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Structural requirements for bioactivation of anticonvulsants to cytotoxic metabolites *in vitro*

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The formation of cytotoxic metabolites from the anticonvulsants phenytoin and carbamazepine was investigated *in vitro* using a hepatic microsomal enzyme system and human mononuclear leucocytes as target cells. Both drugs were metabolised to cytotoxic products. In order to assess the structural requirements for this bioactivation, a series of structurally related compounds was investigated. It was found that molecules which contain either an amide function or an aryl ring may undergo activation *in vitro*, but only the metabolism-dependent toxicity of the latter is potentiated by pre-treatment of the target cells with an epoxide hydrolase inhibitor. Taken collectively, these data are consistent with the concept that reactive epoxide metabolites of both phenytoin and carbamazepine may produce toxicity in individuals with an inherited deficiency in epoxide hydrolase.

Keywords phenytoin carbamazepine bioactivation structural requirements

Introduction

Severe adverse reactions occasionally encountered during phenytoin and carbamazepine therapy, such as hepatic necrosis (Spielberg, 1986) and aplastic anaemia (Gerson *et al.*, 1983), are thought to be mediated by chemically reactive epoxides formed by cytochrome P-450-dependent enzymes. Such epoxides could, in theory, bind covalently to cell macromolecules and cause genetic and cytotoxic damage and, by acting as haptens, lead to secondary immune reactions (Jerina & Daly, 1974; Park *et al.*, 1987). Predisposition to the toxic effects of phenytoin and carbamazepine is presumed to be a consequence of an inherited deficiency in the detoxifying enzyme(s), epoxide hydrolase (Spielberg, 1986; Gerson *et al.*, 1983). We have recently shown that adverse reactions to sorbinil, an aldose reductase inhibitor with a similar structure and toxicity profile to phenytoin (Jaspan, 1986), may have a similar biochemical basis (Riley *et al.*, 1988).

Phenytoin, carbamazepine and sorbinil share several structural components, namely one or more aromatic moieties and an amide function. Oxidation at nitrogen centres by microsomal mixed function oxygenases is known to result in the formation of chemically reactive *N*-hydroxy intermediates (Guengerich & Liebler, 1985). Furthermore, *N*-hydroxylation has been implicated in the formation of cytotoxic metabolites from dapsone (Grossman & Jollow, 1988; Uetrecht *et al.*, 1988) and genotoxic intermediates from various compounds, including 2-acetylaminofluorene (Holme & Søderlund, 1985). It is therefore conceivable that the metabolic activation of phenytoin, sorbinil and carbamazepine may also involve metabolism at the nitrogen atoms of the drugs. In the present study, we have investigated further the activation of phenytoin and carbamazepine to cytotoxic metabolites using an *in vitro* test system in which human mononuclear leucocytes (MNL) are exposed to toxic intermediates generated from drugs by phenobarbitone-induced mouse liver microsomes (Riley *et al.*, 1988; Spielberg, 1986). In addition, we have attempted to assess whether metabolism by cytochrome P-450 enzymes at sites other than their phenyl rings may significantly contribute to

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their adverse effects by comparing their metabolite-mediated toxicity with that of a series of urea analogues, which possess similar chemical groups to these two anticonvulsants.

Methods

Chemicals

5,5-Diphenylhydantoin (phenytoin), 5-(*p*-hydroxyphenyl)-5-phenyl hydantoin (*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, U.K.). Reduced nicotinamide adenine dinucleotide phosphate (NADPH; tetrasodium salt) was obtained from BDH Chemicals Ltd (Poole, Dorset, U.K.). All other chemicals were purchased from Aldrich Chemical Company Ltd (Gillingham, Dorset, U.K.). All solvents used were of h.p.l.c. grade and were products of Fisons plc (Loughborough, Leics., U.K.).

Preparation of murine hepatic microsomes

Male CBA/CA mice (25–30 g) were induced by treatment with phenobarbitone (Riley *et al.*, 1988) and microsomes were prepared from pooled livers as described previously for rat liver microsomes (Maggs *et al.*, 1983). Microsomes were also prepared from a sample of human liver as reported previously (Purba *et al.*, 1987). The microsomes were either used immediately or stored for up to 48 h at −80° C as an intact pellet. Cytochrome P-450 content was measured by the method of Omura & Sato (1964) and microsomal protein was determined by the method of Lowry *et al.* (1951). The cytochrome P-450 content of the induced mouse microsomes and human liver microsomes were 1.48 ± 0.19 nmol mg^{-1} microsomal protein (mean ± s.d.; $n = 4$ batches of pooled microsomes) and 0.67 ± 0.06 nmol mg^{-1} microsomal protein (mean ± s.d.; $n = 4$), respectively.

Isolation of mononuclear leucocytes from human blood

Human mononuclear leucocytes (MNL) were isolated from blood freshly drawn from a single healthy volunteer (in order to eliminate inter-individual variability in detoxication pathways), using a method similar to that described by Boyum (1984) as modified by Riley *et al.* (1988). Their viability, upon isolation, as determined by trypan blue exclusion was > 95%. Isolated cells were kept at room temperature and incubated within 3 h.

Determination of direct and metabolism-dependent cytotoxicity

To determine the effects of phenytoin and carbamazepine on MNL viability in the absence of a metabolising system, the target cells (1×10^6 in HEPES-buffered medium) were incubated with varying concentrations of either drug (0–150 μM) for 2 h at 37° C in 10 ml polystyrene tubes left open to air. The drugs were added in 10 μl h.p.l.c.-grade methanol to give a final concentration of solvent of 1% (v/v). The solvent itself was non-toxic at this dilution and hence incubations consisting of MNL and methanol served as controls. The total incubation volume was 1 ml.

Following the 2 h incubation, the cells were sedimented by centrifugation and resuspended in 1 ml of drug-free medium (HEPES-buffered medium containing 5 mg/ml human serum albumin, fraction V). Incubations were then continued without agitation at 37° C for 16 h and aliquots removed in order to determine cell viability by trypan blue dye exclusion (0.1% trypan blue for *circa* 5 min). Failure to exclude the dye was taken as an index 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.).

Metabolism-dependent toxicity was assessed by incubating MNL and drug (0–150 μM) in the presence of an activating system (0.5 mg microsomal protein and 1 mM NADPH) for 2 h at 37° C. MNL were pre-treated with the epoxide hydrolase inhibitor TCPO (30 μM for 10 min) in some incubations to characterise further the cytotoxic metabolite(s). Cytotoxicity was again assessed by trypan blue exclusion following the 16 h incubation.

In addition to phenytoin and carbamazepine, a number of their derivatives and a series of urea analogues (structurally related to the two anticonvulsants) were investigated. Incubations conducted with these other chemicals were identical to those described earlier for phenytoin and carbamazepine, using an initial concentration of 150 μM (at which the metabolism-dependent toxicity of phenytoin and carbamazepine was clearly discernable). Data shown in Figure 1 and Table 1 are replicates obtained with the same batch of pooled liver microsomes, thereby placing emphasis on the chemical (rather than biochemical) nature of bioactivation.

Statistical analyses were initially performed by one way analysis of variance. Significance

levels were then determined using a non-paired Student's *t*-test.

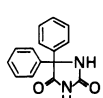
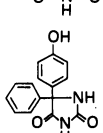
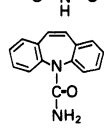
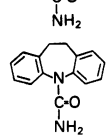
Results and discussion

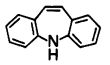
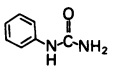
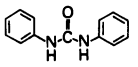
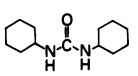
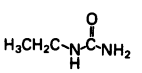
Phenytoin is predominantly metabolised *in vitro* and *in vivo* by cytochrome P-450-dependent enzymes to *p*-hydroxyphenytoin (Chow & Fischer, 1982; Steiner *et al.*, 1987). This biotransformation is thought to proceed via a 3,4-epoxide intermediate. This putative toxic metabolite has been implicated in the manifestation of a variety of adverse reactions to phenytoin, including teratogenicity (Martz *et al.*, 1977), gingival hyperplasia (Wortel *et al.*, 1979), and hepatotoxicity (Spielberg, 1986). The results obtained in the present study support the hypothesis that adverse reactions to phenytoin may arise as a consequence of the generation of chemically reactive epoxides by cytochrome P-450 enzymes. Phenytoin was not cytotoxic *per se* at concentrations up to 150 μM but was metabolised by phenobarbitone-induced mouse liver microsomes to a product which manifested significant death of MNL *in vitro* (Table 1). This toxicity was dependent on the presence of NADPH and the concentration of the drug. In addition, cell death was significantly enhanced by pre-treatment of the target cells with TCPO ($P < 0.001$) (Figure 1). Previous studies have shown that TCPO is not toxic *per se* (Spielberg, 1986; Riley *et al.*, 1988), in the absence or presence of NADPH and hepatic microsomes. Futhermore, *p*-HPPH was also metabolised within this *in vitro* system to a product whose cytotoxicity towards MNL was enhanced by inhibition of epoxide hydrolase within these cells (Table 1). Collectively, these data indicate that phenytoin toxicity is primarily mediated by an epoxide intermediate *in vitro*. In addition, the data obtained with *p*-HPPH support an earlier hypothesis that the cytotoxic epoxide generated during the metabolism of phenytoin by phenobarbitone-induced mouse microsomes may be an intermediate other than that produced during the initial *p*-hydroxylation of the drug *in vitro* (Riley *et al.*, 1988).

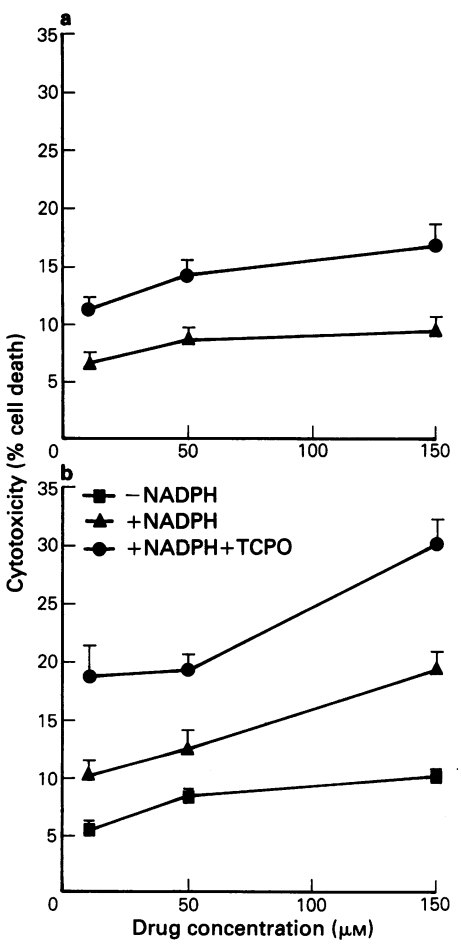
Adverse reactions to carbamazepine (CBZ) are also thought to arise as a consequence of an inherited defect in the detoxication of epoxide intermediates produced during the phase 1 metabolism of the drug (Gerson *et al.*, 1983). Indeed, it has been suggested that predisposition to carbamazepine toxicity may have a similar underlying biochemical basis to that postulated for phenytoin.

Carbamazepine and its derivatives were found to be cytotoxic *per se* ($P < 0.01$ compared with vehicle alone). Carbamazepine exhibited an *in vitro* toxicity profile similar to phenytoin and *p*-HPPH. Phenobarbitone-induced mouse liver microsomes readily activated carbamazepine to a cytotoxic metabolite(s) (Figure 1). As for phenytoin, this toxicity was concentration-

Table 1 Activation of compounds studied to cytotoxic products by phenobarbitone-induced mouse liver microsomes. Values are mean ± s.d. for quadruplicate determinations. Significant differences from controls (−NADPH) are: $*P < 0.01$, $**P < 0.001$ (non-paired Student's *t*-test). Background cytotoxicity (for cells incubated with vehicle alone) was 3.5 ± 0.3% (mean ± s.d. for 4 experiments).

Compound		*Concentration (μM)*	*Cytotoxicity (% cell death)* −NADPH	+NADPH	+NADPH + TCPO
Phenytoin		150	3.3 ± 0.3	9.4 ± 1.1**	16.8 ± 1.9**
p-HPPH		150	3.6 ± 0.8	6.1 ± 0.3**	14.9 ± 2.8**
CBZ		150	10.3 ± 0.6	19.6 ± 1.3**	30.4 ± 2.2**
DihydroCBZ		150	10.4 ± 0.3	18.8 ± 0.9**	27.3 ± 1.6**

Iminostilbene		150	16.4 ± 3.2	27.5 ± 4.6*	39.9 ± 2.4**
Phenylurea		150	3.9 ± 0.9	5.6 ± 1.2	11.4 ± 1.8**
Diphenylurea		10	5.3 ± 0.5	27.8 ± 2.3**	33.2 ± 1.6**
Dicyclohexylurea		10	5.1 ± 0.9	17.0 ± 1.5**	17.7 ± 1.7**
Ethylurea		150	3.3 ± 0.5	3.9 ± 0.5	4.7 ± 1.3



dependent and significantly potentiated by pre-treatment of the target cells with TCPO ($P < 0.001$), suggesting that an epoxide may be responsible for the cytotoxicity (Table 1). Multiple epoxide intermediates have been implicated as products of carbamazepine metabolism, although only the relatively stable 10,11-epoxide has been isolated (Lertratanangkoon & Horning, 1982). Therefore, one possibility is that carbamazepine is metabolised *in vitro* to the stable 10,11-epoxide, which has anticonvulsant properties similar to the parent drug in experimental animals and the formation of which is inducible by carbamazepine and phenobarbitone (Tybring *et al.*, 1981). This stable epoxide may then translocate to the target cells and produce cytotoxicity, which is exacerbated by TCPO. However, the data obtained with dihydrocarbamazepine (Table 1) shows that the cytotoxic epoxide arises via epoxidation at an alternative position in the tricyclic nucleus. Iminostilbene (a metabolite of carbamazepine; Csetenyi *et al.*, 1973) was metabolised *in vitro* to products which exhibited a similar pattern of toxicity to carbamazepine and dihydrocarbamazepine (Table 1). This finding suggests that activation at the terminal amino group in the side chain does not significantly contribute to the

Figure 1 The concentration-dependent activation of a) diphenylhydantoin (phenytoin) and b) carbamazepine to cytotoxic epoxides by phenobarbitone-induced mouse liver microsomes. Values represent mean ± s.d. for quadruplicate determinations. (Phenytoin was not toxic *per se* up to 150 μM). Cell death observed in control incubations (1×10^6 MNL and vehicle) was 3.5 ± 0.3% (mean ± s.d. for 4 experiments).

toxicity of the drug *in vitro* and that the amide side chain is not necessary for interaction with the active site of cytochrome P-450.

In order to assess further the relative contributions of the common structural features of phenytoin and carbamazepine to their toxicity, a number of chemically related compounds (urea analogues) were investigated (Table 1). Only phenylurea appeared to be metabolised by induced mouse microsomes to a cytotoxic epoxide. Indeed, the metabolism-dependent toxicity of this compound was entirely dependent on pre-treatment of MNL with TCPO. Diphenylurea and dicyclohexylurea were readily activated to a cytotoxic product. At 150 μM, cell death was too extensive (> 95%) to quantify accurately the metabolism-dependent cytotoxicity of these compounds (unpublished data). Therefore, the toxicity profiles of diphenylurea and dicyclohexylurea and dicyclohexylurea were investigated at a lower concentration (10 μM). Both compounds were activated to products which caused death of MNL in the presence of an induced mouse microsomal enzyme system (Table 1). The cytotoxicity of these compounds was unaffected by TCPO, suggesting that metabolic activation at the urea function (possibly *N*-hydroxylation) can occur *in vitro*. This was most obvious with *N*-dicyclohexylurea, which does not possess an aryl group that can form an epoxide. Using a similar test system in which human liver microsomes are employed (Riley *et al.*, 1988), preliminary data suggest that diphenylurea is also activated to a cytotoxic metabolite by human hepatic microsomal enzymes (% cell death = 5.6 ± 1.8 without NADPH and 15.7 ± 2.1 with NADPH; mean ± s.d. for quadruplicate determinations with a single human liver). However, phenylurea, *p*-HPPH and phenytoin (Riley *et al.*, 1988) were not activated within this system to cytotoxic products, which suggests that these compounds may undergo different patterns of activation and detoxication *in vitro*. Ethylurea was not activated to a cytotoxic product by phenobarbitone-induced mouse microsomes. This may be a consequence of the relatively low lipophilicity of this compound, which may limit its access to microsomal enzymes and/or the access of any potentially toxic products to critical target macromolecules within the MNL.

In conclusion, it would appear that chemicals possessing aryl and/or amide groups may be activated to cytotoxic metabolites *in vitro*. For phenytoin and carbamazepine, metabolic activation to epoxide intermediates appears to be primarily responsible for their toxicity. Hence, our data support earlier suggestions that, although alternative routes of activation of these compounds may occur (e.g. *N*-chlorination of phenytoin by activated polymorphonuclear cells; Uetrecht & Zahid, 1988), predisposition to phenytoin and carbamazepine toxicity is principally governed by the balance between the metabolic activation and detoxication of reactive epoxides (Gerson *et al.*, 1983).

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