μL) that contained AzddU analogs, UDPGA, and human liver microsomes were incubated for 90 min (4 hr for AzddU) at 37°, quenched with 0.5 mL acetonitrile plus 0.5 mL water, and treated as described above except that the samples were resuspended in 85 μL of 100 mM KH_2PO_4, pH 6.8. Samples (25 μL, 68 nmol nucleoside plus nucleoside metabolites) of each reaction were then incubated for 60 min at 37° in 100 mM KH_2PO_4, pH 6.8, with 170 units β-glucuronidase in the presence or absence of 50 mM D-saccharic acid-1,4-lactone, a specific inhibitor of β-glucuronidase [30]. The final reaction volume was 90 μL. Control reactions contained no β-glucuronidase. Reactions were quenched and treated as described above.

HPLC analysis. All samples were ultrafiltered through a Centrifree micropartition system (Amicon Corp., Danvers, MA) and were analyzed by an HPLC method described previously [10] with some modifications. Samples (100 μL) of the nucleosides and their glucuronides were separated by reversed-phase HPLC on a 4 × 250 mm Hibar® LiChrospher 100 (5 μm) RP-18 column (EM Science, Gibbstown, NJ) at a flow rate of 1 mL/min for 30 min with a mobile phase of 25 mM $NH_4H_2PO_4$, pH 7.2, and a linear gradient of 0 to 45% CH_3CN. The UV absorbance of the column effluent was monitored from 220 to 300 nm with an LKB 2140 Rapid Spectral UV Detector (Pharmacia LKB Biotechnology, Piscataway, NJ). Analyte peak areas, measured at 267 nm, were digitized and integrated by a DS-80Z microcomputer (Digital Specialties, Chapel Hill, NC). Peak areas were linearly related to the concentrations of standards. The lower limit of detection was 0.5 nmol of nucleoside or nucleoside glucuronide. Retention times (min) for the compounds analyzed were: AzddU, 19.7; AzddU-glucuronide, 15.2; AZT, 21.8; GAZT, 16.3; 5-ethyl-AzddU, 24.9; 5-ethyl-AzddU-glucuronide, 17.9; 5-propyl-AzddU, 28.1; and 5-propyl-AzddU-glucuronide, 19.9. Since authentic samples of AzddU-glucuronide, 5-ethyl-AzddU-glucuronide, and 5-propyl-AzddU-glucuronide were not available, assignments of their retention times were based on the retention times of the new product peaks.

Data analysis. Reaction velocities were obtained from the initial, linear portion of the reactions. Background radioactivity associated with GAZT from control reactions that contained no UDPGT (less than 0.5% of the total radioactivity) was subtracted from the radioactivity associated with GAZT from complete reactions. Velocities obtained for AzddU analogs are reported as nanomoles glucuronide produced per hour per unit of UDPGT. One unit of UDPGT was defined as that amount of enzyme which glucuronidates 1 nmol of 1.0 mM AZT/hr at 37° at pH 8.5. The apparent kinetic constants K_i, K_m, and V_{max} were determined from a direct fit of the data to a hyperbola [31]. The mechanism of inhibition by pharmaceuticals and nucleosides was confirmed by statistical conformity of the data of the competitive model [32]. For pharmaceuticals with limited solubilities, apparent K_i values were estimated from Dixon plots [33]. Inhibition was assumed to be competitive. For nucleoside analogs, the equation $K_i = ([I]/i - [I])/(1 + [S]K_m)$, where $i = 1 - (v_i/v_o)$, was used to calculate apparent K_i values from fractional inhibition data obtained at single substrate and inhibitor concentrations. Inhibition was assumed to be competitive.

Hydrophobicity constant determinations. A modified reversed-phase HPLC method designed to model octanol/water partitioning [34] was used to estimate hydrophobicity constants (log k'_w). Since the nucleoside analogs that were studied had short retention times, log k'_w determinations were highly influenced by small changes in the column void that occurred with changes in mobile phase composition. Therefore, column void volumes were determined at each volume fraction of methanol (ϕ_{MeOH} = 0.20, 0.25, 0.30, 0.35, and 0.40) used as the mobile phase, and these column void volumes rather than a single column void volume were used to derive hydrophobicity constants as described previously [34].

RESULTS

Inhibition of AZT glucuronidation by nucleoside analogs. To determine whether hydrophobicity was correlated with the affinity of a nucleoside for UDPGT, hydrophobicity (log k'_w) and inhibition (K_i) constants were measured for 17 nucleoside analogs (Table 1). Since these compounds were structural analogs of AZT, they were assumed to be competitive inhibitors of AZT glucuronidation. Their K_i values were estimated from fractional inhibition data obtained at 0.1 mM AZT and adequate inhibitor to produce 30–80% inhibition. Thymidine, d4T, and 3′-amino-2′,3′-ddT, at 10 mM, produced less than 10% inhibition. Therefore, K_i values could not be estimated for these compounds. In addition, hydrophobicity constants were not determined for these three compounds because they were too hydrophilic and were not retained on the C_8 reversed-phase column used to assess hydrophobicity.

To support the assumption that K_i values could be determined from fractional inhibition data, the mechanism of inhibition of three nucleoside inhibitors of AZT glucuronidation, AzddU, 5-ethyl-AzddU and 5-propyl-AzddU, was studied. All three compounds were competitive inhibitors of AZT glucuronidation. The K_i values (Table 2) obtained for AzddU, 5-ethyl-AzddU, and 5-propyl-AzddU from these competitive inhibition studies (9.1, 0.36 and 0.21 mM, respectively) were in good agreement with those estimated from fractional inhibition data (6.8, 0.36 and 0.15 mM, respectively). Results obtained with 5-ethyl-AzddU and 5-propyl-AzddU are exemplified in Fig. 1.

A least squares linear regression analysis of the plot log K_i versus log k'_w showed a strong correlation ($r^2 = 0.90$, N = 13) between inhibition of AZT glucuronidation and hydrophobicity (Fig. 2). Since

Table 1. Correlation between inhibition and hydrophobicity constants for nucleoside inhibitors of AZT glucuronidation

	Compound	K_i* (mM)		
		UDPGT		
		Microsomal	Solubilized	log k'_w†
(I)	5-Substituent modifications of AzddU			
	5-Propyl-AzddU	0.15 ± 0.01	0.63	0.595
	5-Ethyl-AzddU	0.36 ± 0.04	1.1	0.209
	5-Ethynyl-AzddU	0.75 ± 0.04	1.4	0.186
	5-Methyl-AzddU (AZT)	1.2 ± 0.1	3.3	0.063
	5-Azido-AzddU	3.1 ± 0.6	7.8	−0.031
	AzddU	6.8 ± 0.1	19	−0.111
	5-Amino-AzddU	12 ± 1	35	−0.273
	Plot‡ of log K_i vs log k'_w: (r^2)	(0.94)	(0.87)	
	(slope)	(−2.27)	(−2.20)	
(II)	Sugar modifications of ddT§			
	3′-Azido-2′,3′-ddT (AZT)	1.2 ± 0.1		0.063
	2′-Azido-2′,3′-ddT	2.2 ± 0.4		0.030
	3′-(xylo)-Azido-2′,3′-ddT	5.2 ± 1		−0.038
	3′-fluoro-2′,3′-ddT	7.8 ± 0.9		−0.201
	2′,3′-ddT	23 ± 1		−0.336
	Plot of log K_i vs log k'_w: (r^2)	(0.85)		
	(slope)	(−2.44)		
(III)	Nucleobase modifications of AZT			
	AzddG	0.98 ± 0.2		0.073
	AZT	1.2 ± 0.1		0.063
	AzddU	6.8 ± 0.1		−0.111
	AzddC	24 ± 1		−0.121
	Plot of log K_i vs log k'_w: (r^2)	(0.87)		
	(slope)	(−4.96)		
	AzddA‖	4.8 ± 0.5		0.230

* K_i values were calculated from inhibition data obtained at 0.1 mM AZT and inhibitor concentrations that inhibited AZT glucuronidation 30–80% as described in Materials and Methods. Results obtained with microsomal UDPGT are reported as the means ± SD (N = 3). Results obtained with solubilized UDPGT are the average of two determinations.

† Hydrophobicity constants (log k'_w) were determined as described in Materials and Methods.

‡ Correlation coefficients (r^2) and slopes were determined from least squares linear regression analysis of plots of log K_i vs log k'_w.

§ Thymidine, d4T, and 3′-NH_2-ddT (at 10 mM) inhibited AZT glucuronidation less than 10%. Hydrophobicity constants could not be measured for these three compounds due to their hydrophilic nature.

‖ AzddA, an apparent outlier, was omitted from the calculations of correlation coefficient and slope.

Table 2. Kinetic constants for the enzymatic glucuronidation of AzddU analogs

Compound	K_m or K_i* (mM)	Velocity at 1.0 mM† (nmol/hr/unit)	V_{max}‡ (nmol/hr/unit)	V_{max}/K_m (relative)
AzddU	9.1 ± 0.8	0.61 ± 0.05	6.2	48
5-Methyl-AzddU (AZT)	2.4 ± 0.9	1.0	3.4	100
5-Ethyl-AzddU	0.36 ± 0.04	1.8 ± 0.1	2.5	470
5-Propyl-AzddU	0.21 ± 0.03	3.9 ± 0.2	4.7	1600
Plot§ of log (V_{max}/K_m) vs log k'_w: slope = 2.20, r^2 = 0.94				

* The K_m for AZT is a mean ± SD (N = 25). The K_i values for AzddU, 5-ethyl-AzddU, and 5-propyl-AzddU (K_i ± SE) were determined from competitive inhibition studies.

† Velocities are means ± SD (N = 5).

‡ V_{max} values were calculated from the equation $V_{max} = (v)(K_m + [S])/[S]$. K_i was assumed to equal K_m [35].

§ The slope and correlation coefficient (r^2) were determined from least squares linear regression analysis of a plot of log (V_{max}/K_m) vs log k'_w.

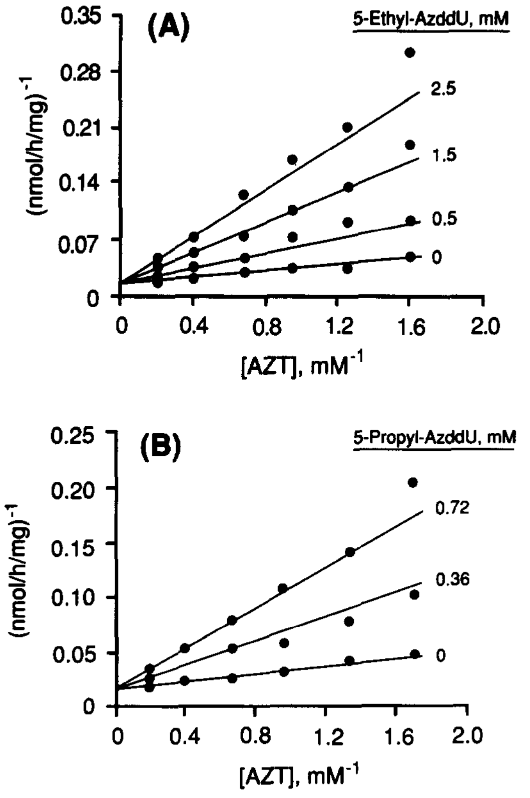

Fig. 1. Inhibition of AZT glucuronidation by (A) 5-ethyl-AzddU and (B) 5-propyl-AzddU. The K_i (±SE) values for 5-ethyl- and 5-propyl-AzddU are 0.36 ± 0.04 and 0.21 ± 0.03 mM, respectively. Inhibition was statistically consistent with the competitive model [32].

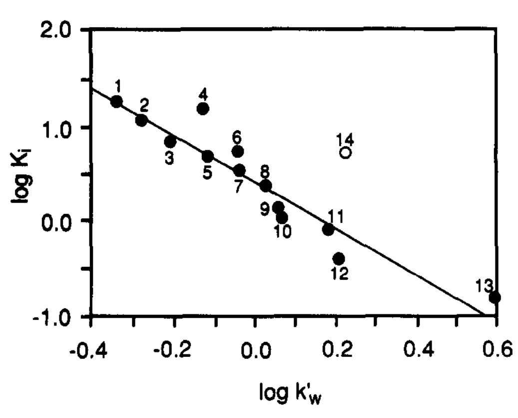

Fig. 2. Plot of log K_i versus log k'_w for a series of nucleoside analogs assayed as inhibitors of AZT glucuronidation. Least squares linear regression analysis indicated that binding affinity (K_i) correlated well with hydrophobicity ($r^2 = 0.90$, slope $= -2.50$). AzddA, an apparent outlier, was not included in the correlation analysis. Key: (1) ddT, (2) 5-amino-AzddU, (3) 3′-fluoro-ddT, (4) AzddC, (5) AzddU, (6) 3′-xylo-azido-ddT, (7) 5-azido-AzddU, (8) 2′-azido-ddT, (9) AZT, (10) AzddG, (11) 5-ethynyl-AzddU, (12) 5-ethyl-AzddU, (13) 5-propyl-AzddU, and (14) AzddA.

AzddA appeared to be an outlier, it was not included in the linear regression analysis. Thymidine, d4T, and 3′-amino-2′,3′-ddT were also omitted from the analysis since accurate estimates of K_i values could not be obtained for these weak inhibitors. However, since these weak inhibitors were also very hydrophilic, they supported the correlation between inhibition potency and hydrophobicity.

Strong correlations between log K_i and log k'_w were also obtained when the effects of substitutions at the 5-position of the pyrimidine ring of AZT or nucleobase or sugar substitutions were analyzed independently (Table 1).

The correlation between inhibition of AZT glucuronidation and hydrophobicity was also studied with solubilized UDPGT to determine whether the affinity of a given nucleoside for the enzyme or the ability of a compound to access the membrane-bound enzyme was being assessed. Although the K_i values were higher with solubilized enzyme (Table 1), the correlation between log K_i and log k'_w ($r^2 = 0.87$, slope $= -2.20$, $N = 7$) was similar to the correlation obtained in studies with microsomal UDPGT ($r^2 = 0.94$, slope $= -2.27$, $N = 7$). Therefore, the affinity of a nucleoside for UDPGT was assessed in these studies.

AzddU analogs as substrates of UDPGT. To test whether substrate efficiency (V_{max}/K_m) also correlated with hydrophobicity, a series of three homologous AZT analogs were studied as substrates of UDPGT. HPLC analysis (see Materials and Methods) of reaction mixtures indicated that AzddU, 5-ethyl-AzddU, and 5-propyl-AzddU were converted to new UV-absorbing species. Product formation increased with reaction time and was dependent on UDPGT and UDPGA (data not shown). In addition, the UV spectrum of each reaction product was similar to the spectrum of the corresponding parent compound. No differences in absorption spectra are expected between the parent compounds and their 5′-*O*-glucuronides. Furthermore, reaction products were reconverted to their respective parent compounds by β-glucuronidase, and D-saccharic acid, 1,4-lactone, an inhibitor of β-glucuronidase [30], prevented this reconversion (data not shown). These data demonstrate that AzddU, 5-ethyl-AzddU, and 5-propyl-AzddU were substrates for UDPGT.

Reaction rates for the glucuronidation of AzddU, AZT, 5-ethyl-AzddU, and 5-propyl-AzddU were determined (Table 2), and V_{max} values were calculated according to the Michaelis–Menten equation, $V_{max} = (v)(K_m + [S])/[S]$. Since the nucleoside analog were substrates of UDPGT as well as competitive inhibitors of AZT glucuronidation, K_i values were assumed to equal K_m values [35]. Least squares linear regression analysis of a plot of log (V_{max}/K_m) versus log k'_w for these analogs plus AZT indicated that substrate efficiency (V_{max}/K_m) correlated well with hydrophobicity ($r^2 = 0.94$).

Xenobiotics as inhibitors of AZT glucuronidation. Sixteen structurally unrelated pharmaceuticals that are glucuronidated in humans were tested as inhibitors of AZT glucuronidation. Their apparent K_i values were estimated from Dixon plots (Table 3). Inhibition was assumed to be competitive.

Table 3. Inhibitors of AZT glucuronidation

Inhibition	K_i* (Dixon plot) (mM)	K_i† (Competitive) (mM)	Peak plasma levels‡ (μM)		Potential inhibition§ (%)
AZT		$K_m = 2.4$	2–10	[10]	—
Probenecid	1.6 ± 0.06	2.0 ± 0.3	700	[18, 36]	30
Chloramphenicol	0.25 ± 0.03	0.28 ± 0.01	25–60	[37, 38]	20
(+)-Naproxen	1.1 ± 0.05	1.2 ± 0.1	50–180	[39]	10
Phenylbutazone	1.4 ± 0.3		120	[40]	8
Dapsone	1.7 ± 0.2		5–12	[37, 41]	1
Zomepirac‖	2.1 ± 0.2		15	[42, 43]	0.7
Oxazepam	0.58 ± 0.04	0.65 ± 0.6	1.8–4.0	[37, 44]	0.6
Furosemide	1.7 ± 0.3		7	[37, 45]	0.4
Acetaminophen	14 ± 6	20 ± 4	30–80	[19]	0.4
Sulfinpyrazone	2.1 ± 0.2		3–6	[46]	0.3
Sulfathiazole	24 ± 5		75	[47]	0.3
Sulfisoxazole	47 ± 8		50–80	[37]	0.2
Lorazepam	0.16 ± 0.009	0.25 ± 0.02	0.06–0.30	[37, 44]	0.1
Cimetidine	11 ± 0.2		3–12	[48]	0.1
Morphine	0.97 ± 0.06	0.88 ± 0.06	1.2 (i.v.)	[49, 50]	0.1
			0.04	[49]	0.005
Ethinylestradiol	0.15 ± 0.05		0.0004	[51]	<0.001
Acyclovir¶	>50		4–13	[52, 53]	<0.03

* Apparent K_i values (K_i ± SE) were determined from Dixon plots as described in Materials and Methods. Inhibition was assumed to be competitive. The AZT concentration was 0.1 mM.

† K_i values (K_i ± SE) were determined from competitive inhibition studies as described in Materials and Methods.

‡ Peak plasma levels were obtained *in vivo* after oral administration except where noted. References for these values are in brackets.

§ Theoretical percent inhibition obtainable *in vitro* at physiological concentrations (peak plasma levels) of inhibitor and AZT. The equation $i = 100 \cdot [I]/(K_i(1 + [S]/K_m) + [I])$, where i = percent inhibition, was used to calculate theoretical percent inhibition. Inhibition was assumed to be competitive for all compounds tested. Since binding of compounds to plasma proteins was not taken into account, the percent inhibition values represent the theoretical maximum inhibition obtainable.

‖ Assays were done in the absence of Mg^{2+}. Zomepirac forms a precipitate in the presence of divalent metal cations.

¶ Acyclovir, at a concentration of 6.4 mM, inhibited AZT glucuronidation less than 10%.

Acyclovir, a nucleoside analog that is not glucuronidated, but is being studied in combination with AZT for the treatment of AIDS [53], was also tested. Acyclovir, at a concentration 60-fold greater than the concentration of AZT, did not inhibit AZT glucuronidation.

The mechanism of inhibition was studied for all compounds with adequate solubilities (Table 3). The seven compounds tested were competitive inhibitors of AZT glucuronidation. The K_i values obtained were in good agreement with the K_i values estimated from Dixon plots. Results obtained with morphine and chloramphenicol are exemplified in Fig. 3.

DISCUSSION

The present study used human liver microsomes as a source of UDPGT to determine whether hydrophobicity is a factor in the glucuronidation of AZT and other nucleoside analogs and whether other xenobiotics could inhibit AZT glucuronidation. These *in vitro* studies have advantages over metabolism studies in laboratory animals for assessing glucuronidation of nucleosides and for studying inhibition of AZT glucuronidation because major differences in AZT metabolism have been observed among humans and other species [6–10]. Results obtained *in vitro* with microsomes prepared from rat liver and human liver as the UDPGT source accurately reflect the limited and extensive glucuronidation of AZT observed in rats and humans, respectively [12, 13]. In addition, studies with microsomal UDPGT have a potential advantage over studies with purified UDPGT as microsomes contain all isozymic forms of UDPGT and may better reflect overall *in vivo* metabolism.

Previous studies have shown that hydrophobicity is a factor in the glucuronidation of phenolic compounds catalyzed by rat liver UDPGT [54] and the glucuronidation of bile acids catalyzed by human liver UDPGT [55]. Both the affinity and substrate efficiency of bile acids appear to increase with increasing bile acid hydrophobicity.

Results obtained from these studies indicate that the binding affinity (K_i) of nucleoside analogs for UDPGT also correlates strongly with nucleoside hydrophobicity. Hydrophilic nucleosides such as thymidine, d4T, and 3′-NH_2-2′,3′-ddT do not appear to bind to UDPGT, whereas hydrophobic nucleosides such as 5-ethynyl-AzddU, 5-ethyl-AzddU, and 5-propyl-AzddU bind more tightly to the enzyme than does AZT. Therefore, hydrophobicity may be an indicator of whether a nucleoside can bind to UDPGT and potentially serve as a substrate of the enzyme.

The substrate efficiency (V_{max}/K_m) of four

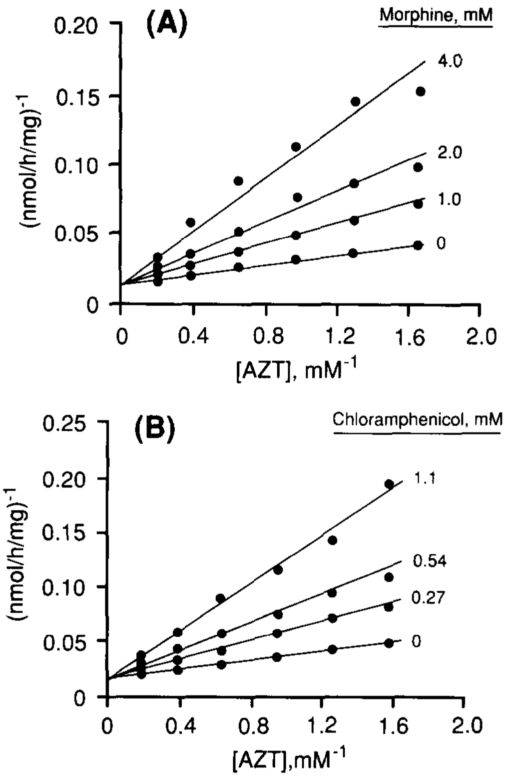

Fig. 3. Inhibition of AZT glucuronidation by (A) morphine and (B) chloramphenicol. The K_i (±SE) values for morphine and chloramphenicol are 0.88 ± 0.06 and 0.28 ± 0.01 mM, respectively. Inhibition was statistically consistent with the competitive model [32].

nucleoside analogs also correlates strongly with hydrophobicity. 5-Ethyl- and 5-propyl-AzzdU are both more hydrophobic than AZT and are more efficient substrates of UDPGT. AzddU, on the other hand, is less hydrophobic and is a less efficient substrate. Variability in K_m values rather than V_{max} values accounts for these substrate specificity differences. K_m values differed by approximately 45-fold, ranging from 0.2 mM (5-propyl-AzddU) to 9 mM (AzddU), whereas V_{max} values varied by less than 3-fold.

Nucleoside analogs that are more hydrophobic than 5-propyl-AzddU may be better substrates for UDPGT. Since the efficient penetration of AZT into the central nervous system is presumably crucial to the ability of this agent to reverse the neurological dysfunction due to HIV infection [56], the synthesis of anti-HIV nucleoside analogs which are more hydrophobic than AZT may be considered. Unfortunately, the results from these studies indicate that more hydrophobic nucleoside analogs may be rapidly inactivated due to extensive first pass glucuronidation.

Several recent studies indicate that monkeys glucuronidate AZT more efficiently than do non-primates [6–9, 11, 57], and the pattern of glucuronidation is similar to that observed in humans [10]. The metabolism of several other nucleoside analogs has also been investigated in monkeys. No glucuronide metabolite of d4T is detected in the urine of rhesus monkeys administered d4T [58], whereas the glucuronide metabolites of 3′-fluoro-2′,3′-ddT [59] and AzddU [57] are readily detected in the urine of monkeys administered 3′-fluoro-2′,3′-ddT or AzzdU. The molar ratio of glucuronide metabolite to unchanged 3′-fluoro-2′,3′-ddT, 1.5, is similar to the ratio of GAZT to AZT recovered in cynomolgus monkey urine [7, 11]. In humans AZT is glucuronidated more extensively, and the urinary GAZT/AZT ratio is approximately 5–6 [10]. Unfortunately, the molar ratio of AzddU-glucuronide to AzddU recovered in the urine was not reported. Since AzddU and 3′-fluoro-2′,3′-ddT are significantly more hydrophobic than d4T, the results obtained from our correlation studies would have predicted that AzddU and 3′-fluoro-2′,3′-ddT were more likely than d4T to be glucuronidated in primates.

Nucleoside analogs are not the only compounds that can inhibit AZT glucuronidation. The effect of probenecid on the disposition of AZT in humans [20–22] suggested that other drugs that undergo significant glucuronidation may inhibit the conjugation of AZT and lead to an increase in AZT plasma levels. Inhibition of AZT glucuronidation would depend, however, on the substrate specificities of the UDPGT isozymes found in humans [60–63]. Overlapping isozyme specificities toward a number of xenobiotics have been observed. The studies presented here indicate that a number of structurally unrelated xenobiotics that form either *O*-,*N*- or *C*-glucuronides can inhibit AZT glucuronidation.

Morphine, chloramphenicol, and several other drugs are competitive inhibitors of AZT glucuronidation. The K_i (0.9 mM) determined for morphine in these studies is in good agreement with a K_m (0.7 to 1.7 mM) determined previously for the low affinity morphine UDPGT from human liver [64, 65]. The K_i (0.15 mM) estimated for ethinylestradiol is also in good agreement with a K_m (0.2 mM) measured by Pacifici and Back [66]. These results suggest that AZT may be glucuronidated by an isozyme(s) of UDPGT that also glucuronidates morphine and ethinylestradiol.

The kinetic constants determined in this study were used to estimate the percent inhibition of AZT glucuronidation that could be expected at peak physiological drug concentrations (Tinle 3). None of the compounds tested are predicted to produce a strong inhibition of AZT glucuronidation. However, some small effects on AZT metabolism and pharmacokinetics may be observed in humans.

The competitive inhibitor probenecid is estimated to inhibit AZT glucuronidation by approximately 30% at peak plasma concentrations of probenecid and AZT. Acetaminophen, another competitive inhibitor, is estimated to inhibit AZT glucuronidation by less than 1%. Unadkat *et al.* [67] also reported that acetaminophen is a much weaker inhibitor of AZT glucuronidation *in vitro* than is probenecid. Recent clinical data are consistent with the results obtained in these *in vitro* studies. Probenecid increases the area under the plasma concentration–time curve for AZT by approximately 2-fold [20–22], whereas acetaminophen has no effect on

AZT pharmacokinetics [23–25]. Inhibition of AZT glucuronidation is, therefore, not likely to be the mechanism for the increase in hematologic toxicity observed upon concurrent use of acetaminophen and AZT [16].

Of the other pharmaceuticals studied *in vitro*, only chloramphenicol and naproxen are estimated to inhibit AZT glucuronidation by 10% or more at peak physiological drug concentrations. Although other compounds such as furosemide, lorazepam, morphine, oxazepam, and zomepirac are excreted almost exclusively as *O*-glucuronide conjugates [37, 42, 43, 49], these compounds are estimated to inhibit AZT glucuronidation by less than 1% since the peak physiological drug concentrations of these inhibitors are considerably less than their K_i values. Dapsone, cimetidine, sulfisoxazole, and sulfathiazole are also estimated to inhibit AZT glucuronidation less than 1%. However, this result is not surprising since these compounds are not glucuronidated extensively *in vivo*. Only 20–30% of a dose of cimetidine [68] or dapsone [69] and less than 5% of a dose of sulfisoxazole [14, 37, 70] or sulfathiazole [14, 37, 71] are excreted as *N*-glucuronide conjugates in humans. Phenylbutazone [40] and sulfinpyrazone [46], compounds which can be excreted as *C*-glucuronides in humans, are estimated to inhibit AZT glucuronidation by 5 and 0.3%, respectively. No systematic clinical study of the interaction of AZT with any of these compounds has been reported. Acyclovir, a nucleoside analog that is being studied in combination with AZT for the treatment of AIDS [53], does not inhibit AZT glucuronidation *in vitro* and does not modify AZT pharmacokinetics upon co-administration in humans [53, 72].

To date, a limited number of *in vivo* interactions between AZT and other drugs have been reported. However, as new therapies are developed to treat AIDS, associated opportunistic infections, and other medical complications, pharmacokinetic interactions between these new therapies and AZT may be observed. *In vitro* studies with human liver UDPGT may indicate whether these therapies have the potential to inhibit AZT glucuronidation in humans. In addition, since results from these and other studies [12, 13] indicate that *in vitro* studies with human liver UDPGT are consistent with results obtained in humans, further studies with this model system may increase our understanding of nucleoside analogs as a new class of UDPGT substrate.

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