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The effect of enzyme inhibition on the metabolism and activation of tacrine by human liver microsomes

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1 Tacrine (1,2,3,4-tetrahydro-9-aminoacridine-hydrochloride: THA) underwent metabolism *in vitro* by a panel (n = 12) of human liver microsomes genotyped for CYP2D6, in the presence of NADPH, to both protein-reactive and stable metabolites.

2 There was considerable variation in the extent of THA metabolism amongst human livers. Protein-reactive metabolite formation showed a 10-fold variation (0.6 ± 0.1%–5.2 ± 0.8% of incubated radioactivity mg^{-1} protein) whilst stable metabolites showed a 3-fold variation (24.3 ± 1.7%–78.6 ± 2.6% of incubated radioactivity).

3 Using cytochrome P450 isoform specific inhibitors CYP1A2 was identified as the major enzyme involved in all routes of THA metabolism.

4 There was a high correlation between aromatic and alicyclic hydroxylation (r = 0.92, $P < 0.0001$) consistent with these biotransformations being catalysed by the same enzymes.

5 Enoxacin (ENOX), cimetidine (CIM) and chloroquine (CQ) inhibited THA metabolism by a preferential decrease in the bioactivation to protein-reactive, and hence potentially toxic, species. The inhibitory potency of ENOX and CIM was increased significantly upon pre-incubation with microsomes and NADPH.

6 Covalent binding correlated with 7-OH-THA formation before (r = 0.792, $P < 0.0001$) and after (r = 0.73, $P < 0.0001$) inhibition by CIM, consistent with a two-step mechanism in the formation of protein-reactive metabolite(s) via a 7-OH intermediate.

7 The use of enzyme inhibitors may provide a useful tool for examining the relationship between the metabolism and toxicity of THA *in vivo*.

Keywords tacrine bioactivation CYP1A2 inhibition

Introduction

Tacrine (1, 2, 3, 4-tetrahydro-9-aminoacridine-hydrochloride: THA), a centrally acting anti-cholinesterase, has recently been introduced for use in the treatment of Alzheimer's disease. Although a beneficial effect has been demonstrated [1–3] , its use has been carefully monitored due to reported elevations in serum transaminase levels associated with the drug [4, 5]. It has been estimated that 20–50% of patients taking tacrine develop dose-dependent, asymptomatic elevations in serum enzymes associated with adverse liver function. This effect is reversible on withdrawal of the drug [6]. Although the mechanism of this adverse effect is still undefined *in vivo*, it has been shown *in vitro* that tacrine undergoes metabolic activation to protein-reactive and cytotoxic metabolites when incubated with human liver microsomes [7, 8].

It is well established that oxidative drug metabolism may be inhibited both *in vitro* and *in vivo* by a range of therapeutic agents such as antifungals (e.g. ketoconazole [9]), oral contraceptive steroids (e.g. ethinyloestradiol [10]), sulphonamides (e.g. sulphaphenazole [9]) and neuroleptics (e.g. thioridazine [11]). Furthermore, certain drugs are selective inhibitors of particular cytochrome P450 (CYP)

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enzymes [12]. For example, the fluoroquinolone antibacterials such as enoxacin are well documented as being inhibitors of CYP1A2 [13–15]. Recently, Fuhr *et al.* [16] investigated the effects of a number of drugs in this class on the *in vitro* metabolism of the CYP1A2 substrate caffeine. Of all the quinolones tested enoxacin was the most potent inhibitor. Inhibition of the metabolism of a given substrate by these specific CYP inhibitors may therefore allow identification of the P450(s) involved. Indeed, we have previously investigated the effects of enoxacin on THA metabolism [7]. Inhibition of all routes of THA metabolism was evident, indicating the involvement of CYP1A2 in the metabolism of tacrine.

Cimetidine has been reported to interfere with CYP-mediated metabolism *in vivo* but it is only a weak inhibitor *in vitro* [17–19]. Its use *in vitro* demands concentrations 10–100-fold greater than can be achieved systemically during normal therapeutic use [20, 21]. It has been reported recently, however, that inhibition *in vitro* can be enhanced by pre-incubation of cimetidine with microsomes and NADPH. Wild & Back [22] demonstrated enhanced inhibition of oestrogen-2-hydroxylase *in vitro* whilst Tingle *et al.* [23] reported increased inhibition of *N*-hydroxylation and methaemoglobinaemia associated with dapsone after employing these pre-incubation procedures. This may be explained by the hypothesis that the inhibitory potential of cimetidine is determined by an 'activated' complex of cimetidine and CYP [21]. Alternatively pre-incubation may lead to the formation of a metabolite with greater inhibitory potential than cimetidine itself.

The aminoquinoline derivative chloroquine has been less well defined as an inhibitor of CYP mediated metabolism. In rat, inhibition of aminopyrine-*N*-demethylase activity has been described [24, 25] whilst Tingle & Park [26] have reported inhibition of ciamexon hydroxylation by chloroquine in human liver microsomes. Recently it has been suggested that chloroquine is a potent inhibitor of metoprolol α-hydroxylation *in vitro* [27]. This reaction is mediated by CYP2D6 in man and CYP2D1 in rats, and is subject to a genetic polymorphism in man whereby approximately 10% of a Caucasian population lack a functional form of the enzyme [28].

The aim of this study was to examine the inhibitory effects of various drugs on the *in vitro* metabolism and bioactivation of THA. The results might then be used to examine the relationship between the metabolism and toxicity of THA *in vivo*.

Methods

Chemicals

THA, 1-OH THA, 2-OH THA, 4-OH THA, 7-OH THA, [^{14}C]-THA (11.47 mCi mmol^{-1}), [^{14}C]-7-OH THA (7.51 mCi mmol^{-1}), furafylline (FUR) and enoxacin (ENOX) were all gifts from Parke Davis Pharmaceutical Division (Michigan, USA). Chloroquine (CQ), *para*nitro phenol (*p*-NP) and quinidine (QUIN) were purchased from Sigma Chemical Co. (Poole, UK). Cimetidine (CIM) was obtained from Aldrich Chemical Co. (Poole, UK). Gestodene (GEST) was from Schering (Berlin, Germany) and sulphaphenazole was from Ciba Geigy (Basle, Switzerland). All solvents were of h.p.l.c. grade and were supplied by Fisons PLC (Loughborough, UK). All other chemicals were of the purest grade available and were purchased from either Sigma Chemical Co. or BDH Chemicals Ltd (Poole, UK).

Preparation of human liver microsomes

Washed human liver microsomes, from histologically normal livers obtained from renal transplant donors, were prepared as described previously [29]. Ethical approval for the study was obtained from the local ethics committee. Once prepared the microsomal pellets were stored at −80° C until use. The microsomal protein content was measured by the method of Lowry *et al.* [30], and the cytochrome P450 content was estimated by the method of Omura & Sato [31].

Metabolism of THA by human hepatic microsomes

Incubations at 37° C contained [^{14}C]-THA (25 μM; 0.5 μCi) or [^{14}C]-7-OH THA (25 μM; 0.2 μCi), human liver microsomal protein (2 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 (1 mM) and terminated after 1 h by the addition of acetonitrile (5 ml). Some incubations also contained an alleged inhibitor, ENOX (0–300 μM), CIM (0–1 mM) or CQ (0–1 mM). The effect of pre-incubation (0, 15, 30 min) of inhibitors with microsomes and NADPH (1 mM) prior to the addition of THA was also investigated.

Isoenzyme specific inhibition of THA metabolism

Incubations were performed as described above in the presence of CYP isoenzyme specific inhibitors: FUR (CYP1A2; 10 μM), SULPH (CYP2C9/10; 5 μM), QUIN (CYP2D6; 10 μM), *p*-NP (CYP2E1; 50 μM) and GEST (CYP3A4; 20 μM).

Analysis of stable metabolites

The reaction mixture with added acetonitrile was left overnight at 4° C to precipitate the microsomal protein which was sedimented by centrifugation (2500 rev min^{-1} for 15 min). The supernatants were removed, evaporated to dryness and resuspended in a small volume of methanol for h.p.l.c. analysis as described previously [7]. The coefficient of variation for the measurement of THA and its metabolites by this method was less than 5%.

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 dH_2O (v/v; 5 ml) washes). The protein was dissolved in 1 M NaOH (2 ml) and aliquots taken for liquid scintillation counting (500 µl) and protein estimation (10 µl). The amount of radiolabelled material bound is expressed as a percentage of the total radioactivity in the original incubation per mg of protein.

Genotyping of human livers

The panel of human livers was genotyped for CYP2D6 by PCR according to the method of Smith *et al.* [32]. Using this method the wild type allele (D6-WT) and the splice-site mutation allele (D6-B) could be identified.

Statistical analysis

Statistical analyses were performed by one way analysis of variance or by Student's *t*-test for unpaired samples accepting $P < 0.05$ as significant. Regression analysis was performed using Pearson's coefficient of correlation. Data are presented as mean ± s.d. of quadruplicate determinations.

Results

In microsomes from liver L29, THA underwent NADPH dependent metabolism to form protein-reactive and stable metabolites. These accounted for 13.0 ± 1.2% and 65.5 ± 1.9% of incubated radioactivity respectively (Figure 1a,b). Stable metabolites were identified as 1-OH, 2-OH, 4-OH and 7-OH THA by co-chromatography with authentic standards. The effect of concentration and pre-incubation time on the inhibition by ENOX was investigated. In the presence of 30 and 300 µM ENOX, protein-reactive metabolite formation was decreased to 81% (10.5 ± 0.6%, $P < 0.05$) and 24% (3.1 ± 0.2%, $P < 0.001$) of control (13.0 ± 1.2%), respectively (Figure 1a). Pre-incubation of ENOX with microsomes and NADPH prior to addition of THA potentiated the inhibition of protein-reactive metabolite formation. Following a 15 min pre-incubation covalent binding was decreased to 72% (7.5 ± 0.3%, $P < 0.001$), 43% (4.5 ± 0.4, $P < 0.001$) and 14% (1.5 ± 0.2, $P < 0.001$) of control (10.4 ± 0.5%) in the presence of 3, 30 and 300 µM ENOX respectively. When the pre-incubation time was increased to 30 min a further enhancement of inhibitory potency was observed. Stable metabolite formation was also inhibited by ENOX (Figure 1b). In the absence of pre-incubation stable metabolite concentrations were decreased in the presence of 30 µM and 300 µM ENOX ($P < 0.001$). With pre-incubation the inhibitory potency towards stable metabolite formation was increased. After 15 min pre-incubation significant inhibition was seen in the presence of 30 and 300 µM ENOX, stable metabolite concentrations being decreased to 79% ($P < 0.001$) and 46% ($P < 0.001$) of control respectively. The degree of inhibition was further increased when the pre-incubation

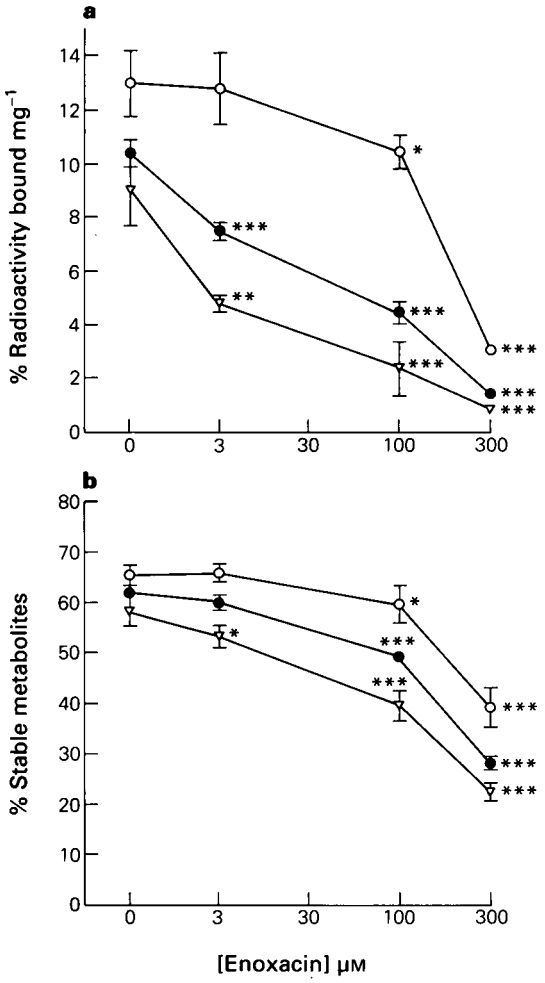

Figure 1 The formation of protein-reactive (a) (expressed as % of incubated radioactivity bound mg^{-1} microsomal protein) and stable (b) metabolites from THA (25 µM) when incubated in the presence of NADPH (1 mM) and ENOX (0–300 µM) with liver microsomes (from L29). Microsomes were pre-incubated with ENOX and NADPH for 0 (○), 15 (●) and 30 (▽) min prior to the addition of THA. Values are expressed as mean ± s.d. of four determinations, statistical analysis was by ANOVA followed by a modified *t*-test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

period was 30 min. Inhibition of stable metabolite formation was not specific to any single metabolite. During the pre-incubation periods a slight reduction in control enzyme activity was observed, falling to 69% of control after 30 min (Figure 1a).

The effect of isozyme specific inhibitors on the metabolism of THA was examined. The greatest inhibition was seen in the presence of the CYP1A2 specific inhibitor FUR. At 10 µM protein-reactive metabolite formation was inhibited completely whilst formation of stable metabolites was decreased to 13.0 ± 2.1% ($P < 0.0001$) of control. Of the other inhibitors tested THA metabolism was inhibited significantly only by QUIN. In the presence of 10 µM QUIN protein-reactive metabolite formation was decreased to 89.8 ± 2.3% ($P < 0.005$) of control and stable metabolite formation was decreased to 94.1 ± 2.1% ($P < 0.05$) of control. GEST, *p*-NP and SULPH were without effect.

In the absence of CIM protein-reactive and stable metabolites accounted for 6.0 ± 1.0% and 79.6 ± 1.5% of incubated radioactivity (Figure 2a,b). CIM produced a concentration dependent and pre-incubation dependent inhibition of THA metabolism. Without pre-incubation only protein-reactive metabolite formation was found to be decreased by CIM, at the highest concentration tested (1 mM), to 77% of the control value (4.6 ± 0.8% *c.f.* 6.0 ± 1.0%). Following pre-incubation for 15 and 30 min inhibition of covalent binding was evident in the presence of both 300 μM and 1 mM CIM. At 300 μM covalent binding was decreased to 65% (3.4 ± 0.3% *c.f.* 5.2 ± 0.8% and 2.8 ± 0.4% *c.f.* 4.3 ± 0.6%, $P < 0.001$) of control after 15 and 30 min pre-incubation, whilst in the presence of 1 mM CIM protein-reactive metabolite formation was decreased to 40% (2.1 ± 0.3%, $P < 0.001$) and 30% (1.3 ± 0.1%, $P < 0.001$) of control following the pre-incubation periods. At these time-points there was also a significant, although much lower, inhibition of stable metabolite formation at both 300 μM and 1 mM CIM (Figure 2b). Inhibition of stable metabolite formation was not exclusive to a single metabolite.

Inhibition of protein-reactive metabolite formation was also seen in the presence of CQ (Figure 3a). Without pre-incubation covalent binding was decreased to 84% and 72% ($P < 0.01$) of control (6.8 ± 0.4%) in the presence of 300 μM and 1 mM CQ, respectively, however, pre-incubation had no effect on the inhibitory potency. CQ had little inhibitory effect on stable metabolite formation (Figure 3b).

In the second part of the study the effect of CIM on THA metabolism was investigated in microsomes prepared from 12 human livers that had been genotyped for CYP2D6. For this part of the study a CIM

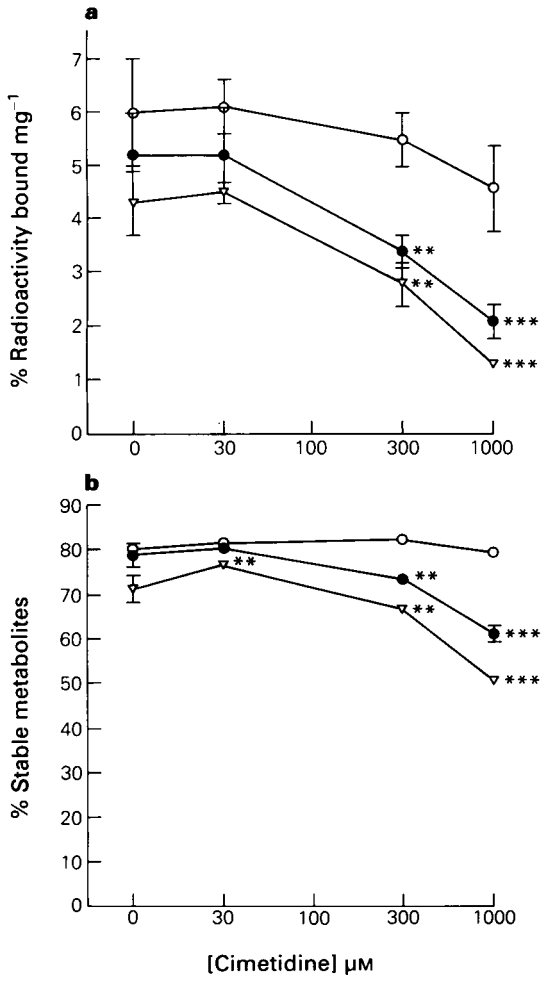

Figure 2 The formation of protein-reactive (a) (expressed as % of incubated radioactivity bound mg^{-1} microsomal protein) and stable (b) metabolites from THA (25 μM) when incubated in the presence of NADPH (1 mM) and CIM (0–1 mM) with liver microsomes (from L29). Microsomes were pre-incubated with CIM and NADPH for 0 (○), 15 (●) and 30 (▽) min prior to the addition of THA. Values are expressed as mean ± s.d. of four determinations, statistical analysis was by ANOVA followed by a modified *t*-test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

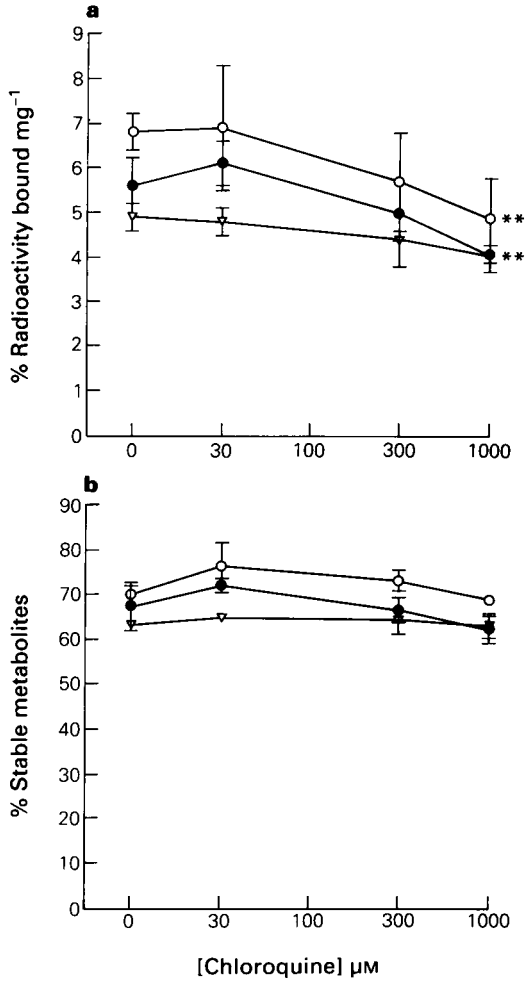

Figure 3 The formation of protein-reactive (a) (expressed as % of incubated radioactivity bound mg^{-1} microsomal protein) and stable (b) metabolites from THA (25 μM) when incubated in the presence of NADPH (1 mM) and CQ (0–1 mM) with liver microsomes (from L29). Microsomes were pre-incubated with CQ and NADPH for 0 (○), 15 (●) and 30 (▽) min. prior to the addition of THA. Values are expressed as mean ± s.d. of four determinations, statistical analysis was by ANOVA followed by a modified *t*-test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

concentration of 300 μM and a pre-incubation time of 15 min were used. In all 12 livers THA was converted to both stable and protein-reactive metabolites. There was considerable variation in the extent of metabolism across the 12 livers (Table 1). In the study livers were obtained from seven male and five female donors. There was no significant effect ($P > 0.05$) on the metabolic profile obtained of either sex or age. Stable metabolite formation ranged from 24.3 ± 1.7% to 78.7 ± 2.6% and protein-reactive metabolites ranged from 0.6 ± 0.1% to 5.2 ± 0.8%. Aromatic hydroxylation (7-hydroxylation + covalent binding) showed a significant correlation with alicyclic (1-, 2 and 4-) hydroxylation ($r = 0.92$; $P < 0.0001$). CYP2D6 genotype had no significant effect on the bioactivation of THA. Of the stable metabolites, only 2-OH THA was found to be significantly lower ($P < 0.05$) in microsomes with the D6-B allele. In the presence of CIM, THA metabolism was perturbed in all 12 livers (Table 2). Covalent binding was decreased by a mean value of 28.6 ± 8.1%, stable metabolite formation was inhibited to a lesser, and statistically insignificant degree. In all 12 livers protein-reactive metabolite formation was inhibited to a significantly greater degree than 1- ($P < 0.001$), 2- ($P < 0.01$), 4- ($P < 0.001$) or 7- ($P < 0.001$) hydroxy formation. There was a significant correlation between 7-OH THA formation and reactive metabolite formation both before ($r = 0.79$; $P < 0.0001$) and after ($r = 0.73$; $P < 0.0001$) pre-incubation of microsomes with CIM.

Protein-reactive metabolite formation was the major route of metabolism of 7-OH THA. In the presence of NADPH and microsomal protein this accounted for 4.1 ± 0.4% of incubated radioactivity. At the concentration investigated (25 μM) stable metabolites accounted for less than 1% of incubated radioactivity. No single stable metabolite could be identified. In the presence of THA the bioactivation of 7-OH THA was significantly decreased (Figure 4). The concentration of THA required to inhibit activation by 50% was estimated to be 68 μM. In the presence of ENOX (300 μM: 15 min pre-incubation) the bioactivation of 7-OH THA was decreased to 57.0 ± 3.4% of control ($P < 0.0001$).

Discussion

Due to the complexity of the metabolism and bioactivation of THA [7, 8] rigorous investigations of biochemical and kinetic parameters relating to these biotransformations were not performed. The experiments were carried out under conditions which were found to give consistently reproducible metabolite profiles in each of the livers tested.

In this present study we have investigated the effects of pre-incubation of ENOX with NADPH and microsomes on its inhibitory potency towards THA. In agreement with our previous findings [7] we observed concentration dependent inhibition of THA metabolism by ENOX in the absence of pre-incubation. We have now demonstrated that this inhibition may be enhanced by pre-incubation of ENOX with the metabolizing system prior to the addition of THA. At lower concentrations the inhibition is selective towards protein-reactive metabolite formation. The effect of pre-incubation on the extent of inhibition produced by ENOX is indicative of mechanism based inhibition.

Inhibition of CYP1A2 mediated reactions by ENOX is well established [33–35], although its specificity for this isoenzyme has not been characterised. We, therefore, investigated the effects of other well characterised CYP isoenzyme selective inhibitors on the metabolism and bioactivation of THA. The individual inhibitors were used at concentrations that have previously been shown to cause inhibition of specific CYP activities [12, 36–38]. FUR the specific CYP1A2 inhibitor caused complete inhibition of reactive metabolite formation and 87% inhibition of stable metabolite formation at the concentration tested. Of the other inhibitors tested the

Table 1 The formation of protein-reactive (expressed as % incubated radioactivity bound mg^{-1} microsomal protein) and stable metabolites (as a % of incubated radioactivity) from THA (25 μM) when incubated in the presence of NADPH (1 mM) with microsomes prepared from 12 histologically normal livers. Microsomes were pre-incubated with NADPH for 15 min prior to the addition of THA (25 μM). Values are expressed as mean ± s.d. of four determinations

Liver	*Sex*	*Age (years)*	*Smoking status*	*CYP2D6 allele*	*Protein-reactive*	*THA*	*1-OH*	*2-OH*	*4-OH*	*7-OH*
29	F	61	S	D6-WT	5.2 ± 0.8	12.9 ± 4.0	32.6 ± 1.8	10.7 ± 0.8	4.3 ± 0.6	23.2 ± 0.8
22	M	32	S	D6-WT	4.0 ± 0.3	46.2 ± 1.0	20.0 ± 0.4	5.9 ± 0.8	3.7 ± 0.8	18.6 ± 0.3
13	M	38	NS	D6-WT	3.5 ± 0.4	44.2 ± 0.1	20.9 ± 0.6	6.0 ± 0.4	4.6 ± 0.1	17.4 ± 0.3
19	M	45	NS	D6-WT	2.7 ± 0.3	59.1 ± 4.8	15.8 ± 0.6	4.9 ± 0.9	2.2 ± 0.2	14.0 ± 1.5
30	M	10	NS	D6-WT	2.6 ± 1.8	41.7 ± 1.6	21.7 ± 0.4	5.8 ± 0.6	4.4 ± 0.2	18.8 ± 0.6
7	M	29	NS	D6-B	1.8 ± 0.2	46.7 ± 1.8	22.2 ± 1.5	1.9 ± 1.5	3.1 ± 1.3	14.0 ± 0.5
20	F	49	NS	D6-B	1.7 ± 0.2	52.0 ± 0.4	19.0 ± 0.5	1.7 ± 0.2	3.3 ± 0.7	14.8 ± 0.7
32	F	36	NS	D6-WT	1.4 ± 0.2	51.0 ± 5.0	21.6 ± 1.7	3.9 ± 0.9	3.3 ± 0.5	17.4 ± 1.8
25	M	56	NS	D6-WT	1.3 ± 0.1	57.2 ± 1.1	16.7 ± 1.3	3.4 ± 0.2	3.1 ± 0.4	16.1 ± 0.5
14	M	32	NS	D6-WT	1.3 ± 0.2	61.9 ± 1.5	15.2 ± 1.0	4.3 ± 0.2	3.2 ± 0.4	12.8 ± 0.5
17	F	46	NS	D6-WT	1.0 ± 0.1	67.8 ± 1.5	13.0 ± 1.0	3.2 ± 0.5	2.8 ± 0.2	11.5 ± 0.5
18	F	40	NS	D6-B	0.6 ± 0.1	74.7 ± 1.7	11.4 ± 1.6	3.8 ± 0.5	2.7 ± 0.1	7.4 ± 1.3

Table 2 The formation of protein-reactive and stable metabolites (expressed as % of control) from THA (25 μM) when incubated in the presence of CIM (300 μM) and NADPH (1 mM) with microsomes prepared from 12 histologically normal livers. Microsomes were pre-incubated with CIM and NADPH for 15 min prior to the addition of THA (25 μM). Values are expressed as mean ± s.d. of four determinations, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

Liver	*Covalent binding as a % of control*	*Stable metabolites as a % of control*			
		1-OH	*2-OH*	*4-OH*	*7-OH*
29	65***	91**	83**	100	103
22	80**	99	90	86	90***
13	69**	83***	80	80***	88***
19	78*	97	84	100	94
30	53**	86	93	91**	96
7	69**	91	79	122	96
20	81**	103	82	100	100
32	79*	92	100	91	93
25	77*	105	94	97	100
14	69*	82**	86**	84*	86**
17	70**	87**	100	104	90*
18	67***	78	55***	63	116

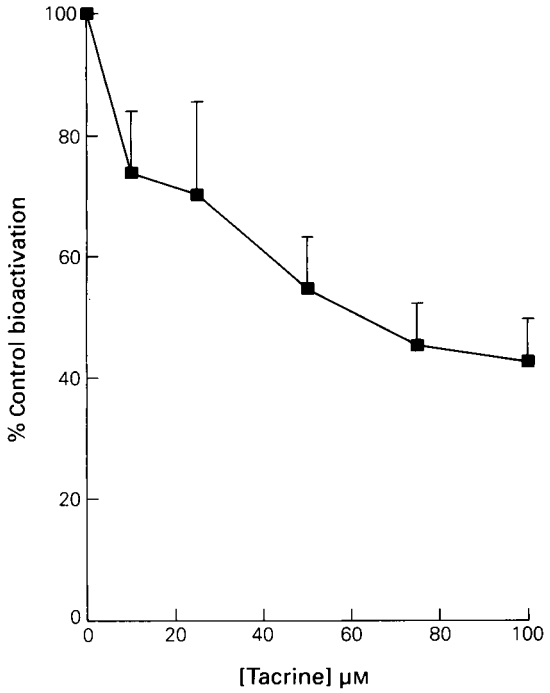

Figure 4 The effect of increasing concentrations of THA (0–100 μM) on the bioactivation (expressed as a % of control) of 7-OH THA (25 μM) when incubated with microsomes (from L29) and NADPH (1 mM).

CYP2D6 inhibitor, QUIN, had a minor inhibitory effect, indicating that, although the metabolism of THA is a complex process involving the generation of both stable and reactive metabolites, a single isoform of CYP, CYP1A2, is responsible for the majority of the biotransformations taking place.

Inhibition of THA metabolism by co-incubation with CIM was achieved only at the highest concentration tested, 1 mM, and was restricted to inhibition of protein-reactive metabolite formation. As found previously [23] pre-incubation of CIM with microsomes and NADPH prior to addition of substrate greatly enhanced inhibitory potency. Recently Faux & Combes [39] have demonstrated enhanced inhibition with CIM when microsomes were pre-incubated with only NADPH, suggesting that CIM forms a complex with the reduced form of P450. Although inhibition of both stable metabolite and protein-reactive metabolite generation was achieved it is important to note that the degree of inhibition of stable metabolite formation was considerably less than the inhibition of protein-reactive metabolite formation. Therefore, in our *in vitro* test system at least, it would appear that CIM may be used selectively, but not exclusively, to inhibit activation of THA to chemically reactive, and hence potentially toxic, metabolites.

Co-incubation of CQ with THA caused a modest, but significant, decrease in THA metabolism. The major inhibitory effect was a decrease in the levels of protein-reactive metabolite(s), with stable metabolites being little affected. At both 300 μM and 1 mM CQ produced greater inhibition than CIM. However, pre-incubation of CQ prior to the addition of substrate had no effect on the inhibitory potency, indicating that the pre-incubation of the microsomes does not itself make them more susceptible to inhibition. This is in contrast to the increased inhibition obtained for ENOX and CIM under identical experimental conditions. Thus, although CQ is a more potent inhibitor of THA metabolism than CIM upon co-incubation, when a pre-incubation period is used the inhibition by CIM then becomes the more potent compound.

THA underwent metabolism to yield protein-reactive and stable metabolites in microsomes from each of the 12 livers studied. There was considerable variation in the extent of metabolism between the 12 livers, protein-reactive metabolite formation showed a 10-fold variation, whilst stable metabolites showed a 3-fold variation. CYP2D6 status of the livers used in the study appeared to have no influence on the degree of bioactivation of THA. Taken with the modest inhibition by QUIN this indicates that CYP2D6 may only have a very minor role to play in the metabolism of THA.

Despite the complexity of the metabolism of THA, CIM produced consistent inhibition using microsomes from each of the 12 livers studied. The major inhibitory effect was again seen in the generation of protein-reactive metabolites with significantly less inhibition of stable metabolite formation being achieved. Indeed, the reduction in stable metabolites failed to reach significance in several of the livers.

We have demonstrated that bioactivation is the major route of metabolism of 7-OH THA. Coupled with the greater effect CIM has on covalent binding this is consistent with a two-step mechanism in the activation of THA to a protein-reactive metabolite proceeding via a 7-OH THA intermediate. This proposed mechanism of reactive metabolite formation is shown in Figure 5 [40]. The significant correlation between aromatic and alicyclic hydroxylation of THA suggests that all of these routes of metabolism are performed predominately by CYP1A2. This is further

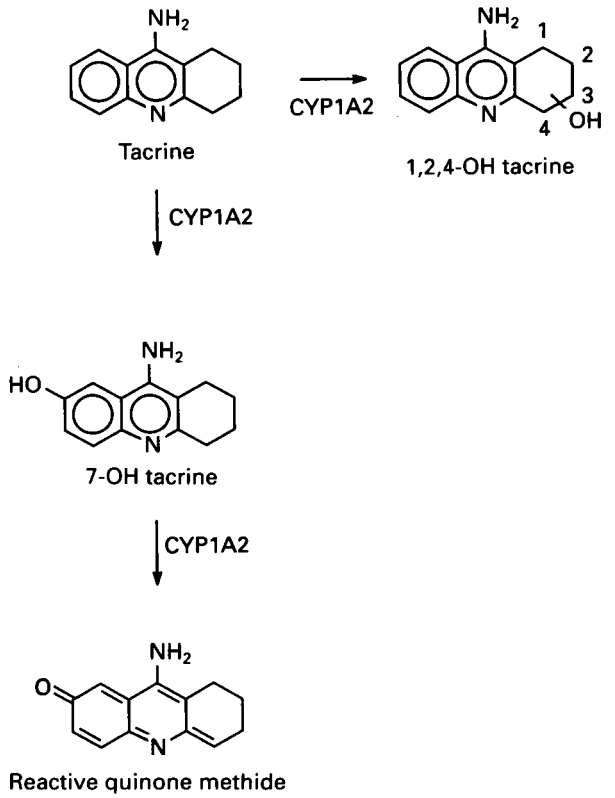

Figure 5 A scheme for the metabolic activation of THA to stable and protein-reactive metabolites by human liver microsomes *in vitro*.

indicated by the inhibition seen in the bioactivation of 7-OH THA in the presence of ENOX. In agreement with this single isoenzyme being involved in all routes of THA metabolism, THA itself was found to inhibit the bioactivation of the 7-OH intermediate significantly, indicating that THA may also be a better substrate for the enzyme.

Most attention concerning enzyme inhibition *in vivo* has been focused on adverse reactions caused by drug interactions. However, administration of inhibitors of oxidative metabolism has in some instances proven useful. First *et al.* [41] reported the effective use of ketoconazole to inhibit cyclosporin metabolism in renal transplant patients. As the metabolite(s) of cyclosporin is nephrotoxic and the parent drug has greater immunosuppressive properties the concomitant administration of these two drugs has proven beneficial from a pharmacological viewpoint. Cimetidine, which is structurally related to ketoconazole, has been observed to reduce the *N*-hydroxylation and increase the AUC of dapsone *in vivo* [42]. This concurrent treatment is of value in reducing the haematological side effects imposed by the hydroxylamine and in increasing the efficacy of the drug. Co-administration with cimetidine has also been shown to decrease the anticholinesterase activity of the insecticide carbaryl, by inhibition of the formation of a pharmacologically active metabolite [43, 44], suggesting the possible use of CIM in carbaryl overdose. Clearly if the *in vitro* inhibition of THA reactive metabolite formation by CIM is also observed *in vivo* it might limit elevations in serum transaminase levels associated with THA therapy.

In conclusion, we have demonstrated that THA undergoes metabolism to protein-reactive and stable metabolites in human liver microsomes. Enzyme inhibition studies have indicated that CYP1A2 is the major isoenzyme involved in the metabolism of THA. Cimetidine and enoxacin inhibit the metabolism and bioactivation in a consistent concentration-dependent and (pre-incubation) time-dependent manner, illustrating the need for metabolic activation of both inhibitors. Preferential inhibition of reactive metabolite formation by ENOX and CIM may provide a method for examination of the relationship between metabolism and toxicity of THA *in vivo*.

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