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METABOLIC INTERACTIONS OF METHOXSALEN AND COUMARIN IN HUMANS AND MICE

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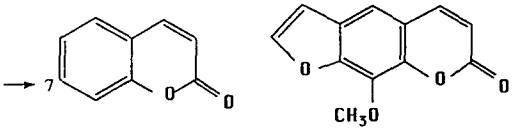
Abstract—Methoxsalen (8-methoxypsoralen) is a very potent inhibitor of human cytochrome P450 2A6 (CYP2A6) and mouse Cyp2a-5-mediated coumarin 7-hydroxylation *in vitro*. To determine the effect of methoxsalen on coumarin 7-hydroxylation in humans *in vivo*, five subjects were given 45 mg of methoxsalen and 5 mg of coumarin. Methoxsalen inhibited *in vivo* coumarin metabolism by 47 ± 9.2% (mean ± SEM). Methoxsalen was metabolized in human liver microsomes at the rate of 50–100 pmol/mg protein/min (approx. 30% of the activity in mouse liver microsomes). Metabolism was not inhibited by the anti-Cyp2a-5 antibody in human liver microsomes. NIH 3T3 cells stably expressing catalytically active CYP2A6 enzyme did not metabolize methoxsalen, indicating that CYP2A6 does not accept methoxsalen as a substrate. In pyrazole-induced mouse liver microsomes, methoxsalen metabolism was inhibited by the anti-Cyp2a-5 antibody. Cyp2a-5 protein expressed in the yeast *Saccharomyces cerevisiae* was capable of metabolizing methoxsalen, indicating that methoxsalen is a substrate of Cyp2a-5. Although kinetic studies indicated that the inhibition of coumarin 7-hydroxylation by methoxsalen is competitive in human liver microsomes, methoxsalen does not appear to be a substrate for CYP2A6. Methoxsalen and coumarin have the potential of strong metabolic interactions in man.

Key words: coumarin; methoxsalen; cytochrome P450; drug interactions; CYP2A subfamily; 8-methoxypsoralen

Methoxsalen§ is a furanocoumarin derivative (Fig. 1) that is widely used in the treatment of psoriasis, vitiligo and cutaneous T-cell lymphoma [2–4]. Methoxsalen is metabolized extensively in rats and humans [5, 6]. Large inter-individual variations exist in the plasma levels of methoxsalen in humans [7]. The metabolism of methoxsalen is catalysed by P450 enzymes in rodents [8, 9]. In humans, methoxsalen inhibits the elimination of caffeine and theophylline [10, 11]. Methoxsalen is also an inhibitor of several P450-mediated reactions in human liver microsomes *in vitro* [12].

Coumarin (Fig. 1) is being actively tested as a drug for lymphedema, various infections and cancer [13]. In humans, coumarin is 7-hydroxylated effectively *in vitro* and *in vivo* [14–16]. Great inter-individual variability exists in hepatic coumarin metabolism activity *in vitro* and *in vivo* [16–18]. COH activity is catalysed almost exclusively by the CYP2A6‖ isoform in humans and Cyp2a-5 in mice [15, 19–21]. Coumarin is the only clinically used agent shown to have high affinity for CYP2A6. In a survey of CYP2A6 and Cyp2a-5 inhibitors [22] methoxsalen turned out to be the most potent inhibitor *in vitro*. This finding indicates that methoxsalen interacts with Cyp2a-5 and CYP2A6, but it is not known whether methoxsalen metabolism is also catalysed by these P450 isoforms. This study was designed to elucidate further the metabolic interactions of methoxsalen and coumarin in humans and mice and to find out whether methoxsalen is a substrate of CYP2A6 and Cyp2a-5.



Coumarin Methoxsalen

Fig. 1. Chemical structures of coumarin and methoxsalen. The arrow indicates the carbon atom preferentially hydroxylated in coumarin.

MATERIALS AND METHODS

Biological material. Liver microsomes from control, 3-methylcholanthrene, pyrazole and phenobarbital pretreated 8–12-week-old DBA/2N male mice were used. 3-Methylcholanthrene (80 mg/kg) was given as single i.p. injection and pyrazole

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§ Abbreviations: P450, cytochrome P450; COH, coumarin 7-hydroxylase; ECOD, ethoxycoumarin *O*-deethylase; 7OHC, 7-hydroxycoumarin; EROD, 7-ethoxyresorufin *O*-deethylase; PROD, 7-pentoxyresorufin *O*-deethylase; AHH, aryl hydrocarbon (benzo(*a*)pyrene) hydroxylase; IC_{50}, inhibitor concentration of the drug to achieve a 50% inhibition of the activity; methoxsalen, 8-methoxypsoralen; [^{3}H]methoxsalen, [^{3}H-methoxyl]methoxsalen.

‖ The recommended P450 nomenclature [1] will be used in this article.

(200 mg/kg) was given as single i.p. injections for 3 consecutive days. The mice were killed 24 hr after treatment. Phenobarbital was administered in drinking water (500 mg/L) for a 1 week period. Human liver samples were obtained from renal transplant donors from the University Hospital of Oulu and from the tissue bank of the Biocenter of the University of Basel, Switzerland. The collection of surplus liver samples has been approved by the Ethics Committee of the Medical Faculty, University of Oulu. Liver samples were stored at −70° until use. 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 at 100,000 *g* for 1 hr. The microsomal pellet was washed and dissolved in 0.1 M sodium–potassium phosphate buffer, pH 7.4. The protein contents were determined according to Bradford [23].

Chemicals. Coumarin was from Sigma (St Louis, MO, U.S.A.). Methoxsalen for *in vitro* studies was from Roth (Leverkusen, Germany). [^{3}H]-methoxsalen (85 Ci/mmol; Amersham, U.K.) was 98.7% pure by HPLC. Venalot® capsules (containing 5 mg coumarin) were from Schaper & Brummer (Ringelheim, Germany), and Puvaderm® (containing 15 mg methoxsalen) tablets were from Leiras (Turku, Finland).

Preparation of the antibody. Cyp2a-5 was purified as described elsewhere [19] and a polyclonal antibody against it was raised in rabbits [24].

Inhibition of urinary excretion of 7OHC by methoxsalen in humans. Five healthy volunteers took a single 5 mg dose of coumarin (a Venalot® capsule) orally after overnight fasting as described in detail previously [16]. Urine samples were collected at 4 and 8 hr. The excretion of 7OHC was quantitated by an HPLC method [16]. The intra-subject variability of 7OHC excretion has been shown to be small [16]. The effect of methoxsalen on 7OHC excretion was studied after a minimum of 6 days. In the interaction experiment, 45 mg methoxsalen (Puvaderm®) was ingested 1 hr before coumarin and the urine samples were collected at 4 and 8 hr after coumarin. Control urine samples were obtained just before coumarin or methoxsalen administration.

Enzyme assays. COH and ECOD assays were determined according to Aitio [25]. EROD and PROD activities were measured as described [26]. AHH was measured by the method of Nebert and Gelboin [27]. The metabolism of methoxsalen was measured by the method of Mays *et al.* [8]. Briefly, incubations consisted of 0.7–1.2 mg/mL microsomal protein and the NADPH regenerating system and 20 μM final concentration of [^{3}H]methoxsalen. Incubation times for mouse and human liver microsomes were 10 and 20 min, respectively. Incubation volume was 500 μL. The reaction was stopped by adding 0.4 mL 3 M phosphate buffer (pH 6.8) and 12 mL hexane. Ten millilitres of the hexane phase was mixed with 10 mL Aqualuma (Lumac, Groningen, The Netherlands) for direct quantitation of the disappearance of the substrate (the limit of detection was 10 pmol/mg protein/min).

The appearance of the metabolites was assayed by the acetone extraction procedure [8]. The hexane phase was removed and 0.8 mL 3 M phosphate buffer and 4 mL acetone were added. The sample was centrifuged for 10 min, then 3.5 mL of the acetone phase were transferred to a scintillation vial and evaporated for 20 min. One millilitre of water and 15 mL of scintillation mixture were added for direct quantitation of the appearance of polar metabolites.

Spectral studies. Determination of P450–substrate interaction spectra was done with a Jasco Uvidec-610 double beam spectrophotometer as described earlier [19].

Methoxsalen and coumarin metabolism by CYP2A6 and Cyp2a-5. CYP2A6 cDNA was cloned and expressed in NIH 3T3 cells by retrovirus-mediated gene transfer as described in detail elsewhere [28]. The culture medium contained Dulbecco-modified Minimal Essential Medium/Nutrient F-12 (Flow Laboratories, Irvine, U.K.) supplemented with 10% newborn calf serum (Gibco, U.K.) and 50 μg/mL of gentamycin. COH activity in NIH 3T3 cells was determined using a 10 μM coumarin concentration [28]. The inhibition of COH activity by methoxsalen was determined using three different concentrations of methoxsalen (0.5, 5.0 and 50.0 μM).

The metabolism of methoxsalen was determined directly in the cells after they were grown in 30 mm Petri dishes. Methoxsalen was added to the medium at a final concentration of 10, 50 and 100 μM. The medium samples (500 μL) were removed 18 hr after adding methoxsalen.

Cyp2a-5 cDNA was isolated and expressed in the yeast *Saccharomyces cerevisiae* AH22 cells [29]. The Cyp2a-5 protein produced by these cells was purified and used in reconstitution experiments. The reconstitution mixture (500 μL) contained 40 pmol Cyp2a-5, 0.15 nmol P450 reductase, 48 μM dilauroylphosphatidylcholine and 1 μM methoxsalen in 0.1 M phosphate buffer (pH 7.4) [19]. Methoxsalen metabolism was determined as described above.

Inhibition studies. In immunoinhibition studies a maximally inhibiting ratio of 1:1 antibody: microsomal protein was used [15]. The microsomes were preincubated with the antibody for 10 min. In chemical inhibition assays, four different methoxsalen and coumarin concentrations (in DMSO) were used (0.5, 5, 50 and 500 μM). IC_{50} values were determined graphically. Control experiments were performed with the same concentration of preimmune IgG or DMSO (5 μL).

RESULTS

Inhibition of 7OHC excretion by methoxsalen in humans

The effect of methoxsalen on coumarin metabolism was studied in five volunteers. Urine samples were taken before coumarin or methoxsalen was ingested and 4 and 8 hr after coumarin. Methoxsalen inhibited coumarin metabolism effectively in every subject (Table 1). The degree of inhibition 8 hr after coumarin administration was 47 ± 9.2% (mean ± SEM, range 20–70%) compared to the amount of 7OHC recovered in the coumarin control test.

Table 1. Inhibition of coumarin metabolism by methoxsalen in humans *in vivo**

Subject	Control	Methoxsalen	Control	Methoxsalen
	4 hr		8 hr†	
	mg 7OHC excreted (% of total coumarin given)			
1	4.36 (87)	1.68 (34)	4.54 (91)	1.80 (36)
2	3.50 (70)	1.88 (38)	4.47 (89)	1.88 (38)
3	3.07 (61)	0.94 (19)	3.13 (62)	1.07 (21)
4	3.61 (72)	2.43 (49)	3.78 (76)	2.60 (52)
5	3.83 (77)	3.17 (63)	4.22 (84)	3.41 (68)
Mean ± SEM	3.67 ± 0.21	2.02 ± 0.38‡	4.03 ± 0.26	2.15 ± 0.40‡

* Each subject was given 5 mg coumarin (control) and 1 week later 45 mg methoxsalen (methoxsalen) 1 hr before coumarin.

† Cumulative excretion.

‡ $P < 0.01$, significantly different vs control (Student's paired *t*-test).

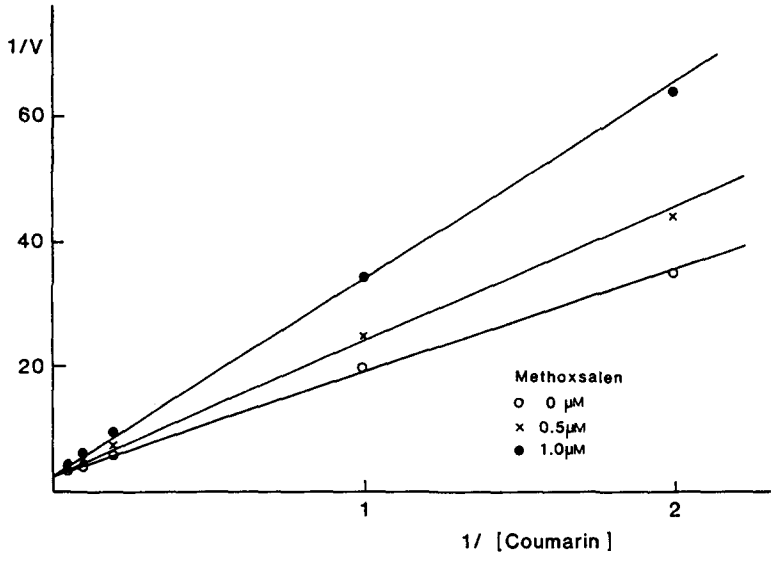

Fig. 2. Lineweaver–Burk analysis of the type of methoxsalen-caused inhibition on COH activity in human liver microsomes.

Kinetics of COH inhibition

The type of inhibition caused by methoxsalen on COH activity was determined by Lineweaver–Burk analysis in pyrazole-induced mouse liver microsomes and human liver microsomes (Fig. 2). Five concentrations of coumarin (0.5–20 μM) and two concentrations of methoxsalen (0.5 and 1.0 μM) were used. The V_{max} values remained unchanged and K_m was increased when methoxsalen was added in human (Fig. 2) and mouse (data not shown) liver microsomes, indicating that the inhibition is competitive. The apparent K_i values for COH inhibition by methoxsalen in mouse and human liver microsomes were 1.2 and 1.5 μM, respectively (data not shown). They were determined from plots of K'_m values with inhibitor concentrations of 0, 0.5 and 1.0 μM [30].

Methoxsalen metabolism in mouse and human liver microsomes

Microsomes from variously pretreated mice metabolized methoxsalen (Table 2). Pyrazole and phenobarbital are known to induce COH activity in the mouse liver [31]. Pyrazole and phenobarbital increased the metabolism of methoxsalen (1.5-fold and 3.4-fold above control level, respectively) (Table 2). Methylcholanthrene slightly decreased methoxsalen metabolism (data not shown). The human liver microsomes also metabolized methoxsalen at a rate of approx. 30% of that in control mouse liver microsomes.

The anti-Cyp2a-5 antibody effectively inhibits COH activity in mouse and human liver microsomes [15]. The antibody produced a weak (32%) inhibition on methoxsalen metabolism in pyrazole-induced

Table 2. The effect of anti-Cyp2a-5 antibody and coumarin on methoxsalen metabolism in mouse and human liver microsomes

	Activity*	Inhibition by Anti-Cyp2a-5 antibody†	Coumarin IC_{50}(μM)	Spectral interaction type‡	K_m (μM)
Control	258 ± 31§	121	>500	I	10.3
Pyrazole	376 ± 21§	68	>500	I	11.2
PB‖	875 ± 39§	98	>500	I	5.9
HL (5)	76 ± 11¶	117**	>500**	I**	41.0

* Activities are given in pmol/mg protein/min.
† Percentage of control activity.
‡ The type of interaction spectra of methoxsalen and P450.
§ Mean activities (±SEM) of four experiments (pooled microsomes). The disappearance of methoxsalen in incubation mixture was determined.
‖Phenobarbital-induced mouse liver microsomes.
¶ Mean activity (±SEM) for five human liver samples.
** Studied in liver HL16.

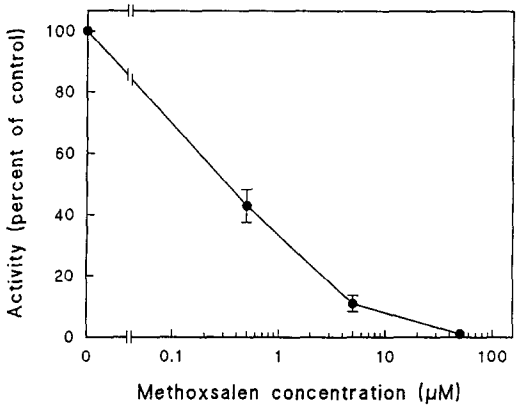

Fig. 3. Inhibition of COH activity in NIH 3T3 cells by methoxsalen. The values are means (±SEM) of three separate experiments.

Table 3. The effect of methoxsalen on ECOD, EROD, PROD and AHH activities in mouse liver

	Activity*	IC_{50} (μM)
ECOD†		
Control	0.53	30
Pyrazole	4.69	2
Phenobarbital	2.76	5
EROD†		
Control	0.31	3
Pyrazole	0.26	15
Phenobarbital	2.01	1
PROD†		
Control	0.08	2
Phenobarbital	0.47	2
AHH†		
Control	0.16	10
Pyrazole	0.03	>500
Phenobarbital	0.62	40

* Activities are given nmol/mg protein/min.
† Substrate concentrations used in the assay were 1 mM (ECOD), 1 μM (EROD), 2 μM (PROD) and 70 μM (AHH).

mouse liver microsomes. It had, however, no effect on methoxsalen metabolism in control or phenobarbital-induced mouse liver microsomes. The antibody did not inhibit methoxsalen metabolism in human liver microsomes (Table 2). Coumarin did not significantly inhibit methoxsalen metabolism in human and mouse liver microsomes (Table 2).

In mouse liver microsomes (control, pyrazole and phenobarbital groups) as well as in human liver microsomes methoxsalen bound to P450 (K_d values 5.9–41.0 μM) and caused a type I binding spectra with an absorption peak at approx. 390 nm (Table 2).

Methoxsalen and coumarin metabolism by CYP2A6 and Cyp2a-5

The effect of methoxsalen on COH activity was studied in NIH 3T3 cells producing the CYP2A6 enzyme (Fig. 3). Methoxsalen was a very potent inhibitor of COH activity; 50 μM methoxsalen in the culture medium caused a 99% reduction in said activity (IC_{50} = 0.3 μM).

Methoxsalen metabolism was also determined in these cells. Methoxsalen did not disappear from the hexane phase and no metabolites appeared in the acetone phase (data not shown), indicating that the NIH 3T3 2A6 cells did not metabolize methoxsalen.

In the reconstitution experiment, methoxsalen (1.0 μM) was metabolized by the purified Cyp2a-5 protein to polar metabolites at a rate of 590 pmol/min/nmol P450 (data not shown).

Effect of methoxsalen on other monooxygenases in mouse liver microsomes

Methoxsalen was a potent inhibitor of certain enzyme activities in variously pretreated mouse liver microsomes (Table 3). Methoxsalen inhibited

ECOD, EROD, PROD and AHH activities by 85–100% regardless of the pretreatment employed. IC_{50} values ranged from 1 to 40 μM except for AHH activity in pyrazole-treated mouse liver microsomes (56% activity left with 500 μM methoxsalen).

DISCUSSION

This study indicates that methoxsalen is a potent inhibitor of coumarin metabolism in humans *in vivo*. Our earlier study showed that methoxsalen efficiently blocks COH activity in human and mouse liver microsomes [22]. The COH enzymes (human CYP2A6 and mouse Cyp2a-5) are immunologically very similar and also share 83% similarity in their amino acid sequences [15, 19, 20, 32]. There are, however, marked differences in their inhibitor preferences [22]. For example, metyrapone and the coumarin derivatives bergapten and isopimpinellin are potent inhibitors of the mouse, but not the human, enzyme.

The key question posed in this work was whether methoxsalen is also accepted as a substrate by CYP2A6. Methoxsalen is clearly metabolized by human liver microsomes. The spectral studies also showed that it interacts with P450s. Since CYP2A6 is almost totally responsible for coumarin 7-hydroxylation in humans [21], the competitive nature of COH inhibition by methoxsalen in human liver microsomes suggests that methoxsalen might serve as a substrate of CYP2A6. However, this does not appear to be the case. First, the anti-Cyp2a-5 antibody, which totally blocks CYP2A6 enzyme function [15], did not affect methoxsalen metabolism in human liver microsomes. Second, the CYP2A6 substrate coumarin did not significantly inhibit methoxsalen metabolism. Third, NIH 3T3 cells expressing the active CYP2A6 enzyme did not metabolize methoxsalen at all. Fourth, COH and methoxsalen metabolism activities do not correlate in human and mouse [15, this paper], suggesting that different P450 isoforms participate in coumarin and methoxsalen metabolisms in human and mouse.

All these data indicate that although methoxsalen is capable of interacting with CYP2A6, it is not a substrate of CYP2A6. This is analogous to the situation with quinidine, which is a potent inhibitor of CYP2D6 but is metabolized by CYP3A isoforms [33, 34]. It still remains unclear which clinically important drugs are substantially metabolized by CYP2A6. Aflatoxin B1, *N*-nitrosodiethylamine, tobacco-related nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and nicotine are metabolized by CYP2A6 to a lesser extent [28, 35–38]. Cyp2a-4 and Cyp2a-5 in mouse liver are able to catalyse testosterone 15α-hydroxylation [39], whereas CYP2A6 does not hydroxylate testosterone [21, 28, 36, 40]. Preliminary results from our laboratory suggest that some other furanocoumarins also serve as substrates for Cyp2a-5 but not for CYP2A6.

It has been suggested that coumarin may cause liver damage at higher doses [13]. Therefore, it is important to determine which substances may interact with coumarin. According to the present study methoxsalen is a potent inhibitor of coumarin metabolism in humans, and co-administration of these drugs may generate toxic plasma levels of coumarin. However, this study as well as previous findings [10, 11] suggest that caution should be exercised when methoxsalen is given concurrently with other drugs.

The potent COH inducers in mouse liver, pyrazole and phenobarbital induced methoxsalen metabolism 1.5- and 3.4-fold, respectively, although their inducing effect on methoxsalen metabolism was much weaker than on COH. Pyrazole is an even more potent inducer of COH than phenobarbital [31], and pyrazole microsomes contain a high proportion of Cyp2a-5 [15]. Interestingly, the immunoinhibition studies with the anti-Cyp2a-5 antibody suggested that Cyp2a-5 catalyses methoxsalen metabolism (approx. 30%) only in pyrazole-induced mouse liver. The reconstitution studies with purified Cyp2a-5 protein indicated that Cyp2a-5 metabolizes methoxsalen. Phenobarbital principally increases other P450 isoforms mainly responsible for methoxsalen metabolism in mouse liver, whereas pyrazole has less effect on these isoforms. These data suggest that P450 isoforms other than Cyp2a-5 predominate in methoxsalen metabolism at least in control and phenobarbital-induced mouse liver.

Besides COH, methoxsalen has been shown to inhibit ECOD and AHH activities in rats and humans *in vitro* [12, 41]. According to the present study methoxsalen inhibits ECOD, EROD, PROD and AHH activities in mice. These findings suggest that methoxsalen inhibits the members of Cyp1a, Cyp2a, Cyp2b and Cyp2c subfamilies [42] in mouse liver.

Which P450 isoforms catalyse methoxsalen metabolism in human and mouse liver? According to preliminary data from our laboratory, members of the CYP3A subfamily may be partly involved in methoxsalen metabolism in human liver microsomes. Mays *et al.* [9] showed that the anti-CYP1A1/2 antibody inhibits methoxsalen metabolism in β-naphthoflavone-induced mouse liver. Phenobarbital also induces P450 isoforms within Cyp2b and Cyp2c subfamilies and the Cyp3 family in mouse liver [42, 43], suggesting that members of these subfamilies might participate in methoxsalen metabolism in phenobarbital-induced mouse liver.

In summary, this study shows that methoxsalen is a very potent inhibitor of coumarin 7-hydroxylation in humans *in vivo*. Although methoxsalen is also a potent COH inhibitor and methoxsalen and coumarin share structural similarities they are not metabolized by the same P450 isoforms in human liver microsomes. Even though Cyp2a-5 metabolizes methoxsalen in mouse liver microsomes, CYP2A6 does not catalyse methoxsalen metabolism in human liver.

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