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AN INVESTIGATION INTO THE FORMATION OF STABLE, PROTEIN-REACTIVE AND CYTOTOXIC METABOLITES FROM TACRINE *IN VITRO*

STUDIES WITH HUMAN AND RAT LIVER MICROSOMES

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Abstract—Tacrine (1,2,3,4-tetrahydro-9-aminoacridine hydrochloride; THA) is known to undergo extensive oxidative metabolism to a variety of mono- and dihydroxylated metabolites in animals and humans. The potential for tacrine to undergo metabolism to stable, protein-reactive and cytotoxic metabolites has been investigated in incubations with human and rat liver microsomes. Using lymphocytes as sensitive markers to quantify cytotoxicity, THA (50 μM) underwent NADPH-dependent bioactivation to a cytotoxic metabolite(s). NADPH-dependent cytotoxicity in the presence of rat and human microsomes was 9.8 ± 3.1% ($P < 0.05$ cf. −NADPH control) and 6.2 ± 2.0% ($P < 0.05$ cf. −NADPH control), respectively. Stable and protein-reactive metabolites were also formed in microsomes from both species. These accounted for 28.2 ± 12.7% and 1.22 ± 0.79% of incubated radioactivity in human microsomes and 6.4 ± 2.2% and 0.4 ± 0.1% of incubated radioactivity in rat microsomes. In microsomes pooled from six human livers the NADPH-dependent cytotoxicity was 9.4 ± 1.1%. Formation of stable and protein-reactive metabolites accounted for 29.2 ± 2.3% and 1.2 ± 1.0% of incubated radioactivity. Reduced glutathione (500 μM) completely blocked NADPH-dependent cytotoxicity and inhibited protein-reactive metabolite formation by 60% ($P < 0.05$). Ascorbic acid (500 μM) inhibited the generation of cytotoxic and protein-reactive metabolites by 75% ($P < 0.05$) and 35% ($P < 0.05$), respectively. Cyclohexene oxide was without effect. Human serum albumin was found to protect the lymphocytes against toxicity. In microsomes prepared from the livers of four donors known to have been smokers there were no significant differences in the generation of metabolites from THA compared with microsomes prepared from livers of non-smokers. Enoxacin, a specific inhibitor of cytochrome P450 1A2 significantly inhibited all routes of THA metabolism. We have therefore demonstrated that THA may be oxidatively metabolized to stable, protein-reactive and cytotoxic metabolites in human and rat liver microsomes. A number of inhibitors may affect these processes, whilst inhibition by enoxacin indicates a role for cytochrome P450 1A2 in THA metabolism.

Tacrine (1,2,3,4-tetrahydro-9-aminoacridine hydrochloride; THA§) is a potent centrally acting anticholinesterase [1] which is currently under investigation for the treatment of Alzheimer's disease. Whilst several investigators have reported a beneficial effect of THA in Alzheimer's patients [2–4] there have also been a number of reports of the high incidence of elevations in liver function enzymes in patients undergoing THA therapy [4–6]. These elevations were not found in any of the animals used in preclinical safety evaluation studies but are dose-dependent and reversible upon reduction of the dose or withdrawal of THA in the patients [4].

In general phase I (or oxidative) drug metabolism results in the formation of polar metabolites facilitating excretion from the body. The majority of these oxidations are performed by the cytochrome P450 mixed function oxidase system of the endoplasmic reticulum [7]. For some drugs such as paracetamol [8, 9], carbamazepine [10], dapsone [11, 12] and amodiaquine [13, 14] oxidative metabolism may result in the generation of chemically reactive metabolites. Formation of covalent adducts between these reactive metabolites and cellular macromolecules such as protein or DNA can lead to toxicity [15] which may manifest itself as carcinogenicity [16], direct tissue damage [17] or as an indirect secondary immune response [18].

THA is known to undergo extensive oxidative metabolism in rat and humans *in vivo*, in both species the major metabolites were identified as 1-OH THA, 2-OH THA and 4-OH THA [19, 20]. However, no information is available on the propensity of THA to undergo metabolic biotransformation to toxic species. In view of this, the purpose of this report is to describe a series of investigations into the formation of stable, chemically reactive and cytotoxic metabolites from THA by microsomes from human and rat liver in an *in vitro* test system in which human mononuclear leucocytes are used as simple general target cells for cytotoxicity.

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§ Abbreviations: ASC, ascorbic acid; CHO, cyclohexene oxide; CYP1A2, cytochrome P450IA2; dH_2O, distilled water; GSH, reduced glutathione; HSA, human serum albumin (fraction V); MNL, mononuclear leucocytes; THA, tacrine; TCPO, 1,1,1-trichloropropaneoxide.

These cells are chosen because they are easily obtained in large numbers, contain certain detoxication enzymes in the form of epoxide hydrolase [21] and glutathione-*S*-transferase [22] which may be manipulated independently of the microsomal activating system and under our experimental conditions there is no bioactivation of the drug by the lymphocyte. Furthermore lymphocytes have been successfully employed as target cells to assess the toxicity of a wide range of drugs such as phenytoin, paracetamol, carbamazepine, ciamexon, sorbinil and mianserin [10, 23–25].

MATERIALS AND METHODS

Chemicals. THA, 1-OH THA, 2-OH THA, 4-OH THA, 7-OH THA, [^{14}H]THA (11.47 Ci mmol^{-1}) and enoxacin were all gifts from the Parke-Davis Pharmaceutical Research Division (MI, U.S.A.). Reduced glutathione (GSH), ascorbic acid (ASC), cyclohexene oxide (CHO) and human serum albumin (HSA: fraction V) were purchased from the Sigma Chemical Co. (Poole, U.K.). Reduced NADPH (tetrasodium salt) was obtained from BDH Chemicals Ltd (Poole, U.K.) and Lymphoprep was purchased from Nycomed (Birmingham, U.K.). All solvents were of HPLC grade and were products of Fisons plc (Loughborough, U.K.). All other chemicals were of the purest grade available and were purchased from either Sigma or BDH.

Preparation of human and rat liver microsomes. Washed human liver microsomes, from histologically normal livers obtained from renal transplant donors, were prepared as described previously [26]. Ethical approval for the study was obtained from the local ethics committee. Rat microsomes were prepared by the same centrifugation procedure used to obtain the washed human microsomes. Once prepared the microsomal pellets were stored at −80° until use. The microsomal protein content was measured by the method of Lowry *et al.* [27], and the cytochrome P450 content estimated by the method of Omura and Sato [28].

Isolation of human mononuclear leucocytes (MNL). Whole blood was obtained from healthy male volunteers (21–30 years) and MNL prepared as described previously [23]. Cell viability upon isolation was >98% as assessed by Trypan blue exclusion.

Determination of metabolism dependent cytotoxicity of THA. Isolated MNL (1×10^6) in 15 mM HEPES-buffered salt medium [1 mL; containing NaCl (125 mM), KCl (0.6 mM), $MgSO_4$ (0.25 mM), NaH_2PO_4 (1.3 mM), $CaCl_2$ (1.3 mM) and glucose (0.4 mM), pH 7.4] were incubated with human or rat liver microsomes (1 mg) in the presence and absence of NADPH in a shaking water bath at 37° for 1 hr. Some incubations also contained a putative modifier [GSH (500 μM), ASC (500 μM), CHO (30 μM), HSA (0.5–2.0 mg) enoxacin (50–250 μM)]. After 1 hr the cells were sedimented and resuspended in drug free buffer (HEPES-buffered salt medium containing HSA: 5 mg/mL) and incubated at 37° for a further 16 hr. Aliquots were then removed to determine cell viability by Trypan blue exclusion. Cells were diluted 1:0.2 with dye prior to counting; in the absence of drug cell death was 4.2 ± 0.5% after the 16 hr incubation.

Metabolism of THA by human and rat hepatic microsomes. Incubations at 37° contained [^{14}C]THA (50 μM; 0.2 μCi), human or rat liver microsomal protein (0.5–2.0 mg) and 0.067 M ammonium phosphate buffer (pH 7.4) to give a final volume of 1 mL. The reaction was initiated by the addition of NADPH and terminated after 1 hr by the addition of ice-cold acetonitrile (5 mL). Some incubations also contained an alleged inhibitor [GSH (500 μM), ASC (500 μM), CHO (30 μM) enoxacin (50–250 μM)].

Analysis of stable metabolites. The reaction mixture with added acetonitrile was left overnight at 4° to precipitate the microsomal protein which was sedimented by centrifugation (2500 rpm for 15 min). The supernatants were removed, evaporated to dryness and resuspended in a small volume of methanol for HPLC analysis using a Spectra Physics 8800 model linked to a radiometric detector (Flo-one βeta, Canberra Packard). Chromatographic separation was performed on a nucleosil 5 C8 column (25 cm: HPLC technology, Macclesfield, U.K.) protected by an on-line guard column. THA and metabolites were eluted from the column using a gradient solvent system of acetonitrile in 0.1 M ammonium acetate buffer (pH 6.9). The acetonitrile content of the mobile phase was 15% (v/v) for 15 min followed by a linear increase to 35% and maintained at this for a further 15 min. The flow rate was 1.25 mL/min. THA and metabolites were identified by co-chromatography with co-injected authentic standards using UV detection at 254 nm. Radioactivity was monitored throughout each run and the peaks were integrated and expressed as a percentage of the total radioactivity eluting from the column.

Determination of protein-reactive metabolite formation. The formation of protein-reactive metabolites was determined as the amount of radiolabelled material irreversibly bound to the microsomal protein after exhaustive solvent extraction [2 × methanol (5 mL) and 2 × 70% methanol in distilled water (dH_2O) (v/v; 5 mL) washes]. The protein was dissolved in 1 M NaOH (1 mL) and aliquots taken for liquid scintillation counting (500 μL) and protein estimation (100 μL).

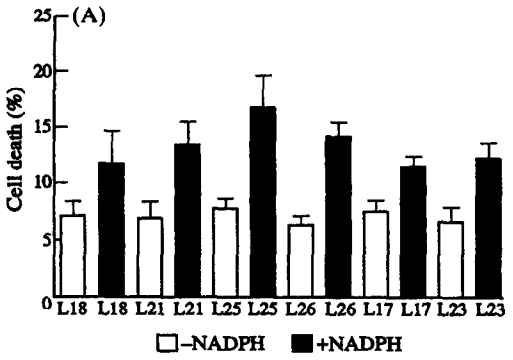
The amount of radiolabelled material bound is expressed as a percentage of the total radioactivity in the original incubation.

Statistical analysis. Statistical analyses were performed by one way analysis of variance or by a Student's *t*-test for unpaired samples accepting $P < 0.05$ as significant. Data are presented as the mean ± SD of quadruplicate determinations.

RESULTS

Activation of THA to a cytotoxic metabolite by human and rat liver microsomes

The activation of THA (50 μM) to a cytotoxic metabolite was assessed in the presence of microsomes prepared from the livers of six humans and six rats. There was bioactivation of THA in microsomes from all six human livers (Fig. 1A), with a mean NADPH-dependent cytotoxicity of



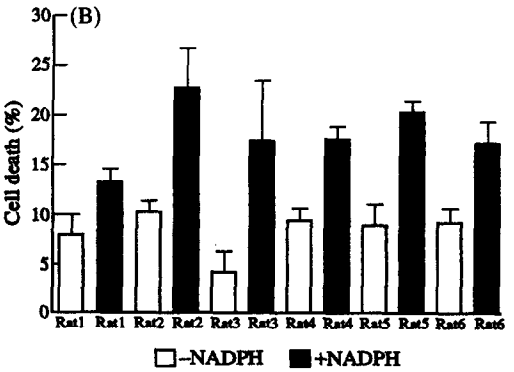


Fig. 1. The formation of cytotoxic metabolites from THA (50 μM) by human (A) and rat (B) liver microsomes in the absence and presence of NADPH (1 mM). The values represent the means ± SD of four determinations.

6.2 ± 2.0% ($P < 0.05$ cf. −NADPH) being produced. Microsomes from all six rat livers also converted THA to a cytotoxic metabolite in the presence of NADPH (Fig. 1B), again to varying degrees with a mean NADPH-dependent cytotoxicity of 9.8 ± 3.1% ($P < 0.05$ cf. −NADPH) being achieved.

Table 2. Formation of protein-reactive and stable metabolites (expressed as a % of incubated radioactivity) from THA in human and rat microsomes in the presence of NADPH (1 mM) or an NADPH regenerating system

	Protein reactive		Stable	
Liver	+NADPH	+RS	+NADPH	+RS
L17	0.58 ± 0.08	0.34 ± 0.05	18.7 ± 4.2	18.6 ± 4.9
L26	1.69 ± 0.26	1.10 ± 0.05	44.3 ± 2.3	37.8 ± 2.9
Rat 3	0.50 ± 0.14	0.49 ± 0.10	9.0 ± 2.2	5.6 ± 0.8

Values are the means ± SD of four determinations. RS, regenerating system.

Formation of stable and protein-reactive metabolites of THA by human and rat liver microsomes

THA was metabolized in an NADPH-dependent manner to stable and protein-reactive metabolites in the presence of both human and rat liver microsomes (Table 1). In microsomes from five human livers 28.2 ± 12.7% and 1.22 ± 0.79% of incubated radioactivity was present as stable metabolites and protein-reactive metabolites, respectively. In microsomes from livers of three rats formation of stable and protein-reactive metabolites was significantly lower than that obtained in human microsomes. Only 6.4 ± 2.2% and 0.4 ± 0.1% of incubated radioactivity was present as stable and protein-reactive metabolites, respectively. Use of a regenerating system in preference to NADPH had no effect on either parameter in human or rat microsomes (Table 2).

Effect of GSH, ASC and CHO on the formation of cytotoxic, stable and protein-reactive metabolites from THA

Microsomes were pooled from six human livers for this part of this study. In the absence of the putative modifiers NADPH-dependent cytotoxicity in the presence of THA was 9.4 ± 1.1%. GSH (500 μM) completely blocked NADPH-dependent

Table 1. Formation of protein-reactive and stable metabolites (expressed as a % of incubated radioactivity) from THA in human and rat microsomes in the absence and presence of NADPH (1 mM)

	Protein-reactive		Stable	
Liver	−NADPH	+NADPH	−NADPH	+NADPH
L17	0.02 ± 0.01	0.58 ± 0.08*	0	18.7 ± 4.2*
L18	0.03 ± 0.01	0.68 ± 0.04*	0	15.6 ± 1.7*
L21	0.02 ± 0.01	0.76 ± 0.05*	0	25.7 ± 4.0*
L25	0.02 ± 0.01	2.40 ± 0.60*	0	39.8 ± 7.4*
L26	0.02 ± 0.01	1.69 ± 0.26*	5.8 ± 3.8	44.3 ± 2.3*
Rat 1	0.06 ± 0.01	0.40 ± 0.04*	0	5.4 ± 0.5*
Rat 2	0.05 ± 0.01	0.29 ± 0.04*	0	4.9 ± 0.1*
Rat 3	0.08 ± 0.02	0.50 ± 0.14*	0	9.0 ± 2.2*

Values are the means ± SD of four determinations, *$P < 0.05$ compared to −NADPH.

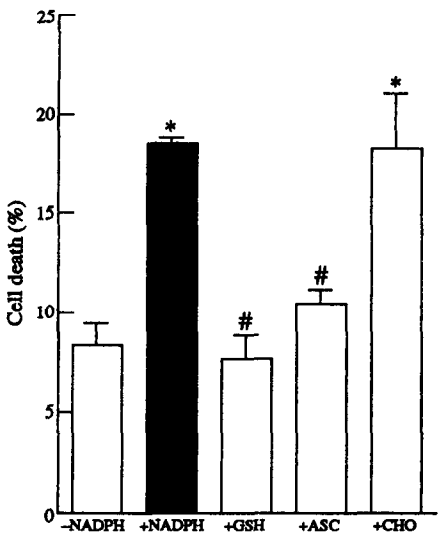


Fig. 2. A comparison of the effects of GSH (500 μM), ASC (500 μM) and CHO (30 μM) on the formation of cytotoxic metabolites from THA (50 μM) by human liver microsomes in the absence and presence of NADPH (1 mM). The values represent the means ± SD of four determinations, *P < 0.05 compared to −NADPH, #P < 0.05 compared to +NADPH.

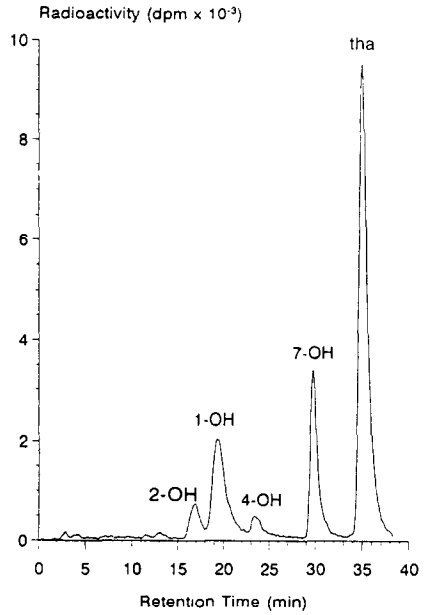


Fig. 3. A representative HPLC chromatograph of an acetonitrile extract of a microsomal incubation with THA (50 μM) in the presence of NADPH (1 mM). Metabolites were identified by co-chromatography with authentic standards.

cytotoxicity. A 75% reduction was seen in the presence of ASC (500 μM), with no effect being observed in the presence of CHO (30 μM) (Fig. 2). Similar, but less pronounced, effects were seen on the formation of protein-reactive metabolites. GSH and ASC caused a 60 and 35% reduction, respectively, CHO again having no effect. Stable metabolites of THA were identified as 2-OH, 1-OH, 4-OH and 7-OH THA (Fig. 3) by co-chromatography with authentic standards. Co-incubation with the three modifiers had no effect on the formation of stable metabolites (Table 3).

Effect of HSA on THA cytotoxicity

Using microsomes pooled from six human livers addition of HSA (0.5–2.0 mg) produced a dose-dependent decrease in cytotoxicity (Fig. 4). In the absence of HSA an NADPH-dependent cytotoxicity of 7.8 ± 2.1% was observed, this was reduced by 31% in the presence of 0.5 mg HSA and by 100% in the presence of 1.0 and 2.0 mg HSA.

Comparison of THA metabolism in microsomes from livers of smokers and non-smokers

By comparing the data from Tables 1 and 4 and Figs 1A and 5 it can be seen that smoking status appears to have little effect on THA metabolism *in vitro*. NADPH-dependent cytotoxicity was 6.2 ± 2.0% (Fig. 1) with microsomes from non-smokers compared to 6.4 ± 1.1% (Fig. 5) in smokers (P > 0.05). Formation of stable metabolites was 28.8 ± 12.7 (Table 1) in non-smokers compared to 32.3 ± 23.4% in smokers (Table 5: P > 0.05). Similarly generation of protein reactive metabolites was increased slightly in microsomes from smokers

Table 3. Formation of protein-reactive and stable metabolites (expressed as a % of incubated radioactivity) from THA in incubations with microsomes pooled from six human livers in the presence of NADPH (1 mM) and modifiers [GSH (500 μM), ASC (500 μM) and CHO (30 μM)]

	Protein-reactive	2-OH THA	1-OH THA	4-OH THA	7-OH THA	THA
Control	1.20 ± 0.10	4.06 ± 0.17	12.77 ± 1.02	0.82 ± 1.63	10.34 ± 0.44	70.81 ± 2.30
+GSH	0.46 ± 0.04*	4.19 ± 0.64	12.17 ± 0.39	1.58 ± 1.89	11.71 ± 0.84*	69.86 ± 1.93
+ASC	0.78 ± 0.08*	5.75 ± 2.09	13.02 ± 1.39	0.52 ± 1.04	13.81 ± 3.20	66.11 ± 5.23
+CHO	1.13 ± 0.05	4.80 ± 0.83	13.56 ± 1.23	0.55 ± 1.09	13.08 ± 3.22	66.88 ± 3.59

Values are the means ± SD of four determinations, *P < 0.05 compared to control.

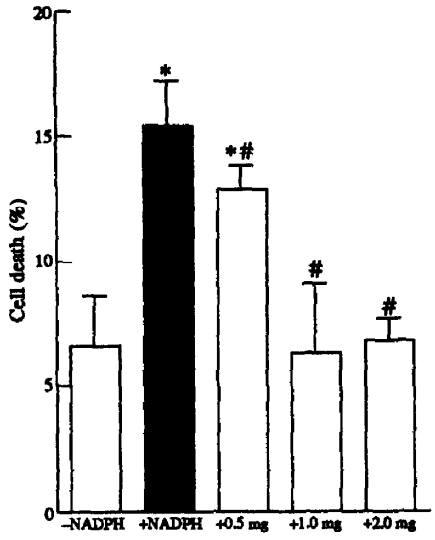


Fig. 4. A comparison of the effects of increasing concentrations of HSA on the formation of cytotoxic metabolites from THA (50 μM) by human liver microsomes in the absence and presence of NADPH (1 mM). Results are expressed as the means ± SD of determinations, *P < 0.05 compared to −NADPH, #P < 0.05 compared to +NADPH.

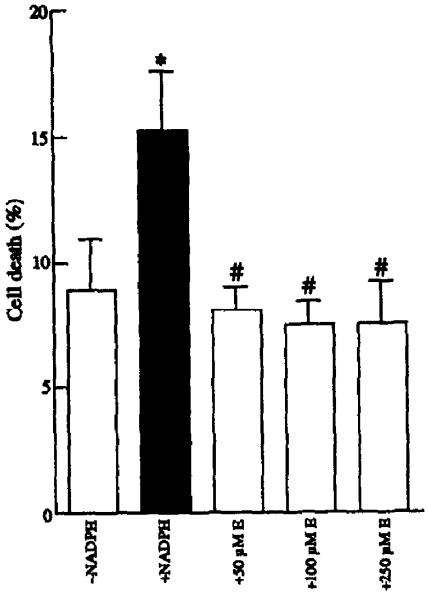


Fig. 5. A comparison of the effects of increasing concentration of enoxacin on the formation of cytotoxic metabolites from THA (50 μM) by human liver microsomes in the absence and presence of NADPH (1 mM). Results are expressed as the means ± SD of four determinations in four separate microsomal preparations, *P < 0.05 compared to −NADPH, # P < 0.05 compared to +NADPH.

Table 4. Formation of protein-reactive and stable metabolites (expressed as a % of incubated radioactivity) from incubations with THA and microsomes from four livers in the presence of NADPH (1 mM) and enoxacin (E; 50–250 μM)

	Protein-reactive	2-OH THA	1-OH THA	4-OH THA	7-OH THA	THA
L10 control	2.57 ± 0.10	7.63 ± 1.08	31.27 ± 1.10	3.85 ± 0.44	13.49 ± 0.52	41.19 ± 0.98
L10 + 50 μM E	2.26 ± 0.20*	7.17 ± 0.96	29.05 ± 2.53	4.16 ± 0.12	12.76 ± 0.87	42.89 ± 0.23*
L10 + 100 μM E	1.83 ± 0.13*	7.07 ± 1.25	27.04 ± 1.48*	4.45 ± 0.45	12.49 ± 0.54*	47.14 ± 1.53*
L10 + 250 μM E	1.16 ± 0.05*	5.17 ± 0.42*	24.52 ± 1.12*	3.22 ± 0.44*	10.46 ± 0.65*	55.36 ± 0.56*
L22 Control	1.16 ± 0.06	3.11 ± 0.26	8.50 ± 0.31	1.24 ± 0.92	9.00 ± 1.12	76.98 ± 1.50
L22 + 50 μM E	1.00 ± 0.11*	3.12 ± 0.36	7.87 ± 0.88	1.18 ± 0.81	8.49 ± 1.32	77.60 ± 2.79
L22 + 100 μM E	0.85 ± 0.10*	2.65 ± 0.43	6.46 ± 1.10*	N.D.*	6.48 ± 0.86*	83.57 ± 1.87*
L22 + 250 μM E	0.55 ± 0.03*	2.03 ± 0.42*	5.27 ± 0.35*	N.D.*	5.09 ± 0.70*	87.05 ± 0.56*
L29 Control	6.11 ± 0.50	3.58 ± 0.41	13.21 ± 0.49	3.08 ± 0.46	19.10 ± 1.31	54.82 ± 1.86
L29 + 50 μM E	4.41 ± 0.63*	3.73 ± 0.96	12.76 ± 1.83	2.61 ± 0.59	15.89 ± 1.10*	60.42 ± 2.78*
L29 + 100 μM E	4.27 ± 0.50*	3.67 ± 1.30	12.43 ± 0.93	2.42 ± 0.44	15.64 ± 1.09*	61.75 ± 2.07*
L29 + 250 μM E	2.42 ± 0.17*	2.67 ± 0.60	10.12 ± 0.69*	1.60 ± 0.38*	12.43 ± 0.76*	70.75 ± 1.36*
L31 Control	1.54 ± 0.10	1.72 ± 0.14	4.49 ± 0.31	0.22 ± 0.26	4.26 ± 0.40	87.78 ± 0.66
L31 + 50 μM E	1.20 ± 0.26*	1.71 ± 0.36	4.51 ± 0.45	0.39 ± 0.45	3.64 ± 0.61	88.54 ± 1.63
L31 + 100 μM E	1.03 ± 0.16*	1.67 ± 0.39	4.60 ± 0.47	N.D.	3.79 ± 0.46	88.80 ± 1.05
L31 + 250 μM E	0.89 ± 0.07*	1.27 ± 0.78	4.10 ± 0.29	0.19 ± 0.38	3.35 ± 0.54*	90.22 ± 1.50*

Values are the means ± SD of four determinations, *P < 0.05 compared to control.

(2.8 ± 2.2% cf. 1.2 ± 0.8% Tables 1 and 5) but this increase was not significant (P > 0.05).

Effect of enoxacin on THA metabolite formation

Enoxacin significantly reduced the NADPH-dependent activation of THA to a cytotoxic metabolite in microsomes prepared from the livers of four donors who were known to be smokers. At a concentration of 50 μM enoxacin completely blocked NADPH-dependent cytotoxicity. This reduction was maintained up to a concentration of 250 μM (Fig. 5). A much reduced effect of enoxacin on the formation of protein-reactive and stable metabolites was observed (Table 4). At 50 μM protein-reactive metabolite formation was reduced by 18%, at 250 μM a reduction of 52% was achieved.

Stable metabolites were also reduced by enoxacin, at 250 μM these were reduced by 10%. This reduction was not specific to any one metabolite.

DISCUSSION

The cholinesterase inhibitor THA is currently being developed for use in patients with Alzheimer's disease. Despite a beneficial effect of THA being reported in Alzheimer's patients, its use has been restricted because of the finding of increased liver function enzymes associated with THA therapy [2–4]. In clinical studies these increases have been reported in between 20 and 50% of patients. Although the mechanism is unclear it has been shown to be both dose-dependent and reversible. In this present study we have investigated the role of metabolism in the toxicity of THA in an *in vitro* system.

Since toxic metabolites are often short lived and highly reactive, precluding identification by conventional means, we have employed an indirect method for assessing their formation, in which MNL are used as target cells for toxicity. As well as being easily obtained in large quantities this cell type also has its own defence mechanism for detoxication in the form of glutathione transferase and epoxide hydrolase. Furthermore, their use allows modification of the activating system (human or rat liver microsomes) without altering the target cells.

In initial experiments it was demonstrated that THA underwent NADPH-dependent metabolism to stable, protein-reactive and cytotoxic metabolites in both human and rat microsomes. There was considerable variation in the extent of THA metabolism in microsomes from both species. The mean NADPH-dependent cytotoxicity in the presence of human and rat microsomes was similar, suggesting comparable formation of cytotoxic metabolite(s). The generation of stable and protein-reactive (as estimated by the amount of radioactivity irreversibly bound to protein) metabolites was significantly greater in human microsomes, although the fraction of metabolite present as protein-reactive species was highest in the rat. However, *in vivo* the absolute amount of reactive metabolite formed would be of most relevance in determining the likelihood of a toxic response. Therefore, despite being a good model for the generation of cytotoxic metabolites from THA, the rat is not a good *in vitro* model for the overall metabolite profile. In view of this further studies were carried out using human microsomes alone.

GSH is a nucleophilic scavenger that can become conjugated to a variety of electrophiles leading to their detoxication. Maintenance of the GSH redox cycle is therefore an important protection mechanism for the cell [29]. Indeed, depletion of cellular GSH has been demonstrated in the toxicity associated with paracetamol [30] and ciamexon [31]. Using a sub-physiological concentration of GSH (500 μM) the NADPH-dependent cytotoxicity of THA was completely abolished (Fig. 2). Generation of protein-reactive metabolites was also significantly reduced by GSH (Table 3). Therefore, an electrophilic metabolite of THA seems to be involved in both cytotoxicity and in covalent binding to cellular protein. Further indications to the nature of the toxic metabolite(s) were obtained by analysing the effects of ASC and CHO on THA metabolite generation. ASC has been shown to inhibit the activation of dapsone [11] and sulphapyridine [32] in this test system. The toxicity of these two drugs is known not to be mediated via epoxide formation. CHO on the other hand is an inhibitor of epoxide hydrolase, an enzyme that hydrolyses potentially toxic epoxides to yield the corresponding dihydrodiol. In this study ASC caused marked inhibition of both cytotoxicity and protein-reactive metabolite formation, whilst CHO was without effect on either parameter, a similar lack of effect was seen with another epoxide hydrolase inhibitor, TCPO (results not shown). Thus, it seems unlikely that an epoxide is the chemically reactive metabolite generated from THA. Stable metabolites of THA were identified as 2-OH, 1-OH, 4-OH and 7-OH THA (Fig. 3). GSH, ASC and CHO were without effect on the formation of any of these metabolites.

It has been suggested that the cytochrome P450IA2 (CYP1A2) may play an important role in the oxidative metabolism of THA [33]. This isozyme of cytochrome P450 can be induced by a variety of polycyclic aromatic hydrocarbons such as 3-methyl cholanthrene and isosafrole [34]. In humans, exposure to cigarette smoke may also be important in determining levels of CYP1A2 since constituents of the smoke are known to induce the CYP1A family of enzymes [35]. Using microsomes prepared from the livers of donors known to have been smokers we have demonstrated metabolic activation of THA. The extent of conversion of THA to stable, cytotoxic and protein-reactive metabolites was not significantly different in this group of microsomes (Fig. 5, Table 4) when compared to those microsomes prepared from livers of non smokers (Fig. 1, Table 1). However, it should be noted that although the donors of these livers have a medical history of smoking no data is available to their exact exposure to cigarette smoke. In the presence of enoxacin, a specific inhibitor of CYP1A2 [36–38], metabolism of THA was significantly reduced in microsomes from the livers of smokers. Formation of cytotoxic metabolite(s) was most sensitive to enoxacin. At 50 μM there was complete inhibition of NADPH-dependent cytotoxicity. Formation of stable and protein-reactive metabolites was considerably less sensitive to enoxacin. Even at 250 μM, protein-reactive metabolite formation was reduced by only 52%. This differential inhibition of cytotoxicity and covalent binding may indicate that an uncoupling CYP1A2 by THA results in oxygen radical mediated toxicity, however catalase had no effect on THA toxicity (unpublished data) suggesting that this is unlikely. Different metabolites may be involved in the two processes or it may be a result of microsomal protein having a certain capacity to bind the reactive species. Only when this is saturated can the toxic metabolite escape to interact with the MNL and cause cell death. Inclusion of a soluble protein (HSA) in our cytotoxicity assay acted to protect the cells against toxicity (Fig. 4). One possible mechanism for this protection by HSA (and also that seen in

the presence of GSH) is that the reactive metabolite that has escaped from the microsomes may become covalently associated with the HSA (or GSH) preventing interaction with cellular proteins of the MNL and hence reducing cell death. Since THA does not become covalently bound to microsomes in the absence of NADPH it is equally unlikely to bind to HSA, therefore reduction in substrate concentration by binding to HSA would not account for the effects of HSA on cytotoxicity. Inhibition of stable metabolite formation was least sensitive to enoxacin. The inhibition observed was not specific to any one metabolite suggesting that formation of 2-OH, 1-OH, 4-OH and 7-OH THA from THA is mediated by CYP1A2.

In summary, therefore, we have demonstrated that THA can be metabolized to stable, protein-reactive and cytotoxic metabolites in the presence of NADPH and an activating system (human or rat hepatic microsomes). A number of modifiers were seen to affect this metabolism. In all cases inhibition of cytotoxicity was more sensitive than protein-reactive or stable metabolite formation. CYP1A2 seems to be intimately involved in all routes of THA metabolism. Studies are presently underway to define the chemical nature of the chemically reactive metabolite(s), and thus discern more clearly the role of metabolism in enzyme changes induced by THA.

REFERENCES

1. Maayani S, Weinstein H, Ben-Zui N, Cohen S and Sokolovsky M, Psychotomometics as anticholinergic agents. I. Cyclohexylpiperidene derivatives: anti-cholinesterase activity and antagonistic activity to acetylcholine. *Biochem Pharmacol* **23**: 1263–1281, 1974.
2. Summers WP, Majouski LV, Marsh GM, Tachiki K and Kling A, Oral tetrahydroaminoacridine in long term treatment of senile dementia, Alzheimer type. *N Engl J Med* **315**: 1241–1245, 1986.
3. Gauthier S, Masson H and Gauthier R, Tetrahydroaminoacridine and lecithin in Alzheimer's disease. In: *Current Research in Alzheimer Therapy* (Eds. Giacobini E and Becker R), pp. 237–245. Taylor and Francis, London, 1988.
4. Farlow M, Gracon SI, Heshey LA, Lewis KW, Sadowsky CH and Dolan-Ureno J, A controlled trial of tacrine in Alzheimer's patients. *JAMA* **268**: 2523–2529, 1992.
5. Forsyth DR, Sormon DJ, Morgan RA and Wilcock GK, Clinical experience with and side effects of tacrine hydrochloride in Alzheimer's disease: a pilot study. *Age Ageing* **18**: 223–229, 1989.
6. O'Brien JT, Eagger S and Levy R, Effects of tetrahydroaminoacridine on liver function in patients with Alzheimer's disease. *Age Ageing* **20**: 129–131, 1991.
7. Park BK, Metabolic basis of adverse drug reactions. *J R Coll Physicians Lond* **20**: 195–200, 1986.
8. Dahlin DC , Miwa GT, Lu AHY and Nelson SD, *N*-acetyl-*p*-benzoquinone imine: a cytochrome P450 mediated oxidation of acetaminophen. *Proc Natl Acad Sci USA* **81**: 1327–1331, 1984.
9. Dahlin DC and Nelson SD, Synthesis, decomposition kinetics, and preliminary toxicological studies of *N*-acetyl-*p*-benzoquinone imine, a proposed toxic metabolite of acetaminophen. *J Med Chem* **25**: 885–886, 1982.
10. Pirmohamed M, Kitteringham NR, Guenther TM, Breckenridge AM and Park BK, An investigation of the formation of cytotoxic, protein-reactive and stable metabolites from carbamazepine *in vitro*. *Biochem Pharmacol* **43**: 1675–1682, 1992.
11. Coleman MD, Breckenridge AM and Park BK, Bioactivation of dapsone to a cytotoxic metabolite by human hepatic microsomal enzymes. *Br J Clin Pharmacol* **28**: 389–395, 1989.
12. Coleman MD, Scott AK, Breckenridge AM and Park BK, The use of cimetidine as a selective inhibitor of dapsone N-hydroxylation in man. *Br J Clin Pharmacol* **30**: 761–767, 1990.
13. Maggs JL, Tingle MD, Kitteringham NR and Park BK, Drug-protein conjugates XIV. Mechanisms of formation of protein-arylating intermediates from amodiaquine, a myelotoxin and hepatotoxin in man. *Biochem Pharmacol* **37**: 303–311, 1988.
14. Harrison AC, Kitteringham NR, Clarke JB and Park BK, The mechanism of bioactivation and antigen formation of amodiaquine in the rat. *Biochem Pharmacol* **43**: 1421–1430, 1992.
15. Parke DV, Activation mechanisms to chemical toxicity. *Arch Toxicol* **60**: 5–15, 1987.
16. Campbell TC and Hayes JR, The role of aflatoxin metabolism in its toxin lesion. *Toxicol Appl Pharmacol* **35**: 199–222, 1976.
17. Mitchell JR, Jollow DJ, Potter WZ, Davis DC, Gillette JR and Brodie BB, Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. *J Pharmacol Exp Ther* **198**: 185–194, 1973.
18. Neuberger J and Kenna JG, Halothane hepatitis. A model of immune mediated drug hepatotoxicity. *Clin Sci* **72**: 263–270, 1987.
19. Hsu RS, Shutzke GM, Dileo EM, Chesson SM, Linville AR and Allen RC, Identification of the urinary metabolites of tacrine in the rat. *Drug Metab Dispos* **18**: 779–783, 1990.
20. Hsu RS, DiLeo GM and Chesson SM, High performance liquid chromatography for the determination of tacrine and its metabolites in plasma. *J Chromatogr Biomed Appl* **530**: 170–176, 1990.
21. Siedegard J, De Pierre JW and Pero RW, Measurement and characterisation of membrane bound and soluble epoxide hydrolase activities in resting mononuclear leukocytes from human blood. *Cancer Res* **44**: 3654–3660, 1984.
22. Singh N and Clausen J, Mixed function oxidase and glutathione *S*-transferase activities in normal human lymphocytes. *Cancer Lett* **13**: 52–61, 1981.
23. Riley RJ, Maggs JL, Lambert C, Kitteringham NR and Park BK, An *in vitro* study of the microsomal and cellular toxicity of phenytoin, sorbinil and mianserin. *Br J Clin Pharmacol* **26**: 577–588, 1988.
24. Spielberg SP, Acetaminophen toxicity in human lymphocytes *in vitro*. *J Pharmacol Exp Ther* **213**: 395–398, 1980.
25. Tingle MD and Park BK, Metabolism of ciamexon by human liver microsomes: an investigation into the formation of stable, chemically reactive and cytotoxic metabolites. *Br J Clin Pharmacol* **29**: 549–556, 1990.
26. Madden S, Back DJ and Orme ML'E, Metabolism of the contraceptive steroid desogestrel by human liver *in vitro*. *J Steroid Biochem* **35**: 281–288, 1990.
27. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
28. Omura T and Sato R, The carbon monoxide binding pigment of liver microsomes. *J Biol Chem* **239**: 2370–2378, 1964.
29. Comporti M, Maellaro B, Del Bello B and Casini AF, Glutathione depletion: its effect on other antioxidant

systems and hepatocellular damage. *Xenobiotica* **21**: 1067–1076, 1991.

30. Moore M, Thor H, Moore G, Nelson S, Moldeus P and Orrenius S, The toxicity of acetaminophen and *N*-acetyl-*p*-benzoquinone imine in isolated hepatocytes is associated with thiol depletion and increased cytosolic Ca^{2+}. *J Biol Chem* **260**: 13035–13040, 1985.
31. Tingle MD, Lavens SE and Park BK, Depletion of glutathione by ciamexon in the rat. *Hum Exp Toxicol* **19**: 489, 1991.
32. Pirmohamed M, Coleman MD, Hussain F, Breckenridge AM and Park BK, Direct and metabolism dependent toxicity of sulphasalazine and its principal metabolites towards human erythrocytes and leucocytes. *Br J Clin Pharmacol* **32**: 303–310, 1991.
33. Kukan M, Bezek S, Pool W and Woolf TF, *In vitro/in vivo* correlations in tacrine metabolism and its stimulation by 3-methylcholanthrene. *Proc Fourth Eur ISSX Meeting* **1**: 35, 1992.
34. Sesardic D, Cole KJ, Edwards RJ, Davis DS, Thomas PE, Levin W and Boobis AR, The inducibility and catalytic activity of cytochromes P450c (P450IA1) and P450d (P450IA2) in rat tissues. *Biochem Pharmacol* **39**: 499–506, 1990.
35. Kalow W and Tang B-K, Use of caffeine metabolite ratios to explore CYP1A2 and xanthine oxidase activities. *Clin Pharmacol Ther* **50**: 508–519, 1991.
36. Davies BI and Maesan FF, Drug interaction with quinolones. *Rev Infect Dis* **11**: S1083–S1090, 1989.
37. Harder S, Fuhr U, Staib AH and Wolff T, Ciprofloxacin-caffeine: a drug interaction using *in vivo* and *in vitro* investigations. *Am J Med* **87**: 89S–91S, 1989.
38. Fuhr U, Stobl G, Francesc M *et al.*, Quinolone antibacterial agents: relationship between structure and *in vitro* inhibition of human cytochrome P450 isoform CYP1A2. *Mol Pharmacol* **87**: 191–200, 1993.