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Effect of cimetidine on the metabolism of coumarin by rat, gerbil and human liver microsomes

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Coumarin (2*H*-1-benzopyran-2-one), in combination with the H_2-receptor antagonist cimetidine, is currently undergoing clinical trials for the treatment of various malignancies [1–4]. Both of these drugs have been shown to have immunomodulatory effects which may mediate the objective tumour regressions observed in some patients. Coumarin is a substrate for the cytochrome P450-dependent monooxygenase system, and can be hydroxylated at all six possible ring positions [5]. There are marked species differences in coumarin metabolism and hepatotoxicity. In man 7-hydroxylation is the major route of metabolism [6], but this pathway is negligible in the rat (in which coumarin is markedly toxic) which primarily metabolizes coumarin via an initial 3-hydroxylation reaction [5]. We have previously suggested that, due to its high coumarin 7-hydroxylase activity, the Mongolian gerbil may be a more appropriate species than the rat as a model for man with respect to coumarin metabolism and toxicity [7]. Inhibition of the hepatic microsomal metabolism of certain drugs by cimetidine, attributed to its binding to cytochrome P450, has been well-documented [8–11]. It is possible that cimetidine could inhibit coumarin metabolism *in vivo*, and therefore co-administration of these two drugs to cancer patients may potentially affect the efficacy and/or toxicity of coumarin.

In this study the interaction of cimetidine with coumarin was examined *in vitro*. We have investigated the effect of cimetidine on the metabolism of coumarin by liver microsomes prepared from untreated and phenobarbitone-treated rats and gerbils, and from four human liver samples.

Materials and Methods

Coumarin, 7-hydroxycoumarin (7-HC*), cofactors and enzymes were obtained from Sigma Chemical Co. (Poole, U.K.), sodium phenobarbitone (PB) was from BDH Chemicals (Poole, U.K.) and 3-hydroxycoumarin (3-HC) was purchased from APIN Chemicals Ltd (Oxon, U.K.). 5-Hydroxycoumarin (5-HC) was synthesized by the method of Kerékjártó [12] and purified by preparative TLC. 6-Hydroxycoumarin (6-HC) and 8-hydroxycoumarin (8-HC) were synthesized by modifications of the Knoevenagel condensation procedure reported by Murayama *et al.* [13]. Cimetidine (originally from Smith, Kline and French, Herts, U.K.) was a gift from Mr C. Richmond (Department of Therapeutics, Queen's Medical Centre, Nottingham, U.K.).

Adult male Wistar rats (150 g) and Mongolian gerbils (*Meriones unguiculatus*; 50 g) were obtained from the University of Nottingham Medical School Animal Unit. They had access to standard laboratory diet and tap water at all times. PB was administered as a 0.1% (w/v) solution in drinking water for 7 days (rats), or injected i.p. (80 mg/kg, in isotonic saline) once daily for 3 days (gerbils). Control animals were untreated. The effectiveness of the PB induction procedure with respect to cytochrome P450 contents and a range of monooxygenase activities of rat and gerbil liver microsomes has previously been reported [14].

Microsomal fractions from three human liver samples (with normal histology) obtained from renal transplant donors were generously provided by A. R. Boobis and B. P. Murray (Department of Clinical Pharmacology, Royal Postgraduate Medical School, London, U.K.). A fourth liver sample was obtained from a patient undergoing lobectomy. Liver microsomes were prepared from this liver, and from pooled livers of six rats or ten gerbils, by the calcium aggregation technique [15]. They were stored at −80° until required. Protein content was measured by the method of Lowry *et al.* [16].

The microsomal incubation mixture used has been described previously [14]. Briefly, it contained, in a total volume of 1 mL, phosphate buffer, pH 7.4 (100 mM), $MgSO_4$ (5 mM), glucose 6-phosphate (5 mM), NADP (0.5 mM), glucose 6-phosphate dehydrogenase (1 unit) and microsomal suspension (approx. 1 mg protein). Cimetidine (0.1–1.0 mM final concentration) was added in 5 μL methanol. The reaction was started by the addition of coumarin (1.0 mM final concentration, in 5 μL methanol) and samples incubated for 10 min at 37°. Reactions were terminated with 0.5 mL 25% (w/v) trichloroacetic acid. Coumarin metabolites present in the deproteinized supernatant were analysed directly by a reversed-phase HPLC assay developed for the separation of coumarin and 12 of its reported metabolites [14], based on the method of Vande Casteele *et al.* [17]. Hydroxycoumarins were quantified by comparison of peak heights with those of standards prepared in aqueous methanol. The presence of cimetidine in the incubations did not interfere with the quantification of the coumarin metabolites.

Statistical analysis was undertaken using paired (human liver microsomes) or unpaired Student's *t*-tests as appropriate.

Results and Discussion

The effect of cimetidine on the metabolism of coumarin by rat, gerbil and human liver microsomes is shown in Table 1. The effects were concentration-dependent over the range investigated (0.1–1.0 mM cimetidine); only the values obtained using a cimetidine concentration of 1 mM are given.

In liver microsomes from control and PB-treated rats 3-HC was the only characterized coumarin metabolite identified; cimetidine inhibited its formation by 60–70%. A second, as yet unidentified, metabolite [18] was also detected (*X*). The production of *X* was inhibited by 35–50% by cimetidine, as determined using the HPLC peak height data.

Coumarin metabolism by gerbil liver microsomes was extensive, with a range of hydroxy metabolites and small amounts of *X* detected. Treatment of gerbils with PB altered the relative amounts of the metabolites formed. The production of 3-HC, 5-HC, 8-HC and *X* was reduced, whereas that of 6-HC and, in particular, 7-HC was increased compared with that for microsomes from control gerbils. Coumarin 7-hydroxylation is also induced by PB in mouse liver [19]. Cimetidine inhibited 3-hydroxylation and the formation of *X* to similar extents (55–70%) in liver microsomes from both control and PB-treated gerbils. In microsomes prepared from control gerbils 7-hydroxylation of coumarin was inhibited by 70% by cimetidine. However,

* Abbreviations: HC, hydroxycoumarin; HPLC, high-performance liquid chromatography; PB, phenobarbitone; TLC, thin layer chromatography.

Table 1. Effect of cimetidine on the hepatic microsomal metabolism of coumarin

Microsomes	Cimetidine (mM)	Metabolites (nmol/mg protein/10 min) 3-HC	5-HC	6-HC	7-HC	8-HC	*X*
Rat							
Control	0	2.0 ± 0.1	ND	ND	ND	ND	
	1.0	0.6 ± 0.0‡	ND	ND	ND	ND	
		(30)					(65)
Phenobarbitone	0	3.3 ± 0.1	ND	ND	ND	ND	
	1.0	1.3 ± 0.1‡	ND	ND	ND	ND	
		(39)					(50)
Gerbil							
Control	0	8.5 ± 0.6	7.4 ± 0.3	2.9 ± 0.1	9.1 ± 0.6	13.8 ± 0.6	
	1.0	2.6 ± 0.2‡	7.3 ± 0.5	2.2 ± 0.3	2.7 ± 0.5†	12.8 ± 1.0	
		(31)	(99)	(76)	(30)	(93)	(45)
Phenobarbitone	0	3.4 ± 0.1	1.1 ± 0.0	4.5 ± 0.1	18.5 ± 0.5	4.8 ± 0.1	
	1.0	1.4 ± 0.1‡	1.6 ± 0.0‡	4.4 ± 0.1	24.0 ± 0.5†	7.3 ± 0.1‡	
		(41)	(145)	(98)	(130)	(152)	(38)
Human	0	0.8 ± 0.3	ND	ND	2.3 ± 0.9	ND	
	1.0	0.5 ± 0.2†	ND	ND	2.8 ± 0.9*	ND	
		(63)			(122)		(63)

Values are means ± SEM for three separate experiments using pooled liver microsomes from rats and gerbils, and for microsomes from four human liver samples. Figures in parentheses are the amounts of metabolites formed in the presence of cimetidine as percentages of those obtained for coumarin alone. For *X*, an unidentified metabolite, percentages have been calculated using the HPLC peak height data.

Where indicated, values obtained with cimetidine present are significantly different from those for coumarin alone at * $P < 0.05$, † $P < 0.01$ and ‡ $P < 0.001$.

HC, hydroxycoumarin; ND, not detected.

cimetidine did not inhibit the formation of 7-HC in liver microsomes from PB-treated gerbils suggesting the involvement of different cytochrome P450 isozymes in the coumarin 7-hydroxylase activities of untreated and PB-treated gerbils. No significant inhibition of 5-, 6- or 8-hydroxylation was observed, with cimetidine actually causing an increase in the production of 5-HC and 8-HC (and 7-HC) by hepatic microsomes from PB-treated gerbils.

7-HC was the major coumarin metabolite formed by human liver microsomes; small amounts of 3-HC and *X* were also produced. With microsomes from one of the human liver samples trace amounts of 5-HC, 6-HC and 8-HC were detected which were not affected by cimetidine. Cimetidine inhibited both 3-hydroxylation and the formation of *X* by about 40% but had a slight stimulatory effect on the 7-hydroxylation of coumarin.

Cimetidine has been shown to inhibit various rat hepatic microsomal monooxygenase activities [8]. It also binds strongly to human hepatic microsomal cytochrome P450 [9], inhibiting several monooxygenase activities. Puurunen *et al.* [20] reported that coumarin 7-hydroxylase activities of homogenates prepared from two human liver biopsy samples were inhibited by about 30% by 10 mM cimetidine. The metabolism of cimetidine in the gerbil has not been investigated but it is likely to be metabolized in part by cytochrome P450-dependent enzymes, as has been demonstrated for other species [8].

In this study we have shown that cimetidine significantly affects the metabolism of coumarin by rat, gerbil and human liver microsomes. It inhibited the 3-hydroxylation of coumarin, the pathway postulated to be responsible for coumarin-induced hepatotoxicity in the rat [21], in liver microsomes prepared from all three species. Inhibition of 7-hydroxylation, the major route of coumarin metabolism in man, was only observed with microsomes from control gerbils. Coumarin 7-hydroxylase activity was not inhibited in human liver microsomes or in microsomes from PB-treated gerbils. On this basis, the PB-treated gerbil would be a better system to model coumarin metabolism in man than would the untreated gerbil. It is likely that differences in the P450 isozyme pattern explain the differences between species, route of metabolism and treatment. Coumarin has been postulated to be a pro-drug with the 7-hydroxy derivative being the pharmacologically-active agent (M. E. Marshall, personal communication). If this is the case then cimetidine should not affect the efficacy of coumarin upon joint therapy with these two drugs. Furthermore, cimetidine, via its effects on coumarin metabolism, is unlikely to potentiate coumarin-induced hepatotoxicity if this, as has been suggested, is due to the production of a reactive intermediate during 3-hydroxylation; cimetidine may in fact protect against toxicity by inhibiting coumarin 3-hydroxylase activity. It is accepted that these conclusions are derived from *in vitro* studies. Nevertheless, good correlations between *in vitro* and *in vivo* inhibition of cytochrome P450 activity (and associated drug–drug interactions *in vivo*) have been reported for a variety of drugs [22, 23]. In conclusion, the *in vitro* hepatic microsomal data suggest that pharmacokinetic interactions between cimetidine and coumarin are unlikely to be of major clinical significance.

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