Biochemical Pharmacology, Vol. 45, No. 5, pp. 1035–1042, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

DIFFERENTIAL INHIBITION OF COUMARIN 7-HYDROXYLASE ACTIVITY IN MOUSE AND HUMAN LIVER MICROSOMES

JUKKA MÄENPÄÄ,*† HOLGER SIGUSCH,*‡ HANNU RAUNIO,* TUULA SYNGELMÄ,* PIA VUORELA,§ HEIKKI VUORELA§ and OLAVI PELKONEN*

*Department of Pharmacology and Toxicology, University of Oulu, SF-90220 Oulu; and §Department of Pharmacy, Pharmacognosy Division, University of Helsinki, SF-00170 Helsinki, Finland

(*Received* 7 *July* 1992; *accepted* 8 *December* 1992)

Abstract—Coumarin is 7-hydroxylated by the P450 isoform Cyp2a-5 in mice and CYP2A6 in humans. Various drugs, endogenous substances, plant substances and carcinogens, altogether about 90 chemicals, were evaluated as possible inhibitors of coumarin 7-hydroxylase (COH) activity in mouse microsomes. The effects of selected compounds on COH activity in human liver microsomes were also tested. The furanocoumarin derivatives methoxsalen (8-methoxypsoralen) and psoralen proved to be the most potent inhibitors of mouse COH activity (IC_{50} values 1.0 and 3.1 μM, respectively). The furanocoumarins bergapten (5-methoxypsoralen), isopimpinellin (5,8-dimethoxypsoralen), imperatorin and sphondin also effectively inhibited mouse COH activity (IC_{50} values 19–40 μM). Methoxsalen, isopimpinellin and metyrapone were also inhibitors in mice *in vivo*. Methoxsalen was a potent inhibitor of COH activity also in human liver microsomes (IC_{50} value 5.4 μM), whereas bergapten, isopimpinellin and imperatorin had no effect. The imidazole antimycotic miconazole was a potent but non-specific inhibitor of COH activity. Several known substrates and inhibitors of members in the CYP1A, CYP2B, CYP2C, CYP2D and CYP3A subfamilies were poor inhibitors of COH activity. These results suggest that (i) the coumarin-type compounds in particular interact with the active sites of Cyp2a-5 and CYP2A6, and (ii) the active sites of Cyp2a-5 and CYP2A6 are structurally different, since a number of compounds inhibited mouse, but not human COH activity.

Cytochrome P450 isoforms catalyse the oxidation of numerous endogenous and exogenous compounds [1]. The P450 gene family consists of many subfamilies [2] with various members having different substrate preferences. Coumarin is a naturally occurring substance in many plants and it has been widely used as a sweetener, fixative, stabilizer and food additive [3]. The metabolism of coumarin has been found to display a large interspecies variation [3–5]. Coumarin 7-hydroxylation is catalysed by the products of the murine *Cyp2a-5* and human *CYP2A6* genes. The activity of the Cyp2a-5‖ isoform is inducible by phenobarbital and pyrazole [6, 7]. The primary structures of the Cyp2a-5 and CYP2A6 proteins are remarkably similar (83% amino acid similarity) [8, 9], and they also share immunological properties [10].

Although it is well established that Cyp2a-5 and CYP2A6 catalyse coumarin 7-hydroxylation it is not obvious as to whether this is their principal biological role. The purpose of this study was to determine which types of compound affect the Cyp2a-5 and CYP2A6-mediated coumarin 7-hydroxylation reaction. By screening about 90 different compounds, including several coumarin derivatives, clinical drugs, carcinogens and endogenous agents, we were able to identify (i) potential coumarin-type substrates of these isoforms and (ii) differences in the active sites of Cyp2a-5 and CYP2A6. To determine the isoform specificity [11] of the Cyp2a-5 inhibitors, they were also tested for their effect on testosterone hydroxylation patterns.

MATERIALS AND METHODS

Chemicals. All chemicals were of the highest purity grade. Testosterone was from Merck (Darmstadt, Germany), [4-^{14}C]testosterone (57 mCi/mmol) from Amersham (Amersham, U.K.) and pyrazole from Fluka Chemical AG (Buchs, Switzerland). Angelicin, apterin, bergapten (5-methoxypsoralen), columbianadin, methoxsalen (8-methoxypsoralen or xanthotoxin), imperatorin, isobergapten, isopimpinellin (5,8-dimethoxypsoralen), ostruthol, pimpinellin and sphondin were isolated or obtained as reported elsewhere [12]. Other drugs tested were obtained from the following sources: aminoglutethimide,

† Corresponding author. Tel. (358) 81 537 5252; FAX (358) 81 330 687.

‡ Permanent address: Department of Clinical Pharmacology, University of Jena, O-6900 Jena, Germany.

‖ Abbreviations: COH, coumarin 7-hydroxylase; 7OHC, 7-hydroxycoumarin; XαOH and XβOH, testosterone hydroxylase activities with X denoting the carbon atom to be oxidized and α/β the stereospecificity of the reaction; Cyp2a-5, mouse P450 isoform catalysing coumarin 7-hydroxylation; CYP2A6, human P450 isoform catalysing coumarin 7-hydroxylation; DEN, diethylnitrosamine.

carbamazepine and glutethimide from Ciba Geigy (Basel, Switzerland); mephenytoin, isradipine and cyclosporin A from Sandoz (Basel, Switzerland); estradiol from Organon (Oss, Holland); debrisoquine from Hoffmann La Roche (Basel, Switzerland); diltiazem hydrochloride and furosemide from Farmos-Group Ltd (Turku, Finland); glibenclamide, temazepam and verapamil from Orion Pharmaceuticals Inc. (Espoo, Finland); nifedipine from Bayer AG (Leverkusen, Germany); cimetidine and SKF 525A from Smith Kline & French Laboratories Ltd (Welwyn Garden City, U.K.); quinidine sulphate and quinine sulphate from Tamro (Finland); cocaine, meperidine and phenylbutazone from Yliopiston Apteekki (Helsinki, Finland); phenytoin and theophylline from Leiras (Turku, Finland); propranolol from Lääke (Turku, Finland); progesterone from Fluka Chemical AG; cholesterol and 1-naphthol from Merck; antipyrine, 4-OH-antipyrine and 1-nitropyrene from Aldrich (Steinheim, Germany); ibuprofen from Boots Co. Ltd (Nottingham, U.K.); lidocaine from Astra (Södertälje, Sweden); digoxin and medroxyprogesterone acetate from Medipolar (Oulu, Finland); 4-methylpyrazole from Labkemi Ab (Göteborg, Sweden). The following agents were from the Sigma Chemical Co. (St Louis, MO, U.S.A.): aflatoxin B_1, aniline, benzo(*a*)pyrene, caffeine, cannabidiol, cannabinol, captopril, cinnamic acid, chlorzoxazone, clotrimazole, coumarin, cyclophosphamide, dexamethasone, dicumarol, diethylnitrosamine (DEN), dimethylnitrosamine, flurbiprofen, histamine, imidazole, 4-iodopyrazole, ketoprofen, khellin, menadione sodium bisulfite, metronidazole, metyrapone, miconazole, α-naphthoflavone, novobiocin, phenylalanine, psoralen, quercetin, rifampicine, sparteine, theobromine, tolbutamide, vitamin D_3, vitamin K_1 and warfarin.

Mouse liver samples. Liver microsomes from pyrazole-pretreated 8–12-week-old male DBA/2N mice were used. Pyrazole was given as single daily i.p. injections (200 mg/kg dissolved in physiological saline) for 3 consecutive days. All the livers were pooled and microsomes were prepared as described below.

Human liver samples. Human liver samples were obtained either from renal transplant donors or liver biopsies taken in connection with abdominal surgery. The use of surplus liver samples was approved by the Ethics Committee of the Medical Faculty, University of Oulu. The microsomal fractions were prepared by homogenizing the tissue in a glass homogenizer in 4 vol. of 0.1 M sodium–potassium phosphate buffer (pH 7.4). Homogenates were centrifuged at 10,000 *g* for 30 min and the supernatant obtained was centrifuged at 100,000 *g* for 1 hr. The microsomal pellets were washed and homogenized in 0.1 M sodium–potassium phosphate buffer pH 7.4 to give a protein concentration of approximately 15 mg/mL. Microsomal protein concentration was measured by the procedure of Bradford [13].

Enzyme assays. Coumarin 7-hydroxylase (COH) activity was determined according to Aitio [14] and testosterone hydroxylase activities by the TLC method of Waxman *et al.* [15] with minor modifications [16]. Some of the studied substances (aflatoxin B_1, apterin, bergapten, columbianadin, isobergapten, 1-naphthol, pimpinellin and psoralen) were metabolized to fluorescent products. Therefore, their effects on COH activity were studied by HPLC according to the method originally developed for the detection of warfarin metabolites [17]. The incubations and centrifugations were performed in the same way as with the fluorometric method. The supernatants were filtered through an 0.2 μm Spartan 13/30 filter (Schleicher & Schuell). Aliquots of the supernatants were injected into an HPLC system consisting of an Interchrom Nucleosil C18 column (5 μm particle size), an Altex model 110 pump, a Cecil Ce 2112 UV-monitor and a Hitachi D-2000 integrator. For elution a 1:5 mixture of buffer A (1.5% acetate, pH 4.85) and buffer B (buffer A + 50% acetonitrile) were used. The flow rate was 1 mL/min and elution was monitored at 313 nm. The retention time of the metabolite 7-hydroxycoumarin (7OHC) was 6 min. Authentic 7OHC was used as an external standard. In every assay the control activity was determined in the presence of pure diluent for the chemical in question. The inhibitory effect of each chemical was determined by using four different concentrations (0.5, 5, 50 and 500 μM). The IC_{50} values were determined graphically.

Urinary excretion of 7OHC in mice. The method of Lush and Andrews [18] was used with slight modifications. Because the basal excretion varied between 3 and 8% in different animals, we adopted a longitudinal experimental plan, employing the animals as their own controls. In each experiment, four male DBA/2 mice first received 3 μmol of coumarin in 100 μL dimethyl sulphoxide : H_2O (1 : 1) to determine the basal excretion of 7OHC. For urine collection, animals were kept in plastic cages and the urine was collected at 6 hr. No food was allowed during collection. Urine was diluted to a suitable final volume for the determination of 7OHC. After an interval of at least 2 days, the inhibitors (10 mg/kg i.p. of methoxsalen, bergapten, isopimpinellin, metyrapone, miconazole, clotrimazole, menadione and 1-naphthol) were administered (in 50 or 100 μL of dimethyl sulphoxide) 30 min before the administration of 3 μmol coumarin. Urine aliquots of 0.5 mL were mixed with 0.5 mg β-glucuronidase/aryl sulphatase (Type H-1, Sigma Chemical Co.) in 0.5 mL 1 N acetate buffer and incubated for 2 hr at 37°. After the incubation an aliquot (0.05 mL) was extracted for 10 min with water (0.45 mL) and chloroform (2.0 mL). One millilitre of the chloroform phase was taken to 1.6 M glycine buffer (1.5 mL), vortexed briefly and the fluorescence of the aqueous phase was measured immediately with a spectrophotofluorometer (excitation at 365 nm and emission at 454 nm). The effects of inhibitors having their own strong fluorescence was studied by the HPLC method described above.

RESULTS

Inhibition of COH by different chemicals in mouse and human liver microsomes

Table 1 gives the IC_{50} values and the extent of inhibition of COH activity by various compounds in mouse and human liver microsomes. The chemical structures of selected furanocoumarins, miconazole,

Table 1. Effect of various compounds on COH activity in mouse and human liver microsomes

Substances	Mouse IC_{50} (μM)	(%*)	Human IC_{50} (μM)	(%*)
Furanocoumarins				
Linear:				
Bergapten	24	24	>500	
Imperatorin	40	25	>500	
Isopimpinellin	25	16	>500	
Methoxsalen	1.0	0	5.4	4
Ostruthol	>500		>500	
Psoralen	3.0	5	110	22
Angular:				
Angelicin	>500		160	33
Apterin	>500		>500	
Columbianadin	>500		>500	
Isobergapten	>500		>500	
Pimpinellin	82	10	>500	
Sphondin	18	17	90	30
Other coumarins				
Dicumarol	>500		>50†	
Warfarin	>500		>500	
Imidazole antimycotics				
Miconazole	10	0	20	2
Clotrimazole	29	17	71	22
Carcinogens				
Benzo(*a*)pyrene	>500		>500	
Diethylnitrosamine	>500		>500	
Isoform-specific inhibitors				
2B—metyrapone	7.0	2	>500	
Miscellaneous substances				
1-Naphthol	72	9	210	30
Vitamin K_3 (menadione)	28	1	12	0

* % of control activity with the highest concentration used (500 μM). Only values lower than 50% are expressed.

† About 80% activity left with the 50 μM concentration (Pearce *et al.* [21]).

metyrapone, menadione and 1-naphthol are shown in Fig. 1. The most potent inhibitor in mouse liver microsomes was methoxsalen (8-methoxypsoralen or xanthotoxin), which caused a 100% inhibition of COH activity (IC_{50} value 1.0 μM). The furanocoumarins psoralen, bergapten (5-methoxypsoralen), imperatorin, pimpinellin, isopimpinellin (5,8-dimethoxypsoralen) and sphondin inhibited COH activity to a lesser extent and with higher IC_{50} values (3.0–82 μM) compared with methoxsalen. Also metyrapone, 1-naphthol and menadione (IC_{50} values 7.0, 72 and 28 μM, respectively) were inhibitory. The antimycotic substance miconazole inhibited COH activity to a similar degree as methoxsalen. The dose-dependent inhibition of methoxsalen, bergapten and miconazole in mouse liver microsomes is shown in Fig. 2.

All furanocoumarins and a selection of other inhibitors were studied also in human liver microsomes (Table 1). Methoxsalen was the most potent inhibitor also in human liver microsomes, producing a 96% inhibition at 500 μM concentration (IC_{50} 5.4 μM). Sphondin and miconazole were less efficient as inhibitors. Bergapten, imperatorin, isopimpinellin, pimpinellin and metyrapone, all inhibitors of mouse COH, did not affect human COH activity at all. The inhibitory effect of the difuranocoumarin derivative aflatoxin B_1 on human COH could not be determined due to production of interfering metabolites.

The effects of a number of different compounds on mouse COH activity are listed in Table 2. The COH activity-inducing agents pyrazole and cocaine [7] (Pellinen *et al.*, unpublished) were not inhibitory. Nifedipine, 4-iodopyrazole, aminoglutethimide, 4-OH-antipyrine, quercetin, testosterone and progesterone had a slight inhibiting effect. All the other compounds tested produced a less than 50% inhibition at the highest dose of inhibitor used (500 μM).

Effect on testosterone hydroxylations by selected COH inhibitors in mouse liver microsomes

The inhibitory effect of methoxsalen, bergapten and miconazole on testosterone hydroxylations was studied in pyrazole-treated mouse liver (Table 3, Fig. 2). All these compounds effectively inhibited testosterone 15α-hydroxylation (15αOH) with the IC_{50} values ranging from 1.1 to 9.0 μM. Methoxsalen and bergapten inhibited only marginally 6βOH (Fig.

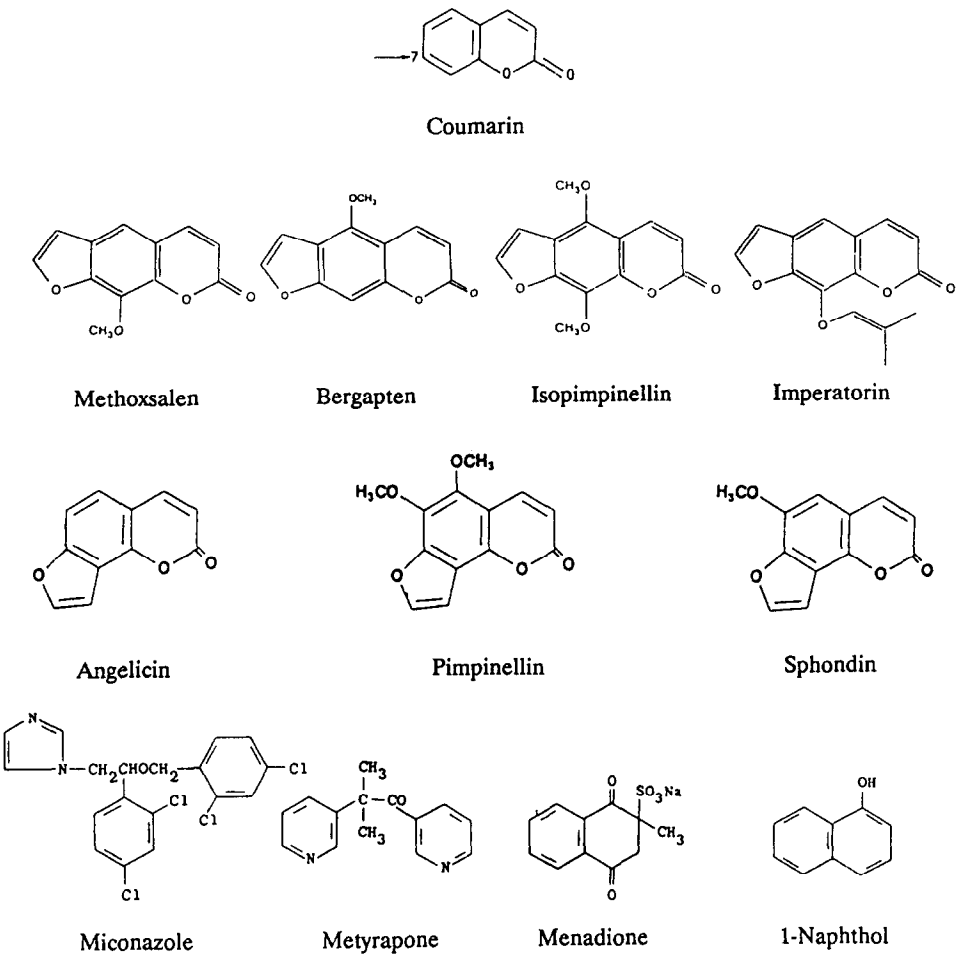

Fig. 1. Chemical structures of coumarin, linear types of furanocoumarin (methoxsalen, bergapten, isopimpinellin and imperatorin), angular types of furanocoumarin (angelicin, pimpinellin and sphondin), miconazole, metyrapone, menadione (menadione sodium bisulphite) and 1-naphthol. The arrow indicates the carbon atom preferentially hydroxylated in coumarin.

2). Methoxsalen and bergapten had only a marginal or no effect on 16αOH and 7αOH activities or on androstenedione formation (Table 3). Miconazole was a very potent and non-selective inhibitor of testosterone oxidations. It had the greatest effect on 6βOH (IC_{50} 1.2 μM) and 16αOH activities, but 15αOH and 7αOH activities, and androstenedione formation were also inhibited effectively by miconazole (Table 3).

Inhibition of 7OHC excretion by selected compounds in mice in vivo

Methoxsalen, the strongest inhibitor *in vitro*, proved to be the most effective one also *in vivo* (Table 4). Also, another psoralen-type compound isopimpinellin inhibited 7OHC excretion by 67%. Metyrapone inhibited the excretion by 80%. The *in vitro* inhibitors miconazole, clotrimazole, bergapten, menadione and 1-naphthol did not inhibit the appearance of 7OHC in urine *in vivo* at the dose administered (10 mg/kg body weight). Diethylnitrosamine, which was not an *in vitro* inhibitor, actually increased the excretion of 7OHC into urine by 63%.

DISCUSSION

This study demonstrates that several furanocoumarin derivatives had a differential inhibitory effect on mouse and human hepatic COH activity *in vitro*. The potent inhibition caused by several coumarin derivatives suggests (but does not directly prove) that they could be possible substrates for Cyp2a-5 and CYP2A6. None of the endogenous substances studied were found to inhibit COH activity appreciably compared with the furanocoumarins. Mouse studies *in vivo* confirmed the *in vitro* results in the case of methoxsalen, isopimpinellin and metyrapone. The lack of inhibition of other *in vitro* inhibitors might be due to slower absorption and/or faster metabolism, resulting in low, ineffective concentrations in the liver.

Furanocoumarins are structurally either linear or angular [3, 19]. Psoralen is a model substance for a

Table 2. Effect of different substances on COH activity studied only in mouse liver microsomes

Substances	IC_{50} (μM)	Inhibition* (%)	Substances	IC_{50} (μM)	Inhibition* (%)
Coumarins			Pyrazole derivatives		
Khellin	>500		4-Iodo-pyrazole	160	26
Novobiocin	>500		4-Methyl-pyrazole	>500	
Coumarin precursors			Pyrazole	>500	
Cinnamic acid	>500		Miscellaneous substances		
Phenylalanine	>500		Aminoglutethimide	120	31
Carcinogens			Aniline	>500	
Aflatoxin B_1	>500		Antipyrine	>500	
Dimethylnitrosamine	>500		4-Hydroxyantipyrine	360	48
1-Nitropyrene	>500		Cannabidiol	>500	
Calcium antagonists			Cannabinol	>500	
Diltiazem	>500		Captopril	>500	
Isradipine	>500		Carbamazepine	>500	
Nifedipine	350	43	Cimetidine	>500	
Verapamil	>500		Cocaine	>500	
Steroids			Cyclophosphamide	>500	
Cholesterol	>500		Digoxin	>500	
Dexamethasone	>500		Flurbiprofen	>500	
Estradiol	>500		Furosemide	>500	
Medroxyprogesterone-acetate	>500		Glibenclamide	>500	
Progesterone	100	14	Glutethimide	>500	
Testosterone	320	43	Histamine	>500	
Isoform-specific inhibitors			Ibuprofen	>500	
1A—α-naphthoflavone	>500		Imidazole	>500	
1A—caffeine	>500		Ketoprofen	>500	
1A—theophylline	>500		Metronidazole	>500	
2B—SKF 525A	>500		Meperidine	>500	
2C—mephenytoin	>500		Phenylbutazone	>500	
2C—tolbutamide	>500		Phenytoin	>500	
2D—debrisoquine	>500		Propranolol	>500	
2D—sparteine	>500		Quercetin	200	43
2D—quinidine	>500		Quinine	>500	
2E—chlorzoxazone	>500		Rifampicin	>500	
3A—lidocaine	>500		Temazepam	>500	
3A—cyclosporin A	>500		Theobromine	>500	
			Vitamin D_3	>500	
			Vitamin K_1	>500	

* % of control activity with the highest concentration used (500 μM). Only values lower than 50% are expressed.

linear type and angelicin for an angular type. Among the linear furanocoumarins, psoralen, 8-methoxypsoralen (methoxsalen), 5-methoxypsoralen (bergapten), 5,8-dimethoxypsoralen (isopimpinellin) and imperatorin proved to be potent inhibitors of COH activity in mouse liver, whereas ostruthol had no effect. Only two angular substances out of the six tested here were COH inhibitors, namely sphondin (6-methoxyangelicin) and pimpinellin (5,6-dimethoxyangelicin). The other four, angelicin, apterin, columbianadin and isobergapten, were without effect. It is of interest that the coumarin anticoagulants warfarin and dicumarol do not inhibit COH activity in human or mouse liver microsomes [20, 21]. Recently, warfarin has been shown to be a substrate for CYP2C9 [22]. Together with previous studies in which the effect of several inhibitors of P450s on mouse COH has been tested [23–25], we have now a relatively extensive view of the types of compounds that are able to interact with the active centres of Cyp2a-5 and CYP2A6.

The mouse Cyp2a-5 and human CYP2A6 enzymes are highly similar in their primary structure (83% overall amino acid similarity) and they both have a high capacity for coumarin 7-hydroxylation [5, 9, 26]. There are, however, clear differences between these two enzymes, e.g. in substrate preferences [27, 28]. The present study demonstrated that the mouse and human isoforms have striking differences in their sensitivity to the inhibitory effect of coumarin derivatives. Bergapten, isopimpinellin, imperatorin and pimpinellin did not inhibit COH in human liver microsomes, although they were rather potent inhibitors of the mouse enzyme. Metyrapone has been shown to inhibit COH activity effectively in mouse liver microsomes [23, 24], whereas it had either a small or no effect on COH in human liver microsomes [5, 16]. According to the present results there may be differences in the conformation of the active sites of human CYP2A6 and mouse Cyp2a-5. It is of interest that Wood [23] also reported that metyrapone and aniline inhibit differently COH in

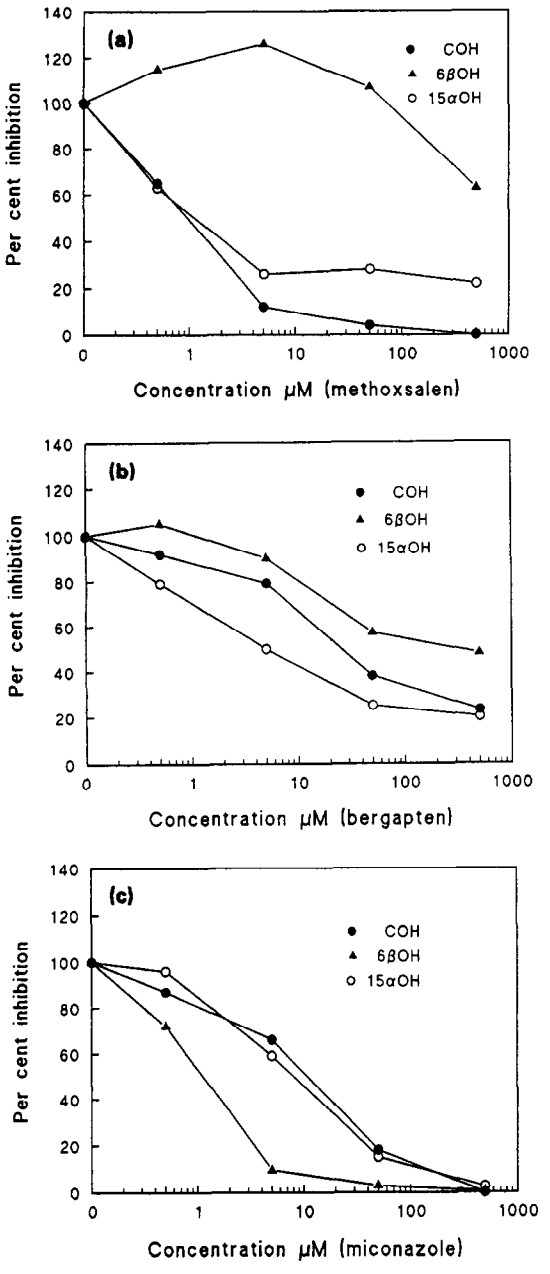

Fig. 2. Inhibition *in vitro* of COH, 15αOH and 6βOH in pyrazole-treated mouse liver microsomes by methoxsalen (a), bergapten (b) and miconazole (c).

DBA/2J and AKR/J mouse liver microsomes, indicating subtle differences in the enzyme even between these two inbred strains. Recently, Lindberg *et al.* [29] demonstrated that only a single amino acid difference in the Cyp2a-5 protein results in a high (DBA/2J) or low (AKR/J) constitutive COH activity. Only a single amino acid substitution is able to change the substrate specificity of Cyp2a-5 to Cyp2a-4-like [30] and certain amino acid residues are critical in determining the substrate binding features and spectral properties of Cyp2a-5 [25, 31]. Since the Cyp2a-5 and CYP2A6 proteins differ by about 80 out of 490 amino acids, it is likely that the active centre of CYP2A6 differs structurally from that of Cyp2a-5. The common feature among the coumarin derivatives that did not inhibit CYP2A6 is the substitution in carbon atom 5 of the coumarin ring. It can thus be speculated that the active site of CYP2A6 is less accessible to these compounds than that of Cyp2a-5.

It seems that the P450 isoform selectivity of the most potent furanocoumarins is rather strict. Methoxsalen and bergapten inhibited the closely related isoform 15αOH (Cyp2a-4) quite selectively compared with other testosterone hydroxylases (7αOH, 16αOH and 6βOH). In the rat liver the P450 isoforms mainly catalysing these reactions have been shown to be members of the 2A, 2C and 3A subfamilies [11]. Metyrapone also inhibits selectively 15αOH in mouse liver microsomes [16]. Coumarin has been shown to be able to inhibit 15αOH to less than 10% of control levels [8].

This study further confirmed earlier findings that imidazole antimycotics are rather potent but non-selective inhibitors of P450 reactions [32–34]. In the present study, miconazole was a very potent inhibitor of COH (IC_{50} 10 μM) in mouse and human liver microsomes, but it also inhibited with an equal potency all the studied testosterone hydroxylations and androstenedione formation in mouse liver microsomes, as described also earlier [35]. However, these antimycotics did not affect coumarin metabolism *in vivo* at the dose used. The different inhibitory effects of psoralens and antimycotics on COH indicate that their mechanisms of action may not be the same which is quite possible in view of their essentially different structures.

Aflatoxin B_1, also a coumarin derivative [19], and DEN have been shown to be activated to cytotoxic and mutagenic species by CYP2A6 [36]. In this study they had no effect on COH activity in mouse liver microsomes. In fact DEN slightly increased the urinary excretion of 7OHC *in vivo* in the mouse.

Table 3. The effect of methoxsalen, bergapten and miconazole on testosterone oxidations in mouse liver microsomes

	Testosterone hydroxylase		
	7α	16α	Androstenedione formation
	IC_{50} (μM)		
Methoxsalen	>500	>500	>500
Bergapten	>500	>500	>500
Miconazole	10	1.0	6.3

Table 4. The effect of various inhibitors on the urinary excretion of 7OHC in mice *in vivo*

Drug	Excretion of 7OHC (% of control)
Methoxsalen	9.2 ± 1.3
Bergapten	93 ± 26
Isopimpinellin	36 ± 16
Metyrapone	21 ± 7.8
Menadione	125 ± 25
1-Naphthol	178 ± 152
Clotrimazole	121 ± 68
Miconazole	104 ± 83
Diethylnitrosamine	163 ± 90

The inhibitors (10 mg/kg) were administered 30 min before coumarin injection (3 μmol). The urine was collected for 6 hr. The control excretion was estimated without inhibitors.

DEN did not affect COH activity in human liver microsomes. The effect of aflatoxin B_1 could not be determined. Since both aflatoxin B_1 and DEN are substrates for CYP2A6, they might have a much lower affinity for the enzyme compared with coumarin. A minor part of 1-nitropyrene has been shown to be metabolized by CYP2A6 and it is mainly metabolized by CYP3A3 and CYP3A4 [37]. Nevertheless, in the present work, 1-nitropyrene did not inhibit COH activity in the mouse liver microsomes. Also, benzo(*a*)pyrene did not inhibit COH activity in the mouse and human liver microsomes. Yun *et al.* [28] have reported that purified CYP2A6 does not have benzo(*a*)pyrene 3-hydroxylation activity, suggesting benzo(*a*)pyrene is not a CYP2A6 substrate.

A number of substrates of other P450 isoforms were also investigated. Mephenytoin is a model substrate of the CYP2C subfamily [1] and was found not to inhibit COH activity significantly. Tolbutamide is metabolized by P450s belonging to the CYP2B (rat) and CYP2C (human) subfamilies [38], but no inhibition of COH activity could be detected. Sparteine and debrisoquine, substrates for CYP2D6 [1] also did not inhibit COH activity. Chlorzoxazone, a substrate for CYP2E1 [39], did not inhibit COH activity. Some CYP3A substrates [40] (nifedipine, progesterone and testosterone) inhibited COH activity by about 50%, whereas others (estradiol, dexamethasone, lidocaine, rifampicin and cyclosporin A) had no effect.

A note of caution is warranted about the relationship between inhibitors and substrates of the same isoform. Although quinidine is a strong inhibitor of bufuralol 1′-hydroxylation, it is not metabolized by the purified CYP2D6 that oxidizes bufuralol [41]. Coumarin blocks efficiently 15αOH in mice, but it is not 7-hydroxylated by Cyp2a-4 [8]. We have, however, preliminary evidence that some of the furanocoumarin-type inhibitors reported here are metabolized by Cyp2a-5.

Acknowledgements—The useful comments of Risto Juvonen and Matti Lang are gratefully acknowledged. We thank Arja Rautio, Pentti Arvela, Ritva Tauriainen and Päivi Kylli for their contribution to this study. 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.

REFERENCES

1. Gonzalez FJ, The molecular biology of cytochrome P450s. *Pharmacol Rev* **40**: 243–288, 1989.
2. Nebert DW, Nelson DR, Coon MR, Estabrook RW, Feyereisen R, Fujii-Kuriyama Y, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Loper JC, Sato R, Waterman MR and Waxman DJ, The P450 superfamily: update on new sequences, gene mapping and recommended nomenclature. *DNA Cell Biol* **10**: 1–14, 1991.
3. Egan D, O'Kennedy R, Moran E, Cox D, Prosser E and Thornes RD, The pharmacology, metabolism, analysis, and applications of coumarin and coumarin-related compounds. *Drug Metab Rev* **22**: 503–529, 1990.
4. Cohen AJ, Critical review of the toxicology of coumarin with special reference to interspecies differences in metabolism and hepatotoxic response and their significance to man. *Fd Cosmet Toxicol* **17**: 277–289, 1979.
5. Pelkonen O, Sotaniemi EA and Ahokas JT, Coumarin 7-hydroxylase activity in human liver microsomes. Properties of the enzyme and interspecies comparisons. *Br J Clin Pharmacol* **19**: 59–66, 1985.
6. Raunio H, Kojo A, Juvonen R, Honkakoski P, Järvinen P, Lang MA, Vähäkangas K, Gelboin HV, Park SS and Pelkonen O, Mouse hepatic cytochrome P-450 isozyme induction by [1,4-bis 2-(3,5-dichloropyridyloxy)] benzene, pyrazole, and phenobarbital. *Biochem Pharmacol* **37**: 4141–4147, 1988.
7. Juvonen RO, Kaipainen PK and Lang MA, Selective induction of coumarin 7-hydroxylase by pyrazole in D_2 mice. *Eur J Biochem* **152**: 3–8, 1985.
8. Negishi M, Lindberg R, Burkhart B, Ichikawa T, Honkakoski P and Lang M, Mouse steroid 15α-hydroxylase gene family: identification of type II P-$450_{15\alpha}$ as coumarin 7-hydroxylase. *Biochemistry* **28**: 4169–4172, 1989.
9. Miles JS, McLaren AW, Forrester LM, Glancey MJ, Lang MA and Wolf CR, Identification of the human liver cytochrome P-450 responsible for coumarin 7-hydroxylase activity. *Biochem J* **267**: 365–371, 1990.
10. Raunio H, Syngelmä T, Pasanen M, Juvonen R, Honkakoski P, Kairaluoma MA, Sotaniemi E, Lang MA and Pelkonen O, Immunochemical and catalytical studies on hepatic coumarin 7-hydroxylase in man, rat, and mouse. *Biochem Pharmacol* **37**: 3889–3895, 1988.
11. Waxman DJ, Interactions of hepatic cytochromes P-450 with steroid hormones, regioselectivity and stereospecificity of steroid metabolism and hormonal regulation of rat P-450 enzyme expression. *Biochem Pharmacol* **37**: 71–84, 1988.
12. Härmälä P, Study on the isolation and chromatographic behavior of coumarins from Angelica (*Angelica archangelica*) roots. Academic thesis, The University of Helsinki, 1991.
13. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
14. Aitio A, A simple and sensitive assay of 7-ethoxycoumarin O-deethylation. *Anal Biochem* **85**: 488–491, 1978.
15. Waxman DJ, Ko A and Walsh C, Regioselectivity and

stereoselectivity of androgen hydroxylations catalyzed by cytochrome P-450 isozymes purified from phenobarbital-induced rat liver. *J Biol Chem* **258**: 11937–11947, 1983.
16. Mäenpää J, Syngelmä T, Honkakoski P, Lang MA and Pelkonen O, Comparative studies on coumarin and testosterone metabolism in mouse and human livers: differential inhibitions by the anti-P450Coh antibody and metyrapone. *Biochem Pharmacol* **42**: 1229–1235, 1991.
17. Fasco MJ, Cashin MJ and Kaminsky LS, A novel method for the quantitation of warfarin and its metabolites in plasma. *J Liq Chromatogr* **2**: 565–575, 1979.
18. Lush IE and Andrews KM, Genetic variation between mice in their metabolism of coumarin and its derivatives. *Genet Res* **31**: 177–186, 1978.
19. Murray RDH, Mendéz J and Brown SA, *The natural Coumarins. Occurrence, Chemistry and Biochemistry*. John Wiley & Sons, New York, 1982.
20. Honkakoski P, Arvela P, Juvonen R, Lang MA, Kairaluoma M and Pelkonen O, Human and mouse liver coumarin 7-hydroxylases do not metabolize warfarin *in vitro*. *Br J Clin Pharmacol* **33**: 313–317, 1992.
21. Pearce R, Greenway D and Parkinson A, Species differences and interindividual variation in liver microsomal cytochrome P450 2A enzymes: effects on coumarin, dicumarol, and testosterone oxidation. *Arch Biochem Biophys* **298**: 211–225, 1992.
22. Rettie AE, Korzekwa KR, Kunze KL, Lawrence RF, Eddy AC, Aoyama T, Gelboin HV, Gonzalez FJ and Trager WF, Hydroxylation of warfarin by human cDNA-expressed cytochrome P-450: a role for P-4502C9 in the etiology of (*S*)-warfarin-drug interactions. *Chem Res Toxicol* **5**: 54–59, 1992.
23. Wood AW, Genetic regulation of coumarin hydroxylase activity in mice. Biochemical characterization of the enzyme from two inbred strains and their F1 hybrid. *J Biol Chem* **254**: 5641–5646, 1979.
24. Kojo A, Honkakoski P, Järvinen P, Pelkonen O and Lang M. Preferential inhibition of mouse hepatic coumarin 7-hydroxylase by inhibitors of steroid metabolizing monooxygenases. *Pharmacol Toxicol* **65**: 104–109, 1989.
25. Juvonen RO, Iwasaki M and Negishi M, Structural function of residue-209 in coumarin 7-hydroxylase (P450coh): enzyme-kinetic studies and site-directed mutagenesis. *J Biol Chem* **266**: 16431–16435, 1991.
26. Yamano S, Tatsuno J and Gonzalez FJ, The CYP2A3 gene product catalyzes coumarin 7-hydroxylation in human liver microsomes. *Biochemistry* **29**: 1322–1329, 1990.
27. Juvonen R, Shkumatov V and Lang M, Purification and characterization of a liver microsomal P-450 isoenzyme with a high affinity and metabolic capacity for coumarin from pyrazole treated D2 mice. *Eur J Biochem* **171**: 205–211, 1988.
28. Yun C-H, Shimada T and Guengerich FP, Purification and characterization of human liver microsomal cytochrome P-450 2A6. *Mol Pharmacol* **40**: 679–685, 1991.
29. Lindberg RLP, Juvonen R and Negishi M, Molecular characterization of the murine Coh locus: an amino acid difference at position 117 confers high and low coumarin 7-hydroxylase activity in P450Coh. *Pharmacogenetics* **2**: 32–37, 1992.
30. Lindberg RLP and Negishi M, Alteration of mouse cytochrome P450coh substrate specificity by mutation of a single amino acid residue. *Nature* **336**: 632–634, 1989.
31. Iwasaki M, Juvonen R, Lindberg R and Negishi M, Alteration of high and low spin equilibrium by a single mutation of amino acid 209 in mouse cytochromes P450. *J Biol Chem* **266**: 3380–3382, 1991.
32. Sheets JJ, Mason JI, Wise CA and Estabrook RW, Inhibition of rat liver microsomal cytochrome P-450 steroid hydroxylase reactions by imidazole antimycotic agents. *Biochem Pharmacol* **35**: 487–491, 1986.
33. Ritter JK and Franklin MR, Induction and inhibition of rat hepatic drug metabolism by N-substituted imidazole drugs. *Drug Metab Dispos* **15**: 335–343, 1987.
34. Pasanen M, Taskinen T, Iscan M, Sotaniemi EA, Kairaluoma M and Pelkonen O, Inhibition of human hepatic and placental xenobiotic monooxygenases by imidazole antimycotics. *Biochem Pharmacol* **37**: 3861–3866, 1988.
35. Ballard SA, Lodola A and Tarbit MH, A comparative study of 1-substituted imidazole and 1,2,4-triazole antifungal compounds as inhibitors of testosterone hydroxylations catalyzed by mouse hepatic microsomal cytochromes P-450. *Biochem Pharmacol* **37**: 4643–4651, 1988.
36. Crespi CL, Penman PW, Leakey JAE, Arlotto MP, Stark A, Parkinson A, Turner T, Steimel DT, Rudo K, Davies RL and Langenbach R, Human cytochrome P450IIA3: cDNA sequence, role of the enzyme in the metabolic activation of promutagens, comparison to nitrosamine activation by human cytochrome P450IIE1. *Carcinogenesis* **11**: 1293–1300, 1990.
37. Howard PC, Aoyama T, Bauer SL, Gelboin HV and Gonzalez FJ, The metabolism of 1-nitropyrene by human cytochromes P450. *Carcinogenesis* **11**: 1539–1542, 1990.
38. Veronese ME, McManus ME, Laupattarakasem P, Miners JO and Birkett DJ, Tolbutamide hydroxylation by human, rabbit and rat liver microsomes and by purified forms of cytochrome P-450. *Drug Metab Dispos* **18**: 356–361, 1990.
39. Peter R, Böcker R, Beaune PH, Iwasaki M, Guengerich FP and Yang CS, Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1. *Chem Res Toxicol* **3**: 566–573, 1990.
40. Wrighton SA and Stevens JC, The human hepatic cytochromes P450 involved in drug metabolism. *Crit Rev Toxicol* **22**: 1–21, 1992.
41. Guengerich FP, Müller-Enoch D and Blair IA, Oxidation of quinidine by human liver cytochrome P-450. *Mol Pharmacol* **30**: 287–295, 1986.