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Induction of Cytochrome P450 1A and 2B by α- and β-Ionone in Sprague Dawley Rats

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β-Ionone has been reported to induce the cytochrome P450 (P450) 2B1 in rats. In this study, the effects of β-ionone and an isomer, α-ionone, on liver P450 1A and 2B expression in Sprague Dawley rats were investigated. Subcutaneous administration of α- and β-ionone 72 and 48 hr prior to sacrificing the animals induced the liver microsomal P450 1A and 2B proteins. P450 2B1 induction was associated with the accumulation of its corresponding mRNA. Induction by β-ionone was much higher than that by α-ionone in both the mRNA and protein levels. When the route of administration was compared, P450 2B was induced more strongly after oral administration compared to that after subcutaneous injection. A single oral dose of 100, 300 and 600 mg/kg of α- and β-ionone for 24 h induced P450 2B1-selective pentoxyresorufin O-depentylase activity comparably in a dose-dependent manner. In addition, α- and β-ionone induced the P450 1A and 2B proteins. These results suggest that α- and β-ionone might be potent P450 2B1 inducers in rats, and that both ionones may be useful for examining the role of metabolic activation in chemical-induced toxicity where metabolic activation is required.

Key words: Induction, Cytochrome P450 2B, Ionones

INTRODUCTION

Cytochrome P450s (P450s) function not only in drug, chemical and xenobiotic detoxification, but also in activating indirect toxins that require metabolic activation to exert their toxicity (Wrighton and Stevens, 1992). Recent progress in molecular biology revealed that the P450s consist of a variety of isozymes with selective substrate specificities. Many reports suggested that natural products consumed daily might be able to interact with the P450 enzymes, which may cause severe drug-drug interactions. Therefore, it is important to investigate the effects of these natural products on P450 expression *in vivo*.

α- and b-Ionone have been found in various plants and are used in the perfumery industry. For example, α-ionone occurs in the essential oils of oranges, tangelos and grapes, and in the flavors of tea (Naves, 1971). Parke and Rahman (1969) originally found that a treatment with β-ionone induced biphenyl 4-hydroxylase activity and the total P450 concentration, and that the hexobarbital sleeping time was reduced by β-ionone. Subsequently, β-ionone has been introduced as a model P450 inducer for investigating the potential role of metabolic activation by the P450 enzymes in cocaine-induced hepatotoxicity (Roth *et al.*, 1992). Recently, β-ionone, a volatile oil originally isolated from the concrete of *Boronia megastigma* Nees (Rutaceae) and also found in tomatoes, was found to induce P450 2B1 in mice and rats (Jeong *et al.*, 1995a; 1995b; 1996; 1998). Because β-carotene and vitamin A possess a β-ionone moiety in its structure, and because vitamin A has also been suggested to induce certain P450 isozymes (Sauer and Sipes, 1995; Badger *et al.*, 1996), it is of interest to investigate the effects of β-ionone on P450 expression in order to investigate the mechanism of P450 induction by vitamin A. As a part of these

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studies, the effects of β-ionone and its isomer, α-ionone, on P450 1A and 2B expression were investigated in Sprague Dawley rats. These studies focused only on P450 1A and 2B, because previous studies suggested that these two isozymes were induced more than other isozymes (Jeong *et al.*, 1995b; 1998). To demonstrate the induction of the P450 proteins, Western immunoblotting analysis was employed, and a reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect the expression of the corresponding mRNAs.

MATERIALS AND METHODS

Animals

Specific pathogen-free male Sprague Dawley rats were obtained from the animal-breeding laboratory at KRICT The animals were 4-5 weeks of age and were acclimated for at least 1 week prior to the experiments. Upon arrival, the rats were randomized and housed four or five per cage. All animals were maintained on gamma-irradiated Jeil Lab Chow (Taejon, Korea) and UV-irradiated tap water *ad libitum*. Six to eight week old animals were used in these studies. The animal quarters were strictly maintained at 23 ± 3°C and 40-60% relative humidity. A 12-hr light/dark cycle was used with an intensity of 150-300 Lux.

Materials

The α-ionone (4-[2,6,6-trimethyl-2-cyclohexen-1-yl]-3-buten-2-one; purity, 90%), β-ionone (4-[2,6,6-trimethyl-1-cyclohexen-1-yl]-3-buten-2-one; purity, 95%), ethoxyresorufin, pentoxyresorufin, resorufin, dicumarol, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NADPH and nitrocellulose filters were purchased from Sigma Chemical Company (St. Louis, MO, USA). Alkaline phosphatase-conjugated goat anti-rabbit IgG antibody, the alkaline phosphatase substrate kit and a kit for protein determination were purchased from the Bio-Rad Laboratory (Richmond, CA, USA). The mRNA purification and first-strand cDNA synthesis kits were purchased from Pharmacia Biotech (Uppsala, Sweden). All other chemicals used were of reagent grade.

Animal treatment and microsome preparation

Unless otherwise specified, the animals were treated subcutaneously with the ionones in corn oil at 100, 300 and 600 mg/kg 72 and 48 hr prior to sacrifice. The dose and times were selected according to previous reports (Jeong *et al.*, 1995b; Roth *et al.*, 1992). The microsomes were prepared from the livers, as described elsewhere (Kim *et al.*, 1995) and were resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. Aliquots of the liver microsomes were stored at -80°C until used. The microsomal protein concentration was determined according to the method reported by Bradford using bovine serum albumin as a standard (Bradford, 1976). To examine mRNA expression the male rats were treated with 100, 300 and 600 mg/kg of β-ionone subcutaneously once for 6 h because previous studies have shown that the maximum increase in the mRNA level was observed 6 h after treatment (Jeong *et al.*, 1995b). For assaying the P450-associated monooxygenase activities, the rats were treated orally with 100, 300 and 600 mg/kg of either ionone for 24 h.

Monooxygenase assays

The ethoxyresorufin O-deethylase (EROD) and pentoxyresorufin O-depentylase (PROD) activities were determined as described in previous studies (Jeong *et al.*, 1995b; 1998). The reaction mixture (2 ml) consisted of 0.1 M potassium phosphate buffer, pH 7.4, containing 2 mg/ml of bovine serum albumin, 10 μM dicumarol, 5 mM glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase and 5 μM NADPH, 2.5 μM of ethoxyresorufin and 2.0 μM pentoxyresorufin were used as substrates for EROD and PROD, respectively. Resorufin formation was monitored fluorometrically at an excitation maximum of 550 nm and an emission maximum of 585 nm.

Western immunoblotting analyses

The microsomal proteins (10 μg/well) were resolved on 10% SDS-PAGE and were transferred to nitrocellulose filters. The filters were incubated with 2.5% non-fat dry milk for 30 min in order to block the nonspecific binding. They were then incubated with rabbit polyclonal antibodies against either rat P450 1A1/2 or P450 2B1/2, followed by alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies. The primary antibodies against individual isozymes were prepared as described previously (Kaminsky *et al.*, 1981). For immunoblotting, the nitrocellulose filters were developed with a mixture of 5-bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium and 0.1 M Tris buffer (1:1:10) according to the manufacturers instructions.

mRNA extraction and RT-PCR

Cellular poly(A^+) mRNA was isolated and reversely transcribed using a commercially available kit according to the manufacture's instruction. The reaction was quenched by incubating the mixture in a boiling water bath for 5 min. A 5 μl aliquot of cDNA corresponding to approximately 20 ng of the mRNA from each sample was amplified for 30

cycles by PCR in a 50 μl mixture containing 50 pmole of the specific primers, 1 mM dNTPs, 10x PCR buffer (100 mM Tris-HCl with 500 mM KCl, pH 8.3), and 0.5 μl of *Taq* polymerase (Pharmacia Biotech). The rat P450 2B1-specific primers producing the 443-bp PCR products were synthesized at Genosys Biotechnologies, Inc. (Woodlands, TX, USA). The sequences for P450 2B1 mRNA were designed around the active center (Jeong *et al.*, 1998): sense primer, 5'-TTGTTTGGTGCTGGGACAGAG-3'; antisense primer, 5'-GGCTAGGCCCTCTCCTGCACA-3'. The RNA concentration and PCR cycle were titrated to obtain a linearity of the signal strength. The PCR products (10 μl/well) were visualized by UV illumination following electrophoresis through a 1.5% agarose gel in Tris-borate-EDTA buffer containing 0.5 μg/ml ethidium bromide.

Statistics

The monooxygenase activity is expressed as a mean ± S.E. and a Dunnett's t-test was used to compare the significance of the data obtained. The asterisks indicate the values significantly different from the vehicle control at P<0.05 (*) or P<0.01 (**).

RESULTS

In this study, the inductive effects of α- and β-ionone on P450 1A and 2B expression in Sprague Dawley rats were compared. Fig. 1A shows that α- and β-ionone induced the P450 2B proteins in rat liver microsomes. When the rats were given the ionones subcutaneously, the P450 2B proteins were induced in the liver microsomes in a dose dependent manner. P450 2B induction by β-ionone was much greater than that by α-ionone.

Following these observations, the effects of α- and β-ionone on P450 2B1 mRNAs were further examined by using RT-PCR of the mRNAs isolated from the α- and β-ionone-treated male rat livers. Either α- or β-ionone was treated for 6 h, because a previous study suggested that P450 2B1 mRNA expression reached a maximum 6 h after the treatment with β-ionone (Jeong *et al.*, 1995b). As shown in Fig. 1B, the P450 2B1 mRNA levels was higher after treatment with the ionones. As expected, the increase in the P450 2B1 mRNA level induced by β-ionone was higher than that induced by α-ionone.

In subsequent studies, P450 2B and 1A induction by a- and β-ionone was further characterized. In Fig. 2, the effects of the route of administration were compared. Originally, β-ionone was administered subcutaneously 72 and 48 h prior to sacrifice without characterizing the induction profile by β-ionone (Thompson *et al.*, 1984; Roth *et al.*, 1992). When the original subcutaneous and the oral administrations of β-ionone were compared with other classical P450 inducers, oral administration (lane 6) was found to be better than a subcutaneous injection in inducing P450 2B (lane 5). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), a well-known P450 1A1/2 inducer, and

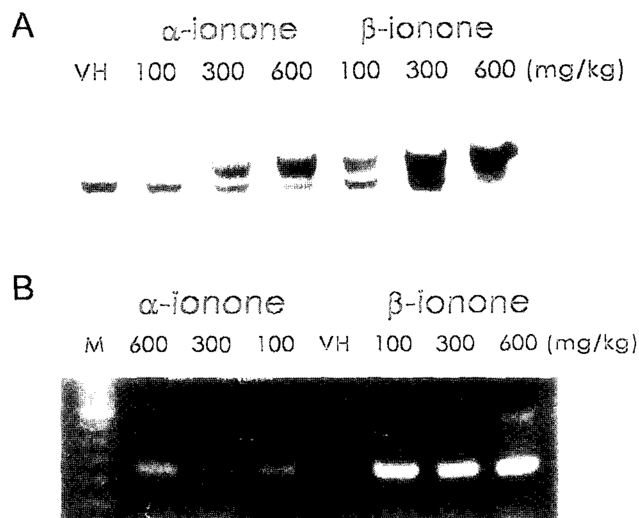

Fig. 1. A. Induction of P450 2B by α- and β-ionone in liver microsomes of male Sprague Dawley rats. Either α- or β-ionone in corn oil was injected subcutaneously in the rats 72 and 48 hr prior to sacrifice. The microsomal proteins (10 μg/well) were resolved on a 10% SDS-PAGE and transferred onto a nitrocellulose filter for the Western immunoblotting. The filter was incubated with the primary antibodies against P450 2B proteins. This was followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG. The filter was then developed using a 1:1:10 mixture of 5-bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium and 0.1 M Tris buffer. The upper band indicates the P450 2B proteins and the lower band indicates non-specific binding which was reported in previous studies (Jeong *et al.*, 1995b). B. RT-PCR amplification of the P450 2B1 mRNAs from the rat livers treated with either α- or β-ionone. The rats were treated subcutaneously with the ionones for 6 h, M, molecular weight size markers showing 100-bp ladders; VH, corn oil-treated vehicle control.

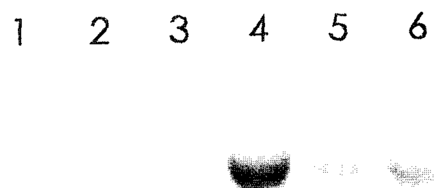

Fig. 2. Effects of the routes of administration on P450 2B induction by β-ionone. β-Ionone was administered either subcutaneously or orally at 300 mg/kg 72 and 48 h before sacrificing animals. The liver microsomal proteins (10 μg/well) prepared from individual inducers-administered male Sprague Dawley rats were resolved on a 10% SDS-PAGE. Lane 1, untreated control; lane 2, 10 μg/kg TCDD-treated once for 3 days, ip; lane 3, 5 ml/kg ethanol-treated daily for 3 days, po; lane 4, 80 mg/kg phenobarbital-treated daily for 3 days, ip; lane 5, β-ionone treated subcutaneously; lane 6, β-ionone treated orally.

ethanol, a P450 2E1 inducer, did not induce P450 2B (lanes 2 and 3, respectively). Meanwhile, phenobarbital sodium, a well-known P450 2B inducer, was able to stimulate P450 2B the most when compared to other treatments modalities (lane 4).

In Fig. 3, inductive effects of α- and β-ionone on the P450 1A- and P450 2B-selective enzyme activities were compared, following an oral administration at 100, 300 and 600 mg/kg for 24 h. The EROD activities were induced slightly by both ionones without showing a dose dependency. Meanwhile, the PROD activities were induced comparably by both ionones with a clear dose dependency. The effects of the oral administration of ionones on P450 1A and 2B expression are shown in Fig. 4A and 4B, respectively. When 100 mg/kg of either one of the ionones was administered orally, the P450 1A proteins were induced slightly in the rat liver microsomes (Fig. 4A). The inductive effects of β-ionone on the P450 2B proteins were much greater than those caused by α-ionone (Fig. 4B).

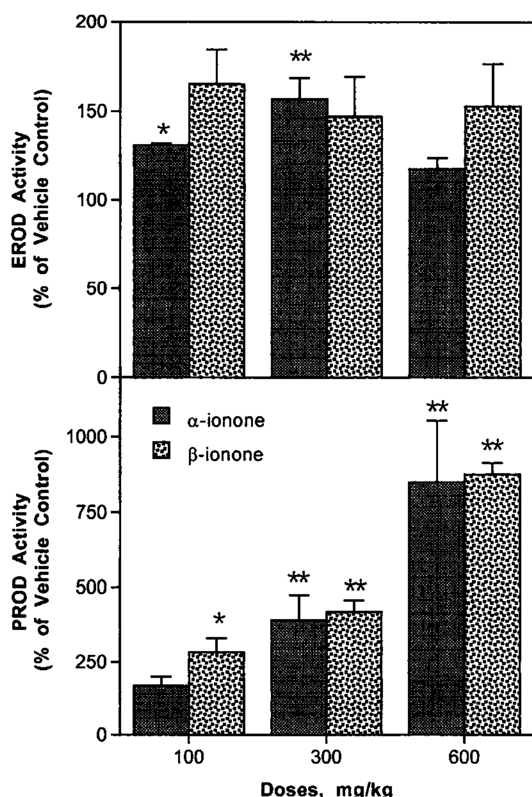

Fig. 3. Effects of α- and β-ionone on liver microsomal P450 enzyme activities. The rats were given either α- or β-ionone in corn oil orally for 24 h. Each bar represents the mean percentage of the vehicle control ± S.E. of three animals. The asterisks indicate the values significantly different from corn oil-treated vehicle control at $P<0.05$ (*) or $P<0.01$ (**). As a reference, the EROD and PROD activities were 250% and 626% of the control, respectively, when 80 mg/kg of phenobarbital was given intraperitoneally for 3 days.

DISCUSSION

Many chemicals isolated from plants have been characterized to either induce or inhibit the expression of certain P450 enzymes that may cause a significant interaction with drugs. In addition, these compounds are not only abundant in plants but are also contained in the diet. They may be freely taken and modulate P450 expression. Therefore, it is very important to characterize the effects of plant-derived compounds on the expression of the P450 enzymes. Among those compounds, β-ionone has recently been reported to induce P450 2B1 in rats (Jeong *et al.*, 1995b) and P450 1A and 2B in mice (Jeong *et al.*, 1995a; 1996). Interestingly, β-ionone has only been used as a model P450 enzyme inducer in cocaine-induced hepatotoxicity, in which the metabolic activation by P450 2B1 is necessary to form toxic metabolites (Thompson *et al.*, 1984; Roth *et al.*, 1992; Boelsterli *et al.*, 1992). Most recently, the inductive effect of β-ionone on P450 2B1 was also demonstrated in Sprague Dawley rats (Jeong *et al.*, 1995b; 1998). In these studies, P450 2B1 induction by β-ionone was due to the accumulation of the mRNA encoding P450 2B1. Similarly, the induction of P450 1A by β-ionone was also due to mRNA accumulation (Jeong *et al.*, 1998).

In this study, α- and β-ionone clearly induced both the P450 1A and 2B proteins in rats (Fig. 1A, 2, 4A and 4B). In addition, both ionones could induce both the P450 1A- and 2B-associated microsomal activities of EROD and PROD. In particular, the induction of PROD activity by both ionones was comparable with a clear dose dependency (Fig. 3). Because the induction of rat P450 2B might be closely related to the increase in P450 2B1 mRNA

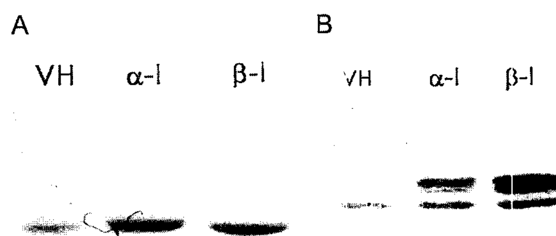

Fig. 4. A. Effects of α- and β-ionone on the expression of the P450 1A proteins in male Sprague Dawley rats. The ionones were administered orally at 100 mg/kg for 24 h. The liver microsomal proteins (10 μg/well) were resolved on a 10% SDS-PAGE and transferred onto a nitrocellulose filter for the Western immunoblotting. α-I, α-ionone; β-I, β-ionone. B. Effects of α- and β-ionone on the expression of the P450 2B proteins in male Sprague Dawley rats. Ionones were administered orally at 100 mg/kg for 24 h. The liver microsomal proteins (10 μg/well) were resolved on a 10% SDS-PAGE and transferred onto a nitrocellulose filter for the Western immunoblotting. α-I, α-ionone; β-I, β-ionone.

transcription (Jeong *et al.*, 1998; Fig. 1B), α- and β-ionone may induce P450 enzymes through the accumu-lation of the P450 mRNAs. Of equal importance is that the oral administration of β-ionone was better in inducing the P450 2B proteins than to the original subcutaneous injec-tion (Fig. 2), and that a single oral dose of both ionones could induce the P450 2B enzymes significantly with a slight induction of the P450 1A proteins (Fig. 3, 4A and 4B). Because the P450 1A1 protein is not expressed in normal rats, and because a band was detected in the vehicle control in Figure 4A, it is believed that P450 1A2 was induced by β-ionone. Overall, these results suggest that a single oral dose of β-ionone may induce the P450 2B proteins more selectively than the original subcutaneous injection. Indeed, the subcutaneous injection induced other P450 isozymes too in our previous studies (Jeong *et al.*, 1998). A selective inducer is quite useful for investigating the possible role of metabolic activation by individual P450 isozymes in chemical-induced toxicity both *in vivo* and *in vitro*.

In conclusion, α- and β-ionone induce the P450 1A and 2B proteins in Sprague Dawley rats, and that the inductive effect of β-ionone was much greater than that of α-ionone. Since β-ionone itself was neither hepatotoxic nor immunotoxic at the dose showing P450 induction (Thompson *et al.*, 1984; Roth *et al.*, 1992; Jeong *et al.*, 1995a), α- and β-ionone may be useful model P450 inducers for investigating the possible role of the metabolism in chemical-induced toxicity. In this regard, we have recently demonstrated that pretreatment of male BALB/c mice with β-ionone potentiated the hepatotoxicity induced by thioacetamide, which requires metabolic activation by P450 2B1 to form the hepatotoxic metabolites (Jeong *et al.*, 1999). However, the underlying mechanism of P450 induction by α- and β-ionone remains to be clarified.

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REFERENCES

Badger, D. A., Sauer, J. -M., Hoglen, N. C., Jolley, C. S., and Sipes, I. G., The role of inflammatory cells and cytochrome P450 in the potentiation of CCl_4-induced liver injury by a single dose of retinol. *Toxicol. Appl. Pharmacol.,* 141, 507-519 (1996).

Boelsterli, U. A., Lanzotti, A., Göldlin, C., and Oertle, M., Identification of cytochrome P-450IIB1 as a cocaine-bioactivating isoform in rat hepatic microsomes and in cultured rat hepatocytes. *Drug Metabol. Dispos.,* 20, 96-101 (1992).

Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the protein-dye binding. *Anal. Biochem.,* 72, 248-254 (1976).

Jeong, T. C., Gu, H. K., Chun, Y. J., Yun, C. -H., Han, S. S., and Roh, J. K., Effects of β-ionone on the expression of cytochrome P450s and NADPH-cytochrome P450 reductase in Sprague Dawley rats. *Chem. Biol. Interact.,* 114, 97-107 (1998).

Jeong, T. C., Gu, H. K., Park, J. -I., Yun, H. -I., Kim, H. C., Ha, C. S., and Roh, J. K., Pretreatment of male BALB/c mice with β-ionone potentiates thioacetamide-induced hepatotoxicity. *Toxicol. Lett.,* 105, 39-46 (1999).

Jeong, T. C., Jordan, S. D., Matulka, R. A., Stanulis, E. D., Kaminski, E. J., and Holsapple, M. P., Role of metabolism by esterase and cytochrome P-450 in cocaine-induced suppression of the antibody response. *J. Pharmacol. Exp. Ther.,* 272, 407-416 (1995a).

Jeong, T. C., Jordan, S. D., Matulka, R. A., Stanulis, E. D., Park, S. S, and Holsapple, M. P., Immunosuppression by acute exposure to cocaine is dependent on metabolism by cytochrome P-450. *J. Pharmacol. Exp. Ther.,* 276, 1257-1265 (1996).

Jeong, T. C., Kim, H. J., Yun, C. -H., Lee, S. S., Yang, K. H., Han, S. S., and Roh, J. K., Induction of liver cytochrome P450 2B1 by β-ionone in Sprague Dawley rats. *Biochem. Biophys. Res. Commun.,* 216, 198-202 (1995b).

Kaminsky, L. S., Fasco, M. J. and Guengerich, F. P., Production and application of antibodies to rat liver cytochrome P-450. *Meth. Enzymol.,* 74, 262-272 (1981).

Kim, D. H., Kim, E. J., Han, S. S., Roh, J. K., Jeong, T. C., and Park, J. H., Inhibitory effects of H_2-receptor antagonists on cytochrome P450 in male ICR mice. *Human Exp. Toxicol.,* 14, 623-629 (1995).

Naves, Y. R., Some developments in the chemistry of ionones and their derivatives- a subject review. *J. Soc. Cosm. Chem.* 22, 439-456 (1971).

Parke, D. V. and Rahman, H., The effects of some terpenoids and other dietary nutrients on hepatic drug-metabolizing enzymes. *Biochem. J.,* 113, 12 (1969).

Roth, L., Harbison, R. D., James, R. C., Tobin, T., and Roberts, S. M., Cocaine hepatotoxicity: influence of hepatic enzyme inducing and inhibiting agents on the site of necrosis. *Hepatology,* 15, 934-940 (1992).

Sauer, J. -M. and Sipes, I. G., Modulation of chemical-induced lung and liver toxicity by all-*trans*-retinol in the male Sprague-Dawley rats. *Toxicology,* 105, 237-249 (1995).

Thompson, M. L., Shuster, L., Casey, E., and Kanel, G. C., Sex and strain differences in response to cocaine. *Biochem. Pharmacol.,* 33, 1299-1307 (1984).

Wrighton, S. A. and Stevens, J. C., The human hepatic cytochrome P450 involved in drug metabolism. *Crit. Rev. Toxicol.,* 22, 1-21 (1992).