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FORMATION OF CYTOTOXIC METABOLITES FROM PHENYTOIN, IMIPRAMINE, DESIPRAMINE, AMITRIPTYLINE AND MIANSERIN BY MOUSE AND HUMAN HEPATIC MICROSOMES

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Abstract—The effects of enzyme induction on the generation of cytotoxic metabolites from phenytoin, mianserin, imipramine, desipramine and amitriptyline by mouse liver microsomes has been investigated and then compared with the bioactivation mediated by human hepatic microsomes. Cytotoxicity was assessed by co-incubation of drug and microsomes with human mononuclear leucocytes which served as target cells. Enzyme induction was assessed by measurement of hepatic cytochrome P-450 content, and determination of alkoxycoumarin *O*-dealkylase activity. None of the compounds investigated were metabolized to cytotoxic metabolites in the presence of control mouse microsomes. However, significant bioactivation could be observed for each drug when incubated with microsomes prepared from mice pretreated with either phenobarbitone (60 mg/kg) or β-naphthoflavone (75 mg/kg). The rank order for metabolism-dependent cytotoxicity with phenobarbitone-induced mouse microsomes (expressed as % cell death) was phenytoin (14.6%) > desipramine (10.5%) > imipramine (7.5%) > mianserin (3.4%) > amitriptyline (3.1%). Expression of cytotoxicity with phenytoin required pre-exposure of the target cells to trichloropropane oxide, an epoxide hydrolase inhibitor. Only mianserin and desipramine were activated to cytotoxic metabolites by human liver microsomes. Analysis of stable metabolites revealed that mianserin underwent extensive (>80%) metabolism by both control and induced mouse microsomes and that the principal metabolites, 8-hydroxymianserin, desmethylmianserin and mianserin *N*-oxide, were the same as those produced by human liver microsomes. These data suggest that mianserin is activated to a cytotoxic metabolite selectively by a constitutive form of human cytochrome P-450, whereas phenytoin, amitriptyline and imipramine are selectively activated by forms of mouse cytochrome P-450 which are induced by either phenobarbitone or β-naphthoflavone.

The formation of chemically reactive metabolites from xenobiotics is thought to be involved in the pathogenesis of a variety of adverse drug reactions [1, 2]. These highly reactive intermediates may combine covalently with critical cellular macromolecules, such as nucleic acids and protein, to initiate tissue damage. The ability of a drug to generate reactive metabolites *in vitro*, however, is not sufficient to predict its toxicological characteristics *in vivo* [3, 4] as compounds which yield protein-reactive intermediates are not invariably toxic [5] and a toxic reaction may only be manifested once deactivation (or detoxication) mechanisms [6] or means for repair and disposal of damaged or chemically modified macromolecules [7, 8] have been overwhelmed.

It has been proposed that sub-groups of the population may be predisposed to idiosyncratic drug reactions and that this increased risk of toxicity may in part be due to genetic differences in the metabolism and response to drugs [1, 9]. In order to investigate this phenomenon, Spielberg and co-workers have described an *in vitro* test system in which human mononuclear leucocytes are exposed to chemically reactive metabolites generated from various compounds by induced murine hepatic microsomes [10]. This work has largely focused on genetically determined inter-individual differences in detoxication pathways mediated by epoxide hydrolases and glutathione metabolizing enzymes. It was concluded that certain individuals have inherited deficiencies in these detoxication enzymes and are therefore predisposed to toxic reactions mediated by chemically reactive metabolites of certain compounds [11].

Using a similar *in vitro* test system, we have recently shown that toxicity is also governed by the degree and nature of the bioactivation of drugs and chemicals [12]. Hence quantitative and qualitative differences in the activation of drugs to chemically reactive metabolites may also play a role in the predisposition of certain individuals to adverse drug reactions. These differences were best exemplified by mianserin and phenytoin. Mianserin was activated to a cytotoxic metabolite more readily by human liver microsomes than by mouse liver microsomes following enzyme induction by phenobarbitone, whereas phenytoin was only activated to a toxic metabolite by phenobarbitone-induced mouse microsomes. However, it was not established whether these differences were due to inter-species variation in activation pathways or whether they were a consequence of hepatic enzyme induction.

In the present study, we have investigated the

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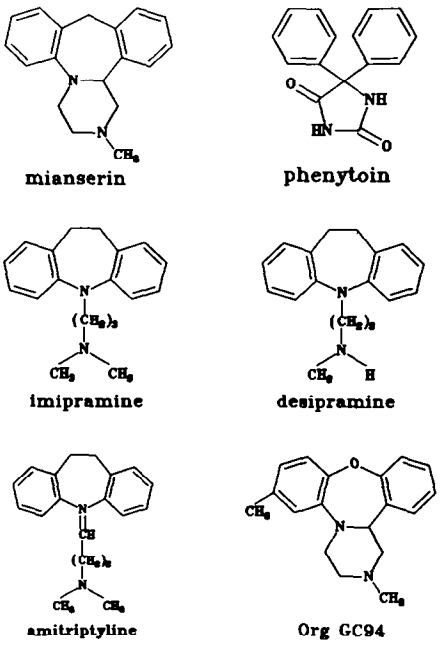

Fig. 1. Structures of the drugs investigated in the present study.

effects of hepatic enzyme induction on the *in vitro* cytotoxicity of metabolites generated from phenytoin and mianserin, in order to gain further insight into the biochemical mechanisms of drug activation. In an attempt to investigate the structural requirements for the bioactivation of mianserin, we have studied imipramine, desipramine and amitriptyline (antidepressants which undergo similar routes of metabolism to mianserin, namely ring hydroxylation and N-demethylation), and also ORG GC94, which is a structural congener of this tetracyclic antidepressant (Fig. 1).

MATERIALS AND METHODS

Chemicals. [13-^{3}H]Mianserin (20.4 Ci/mmol) was a gift from Organon International B.V. (Oss, Netherlands) and was found to be radiochemically pure when analysed by HPLC. Unlabelled mianserin, desmethylmianserin, 8-hydroxymianserin, mianserin 2-oxide and ORG GC94 were also donated by Organon. 5,5-Diphenyl [14-^{14}C]hydantoin (58 mCi/mmol, >98% pure) was supplied by Amersham International plc (Amersham, U.K.). Unlabelled phenytoin (5,5-diphenylhydantoin, 5-(4-hydroxyphenyl)-5-phenylhydantoin; 4-HPPH), human serum albumin (HSA; fraction V) and 1,2-epoxy-3,3,3-trichloropropane (TCPO†) were purchased from the Sigma Chemical Co. (Poole, U.K.). Reduced nicotinamide adenine dinucleotide phosphate (NADPH: tetrasodium salt) was obtained from BDH Chemicals Ltd (Poole, U.K.). Other reagents were purchased from the Sigma Chemical Co. All solvents used were of HPLC grade and were products of Fisons plc (Loughborough, U.K.). Scintillation fluid (Aqua Luma Plus) was from Lumac/3M B.V. (Schaesberg, Netherlands).

Preparation of human and murine hepatic microsomes. Washed human liver microsomes were prepared as reported previously [13]. Ethical approval for removing the liver was granted by the Mersey Regional Ethical Committee and consent was obtained from the donor's relatives. The cytochrome P-450 content of these microsomes was 0.41 ± 0.06 nmol/mg protein (mean ± SD; N = 4).

Male CBA/ca mice (25–30 g) (outbred from Departmental stock; maintained on Diet CRM, Labsure, Manea, U.K.) were administered phenobarbitone once daily for 3 days (60 mg/kg), by i.p. injection (0.15 mL) in 0.9% saline. Another group of mice received i.p. injections (0.20 mL) of β-naphthoflavone suspended in corn oil at a dose of 75 mg/kg for 3 days. Control mice were either untreated or received vehicle [saline (0.15 mL) or corn oil (0.20 mL)] once a day for 3 days. Following the final set of injections, the mice were fasted for 24 hr. The animals were then killed by cervical dislocation, their livers removed and weighed and microsomes were prepared as described previously [14]. The microsomes were either used immediately or stored for up to 48 hr at −80° as an intact pellet. Cytochrome P-450 content was measured by the method of Omura and Sato [15] and microsomal protein was determined by the method of Lowry *et al.* [16].

Characterization of hepatic enzyme induction produced by treatment of CBA/CA mice with phenobarbitone or β-naphthoflavone. 7-Ethoxycoumarin (7-EC) *O*-deethylase activity and 7-methoxycoumarin (7-MC) *O*-demethylase activity were determined for microsomes prepared from control (untreated) animals under conditions shown to be linear with respect to time and protein concentration. Activities in microsomes prepared from phenobarbitone or β-naphthoflavone-treated animals were determined under identical conditions. NADPH concentration was non-limiting. Incubations of either 7-EC or 7-MC (100 μM) were conducted in round bottomed 20-mL glass tubes containing 1 mL sodium potassium phosphate buffer (pH 7.4; 1/15 M) 150 μg microsomal protein and NADPH (1 mM). Reactions were carried out in a shaking water bath at 37° for 5 min and were initiated by the addition of NADPH. Control incubations contained no NADPH. Extraction and quantification of the product, 7-hydroxycoumarin (7-OH-C), were performed as reported by Greenlee and Poland [17].

Isolation of mononuclear leucocytes from human blood. Human mononuclear leucocytes (MNL) were isolated from blood freshly drawn from healthy volunteers, using a method similar to that described by Boyum [18]. The blood (30–40 mL) was diluted (1:1, v/v) with phosphate-buffered saline (PBS; NaCl 8 g/

† Abbreviations: TCPO, 1,2-epoxy-3,3,3-trichloropropane; NADPH, reduced nicotinamide adenine dinucleotide phosphate; MNL, mononuclear leucocytes; HEPES, 4-(2-hydroxyethyl)1-piperazine-ethane sulphonic acid; HSA, human serum albumin; PB, phenobarbitone; BNF, β-naphthoflavone; 7-EC, 7-ethoxycoumarin; 7-MC, 7-methoxycoumarin; 7-OH-C, 7-hydroxycoumarin; 4-HPPH, 5-(4-hydroxyphenyl)-5-phenylhydantoin; 8-OH-M, 8-hydroxymianserin; DM-M, desmethylmianserin; M-NO, mianserin *N*(2)-oxide.

L; KCl, 0.2 g/L; Na_2HPO_4, 0.2 g/L; pH 7.2) containing lithium heparin and MNL prepared by centrifugation through a Ficoll/sodium metrizoate density gradient as described previously [12]. Contaminating erythrocytes were removed by lysis with ammonium chloride (0.85% for 5 min) following a washing phase in RPMI 1640 medium. Finally, the MNL were resuspended in 15 mM 4-(2-hydroxyethyl)1-piperazine-ethanesulphonic acid (HEPES)-buffered balanced salt medium to obtain *ca.* 1.5×10^6 cells/mL. Their viability, upon isolation, as determined by trypan blue exclusion was >95%. Isolated cells were kept at room temperature and incubated within 3 hr.

Determination of the cytotoxicity of metabolites of phenytoin and mianserin generated by murine liver microsomes. For the determination of metabolism (NADPH)-dependent cytotoxicity, incubations were performed using concentrations at which the drugs investigated were not cytotoxic *per se* [12]. Hence, 1×10^6 MNL were incubated with phenytoin (150 μM) or mianserin (10 μM), and control or induced murine hepatic microsomes (0.5 mg protein) in the presence or absence of NADPH (1 mM) for 2 hr in HEPES-buffered medium at 37°. MNL were pre-treated with the epoxide hydrolase inhibitor TCPO (30 μM for 10 min) prior to incubation with phenytoin as this procedure has previously been shown to enhance significantly the cytotoxicity of phenytoin metabolites [19]. The drugs were added in 10 μL MeOH, which, as a 1% (v/v) solution, was non-toxic. The total incubation volume was 1 mL. To eliminate variability caused by inter-individual differences in detoxifying enzymes, MNL from the same donor were used for all incubations conducted with each compound.

Following the 2 hr incubation, the cells were sedimented and re-suspended in a drug-free medium (HEPES-buffered medium containing 5 mg/mL HSA). Incubations were then continued without agitation at 37° for 16 hr and aliquots removed in order to determine cell viability by trypan blue dye exclusion (0.1% trypan blue for *ca.* 5 min). Failure to exclude the dye was taken as an indication of cell death. The cells (>200) were examined on a Neubauer haemocytometer under a Model Wilovert II microscope (Will Wetzlar GmBH, Nauborn, F.R.G.).

Determination of the metabolism-dependent cytotoxicity of compounds related to mianserin. Three tricyclic antidepressants, imipramine, desipramine and amitriptyline were investigated in order to compare their activation by mouse liver microsomes to cytotoxic metabolites with that of mianserin. Incubations were performed as described above for mianserin with a final drug concentration of 10 μM, at which the drugs themselves were non-toxic (unpublished data).

The ability of *human* liver microsomes to generate toxic species from mianserin and these compounds, as well as from ORG GC94 (a structural congener of mianserin) and the major phase one metabolites of mianserin (8-hydroxymianserin, desmethylmianserin and mianserin *N*(2)-oxide was also assessed.

Metabolism of drugs by murine hepatic microsomes. [^{14}C]Phenytoin (150 μM, 1 μCi) and [^{3}H]mianserin (10 μM, 0.25 μCi) were incubated with either control or induced murine hepatic microsomes (2.0 mg protein), 1×10^6 MNL and 1 mM NADPH in 15 mM HEPES-buffered medium, pH 7.4 (final volume, 4 mL). Reactions were initiated by addition of NADPH and were performed in 25-mL Ehrlenmeyer flasks in a shaking water bath at 37° for 2 hr as described by Spielberg [11]. NADPH was omitted from control incubations. TCPO (30 μM) was included in incubations conducted with phenytoin. Reactions were terminated by cooling the incubation mixture to 0° and extracting with organic solvent. Ascorbic acid was added (final concentration 10 mM) following termination of the reactions to stabilize any oxygen-sensitive metabolites e.g. catechols [13] which may have been formed. Stable metabolites were recovered from incubations of [^{14}C]phenytoin by extraction into ethyl acetate (1:1, v/v, twice). Thereafter, the microsomal and cellular protein was precipitated by adding acetone (1:1, v/v) and leaving the mixtures overnight at 4°. The protein in incubations of [^{3}H]mianserin was precipitated with methanol (1:1, v/v) immediately after termination of the reactions to enable quantification of stable metabolites by HPLC (as not all of the metabolites of mianserin are extracted into ethyl acetate).

Irreversible binding of radiolabelled material. Radiolabelled material irreversibly bound to precipitated protein was determined by exhaustive solvent extraction. The protein was successively extracted with methanol (5 mL × 2) and 70% (v/v) methanol (5-mL aliquots) by vigorous vortex mixing until no further radioactivity was extractable. The protein was dissolved in 3 mL sodium hydroxide (0.5 M) at 60°. Aliquots of the solubilized protein (0.5 mL) were neutralized with glacial acetic acid (100 μL) and radioactivity was quantified in 4 mL scintillant. Further aliquots were taken for protein determination [16].

Analysis of metabolites. Stable metabolites generated from phenytoin and mianserin in the microsomes-cell incubations were analysed by radiometric HPLC. They were chromatographically identified by co-chromatography with authentic standards. Pooled ethyl acetate extracts of [^{14}C]phenytoin were evaporated to dryness under N_2 with warming and reconstituted in methanol (400 μL). Mianserin and its metabolites were analysed directly in the supernatant following precipitation of protein. [^{14}C]Phenytoin, [^{3}H]mianserin and their respective metabolites were chromatographed as described previously [4, 20]. Eluate was monitored at either 225 nm (phenytoin) or 254 nm (mianserin), and collected in 30 s fractions which were mixed with 4 mL of scintillant for measurement of radioactivity. Recoveries of chromatographed radioactivity were 94 ± 9% (mean ± SD, N = 12) for phenytoin (20-μL aliquots injected; *ca.* 100,000 dpm) and 93 ± 6% (mean ± SD, N = 12) for mianserin (200-μL aliquots injected; *ca.* 10,000 dpm).

Statistical analyses were performed using a Student's *t*-test, non-paired or paired format as appropriate.

RESULTS

Treatment of male CBA/ca mice with phenobarbitone (PB) and β-naphthoflavone (BNF)

Table 1. Effects of various treatments on the mass, yield of microsomal protein and cytochrome P-450 content of male CBA/CA mouse livers

Treatment	Liver mass (g)	Yield of microsomal protein (mg/g liver)	Cytochrome P-450 content (nmol/mg microsomal protein)
None	1.13 ± 0.12	16.30 ± 2.77	0.63 ± 0.10
Saline	1.37 ± 0.14	17.07 ± 2.89	0.61 ± 0.11
Phenobarbitone	1.63 ± 0.05*	26.00 ± 1.64†	1.34 ± 0.14‡
Corn oil	1.28 ± 0.22	14.25 ± 3.08	0.68 ± 0.08
β-Naphthoflavone	1.23 ± 0.13	16.50 ± 1.47	0.98 ± 0.12‡

Values are mean ± SD for four animals.
Significant differences between values for control and induced groups are: * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ (non-paired Student's t-test).

Table 2. The metabolism of phenytoin (150 μM) to stable and chemically reactive metabolites by control and induced mouse liver microsomes. Incubations were conducted in the presence of TCPO (30 μM)

Treatment	Irreversible binding (pmol bound/mg protein) −NADPH	Irreversible binding (pmol bound/mg protein) +NADPH	Total stable metabolites* (4-HPPH as % incubated radioactivity)
Saline	111 ± 15 (0.04)	315 ± 51 (0.11)	0.24 ± 0.05
Phenobarbitone	66 ± 15 (0.02)	465 ± 39 (0.16)	0.49† ± 0.05
Corn oil	159 ± 39 (0.05)	408 ± 78 (0.14)	0.31 ± 0.06
β-Naphthoflavone	135 ± 30 (0.04)	435 ± 54 (0.15)	0.35 ± 0.04

* Values from control incubations (without NADPH) have been subtracted.
Data are expressed as means ± SD of three (NADPH-dependent metabolism) or four (irreversible binding) determinations. Each determination representing a different animal.
Numbers in parentheses are mean values of binding data expressed as % incubated radioactivity.
† Statistically different from saline-treated control ($P < 0.001$).

resulted in a significant increase in the cytochrome P-450 content of their hepatic microsomal fraction compared with the levels in vehicle-treated animals ($P < 0.001$, Table 1). The increase in P-450 content induced by BNF was paralleled by a spectral shift in the position of the Soret maximum of the reduced cytochrome-carbon monoxide complex by 1–2 nm towards the blue, consistent with the findings of other workers [21]. PB also produced a significant increase in the mass of excised livers ($P < 0.05$) and the yield of microsomal protein (mg/g liver; $P < 0.01$) compared with the values obtained from saline-treated animals.

Phenytoin, in accordance with previous reports [12] and the findings of other workers [22, 23] was poorly metabolized *in vitro* to 4-HPPH, its sole detectable metabolite (Table 2). Induction by PB was associated with an increase in 4-HPPH formation (0.49% compared with 0.24% in saline-treated controls). BNF did not affect the metabolism of phenytoin to 4-HPPH. Despite this limited metabolic turnover, phenytoin was activated in the presence of NADPH to a chemically reactive intermediate which became irreversibly bound to protein (Table 2). The NADPH-dependent activation of phenytoin to a chemically reactive metabolite, as assessed by irreversible binding of phenytoin to protein, was significantly enhanced by PB induction (0.14% compared with 0.07% in saline-treated controls; $P < 0.001$).

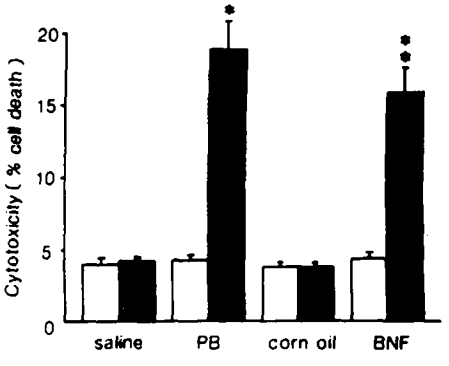

Fig. 2. The metabolism of phenytoin (150 μM) to cytotoxic metabolites by control and induced mouse microsomes in the presence (▨) and absence (□) of NADPH (1 mM). Data are means ± SD for four animals. * Significantly different from controls (−NADPH), $P < 0.01$; ** Significantly different from controls (−NADPH), $P < 0.001$.

Metabolism (NADPH)-dependent cytotoxicity, as assessed by death of MNL, produced by phenytoin was entirely dependent on hepatic enzyme induction (Fig. 2). Both PB- and BNF-induced murine hepatic microsomes activated phenytoin to a cytotoxic species in the presence of NADPH. Microsomes from either vehicle-treated group did not metabolize

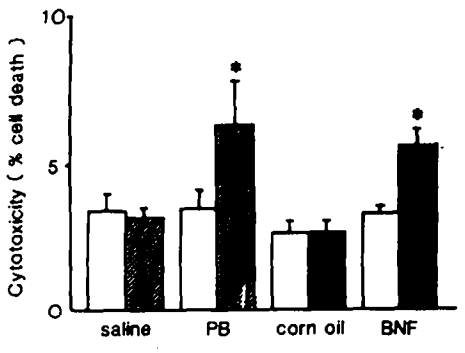

Fig. 3. The metabolism of mianserin (10 μM) to cytotoxic metabolites by control and induced mouse microsomes in the presence (▨) and absence (□) of NADPH (1 mM). Data are means ± SD for four animals. * Significantly different from controls (−NADPH), $P < 0.05$.

phenytoin to a metabolite which produced significant toxicity, although they activated phenytoin to a chemically reactive intermediate.

Mianserin was extensively metabolized *in vitro* by hepatic microsomes from vehicle-treated (control) and induced mice (Table 3). Three major oxidation products could be identified by HPLC analysis: 8-hydroxymianserin (8-OH-M), desmethylmianserin (DM-M) and mianserin *N*(2)-oxide (M-NO). All three metabolites have been identified as products of the drug's biotransformation *in vivo* [24]. Treatment by both PB and BNF increased the overall metabolism of mianserin *in vitro* compared with their respective vehicle controls. As a result of the extensive metabolism of mianserin in this system, there are a number of unidentified products in the metabolic profile. In addition, both PB and BNF significantly increased the formation of chemically reactive metabolites from this tetracyclic antidepressant ($P < 0.001$ for PB, $P < 0.05$ for BNF compared with their respective vehicle controls; Table 3). Furthermore, microsomes from vehicle-treated animals did not metabolize mianserin to a cytotoxic species (Fig. 3). Induction by PB or BNF, however, produced a small but significant level of cell death, as assessed by trypan blue exclusion. Hence, for mianserin, NADPH-dependent cytotoxicity was dependent on hepatic enzyme induction and correlated well with the total formation of chemically reactive metabolites by mouse liver microsomes.

To elucidate further the nature of the toxic metabolite of mianserin, the metabolism-dependent cytotoxicity produced by its major phase I metabolites and a structural congener (ORG GC94) was measured. Human liver microsomes were used in this study as this was found to be the optimal activating system for mianserin. At a concentration of 10 μM, all of these compounds were activated to cytotoxic species by human liver microsomes (% NADPH-dependent cell death = 8.3, 10.0, 7.2 and 6.5 for 8-OH-M, DM-M, M-NO and ORG GC94, respectively; mean values of quadruplicate determinations). The metabolism of these compounds was not measured as no radiolabelled material was available to allow estimation of irreversible binding, the index necessary to quantify reactive metabolite formation.

Table 4 shows that imipramine, desipramine and amitriptyline, like mianserin, were metabolized by induced, but not vehicle-treated (control), mouse microsomes to products which caused significant cytotoxicity. However, of these other antidepressants, only desipramine was activated to a cytotoxic metabolite by human liver microsomes, although the degree of metabolism-dependent toxicity was less than that observed for mianserin at the concentration used (10 μM).

DISCUSSION

The aim of the present study was to define the biochemical basis for the previously reported differential activation of phenytoin and mianserin to toxic metabolites by human and phenobarbitone-induced mouse liver microsomes [12]. In order to investigate further these differences, we have examined the effects of hepatic enzyme induction in mice on the metabolism of phenytoin and mianserin to stable, chemically reactive and cytotoxic products *in vivo*. Furthermore, we have compared the activation of mianserin to cytotoxic metabolites with that of other antidepressants, which share similar structural features and/or routes of biotransformation with mianserin, to gain further insight into the structural requirements for the activation of this xenobiotic.

In agreement with the findings of other workers

Table 3. The metabolism of mianserin (10 μM) to stable and chemically reactive metabolites by control and induced mouse liver microsomes

Treatment	Irreversible binding (pmol bound/mg protein) −NADPH	Irreversible binding (pmol bound/mg protein) +NADPH	Total stable metabolites (% incubated radioactivity)* 8-OH-M	DM-M	M-NO	M
Saline	98 ± 2 (0.49)	1054 ± 246 (5.27)	18.56 ± 0.2	27.2 ± 1.9	10.8 ± 13.7	17.0 ± 4.7
Phenobarbitone	146 ± 14 (0.73)	2316 ± 260 (11.58)	34.6 ± 1.1	16.3 ± 3.6	6.6 ± 0.6	3.1 ± 0.2
Corn oil	110 ± 10 (0.55)	1398 ± 174 (6.99)	24.0 ± 3.6	19.7 ± 2.1	4.9 ± 0.7	5.7 ± 0.6
β-Naphthoflavone	110 ± 4 (0.55)	1720 ± 56 (8.60)	32.2 ± 2.7	23.9 ± 2.4	3.9 ± 0.6	4.4 ± 0.2

* Values from control incubations (without NADPH) have been subtracted.

Data are expressed as mean ± SD of three (NADPH-dependent metabolism) or four (irreversible binding) determinations. Each determination representing a different animal.

Numbers in parentheses are mean values of binding data expressed as % incubated radioactivity.

Table 4. A comparison of the metabolism of mianserin and related compounds to cytotoxic metabolites by human and mouse liver microsomes

	Mianserin		Cytotoxicity (% cell death)* Imipramine		Desipramine		Amitriptyline	
	−NADPH	+NADPH	−NADPH	+NADPH	−NADPH	+NADPH	−NADPH	+NADPH
Human	2.3	16.9§	3.0	3.9	3.2	5.7‡	4.2	5.7
Mouse								
Saline	3.3	3.2	6.1	6.7	6.1	6.5	3.9	5.5
Phenobarbitone	3.0	6.4‖	5.7	13.2†	5.7	15.2§	5.4	8.6‡
Corn oil	2.9	2.6	5.1	6.9	5.0	5.7	4.1	6.6
β-Naphthoflavone	3.2	5.5‖	3.7	10.1‡	5.0	12.3‡	5.6	9.1‡

* Observed after the 16 hr incubation in drug (and microsome)-free medium.
Values are means of four determinations (pooled microsomes) or means of four determinations for four animals (mianserin data).
Significant differences between control (−NADPH) and test (+NADPH) values are as follows: † $P < 0.05$; ‡ $P < 0.01$, § $P < 0.001$ (non-paired Student's *t*-test); ‖ $P < 0.05$ (paired Student's *t*-test).

[15, 17, 25], both phenobarbitone and β-naphthoflavone treatment increased 7-EC O-deethylation. Treatment with phenobarbitone also significantly increased 7-MC O-demethylation, whereas treatment with β-naphthoflavone did not increase the rate of O-dealkylation of this substrate, in agreement with the findings of Matsubara *et al.* [26] and Paterson *et al.* [25]. It is therefore apparent that phenobarbitone and β-naphthoflavone produce different patterns of hepatic enzyme induction in male CBA/ca mice.

Phenytoin is metabolized by phenobarbitone-induced mouse liver microsomes to a cytotoxic metabolite *in vitro*, which is thought to be an epoxide [19]. In contrast, human liver microsomes do not metabolize phenytoin to a cytotoxic product, which suggests either, that a threshold concentration of the epoxide is required for cytotoxicity that is only achieved after induction, or that a species difference exists with respect to epoxide formation [12]. However, the results presented here clearly show that hepatic enzyme induction by phenobarbitone or β-naphthoflavone is necessary for the generation of the cytotoxic metabolite from phenytoin by liver microsomes of CBA/ca mice (Fig. 2).

It has been proposed that the epoxide responsible for mediating the toxicity of phenytoin both *in vitro* [19] and *in vivo* [27] is the 3,4-epoxide produced during 4-hydroxylation, the major route of phase I metabolism of the drug in mice and humans [20, 21]. Our findings show that, whereas induction by both phenobarbitone and β-naphthoflavone results in the formation of a cytotoxic epoxide by mouse liver microsomes, only phenobarbitone increased the formation of 4-HPPH and, therefore, the intermediate 3,4-epoxide. Thus, it appears that both phenobarbitone and β-naphthoflavone induce the formation of an additional cytotoxic metabolite, possibly a further metabolite of the phenol, 4-HPPH [30] which, although produced in insufficient amounts (or too unstable) to be accurately detected by HPLC analysis, mediates the cytotoxic effect. Indeed, it has been suggested that the species- and strain-dependent effect of 3-methylcholanthrene-like inducers (e.g. β-naphthoflavone) to increase the generation of chemically reactive metabolites from phenytoin [31] occurs independently of any effect on its major route of metabolism, 4-hydroxylation [23]. Hence, the differential activation of phenytoin to a cytotoxic epoxide by human and phenobarbitone-induced mouse liver microsomes [12] may be explained by lower levels of the necessary isozyme(s) of cytochrome P-450 within human liver. Predisposition to the adverse effects associated with phenytoin may therefore be a consequence of both enhanced metabolic activation (by autoinduction, for example) by cytochrome P-450 and decreased detoxication by epoxide hydrolase [19].

Mianserin is metabolized to a cytotoxic metabolite by human liver microsomes [12] but not by control mouse liver microsomes (Table 4). However, chromatographic analysis of incubation extracts clearly showed that mianserin is extensively metabolized by mouse liver microsomes (>80% turnover) and that the principal metabolites were 8-hydroxymianserin, desmethylmianserin and mianserin *N*(2)-oxide,

which are also formed by human liver microsomes [4, 12]. Therefore, the observed species difference in the activation of mianserin indicates that human liver contains a specific form of cytochrome P-450 which is capable of transforming mianserin into a cytotoxic metabolite, that is thought to be an iminium ion [32].

Further experiments showed that microsomes from mice induced with either phenobarbitone or β-naphthoflavone can produce a cytotoxic metabolite from mianserin. However, the degree of cytotoxicity produced was significantly less than that observed with human liver microsomes ($P < 0.001$). HPLC analysis of incubation extracts showed that the overall metabolism of mianserin was greater with microsomes from induced animals and that the chromatographic profile was more complex than that initially anticipated. It is therefore possible that in this situation, cytotoxic metabolites are formed not only from mianserin itself but also from its phase I metabolites. Indeed we have found that human liver microsomes activate mianserin, 8-hydroxymianserin, desmethylmianserin and mianserin *N*-oxide to cytotoxic metabolites with similar facility [20].

Imipramine, desipramine and amitriptyline were metabolized to cytotoxic products in the presence of hepatic microsomes from mice induced with either phenobarbitone or β-naphthoflavone (Table 4). However, like mianserin, none of these drugs were readily activated by control mouse liver microsomes to a form which manifested significant death of MNL. Unlike the cell death produced by the metabolism of phenytoin, the cytotoxicity associated with these three drugs was not dependent on pre-treatment of the target cells by TCPO (pretreatment of MNL with TCPO having no effect on the cytotoxicity measured; data not shown), suggesting that, in each case, the toxic metabolite was not an epoxide. Previous studies have shown that all three compounds may be metabolized to hydroxylamines [33] and hence it is possible that this route of biotransformation may be responsible for their toxicity *in vitro*. Indeed, as a result of similar *in vitro* studies, hydroxylamines derived from various sulphonamides have been implicated as the cytotoxic form of this class of antibiotics [34]. Clearly, further work is required to elucidate the chemical nature of the cytotoxic metabolites derived from these three antidepressants, including an examination of their metabolism under the conditions of the *in vitro* cytotoxicity assay.

In summary, all of the compounds investigated in the present study possess functional groups which can be activated to cytotoxic products by cytochrome P-450 enzymes present in induced mouse liver microsomes. However, only mianserin was readily activated to a toxic metabolite in the presence of a human microsomal system. Hence, it would appear that mianserin is specifically activated by one or more forms of cytochrome P-450 found in human, but not mouse, liver microsomes to a cytotoxic metabolite. Furthermore, as the other antidepressants possess tricyclic moieties similar to that found in mianserin, the fourth (piperazine) ring present in mianserin may play an important role in the generation of its cytotoxic metabolite. This is consistent with our hypothesis that mianserin toxicity may be mediated by an iminium derivative [32] and is supported by the observation that cytotoxicity is still prevalent with ORG GC94, as all structural differences between this compound and mianserin occur at areas of the molecule distal to the proposed site of iminium ion formation.

A species difference in the activation of a drug to a toxic metabolite has also recently been reported with bromobenzene [35], which forms electrophilic epoxide intermediates. This result, along with the species difference reported here with mianserin, suggests that, for certain drugs, man may be more susceptible to metabolism-dependent toxicity than other species commonly used in toxicology studies, such as the mouse. Collectively, these data emphasise that human tissue is indispensable for *in vitro* drug metabolism studies [36] and that the *in vitro* toxicity assay outlined here may prove useful in the selection of an appropriate animal model with which to investigate the relationship between the metabolism and toxicity of a given drug *in vivo*.

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