Human and mouse liver coumarin 7-hydroxylases do not metabolize warfarin *in vitro*

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- 1 The contribution of human P450 2A6 and mouse P450 2a-5 isoenzymes, both highly active in coumarin 7-hydroxylation, to the metabolism of warfarin was studied in several *in vitro* systems with human and mouse liver preparations.
- 2 The reconstituted P450 2a-5 purified from DBA/2 mouse liver did not metabolize warfarin.
- 3 An anti-P450 2a-5 antibody did not consistently inhibit any of the warfarin biotransformation reactions catalyzed by human or mouse liver microsomes, although coumarin 7-hydroxylation was inhibited by over 90%.
- 4 In some human microsomal samples, 4- and 8-hydroxylations of warfarin were inhibited to some extent by the anti-P450 2a-5 antibody.
- 5 Warfarin (<1 mM) did not inhibit coumarin 7-hydroxylation by human or mouse liver microsomes *in vitro*.
- 6 We conclude that mouse and human coumarin 7-hydroxylases do not oxidise warfarin.

Keywords warfarin coumarin hydroxylation P450 2A6 P450 2a-5 drug metabolism

Introduction

Warfarin, a coumarin-type anticoagulant, is extensively oxidized in human and rodent liver microsomes, principally by P450-mediated reactions, to various hydroxymetabolites, and to several further oxidized products (Figure 1; Sutcliff *et al.*, 1987). Human and mouse liver contain a specific P450 isoenzyme which oxidizes/metabolizes coumarin and 7-alkoxycoumarins at the 7-position (Juvonen *et al.*, 1987, 1990; Pelkonen *et al.*, 1985; Raunio *et al.*, 1988). It is possible that this P450 form* also metabolizes coumarin anticoagulants, because they possess a free 7-position (Miles *et al.*, 1990; Yamano *et al.*, 1990). In the present study the purified P450 2a-5 isoenzyme (Juvonen *et al.*, 1988) and a specific antibody (Lang *et al.*, 1989) were used to study the role of mouse P450 2a-5 and human P450 2A6 in warfarin metabolism directly.

Methods

Human liver specimens

Liver samples ($n = 7$) were obtained from two female and five male (mean age 59 years, range 40–72) patients undergoing surgery for either primary or metastatic liver tumors. Only tumour-free tissue was used. The samples were stored as microsomal suspensions at -80°C for 3 to 12 months. The use of surplus liver tissue was approved by the Ethics Committee of the Medical Faculty, University of Oulu.

Mouse liver samples

Male, 8–10 weeks old DBA/2N//Kuo mice were obtained from the National Laboratory Animal Centre in Kuopio, Finland. The animals were maintained as described by

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*According to latest recommendations (Nebert *et al.*, 1991) the murine P450Coh (Juvonen *et al.*, 1988) and the corresponding human P450 isoenzyme (Miles *et al.*, 1990; Yamano *et al.*, 1990) have been assigned as the products of the genes Cyp2a-5 and CYP2A6 (previously 2A3), respectively.

Figure 1 Structures of coumarin and warfarin and the principal sites of P450 catalyzed hydroxylations (arrows).

Honkakoski & Lang (1989) and were given intraperitoneal injections of either pyrazole (200 mg kg⁻¹ day⁻¹) or 0.9% w/v NaCl vehicle (0.5 ml/20 g) for 3 days.

Reconstitution experiments

Electrophoretically homogeneous P450 2a-5 was prepared from pyrazole-treated DBA/2N mouse livers, and NADPH-cytochrome P450 reductase was prepared from phenobarbitone-treated rat livers, as described by Juvonen *et al.* (1988). The monooxygenase complex was reconstituted by mixing 50 pmol P450 2a-5, 300 pmol reductase and 20 nmol dilauroylphosphatidylcholine. After a 10 min preincubation, 50 mM Tris-HCl buffer pH 7.4, containing 5 mM MgCl₂ and 0.8 mM warfarin (racemic, R- and S-warfarin were studied separately in each experimental system; Kaminsky *et al.*, 1984) were added. The reaction was started with 2 mM NADPH. The final concentrations were in a volume of 0.5 ml. The mixture was incubated for 30 min at 37°C and the reaction was stopped by boiling for 5 min. The deproteinized mixture was stored at -80°C until assay of the metabolites. Coumarin 7-hydroxylase activity (100 µM substrate) was measured as described previously (Aitio, 1978; Honkakoski & Lang, 1989).

Microsomal incubations

Microsomes from human and mouse liver (pooled from 3–10 animals) were prepared by standard differential centrifugation (Raunio *et al.*, 1988, 1990). The incubation mixture was similar to that used in the reconstitution system, except that 0.5–1.0 mg of microsomal protein from control or pyrazole-pretreated mouse liver or from human liver replaced the reconstituted monooxygenase complex. Microsomal protein was measured according to Bradford (1976).

Antibody and substrate inhibition

Polyclonal antibodies were raised against the purified P450 2a-5 as described by Lang *et al.* (1989). The purified IgG efficiently inhibits only P450 2a-5 and P450 2a-4 mediated activities in mouse liver (Honkakoski & Lang, 1989; Negishi *et al.*, 1989). To the warfarin or coumarin hydroxylation incubation mixtures described above, 0.45–0.9 mg of the anti-P450 2a-5 or the pre-immune IgG was added 10 min before starting the reaction. This was sufficient to inhibit >90% of coumarin hydroxylase activity in human and mouse livers (Raunio *et al.*, 1988).

10–1000 µM R- or S-warfarin was added to the coumarin 7-hydroxylation mixtures described above 5 min prior to the start of the reaction. Control incubations contained an equal volume of diluent.

Measurement of warfarin metabolites

The warfarin metabolites were extracted and concentrated by Sep-Pak C18 chromatography and measured by h.p.l.c. as described by Fasco *et al.* (1979) using a Spherisorb C8 column (15 cm × 4.6 mm) with a particle size of 5 µm. An external standardization method was used to calculate the concentrations of 4'-, 6-, 8- and 7-hydroxywarfarin (gifts from Dr Eino Hietanen, I.A.R.C., Lyon, France). A linear gradient elution was used. Solvent A was 1.5% v/v acetic acid with pH adjusted to 4.85 with ammonium hydroxide and solvent B was prepared by diluting solvent A with an equal volume of acetonitrile. The flow rate was 1.0 ml min⁻¹ and the proportion of solvent B was 20% at the beginning and 90% at the end of the run. The eluent was monitored at 313 nm and the retention times for 4'-, 6-, 8-, and 7-hydroxyderivatives and the parent compound were 18.3, 19.0, 19.8, 20.3 and 23.0 min, respectively.

Results

Warfarin and coumarin metabolism by human and mouse liver microsomes

The warfarin metabolites produced by mouse and human liver samples are indicated in the chromatograms in Figure 2. The major position of hydroxylation catalyzed by human liver microsomes was at carbon atom 6, whereas hydroxylations at positions 4', 8, and 7 proceeded at a slower rate, confirming the findings of Rettie *et al.* (1989). Similar regioselectivity was observed with mouse liver microsomes. Other metabolites, which could not be assayed because of the lack of reference compounds, were detectable in the chromatograms (Figure 2). Correlations between the extents of production of hydroxywarfarin metabolites and coumarin 7-hydroxylase activity in human liver microsomes were weak and statistically insignificant (0.14 > *r* > -0.44; Table 1).

Pyrazole pretreatment of mice decreased warfarin hydroxylations, as compared with control animals, despite a 6-fold increase in coumarin 7-hydroxylase activity (Table 2).

Figure 2 Chromatograms (h.p.l.c.) of warfarin metabolites produced in the *in vitro* incubations with purified, reconstituted mouse liver P450 2a-5 (a), reconstitution blank without P450 (b), human liver microsomes (c) and DBA/2 control mouse liver microsomes (d). The absorbance scales are identical in all four panels.

The retention times of 4-hydroxy, 6-hydroxy, 8-hydroxy, 7-hydroxy and the parent warfarin were 18.25, 18.98, 19.78, and 23.04 min, respectively in panel c (corresponding peaks in d were slightly retarded). The peaks eluting between 13.65 and 16.28 probably correspond to dehydrowarfarin and warfarin alcohol.

Effect of the anti-P450Coh antibody

In two human liver samples, 4'-hydroxylation and in two others, 8-hydroxylation, were inhibited by more than 30% in the presence of the anti-P450 2a-5 antibody (Table 1). 8-hydroxylation was inhibited in two other samples, but the extent of inhibition could not be determined. Metabolite formation was apparently activated in the other samples. 7-hydroxylation of warfarin was not inhibited in any of the liver microsomes, while coumarin 7-hydroxylase activity was almost totally inhibited in all cases. In mouse liver microsomes, none of the hydroxylation pathways of warfarin were significantly affected by the antibody (Table 2). Also, the unmeasured metabolites were not affected (data not shown).

Metabolism of warfarin by purified, reconstituted mouse liver P450Coh

No warfarin metabolites, either hydroxy products or other, non-quantifiable metabolites, were produced by the purified P450 2a-5 (Figure 1). The detection limit of the assay for different metabolites is around 5 ng

Table 1 The effect of anti-P450 2a-5 antibody on the formation of hydroxylated warfarin metabolites and coumarin 7-hydroxylation (COH) by human liver microsomes

Liver	Specific activity (pmol min ⁻¹ mg protein ⁻¹) Hydroxylational position				
	4'-OH	6-OH	8-OH	7-OH	COH
1	3.29 (191) ¹	52.4 (135)	15.4 (136)	11.3 (122)	390 (2)
2	4.46 (71)	51.7 (119)	18.3 (36)	12.1 (89)	370 (2)
4	4.63 (173)	16.9 (173)	9.74 (63)	5.56 (144)	890 (9)
5	4.01 (71)	16.1 (129)	16.1 nd ²	3.37 (125)	370 (0)
6	4.84 (97)	23.7 (149)	19.1 (103)	7.20 (124)	730 (1)
7	8.37 (158)	82.9 (124)	20.6 (135)	24.4 (129)	520 (1)
8	5.25 (120)	13.7 (112)	9.91 nd	7.34 (106)	450 (1)

¹indicates the inhibition (% of control activity) in the presence of the anti-P450 2a-5 antibody at saturating concentrations (1:1 antibody/microsomal protein ratio).

²not determinable because of the low amount of the 8-hydroxy metabolite formed and of the presence of the interfering 7-hydroxywarfarin peak.

Table 2 The effect of anti-P450 2a-5 antibody on the formation of hydroxylated warfarin metabolites and coumarin 7-hydroxylation (COH) by mouse liver microsomes from control and pyrazole-treated animals

Liver	Specific activity (pmol min ⁻¹ mg protein ⁻¹) Hydroxylational position				
	4'-OH	6-OH	8-OH	7-OH	COH
Control	20.6 (91) ¹	37.6 (100)	54.6 (103)	47.8 (97)	229 (1)
Pyrazole	12.8 (100)	6.40 (100)	15.4 (83)	12.8 (100)	1323 (1)

Values are means of determinations on microsomal preparations from 2 to 3 pools of livers from 3 to 6 female DBA/2 mice.

¹indicates the inhibition (% of control activity) in the presence of the anti-P450 2a-5 antibody at saturating concentrations (1:1 antibody/microsomal protein ratio).

hydroxywarfarin. Thus, the turnover number of P450 2a-5 for warfarin metabolite production would be less than 0.01 nmol min⁻¹ nmol⁻¹ P450, which is more than three orders of magnitude lower than the turnover number for coumarin 7-hydroxylation (20 nmol min⁻¹ nmol⁻¹ P450).

Inhibition by warfarin of mouse and human coumarin 7-hydroxylase

Warfarin concentrations of 10–1000 µM did not inhibit significantly the reconstituted or microsomal mouse or human coumarin 7-hydroxylase activity (data not shown). This suggests that warfarin does not compete with coumarin for the same binding or catalytic site on P450 2a-5.

Discussion

Despite the suggestions of Miles *et al.* (1990) and Yamano *et al.* (1990), warfarin does not seem to be metabolized by coumarin 7-hydroxylase in either human or mouse liver microsomes. Firstly, the anti-P450 2a-5 antibody did not consistently inhibit any of the four warfarin hydroxylations studied in mouse liver microsomes (and in most human livers), nor did it inhibit the formation of the other detectable metabolite. Secondly, warfarin is not metabolized by reconstituted P450 2a-5. Thirdly, warfarin does not inhibit either reconstituted or microsomal coumarin 7-hydroxylase activity. It is also relevant that other coumarin anticoagulants, dicoumarol and phenprocoumon, have not been found to inhibit microsomal coumarin 7-hydroxylase activity (Mäenpää *et al.*, unpublished work). Finally, warfarin hydroxylation was correlated poorly with coumarin 7-hydroxylase activity.

The anti-P450 2a-5 antibody inhibited warfarin 4- and 8-hydroxylations to a small extent in some human liver preparations, but caused an apparent activation or no

change in most samples. Whether there is a human P450 isoenzyme recognized by the antibody which catalyzes warfarin hydroxylations, is unclear.

The present findings provide mechanistic explanations of some earlier, indirect observations. Thus, coumarin 7-hydroxylase activity does not protect mice from warfarin toxicity (Lush & Arnold, 1975), apparently because enzymes other than P450 2a-5 eliminate warfarin. Furthermore, coumarin 7-hydroxylase induction by cobalt does not affect warfarin metabolism (Kling & Legrum, 1981). Cobalt, although inducing P450 2a-5, does not induce those enzymes which are responsible for warfarin oxidation. Finally, Kling *et al.* (1985) demonstrated that warfarin pretreatment of mice caused a P450 induction pattern similar to that produced by phenobarbitone, but different from that caused by cobalt.

Financial support was received from the Academy of Finland, Medical Research Council (contract no. 1051029). This study was performed within the framework of the Action COST B1.

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(Received 19 March 1991,
accepted 21 October 1991)