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ORIGINAL ARTICLE

Merrill J. Egorin · D. Marc Rosen · Sara E. Benjamin
Patrick S. Callery · Dorothy L. Sentz · Julie L. Eiseman

In vitro metabolism by mouse and human liver preparations of halomon, an antitumor halogenated monoterpene

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Abstract *Objectives*: To characterize the enzymes responsible for and metabolites produced from the metabolism of halomon, a halogenated monoterpene that is isolated from the red algae *Portieria hornemanii* and has in vitro activity in the NCI screen against brain, renal, and colon cancer cell lines. *Materials and methods*: Mouse and human liver fractions, prepared by homogenization and differential centrifugation, were incubated with halomon, extracted with toluene, and analyzed by gas chromatography. *Results*: In the presence of NADPH, mouse-liver 9,000-*g* supernatant (S9) fractions metabolized halomon, but boiled S9 fractions did not. NADH could not substitute for NADPH. Further separation of murine hepatic S9 fractions produced a microsomal fraction that contained all of the halomon-metabolizing activity; cytosol had none. Carbon monoxide reduced murine hepatic microsomal metabolism of halomon, whereas an anaerobic, N_2 environment greatly accelerated the disappearance of halomon. Human hepatic microsomes metabolized halomon and required NADPH to do so. Carbon monoxide completely inhibited human hepatic microsomal metabolism of halomon. Unlike murine hepatic microsomal metabolism of halomon, anaerobic conditions did not enhance the metabolism of halomon by human hepatic microsomes. Neither 100 μM diethyldithiocarbamate, 1 μM quinidine, 100 μM ciprofloxacin, 3 μM ketoconazole, nor 100 μM sulfinpyrazone inhibited the metabolism of halomon by human hepatic microsomes. Both murine and human hepatic microsomes produced a metabolite of halomon. The mass spectrum of this metabolite indicated the loss of one chlorine atom and one bromine atom. *Conclusions*: Halomon is metabolized by mouse and human hepatic cytochrome P-450 enzymes, the identities of which remain unknown. Hepatic metabolism of halomon is very consistent with the concentrations of halomon measured in mouse tissues and urine after i.v. administration of the drug.

Key words Halomon · Natural products · Halogenated monoterpenes · Cytochrome P450

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M.J. Egorin (✉) · D.M. Rosen · S.E. Benjamin · P.S. Callery · D.L. Sentz · J.L. Eiseman
Division of Developmental Therapeutics,
University of Maryland Cancer Center,
Bressler 9-024, 655 West Baltimore Street,
Baltimore, MD 21201, USA

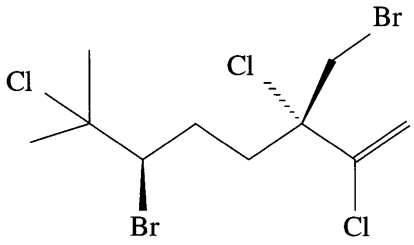
M.J. Egorin
Division of Hematology/Oncology,
Department of Medicine,
University of Maryland School of Medicine,
Baltimore, Maryland, USA

P.S. Callery
Department of Pharmaceutical Sciences,
University of Maryland School of Pharmacy,
Baltimore, Maryland, USA

J.L. Eiseman
Department of Pathology,
University of Maryland School of Medicine,
Baltimore, Maryland, USA

Introduction

Rhodophyta (red algae) are known to produce halogenated monoterpenes [11]. One of these, 6(*R*)-bromo-3(*S*)-(bromomethyl)-7-methyl-2,3,7-trichloro-1-octene-(halomon, NSC650893; Fig. 1), has been isolated from the red algae *Portieria hornemannii* [6] and has demonstrated interesting activity when tested in the National Cancer Institute's (NCI) human tumor, disease-oriented in vitro screen [5]. When analyzed in computerized pattern-recognition studies using the COMPARE algorithm [1, 10], these in vitro studies, indicated that the mechanism of action of halomon was different from that of an electrophilic alkylating agent. In addition, the screening profile of halomon had little, if any, substantial resemblance to that of any known mechanistic or structural class of cytotoxic agents in the NCI data base.



6(*R*)-Bromo-3(*S*)-bromomethyl-7-methyl-2,3,7-trichloro-1-octene

Fig. 1 Chemical structure of halomon

Moreover, the observation that cell lines relatively more sensitive to halomon clustered among renal, brain, colon, and non-small-cell lung cancer panels, whereas relatively less sensitive lines clustered among leukemia and melanoma panels was highly unusual. The combination of a potentially unique mechanism of action and preferential cytotoxicity to cell lines derived from highly chemoresistant human tumors led to in vivo evaluation of the antitumor activity and pharmacokinetics of halomon in mice. Previous studies in our laboratory have demonstrated minimal urinary excretion of halomon by mice given large i.v. doses of the compound [4]. In addition, after i.v. injection of halomon, concentrations of halomon in liver were low relative to those in other tissues [4]. These observations and the structure of halomon led us to investigate the potential for hepatic metabolism of halomon. Those studies form the basis of the current report.

Materials and methods

Reagents

Halomon was obtained from the Developmental Therapeutics Program, NCI (Bethesda, Md., USA). NADPH, NADH, (+)3,9-dibromocamphor (internal standard), sodium diethyldithiocarbamate, quinidine hydrochloride monohydrate, and (±)sulfinpyrazone were obtained from Sigma Chemical Co. (St. Louis, Mo.). Ciprofloxacin hydrochloride was obtained from Bayer Corp., Diagnostics Division (Kankakee, Ill.). Toluene, Baker analyzed grade, was obtained from J.T. Baker (Phillipsburg, N.J.). Ketoconazole was graciously provided by Dr. Jerry Collins, CDER, FDA (Rockville, Md.). Coomassie blue protein-determination kits were purchased from Pierce (Rockford, Ill.).

Mice

Specific-pathogen-free, adult, male CD_2F_1 mice (5–6 weeks of age) were obtained from the Animal Program administered by the Animal Genetics and Production Branch of the NCI. Mice were allowed to acclimate to the University of Maryland Animal Facility for at least 1 week before studies were initiated. To minimize exogenous infection, mice were maintained in conventional cages in a separate room and handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH 85-23, 1985). Ventilation and air flow in the Animal Facility were set to 12 changes/h. Room temperatures were regulated at 72 ± 2 °F, and the rooms were kept on automatic 12-h light/dark cycles. Mice received Purina 5001 Chow and water ad libitum. Sentinel mice (CD-1 mice housed in cages containing one-fifth of the bedding removed from study-mouse cages at cage change) were maintained in the animal room and assayed at monthly intervals for specific murine pathogens by the murine antibody profile (MAP) test (Litton Bionetics, Charleston, S.C.). These mice remained free of specific pathogens throughout the study period, indicating that the study mice were free of specific pathogens.

Human livers

Human liver samples (HL-5, HL-6, HL-7, and HL-13), medically unsuitable for transplantation, were acquired under the auspices of the Washington Regional Transplant Consortium (Washington, D.C.). Upon acquisition, livers were immediately sectioned and stored at −70 °C until used. All results in this report were generated with liver HL-13.

Preparation of mouse hepatic fractions

Mice were euthanized by carbon dioxide asphyxiation and exsanguinated by cardiac puncture, and their livers were rapidly removed and placed onto ice. Mouse livers were aseptically homogenized in sterile 100 m*M* sodium-potassium phosphate buffer (pH 7.4; 3 ml buffer: 1 g liver) using a Dounce homogenizer. The homogenate was transferred to 14-ml polypropylene test tubes and centrifuged at 9,000 *g* for 20 min at 4 °C. The resulting supernatant was decanted, returned to its original volume with 100 m*M* sodium-potassium phosphate buffer, and stored on ice until used. For some experiments, portions of the mouse 9000-*g* supernatant fraction were transferred to sterile glass test tubes measuring 16 × 150 mm, which were capped with parafilm and placed in a boiling water bath for 10 min. Microsomal and cytosolic fractions of murine livers were prepared by centrifugation of the mouse 9,000-*g* supernatant fraction at 100,000 *g* for 60 min at 4 °C. The supernatant, designated as the cytosolic fraction, was decanted into 14-ml polypropylene tubes and placed onto ice. The microsomal pellet was resuspended to its original volume in 100 m*M* sodium-potassium phosphate buffer and placed onto ice. The protein concentration of each hepatic fraction was determined by the Coomassie blue method [2]. All mouse hepatic fractions were either used on the same day on which they were prepared or stored overnight at −70 °C.

Preparation of human liver microsomes

Human liver microsomes were prepared as described by Jamis-Dow et al. [8]. In brief, 70 g liver was mixed with 450 ml buffer A (154 m*M* KCl, 100 m*M* sodium phosphate, 1 m*M* EDTA, pH 7.4) and homogenized in a blender. After filtration through gauze, the preparation was homogenized with a Dounce homogenizer and centrifuged twice at 13,000 *g* for 20 min at 4 °C, the pellet being discarded after the first centrifugation. The resulting supernatant was filtered through gauze and centrifuged at 105,000 *g* for 60 min at 4 °C. The microsomal pellet produced was resuspended in buffer B (5 m*M* $MgCl_2$, 100 m*M* sodium phosphate, 1 m*M* EDTA, pH 7.4), centrifuged for a second time at 105,000 *g* for 60 min at 4 °C, and resuspended again in buffer B. The protein concentration of the microsomal fraction was determined by the Coomassie blue method [2], after which it was adjusted to 10 mg/ml with buffer B. The final microsomal preparation was dispensed into single-use vials and stored at −70 °C until use.

Incubation of hepatic preparations with halomon

Aerobic incubations were performed in uncapped, 20-ml polyethylene scintillation vials, whereas anaerobic or CO-poisoned incubations were performed in 10-ml glass serum vials that had been fitted with rubber stoppers after being percolated and prefilled with N_2 or CO, respectively. Incubation vessels were arranged in a Dubnoff shaking water bath set at 37 °C and 32–36

oscillations/min. Microsomal fractions used in anaerobic or CO-poisoned incubations were percolated with N_2 or CO, respectively, for 5 min before being mixed with other incubation constituents. Incubation mixtures were of 1 ml volume and contained 0.5 ml mouse hepatic fraction or 0.1 ml human microsomes (diluted to 0.5 ml with 100 m*M* sodium-potassium phosphate buffer, pH 7.4), 0.1 ml 100 m*M* $MgCl_2$, 0.1 ml 10 m*M* NADPH or 10 m*M* NADH, and 0.3 ml 100 m*M* sodium-potassium phosphate buffer (pH 7.4). Reaction mixtures were prewarmed for 5 min, after which reactions containing mouse hepatic fractions were initiated by the addition of 50 µl of a 200-µg/ml (0.5 m*M*) solution of halomon in ethanol and reactions containing human microsomes were initiated by the addition of 1 µl of a 1- or 3-mg/ml (2.5 or 7.5 m*M*) solution of halomon in dimethylsulfoxide. After incubation for 0, 5, or 10 min, reactions were terminated by the addition of 0.1 ml internal standard (4 µg/ml in 50% methanol) and 1 ml toluene. After being shaken for 5 min, the entire contents of each vial were transferred to 2.0-ml polypropylene microfuge tubes and centrifuged at 13,000 *g* for 5 min. The resulting upper organic layers were transferred with glass Pasteur pipets into glass autosampler vials, and 1 µl of each supernatant was injected by autosampler into the gas chromatography system described below.

In experiments evaluating the effects of specific inhibitors on the metabolism of halomon by human hepatic microsomes, 100 µ*M* diethyldithiocarbamate, 100 µ*M* ciprofloxacin, or 1 µ*M* quinidine was introduced into reaction mixtures by the addition of 10 µl of 100-fold concentrated solutions of drug in distilled water. Similarly, 3 µ*M* ketoconazole was introduced by the addition of 10 µl of a 100-fold concentrated solution of drug in 0.05 *M* HCl, and 100 µ*M* sulfinpyrazone was introduced by the addition of 1 µl of a 1,000-fold concentrated solution in dimethylsulfoxide [8]. Inhibitors were incubated with the reaction mixture for at least 5 min before the introduction of halomon. Parallel control incubations involved appropriate volumes of vehicle without drug addition.

Analysis of halomon

Toluene extracts were analyzed with a Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, Palo Alto, Calif.) fitted with a 10-m HP-5 (cross-linked 5% phenyl methyl silicone) column (0.53-mm inside diameter, 2.65-µm film thickness) and a Hewlett-Packard 7673A autosampler. Injection was splitless, with a purge time of 1 min, and the injector port was maintained at 250 °C. The oven temperature was maintained at 100 °C for 1 min and then increased at 20 °C/min to 250 °C, which was held for 3 min. The carrier gas was helium at 3 psig, and the makeup gas was argon:methane (95:5). Column effluent was monitored with an electron capture detector maintained at 300 °C, and the detector signal was processed with a Hewlett-Packard 3392A integrator so as to integrate the area under each peak. The halomon concentration in each sample was calculated by determination of the ratio of halomon peak area to that of the corresponding internal standard peak and comparison of that ratio with a concomitantly performed standard curve prepared in the appropriate matrix. Under these conditions, the retention times of internal standard and halomon were approximately 6.8 and 7.9 min, respectively (Fig. 2). The quantitative performance of this assay in our laboratory has been described elsewhere [4].

Calculation of rates of halomon disappearance

Halomon concentration versus time data were plotted and fit with linear regression analysis. The rate of halomon disappearance was defined as the slope of the line determined by this linear regression analysis. Rates of halomon disappearance were normalized on a milligram-of-protein basis.

Analysis of a potential halomon metabolite

Toluene extracts of incubation mixtures were analyzed with a Hewlett-Packard 5890 gas chromatograph fitted with a 12-m HP-1 (cross-linked methyl silicone gum) column (0.2-mm inside diameter, 0.33-µm film thickness) and an HP 5970 Mass Selective Detector. Injection was splitless and the injector port temperature was maintained at 250 °C. The oven temperature was programmed to increase from 100° to 250 °C at 20 °C/min, the carrier gas was helium, and the transfer line temperature was 250 °C. The Mass Selective Detector operated over a scan range of 50–500 amu with the electron multiplier set at 1,800 V.

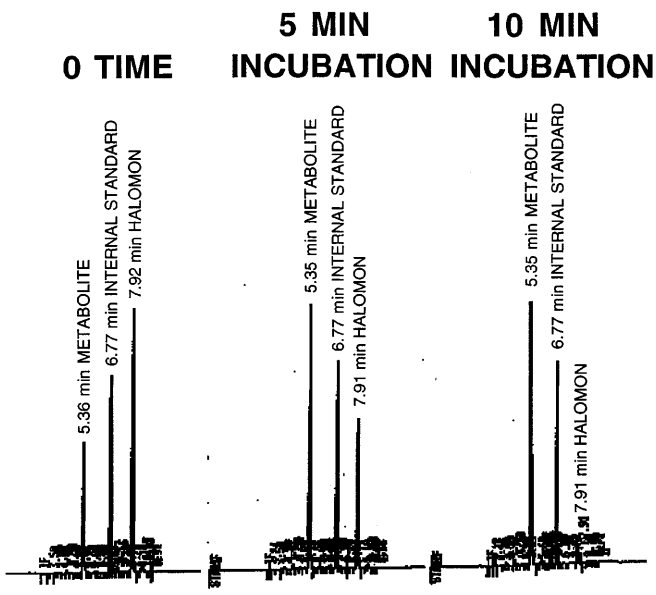


Fig. 2 Gas chromatography tracings of toluene extracts of mixtures of halomon and mouse hepatic microsomes incubated under anaerobic conditions for 0, 5 or 10 min

Results

Halomon metabolism by mouse hepatic preparations

Under aerobic conditions, murine hepatic 9,000-*g* supernatant fractions caused the disappearance of halomon from the incubation medium (Table 1). This disappearance required the presence of NADPH. NADH could not substitute for NADPH. When serially diluted 9,000-*g* supernatant fractions were used as the enzyme source in reaction mixtures, the rate of disappearance of halomon decreased in a manner roughly proportional to dilution (data not shown). Boiling of the 9,000-*g* supernatant fractions destroyed their ability to metabolize halomon (Table 1). Further separation of murine hepatic preparations into microsomal and cytosolic fractions showed halomon metabolism to reside entirely in the microsomal fraction (Table 1). Microsomally mediated disappearance of halomon was inhibited by CO (Table 2) but was accelerated under anaerobic (N_2) conditions (Table 2).

Under both aerobic and anaerobic conditions, a peak with a retention time of approximately 5.4 min was detected in the gas chromatogram (Fig. 2). Under aerobic conditions, the area of this peak increased slightly with time, even though the size of the corresponding halomon

Table 1 Aerobic metabolism of halomon by mouse hepatic fractions[a]

Hepatic fraction	Cofactor	Rate[b] (nmol min^{-1} mg $protein^{-1}$)
None	NADPH	0
9,000-*g* supernatant	None	0
9,000-*g* supernatant	NADPH	0.12–0.24
9,000-*g* supernatant	NADH	0
9,000-*g* supernatant (boiled)	NADPH	0
Microsomes	NADPH	1.44–2.48
Microsomes	None	0
Cytosol	NADPH	0

[a]Incubation reactions contained halomon at 10 μg/ml (25 μ*M*), 0.5 ml hepatic fraction, 0.1 ml 100 m*M* $MgCl_2$, 0.1 ml 10 m*M* NADPH or NADH, and 0.3 ml 100 m*M* sodium-potassium phosphate buffer (pH 7.4)

[b]Data represent the range of values from 2–4 experiments, each performed in triplicate

Table 2 Halomon metabolism by mouse hepatic microsomes in aerobic, carbon monoxide, or nitrogen atmospheres

Condition[a]	Rate[b] (nmol min^{-1} mg $protein^{-1}$)
Aerobic	0.54, 0.60
CO	0.12, 0.15
Anaerobic (N_2)	1.09, 1.14

[a]Incubation reactions contained halomon at 10 μg/ml (25 μ*M*), 0.5 ml microsomes, 0.1 ml 100 m*M* $MgCl_2$, 0.1 ml 10 m*M* NADPH, and 0.3 ml 100 m*M* sodium-potassium phosphate buffer (pH 7.4)

[b]Values represent the results of 2 experiments, each performed in triplicate

peak decreased markedly. In contrast, not only were anaerobic conditions associated with an increased rate of halomon disappearance as noted above, but the size of the peak at 5.4 min increased at a rate roughly proportional to the rate of decrease in halomon (Fig. 2). Carbon monoxide inhibited both the disappearance of halomon and the appearance of the 5.4-min peak. Mass spectral analysis of the potential halomon metabolite appearing at 5.4 min provided a molecular ion at m/z 284 with a characteristic isotope triplet that would be observed for a compound containing one bromine and two chlorine atoms. The mass spectrum of the proposed metabolite indicated the loss of one chlorine and one bromine from the parent compound and was consistent with two dehalogenated structures (Fig. 3).

Halomon metabolism by human hepatic microsomal preparations

Under aerobic conditions, human hepatic microsomes caused the disappearance of halomon from the incubation medium, with NADPH being required for this activity. Rates of disappearance ranged from 0.08–0.16 nmol min^{-1} mg $protein^{-1}$ at halomon substrate concentrations of 1 μg/ml (2.5 m*M*; $n = 10$) to 0.33–0.41 nmol min^{-1} mg $protein^{-1}$ at halomon substrate concentrations of 3 μg/ml (7.5 m*M*; $n = 10$). As in murine hepatic microsomal experiments, carbon monoxide inhibited, in this case completely, microsomally mediated disappearance of halomon ($n = 2$). In contrast to experiments using murine hepatic microsomal preparations, the rate of halomon disappearance in incubations of halomon at 3 μg/ml (7.5 m*M*) with human hepatic microsomal preparations was comparable under both aerobic (0.40 ± 0.10 nmol min^{-1} mg $protein^{-1}$; $n = 10$) and anaerobic (0.33 ± 0.09 nmol min^{-1} mg $protein^{-1}$; $n = 10$) conditions. The same potential halomon metabolite produced by mouse hepatic fractions was detected in incubation mixtures with human hepatic microsomes, but the rate of increase in the potential metabolite was not accelerated under anaerobic conditions.

Preliminary efforts to identify the human P450 responsible for the metabolism of halomon used known inhibitors of specific P450 isoenzymes (Table 3). The disappearance of halomon was not inhibited under either aerobic or anaerobic conditions by 100 μ*M* diethyldithiocarbamate, 1 μ*M* quinidine, 100 μ*M* ciprofloxacin, 3 μ*M* ketoconazole, or 100 μ*M* sulfinpyrazone.

Discussion

Halomon, a natural product isolated from red algae [6], possesses a number of characteristics that make it an interesting candidate for development as a potential antineoplastic chemotherapeutic agent [1, 5, 10]. We previously showed that after i.v. injection of halomon, hepatic concentrations of the compound decreased rapidly and were relatively low as compared with those in other tissues and plasma [4]. Furthermore, urinary excretion of halomon was negligible [4]. These data and the recognized importance of hepatic cytochrome P450 isoforms in the metabolism of halogenated compounds [7, 9] led to the current studies evaluating the potential for hepatic metabolism of halomon. Our data clearly demonstrate the ability of hepatic preparations to metabolize halomon. The data from both murine and human hepatic studies indicate that this activity resides in a microsomal and, more specifically, cytochrome P450

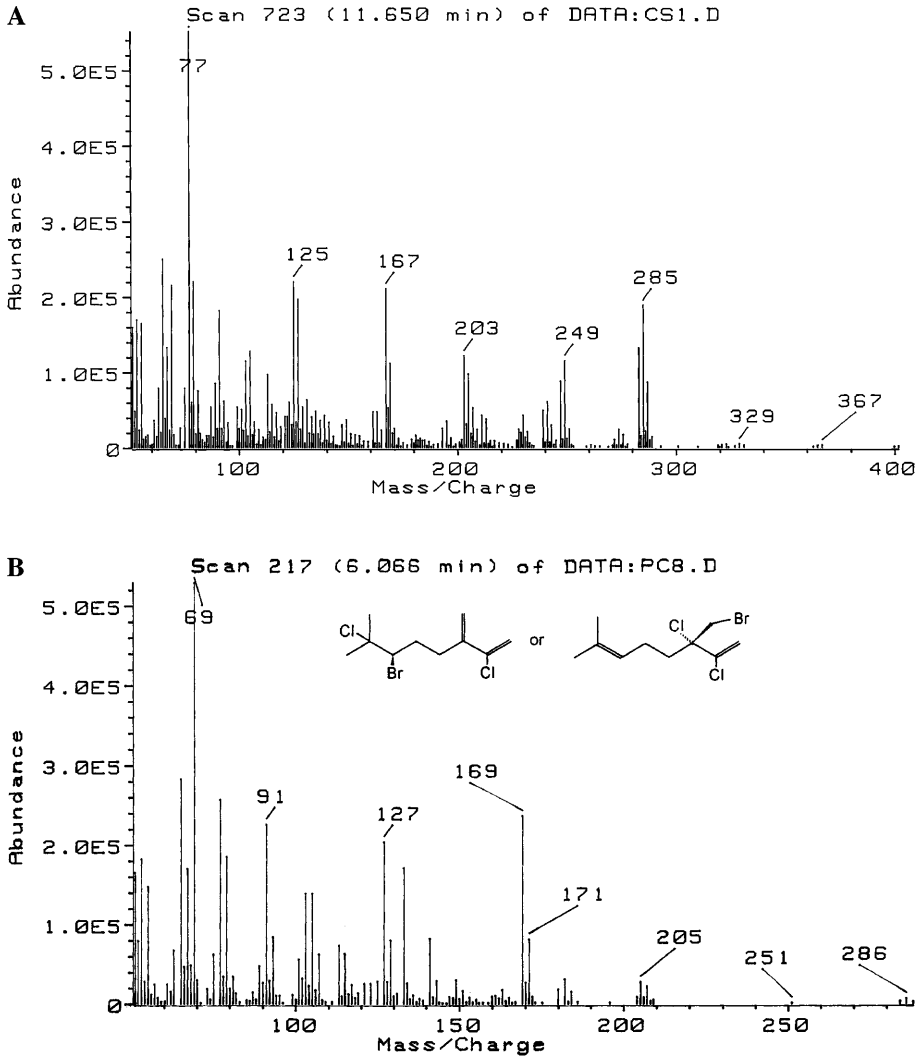


Fig. 3 **A** Mass spectrum of halomon. **B** Mass spectrum of the proposed metabolite of halomon and the two structures with which it is compatible

Table 3 Effect of inhibitors on the in vitro metabolism of halomon by human hepatic microsomes[a]

Inhibitor	Target P450	Condition	Rate (% control)[b,c]
Diethyldithiocarbamate (100 μ*M*)	2E	Aerobic	125
		Anaerobic	85
Quinidine (1 μ*M*)	2D6, 3A	Aerobic	130
		Anaerobic	100
Ciprofloxacin (100 μ*M*)	1A2	Aerobic	91
		Anaerobic	115
Ketoconazole (3 μ*M*)	3A	Aerobic	102
		Anaerobic	102
Sulfinpyrazone (100 μ*M*)	2C9	Aerobic	134
		Anaerobic	88

[a]Incubation reactions contained halomon at 1 μg/ml (2.5 μ*M*) or 3 μg/ml (7.5 μ*M*), 0.1 ml of a 10-mg/ml human microsomal preparation, 0.1 ml 100 m*M* $MgCl_2$, 0.1 ml 10 m*M* NADPH, and 0.7 ml 100 m*M* sodium-potassium phosphate buffer (pH 7.4)
[b]Control rates ranged between 0.1 and 0.4 nmol min^{-1} mg $protein^{-1}$ in separate experiments displayed in this table
[c]Values represent the means of 2 experiments, each performed in triplicate

enzyme and that halomon metabolism requires NADPH as a cofactor.

Although the proposed dehalogenated metabolite produced by both species was the same, there were some noteworthy differences in the ability of hepatic preparations from mice and humans to metabolize halomon. Our data do not explain why anaerobic conditions enhanced halomon disappearance when mouse hepatic

microsomes were used as the enzyme source but not when human microsomes were used. Furthermore, whereas carbon monoxide decreased the metabolism of halomon by murine hepatic microsomes by approximately 85%, it completely inhibited the metabolism of halomon by human microsomes. These results may indicate the metabolism of halomon by a murine microsomal enzyme other than a cytochrome P450 as opposed to only cytochrome P450-mediated halomon metabolism in human hepatic microsomes. In view of the above-referenced ability of cytochrome P450 2E1 to catalyze dehalogenation reactions under both oxidative and reductive conditions [9], the failure of diethyldithiocarbamate to inhibit halomon metabolism by human hepatic microsomes was somewhat surprising. Moreover, the failure of any of the other four specific P450 inhibitors to decrease halomon metabolism by human hepatic microsomes leaves the identity of the specific human isoenzyme responsible for the metabolism of halomon unknown. Our results may reflect that for consistency we chose to use only one human liver in our metabolism studies. This limited sample size may have decreased our ability to define the P450 isoform responsible for the metabolism of halomon because the contribution of specific P450 isoforms is known to vary between livers [3]. Lacking any guidance from the inhibitor studies described, we chose not to screen recombinant expressed human P450 isoforms for their ability to metabolize halomon. The possible implications of halomon metabolism or metabolites in the mechanisms of action and toxicity of halomon remain to be defined.

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