Biochemical Pharmacology, Vol. 38, No. 10, pp. 1647–1655, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00
© 1989. Pergamon Press plc

ACTIVATION OF 8-METHOXYPSORALEN BY CYTOCHROME P-450

ENZYME KINETICS OF COVALENT BINDING AND INFLUENCE OF INHIBITORS AND INDUCERS OF DRUG METABOLISM

DENNIS C. MAYS,* JAY B. HILLIARD, DORA D. WONG and NICHOLAS GERBER
Departments of Family Medicine and Pharmacology, College of Medicine, The Ohio State University, Columbus, OH, U.S.A.

(*Received* 6 *June* 1988; *accepted* 26 *September* 1988)

Abstract—The kinetics of covalent binding of reactive metabolites of 8-methoxypsoralen (8-MOP) to protein were measured in incubations of liver microsomes of rats pretreated for 3 days with i.p. injections of 80 mg/kg/day of β-naphthoflavone (BNF), phenobarbital (PB), 8-MOP, or vehicle. Covalent binding of radioactivity derived from [^{14}C]8-MOP (labeled at the metabolically stable 4-position in the coumarin ring) required NADPH, obeyed classical Michaelis–Menten kinetics, and was inducible by both PB and BNF. Plots of V versus $V/[S]$ were linear in liver microsomes of rats pretreated with vehicle, PB, or 8-MOP; respective values for K_m were 26, 24 and 13 μM and for V_{max} were 0.61, 1.70 and 0.50 nmol bound/min/mg protein. In microsomes of rats pretreated with BNF, high- and low-affinity components of covalent binding were observed with respective values for K_m of 4.7 and 117 μM and for V_{max} of 0.77 and 1.71 nmol bound/min/mg protein. Addition of glutathione and cysteine to the incubations decreased covalent binding by 33 and 67%, respectively, presumably by trapping reactive electrophilic metabolites. Inhibition of epoxide hydrolase with 1,1,1-trichloropropene-2,3-oxide did not affect covalent binding of reactive metabolites of 8-MOP. SKF-525A was a potent inhibitor of both the metabolism of 8-MOP and covalent binding in microsomes from rats pretreated with PB, but had only a slight effect in microsomes from rats pretreated with BNF. In contrast, α-naphthoflavone almost completely inhibited metabolism of 8-MOP and covalent binding in BNF-induced microsomes but had no effect in PB-induced microsomes. Apparent covalent binding was reduced by 39% in incubations with 8-MOP labeled with tritium in the metabolically labile methoxy group. Collectively, these results indicate that 8-MOP is biotransformed by two or more isozymes of cytochrome P-450 to reactive electrophiles capable of binding to tissue macromolecules.

Psoralens are a class of naturally occurring furocoumarins present in many edible plants such as celery, limes, parsnips and figs [1, 2]. Certain photoactive psoralens, including 8-methoxypsoralen (8-MOP)†, 5-methoxypsoralen and 4,5′-8-trimethylpsoralen, are used in combination with long wavelength ultraviolet irradiation to treat psoriasis, vitiligo and cutaneous T-cell lymphoma [3, 4].

Early work from this laboratory showed that, in incubations with liver microsomes of rats, 8-MOP is metabolically activated and its metabolites irreversibly bind to microsomal protein [5]. This covalent binding to protein requires NADPH and oxygen, suggesting that the reaction is mediated by cytochrome P-450 [6, 7]. More recently, we showed that 8-MOP, administered acutely to humans and animals, is a potent inhibitor of drug metabolism *in vivo* [8, 9]. It inhibits the metabolism of caffeine, hexobarbital and phenytoin by > 75% in rats pretreated with 27 mg/kg of 8-MOP [8]. In humans, a single dose of 8-MOP prolongs the half-life of caffeine from 5.6 to 57 hr [9]. Repeated treatment of rats with 8-MOP induces its own metabolism and that of caffeine, but does not change the clearance of hexobarbital or phenytoin [8, 10].

An association between bioactivation of 8-MOP and its inhibition of drug metabolism by inactivation of cytochrome P-450 has been proposed recently by Fouin-Fortunet *et al.* [7]. A similar mechanism for inhibition of drug biotransformation has been proposed for chloramphenicol [11–14] and spironolactone [15]. These drugs are activated to metabolites that bind covalently to cytochrome P-450 and also irreversibly inhibit drug metabolism.

This investigation, to help elucidate the mechanism of inhibition of drug metabolism by 8-MOP, was designed to compare the kinetics of covalent binding of [^{14}C]8-MOP in incubations with liver microsomes of control rats and rats pretreated with inducers of cytochrome P-450. The influence of inhibitors of cytochrome P-450 and agents that can trap reactive electrophiles on the covalent binding of 8-MOP to microsomal protein was also examined.

* Correspondence: Dennis C. Mays, Ph.D., Department of Family Medicine, The Ohio State University, 333 W. 10th Ave., Columbus, OH 43210.

† Abbreviations: 8-MOP, 8-methoxypsoralen; GCMS, gas chromatography-mass spectrometry; PB, phenobarbital; ANF, α-naphthoflavone; BNF, β-naphthoflavone; GSH, reduced glutathione; SKF-525A, β-diethylaminoethyl diphenyl acetate; and TCPO, 1,1,1-trichloropropene-2,3-oxide.

MATERIALS AND METHODS

Chemicals. Unlabeled 8-MOP (Lot No. 29815)

was a gift from Elder Pharmaceutics (Costa Mesa, CA), and [4[14]C]8-MOP (27 mCi/mmol), labeled at the metabolically stable 4-position in the coumarin ring, was from New England Nuclear (Boston, MA). The chemical purity of unlabeled 8-MOP, determined by HPLC and GCMS, was > 99%. The radiochemical purity of [[14]C]8-MOP, measured by countercurrent distribution using the method of Bush [16], was > 98%. 8[*methoxyl*-[3]H]-methoxypsoralen (85 Ci/mmol, Amersham, Arlington Heights, IL), prepared by demethylation of 8-MOP followed by methylation with [[3]H]methyl iodide, was 97% pure by HPLC and contained a small impurity (1.5%) which eluted at 43 min in our HPLC system. Both 5-methoxypsoralen and 5,8-dimethoxypsoralen (isopimpinellin) also elute at 43 min [17] and are often found as impurities in 8-MOP isolated from natural sources. Therefore, it is likely that the starting material for the synthesis of [[3]H]8-MOP was contaminated with one or both of these compounds. Phenobarbital (free acid, PB), β-naphthoflavone (BNF), NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, *N*-acetylcysteine, Trizma base, and 1,1,1-trichloropropene-2,3-oxide (TCPO) were from the Sigma Chemical Co. (St. Louis, MO). Protosol and Atomlight were from New England Nuclear. Glutathione (GSH) and α-naphthoflavone (ANF) were obtained from the Aldrich Chemical Co. (Milwaukee, WI). HPLC grade methanol, acetonitrile, acetone, hexane and reagent grade ascorbic acid were from Fisher Scientific (Cincinnati, OH).

Treatment of animals. Male Sprague–Dawley rats (Harlan Industries, Indianapolis, IN), 320–420 g, were housed in stainless steel cages; allowed free access to Purina Rodent Chow (Ralston–Purina, St. Louis, MO) and tap water; and acclimated for at least 1 week prior to use. To determine the effects of enzyme induction, rats were pretreated once daily for 3 days with i.p. injections of 80 mg/kg/day of PB, BNF or 8-MOP dissolved in corn oil (10 ml/kg). Control rats were treated with vehicle for the same period.

Radiolabel assay for 8-MOP. The concentration of 8-MOP in microsomal incubations was determined by a specific radiochemical assay that separated parent drug from its more polar metabolites [10, 17]. Briefly, 0.4-ml samples of the incubation mixture and 0.4 ml of 4 M phosphate buffer (pH 6.8) were extracted with hexane (12 ml). A 10-ml portion of the hexane extract was mixed with 10 ml of liquid scintillation fluid (Formula 963, New England Nuclear) and counted in a Beckman model LS 6800 liquid scintillation counter. The specificity of the assay for 8-MOP, determined by HPLC and counter current distribution, was > 98% [17].

Covalent binding of [[14]C]8-MOP to microsomal protein was quantified by exhaustive extraction of samples of the incubation mixture with methanol. Each sample (2–3 ml) was shaken vigorously with 10 ml of methanol for 10 min on a mechanical shaker and centrifuged for 10 min at 1500 *g*. The methanol was removed and the protein pellet was resuspended in a fresh 10-ml aliquot of methanol, shaken, and centrifuged again. In the initial experiments, the protein was repeatedly extracted until the methanol contained less than twice background radioactivity (6–12 washes). Later, we discovered that after 6 washes filtration of the methanol through a 0.45 μm Acrodisc filter (Gelman, Ann Arbor, MI) eliminated residual radioactivity apparently by removing suspended particles of protein. The washed protein was dissolved in 0.5 ml of 1 N NaOH and diluted to 5 ml with water. One portion (1 ml) was mixed with 15 ml of Atomlight and counted for radioactivity and another (50 μl) was analyzed for protein by the method of Lowry *et al.* [18] using bovine albumin standards. Covalent binding was expressed as nanomoles of [[14]C]- or [[3]H]8-MOP bound per milligram of protein.

Michaelis–Menten kinetics of in vitro *covalent binding of 8-MOP to liver microsomal protein.* Rats were killed 24 hr after the third pretreatment and liver microsomes were isolated as described previously [10]. Incubations in 25-ml Erlenmeyer flasks were maintained at 37° in a Dubnoff shaking incubator (Precision Instruments, Chicago, IL). Flasks, containing microsomes, were flushed with 100% O_2, and equilibrated in the incubator for 2 min; the reaction was initiated by addition of the NADPH-generating system. The final incubation mixture consisted of 0.6 to 0.9 mg/ml of microsomal protein; 2.3, 4.6, 11.5, 23, 46 or 93 μM [[14]C]8-MOP (0.6 μCi) in 0.1 M phosphate buffer (pH 7.4); 0.4 mM NADPH; 6 mM $MgCl_2$; 10 mM glucose-6-phosphate; 0.8 enzyme units/ml of glucose-6-phosphate dehydrogenase; and enough 0.1 M potassium phosphate buffer (pH 7.4) to bring the final volume to 5 or 10 ml. Samples were removed at 2 min and assayed for covalent binding and 8-MOP. Metabolism of 8-MOP was calculated as the difference between total radioactivity (determined by direct counting) and hexane-extractable radioactivity.

Covalent binding of 8-MOP at 2 min was linear ($r > 0.99$) with microsomal protein concentrations between 0.25 to 1.0 mg/ml in incubations of liver microsomes from animals pretreated with corn oil or BNF (Fig. 1). Therefore, covalent binding of 8-MOP was subsequently measured at 2 min in incubations with protein concentrations of 0.6 to 0.9 mg/ml. Plots of velocity of covalent binding (V), expressed as nmol bound/min/mg protein, versus $V/[S]$ were constructed according to the method of Eadie [19] and Hofstee [20].

Alteration of in vitro *covalent binding of 8-MOP by inhibitors and trapping agents in microsomes of rats pretreated with PB.* The effects of inhibitors and trapping agents on *in vitro* covalent binding of 8-MOP were investigated in incubations with liver microsomes from rats pretreated with PB as described above. Each incubation, containing either 2.3 μM [[14]C]8-MOP (27 mCi/mmol) or [[3]H]8-MOP (235 mCi/mmol), was equilibrated for 2 min at 37° with one of the following: SKF-525A (0.25 mM), glutathione (1 mM), cysteine (5 mM), *N*-acetylcysteine (5 mM) or ascorbic acid (1 mM). Fresh solutions of inhibitors and trapping agents were prepared daily in 0.1 M phosphate buffer (pH 7.4). The enzymatic reaction was started by addition of the NADPH-generating system and stopped after 2 min for measurement of metabolism of 8-MOP and covalent binding of metabolites of 8-MOP.

Differential inhibition by SKF-525A and ANF of

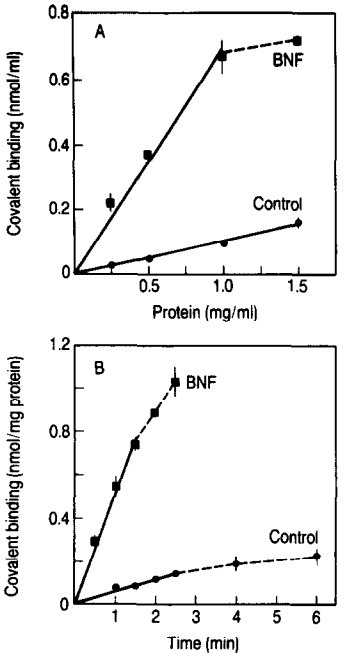

Fig. 1. Covalent binding of 8-MOP to liver microsomal protein: Effects of protein concentration and time. Liver microsomes were isolated and pooled from three rats pretreated for 3 days with daily i.p. injections of BNF (80 mg/kg) or corn oil as described in Materials and Methods. Covalent binding of [^{14}C]8-MOP (2.3 μM, 27 mCi/mmol) in incubations of liver microsomes containing an NADPH-generating system was measured at 2 min with 0, 0.25, 0.5, 1.0 and 1.50 mg/ml of microsomal protein (panel A) or at various times with 0.75 mg of microsomal protein/ml (panel B). Each point is the mean ± SD of three incubations. Covalent binding of 8-MOP at 2 min was linear ($r > 0.99$) with protein concentrations up to 1.0 mg/ml.

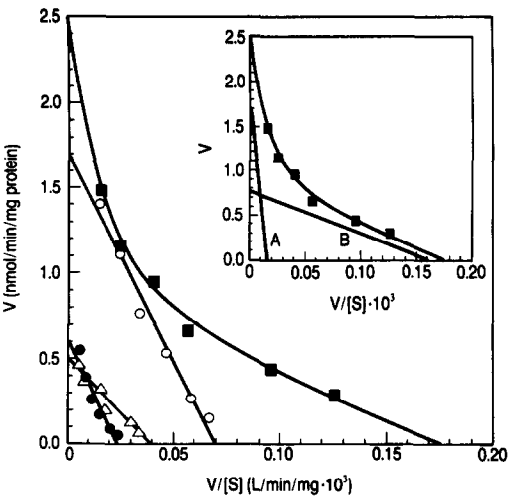

Fig. 2. Eadie–Hofstee plots of covalent binding of 8-MOP to liver microsomal protein. Covalent binding of [^{14}C]8-MOP (0.6 μCi; 2.3, 4.6, 11.5, 23, 46 or 93 μM) was determined at 2 min in incubations containing an NADPH-generating system and liver microsomes from rats pretreated for 3 days with 8-MOP (△), PB (○), BNF (■), or vehicle (●). Each point is the mean of four to six incubations. The inset shows the low-affinity (A) and high-affinity components (B) of covalent binding in microsomes from rats pretreated with BNF. See Table 1 for details.

in vitro *covalent binding of 8-MOP in microsomes of rats pretreated with PB or BNF.* Three rats per group were pretreated with PB or BNF as described above. Duplicate incubations (5 ml final volume) with microsomes prepared separately from each rat were equilibrated for 2 min with SKF-525A (0.25 mM final concentration) added in 0.1 ml of 0.1 M phosphate buffer (pH 7.4) or with ANF (10 μM final concentration) added in 13 μl of absolute ethanol.

Effect of acute in vivo *pretreatment with 8-MOP on covalent binding of 8-MOP metabolites* in vitro. One rat was pretreated with a single i.p. injection of 27 mg/kg of 8-MOP in corn oil and another with corn oil alone. Four hours later, the rats were killed, microsomes were isolated, and triplicate incubations were prepared with 2.3 μM [^{14}C]8-MOP (27 mCi/mmol). Timed samples were removed for measurement of 8-MOP and covalent binding of metabolites.

We considered the possibility that covalent binding of [^{14}C]8-MOP added *in vitro* may be affected by residual unlabeled 8-MOP in the microsomes from the i.p. injection. The results of our previous pharmacokinetic studies of 8-MOP indicated that the concentration of 8-MOP in the liver 4 hr after an i.p. dose of 27 mg/kg could be as high as 16 μg/g, assuming a concentration of 8-MOP in blood of 8 μg/ml and a liver/blood ratio of 2 [8, 17]. The actual concentration of unlabeled 8-MOP in the incubation, before addition of radiolabeled 8-MOP, was 80 ng/ml (0.4 μM). This was determined by HPLC as previously described [17]. Thus, the final concentration of 8-MOP was slightly higher (2.7 vs 2.3 μM) and, correspondingly, the specific activity was slightly lower (23 vs 27 mCi/mmol) in microsomal incubations from rats pretreated 4 hr previously with 8-MOP. Any error in covalent binding resulting from the lower specific activity should be offset somewhat by a higher reaction velocity at the higher substrate concentration and, in any case, should not exceed 15%.

RESULTS

Michaelis–Menten kinetics of covalent binding of 8-MOP to liver microsomal protein. Eadie–Hofstee plots of V versus $V/[S]$ for covalent binding of 8-MOP metabolites to liver microsomal protein are presented in Fig. 2. Covalent binding of 8-MOP in microsomes of rats pretreated for 3 days with vehicle, PB, or 8-MOP followed classical Michaelis–Menten kinetics, each with a single apparent Michaelis constant (K_m) and maximal velocity (V_{max}). The apparent K_m was 26 μM and the maximal velocity (V_{max}) was 0.61 nmol/min/mg protein in control microsomes (Table 1). Prior treatment with PB, an inducer of cytochromes P-450b and P-450e in the rat [23], almost tripled the rate of covalent binding (V_{max} = 1.70 nmol/min/mg protein) but did not significantly affect the K_m.

In contrast, the plot of V versus $V/[S]$ for covalent binding of 8-MOP was nonlinear in microsomes of rats pretreated with BNF, an inducer of cytochromes

Table 1. Kinetics of covalent binding of 8-MOP to liver microsomes

Pretreatment	K_m (μM)	V_{max} (nmol/min/mg protein)
Vehicle	26	0.61
PB	24	1.70
8-MOP	13	0.50
BNF (low affinity)	117	1.71
BNF (high affinity)	4.7	0.77

Covalent binding data (see Fig. 2) in microsomes of rats pretreated with vehicle, PB, or 8-MOP were fit by linear regression to the equation: $V = V_{max} - K_m(V/[S])$. V_{max} and K_m values for the high and low activity components of the reaction in the BNF-induced microsomes were estimated by curve peeling [21]. Using these initial estimates, covalent binding data were fit by nonlinear regression (PCNONLIN [22]) to the equation $V = V_{max}[S]/(K_{m1} + [S]) + V_{max}[S]/(K_{m2} + [S])$.

P-450c and P-450d [23]. Two straight lines, each with an apparent K_m and V_{max} were obtained by nonlinear regression analysis (Fig. 2; Table 1). The rate of covalent binding of the reactive intermediate of 8-MOP was very fast at low concentrations of 8-MOP in microsomes of rats pretreated with BNF, indicating a high-affinity component for covalent binding not found with other pretreatments. The overall V_{max} of the reaction in microsomes from rats treated with BNF was 2.5 nmol/min/mg protein. Pretreatment with 8-MOP, an inducer of its own metabolism *in vivo* in the rat [10], did not change significantly the V_{max} of covalent binding, but decreased the K_m to 13 μM.

Alteration of covalent binding of 8-MOP by inhibitors and trapping agents in microsomes of rats pretreated with PB. Preincubation of hepatic microsomes with SKF-525A for 2 min reduced metabolism and covalent binding of 8-MOP by 71 and 75% respectively (Table 2). It is probable that SKF-525A diminished covalent binding by decreasing cytochrome P-450-mediated activation of 8-MOP. TCPO, a potent inhibitor of epoxide hydrolase [24], did not affect either the disappearance of 8-MOP or covalent binding.

Apparent covalent binding in incubations with]^{3}H-*methoxyl*]8-MOP was 39% lower than corresponding incubations with [^{14}C]8-MOP (Table 2), suggesting that a substantial fraction of 8-MOP was *O*-demethylated before reacting with protein. Although *O*-demethylation is a minor pathway (< 1%) of 8-MOP metabolism in humans [25], it is more important in rats with free and conjugated forms of 8-hydroxypsoralen representing about 8% of the urinary metabolites in unpretreated rats [17].

Cysteine had the most pronounced effect on covalent binding (67% inhibition) of the sulfhydryl-containing trapping agents tested (Table 3). It is well known that nucleophiles such as cysteine can trap reactive electrophiles generated by cytochrome P-450, including those of acetaminophen [26], bromobenzene [27] and naphthalene [28]. Cysteine may be a more efficient trapping agent than *N*-acetylcysteine because it can form a stable cyclic adduct with certain reactive intermediates [29]. The sulfhydryl-containing agents did not affect the rate of metabolism of 8-MOP, which is consistent with inhibition of covalent binding by the trapping of reactive intermediates of 8-MOP after their formation by cytochrome P-450. Ascorbic acid slightly reduced covalent binding (22%) but increased metabolism (29%) of 8-MOP. Ascorbic acid also has been reported to decrease formation of adducts derived from acetaminophen in liver microsomes, presumably by chemically reducing the reactive intermediate, *N*-acetyl-*p*-benzoquinoneimine, to acetaminophen [26].

Differential inhibition by SKF-525A and ANF of metabolism and covalent binding of 8-MOP in microsomes of rats pretreated with PB or BNF. SKF-525A had only a small effect when added to microsomes

Table 2. Effects of inhibitors on metabolism and covalent binding of 8-MOP in liver microsomes from rats pretreated with phenobarbital

Incubation	N*	Covalent binding (nmol/mg protein)	% Inhibition†	Metabolism (nmol/mg protein)	% Inhibition	Binding/ Metabolism	% Inhibition
Complete System	19	0.36 ± 0.04		1.56 ± 0.09		0.23 ± 0.01	
−NADPH	6	0.01 ± 0.00†	97	0.29 ± 0.03‡	81	0.03 ± 0.01‡	87
SKF-525A (0.25 mM)	8	0.09 ± 0.01‡	75	0.46 ± 0.02‡	71	0.20 ± 0.03	13
TCPO (0.1 mM)	6	0.33 ± 0.03	8	1.50 ± 0.11	4	0.23 ± 0.02	0
[^{3}H]*Methoxyl*§	6	0.22 ± 0.01‡	39	1.38 ± 0.09	12	0.15 ± 0.01‡	35

Rats were pretreated for 3 days with PB, and microsomes were prepared 24 hr after the last dose as described in Materials and Methods. Inhibitors were preincubated with microsomes for 2 min prior to the addition of cofactors. All incubations contained 2.3 μM 8-MOP (27 mCi/mmol) and about 0.9 mg/ml of microsomal protein. Metabolism and covalent binding of 8-MOP were determined at 2 min as described in Materials and Methods. Values are means ± SE.

* Number of incubations.

† Percent inhibition compared to incubations with cofactors (complete system).

‡ Significantly different from control incubations (complete system) as determined by the Kruskal–Wallis Test and the Mann–Whitney U-Test ($P < 0.05$).

§ Incubation contained 8-MOP labeled with tritium in the methoxy group.

Table 3. Effects of scavenging agents on metabolism and covalent binding of 8-MOP in liver microsomes of rats pretreated with phenobarbital

Incubation	N*	Covalent binding (nmol/mg protein)	% Inhibition†	Metabolism (nmol/mg protein)	% Inhibition	Binding/ Metabolism	% Inhibition
Complete System	19	0.36 ± 0.04		1.56 ± 0.09		0.23 ± 0.01	
Glutathione (1 mM)	8	0.24 ± 0.01‡	33	1.69 ± 0.07	−8	0.13 ± 0.01‡	43
Cysteine (5 mM)	6	0.12 ± 0.00‡	67	1.48 ± 0.03	5	0.08 ± 0.00‡	65
N-Acetyl cysteine (5 mM)	6	0.22 ± 0.01‡	39	1.45 ± 0.03	7	0.15 ± 0.00‡	35
Ascorbate (1 mM)	8	0.28 ± 0.02‡	22	2.02 ± 0.13‡	−29	0.14 ± 0.01‡	39

Microsomal incubations containing 2.3 μM 8-MOP and scavengers were performed as described in Table 2. Metabolism and covalent binding of 8-MOP were determined at 2 min as described in Materials and Methods. Values are means ± SE.

* Number of incubations.

† Percent inhibition compared to incubations without scavengers (complete system).

‡ Significantly different from control incubations (complete system) as determined by the Kruskal–Wallis Test and the Mann–Whitney U-Test ($P < 0.05$).

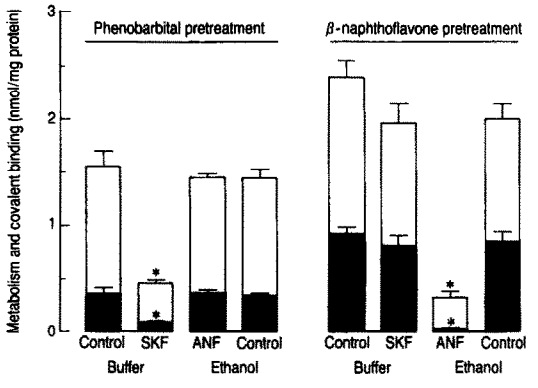

Fig. 3. Selective inhibition of microsomal metabolism and covalent binding of 8-MOP by SKF-525A and ANF. Liver microsomes were isolated separately from three rats pretreated with PB or BNF as described in Materials and Methods. SKF-525A (250 μM final concentration) in phosphate buffer or ANF (10 μM final concentration) in ethanol was preincubated for 2 min with 3.5 mg of microsomal protein in pH 7.4 phosphate buffer (0.1 M, 5 ml final volume) and 2.3 μM [^{14}C]8-MOP (27 mCi/mmol). The reaction was started by adding the NADPH-generating system. Each value is the mean ± SE (N = 6) of covalent binding (filled bars) and metabolism (unfilled bars) of 8-MOP determined at 2 min. Covalent binding and metabolism of 8-MOP in incubations without added cofactors were typically less than 0.01 and 0.30 nmol/mg protein respectively. The differential effects of inhibition by SKF-525A and ANF are consistent with formation of reactive intermediates of 8-MOP by at least two different isozymes of cytochrome P-450. Key: (*) significantly different from the corresponding vehicle control incubation ($P < 0.05$).

of rats pretreated with BNF (Fig. 3). However, when added to incubations of 8-MOP with microsomes of rats pretreated with PB, it markedly inhibited both metabolism and covalent binding of 8-MOP. Preincubation with SKF-525A for 2 min decreased covalent binding of 8-MOP from 0.43 ± 0.09 to 0.09 ± 0.04 nmol/mg protein and metabolism from 1.55 ± 0.08 to 0.46 ± 0.07 nmol/mg protein in microsomes of rats treated previously with PB.

In contrast, ANF had very little effect on covalent binding and metabolism of 8-MOP in microsomes of rats pretreated with PB (Fig. 3). However, when added to incubations of 8-MOP with microsomes of BNF-induced rats, it dramatically reduced both the metabolism and covalent binding of 8-MOP. Preincubation with ANF for 2 min decreased covalent binding from 0.86 ± 0.16 to 0.04 ± 0.01 nmol/mg protein and metabolism from 2.00 ± 0.38 to 0.40 ± 0.06 nmol/mg protein in microsomes from rats pretreated with BNF. In these incubations with a low concentration of 8-MOP (2.3 μM), covalent binding was higher in microsomes from rats induced with BNF than in those induced with PB, which is consistent with the high-affinity component for covalent binding observed for BNF in the kinetic studies (see Table 1 and Fig. 2).

Effects of acute pretreatment with 8-MOP on in vitro covalent binding of 8-MOP to microsomal protein. Pretreatment with 27 mg/kg i.p. of unlabeled 8-MOP, 4 hr before the animals were killed, markedly inhibited subsequent *in vitro* covalent binding and metabolism of [^{14}C]8-MOP (Fig. 4). Covalent binding after incubation for 5 min was only 0.03 ± 0.01 nmol/mg protein in microsomes from the rat pretreated acutely with 8-MOP compared to 0.15 ± 0.02 nmol/mg protein in control microsomes prepared from the rat pretreated with corn oil.

DISCUSSION

These experiments demonstrate that 8-MOP was biotransformed *in vitro* to reactive intermediates that bound covalently to liver microsomes. Covalent binding of these reactive metabolites of 8-MOP was

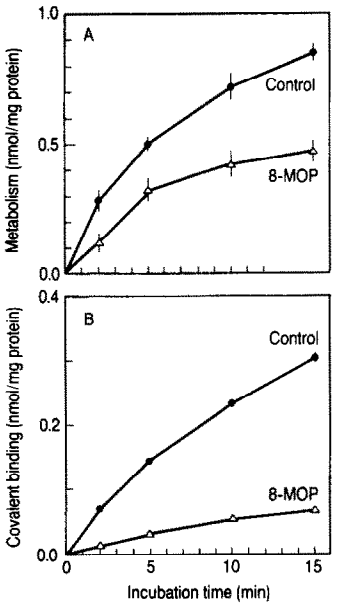

Fig. 4. Effect of acute *in vivo* pretreatment with 8-MOP on the *in vitro* metabolism and covalent binding of [^{14}C]8-MOP in liver microsomes. Liver microsomes, prepared from rats pretreated 4 hr prior to sacrifice with unlabeled 8-MOP (27 mg/kg) or vehicle (control), were incubated with 2.3 μM [^{14}C]8-MOP (27 mCi/mmol) as described in Materials and Methods. Metabolism (panel A) and covalent binding (panel B) of [^{14}C]8-MOP were determined at 2, 5, 10 and 15 min as described in Materials and Methods. Values are the means ± SE of triplicate incubations. In incubations with microsomes from the rat pretreated with 8-MOP, the concentration of residual unlabeled 8-MOP was 0.4 μM, prior to the addition of [^{14}C]8-MOP.

mediated by cytochrome P-450, obeyed Michaelis–Menten kinetics and was enhanced by prior treatment of rats with BNF or PB (Table 1; Fig. 2). The different K_m values for covalent binding after pretreatment of rats with BNF or PB suggest that two or more distinct enzyme systems contribute to the formation of these reactive products.

The studies with inhibitors (Table 2 and Fig. 3) further supported the concept that two or more isozymes of cytochrome P-450 activate 8-MOP. SKF-525A substantially reduced both covalent binding and metabolism of 8-MOP in microsomes from rats pretreated with PB, but had very little effect in microsomes of rats pretreated with BNF. Conversely, ANF almost completely inhibited covalent binding of 8-MOP in microsomes of rats pretreated with BNF, but had no effect in microsomes from rats pretreated with PB. These results are in agreement with previous reports that have also demonstrated different inhibitory effects of ANF and SKF-525A on reactions mediated by cytochrome P-450. Wiebel and coworkers [30, 31] showed that ANF selectively inhibits aromatic hydrocarbon hydroxylase (AHH) activity in microsomes of rats pretreated with 3-methylcholanthrene (3-MC) but not in microsomes from control rats. Similarly, Goujon *et al.* [32] demonstrated that SKF-525A preferentially inhibits phenobarbital-inducible forms of cytochrome P-450. It is not known whether the reactive intermediate(s) of 8-MOP differs with the state of induction. This possibility seems likely in view of the striking difference between the patterns of urinary metabolites of 8-MOP in rats pretreated with BNF and PB [10].

Covalent binding is an experimental measurement providing an estimate of the formation of highly reactive metabolites. Numerous reports have appeared in the literature linking enzymatic activation of acetaminophen, polycyclic aromatic hydrocarbons (PAH), bromobenzene, furosemide and aflatoxins to toxic metabolites that bind to tissue macromolecules [33–35]. The consequences of metabolic activation of 8-MOP are not clear. In spite of the high level of covalent binding of 8-MOP observed in liver microsomes *in vitro* in this investigation and *in vivo* in previous studies [36], evidence for hepatotoxicity of 8-MOP is lacking (unpublished results). Metabolic activation of 8-MOP, *per se*, may not lead directly to hepatic necrosis, but instead may result in inhibition of liver enzymes, including the isozymes of cytochrome P-450 that form the reactive intermediates. For example, chloramphenicol [11–14] and spironolactone [15] are biotransformed to reactive intermediates that irreversibly bind to and inactivate cytochrome P-450. The decrease in the *in vitro* metabolism of 8-MOP and covalent binding of its reactive metabolites after acute pretreatment with 8-MOP *in vivo* suggests that the covalent binding is self-limiting (see Fig. 4). Whether inactivation of cytochrome P-450 by 8-MOP is related to its dose-dependent elimination in the rat [17] and mouse [37] is unclear.

Since our initial discovery that 8-MOP binds covalently to microsomal protein [5, 6], other investigators have shown that 8-MOP also inhibits the *in vitro* activities of hexobarbital hydroxylase, ethoxycoumarin deethylase, benzo[*a*]pyrene hydroxylase and aminopyrine deethylase [7]. Subsequent investigations in our laboratory have shown that 8-MOP also potently inhibits the *in vivo* metabolism of caffeine, hexobarbital and phenytoin in the rat [8]. In contrast, the metabolism of 5-(4′-hydroxyphenyl)-5-phenylhydantoin, a phenytoin metabolite eliminated mostly by conjugation with glucuronic acid, is reduced only slightly by 8-MOP [8]. Further investigations have revealed that other psoralen analogs (5-methoxypsoralen and unsubstituted psoralen, but not 4,5′,8-trimethylpsoralen) also inhibit cytochrome P-450 [38, 39]. We have confirmed that these linear psoralens and, in addition, the angular 5-methylisopsoralen are inhibitors of drug metabolism.*

The structure of the reactive intermediate(s) of 8-MOP is still unknown and is currently being investigated in our laboratory. Certain inferences about the reactive intermediate can be made from the results of the studies reported here and also from our earlier investigations with 8-MOP. The furan ring of 8-MOP is oxidized extensively in the rat to

* Gerber N, Hilliard JB, Pacula CM and Mays DC, Interactions of 8-methoxypsoralen and other psoralen analogs with *in vivo* drug metabolism in the mouse. *Tenth International Congress of Pharmacology*, Sydney, Australia, 1987 (Abstract P73).

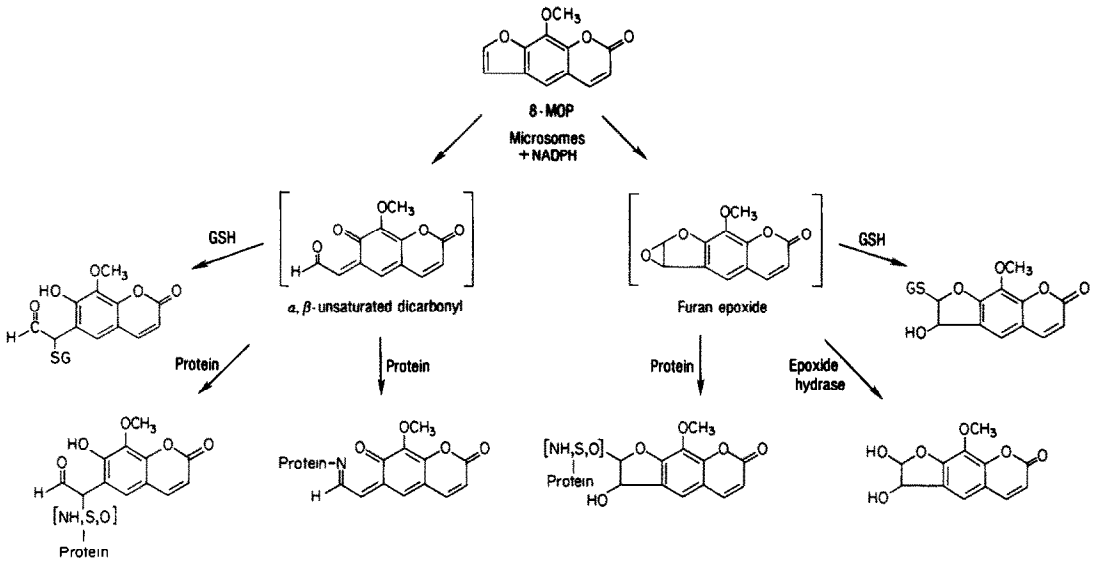

Fig. 5. Proposed pathways of metabolic activation of 8-MOP. The structures shown in brackets are the postulated reactive products of 8-MOP formed by cytochrome P-450 in liver microsomes of the rat. α,β-Unsaturated dicarbonyl intermediates are unstable and can react via Michael addition of the sulfhydryl groups of protein and glutathione (GSH) across the activated double bond, and by nucleophilic addition to the aldehyde forming a Schiff base. Electrophilic furan epoxides can also react with nucleophiles such as GSH and protein. Alternatively, this epoxide can be hydrolyzed by epoxide hydrase.

products possibly arising via a furan epoxide [17] (see Fig. 5). Furan epoxides have been implicated as the ultimate reactive species responsible for the hepatotoxicity of furosemide [40] and the pulmonary toxicity of ipomeanol [41]. In our study, TCPO, an inhibitor of epoxide hydrolase, did not alter covalent binding of 8-MOP which seems to rule out participation of an epoxide in covalent binding. Alternative explanations for this observation include the formation of an unstable epoxide which reacts with protein before hydrolysis by epoxide hydrolase; the intermediate remains active after hydrolysis by epoxide hydrolase; or the reactive intermediate itself may inhibit epoxide hydrolase.

8-MOP may be activated to an α,β-unsaturated dicarbonyl (Fig. 5) by a pathway analogous to that of simple alkyl furans [42]. This reaction scheme is supported by the syntheses of Manfredi *et al.* [43], which showed that alkyl furans react with *m*-chloroperbenzoic acid to form enediones, presumably via an epoxide intermediate. As a consequence of the requisite loss of aromaticity of the furocoumarin in this proposed reaction, more energy would be required for formation of the corresponding quinone methide from 8-MOP. Moreover, this intermediate would be less stable than the analogous intermediate of alkyl furans and would probably react very quickly with protein via either a Michael addition with a sulfhydryl group across the activated double bond or by nucleophilic addition to the aldehyde [29] (see Fig. 5). Hydroxylation of 8-MOP at the 5-position and *O*-demethylation give rise to 5,8-dihydroxypsoralen, a hydroquinone that is readily oxidized to the corresponding quinone, 5,8-dioxopsoralen [17]. Acetaminophen forms an analogous quinoneimine (*N*-acetyl-*p*-benzoquinoneimine), considered to be the reactive intermediate which binds irreversibly to microsomal protein [44]. Covalent binding of radiolabeled acetaminophen and *N*-acetyl-*p*-benzoquinoneimine to microsomal protein is inhibited markedly by ascorbic acid [44]. In our experiments, however, ascorbic acid was a much less potent inhibitor of covalent binding of 8-MOP (Table 3). Thus, the importance of the proposed quinone-hydroquinone redox pair and the quinone methide to covalent binding of 8-MOP remains uncertain.

Recent studies in our laboratory have focused on the targets of covalent binding of 8-MOP in microsomes. In mice pretreated with BNF, over 50% of the radioactivity in sodium dodecyl sulfate-polyacrylamide gel electrophoretograms of liver microsomal protein, incubated with [^{14}C]8-MOP, is bound to protein in the molecular weight regions corresponding to cytochromes P-450 and b_5 [45]. Addition of cysteine to the incubation causes a 73% reduction in covalent binding of 8-MOP without significantly affecting the metabolism of 8-MOP. In these experiments with cysteine, covalent binding to proteins eluting in the region of cytochrome P-450 (mol. wt. 52,000–55,000) is reduced by one-half; very little binding to other proteins, including cytochrome b_5, could be detected. In spite of this pronounced reduction of covalent binding to "nonspecific" protein and to cytochrome P-450 by addition of cysteine, the inhibitory effect of 8-MOP on 7-ethoxycoumarin deethylase activity remains unchanged [45]. This provides unequivocal evidence that the extent of covalent binding of reactive metabolites of 8-MOP to protein does not correlate directly with the inhibition of drug metabolism by 8-MOP. Covalent binding,

per se, is not the determinant of inhibition of drug metabolism by 8-MOP, but instead only an indicator of the degree of metabolic activation of the molecule. The consequences of metabolic activation of 8-MOP, with regard to inhibition of drug metabolism, depend on the specific targets of the reactive molecule and their relative importance to drug metabolism. More detailed investigations into the relationship of metabolic activation of 8-MOP to inhibition of drug metabolism are currently in progress.

Acknowledgements—This work was supported in part by the Samuel J. Roessler Medical Scholarship fund. We thank Ms. Jeannette Climie, Mr. Mark Chambers and Dr. Stephen Hecht for their technical assistance and Dr. Dale Sharp for his thoughtful discussions of this research.

REFERENCES

1. Pathak MA, Daniels F and Fitzpatrick TB, The presently known distribution of furocoumarins (psoralens) in plants. *J Invest Dermatol* **39**: 225–239, 1962.
2. Ivie GW, Hold DL and Ivey MC, Natural toxicants in human foods: Psoralens in raw and cooked parsnip root. *Science* **213**: 909–910, 1981.
3. Parrish JA, Fitzpatrick TB, Tanenbaum L and Pathak MA, Photochemotherapy of psoriasis with oral methoxsalen and longwave ultraviolet light. *N Engl J Med* **291**: 1207–1211, 1974.
4. Edelson R, Berger C, Gasparro F, Jegasothy B, Heald P, Wintroub B, Vonderheid E, Knobler R, Wolff K, Plewig G, McKiernan G, Christiansen I, Oster M, Honigsmann H, Wilford H, Kokoschka E, Rehle T, Perez M, Stingl G and Laroche L, Treatment of cutaneous T-cell lymphoma by extracorporeal photochemotherapy. *N Engl J Med* **316**: 297–303, 1987.
5. Sharp DE, Mays DC, Rogers SL, Guiler RC, Hecht S and Gerber N, *In vitro* metabolism of 8-methoxypsoralen. *Proc West Pharmacol Soc* **27**: 255–258, 1984.
6. Hecht S, Climie JC, Guiler RC, Sharp DE, Mays DC and Gerber N, Demonstration of inducible metabolism of 8-methoxypsoralen in rat liver microsomes and evidence for covalent binding to microsomal proteins. *Fed Proc* **44**: 1467, 1985.
7. Fouin-Fortunet H, Tinel M, Descatoire V, Letteron P, Larrey D, Geneve J and Pessayre D, Inactivation of cytochrome P-450 by the drug methoxsalen. *J Pharmacol Exp Ther* **236**: 237–247, 1986.
8. Mays DC, Nawoot S, Hilliard JB, Pacula CM and Gerber N, Inhibition and induction of drug biotransformation *in vivo* by 8-methoxypsoralen: Studies of caffeine, phenytoin and hexobarbital metabolism in the rat. *J Pharmacol Exp Ther* **243**: 227–233, 1987.
9. Mays DC, Camisa C, Cheney P, Pacula CM, Nawoot S and Gerber N, Methoxsalen is a potent inhibitor of the metabolism of caffeine in humans. *Clin Pharmacol Ther* **42**: 621–626, 1987.
10. Mays DC, Hecht SG, Unger SE, Pacula CM, Sharp DE and Gerber N, Disposition of 8-methoxypsoralen in the rat: Induction of metabolism *in vivo* and *in vitro* and identification of urinary metabolites by thermospray mass spectrometry. *Drug Metab Dispos* **15**: 318–328, 1987.
11. Halpert J and Neal RA, Inactivation of purified rat liver cytochrome P-450 by chloramphenicol. *Mol Pharmacol* **17**: 427–431, 1980.
12. Halpert J, Covalent modification of lysine during the suicide inactivation of rat liver cytochrome P-450 by chloramphenicol. *Biochem Pharmacol* **30**: 875–881, 1981.
13. Halpert J, Further studies of the suicide inactivation of purified rat liver cytochrome P-450 by chloramphenicol. *Mol Pharmacol* **21**: 166–172, 1982.
14. Halpert J, Naslund B and Betner I, Suicide inactivation of rat liver cytochrome P-450 by chloramphenicol *in vivo* and *in vitro*. *Mol Pharmacol* **23**: 445–452, 1983.
15. Sherry JH, O'Donnell JP, Flowers L, Lacagnin LB and Colby HD, Metabolism of spironolactone by adrenocortical and hepatic microsomes: Relationship to cytochrome P-450 destruction. *J Pharmacol Exp Ther* **236**: 675–680, 1986.
16. Bush MT, Design of solvent extraction methods. *Methods Enzymol* **77**: 353–372, 1981.
17. Mays DC, Rogers SL, Guiler RC, Sharp DE, Hecht SG, Staubus AE and Gerber N, Disposition of 8-methoxypsoralen in the rat: Methodology for measurement, dose-dependent pharmacokinetics, tissue distribution and identification of metabolites. *J Pharmacol Exp Ther* **236**: 364–373, 1986.
18. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
19. Eadie GS, On the evaluation of the constants V_m and K_M in enzyme reactions. *Science* **116**: 688, 1952.
20. Hofstee BHJ, On the evaluation of the constants V_m and K_M in enzyme reactions. *Science* **116**: 329–331, 1952.
21. Robard D, Munson PJ and Thakur AK, Quantitative characterization of hormone receptors. *Cancer* **46**: 2907–2918, 1980.
22. Statistical Consultants, Inc., PCNONLIN and NONLIN84: Software for the statistical analysis of nonlinear models. *Am Statistician* **40**: 52, 1986.
23. Levin W, Thomas PE, Reik LM, Wood AW and Ryan DE, Multiplicity and functional diversity of rat hepatic microsomal cytochrome P450 isozymes. In: *IUPHAR 9th International Congress of Pharmacology Proceedings* (Eds. Paton W, Mitchell J and Turner P), Vol. 3, pp. 203–209. Macmillan Press, London, 1984.
24. Oesch F, Kaubisch N, Jerina DM and Daly JW, Hepatic epoxide hydrase. Structure-activity relationships for substrates and inhibitors. *Biochemistry* **10**: 4858–4866, 1971.
25. Schmid J, Prox A, Reuter A, Zipp H and Koss FW, The metabolism of 8-methoxypsoralen in man. *Eur J Drug Metab Pharmacokinet* **5**: 81–92, 1980.
26. Corcoran GB, Mitchell JR, Vaishnav YN and Horning EC, Evidence that acetaminophen and *N*-hydroxyacetaminophen form a common arylating intermediate, *N*-acetyl-*p*-benzoquinoneimine. *Mol Pharmacol* **18**: 536–542, 1980.
27. Lau SS, Monks TJ and Gillette JR, Multiple reactive metabolites derived from bromobenzene. *Drug Metab Dispos* **12**: 291–296, 1984.
28. Smart G and Buckpitt AR, Formation of reactive naphthalene metabolites by target vs non-target tissue microsomes: Methods for the separation of three glutathione adducts. *Biochem Pharmacol* **32**: 943–946, 1983.
29. Esterbauer H, Ertl A and Scholz N, The reaction of cysteine with unsaturated aldehydes. *Tetrahedron* **32**: 285–289, 1975.
30. Wiebel FJ, Leutz JC, Diamond L and Gelboin HV, Aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase in microsomes from rat tissues: Differential inhibition and stimulation by benzoflavones and organic solvents. *Arch Biochem Biophys* **144**: 78–86, 1971.
31. Wiebel FJ, Metabolism of monohydroxybenzo(a)-pyrenes by rat liver microsomes and mammalian cells in culture. *Arch Biochem Biophys* **168**: 609–621, 1975.
32. Goujon FM, Nebert DW and Gielen JE, Genetic expression of aryl hydrocarbon hydroxylase induction. IV. Interaction of various compounds with different forms of cytochrome P-450 and the effect on

benzo[*a*]pyrene metabolism *in vitro*. *Mol Pharmacol* **8**: 667–680, 1972.
33. Guengerich FP and Liebler DC, Enzymatic activation of chemicals to toxic metabolites. *CRC Crit Rev Toxicol* **14**: 259–307, 1985.
34. Nelson SD, Metabolic activation and drug toxicity. *J Med Chem* **25**: 753–765, 1982.
35. Smith CV, Lauterburg BH and Mitchell JR, Covalent binding and acute lethal injury *in vivo*: How has the original hypothesis survived a decade of critical examination? In: *Drug metabolism and disposition: Considerations in Clinical Pharmacology* (Eds. Wilkinson GR and Rawlins MD), pp. 161–181. MTP Press, Boston, 1985.
36. Hilliard JB, Mays DC, Pacula CM and Gerber N, The time course of *in vivo* covalent binding of a reactive metabolite of 8-methoxypsoralen with mouse hepatic microsomal protein. *Fed Proc* **46**: 863, 1987.
37. Cheney P, Pacula CM, Mays DC and Gerber N, Dose-dependent elimination of 8-methoxypsoralen in the mouse. *Fed Proc* **45**: 336, 1986.
38. Letteron P, Descatoire V, Larrey D, Tinel M, Geneve J and Pessayre D, Inactivation and induction of cytochrome P-450 by various psoralen derivatives in rat. *J Pharmacol Exp Ther* **238**: 685–692, 1986.
39. Tinel M, Belghiti J, Descatoire V, Amouyal G, Letteron P, Geneve J, Larrey D and Pessayre D, Inactivation of human liver cytochrome P-450 by the drug methoxsalen and other psoralen derivatives. *Biochem Pharmacol* **36**: 951–955, 1987.
40. Wirth PJ, Bettis CJ and Nelson WL, Microsomal metabolism of furosemide. Evidence for the nature of the reactive intermediate involved in covalent binding. *Mol Pharmacol* **12**: 759–768, 1976.
41. Boyd MR, Burka LT, Wilson BJ and Sasame HA, *In vitro* studies on the metabolic activation of the pulmonary toxin, 4-ipomeanol, by rat lung and liver microsomes. *J Pharmacol Exp Ther* **207**: 677–686, 1978.
42. Ravindranath V, Burka LT and Boyd MR, Reactive metabolites from the bioactivation of toxic methylfurans. *Science* **224**: 884–886, 1984.
43. Manfredi K, Gingerich SB and Jennings PW, Peracid oxidation of 3,4,5-trialkylated furans: A novel ring expansion reaction of 3-methyldecahydrocyclododeca[*b*]furan. *J Org Chem* **50**: 535–537, 1985.
44. Dahlin DC, Miwa GT, Lu AYH and Nelson SD, *N*-acetyl-*p*-benzoquinone imine: A cytochrome P-450-mediated oxidation product of acetaminophen. *Proc Natl Acad Sci USA* **81**: 1327–1331, 1984.
45. Hilliard JB, Wong DD, Mays DC, Park SS, Gelboin HV and Gerber N, Covalent binding of 8-methoxypsoralen to microsomal proteins: Modification with cysteine and monoclonal antibodies to cytochrome P-450. *FASEB J* **2**: A1141, 1988.