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GLUCURONIDATION OF 3'-AZIDO-3'-DEOXYTHYMIDINE: HUMAN AND RAT ENZYME SPECIFICITY

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Since preclinical studies indicated that 3'-azido-3'-deoxythymidine (AZT, zidovudine, Retrovir®, BW A509U), a potent anti-HIV agent, is not metabolized extensively in rats, rabbits, mice, guinea pigs, cats, or dogs [1,2], the extensive biotransformation of AZT observed in humans was not expected. On average, approximately 75% of an oral AZT dose is recovered in human urine as a single metabolite while only 14-18% of the dose is recovered unchanged [1,3]. Ultraviolet, infrared, nuclear magnetic resonance, and mass spectra and enzymatic degradation characterized the isolated major metabolite as a 5'-O-glucuronide (3'-azido-3'-deoxy-5'-β-D-glucopyranuronosylthymidine, GAZT), a very unique nucleoside metabolite [1]. These observations suggest that UDP-glucuronosyltransferase (UDPGT), EC2.4.1.17, mediates the *in vivo* biotransformation of AZT to GAZT. Since glucuronidation is one of the major conjugation reactions involved in the metabolic conversion of xenobiotics to more polar, water-soluble metabolites, it is an important detoxification pathway in humans. Therefore, it is important to understand the enzymatic basis for the discrepancy between metabolism of AZT in laboratory mammals and humans. This is especially relevant in light of the use of laboratory mammals to predict the metabolism of novel pharmaceutical agents in humans. The study presented herein confirms that liver UDPGT does catalyze the glucuronidation of AZT and that the higher substrate efficiency of AZT with human enzyme compared to rodent enzyme may account for metabolic differences observed *in vivo*.

MATERIALS AND METHODS

Materials. *Escherichia coli* β-glucuronidase 4-methylumbelliferone, UDP-glucuronic acid (UDPGA), 3-methylcholanthrene, and phenobarbital were obtained from the Sigma Chemical Co. (St. Louis, MO). AZT, [^{3}H]AZT, and GAZT were provided by B. Sickles, J. Hill, and S. Good of the Wellcome Research Laboratories. All other chemicals were of the purest grade available.

Enzyme induction. Rats were treated with either phenobarbital (100 mg/kg i.p. on day 1 plus 0.1% phenobarbital in drinking water 5 days prior to sacrifice) or 3-methylcholanthrene (40 mg/kg i.p. (dissolved in olive oil) 4 days prior to sacrifice).

Microsome preparation. Microsomes were prepared as described [4] with minor modifications. Livers of adult male Long Evans rats or human liver samples obtained from donated organs or autopsy specimens were used. Hepatic homogenates were prepared in 150 mM

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KCl, 20 mM KH_2PO_4, pH 7.4, with a Polytron homogenizer (2 x 15 sec homogenizations, power setting 2-3).

Protein assays. A refined Coomassie Blue assay was used to quantitate protein [5]. Bovine serum albumin (BSA) was used as the protein standard.

Glucuronidation assays. 4-Methylumbelliferone glucuronidation was assayed spectrofluorometrically as described [6] with some modifications. Glass reaction vials contained 50 mM Tris-HCl, pH 7.5, 16 mM $MgCl_2$, 5 mM UDPGA, 0.36 mM 4-methylumbelliferone, and 0.1 to 0.5 mg microsomal protein/ml. Fluorescence was measured at an excitation maximum of 358 nm and an emission maximum of 448 nm on a Kontron SFM-25 spectrofluorometer. AZT glucuronidation was assayed in glass vials that contained 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM UDPGA, 2-5 mg microsomal protein/ml, and 0.1 to 15 mM [^{3}H]AZT (5-10 μCi/μmol). Reactions were initiated upon the addition of enzyme and were incubated at 37°. At several time-intervals samples were removed and spotted onto silica gel thin-layer chromatography sheets prespotted with AZT and GAZT carriers. Separation of substrate and product was achieved with ascending chromatography in 80% acetonitrile. [^{3}H]AZT (R_f = 0.75) and [^{3}H]GAZT (R_f = 0.44) spots were cut out, placed in 5 ml Scintilene, and quantitated by scintillation counting. Radioactivity in the product spot from mock reactions with UDPGT omitted (about 1% of the total) was subtracted from that obtained with complete reactions. Product identification was confirmed by HPLC analysis [7]. The radiolabeled product cochromatographed with authentic GAZT. Similar rates of glucuronidation were obtained by TLC and HPLC analyses. Moreover, the radiolabeled product was enzymatically degraded to AZT by *E. coli* β-glucuronidase.

Kinetic analysis. The apparent kinetic constants, K_m and V_{max}, were determined from a direct fit of the data to a hyperbola [8]. Velocities were calculated from the initial, linear portion of the reactions.

RESULTS

Both human liver and rat liver microsomes catalyzed the glucuronidation of AZT. Reactions were linear for 2 hr at each concentration of AZT assayed. The plot of velocity versus substrate concentration exhibited typical Michaelis-Menten saturation kinetics when human liver microsomes served as the source of UDPGT (Fig. 1). Very similar results were obtained with solubilized UDPGT (Fig. 2). In contrast, the plot of velocity versus substrate concentration showed no signs of saturation when rat liver microsomes served as the source of UDPGT. The data of Fig. 3 display this linear relationship. Although induction of rat UDPGT with either phenobarbital or 3-methylcholanthrene increased the rate of glucuronidation of AZT, it did not result in saturating kinetics. The K_m and V_{max} values obtained from two different human liver samples are presented in Table 1. Since the solubility of AZT was less than necessary to obtain accurate K_m and V_{max} values with rat liver UDPGT, velocities produced at 0.1 and 1.0 mM AZT are presented for comparative purposes. The velocity of the glucuronidation of 4-methylumbelliferone was also determined as a standardization reference to assure that all enzyme preparations were active.

Preliminary attempts to purify UDPGT from human liver by chromatography on a UDP-hexanolamine agarose affinity column led to an increase in specific activity of >10-fold for 4-methylumbelliferone glucuronidation. This partially purified enzyme also catalyzed the glucuronidation of AZT with a concomitant 15- to 60-fold increase in specific activity.

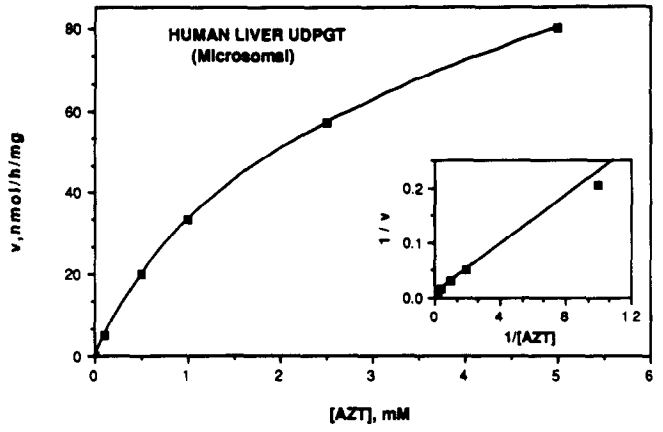

Fig. 1. Effect of AZT concentration on the velocity of glucuronidation catalyzed by human liver microsomal UDPGT.

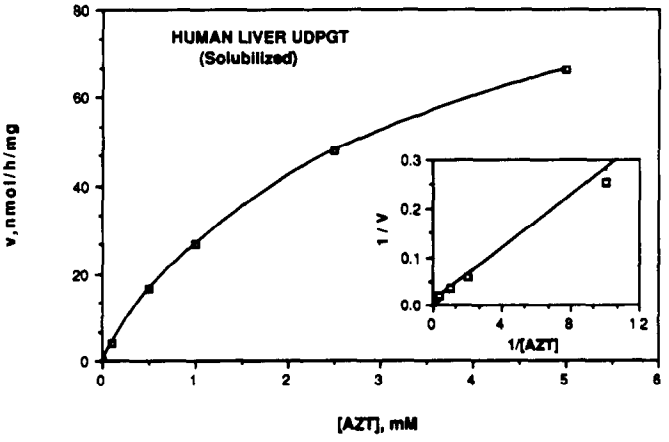

Fig. 2. Effect of AZT concentration on the velocity of glucuronidation catalyzed by detergent-solubilized human liver UDPGT.

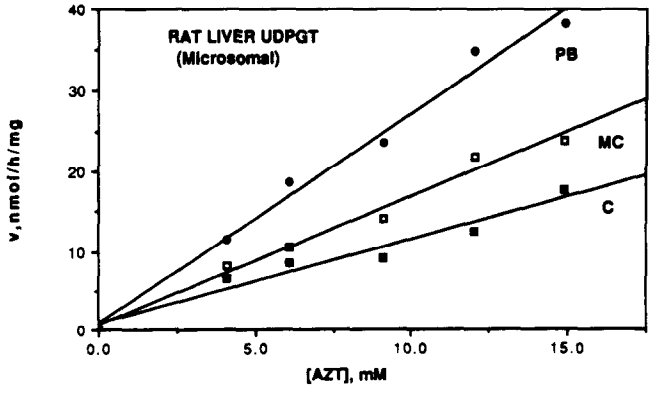

Fig. 3. Effect of AZT concentration on the velocity of glucuronidation catalyzed by rat liver microsomal UDPGT. Rats were either untreated (C) or pretreated with phenobarbital (PB) or 3-methylcholanthrene (MC).

Table 1. Kinetic parameters for the enzymatic glucuronidation of AZT by liver microsomes

Enzyme Source	K_m (mM)	V_{max} (nmol/hr/mg)	Velocity (nmol/hr/mg) [AZT] 0.1 mM	1.0 mM
Human				
#1	2.7 ± 0.2*	122 ± 4	4.4	33
#2	2.2 ± 0.3	58 ± 4	2.5	18
#1 (solubilized)	2.8 ± 0.5	103 ± 9	3.6	27
Rat				
Untreated	>15	>10	0.15	1.5
MC-induced	>15	>20	0.19	1.9
PB-induced	>15	>30	0.31	3.1

*K_m and V_{max} values ± S.E. Other values are an average of two determinations.

DISCUSSION

Rats dosed with 10 mg AZT/ kg body weight excrete 78% of the dose as unchanged drug and <2% as GAZT in the urine (20% of the dose is recovered as 3'-amino-3'-deoxythymidine in the feces)* . This metabolic pattern is in sharp contrast to that observed in humans where only 14-18% of the dose can be recovered in urine as AZT and 72-75% as GAZT [1,3]. These differences in metabolism may be explained by the enzyme studies presented here. Although UDPGT prepared from both rat liver and human liver catalyzed the glucuronidation of AZT, the enzymes from the two sources exhibited large differences in their substrate efficiencies with AZT. The concentrations of AZT used in studies of human UDPGT clearly indicated substrate-saturation kinetics and permitted a K_m value of 2.5 mM to be calculated. On the other hand, there was no indication of hyperbolic kinetics with rat liver UDPGT even at 15 mM AZT, the highest concentration (solubility limitation) tested. It can be concluded that this concentration of AZT must be considerably below its K_m value. In lieu of kinetic constants from both enzyme sources, specific activity velocity measurements at a fixed concentration of AZT provide a direct comparison. Human liver UDPGT catalyzed the glucuronidation of 0.1 mM AZT 10- to 25-fold faster than either induced or constitutive rat liver UDPGT. This difference would be at least as great at AZT's peak plasma concentrations of 3 to 6 μM in humans [3] and 15 μM in rats* and probably accounts for the metabolic differences.

AZT is not the only compound reported to have significantly different glucuronidation patterns in humans and other species. Phenylbutazone, an anti-arthritic agent, is metabolized to a C-glucuronide in humans [9,10], while oxidative metabolism is the primary metabolic route in rats and dogs [9,11]. Tripelennamine, an anti-histaminic, is metabolized to a unique quaternary N-glucuronide in humans [12]; no such metabolite has been observed in rat [13] or guinea pig [14]. These examples and the present study demonstrate that studies *in vitro* with human UDPGT may have advantages over metabolism studies in laboratory animals for predicting patterns of glucuronidation in humans.

*Personal communication, 1988. With permission of S. Good.

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