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In vitro inhibition studies of the glucuronidation of 3′-azido-3′-deoxythymidine catalysed by human liver UDP-glucuronosyl transferase

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3′-Azido-3′-deoxythymidine (zidovudine*), formerly known as azidothymidine (AZT), is at present the drug of choice for the treatment of AIDS and ARC although newly developed drugs such as ddI and 2′,3′-dideoxycytidine are currently being evaluated in clinical trials. Zidovudine is phosphorylated by cellular enzymes to the respective 5′-triphosphate which inhibits HIV, the aetiological agent of AIDS, replication by preferential inhibition of HIV reverse transcriptase and termination of viral DNA chain elongation [1]. In man, zidovudine has a half-life of 1 hr [2] and is metabolized extensively to an ether glucuronide (GAZT; [3]), thereby making the metabolite more polar and hence more suitable for renal excretion. To date, the UDPGT responsible for the metabolism of zidovudine remains unidentified although the $UDPGT_2$ form has been implicated recently [4]. In addition, there is the possibility of extrahepatic metabolism [5].

Because of the nature of the disease, patients with AIDS and ARC will be receiving, in addition to their AZT, a spectrum of other drugs. Whenever there is a multi-drug use there is the potential for drug interaction [6]. Interference with the conjugation of AZT could lead to the enhancement of AZT effect and potentially increased toxicity of the drug. Alternatively, the deliberate administration of a competing drug could have an economic benefit by enabling a reduction in AZT dosage regimens. In this regard, De Miranda *et al.* [7] and Kornhauser *et al.* [8] have advocated the use of probenecid, which inhibits AZT glucuronidation and the renal excretion of GAZT, to reduce the daily requirement of AZT. De Simone *et al.* [9] have suggested the use of inosine pranobex to inhibit the hepatic metabolism of AZT.

From a toxicological perspective it is essential to pinpoint drugs which are capable of interfering with the hepatic metabolism of AZT. Since an important group of patients presenting with AIDS or ARC are drug abusers we have examined the effects of a number of drugs commonly used by such patients (morphine, codeine, methadone and cocaine) on the glucuronidation of AZT by human liver microsomes. Previous work has demonstrated that both morphine and codeine are glucuronidated by human liver microsomes [10]. In addition, a range of other drugs known to be conjugated have been studied.

* Abbreviations: zidovudine, 3′-azido-3′-deoxythymidine; AZT, azidothymidine; AIDS, acquired immune deficiency syndrome; ARC, AIDS-related complex; GAZT, 3′-azido-3′-deoxy-5′-β-D-glucopyranosylthymidine; UDPGT, glucuronosyltransferase; UDPGA, UDP-glucuronic acid; ddI, 2′-3′-dideoxyinosine.

Materials and Methods

Chemicals. AZT and GAZT were gifts from the Wellcome Research Laboratories (Beckenham, U.K.). 2′,3′-Dideoxyinosine was a gift from Bristol-Myers Squibb (Wallingford, USA) and naproxen from Syntex Pharmaceuticals (Maidenhead, U.K.). Morphine sulphate, methadone hydrochloride, codeine, cocaine, diazepam, temazepam, flunitrazepam, probenecid, sulphamethoxazole, estrone, salicylic acid and UDPGA (ammonium salt) were purchased from the Sigma Chemical Co. (Poole, U.K.). HPLC grade acetonitrile was purchased from Fisons plc (Loughborough, U.K.). Orthophosphoric acid (Aristar grade) was purchased from BDH (Poole, U.K.). All other chemicals used were of the highest grade available.

Human liver samples. Samples of histologically normal livers were obtained from kidney transplant donors. Ethical approval for the studies was granted and consent to removal of the liver was obtained from the donors' relatives. Liver 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°, immediately. Liver samples were stored at −80° until required.

Preparation of microsomes. Washed microsomes were prepared by the classical differential centrifugation technique [11]. Microsomal protein yield was determined by the method of Lowry *et al.* [12].

Glucuronidation assay. The glucuronidation of AZT was assayed in 1.5 mL microcentrifuge tubes that typically contained the following: 5–7 mg/mL microsomal protein, 5 mM $MgCl_2$, 50 mM Tris–HCl pH 7.5, 5 mM UDPGA and 0.5–10 mM AZT in a final volume of 0.2 mL. The detergent Brij 58 was used in preliminary experiments to determine optimal activation conditions. For optimal activation, microsomes were preincubated with 0.25 mg Brij 58/mg

Table 1. Effect of various drugs on AZT glucuronidation in human liver microsomes

Compound	K_i (mM)
Diazepam	0.30 ± 0.09 (3)
Temazepam	0.58 ± 0.30 (4)
Flunitrazepam	0.19 ± 0.03 (4)
Morphine	1.01 ± 0.21 (4)
Codeine	3.28 ± 1.88 (4)
Cocaine	3.80 ± 1.85 (4)
Methadone	1.10 ± 0.21 (4)
Probenecid	0.53 ± 0.23 (3)
Naproxen	0.20 ± 0.04 (3)
Sulfamethoxazole*	<20% inhibition at 5 mM (AZT = 2.5 mM)
Estrone*	<20% inhibition at 3 mM (AZT = 2.5 mM)
Salicylic acid*	<30% inhibition at 6 mM (AZT = 2.5 mM)
Dideoxyinosine*	No inhibition at 5 mM (AZT = 2.5 mM)

K_i values (means ± SD) were determined from Dixon plots ($1/V$ vs [I]). Incubations contained 0–10 mM AZT and at least four inhibitor concentrations.

The figure in brackets denotes the number of microsomal preparations from different livers.

* Kinetic studies not performed because of low extent of inhibition.

protein. The concentration of UDPGA used was approximately 3-fold that of its K_m as described previously [13]. Incubations were equilibrated for 10 min prior to the addition of UDPGA, after which incubations were continued for 1 hr at 37° and terminated by the addition of 0.1 mL acetonitrile to precipitate the protein. After centrifugation at 13,000 rpm for 3 min to remove particulate matter, 10–20 μL aliquots were assayed for AZT and GAZT using reverse phase HPLC according to a modified method of Good *et al.* [14] and as described previously [13].

Inhibition studies. The inhibitory effects of the various compounds were assessed in microsomal preparations from four livers using the AZT glucuronidation assay. Initially, generation of IC_{50} data was carried out at an AZT concentration of 2.5 mM whilst varying the inhibitor concentration between 0 and 10 mM. The apparent K_i was determined from graphical (Dixon) plots of $1/V$ against [I] using AZT concentrations varying between 0.25 and 10 mM and inhibitor concentrations between 0 and 10 mM. Results are presented as the means ± SD of four livers. The Michaelis–Menten parameters V_{max} and K_m were determined using an iterative programme (ENZPACK), based on non-linear least squares regression analysis to fit the experimental data to the Michaelis–Menten equation.

RESULTS

All of the livers used in this study were obtained from patients who had normal case histories and to our knowledge had not received UDPGT enzyme-inducing drugs prior to death. The UDPGT activity towards AZT followed Michaelis–Menten kinetics with K_m and V_{max} values of 2.5 ± 1.1 mM and 48 ± 12 nmol/hr/mg, respectively (mean values generated in four livers).

Activation by Brij-58 was maximal (4–5-fold increase) at a concentration of 0.25 mg detergent/mg microsomal protein; thereafter enzyme activity declined. We decided to use non-activated microsomes for inhibition studies for two reasons; firstly, the K_i value obtained for morphine was virtually identical in activated (1.11 mM) and non-activated (1.01 mM) microsomes, and secondly Dixon plots deviated from linearity at high concentrations of probenecid in activated microsomes. We recognise that it is possible theoretically that in using a non-activated system the drugs under test could produce some activation at higher concentrations. The effects of the alleged inhibitors of AZT glucuronidation are shown in Table 1. Based on K_i values the rank order of inhibitory potency is: flunitrazepam > naproxen > diazepam > probenecid > temazepam > morphine > methadone > codeine > cocaine > other drugs tested. Representative Dixon plots for flunitrazepam, diazepam, naproxen and morphine are shown in Fig. 1.

DISCUSSION

UDPGTs are low affinity, high capacity enzymes [15] responsible for the biotransformation of xenobiotics and endogenous compounds. Multiple forms of UDPGT exist in human liver, some with overlapping substrate specificity. Numerous studies have been performed on the glucuronidation of a variety of compounds by human liver microsomes and it is possible to obtain preliminary information about the substrate specificity of human UDPGTs from kinetic-inhibitor studies [16]. We and others [4, 13, 17] have used this approach to try to assess whether or not some commonly used drugs will interfere with the glucuronidation of AZT.

Since drug abusers are a group from which a large number of AIDS and ARC patients are drawn it is possible that they will be particularly susceptible to drug interactions with consequent toxicological sequelae. In addition to being a drug of abuse morphine is also used to alleviate pain in patients with advanced cancer [18]. Both morphine and codeine undergo extensive hepatic glucuronidation [19] and in addition codeine has a minor pathway of conversion to morphine [20]. The conjugation of morphine and codeine has been considered to be under the control of the same or similar UDPGTs [19]. However, Miners *et al.* [21] have reported that morphine glucuronidation is under the control of more than one form of UDPGT and that these isozymes have overlapping substrate specificity. The present study shows morphine to be a more potent inhibitor of AZT conjugation than codeine. The K_i value for morphine (1.01 ± 0.21 mM) is virtually identical to that reported previously (0.97 ± 0.06 mM) [17]. The occurrence of a clinically relevant pharmacokinetic interaction to a large extent depends upon the blood concentration (or more important, the hepatic concentration) achieved after dosing. Established AZT regimens produce peak plasma concentrations of 5 μM [22]. Peak plasma concentrations of morphine and codeine attained after single doses have

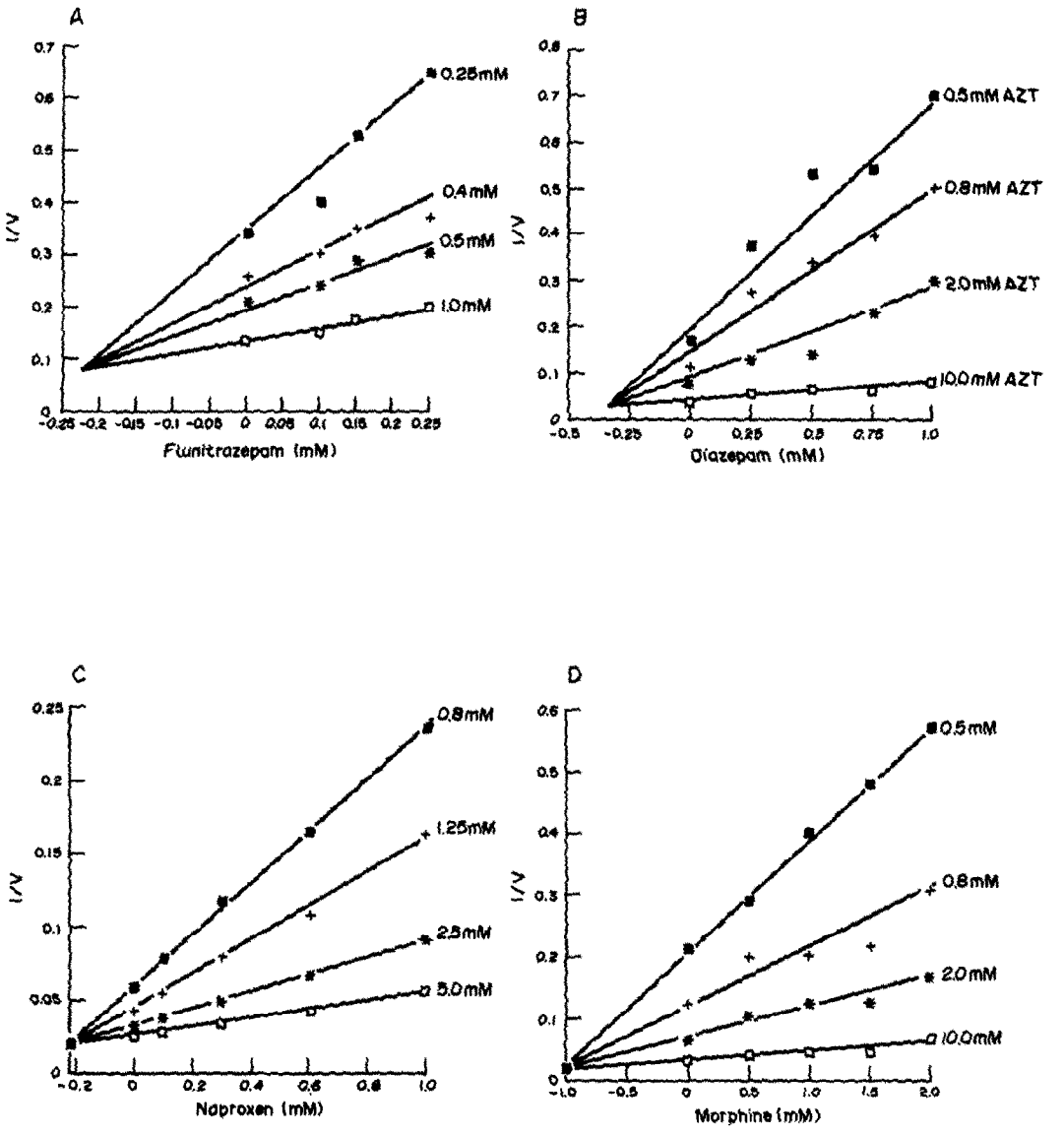

Fig. 1. Representative Dixon plots for the inhibition of AZT glucuronidation in human liver microsomes. Inhibitors were flunitrazepam (A), diazepam (B), naproxen (C) and morphine (D). Velocities are expressed as nmol/hr/mg protein.

been reported as 0.5 μM [23] and 0.4 μM [24], respectively. Clearly to obtain meaningful plasma concentrations from drug abusers is difficult since the lifestyle leads to irregular dosing times and amounts administered. However, concentrations in drug abusers are likely to be higher than reported above and hence AZT, morphine and codeine plasma levels may be comparable. Resetar *et al.* [17] have calculated that the theoretical percentage inhibition obtainable *in vitro* at physiological (peak plasma) concentrations of morphine and AZT is less than 0.1% i.e. morphine is unlikely to produce a clinically important interaction. Using the same equation ($i = 100\ [I]/(K_i(1 + [S]/K_m) + [I])$, where i = percentage inhibition) codeine will also produce less than 0.1% inhibition. Of the other compounds we have studied probenecid is estimated to inhibit AZT glucuronidation by more than 50% at peak plasma concentrations of AZT and probenecid [25], and naproxen to inhibit by 30% at peak concentrations [26]. Although the benzodiazepines diazepam, temazepam and flunitrazepam produce marked inhibition *in vitro*, it is unlikely that this will be translated into an important *in vivo* effect. Taking an extreme example of a benzodiazepine abuser [27] with peak blood diazepam levels of 1.2 μg/mL (4.2 μM), the estimated inhibition of AZT glucuronidation is less than 2%.

It is interesting that we obtained considerable inhibition of AZT glucuronidation in the presence of methadone, a drug which is not metabolized by conjugation but rather undergoes a cytochrome P450 dependent N-demethylation reaction to inactive metabolites [28]. There is also a

clinical report [29] indicating that methadone alters the pharmacokinetics of AZT and gives rise to elevated plasma concentrations. The mechanism for inhibition may be similar to that reported for the inhibition of morphine and codeine glucuronidation by diazepam [10] in that there is an affinity for the binding site of the UDPGTs without actually being a substrate for the enzyme. Cocaine is metabolized by plasma cholinesterases to a ecgonine methyl ester [30] as well as undergoing a cytochrome P450 dependent N-demethylation [31]. It is not metabolized by glucuronidation and appears to be a very weak inhibitor of conjugation.

Finally we examined the effects of ddI. It is very probable that in the near future combinations of AZT and ddI will be in clinical use. Although the metabolic profile of ddI has not been firmly established in man it has been suggested that a glucuronide conjugate may be formed [32]. However, we have found no evidence of an *in vitro* interaction between AZT and ddI.

In summary, until the UDPGT responsible for AZT glucuronidation has been cloned and the resultant protein used to produce specific antibodies, the use of microsomal enzyme assays will prove to be an important experimental approach to gaining an understanding of AZT–drug interactions.

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Modulation of superoxide production from murine macrophages by the antitumour agent flavone acetic acid and xanthenone acetic acid analogues

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Flavone-8-acetic acid (FAA*) is a synthetic flavonoid compound whose antitumour activity against a broad range of experimental solid tumours [1] contrasts sharply with a lack of activity in clinical trials [2]. The mechanism of action of FAA *in vivo* is not fully understood but involves the production of cytokines [3] and nitric oxide [4]. FAA also increases the direct *in vitro* cytotoxicity of murine macrophages against tumour targets [5] and stimulates the formation of active nitrogen intermediates in activated macrophages [6]. Cultures of elicited macrophages release substantial amounts of superoxide when stimulated with PMA [7]. Antiinflammatory agents can inhibit the production of superoxide from appropriately stimulated macrophages or polymorphonuclear leukocytes [8–10] and there is evidence that some flavonoids are antiinflammatory, and modulate the release of reactive oxygen intermediates [11, 12]. XAA, which has been used as the basis for the synthesis in this laboratory of novel compounds with antitumour properties similar to those of FAA [13, 14], is also reported to have antiinflammatory properties [15]. These observations raise the question of whether the antitumour action of FAA and XAA analogues involves modulation of the production of active oxygen intermediates.

In this study, the effect of FAA on superoxide production from murine macrophages in the presence or absence of PMA is described and the effects of FAA with those of XAA derivatives compared. These derivatives include 5,6-MeXAA, the most dose potent of antitumour agents [14], and 8-MeXAA, an inactive analogue [13]. The use of this series with diverse dose potency and activity provides an excellent basis for determining whether superoxide production correlates with antitumour effects. Thus, comparisons are also made here between the effects of these agents on superoxide production and their antitumour effects against the experimental s.c. Colon 38 murine tumour.

* Abbreviations: FAA, flavone-8-acetic acid; PBS, phosphate buffered saline; SPBS, supplemented phosphate buffered saline; PMA, phorbol 12-myristate 13-acetate; XAA, xanthenone-4-acetic acid; 5-MeXAA, 5-methyl XAA; 5,6-MeXAA, 5,6-dimethyl XAA; 8-MeXAA, 8-methyl XAA; DMSO, dimethyl sulphoxide.

Materials and Methods

Materials. α-Minimal essential culture medium (Gibco, Grand Island, NY, U.S.A.) was supplemented with foetal calf serum, 2-mercaptoethanol (50 μM), penicillin (100 units/mL) and streptomycin sulphate (100 μg/mL). SPBS consisted of PBS supplemented with calcium chloride (2.7 mM), magnesium chloride (3.4 mM) and glucose (5.6 mM). FAA (National Cancer Institute, U.S.A.) and XAA derivatives (synthesized as described [13, 14] by Drs W. A. Denny, G. J. Atwell and G. W. Rewcastle) were dissolved immediately prior to use in a minimal amount of 5% (w/v) sodium bicarbonate and diluted in SPBS. Indomethacin (Merck Sharpe and Dohme (NZ) Ltd) was dissolved in a minimal amount of DMSO and diluted in SPBS. Sodium bicarbonate and DMSO at the highest concentrations used (0.001% and 0.2%, respectively) were shown not to alter cell viability or superoxide production in the *in vitro* macrophage cultures. PMA (Sigma Chemical Co., St. Louis, MO, U.S.A.) was prepared as a 1.62 mM stock solution in DMSO and stored as 2.5 μL aliquots in glass vials at −70°. Ferricytochrome *c* (Sigma) was prepared as a 1.5 mM stock solution in SPBS, stored in 1 mL aliquots at −20° and diluted in SPBS immediately prior to use. Superoxide dismutase (Sigma) was prepared in SPBS (7.5 units/μL) and stored at −20°. Thioglycollate (Beckton Dickinson and Co., Cockeysville, U.S.A.) (10% w/v) broth was in sterile water.

Macrophage preparation and culture. C_3H/HeN mice were bred in the laboratory animal facility under constant temperature and humidity with sterile bedding, water and food according to institutional ethical guidelines. Mice between 6 and 12 weeks of age were injected i.p. with thioglycollate broth (0.2 mL/mouse). Mice were killed by cervical dislocation 4–6 days later and peritoneal exudate cells were collected in PBS. Adherent macrophages were collected by plating 1.25×10^5 cells in 0.5 mL culture medium in 24-well plates (or alternatively, 3×10^5 cells in 100 μL culture medium in 96-well flat-bottom microwell trays) and incubating for 2 hr at 37° in 95% air/5% CO_2.