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IN VITRO INHIBITION OF MOUSE HEPATIC MIXED-FUNCTION OXIDASE ENZYMES BY HELENALIN AND ALANTOLACTONE*†

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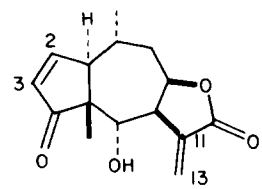
Abstract—The sesquiterpene lactones (STL) helenalin and alantolactone were effective *in vitro* inhibitors of the mouse hepatic microsomal mixed-function oxidase (MFO) enzymes, aminopyrine demethylase (APD), aniline hydroxylase (ANH) and 7-ethoxyresorufin deethylase (ERD). Helenalin and alantolactone concentrations of 0.5 mM produced a 50–60% inhibition of APD and ERD, and a 20–30% inhibition of ANH. An increase in substrate (aminopyrine) concentration from 0.5 to 25 mM decreased STL inhibition of APD by 12–32%. APD was also inhibited at low aminopyrine concentrations (0.5 mM) by the helenalin derivative 2,3,11,13-tetrahydrohelenalin (tetrahydrohelenalin). The STL produced type I binding spectra with oxidized microsomes; K_s values for helenalin and alantolactone were 161 and 9 μM respectively. These results suggest that STL inhibition of the MFO system results, in part, from STL binding to the substrate-binding site of cytochrome P-450. It has been reported that the irreversible alkylation of protein cysteinyl residues is responsible for STL inhibition of several different enzymes, and second-order rate constants for the reaction of helenalin and alantolactone with glutathione were 25.1 and 1.80 $mM^{-1} \cdot hr^{-1}$ respectively. Tetrahydrohelenalin did not react with glutathione. However, the subsequent addition of 3.0 mM thiols, i.e. L-cysteine, *N*-acetylcysteine or glutathione, to STL-treated (0.5 mM) microsomes reversed helenalin and alantolactone inhibition of APD and ERD by 50–80%. The ability of thiols to reverse STL inhibition of APD was decreased 20–43% by the coincubation of STL and microsomes with an NADPH-generating system. In addition, established effects of sulfhydryl-reactive compounds on the MFO system, i.e. inhibition of NADPH–cytochrome *c* reductase and conversion of cytochrome P-450 to cytochrome P-420, were not observed after addition of helenalin (1.0 mM) or alantolactone (0.5 mM) to mouse hepatic microsomes. These results suggest that STL inhibition of MFO enzymes may not be dependent upon the reactivity of the STL towards sulfhydryl groups. Instead, we suggest that STL binding to the substrate-binding site of cytochrome P-450 and subsequent metabolism of the STL may contribute to inhibition of the MFO system.

The sesquiterpene lactones, with over ninety different compounds associated with significant antitumor or cytotoxic activities, comprise one of the largest and most widely distributed groups of plant-derived antitumor compounds [1]. In addition, these compounds are potentially toxic; the consumption of sesquiterpene lactone-containing plants by livestock produces substantial mortality in sheep and cattle [2, 3], and human exposure to sesquiterpene lactones may result in the development of severe allergic contact dermatitis [4]. Investigations in our laboratory have established that treatment with the sesquiterpene lactone helenalin (Fig. 1) and related compounds significantly increase the lifespan of P-388 tumor-bearing mice [5], and inhibit the growth of Walker 256 carcinoma in rats and Ehrlich ascites tumors in mice [6]. These antineoplastic activities of



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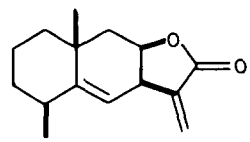

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Fig. 1. Structures of helenalin (I) and alantolactone (II).

the sesquiterpene lactones are dependent upon the presence of a sulfhydryl-reactive, α,β-unsaturated carbonyl group, O=C—C=CH [7, 8]. Reactivity towards sulfhydryl groups also appears to be an important prerequisite for the *in vitro* inhibition of

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phosphofructokinase and glycogen synthase by several different sesquiterpene lactones, and this inhibition is accompanied by a concomitant decrease in free thiol groups in these enzymes [9, 10].

We were interested in determining the effects of helenalin on the hepatic mixed-function oxidase system because of the critical role this system plays in the metabolism and disposition of many xenobiotic compounds. Recent experiments in our laboratory indicated that helenalin is an effective *in vivo* inhibitor of the mouse hepatic mixed-function oxidase system [11], and the sesquiterepene lactone alantolactone has been shown to inhibit the rat hepatic mixed-function oxidase system *in vitro* [12]. Because the mixed-function oxidase system is inhibited by other sulfhydryl-reactive compounds [13], it was assumed that inhibition by helenalin also occurred as a result of its sulfhydryl-reactive properties. However, the helenalin dosages used in our previous *in vivo* study were toxic, and it was unclear whether helenalin directly inhibited the mixed-function oxidase system or reduced mixed-function oxidase activities indirectly as a result of its systemic toxicity.

The present studies were designed to establish and characterize the *in vitro* inhibition of the mouse hepatic mixed-function oxidase system by the sesquiterpene lactones, helenalin and alantolactone. Known effects of sulfhydryl-reactive reagents upon the mixed-function oxidase system, i.e. inhibition of mixed-function oxidase enzyme activities [14], conversion of cytochrome P-450 to cytochrome P-420 [15], and inhibition of cytochrome P-450 reductase [16], were examined. The sulfhydryl-reactive compounds, *N*-ethylmaleimide and *p*-chloromercuribenzoate, were included in some studies because of their established ability to inhibit the mixed-function oxidase system *in vitro* [14]. Structure–activity relationships between helenalin and the helenalin derivatives, 11,13-dihydrohelenalin and 2,3,11,13-tetrahydrohelenalin, provided additional information about the mechanism responsible for sesquiterpene lactone inhibition of the mixed-function oxidase system.

METHODS

Chemicals. Helanalin was isolated from *Helenium microcephalum* [17]. 11,13-Dihydrohelenalin (plenolin) and 2,3,11,13-tetrahydrohelenalin (tetrahydrohelenalin) were prepared by catalytic reduction of helenalin as described previously [18]. The identity and purity of the isolated helenalin and of the prepared helenalin derivatives, plenolin and tetrahydrohelenalin, were established by elemental analysis, thin-layer chromatography, and NMR and IR spectral comparisons. Alantolactone is marketed by the Sigma Chemical Co. (St Louis, MO) as helenin (alantolactone), but will be referred to as alantolactone in this paper because of the similarity between the names helenin and helenalin. Aniline hydrochloride was obtained from the Eastman Kodak Co. (Rochester, NY). 7-Ethoxyresorufin was obtained from the Pierce Chemical Co. (Rockford, IL). 5,5-Dithiobis-(2-nitrobenzoic acid) (DTNB*) and resorufin were obtained from the Aldrich Chemical Co. (Milwaukee, WI). Carbon monoxide was obtained from the Union Carbide Corp. (Raleigh, NC). All other reagents were obtained from the Sigma Chemical Co. or the Fisher Chemical Co. (Raleigh, NC), and were reagent grade or better. All concentrations are final concentrations.

Animals. Male BDF_1 mice, bred from Charles River Breeding Laboratory stock (Wilmington, MA), weighing 20–27 g, were used in all studies. Mice were fed *ad lib.* and were provided with a 12-hr light–dark cycle.

Reactivity towards glutathione. Sulfhydryl-reagents (helenalin, plenolin, tetrahydrohelenalin, alantolactone, pCMB and NEM) were dissolved in 95% ethanol. Reactions were carried out at 22° in 0.1 M phosphate buffer, pH 7.4, and with approximately equal (400 μM) concentrations of both sulfhydryl-reagent and glutathione. After the appropriate incubation time, excess DTNB in phosphate buffer (2.0 mM) was added to oxidize the remaining, unreacted glutathione and stop the reaction. The extent of the reaction was determined spectrophotometrically by measuring the amount of reduced DTNB ($\varepsilon = 13{,}600$ at 412 nm) produced from the oxidation of the unreacted glutathione.

The order of the reaction between glutathione and sulfhydryl-reagent was determined graphically [19], and all sulfhydryl-reagents exhibited second-order kinetics. Except for helenalin, second-order rate constants (k_2) were calculated from the equation: $k_2 = (1/t)[1/(b-a)]\ln[b(a-x)/a(b-x)]$, where b and a are the starting amounts of the reactants and x is the amount of glutathione or sulfhydryl-reagent that has reacted at time (t).

A somewhat different procedure was used for the calculation of k_2 for helenalin. Because helenalin is a bifunctional molecule which can react with glutathione at both the α-methylene-γ-lactone and the α,β-unsaturated ketone moieties, an apparent k_2 for the reaction of helenalin with glutathione was obtained from the theoretical rate equations that describe two pairs of competitive and consecutive second-order reactions [20]. To solve these equations, it was assumed that the reaction of glutathione at one site on the helenalin molecule did not affect the reaction at the second site, and that the k_2 established for plenolin is an accurate estimate of the rate constant for the reaction of glutathione with the α,β-unsaturated ketone moiety of helenalin. Under these conditions, the rate constant for the reaction of glutathione with the α-methylene-γ-lactone moiety of helenalin can be estimated by nonlinear regression analysis of the simplified rate equation. The apparent overall rate constant for the reaction of helenalin with glutathione (k_2) was then calculated as the sum of the individual rate constants for the α-methylene-γ-lactone and α,β-unsaturated ketone moieties.

* Abbreviations: BDF_1, (C57BL/6 female X DBA/2 male)F_1; pCMB, *p*-chloromercuribenzoate; DTNB, 5,5-dithiobis-(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; A_{max}, maximal absorbance difference; and K_s, spectral dissociation constant.

Preparation of microsomes. Livers were perfused for 3–4 min with ice-cold 1.15% KCl, at a delivery rate of 9 ml/min, in a retrograde direction from the right atrium and through the inferior vena cava. Perfused livers were homogenized in 7 vol. of a 0.15 M KCl, 0.05 mM Tris homogenization buffer, pH 7.7. The liver homogenate and subsequent supernatant fractions were centrifuged at 1,000 *g* for 10 min, 24,000 *g* for 10 min, and 159,000 *g* for 45 min. The microsomal pellets from the last centrifugation were resuspended in the homogenization buffer and washed by centrifugation at 159,000 *g* for 45 min. All procedures were carried out at 0–4°. The final microsomal pellet was covered in a minimum volume of 0.1 M Tris buffer, pH 7.7, and stored for a maximum of 24 hr at −20°. Microsomal protein concentrations were determined by the method described previously [21].

Microsomal enzyme assays. Microsomes were resuspended in 0.1 M Tris buffer, pH 7.7, to a final microsomal protein concentration of 1.0 to 1.5 mg/ml. Aminopyrine demethylase and aniline hydroxylase activities were determined by measurement of formaldehyde production from 4-dimethylaminopyrine (aminopyrine) and *p*-aminophenol formation from aniline respectively [22]. 7-Ethoxyresorufin deethylase activities were determined by the fluorometric measurement of resorufin formation from 7-ethoxyresorufin, with the addition of 60 μM EDTA instead of bovine serum albumin [23]. Unless stated otherwise, product formation was measured after 5 min (7-ethoxyresorufin deethylase), 20 min (aniline hydroxylase) or 30 min (aminopyrine demethylase), and mixed-function oxidase activities were supported by an NADPH-generating system that consisted of glucose-6-phosphate (5.0 mM), $NADP^+$ (1.0 mM) and yeast glucose-6-phosphate dehydrogenase (2 units/ml). Final aminopyrine, aniline and 7-ethoxyresorufin concentrations (unless stated otherwise) were 4 mM, 9 mM and 1.5 μM respectively. Sulfhydryl-reagents were dissolved in 95% ethanol, and control incubations received an equal volume of 95% ethanol. Sulfhydryl-reagents, at concentrations used in these experiments, did not inhibit the reduction of $NADP^+$ to NADPH by yeast glucose-6-phosphate dehydrogenase or interfere with the measurement of formaldehyde, *p*-aminophenol or resorufin. However, alantolactone concentrations greater than 0.6 mM inhibited reduction of $NADP^+$ to NADPH by yeast glucose-6-phosphate dehydrogenase, concomitant with the formation of a white precipitate in the incubation medium.

Aminopyrine demethylase and aniline hydroxylase time–course experiments were started by the simultaneous addition of substrate and sesquiterpene lactone (0.5 mM), and the amount of product formed after incubation at 22° for 0–45 min was determined. Because 7-ethoxyresorufin deethylase is inhibited by its product resorufin [23], 7-ethoxyresorufin deethylase time–course experiments were conducted to minimize the amount of resorufin formed. Microsomes, NADPH-generating system, and sesquiterpene lactones were incubated at 22° for 0–45 min, before 7-ethoxyresorufin was added. Resorufin formation was then determined after an additional 5-min incubation with substrate. The time–course effects of sesquiterpene lactones on enzyme activities were compared to control incubations which received an equal volume of 95% ethanol and were incubated for a comparable length of time.

Since time–course experiments indicated that sesquiterpene lactone inhibition of enzyme activity reached a relatively constant level within 15–20 min, concentration–response experiments were started by addition of substrate after preincubation of sulfhydryl-reagents, microsomes, and NADPH-generating system for 15 min (aminopyrine demethylase and 7-ethoxyresorufin assays) or 20 min (aniline hydroxylase assays). The effects of substrate concentration on sesquiterpene lactone inhibition of aminopyrine demethylase followed a similar protocol, i.e. sesquiterpene lactones, NADPH-generating system and microsomes were preincubated at 22° for 15 min before the addition of 0.5 to 25 mM aminopyrine.

Reversibility of enzyme inhibition. Experiments designed to examine thiol reversal of sesquiterpene lactone inhibition of either aminopyrine demethylase of 7-ethoxyresorufin deethylase followed slightly different protocols. In the 7-ethoxyresorufin deethylase experiments, microsomes (1.0 to 1.5 mg protein/ml) in 0.1 M Tris buffer, pH 7.7, and sesquiterpene lactones (0.5 mM) were incubated at 22° for 30 min, thiols (3.0 mM) were then added, and the incubation was continued for an additional 30 min. 7-Ethoxyresorufin deethylase activities were determined after the final 30-min incubation. Sesquiterpene lactones were dissolved in 95% ethanol, and thiols were dissolved in distilled water; control incubations received equal volumes of the appropriate solvents. Aminopyrine demethylase experiments followed a similar protocol, except that microsomes were reisolated by centrifugation (159,000 *g* for 30 min at 4°) after incubation with thiol and before the measurement of aminopyrine demethylase activities. In some aminopyrine demethylase experiments, an NADPH-generating system (described above) was included in the initial 30-min incubation of sesquiterpene lactones with resuspended microsomes. Percent recovery of enzyme activity was calculated by normalizing the inhibition observed after addition of thiol and sesquiterpene lactone to the inhibition observed after addition of sesquiterpene lactone, i.e. $100 \times [1-(\%$ inhibition with sesquiterpene lactone and thiol)/(% inhibition with sesquiterpene lactone)].

Inhibition of NADPH–cytochrome c (*P*-450) *reductase.* The electron receptor cytochrome *c* was used in place of cytochrome P-450, the physiological electron receptor, for the measurement of NADPH–cytochrome *c* (P-450) reductase activity [24]. Microsomes were resuspended in 0.3 M potassium phosphate buffer, pH 7.7, to a final protein concentration of 0.1 to 0.2 mg/ml. Resuspended microsomes, cytochrome *c* (40 μM) and sulfhydryl-reagents were incubated at 22° for 0–45 min before the assay was started by the addition of NADPH (100 μM). Reduction of cytochrome *c* was measured spectrophotometrically at 550 nm ($\varepsilon = 0.021\ mM^{-1}\ cm^{-1}$), and rates of enzyme activity were determined for the first 2 min after addition of NADPH. Sulfhydryl-reagents were dissolved in 95% ethanol (helenalin, alantolactone and NEM) or 0.1 mM NaOH (pCMB), and control

Table 1. Interaction of sulfhydryl-reactive compounds with glutathione

Compound	k_2* ($mM^{-1} \cdot hr^{-1}$)
p-Chloromercuribenzoate	>24,000†
N-Ethylmaleimide	12,900 ± 1,000‡
Helenalin	25.1 ± 1.1§
Plenolin	6.88 ± 0.34
Alantolactone	1.80 ± 0.02
Tetrahydrohelenalin	0.0‖

* Second-order rate constants (k_2) were measured at 22° and pH 7.4.

† The second-order rate constant for *p*-chloromercuribenzoate exceeded the upper limit of detectability.

‡ Values are the means ± SD of three separate determinations.

§ The second-order rate constant for helenalin is an apparent rate constant, calculated from the sum of the rate constants for plenolin and the α-methylene-γ-lactone of helenalin (see Methods).

‖ No interaction of tetrahydrohelenalin with glutathione could be detected after 120 min.

incubations received an equal volume of the appropriate solvent; no difference in enzyme activity was observed between microsomes receiving 95% ethanol or 0.1 mM NaOH.

Sesquiterpene lactone interaction with cytochrome P-450. Cytochrome P-450 concentrations were determined as described previously [25]. Microsomes were resuspended in 0.1 M Tris buffer, pH 7.7, to a final protein concentration of 1.0 to 1.5 mg/ml. Resuspended microsomes were reduced with sodium dithionite and divided equally into sample and reference cuvettes. The sample cuvette was saturated with carbon monoxide, sulfhydryl-reagents were added to both sample and reference cuvettes, and carbon monoxide difference spectra were determined after incubation at 22° for 0–45 min. A 91 $mM^{-1} \cdot cm^{-1}$ millimolar extinction coefficient was used for the calculation of cytochrome P-450 contents. Sulfhydryl-reagents were dissolved in 95% ethanol (helenalin, alantolactone and NEM) or 0.1 mM NaOH (pCMB), and control incubations received an equal volume of the appropriate solvent; no difference in cytochrome P-450 content was observed between microsomes receiving 95% ethanol or 0.1 mM NaOH.

Sesquiterpene lactone binding to oxidized microsomal cytochrome P-450 was determined after resuspension of microsomes to 1.0 to 1.5 mg protein/ml in 0.1 M Tris buffer, pH 7.7. Resuspended microsomes (3 ml) were placed in both sample and reference cuvettes, and 5- to 10-μl aliquots of sesquiterpene lactone (helenalin, plenolin, tetrahydrohelenalin or alantolactone in 95% ethanol) were added sequentially to the sample cuvette; the reference cuvette received an equal volume of 95% ethanol. After each addition of sesquiterpene lactone, difference spectra from 375 nm to 500 nm were recorded. Double-reciprocal plots of sesquiterpene lactone concentration versus differences between absorbance maxima (at approximately 390 nm) and absorbance minima (at approximately 420 nm) of the difference spectra were fit to a straight line by least squares linear regression to obtain estimates of spectral dissociation constants (K_s) and maximal absorbance differences (A_{max}) for the sesquiterpene lactones [26].

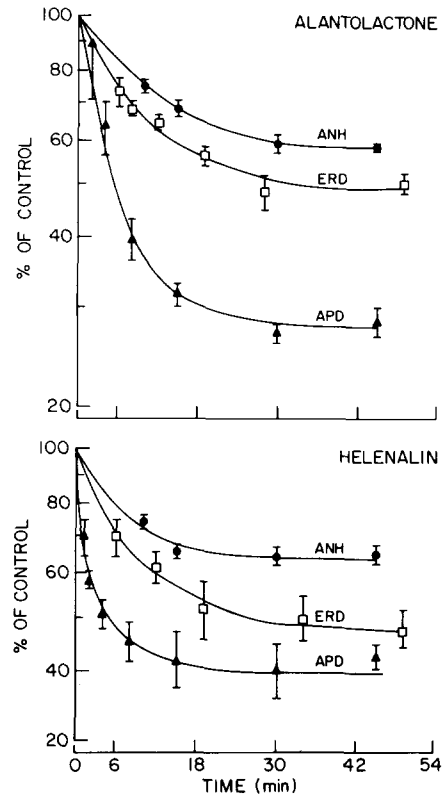

Fig. 2. Inhibition of microsomal enzyme activities by helenalin and alantolactone. Concentrations of helenalin and alantolactone were 0.5 mM. Values are the means ± SE of three determinations for aniline hydroxylase, ANH (●); 7-ethoxyresorufin deethylase, ERD (□); and aminopyrine demethylase, APD (▲). Enzyme activities, at 0 min and without inhibitor; aminopyrine demethylase, 7.83 nmol formaldehyde · min^{-1} · (mg protein)$^{-1}$; aniline hydroxylase, 0.613 nmol *p*-aminophenol · min · (mg protein)$^{-1}$; and 7-ethoxyresorufin deethylase, 72.1 pmol resorufin · min^{-1} · (mg protein)$^{-1}$.

Statistical analysis. Statistical significance was established by analysis of variance followed by Student's *t*-test between means as described by Gad and Weil [27]. Nonlinear regression analysis followed the procedure outlined previously [28].

RESULTS

Interaction with glutathione. NEM was 500- to 5000-fold more reactive towards glutathione than were the sesquiterpene lactones, and helenalin was 4-fold or 14-fold more reactive towards glutathione than were plenolin or alantolactone respectively (Table 1). The rate constant for the reaction of the α-methylene-γ-lactone of helenalin with glutathione (see Methods) was 18.2 $mM^{-1} \cdot hr^{-1}$, which represents less than a 3-fold difference between the

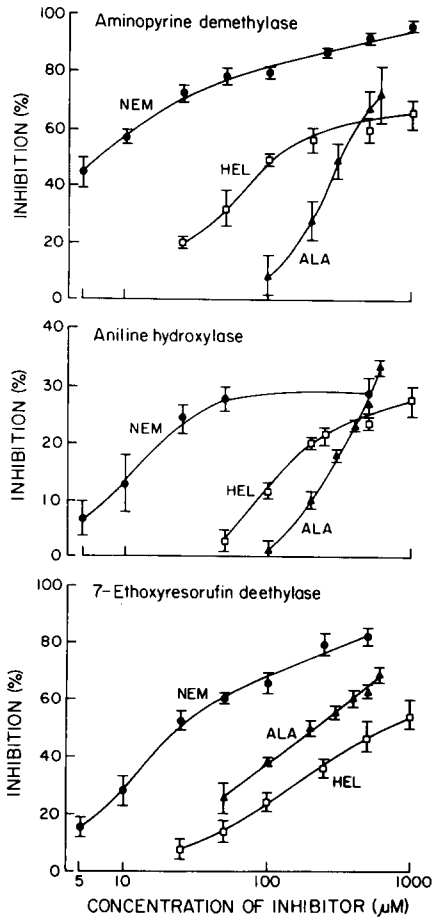

Fig. 3. Inhibition of microsomal enzyme activities by the sulfhydryl-reagents: *N*-ethylmaleimide, NEM (●), helenalin, HEL (□), and alantolactone, ALA (▲). Values are the means ± SE of three to five determinations.

reactivity of the α-methylene-γ-lactone and the α,β-unsaturated ketone ($6.9\ mM^{-1} \cdot hr^{-1}$ for plenolin) moieties of helenalin. Tetrahydrohelenalin did not react with glutathione at a measurable rate, and pCMB reacted at a rate too rapid to measure accurately.

Inhibition of mixed-function oxidase enzymes. Inhibition of mixed-function oxidase enzymes was quite rapid for both helenalin and alantolactone (Fig. 2). Inhibition of aminopyrine demethylase, 7-ethoxyresorufin deethylase and aniline hydroxylase to one-half the maximum inhibition (obtained after incubation for 15–20 min) required 1, 4, and 5 min of incubation for helenalin (0.5 mM), and 3, 6, and 8 min for alantolactone (0.5 mM) respectively.

Some specificity of the sesquiterpene lactones for individual mixed-function oxidase enzymes was apparent (Fig. 3). For example, 0.1 mM helenalin was a better inhibitor of aminopyrine demethylase and aniline hydroxylase, whereas 0.1 mM alantolactone was a better inhibitor of 7-ethoxyresorufin deethylase. In addition, aniline hydroxylase was less sensitive to sesquiterpene lactone inhibition than were aminopyrine demethylase and 7-ethoxyresorufin deethylase. Helenalin inhibition of enzyme activity appeared to reach a maximal percent inhibition (i.e. 60% inhibition of aminopyrine demethylase and 7-ethoxyresorufin deethylase or 30% inhibition of aniline hydroxylase) as helenalin concentrations approached 1.0 mM. However, alantolactone inhibition of enzyme activity continued to increase to the upper limit of alantolactone concentrations tested (0.6 mM). Alantolactone concentrations greater than 0.6 mM were not used in these studies because reduction of $NADP^+$ to NADPH by yeast glucose-6-phosphate dehydrogenase was inhibited significantly by alantolactone concentrations greater than 0.6 mM.

Sesquiterpene lactone inhibition of aminopyrine demethylase decreased approximately 20% when aminopyrine concentrations were increased from 0.5 to 25 mM (Table 2). In addition, the helenalin derivatives tetrahydrohelenalin and plenolin inhibited aminopyrine demethylase in these studies. Tetrahydrohelenalin, which did not react with glutathione (Table 1), inhibited aminopyrine demethylase only at the lowest aminopyrine concentration (0.5 mM). Plenolin, in which the α-methylene-γ-lactone moiety of helenalin was selectively saturated, inhibited aminopyrine demethylase at all substrate concentrations but the amount of inhibition produced by plenolin was 30–60% of that observed with equimolar (0.5 mM) concentrations of helenalin or alantolactone.

Reversibility of enzyme inhibition. Inhibition of both aminopyrine demethylase and 7-ethoxyresorufin deethylase by the sesquiterpene lactones could be partially reversed by the subsequent addition of thiols to sesquiterpene lactone-treated microsomes (Table 3). L-Cysteine, glutathione and *N*-acetylcysteine were all about equally effective, and sesquiterpene lactone inhibition of aminopyrine demethylase and 7-ethoxyresorufin deethylase was reversed by 50–80% in these studies. Initial experiments indicated that the presence of thiol compounds significantly stimulated aminopyrine demethylase but did not affect 7-ethyoxyresorufin deethylase (data not shown). However, when microsomes were separated from the incubation medium by centrifugation, presumably removing most of the added thiol, stimulation of aminopyrine demethylase was minimized.

The addition of an NADPH-generating system to microsomal incubations increased sesquiterpene lactone inhibition of aminopyrine demethylase by 10% (Table 4). In addition, the ability of thiols to reverse sesquiterpene lactone inhibition of aminopyrine demethylase was decreased by 20–43% when an NADPH-generating system was present. The addition of an NADPH-generating system to control microsomal incubations (i.e. incubations without added sesquiterpene lactone or thiol) resulted in a 32% decrease in aminopyrine demethylase activities. This decrease in enzyme activity was partially reversed by cysteine and glutathione, and aminopyrine demethylase activities were decreased by only 12% when thiols and NADPH were present.

Inhibition of NADPH–cytochrome c *(P-450) reductase.* NADPH–cytochrome *c* reductase was not inhibited by 1.0 mM helenalin or 0.5 mM alantolactone (Table 5). However, 0.5 mM NEM and

Table 2. Inhibition of aminopyrine demethylase by sesquiterpene lactones

	Aminopyrine demethylase activities [nmol formaldehyde · min^{-1} · (mg protein)$^{-1}$]			
	Aminopyrine			
	0.5 mM	2.0 mM	10.0 mM	25.0 mM
Control	2.90 ± 0.08*	6.37 ± 0.17	16.84 ± 0.17	21.62 ± 0.67
Helenalin†	0.85 ± 0.04 (29%)‡	2.43 ± 0.05 (38%)	7.41 ± 0.15 (44%)	8.83 ± 0.18 (41%)
Plenolin	1.56 ± 0.06 (54%)	4.11 ± 0.39 (64%)	13.19 ± 0.29 (78%)	16.85 ± 0.17 (78%)
Tetrahydrohelenalin	2.54 ± 0.08 (88%)	6.59 ± 0.10 (103%)	17.29 ± 0.26 (103%)	21.46 ± 0.02 (99%)
Alantolactone	0.40 ± 0.02 (14%)	1.37 ± 0.04 (21%)	6.20 ± 0.09 (37%)	9.84 ± 0.17 (46%)

* Values are the means ± SD of three or four measurements. All values are significantly different from controls at $P < 0.05$, except for tetrahydrohelenalin at 2.0, 10.0 and 25.0 mM aminopyrine.

† Sesquiterpene lactone concentrations were 0.5 mM.

‡ Values in parentheses are percent of control at the appropriate aminopyrine concentration.

0.05 mM pCMB inhibited NADPH–cytochrome *c* reductase by 58 and 100%, respectively. Inhibition of NADPH–cytochrome *c* reductase was evident within 2 or 30 min after the addition of pCMB or NEM respectively (data not shown).

Conversion of cytochrome P-450 to cytochrome P-420. Cytochrome P-450 concentrations were not affected by 1.0 mM helenalin, 0.5 mM alantolactone or 1.0 mM NEM (Table 5). Cytochrome P-450 concentrations were decreased 28% by 0.1 mM pCMB, and this decrease was accompanied by a concomitant increase in cytochrome P-420 concentrations. Decreased cytochrome P-450 concentrations were evident within 8 min after the addition of pCMB (data not shown).

Binding of sesquiterpene lactones to cytochrome P-450. Helenalin, plenolin, tetrahydrohelenalin and alantolactone produced type I difference spectra with oxidized microsomes; representative spectra for helenalin and alantolactone are presented in Fig. 4. Absorbance maxima and minima for type I spectra were observed at 386 to 392 nm and at 416 to 422 nm respectively (Table 6). A_{max} and K_s values, determined from double-reciprocal plots (Fig. 5), are summarized in Table 6. A_{max} values for helenalin, plenolin and tetrahydrohelenalin were very similar, but A_{max} for alantolactone was 1.7-fold higher than for helenalin. The K_s for alantolactone was at least 18-fold lower than K_s values for helenalin, plenolin or tetrahydrohelenalin.

DISCUSSION

The sesquiterpene lactone, helenalin, was an effective *in vitro* inhibitor of the mouse hepatic mixed-function oxidase system. This suggests that the previously reported *in vivo* inhibition of mouse hepatic mixed-function oxidase enzymes by helenalin [11] may have resulted from direct inhibition of enzyme activity, rather than the systemic toxicity associated with *in vivo* exposure to helenalin. Additional *in vivo* studies, in which a single dose of helenalin produced significant decreases in aminopyrine demethylase and aniline hydroxylase activities before overt signs of toxicity were apparent, i.e. within 1–2 hr post-exposure, support this conclusion [29].

Sesquiterpene lactone inhibition of mixed-function oxidase enzymes in the present study is comparable to the previously reported 70% inhibition of rat hepatic benzphetamine *N*-demethylase by 1.0 mM alantolactone [12]. Thus, the *in vitro* sensitivity of mixed-function oxidase enzymes to sesquiterpene lactones appears to be similar to that reported for other enzymes. For example, 0.5 mM helenalin inhibits acid phosphatase and aryl sulfatase by 33 and 76% [30], and sesquiterpene lactone concentrations of 1.0 mM inhibit several glycolytic enzymes by 50–80% [31].

It has been suggested that the biological activities of the sesquiterpene lactones result from the alkylation of critical protein sulfhydryl groups by a Michael-addition reaction [9, 10]. Alkylation of protein cysteinyl residues by the sesquiterpene lactones is assumed to be irreversible, since the subsequent addition of thiols to sesquiterpene lactone-treated enzymes could not reverse enzyme inhibition [9, 31]. Inhibition of the mixed-function oxidase system by various sulfhydryl-reagents, usually as a result of the inhibition of NADPH–cytochrome P-450 reductase [16] or conversion of cytochrome P-450 to cytochrome P-420 [15], has been firmly established. We assumed that sesquiterpene lactone inhibition of mixed-function oxidase enzymes also resulted from the alkylation of sulfhydryl groups critical to mixed-function oxidase activity. However, the present studies suggest that the irreversible alkylation of sulfhydryl groups may not be the sole determinant of sesquiterpene lactone inhibition of the mixed-function oxidase system. This conclusion is based on the following observations: (i) helenalin and alantolactone inhibition of mixed-function oxidase enzymes could be reversed by the addition of thiols to sesquiterpene lactone-treated microsomes, (ii) tetrahydrohelenalin, which was not reactive towards glutathione, inhibited aminopyrine demethylase at low substrate concentrations, and (iii) helenalin and alantolactone did not inhibit NADPH–cytochrome *c* reductase or convert cytochrome P-450 to cytochrome P-420.

The decrease in sesquiterpene lactone inhibition of aminopyrine demethylase produced by increasing aminopyrine concentrations suggested that the sesquiterpene lactones interacted with the substrate-binding site of cytochrome P-450 and that this inter-

Table 3. Reversal of helenalin and alantolactone inhibition of aminopyrine demethylase and 7-ethoxyresorufin deethylase by thiols

	Aminopyrine demethylase Activity [nmol formaldehyde · min^{-1} · (mg protein)$^{-1}$]	Recovery* (%)	7-Ethoxyresorufin deethylase Activity [pmol resorufin · min^{-1} · (mg protein)$^{-1}$]	Recovery (%)
Control	7.78 ± 1.30†		50.4 ± 9.8	
+CYS‡	6.29 ± 1.83 (81%)§		45.2 ± 9.8 (90%)	
+GSH	9.31 ± 1.28 (120%)		46.9 ± 9.6 (93%)	
+NAC	ND‖		48.5 ± 10.1 (96%)	
Helenalin¶	2.89 ± 0.78 (37%)		25.6 ± 4.2 (51%)	
+CYS	4.42 ± 1.72 (70%)	52	40.4 ± 4.3 (89%)	78
+GSH	6.75 ± 2.05 (73%)	57	38.2 ± 5.8 (81%)	61
+NAC	ND		40.4 ± 4.1 (83%)	65
Alantolactone	1.55 ± 0.14 (20%)		24.9 ± 1.9 (49%)	
+CYS	5.02 ± 0.57 (80%)	75	39.0 ± 6.8 (86%)	73
+GSH	6.46 ± 1.12 (69%)	61	38.1 ± 3.3 (81%)	63
+NAC	ND		39.6 ± 5.4 (82%)	65

* Percent recovery of enzyme activity relative to the inhibition observed without added thiol.
† Values are the means ± SD of three separate experiments.
‡ Final L-cysteine (CYS), glutathione (GSH) and *N*-acetylcysteine (NAC) concentrations were 3.0 mM.
§ Values in parentheses are the percent of appropriate control with or without thiol.
‖ ND: not determined
¶ Final sesquiterpene lactone concentrations were 0.5 mM.

Table 4. Reversal of helenalin and alantolactone inhibition of aminopyrine demethylase by thiols: preincubation with NADPH-generating system

	Without NADPH		With NADPH		
	Activity [nmol formaldehyde · min^{-1} · (mg protein)$^{-1}$]	Recovery† (%)	Activity [nmol formaldehyde · min^{-1} · (mg protein)$^{-1}$]	Recovery (%)	Difference* (%)
Control	7.35 ± 0.30‡		4.95 ± 0.44		
+ CYS§	7.25 ± 0.54 (99%)‖		6.35 ± 0.46 (128%)		+29
+ GSH	8.69 ± 0.08 (118%)		7.64 ± 0.51 (154%)		+36
Helenalin¶	2.70 ± 0.05 (37%)		1.39 ± 0.29 (28%)		+9
+ CYS	4.75 ± 0.19 (67%)	46	2.43 ± 0.28 (38%)	14	−32
+ GSH	6.58 ± 0.03 (66%)	62	3.21 ± 0.16 (42%)	19	−43
Alantolactone	3.37 ± 0.05 (46%)		1.76 ± 0.22 (36%)		+10
+ CYS	5.84 ± 0.57 (81%)	65	3.74 ± 0.34 (51%)	36	−29
+ GSH	6.90 ± 1.12 (79%)	61	4.72 ± 0.38 (62%)	41	−20

* Difference between percent recovery (thiol added) with and without NADPH, or difference between percent inhibition (thiol absent) with and without NADPH.

† Recovery of enzyme activity relative to the inhibition observed without added thiol.

‡ Values are the means ± SD of three separate experiments.

§ Final L-cysteine (CYS) and glutathione (GSH) concentrations were 3.0 mM.

‖ Values in parentheses are percent of appropriate control with or without thiol.

¶ Final sesquiterpene lactone concentrations were 0.5 mM.

Table 5. Effects of sulfhydryl-reactive compounds on NADPH–cytochrome *c* reductase and cytochrome P-450*

	Concn (mM)	NADPH–cytochrome *c* reductase [nmol cytochrome *c* reduced·min^{-1} (mg protein)$^{-1}$]	Cytochrome P-450 [nmol·(mg protein)$^{-1}$]
Control		65.6 ± 2.6†	0.641 ± 0.011‡
Helenalin	1.0	59.5 ± 1.4 (91%)§	0.642 ± 0.054 (100%)
Alantolactone	0.5	62.9 ± 0.2 (96%)	0.693 ± 0.044 (108%)
N-Ethylmaleimide			
	0.5	27.2 ± 0.3 (42%)	ND
	1.0	ND‖	0.653 ± 0.036 (102%)
p-Chloromercuribenzoate			
	0.05	0.0 ± 0.0¶ (0%)	ND
	0.1	ND	0.458 ± 0.044 (72%)

* Effects were determined after incubation of mouse hepatic microsomes with sulfhydryl-reactive compounds for 45 min at 22°.

† Values are the means ± range of two determinations.

‡ Values are the means ± SD of four to five determinations.

§ Values in parentheses are percent of appropriate control.

‖ ND: not determined.

¶ *p*-Chloromercuribenzoate inhibited NADPH–cytochrome *c* reductase by 100% within 2 min after addition.

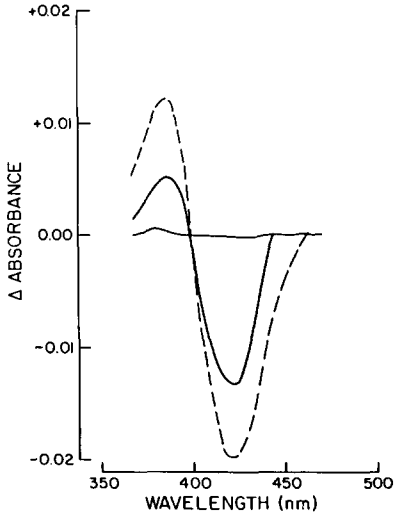

Fig. 4. Interaction of helenalin (——) and alantolactone (-----) with oxidized microsomes. Final sesquiterpene lactone concentrations were 1.5 and 0.025 mM for helenalin and alantolactone respectively. Microsomal protein concentrations were 1.5 mg/ml.

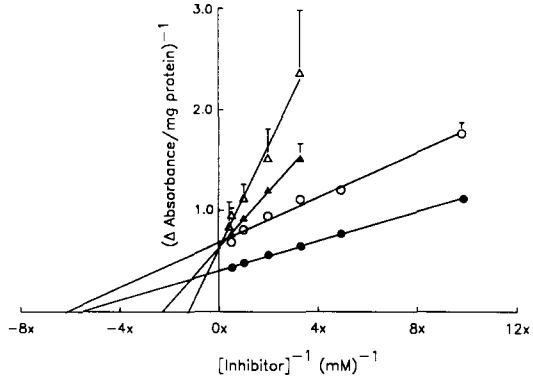

Fig. 5. Double-reciprocal plot of changes in absorbance produced by sesquiterpene lactone interaction with oxidized microsomes. Absorbance differences for helenalin (○), tetrahydrohelenalin (▲), plenolin (△) and alantolactone (●) were calculated between absorbance maxima (386 to 392 nm) and minima (416 to 422 nm). Inhibitor concentration scale: 1 × (mM^{-1}) for helenalin, tetrahydrohelenalin and plenolin; 20 × (mM^{-1}) for alantolactone. Values are the means ± SE of three determinations for helenalin and alantolactone, and the means ± range of two determinations for tetrahydrohelenalin and plenolin. Values without error bars: standard error or range less than size of symbol.

action was responsible, in part, for inhibition of enzyme activity. The interaction of the sesquiterpene lactones with the substrate-binding site of cytochrome P-450 was confirmed by the formation of type I difference spectra between the sesquiterpene lactones and oxidized microsomes. Type I difference spectra are characteristic of substrate binding to cytochrome P-450, and the K_s values of 161 μM for helenalin and 9 μM for alantolactone are comparable to K_s values for a variety of other cytochrome P-450 substrates [32]. The greater affinity of alantolactone for cytochrome P-450, as indicated by the lower K_s value, may have resulted from structural differences between helenalin and alantolactone and a better fit of alantolactone to the substrate-binding site of cytochrome P-450.

Some specificity of the sesquiterpene lactones for cytochrome P-450 isozymes was suggested by the present studies. For example, alantolactone interacted with 70% more cytochrome P-450, based on A_{max} values, than did helenalin, and this may have resulted from the presence of cytochrome P-450 isozymes with which alantolactone but not helenalin interacted. Helenalin and alantolactone also exhibited some selectivity for the *in vitro* inhibition of particular mixed-function oxidase enzyme activities. At low (0.1 mM) sesquiterpene lactone concentrations helenalin was a more effective inhibitor of aminopyrine demethylase, whereas alantolactone was a more effective inhibitor of 7-ethoxyresorufin

Table 6. Spectral constants for the interaction of sesquiterpene lactones with oxidized microsomes*

	K_s (μM)	A_{max} [Δ absorbance · mg protein)$^{-1}$]	Wavelength (nm)	
			Maximum	Minimum
Helenalin	161	1.46	392	422
Plenolin	793	1.57	391	421
Tetrahydrohelenalin	435	1.58	386	416
Alantolactone	9	2.47	390	421

* Spectral constants were derived from the data presented in Fig. 4. K_s, spectral dissociation constant; A_{max}, maximal absorbance differences.

deethylase. This selectivity may also represent a specificity of the sesquiterpene lactones for particular cytochrome P-450 isozymes. Multiple isozymic forms of mouse hepatic cytochrome P-450 which differ in their substrate specificity have been identified [33].

We suggest that sesquiterpene lactone binding to the substrate-binding site of cytochrome P-450 is the initial event leading to inhibition of the mixed-function oxidase system. This interaction appears to be reversible by subsequent addition of thiols and may account for a substantial portion of the total inhibition produced by the sesquiterpene lactones. However, the present results also suggest that sesquiterpene lactone inhibition of the mixed-function oxidase system includes an NADPH-dependent, irreversible component. For example, preincubation of microsomes and sesquiterpene lactones with an NADPH-generating system decreased by approximately 31% the ability of thiols to reverse sesquiterpene lactone inhibition of aminopyrine demethylase. The NADPH-dependent metabolism of the sesquiterpene lactones by cytochrome P-450 to an irreversible inhibitor of the mixed-function oxidase system could explain this effect. Thus, the sesquiterpene lactones may function as suicide substrates for cytochrome P-450, in which the formation of a sesquiterpene lactone metabolite results in the irreversible inhibition of the mixed-function oxidase system.

We cannot exclude the possibility that sesquiterepene lactone alkylation of cysteinyl residues at or near the substrate-binding site of cytochrome P-450 contributes to inhibition of the mixed-function oxidase system. Structure–activity comparisons between helenalin, plenolin and tetrahydrohelenalin suggests a positive relationship between their reactivity towards glutathione and inhibition of aminopyrine demethylase. Microsomal cytochrome P-450 contains seven cysteinyl residues [34], and bacterial and mitochondrial cytochromes P-450 appear to have cysteinyl residues in close proximity to their substrate binding sites [35, 36]. However, it is difficult to explain why the Michael-addition reaction between the sesquiterpene lactones and the mixed-function oxidase system should be uniquely reversible or how NADPH would contribute to this reaction.

In summary, sesquiterpene lactone inhibition of the mixed-function oxidase system appeared to result, in part, from sesquiterpene lactone binding to the substrate-binding site of cytochrome P-450. Circumstantial evidence suggests that the sesquiterpene lactones may also be metabolized to irreversible inhibitors of the mixed-function oxidase system. The nature of the putative sesquiterpene lactone metabolite(s) and the mechanism(s) responsible for the irreversible inhibition of the mixed-function oxidase system are unknown. The hypothesis that a sesquiterpene lactone metabolite contributes to inhibition of the mixed-function oxidase system must be considered tentative at this time. Metabolism of the sesquiterpene lactones has received little attention, presumably because of the assumed dependence of their biological activities on the sulfhydryl-reactive properties of the α,β-unsaturated carbonyl moiety. The further characterization of sesquiterpene lactone inhibition of the mixed-function oxidase system is currently under investigation in our laboratory.

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