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The effect of various drugs on the glucuronidation of zidovudine (azidothymidine; AZT) by human liver microsomes

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1 Zidovudine (3′-azido-3′-deoxythymidine; AZT) is the drug of proven efficacy available for the treatment of patients with AIDS or ARC. It is eliminated mainly by hepatic glucuronidation. Therefore, interference with this metabolic pathway may lead to enhancement of AZT effect or to increased toxicity of the drug. We have examined the effect of a number of drugs which themselves undergo glucuronidation on AZT conjugation by human liver microsomes *in vitro*.

2 AZT glucuronidation followed Michaelis-Menten kinetics. The apparent K_m and V_{max} values (mean ± s.d., $n = 5$), were 2.60 ± 0.52 mM and 68.0 ± 23.4 nmol h^{-1} mg^{-1}, respectively, as determined from Eadie-Hofstee plots.

3 Dideoxyinosine, sulphanilamide and paracetamol were essentially non-inhibitory at concentrations up to 10 mM (4 times the concentration of AZT in the incubation). The most marked inhibitory effects were seen with indomethacin, naproxen, chloramphenicol, probenecid and ethinyloestradiol, with enzyme activity decreased by 97.7, 94.9, 88.7, 83.4% and 79.0%, respectively, at a concentration of 10 mM. Other compounds producing some inhibition of AZT conjugation were oxazepam, salicylic acid and acetylsalicylic acid.

4 Further studies are necessary to characterise the inhibition observed but the method described enables a screen of potentially important drug interactions to be carried out.

Keywords zidovudine glucuronidation liver microsomes

Introduction

Zidovudine (3′-azido-3′-deoxythymidine), formerly known as azidothymidine (AZT) is an analogue of naturally occurring thymidine. It is currently the only drug of proven efficacy available for patients with AIDS or ARC. In man, the drug has a short half-life of approximately 1 h (Cload, 1989), being metabolized extensively to an ether glucuronide (3′-azido-3′-deoxy-5′-β-D-glucopyranuronosylthymidine, GAZT (Good *et al.*, 1990; Figure 1). This metabolite is rapidly excreted in the urine. Interference with the conjugation of AZT or the renal excretion of AZT or GAZT by other drugs could lead to enhancement of AZT effect and/or increased toxicity of the drug. On the other hand, administration of a competing drug could be considered as a strategy for reducing the frequency of AZT administration in AIDS patients. In this regard probenecid has been shown by de Miranda *et al.* (1989) to cause a significant increase in the area under the curve (AUC) and half-life of AZT in patients with AIDS or ARC. Based on changes in pharmacokinetics and urinary excretion of GAZT and AZT,

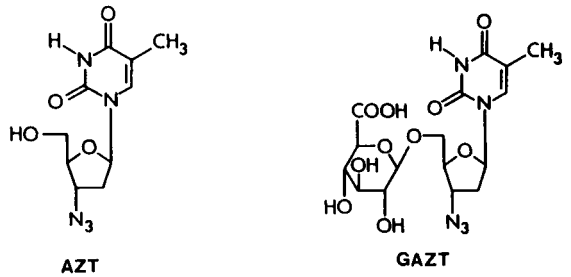

Figure 1 Chemical structures of zidovudine (AZT) and its major metabolite in man (GAZT).

it was concluded that probenecid inhibits AZT glucuronidation and also the renal excretion of GAZT. A similar study by Kornhauser *et al.* (1989) also demonstrated an 80% increase in AUC. This significant increase in the plasma concentration of AZT and the rise in the amount of unchanged AZT recovered in the urine after probenecid, indicated a greatly impaired metabolic

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clearance of AZT. In addition, probenecid also lowered the renal clearance of GAZT by more than 50%, presumably by inhibition of the renal organic anion secretory mechanism.

Both de Miranda *et al.* (1989) and Kornhauser *et al.* (1989) have suggested the use of probenecid to extend the interval between AZT doses and to lower the daily requirement of the drug. De Simone *et al.* (1989) also arrived at a similar conclusion with inosine pranobex (INPX), the *p*-aceto aminobenzoic acid moiety of which is glucuronidated in the liver.

Based on the above findings, it is important to know which drugs, when given to AIDS patients, could promote toxicity by a pharmacokinetic interaction. Also, we need to be able to identify drugs, in addition to probenecid, which could be co-administered deliberately with a lowered dosage of AZT.

We have examined the effects of a number of drugs which themselves undergo glucuronidation on the conjugation of AZT by human liver microsomes.

Methods

Chemicals

Tritiated AZT ([5′-^{3}H]-3′-azido-3′-deoxythymidine, 12.5 Ci mmol^{-1}), AZT and GAZT were gifts from the Wellcome Research Laboratories. Oxazepam was a gift from Wyeth Laboratories, dideoxyinosine from Bristol-Myers International Corporation, naproxen from Syntex and indomethacin from Merck, Sharp and Dohme Research Laboratories. Probenecid, paracetamol, sulphanilamide, acetylsalicylic acid, salicylic acid, 17α-ethinyloestradiol, chloramphenicol and UDP-glucuronic acid (sodium salt) were purchased from the Sigma Chemical Co. H.p.l.c. grade acetonitrile was purchased from Fisons. Orthophosphoric acid (Aristar grade) was purchased from BDH Ltd. All other chemicals were of analytical reagent grade.

Human liver samples

Samples of histologically normal livers were obtained from kidney transplant donors. Ethical approval for the study was granted and consent to removal of the liver was obtained from the donor's relatives. Samples were transferred on ice to the laboratory within 30 min where they were sectioned into 10–20 g portions, placed in plastic vials and frozen in liquid nitrogen at −196° C. Liver was stored at −80° C until required.

Preparation of liver microsomes

Washed microsomes were prepared by the differential centrifugation technique (Purba *et al.*, 1987). The tissue was chopped with scissors and then ground in 67 mM phosphate buffer (pH 7.4), containing 1.15% w/v KCl, with an Ultra-Turrax device. A 25% homogenate was then produced using a Teflon in glass motor drawn homogenization device. The first microsomal pellets (105,000 *g* pellets) were resuspended in buffer and centrifuged to obtain the washed microsomes. Microsomal protein yield was determined by the method of Lowry *et al.* (1951).

Glucuronidation assays

The glucuronidation of AZT by human liver microsomes was assayed in microcentrifuge tubes that typically contained 4.5 to 9.5 mg microsomal protein ml^{-1}, 5 mM $MgCl_2$, 5 mM UDPGA, 50 mM Tris-HCl, pH 7.5 and 2.5 mM AZT in a final volume of 0.2 ml. The concentration of UDPGA used was approximately 3-fold that of its K_m. After equilibrating for approximately 10 min at 37° C, the reaction was started by the addition of UDPGA and the samples were then incubated for 1 h at 37° C. Reaction rates were shown to be linear for incubation times up to at least 1.5 h and for microsomal protein content to at least 8.5 mg ml^{-1}. The incubation was terminated by the addition of 0.1 ml acetonitrile to precipitate the protein. After centrifuging at 12,000 *g* for 3 min to remove particulate matter, 5 to 20 μl aliquots of the supernatant was injected into the chromatograph and assayed for AZT and GAZT according to a modified method of Good *et al.* (1988).

H.p.l.c. assays

The chromatograph used was fitted with a pre-saturation column (25 cm × 4.6 mm i.d., Whatman pre-column gel, 37–53 μm), a guard column (8 cm × 4.6 mm i.d., Whatman C_{18} pellicular ODS, 37–53 μm), and a Radial-Pak LC Cartridge (10 × 8 mm i.d. μBondapak C_{18}, 10 μm, Waters Associates) housed in a Z-module Radial Compression Separation System (Waters Associates). The absorbance of the effluent was monitored at 267 nm using a variable-wavelength detector (Cecil Instruments, CE 112). Samples were eluted at a constant flow rate of 1.0 ml min^{-1} using a mobile phase comprising a 15 : 85 mixture of acetonitrile : ammonium phosphate buffer [0.1% (v/v) phosphoric acid, adjusted to pH 2.70 with ammonium hydroxide]. All chromatography was performed at ambient temperature (approximately 22° C). Under these conditions the retention times of GAZT and AZT were 8.9 min and 14.4 min, respectively.

Peak areas of GAZT and AZT were integrated using a Spectra-Physics integrator (SP4290). The amount of GAZT produced at the end of incubation was calculated from the percentage area of the GAZT peak integrated for each injection, knowing the amount of AZT present at the start of incubation. The validity of using the integrated metabolite peak area percentage method for measuring the amount of GAZT present was checked by injecting aliquots of known proportions of AZT and GAZT standards and measuring the relative percentage of the integrated peaks. For example, if a 50/50 (v/v) mixture was injected comparable peak areas were obtained, whereas if a 90/10 (AZT/GAZT; v/v) mixture was injected the relative areas of the respective peaks was 90 to 10.

In cases where the inhibitor (e.g. paracetamol) or its metabolite interfered with the u.v. detection of GAZT or AZT, it was necessary to utilise radiometric detection. Fractions (0.5 ml) of eluent were collected in scintillation vial inserts to which were added 4 ml of scintillant (Aqua Luma, LKB) and the radioactivity of each fraction was

determined by liquid scintillation spectrometry (Beckman, LS1801).

Kinetic and inhibitor studies

In experiments performed to determine apparent K_m and V_{max} values for AZT glucuronidation, the UDPGA concentration was kept constant (5 mM) and enzyme activity was measured for five AZT concentrations over the range of 0.25–10 mM. To determine the apparent K_m and V_{max} values for UDPGA, the concentration of AZT was kept constant (2.5 mM) and activity was measured for five UDPGA concentrations over the range 0.625–10 mM.

Possible inhibitory effects of probenecid, paracetamol acetylsalicylic acid, salicylic acid, oxazepam, 17α-ethinyloestradiol, naproxen, indomethacin, dideoxyinosine and chloramphenicol were assessed using a fixed AZT concentration (2.5 mM and 1.5 μCi ml^{-1} [^{3}H]-AZT where appropriate) and varying inhibitor concentrations over the range 0.5–10 mM. These experiments were carried out using microsomes from one liver (L8). The inhibitory effects (if any) of the above drugs were confirmed with two other livers (L9 and W1) at inhibitor concentrations of 0.5 and 10 mM only.

Analysis of results

Results are presented as mean ± s.d. The Michaelis-Menten parameters V_{max} and apparent K_m were determined using an iterative programme (ENZPACK), based on non-linear least squares regression analysis.

Results

AZT glucuronide formation followed Michaelis-Menten kinetics in all five livers studied (Figure 2). At a fixed UDPGA concentration of 5 mM, but with variable AZT, the apparent K_m and V_{max} values (mean ± s.d.; $n = 5$) were 2.60 ± 0.52 mM and 68.0 ± 23.4 nmol h^{-1} mg^{-1} respectively (Table 1). The variation in apparent K_m was 1.5 fold. The V_{max} value obtained with the liver (L8) of a donor who had been on long-term phenytoin and phenobarbitone treatment (109 nmol h^{-1} mg^{-1}) was approximately twice that of the other livers studied. Using a fixed concentration of AZT (2.5 mM) and varying UDPGA concentration resulted in linear Eadie-Hofstee plots (data not shown). The mean apparent K_m for UDPGA in four livers was 1.29 ± 0.56 nM, (variation 2.3 fold).

Table 1 Michaelis-Menten parameters of AZT glucuronidation in human liver microsomes

Donor	*Sex*	*Age (years)*	K_m *(mM)*	V_{max} *(nmol h^{-1} mg^{-1})*
L7	M	29	2.28	66.1
L9	M	27	2.21	55.8
L8	F	46	2.97	109.0
W1	F	17	3.32	54.4
WT1	M	18	2.21	54.7
Mean ± s.d.			2.60 ± 0.52	68.0 ± 23.4

Apparent K_m and V_{max} values were calculated by fitting the Michaelis-Menten equation for a single enzyme system to the experimental data.

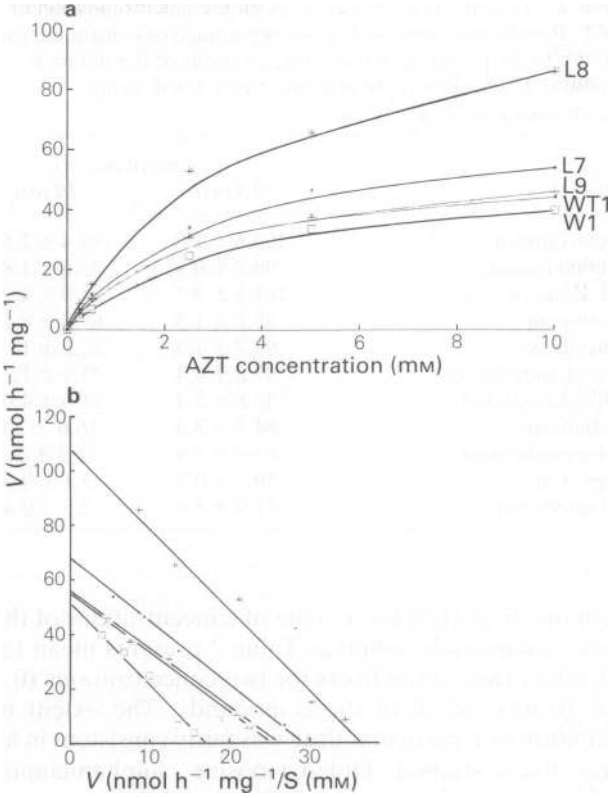

Figure 2 The effect of varying AZT concentration on microsomal glucuronosyltransferase activity (nmol h^{-1} mg^{-1}) of five human livers. a) velocity *vs* substrate concentration, b) Eadie-Hofstee.

The effects of acetylsalicylic acid, chloramphenicol, dideoxyinosine, ethinyloestradiol, indomethacin, naproxen, oxazepam, paracetamol, probenecid, salicylic acid and sulphanilamide on AZT glucuronidation are illustrated in Figure 3 and Table 2. Figure 3 shows data

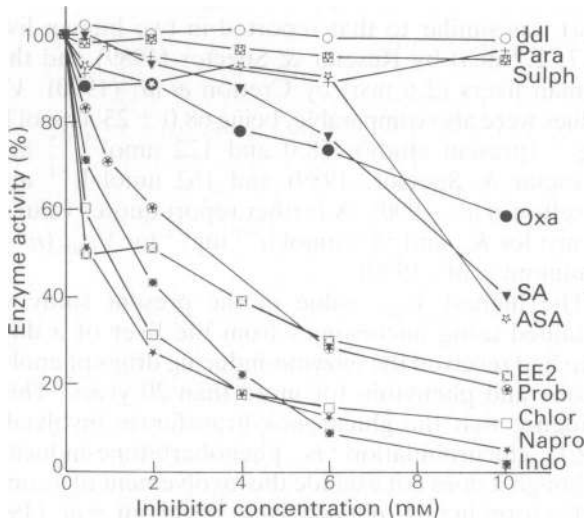

Figure 3 The effect of various drugs on microsomal glucuronosyltransferase activity in a single human liver. ddI, dideoxyinosine; Para, paracetamol, Sulph, sulphanilamide; Ox, oxazepam; SA, salicylic acid; ASA, acetylsalicyclic acid; EE_2, ethinyloestradiol; Prob, probenecid; Chlor, chloramphenicol; Naprox, naproxen; Indo, indomethacin.

Table 2 The effect of various drugs on the glucuronidation of AZT. Results are expressed as the percentage of control enzyme activity in the presence of two concentrations of the putative inhibitor. Each value represents the mean ± s.d. using microsomes from three livers

Drug	% control activity 0.5 mM	10 mM
Dideoxyinosine	100.6 ± 1.8	98.4 ± 2.5
Sulphanilamide	99.2 ± 0.9	93.6 ± 1.8
Paracetamol	101.1 ± 9.7	91.8 ± 4.3
Oxazepam	89.5 ± 1.5	67.9 ± 8.1
Salicylic acid	99.7 ± 1.3	52.2 ± 13.1
Acetylsalicyclic acid	97.8 ± 4.4	43.9 ± 12.1
Ethinyloestradiol	46.8 ± 3.4	21.0 ± 4.0
Probenecid	84.7 ± 3.3	16.6 ± 2.8
Chloramphenicol	63.3 ± 2.6	11.3 ± 2.3
Naproxen	50.2 ± 0.7	5.1 ± 0.3
Indomethacin	71.9 ± 3.6	2.3 ± 0.4

from one liver (L8) for a range of concentrations of the above compounds, whereas Table 2 presents mean (± s.d.) data from three livers for two concentrations (0.5 and 10 mM) of all of the compounds. The extent of inhibition by a particular drug was fairly consistent in all three livers studied. Dideoxyinosine, sulphanilamide and paracetamol were essentially non-inhibitory at concentrations up to 10 mM (i.e. four times the AZT concentration). The most marked inhibitory effects were seen with indomethacin, naproxen, chloramphenicol, probenecid and ethinyloestradiol, with IC_{50} values (concentration of inhibitor producing 50% inhibition of AZT conjugation) of 1.6, 0.5, 1.0, 3.2 and 2.2 mM, respectively.

Discussion

AZT glucuronidation by human liver microsomes has been characterized using a h.p.l.c. procedure for the measurement of GAZT. Conjugation followed Michaelis-Menten kinetics. The apparent K_m value (2.60 ± 0.52 mM) was similar to that reported in two human livers (2.7; 2.2 mM) by Resetar & Spector (1989) and three human livers (2.6 mM) by Cretton *et al.* (1990). V_{max} values were also comparable, being 68.0 ± 23.4 nmol h^{-1} mg^{-1} (present study), 58.0 and 122 nmol h^{-1} mg^{-1} (Resetar & Spector, 1989) and 162 nmol h^{-1} mg^{-1} (Cretton *et al.*, 1990). A further report quotes values of 13 mM for K_m and 58.7 nmol h^{-1} mg^{-1} for V_{max} ($n = 3$; Haumont *et al.*, 1990).

The highest V_{max} value in the present study was obtained using microsomes from the liver of a donor who had received the enzyme-inducing drugs phenobarbitone and phenytoin for more than 20 years. This is evidence that the glucuronosyltransferase involved in AZT glucuronidation is phenobarbitone-inducible, although it does not exclude the involvement of a constitutive form in non-induced liver. Haumont *et al.* (1990) have recently shown that in rats, AZT glucuronidation was stimulated four-fold by phenobarbitone. We have previously shown that the glucuronidation of the steroid ethinyloestradiol was also enhanced in our induced liver (Pacifici & Back, 1988).

Having obtained the basic kinetic data we proceeded to examine the effect of a number of drugs, which themselves undergo glucuronidation, on AZT conjugation. Paracetamol was essentially non-inhibitory, which is consistent with other *in vitro* (Haument *et al.*, 1990) and *in vivo* (Koda *et al.*, 1989; Prazin *et al.*, 1989) findings. Sulphanilamide (which forms an *N*-glucuronide) was also non-inhibitory. In contrast, the non-steroidal anti-inflammatory drugs, naproxen and indomethacin produced marked inhibition such that at a concentration of 10 mM only 5.1 and 2.3% of the enzyme activity remained. Similarly chloramphenicol, ethinyloestradiol and probenecid were very inhibitory. The probenecid data are consistent with the *in vivo* findings of de Miranda *et al.* (1989) and Kornhauser *et al.* (1989). In both of these clinical studies there was good evidence to show that probenecid lowers the metabolic clearance of AZT.

Oxazepam, salicylic acid and acetylsalicylic acid produced some inhibition of AZT conjugation but this was much less than with the other compounds. We excluded the possibility that in the presence of salicylic acid, GAZT may have been hydrolysed because of a lowering of the pH of the incubation medium.

Dideoxyinosine (ddI) is the deamination product of dideoxyadenosine (ddA) and possesses a high level of antiviral activity. Comparatively little is known about the overall metabolism of ddI in humans. A recent paper has speculated that a conjugated metabolite of ddI might exist, similar to AZT glucuronide (Hartman *et al.*, 1990). In our *in vitro* model, ddI failed to produce any inhibition of AZT conjugation.

Using the *in vitro* human liver microsome system it should be possible to screen those drugs that may interact with AZT *in vivo*. However, although we could predict that naproxen, indomethacin, chloramphenicol and ethinyloestradiol, in addition to probenecid (for which clinical findings exist) will alter AZT disposition in AIDS patients, the likelihood of an *in vivo* interaction depends upon the plasma (or more importantly hepatic) concentrations attained by the substrate and inhibitor. After oral administration of 200 mg AZT, peak plasma concentrations are approximately 4–5 μM (Taburet *et al.*, 1990). Peak concentrations of naproxen and indomethacin will be higher after conventional dosage regimens (350 μM after 500 mg and 8 μM after 50 mg twice and three times daily (Van Den Ouweland *et al.*, 1987; Pullar *et al.*, 1988). Although these *in vivo* plasma concentrations are somewhat less than the concentrations used in the *in vitro* system, they indicate that naproxen and indomethacin will be in excess of AZT. If a compound is essentially non-inhibitory in the *in vitro* system, it is unlikely to inhibit *in vivo* (c.f. paracetamol). Rather than quoting IC_{50} values or percentage inhibition at a certain drug concentration, it is important to determine K_i values and the nature of inhibition for each alleged inhibitor. This refinement should be included in future studies. It was not possible to determine any underlying structural similarities between inhibitory or non-inhibitory compounds or the type of conjugate formed. Although AZT forms an ether glucuronide, indomethacin, naproxen and probenecid all form ester conjugates and are inhibitory. In contrast, paracetamol which forms an ether conjugate is non-inhibitory while ethinyloestradiol and chloramphenicol also form ether conjugates but do

inhibit. However, multiple forms of UDP-glucuronosyltransferases (UDPGTs) exist and it is clear that many UDPGTs are capable of reacting with more than one xenobiotic and with different classes of xenobiotic. In addition, numerous xenobiotics react with more than one isoform of UDPGT (Tephly & Burchell, 1990).

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