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SHORT COMMUNICATIONS

The effect of malaria infection on 3'-azido-3'-deoxythymidine and paracetamol glucuronidation in rat liver microsomes

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Abstract—The effect of malaria infection on UDP-glucuronosyltransferase (UDPGT) activity was investigated in rat liver microsomes using 3'-azido-3'-deoxythymidine and paracetamol. The Michaelis–Menten parameters, K_m and V_{max} were calculated and intrinsic clearance values were estimated for normal and infected livers. The results show that malaria infection alters the activity of UDPGT.

Malaria infection is known to affect adversely Phase I drug metabolism [1, 2]. Although Phase II conjugation reactions, particularly glucuronidation, play an important role in detoxification, little is documented concerning UDP-glucuronosyltransferase (UDPGT*) activity in malaria-infected liver. Emudianughe *et al.* [3] observed inhibition of benzoic acid glucuronidation with increasing parasitaemia in mice infected with malaria. Recently, Mansor *et al.* [4] found that malaria infection decreased plasma concentrations of paracetamol glucuronide in rats. We have investigated the effect of infection with the rodent malarial parasite (*Plasmodium berghei*) on UDPGT activity in rat liver microsomes using two test substrates, 3'-azido-3'-deoxythymidine (AZT) and paracetamol, which are metabolized in human and rat liver by direct conjugation with glucuronic acid [5, 6]. Evidence suggests that these substrates are metabolized by different izozymes in human liver; AZT by the $UDPGT_2$ form [7] and paracetamol possibly by the digitoxigenin monodigitoxoside UDPGT [8]. Malaria infection could affect these two enzymes differently, since previous work [9] has shown that *P. berghei*, which produces changes in hepatic ultrastructure that resemble those caused by the human malarial parasite *P. falciparum* [10, 11], can impair selectively the formation of antipyrine metabolites in the rat which are regulated by different forms of cytochrome P450. Thus, the use of substrates which are regulated by different isozymes would permit observation of any selectivity of inhibitory effects.

Materials and Methods

3-Acetamidophenol, paracetamol, Brij 58 (polyoxyethylene 20-cetyl ether) and UDP-glucuronic acid (UDPGA) (sodium salt) were obtained from the Sigma Chemical Co. (Poole, U.K.). Paracetamol glucuronide was a gift from Sterling Winthrop (Alnwick, U.K.) AZT and 3'-azido-3'-deoxy-5'-β-D-glucopyranosylthymidine (azidothymidine glucuronide) (GAZT) were obtained from the Wellcome Research Laboratories (Beckenham, U.K.). All other reagents and solvents, of analytical grade, were supplied by BDH Chemicals (Poole, U.K.).

Infection of animals with malaria. Male Wistar rats (100 ± 20 g) were infected with the rodent-specific malaria parasite *P. berghei* N strain using an inoculation (i.p.) of 10^6 parasitized erythrocytes from previously infected male C.B.A. mice. Blood samples were taken from the tail vein for daily monitoring of the degree of parasitaemia by Giemsa-stained thin blood films evaluated under a light microscope. Rats were killed and their livers removed at two stages of the infection, at a lower parasitaemia phase (10–20% parasitaemia) and at a higher parasitaemia phase (25–35% parasitaemia). Livers from uninfected rats acted as controls. Microsomes from both the infected and control livers were prepared by differential centrifugation [12].

Measurement of AZT glucuronidation by rat liver microsomes. The incubation mixture contained UDPGA (10 mM), $MgCl_2$ (25 mM), microsomal protein (from infected and control livers; 0.8 mg), Brij 58 (detergent to protein ratio 0.1; previously found to be optimal for activation), Tris–HCl buffer (50 mM) and AZT (1–16 mM) in a final volume of 200 μL. Reaction times were demonstrated to be linear up to 1 mg microsomal protein concentration and incubation times up to 1 hr. Incubations were performed at 37° for 1 hr and the reaction was stopped by the addition of acetonitrile (100 μL). The tubes were then centrifuged (13,000 *g*; 7 min) and aliquots (10 μL) were injected on to the chromatograph.

The Spectra Physics (Hemel Hempstead, U.K.) HPLC comprised a Rheodyne 7125 valve injector, an SP8800 ternary pump and an SP8450 UV/vis detector operating at 267 nm. Separation was effected at ambient temperature on a 10 micron C-18, Radial-PAK cartridge (100 cm × 8 mm i.d.; Waters Millipore, Harrow, U.K.). The mobile phase was 85% ammonium acetate buffer and 15% acetonitrile adjusted to pH 2.7 at a flow rate of 1 mL/min [13, 14]. Peak areas of AZT and GAZT were integrated using an SP4100 computing integrator. The retention times for AZT and GAZT were 7 and 12 min, respectively. The retention time of GAZT produced was confirmed by comparison to an authentic standard. The coefficients of variation for determination of AZT and GAZT were less than 5% at 20 μM.

Measurement of paracetamol glucuronidation by rat liver microsomes. The method by Miners *et al.* [8] was used to assay UDPGT activity using paracetamol as the substrate. A standard microsomal incubation contained UDPGA (20 mM), $MgCl_2$ (20 mM), Tris–HCl (50 mM, pH 7.4), microsomal protein (from infected and control livers 0.5 mg), Brij 58 (detergent to protein ratio of 0.15; optimal value for activation) and paracetamol (1–20 mM) in a final volume of 250 μL. Incubations were performed at 37° for 1 hr. Reaction times were linear up to incubation times of 3 hr and up to microsomal protein concentrations of 0.75 mg. The reaction was stopped by the addition of saturated barium hydroxide (100 μL) and 20% zinc sulphate in 50% aqueous methanol (100 μL). The incubation tubes were centrifuged (1500 *g*; 5 min) and an aliquot (100 μL) of the supernatant from each tube was transferred to an Eppendorf tube. NaOH (0.5 M; 50 μL) and the internal standard (3-acetamidophenol; 50 ng) were added and the tubes centrifuged (1500 *g*) for a further 5 min. An aliquot (20 μL) of the supernatant was injected on the HPLC.

* Abbreviations: AZT, 3'-azido-3'-deoxythymidine; GAZT, 3'-azido-3'-deoxy-5'-β-D-glucopyranosylthymidine; UDPGA, UDP-glucuronic acid; UDPGT, UDP-glucuronosyltransferase.

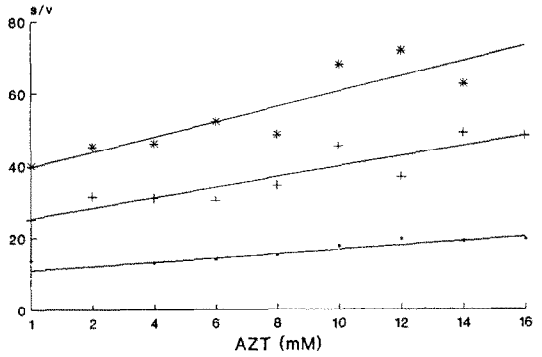

Fig. 1. Hanes–Woolf plots for AZT glucuronidation in (·) controls and malaria-infected livers, (+) low percentage parasitaemia, (*) high percentage parasitaemia.

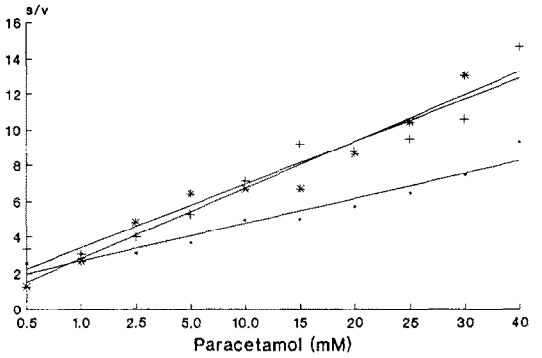

Fig. 2. Hanes–Woolf plots for paracetamol glucuronidation in (·) controls, (+) low percentage parasitaemia and (*) high percentage parasitaemia.

The HPLC system used was as described above and the mobile phase was 98% aqueous phase (sodium acetate buffer; 0.5 M; pH 6) and 2% organic phase (methanol containing 13% ethyl acetate) flowing at 2.5 mL/min [15]. This condition was maintained for the first 7 min; subsequently, the composition was altered to 80% of aqueous phase and 20% organic phase for 5 min. The flow rate was kept constant at 2.5 mL/min. Under these conditions, the retention times for paracetamol glucuronide, paracetamol and the internal standard were 4, 10 and 12 min, respectively. Quantification (250 nm) of both paracetamol and paracetamol glucuronide was done by comparison of measured peak heights to those of a standard curve generated by the addition of known quantities of the standards to a fixed amount of the internal standard (50 ng) in drug-free microsomal suspensions. The interassay coefficients of variation were 5% for the determination of paracetamol at 130 μM and 3.5% for the determination of paracetamol glucuronide at 30 μM.

Protein determination. The method of Lowry *et al.* [16] was used to measure the microsomal protein concentrations.

Analysis of results. Results are presented as the means ± SD of six livers. The Michaelis–Menten parameters V_{max} and apparent K_m of each individual experiment were estimated by linear regression analysis of Hanes–Woolf plots using ENZPACK. Representative plots for controls and malaria-infected livers for both AZT and paracetamol are shown in Figs 1 and 2, respectively. An estimate of intrinsic clearance Cl_{int} was made from the relationship: $Cl_{int} = V_{max}/K_m$ [17]. Statistical significance was assessed by one-way analysis of variance (ANOVA).

Results

AZT and paracetamol glucuronide formation followed Michaelis–Menten kinetics in all the livers studied. The K_m and V_{max} values are shown in Table 1 for AZT and in Table 2 for paracetamol glucuronidation. Decreases in Cl_{int} and V_{max} values ($P < 0.05$) were observed in malaria-infected livers when AZT was the test substrate. K_m values were unchanged. When paracetamol was the test drug, both K_m and V_{max} values ($P < 0.005$) were decreased, whereas Cl_{int} values remained the same.

Discussion

The substrate concentrations used in this study to generate kinetic parameters are in excess of those found in patients receiving doses of either AZT (3 μM after 200 mg [18]) or paracetamol (100 μM after 1000 mg [19]). The range of substrate concentrations must exceed K_m in order to ensure that it is not limiting.

Table 1. Apparent kinetic constants for glucuronidation with AZT in livers of rats experimentally infected with *P. berghei*

	Controls	10–20% parasitaemia	25–35% parasitaemia
K_m (mM)	13.1 ± 8.1	16.1 ± 8.4	18.5 ± 8.6
V_{max} (nmol/mg/min)	1.82 ± 0.90	0.98 ± 0.60	0.93 ± 0.40*
Cl_{int} (μL/mg/min)	0.18 ± 0.10	0.06 ± 0.05*	0.06 ± 0.05*

* $P < 0.05$ significantly different compared to controls.

Table 2. Apparent kinetic constants for glucuronidation with paracetamol in livers of rats experimentally infected with *P. berghei*

	Controls	10–20% parasitaemia	25–35% parasitaemia
K_m (mM)	18.3 ± 4.3	9.04 ± 4.6*	7.57 ± 2.00*
V_{max} (nmol/mg/min)	6.70 ± 1.80	4.03 ± 0.80*	3.53 ± 1.00*
Cl_{int} (μL/mg/min)	0.38 ± 0.10	0.44 ± 0.20	0.50 ± 0.20

* $P < 0.005$ significantly different compared to controls.

For AZT in rat liver microsomes, Cretton *et al.* [20] reported an apparent K_m of 9.1 mM and a V_{max} of 1.6 nmol/mg/min. The present study gives a value of 13.1 ± 8.1 mM for K_m and 1.82 ± 0.9 nmol/mg/min for V_{max} in control rat liver microsomes (see Table 1). A 48% decrease in V_{max} values was seen in both the low and high parasitized livers. This shows that the reduction in V_{max} for AZT glucuronidation is not related to the degree of parasitaemia. The K_m values were not affected by malaria infection. A decrease in V_{max} without any change in the apparent K_m values in infected livers has been observed in studies with liver cirrhosis [21] and liver fluke infection [22]. K_m is inversely related to the affinity of a particular enzyme for a substrate, whereas V_{max} reflects its capacity in catalysing a reaction. Hence, the impairment by malaria infection observed in the AZT glucuronidation is due to a deficiency in enzyme protein and not a change in the affinity of the enzyme for the substrate. An *in vitro* estimate of intrinsic clearance (Cl_{int}), defined as the volume of liver water cleared of drug per unit time, was estimated from the ratio V_{max}/K_m [17]. Since V_{max} decreased while K_m remained unchanged, Cl_{int} was reduced in parasitized livers. This suggests a decrease in the ability of the malaria-infected liver to eliminate AZT by the glucuronidation pathway.

For paracetamol, the apparent kinetic constants K_m and V_{max} reported by Miners *et al.* [8] using human liver microsomes are 7.37 ± 0.99 mM and 4.76 ± 1.35 nmol/mg/min, respectively. Values of K_m and V_{max} for paracetamol glucuronidation using rat liver microsomes have not been reported. Bolanowska and Gessner [23] reported an activity value of 1.48 nmol/mg/min for rat liver microsomal UDPGT with 5 mM paracetamol and 15 mM UDPGA. In the present study, the activity obtained with 5 mM paracetamol and 20 mM UDPGA is 1.4 nmol/mg/min. The percentage decrease in V_{max} in both phases of malaria-infected livers was about the same as that observed for AZT glucuronidation (44%). However, a decrease in K_m due to malaria infection was also observed with paracetamol. It is not clear as to why the K_m values decreased; it is possible that one or more of the pathophysiological changes induced by malaria infection could modulate the glucuronidation process resulting in the apparent increase in the affinity for glucuronidation to paracetamol (Table 2). Since the magnitude of the decrease in K_m is about equal to that of the decrease in V_{max}, the Cl_{int} values do not change for the malaria-infected livers.

In summary, malaria infection affects the glucuronidation reactions of AZT and paracetamol. For AZT, it is evident in the decrease of Cl_{int} and V_{max} values, for paracetamol in the decrease of K_m and V_{max} values. The reason for these different effects is not clear and merits further investigation. This work also suggests that during malaria infection, the ability of the liver to glucuronidate is altered and this factor could be important when administering drugs that are eliminated by this pathway.

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