An *in vitro* study of the microsomal metabolism and cellular toxicity of phenytoin, sorbinil and mianserin

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1 The cytotoxicity of metabolites generated from phenytoin, sorbinil and mianserin by human and mouse liver microsomes was assessed by co-incubation with human mononuclear leucocytes as target cells. Cytotoxicity was determined by trypan blue dye exclusion.

2 Phenytoin and sorbinil were metabolised by NADPH-dependent murine microsomal enzymes to cytotoxic metabolites. Cytotoxicity produced by both drugs was significantly enhanced by the epoxide hydrolase inhibitor trichloropropane oxide (TCPO). No significant cytotoxicity was observed in the presence of human liver microsomes.

3 Mianserin was metabolised by both human and mouse liver microsomes to a cytotoxin. Cytotoxicity was greater in the presence of human liver microsomes ($13.7 \pm 2.2\%$; mean $\pm$ s.d. for four livers, compared with $6.0 \pm 2.4\%$, mean $\pm$ s.d., $n = 4$, with mouse liver microsomes), and was unaffected by pretreatment with TCPO.

4 Stable metabolites were quantified by radiometric high performance liquid chromatography. Phenytoin and sorbinil were metabolised to 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (0.3–0.5% of incubated radioactivity) and 2-hydroxysorbinil (0.4–2.7% of incubated radioactivity), respectively, by both human and mouse liver microsomes.

5 Mianserin was metabolised to 8-hydroxymianserin and desmethylmianserin by both human and mouse liver microsomes. Desmethylmianserin was the major product in incubations with human liver microsomes ($32.3 \pm 12\%$, mean $\pm$ s.d. for four livers), whereas 8-hydroxymianserin was the predominant metabolite generated by mouse liver microsomes ($25.9 \pm 1.5\%$, mean $\pm$ s.d., $n = 4$).

6 Generation of electrophilic metabolites was assessed by determination of the amount of radiolabelled material which became irreversibly bound to protein. Only mouse liver microsomes activated phenytoin to a chemically reactive metabolite, whereas both mouse and human liver microsomes generated reactive metabolites from sorbinil and mianserin.

7 These studies show that drug cytotoxicity can be mediated by low concentrations (*circa* μM) of metabolites generated by NADPH-dependent hepatic microsomal enzymes; however demonstration of cytotoxicity *in vitro* has not been established as a means of predicting *in vivo* toxicity.

Keywords microsomal metabolism toxicity phenytoin sorbinil mianserin

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Introduction

The metabolism of essentially non-toxic drugs to chemically reactive metabolites is thought to initiate a variety of adverse reactions (Park, 1986; Parke, 1987). These reactive species are commonly products of Phase I oxidation reactions mediated by cytochrome P-450-dependent mixed function oxygenases (Guengerich & Liebler, 1985). The generation of such electrophilic metabolites may produce adverse reactions via three inter-related processes, namely formation of free radicals, oxidation of thiols, and covalent reactions with endogenous macromolecules, especially DNA and protein. The structural and metabolic disruptions induced within target cells may eventually be manifested as an overt toxic reaction (Bridges *et al.*, 1983).

The potential of a drug to form chemically reactive metabolites in man may be assessed by measuring the extent of its irreversible binding to protein in a human liver microsomal preparation (Kitteringham *et al.*, 1988). Although this is a useful index of reactive metabolite formation *in vitro*, discrepancies are often observed between such indiscriminate irreversible binding and cytotoxicity (Devalia *et al.*, 1982; Albano *et al.*, 1985). Indeed, compounds which yield protein-reactive intermediates are not invariably toxic (Streeter *et al.*, 1984). Furthermore, toxicity observed *in vivo* will depend on the balance between activation and deactivation pathways and may therefore only ensue once deactivation (or detoxication) mechanisms have been overwhelmed (Jones *et al.*, 1986). Hence, the demonstration of an ability to generate protein-reactive metabolites *in vitro* is not sufficient to predict the toxicological characteristics of a drug *in vivo*.

In order to assess the roles of metabolic activation and deactivation in cytotoxicity, *in vitro* systems have been developed which incorporate both activating systems and target cells which possess defence mechanisms against cytotoxic metabolites (Acosta & Mitchell, 1981; Horner *et al.*, 1987; Spielberg, 1984). Spielberg and co-workers used phenobarbitone-induced mouse liver microsomes to generate reactive metabolites from a number of drugs, including phenytoin (Spielberg *et al.*, 1981). Human mononuclear leucocytes (MNL) were employed as target cells as they are readily obtained in large numbers and contain the detoxifying enzymes epoxide hydrolase (Seidegard *et al.*, 1984) and glutathione transferase (Seidegard *et al.*, 1987). However, although a particular MNL detoxifying enzyme may be identical to that

found in other cells (Seidegard *et al.*, 1987), the considerable qualitative and quantitative differences between the detoxifying enzymes of different cell types (Dorstal *et al.*, 1986) should be taken into account when considering the cytotoxicity of drugs that cause cell-selective toxicity. MNL from patients who had experienced phenytoin-associated adverse reactions (or from certain of these patients' relatives) were more susceptible to phenytoin metabolites than were cells obtained from normal volunteers (Spielberg, 1981). Significant mortality was only observed in volunteers' cells after they had been pretreated with epoxide hydrolase inhibitors, suggesting that a hereditary deficiency of an enzyme which detoxifies arene oxides may predispose individuals to phenytoin toxicity. Although this system clearly demonstrated the formation of a cytotoxic metabolite, the identity of this species, or of other metabolites generated within the microsomal incubations, was not ascertained. In the present study, we have extended this cytotoxicity assay by incorporating human liver microsomes, to create a system more relevant to adverse reactions in man, and by characterisation of the metabolites, both stable and reactive, formed under identical incubation conditions.

Two of the drugs investigated, phenytoin and mianserin, have been shown to undergo metabolic activation by human liver microsomes to chemically reactive metabolites (Kitteringham *et al.*, 1988) whilst the third, sorbinil, does not appear to produce a reactive species capable of covalent binding to proteins under identical conditions (Maggs & Park, 1988). Nevertheless, all three compounds (Figure 1) have been reported to cause adverse reactions in man. Phenytoin has been linked with a life-threatening hypersensitivity reaction (Dhar *et al.*, 1974), mianserin with blood dyscrasias including agranulocytosis (Chaplin, 1986) and sorbinil with a fever-rash syndrome similar to that observed with phenytoin (Jaspan, 1986).

Methods

Chemicals

5,5-Diphenyl[^{14}C]-hydantoin ([^{14}C]-phenytoin; 58 mCi mmol $^{-1}$; radiochemical purity 98%) was purchased from Amersham International plc (Bucks, UK). [13- ^3H]Mianserin (3.5 Ci mmol $^{-1}$) was a gift from Organon International B.V. (Oss, Netherlands), and was found to be radiochemically pure when analysed by h.p.l.c. [8- ^3H]Sorbinil (10 Ci mmol $^{-1}$) was provided by

Figure 1 Drugs used in the present cytotoxicity studies.

Pfizer Central Research (Groton, Conn., USA), and was purified to radiochemical homogeneity (Maggs & Park, 1988). Unlabelled phenytoin, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH), 1,1,1-trichloro-2-propane oxide (TCPO) and human serum albumin (HSA, fraction V) were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Unlabelled mianserin, desmethylmianserin, 8-hydroxymianserin and mianserin *N*-oxide were supplied by Organon. 2-Hydroxysorbinil and 2,4-imidazolidinedione 5-(2-hydroxyethyl)-5-(5-fluoro-2-hydroxyphenyl) (IHFH) were from Pfizer. NADPH (tetrasodium salt) was obtained from BDH Chemicals Ltd (Poole, Dorset, UK). H.p.l.c. grade solvents were products of Fisons plc (Loughborough, Leics, UK).

Isolation of human mononuclear leucocytes

Mononuclear leucocytes (MNL) were isolated (*circa* 1×10^6 ml⁻¹ whole blood) from freshly drawn heparinized (lithium heparin) blood from healthy male volunteers (21–25 years). The blood (30–40 ml) was diluted (1:1, v/v) with phosphate-buffered saline (PBS; NaCl, 8 g l⁻¹; KCl, 0.2 g l⁻¹; Na₂HPO₄, 1.15 g l⁻¹; KH₂PO₄, 0.2 g l⁻¹; pH 7.2). Aliquots (12 ml) were layered onto Lymphoprep (Nyegaard UK Ltd, Birmingham; 9 ml aliquots), and centrifuged at 1,900 rev min⁻¹ (*circa* 650 g) for 13 min in a Centaur 2 centrifuge (MSE, Crawley, Sussex, UK). The interface layer was removed, the suspension of cells diluted with PBS (1:1, v/v) and the cells sedimented by centrifugation. They were gently resuspended in PBS and sedimented at a low speed (800 rev min⁻¹, *circa* 115 g, for 10 min) to reduce contamination by platelets; this procedure was repeated once. Contaminating erythrocytes were removed by hypotonic lysis. Finally, the MNL were resuspended in 15 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES)-buffered balanced salt medium (Spielberg, 1980) to obtain *circa* 2×10^6 cells ml⁻¹. The isolated cells were left at room tem-

perature, and incubated within 3 h. Their viability upon isolation, as determined by trypan blue exclusion, was $\geq 95\%$.

Preparation of hepatic microsomes

Washed human liver microsomes were prepared as reported previously (Purba *et al.*, 1987). Ethical approval for removing the livers was granted by the Mersey Regional Ethics Committee and consent was obtained from the donors' relatives. Donor details, where obtained, are given in Table 1. Microsomes were isolated from the pooled livers of 4–8 phenobarbitone (PB) pre-treated (60 mg kg⁻¹ body weight day⁻¹, i.p., for 3 days) male CBA mice by the centrifugation procedure used to obtain washed human liver microsomes. Their cytochrome P-450 contents were 1.48 ± 0.19 nmol mg⁻¹ protein (mean $\pm$ s.d.; $n = 4$ batches of pooled microsomes). Concentrated suspensions of the microsomes (*circa* 6–8 mg microsomal protein ml⁻¹) in 0.1 M sodium phosphate buffer, pH 7.4, were prepared for incubation with MNL. Aliquots were stored at -70°C ; von Bahr *et al.* (1980) have shown that cytochrome P-450-dependent activities of human liver microsomes stored in suspension are stable over long periods.

Measurement of MNL viability by trypan blue dye exclusion

Cell viability (% excluding dye) was assessed using 100 μ l aliquots of suspensions: they were mixed with 25 μ l of a 0.2% (w/v) solution of trypan blue (dye content $\geq 50\%$; Aldrich Chemical Co., Gillingham, Dorset, UK) in PBS. The cells (≥ 200) were examined on a Neubauer haemocytometer under a Model Wilovert II microscope (Will Wetzlar GmbH, Nauborn, FRG). Cell death could then be expressed as % cells not excluding dye.

Table 1 Details of donors and histological examination of human liver samples

Liver donor's age (years)	Sex	Cause of death	Drugs	Histology	Hepatic microsomal cytochrome P-450 (nmol mg ⁻¹ protein; mean $\pm$ s.d., n = 3–10)*
66	F	Brain haemorrhage	warfarin	normal	0.67 $\pm$ 0.15
29	M	Head injuries	unknown	normal	0.41 $\pm$ 0.06
46	F	Sub-arachnoid haemorrhage	phenobarbitone, phenytoin, dopamine, dobutamine, frusemide	normal	0.67 $\pm$ 0.06
18	M	Head injuries	unknown (smoker)	normal	0.47 $\pm$ 0.06

* Murine hepatic cytochrome P-450 contents in present study were 1.48 ± 0.19 nmol mg⁻¹.

Determination of cytotoxicity

For determination of direct cytotoxicity, MNL (1×10^6 cells in 1 ml HEPES-buffered medium) were incubated with the test compound, added in 10 μ l h.p.l.c.-grade methanol, in 10 ml polystyrene tubes. The tubes, open to the air, were shaken in a water bath at 37° C for 2 h. Control incubations contained methanol alone, which, as a 1% (v/v) solution, was non-toxic: the initial viabilities of cells incubated without additives and in the presence of methanol were $98 \pm 1\%$ (mean $\pm$ s.d., n = 3) and $99 \pm 1\%$, respectively. After 2 h, viability was determined by trypan blue exclusion. The cells were sedimented, and re-suspended in a drug-free medium (HEPES-buffered medium containing 5 mg ml⁻¹ HSA). Incubations were continued without agitation in a humidified air-CO₂ (95:5) incubator at 37° C for 16 h, and aliquots removed for the dye exclusion assay.

Determinations of metabolism (NADPH)-dependent cytotoxicity were performed in the same manner as above except that the 2 h incubations included murine or human hepatic microsomes (0.5 mg protein) together with NADPH (1 mM). MNL epoxide hydrolase was inhibited with 30 μ M TCPO (Oesch *et al.*, 1971) by pre-incubating the cells with inhibitor for 10 min prior to addition of the test compound, microsomes and NADPH (Spielberg *et al.*, 1981). The results shown represent cytotoxicity (% cells not excluding trypan blue) observed after the 16 h incubation, as cell death after 2 h was minimal.

Metabolism of drugs by murine and human liver microsomes

[¹⁴C]-Phenytoin (150 μ M, 1 μ Ci), [³H]-mianserin (10 μ M, 1 μ Ci) and [³H]-sorbiniol (150 μ M, 2 μ Ci)

were incubated with either murine or human liver 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 started by addition of NADPH, and were performed in 25 ml Erlenmeyer flasks in a shaking water bath at 37° C for 2 h. NADPH was omitted from control incubations. TCPO (30 μ M) was included in some incubations. Reactions were terminated by cooling the flasks on ice. HSA in phosphate buffer (15 mg ml⁻¹; 1 ml), containing 50 mM ascorbate to stabilise oxygen-sensitive metabolites (Kitteringham *et al.*, 1988), was added to the incubations to facilitate the estimation of protein recovery following exhaustive extraction. Stable metabolites were recovered from incubations of [¹⁴C]-phenytoin and [³H]-sorbiniol by extraction into diethyl ether and ethyl acetate respectively (1:1, v/v, twice). Thereafter, the soluble and microsomal protein was precipitated by adding acetone (1:1, v/v) and leaving the mixtures at 5° C for *circa* 15 h. The protein in incubations of [³H]-mianserin was precipitated with methanol (1:1, v/v) immediately after termination of the reactions.

Irreversible binding of radiolabelled material

Radiolabelled material irreversibly bound to the precipitated protein was determined by exhaustive solvent extraction. The protein was successively extracted with methanol (5 ml $\times$ 2) and 70% (v/v) methanol (5 ml $\times$ 2) by vigorous vortex mixing. Extracts were assayed for radioactivity to ensure that removal of the reversibly bound material was complete. The protein was solubilized in 3 ml of 0.5 M NaOH by heating at 60° C. Aliquots were taken for protein determination (Lowry *et al.*, 1951) and liquid scintillation counting.

Analysis of metabolites

Stable metabolites in the microsomes-cell incubations were analysed by radiometric h.p.l.c. They were chromatographically identified by comparing their retention times with those of co-injected authentic unlabelled compounds. Pooled ether and ethyl acetate extracts of, respectively, the [^{14}C]-phenytoin and [^3H]-sorbinil incubations were evaporated to dryness under N_2 with warming and reconstituted in methanol. [^{14}C]-Phenytoin and its sole detectable metabolite, HPPH, were chromatographed as described previously (Kitteringham *et al.*, 1988); aliquots containing $40\text{--}150 \times 10^3 \text{ d min}^{-1}$ were injected onto the column for measurement of $0.5\text{--}1.0 \times 10^3 \text{ d min}^{-1}$ associated with HPPH. [^3H]-Sorbinil and 2-hydroxysorbinil were resolved (retention time 4.5 min and 7 min, respectively) on a $\mu\text{Bondapak C}_{18}$ column (30 cm $\times$ 0.39 cm i.d., 10 μM ; Waters Associates, Hartford, Cheshire, UK) using a gradient of acetonitrile (15–30% at $1\% \text{ min}^{-1}$) in 10 mM potassium phosphate buffer, pH 6.0; the flow rate was 1.5 ml min^{-1} ; aliquots containing $200\text{--}250 \times 10^3 \text{ d min}^{-1}$ were analysed for determination of $1.5\text{--}2.0 \times 10^3 \text{ d min}^{-1}$ associated with 2-hydroxysorbinil. [^3H]-Mianserin (retention time 8 min) and its metabolites—desmethylmianserin (retention time 6 min), 8-hydroxymianserin, (retention time 4 min) and mianserin *N*-oxide (retention time 15 min)—were also chromatographed on the $\mu\text{Bondapak}$ column but using an

eluent consisting of acetonitrile and 0.05 M ammonium dihydrogen orthophosphate, pH 4.3, containing 5 mM sodium heptane sulphate (19:31, v/v); the flow rate was 1.5 ml min^{-1} ; aliquots containing $30\text{--}100 \times 10^3 \text{ d min}^{-1}$ were analysed for determination of $4\text{--}40 \times 10^3 \text{ d min}^{-1}$ associated with desmethylmianserin and 8-hydroxymianserin. Eluate was monitored at either 284 nm (sorbinil) or 254 nm (mianserin), and collected in 30 s fractions which were mixed with 4 ml of scintillant for measurement of radioactivity.

Results

Phenytoin and sorbinil produced no cytotoxicity in the absence of a metabolising system at concentrations up to 150 μM , whereas mianserin was directly cytotoxic at concentrations $\geq 25 \mu\text{M}$ (data not shown). In order to investigate cytotoxicity mediated by metabolites, concentrations were chosen for each compound at which the drugs were not toxic *per se*. These were 150 μM for phenytoin and sorbinil and 10 μM for mianserin.

Phenytoin underwent minimal metabolism *in vitro* to its sole detectable stable metabolite, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH) (Figure 2). This is consistent with the findings of other workers (Pantarotto *et al.*, 1982). The extent of hydroxylation was similar for the four human livers studied (Table 2).

Figure 2 Mean values for the formation of stable and chemically reactive metabolites (expressed as % of incubated radioactivity) and cytotoxic metabolites (expressed as % cell death produced) from phenytoin by human and murine liver microsomes. Columns running from front to back represent controls (-NADPH, where appropriate), incubations with NADPH and incubations including NADPH and TCPO. For estimates of variability see Tables 2, 3 and 4.

Table 2 Metabolism of phenytoin, sorbinil and mianserin to stable metabolites by human and murine liver microsomes. Values for human livers represent the means of quadruplicate incubations, and for mouse livers the mean $\pm$ s.d. ($n = 4$)

Human liver	Total stable metabolites (% eluate radioactivity) #									
	Phenytoin		Sorbini		Mianserin		8-OH M		DM-M	
	+NADPH	p-HPPH +NADPH+TCPO	+NADPH	2-OH SB +NADPH+TCPO	+NADPH	2-OH SB +NADPH+TCPO	+NADPH	8-OH M +NADPH+TCPO	+NADPH	DM-M +NADPH+TCPO
1	0.5	0.6	0.7	0.7	0.7	0.7	17.1	14.6	30.8	30.4
2	0.5	0.5	0.7	0.8	0.8	0.8	12.5	11.1	42.8	40.0
3	0.3	0.3	0.9	0.8	0.8	0.8	13.5	12.0	39.5	37.6
4	0.4	0.4	0.4	0.4	0.4	0.4	19.7	16.6	16.0	15.7
Mean $\pm$ s.d.	0.4 $\pm$ 0.1	0.4 $\pm$ 0.1	0.7 $\pm$ 0.2	0.7 $\pm$ 0.2	0.7 $\pm$ 0.2	0.7 $\pm$ 0.2	15.7 $\pm$ 3.3	13.6 $\pm$ 2.5*	32.3 $\pm$ 12.0	30.9 $\pm$ 10.9
Murine liver	Total stable metabolites (% eluate radioactivity) #									
	Phenytoin		Sorbini		Mianserin		8-OH M		DM-M	
	+NADPH	p-HPPH +NADPH+TCPO	+NADPH	2-OH SB +NADPH+TCPO	+NADPH	2-OH SB +NADPH+TCPO	+NADPH	8-OH M +NADPH+TCPO	+NADPH	DM-M +NADPH+TCPO
Mean $\pm$ s.d.	0.5 $\pm$ 0.0	0.5 $\pm$ 0.1	2.7 $\pm$ 0.1	2.4 $\pm$ 0.1	2.7 $\pm$ 0.1	2.4 $\pm$ 0.1	25.9 $\pm$ 1.5	24.9 $\pm$ 1.2	14.3 $\pm$ 0.7	16.0 $\pm$ 1.8

* Significantly less than without TCPO, $P \leq 0.05$ (Student's paired t -test).

Control values (from incubations without NADPH) have been subtracted.

Phenobarbitone-induced mouse liver microsomes and human liver microsomes metabolised phenytoin to a similar extent (Table 2 and Figure 2) even though the former had greater cytochrome P-450 contents (Table 1). However, mouse liver microsomes, unlike human liver microsomes, activated phenytoin to a chemically reactive metabolite (Table 3), although the extent of NADPH-dependent irreversible binding was relatively small ($< 0.1\%$). The epoxide hydrolase inhibitor, TCPO, had no effect on the metabolism of phenytoin by either human or mouse liver microsomes (Table 2). Only mouse liver microsomes were capable of metabolising phenytoin to a product which induced significant death of human MNL (Table 4 and Figure 2). This cytotoxicity was NADPH-dependent and was significantly greater in cells pretreated with TCPO than in untreated cells ($P < 0.001$).

Table 2 shows that human liver microsomes also metabolised sorbinil to its sole detectable stable metabolite, 2-hydroxysorbinil (2-OHSB), to only a small extent; 2-hydroxylation is the major pathway of sorbinil's metabolism *in vivo* (Maggs & Park, 1988). Mouse liver microsomes catalysed this biotransformation more efficiently than human liver microsomes (Table 2).

Both human and mouse microsomes activated sorbinil, in the presence of NADPH, to a chemically reactive metabolite (Figure 3). The extent of irreversible binding appeared unrelated to the total amount of extractable metabolite produced, as observed with phenytoin. TCPO had no effect on the hydroxylation or activation of sorbinil. Significant cytotoxicity was only observed with sorbinil in the presence of NADPH and a phenobarbitone-induced microsomal system (Table 4 and Figure 3). Cell death was significantly greater in human MNL which had been pretreated with TCPO ($P < 0.005$).

Mianserin was extensively metabolised by both mouse and human liver microsomes to 8-hydroxymianserin (8-OH M) and desmethylmianserin (DM-M), which are metabolites of the drug *in vivo* (De Jongh *et al.*, 1981). In general, human liver microsomes produced more of the demethylated metabolite, whereas 8-OH M was the predominant metabolite produced by mouse liver microsomes (Table 2). Figure 4 shows that both mouse and human liver microsomes activated mianserin to a chemically reactive metabolite. TCPO had no effect on the metabolic activation of mianserin (Table 3). Although the extent of irreversible binding of mianserin to protein was greater with mouse liver microsomes, cytotoxicity was greater with a

human liver microsomal system (Figure 4). In the case of mianserin, pretreatment of the target cells with TCPO had no effect on the cytotoxicity produced (Table 4).

Discussion

The purpose of this study was to investigate the roles of metabolic activation and detoxication in the *in vitro* cytotoxicity of three drugs, phenytoin, sorbinil and mianserin, which are associated with adverse reactions in man. Spielberg *et al.* (1981) have described a simple model of metabolism-dependent cytotoxicity in which human MNL were exposed to drug metabolites generated by hepatic microsomes from phenobarbitone-treated mice *in vitro*. Cytotoxicity was assessed by measurement of trypan blue exclusion. This assay correlates well with other indices of cell death such as lactate dehydrogenase release from cells and loss of the cells' ability to respond to concanavalin A (Spielberg, 1980). We have extended this system by employing human, as well as murine, microsomes in an investigation of the direct and metabolism-dependent cytotoxicity of phenytoin, sorbinil and mianserin. These drugs have been associated with a variety of adverse reactions, some of which may have an immunological aetiology (Grob & Herold, 1972; Jaspan, 1986; Stricker *et al.*, 1985). In addition, we have attempted to define the role of metabolism in the cytotoxicity of the drugs *in vitro* by measurement of both stable and chemically reactive metabolites formed under the conditions of the toxicity assay.

In agreement with the findings of Spielberg *et al.* (1981), phenobarbitone-induced murine microsomes metabolised phenytoin to a derivative which was toxic towards human MNL. Cytotoxicity was enhanced by pre-treatment of the target cells with the epoxide hydrolase inhibitor, TCPO. However, under identical conditions, microsomes from four human livers did not produce a cytotoxic metabolite, even though microsomes from both species only metabolised phenytoin to *p*-HPPH, the major phase I metabolite in mice and humans (Chow & Fischer, 1982; Steiner *et al.*, 1987). It has been suggested that the cytotoxic metabolite derived from phenytoin is the electrophilic 3,4-epoxide, which can either rearrange to *p*-HPPH or become covalently bound to protein (Martz *et al.*, 1977; Spielberg *et al.*, 1981; Claesen *et al.*, 1982; Pantarotto *et al.*, 1982). This hypothesis is based largely on the finding that TCPO enhances the cytotoxicity of phenytoin in the *in vitro* test

Table 3 Metabolism of phenytoin, sorbinil and mianserin to chemically reactive metabolites by human and murine liver microsomes. Each value represents mean $\pm$ s.d. of quadruplicate incubations

<i>Human liver</i>	<i>Irreversible binding (pmol bound mg⁻¹ protein)</i>									
	<i>Phenytoin</i>			<i>Sorbinil</i>			<i>Mianserin</i>			
	-NADPH	+NADPH	+NADPH+TCPO	-NADPH	+NADPH	+NADPH+TCPO	-NADPH	+NADPH	+NADPH+TCPO	+NADPH+TCPO
1	126	153	165	255	651	605	52	786		966
2	241	271	241	257	513	443	56	1080		1142
3	182	232	226	106	305	301	64	992		966
4	194	181	214	237	300	284	56	430		404
Mean										
$\pm$ s.d.	186 $\pm$ 47	209 $\pm$ 53	211 $\pm$ 33	214 $\pm$ 72	442 $\pm$ 171*	408 $\pm$ 149	57 $\pm$ 5	822 $\pm$ 289*		869 $\pm$ 321*
<i>Murine liver</i>										
	-NADPH	+NADPH	+NADPH+TCPO	-NADPH	+NADPH	+NADPH+TCPO	-NADPH	+NADPH	+NADPH+TCPO	+NADPH+TCPO
Mean										
$\pm$ s.d.	367 $\pm$ 73	661 $\pm$ 73**	661 $\pm$ 24**	188 $\pm$ 25	366 $\pm$ 14**	365 $\pm$ 25**	60 $\pm$ 12	1512 $\pm$ 154**		1650 $\pm$ 80**

* Significantly greater than controls (-NADPH), $P < 0.05$ (Student's paired t -test).

** Significantly greater than controls (-NADPH), $P < 0.001$ (Student's non-paired t -test).

Table 4 Metabolism of phenytoin, sorbinil and mianserin to cytotoxic metabolites by human and murine liver microsomes

Human ⁺ liver	Cytotoxicity (% cell death)			
	Phenytoin		Sorbinil	
	+NADPH	+NADPH+TCPO	+NADPH	+NADPH+TCPO
1	3.8	4.2	2.2	2.4
2	4.1	4.3	3.0	2.9
3	4.1	4.7	3.1	2.9
4	4.6	4.3	2.4	2.8
Mean				
± s.d.	4.1 ± 0.3	4.4 ± 0.2	2.7 ± 0.4	2.8 ± 0.2
Murine [#] liver	Cytotoxicity (% cell death)			
	Phenytoin		Sorbinil	
	+NADPH	+NADPH+TCPO	+NADPH	+NADPH+TCPO
1	3.8	4.2	2.2	2.4
2	4.1	4.3	3.0	2.9
3	4.1	4.7	3.1	2.9
4	4.6	4.3	2.4	2.8
Mean				
± s.d.	4.1 ± 0.3	4.4 ± 0.2	2.7 ± 0.4	2.8 ± 0.2

* Significantly greater than controls $P < 0.01$ (Student's non-paired *t*-test).** Significantly greater than controls $P < 0.005$ (Student's paired *t*-test).*** Significantly greater than controls (-NADPH), $P < 0.001$ (Student's non-paired *t*-test).+ Control cytotoxicity values for phenytoin, sorbinil and mianserin were $3.2 \pm 1.6\%$, $3.0 \pm 0.5\%$ and $2.6 \pm 0.4\%$ (mean $\pm$ s.d., $n = 4$), respectively.# Control cytotoxicity values were $4.3 \pm 2.1\%$ for phenytoin (mean $\pm$ s.d., $n = 12$), $2.0 \pm 0.5\%$ for sorbinil ($n = 4$) and $2.3 \pm 0.7\%$ for mianserin ($n = 4$).Each value represents the mean $\pm$ s.d. ($n = 4$) except for phenytoin, where $n = 12$.

system. The data from our study show a clear dissociation between the formation of *p*-HPPH and both MNL toxicity and irreversible binding of the drug to protein. It is therefore possible that phenytoin is metabolised to two (regio-isomeric) epoxides, only one of which is cytotoxic. Such a process is observed in the metabolism of the hepatotoxin bromobenzene, where the 3,4-epoxide is more toxic than the 2,3-epoxide towards hepatocytes (Zampaglione *et al.*, 1973; Horner *et al.*, 1987). An alternative cytotoxic metabolite of phenytoin might be an epoxide formed by further oxidation of *p*-HPPH

(Billings & Fischer, 1985). Whatever the structure of the metabolite, it is apparently toxic at sub-micromolar concentrations since only 0.5% of the incubated drug was metabolised *in vitro*.

Sorbinil, an aldose reductase inhibitor, is structurally related to phenytoin in that it contains hydantoin and aromatic moieties (Figure 1). Its side effects (Jaspan, 1986) include a hypersensitivity reaction similar to that associated with phenytoin (Dhar *et al.*, 1974). The toxicity profile for sorbinil was similar to that observed with phenytoin in the *in vitro* system: the formation of a cytotoxic metabolite was only

Figure 3 Mean values for the formation of stable and chemically reactive metabolites (expressed as % of incubated radioactivity) and cytotoxic metabolites (expressed as % of cell death produced) from sorbinil by human and murine liver microsomes. Columns running from front to back represent controls (-NADPH, where appropriate), incubations with NADPH and incubations including NADPH and TCPO. For estimates of variability see Tables 2, 3 and 4.

Figure 4 Mean values for the formation of stable and chemically reactive metabolites (expressed as % of incubated radioactivity) and cytotoxic metabolites (expressed as % of cell death produced) from mianserin by human and murine liver microsomes. Columns running from front to back represent controls (-NADPH, where appropriate), incubations with NADPH and incubations including NADPH and TCPO. For estimates of variability see Tables 2, 3 and 4.

observed with microsomes from phenobarbitone-induced mice and was significantly enhanced in MNL pre-treated with TCPO. The former observation indicates that toxic metabolites from phenytoin and sorbinil may selectively be produced by a cytochrome P-450 present in liver microsomes from phenobarbitone-induced mice. However, it remains to be established whether this is a species difference or a consequence of enzyme induction. The requirement

for TCPO and the low metabolic turnover of sorbinil (< 3%) suggest that its cytotoxic metabolite is also an epoxide which is effective at very low (sub-micromolar) concentrations. For sorbinil, however, there was no simple relationship between cytotoxicity and the irreversible binding of metabolite(s) to protein.

Mianserin has been associated with blood dyscrasias which may result from direct cytotoxicity (O'Donnell *et al.*, 1985) or have an

immunological basis (Stricker *et al.*, 1985). Unlike phenytoin and sorbinil, it was metabolised to a cytotoxic species by both human and murine microsomes, and was directly toxic to MNL at low concentrations ($\geq 25 \mu\text{M}$). The metabolism-dependent cytotoxicity of mianserin also differed in that it did not depend upon pre-treatment with TCPO; hence it was presumably not mediated by an epoxide, although it is conceivable that a toxic mianserin epoxide(s), in common with certain epoxides (Hammock & Hasagawa, 1983), is a poor substrate for epoxide hydrolases. Human microsomes effected the greater toxicity, and principally demethylated mianserin whereas murine microsomes favoured 8-hydroxylation. This implies a link between the *N*-demethylation and the toxicity of mianserin. In addition, the irreversible binding of the drug to protein was greater with murine microsomes, confirming that there is no simple relationship between the total generation of reactive metabolites and toxicity (Streeter *et al.*, 1984). However, in these experiments, it was not possible to distinguish the irreversible binding of drug to microsomes from the binding to cells, which alone might have been related to the

observed cytotoxicity. While the extent of a drug's irreversible binding to microsomes is a useful index of reactive metabolite formation *in vitro*, it is not a reliable indication of the drug's toxicity *in vivo* (Roberts & Jollow, 1979; Kitteringham *et al.*, 1988).

It is clear from this study that both metabolic activation and metabolic detoxication can play an important role in the cytotoxicity of a drug. An individual's susceptibility to adverse drug reactions may result from a combination of these processes, governed by both genetic and environmental factors. Using this simple test system, the effects of factors such as enzyme induction and/or enzyme inhibition on drug toxicity may be investigated. Furthermore, analysis of stable and protein-reactive metabolites should lead ultimately to the definition of the chemical species responsible for toxicity.

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