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Farnesol as an inhibitor and substrate for rabbit liver microsomal P450 enzymes

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Abstract

Farnesol and the related isoprenoids, geranylgeraniol, geranylgeranyl pyrophosphate, and farnesyl pyrophosphate, are produced in the endoplasmic reticulum of hepatocytes in mammals, and each serve important biological functions. Of these compounds, only farnesol was shown to significantly inhibit rabbit liver microsomal cytochrome P450 enzymes. The observed inhibition appeared to be reversible, and was not strictly competitive, but rather mixed in nature. Of the activities examined, ethoxycoumarin de-ethylase and diclofenac-4-hydroxylase activities were most sensitive to farnesol, with K_I and K_I' values between 11 and 40 μM. Caffeine-8-hydroxylation and taxol-6-hydroxylation were not inhibited at all by farnesol. Farnesol appeared to be a P450 substrate, as well as an inhibitor, as indicated by the NADPH-dependent decrease in farnesol concentration in microsomal incubations, and the metabolism was inhibited by CO, which pointed to the involvement of P450 isozymes. © 2002 Elsevier Science (USA). All rights reserved.

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Cytochrome P450 enzymes are well known for their ability to metabolize a broad range of chemical entities, many of which are foreign to the host organism. Due to the prominent role of P450 enzymes in the metabolism of many pharmaceutical agents and activation or de-activation of potential carcinogens, the inhibition or induction of cytochrome P450 enzymes by xenobiotics has received considerable attention [1]. It has also been shown that cytochrome P450 enzymes can metabolize endogenous chemicals as well, resulting in either activation or de-activation of that particular compound. For example, all-trans retinoic acid, the active form of vitamin A, is oxidized to 4-hydroxy and 4-oxo-retinoic acid [2], a process believed to represent de-activation. In comparison, an area that has not been explored to a large extent is the role that endogenous chemicals may have in attenuating cytochrome P450 activity through direct enzyme inhibition. In 1969, DiAugustine and Fouts [3] showed that arachidonic acid was a potent inhibitor of cytochrome P450 activity in the rabbit liver, and it was later determined that arachidonic acid was a substrate for P450 enzymes [4]. More recently, Bestervelt et al. [5] and Kuo et al. [6] have shown that 4-hydroxynonenal, a product of lipid peroxidation, may inhibit several different liver microsomal P450s at micromolar concentrations, and Gervasini et al. [7] recently described the inhibition of $P450_{2C9}$ by 5-hydroxytryptamine and adrenaline. These studies demonstrate the potential significance of P450 inhibition by endogenously produced compounds.

An important class of endogenous compounds called isoprenoids, which have significant biological functions in the areas of cell cycle regulation [8], cholesterol metabolism [9], and gene regulation [10], are produced in nearly all eukaryotic organisms including mammals. Several of these compounds are produced in the endoplasmic reticulum of hepatocytes and are very hydrophobic, raising the question as to the potential for interaction between liver microsomal P450s and certain members of this class of compounds. In particular, farnesol is a 15-carbon isoprenoid alcohol that has been

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implicated in gene regulation [10] and cell differentiation [11], via the nuclear receptors FXR and PPARα, control of cholesterol synthesis via its interaction with HMG-CoA reductase [9], and apoptosis [12]. Esterification of alcohol to the diphosphate, a so-called "salvage reaction" that has been shown to occur in mammalian cells [13], results in farnesyl pyrophosphate (FPP), which is the farnesyl group donor in protein prenylation reactions [8]. The analogous 20-carbon isoprenoid alcohol, geranylgeraniol, has been implicated in apoptosis through its selective activation of caspase-3 [14], and the corresponding diphosphate derivative, GGPP, is a prenyl group donor involved in rab and ras-related small G-protein activation [15].

The goal of this study was to examine potential interactions between specific microsomal P450 isozymes and the endogenous isoprenoids farnesol, FPP, geranylgeraniol, and GGPP. Using uninduced rabbit liver microsomes, various cytochrome P450 substrates were used to probe P450 inhibition by each of the four compounds. A reconstituted system consisting of recombinant $P450_{2E1}$-Δ3-29, NADPH cytochrome P450 reductase and dilauroyl phosphatidylcholine (DLPC) was also examined in order to confirm the results obtained using microsomes. Finally, GC/MS analysis was used to explore the possibility that farnesol was metabolized by P450 enzymes in the rabbit liver microsomes, or by the reconstituted $P450_{2E1}$-Δ3-29 system.

Experimental

The compounds farnesol, geranylgeraniol, farnesyl pyrophosphate, and geranylgeranyl pyrophosphate were purchased from Sigma Chemical, as were the substrates diclofenac, taxol, and *p*-nitrophenol. The standards, 4-hydroxydiclofenac and 6-hydroxytaxol were purchased from Gentest.

Rabbit livers were purchased from Pel-Freez (Rogers, Arkansas) and rabbit liver microsomes were prepared by a variation of a published protocol [16]. Procedures in the current study were identical to the published ones with the exception that the final centrifugation step was carried out for 2 h at 45,000*g* rather than 60 min at 100,000*g*. The resulting pellet from this spin was suspended in 10.0 mM Tris–acetate buffer containing 1.0 mM EDTA and 20% glycerol. NADPH oxidation rates, protein content, and reduced-CO difference spectra were measured using established protocols [16].

Cytochrome $P450_{2E1}$-Δ3-29 was expressed in *Escherichia coli* using a clone provided by Dr. Minor Coon (The University of Michigan, Medical School) using a published procedure [17]. The expressed enzyme was partially purified by lysing the *E. coli* cells enzymatically and with a sonic dismembrator, in 30 mM phosphate buffer, pH 7.7 containing 0.50 mM EDTA, 50 mM imidazole, and 20% glycerol. Following sonication, 1% tergitol was added, mixed well, and the solution centrifuged at 42,000*g* for 2 h. The supernatant was diluted with 30 mM phosphate buffer, pH 7.7, containing 50 mM imidazole and 20% glycerol to give a final tergitol concentration of 0.2%. This was loaded onto a hydroxyapatite column equilibrated in the same buffer, and washed with 5 column volumes of the same buffer without tergitol. The enzyme was eluted from the column using a minimum volume of 400 mM potassium phosphate buffer, pH 7.4, 0.1 mM EDTA, and the sample was dialyzed to remove imidazole. Reductase was expressed and purified by a published procedure [18] using a clone produced by Dr. Charles Kasper (The University of Wisconsin, Madison) and provided to us by Dr. Minor J. Coon (The University of Michigan, Medical School).

The assays used in this study have all been described previously. The oxidation of *p*-nitrophenol (10–100 μM) was used to monitor Cytochrome $P450_{2E1}$ activity [19]. Caffeine-N-demethylation was used to probe $P450_{1A2}$ activity at concentrations between 1.0 and 10.0 mM [20]. $P450_{1A1}$ activity was examined using a spectrofluorometric assay involving O-de-ethylation of ethoxy coumarin (EROD) [16]. Taxol-6-hydroxylation activity of the rabbit liver microsomes, an activity that is typically associated with the human $P450_{2C8}$ isoform, was measured using an HPLC assay, where taxol concentrations between 5 and 100 μM were used [21] and detection of the product was at 229 nm. Diclofenac-4-hydroxylation activity, over a range of concentrations from 40 to 120 μM, was measured for the rabbit liver microsomes using a published protocol [22]. In humans, this reaction is catalyzed primarily by $P450_{2C9}$. Finally, caffeine-8-hydroxylation was monitored using caffeine concentrations in the range of 1–60 mM. This activity in the rabbit liver is most likely related to the $P450_{3A6}$ isoform, by analogy with human $P450_{3A4}$. All HPLC product analyses were performed on a Shimadzu HPLC system with diode array detection that has been described previously [23]. An ISA Jobin Yvon-Spex FlouroMax-2 spectrofluorimeter was used for the analysis of EROD activity.

Inhibition experiments were performed in which 5–180 μM inhibitor was included in the reaction mixtures and activities were compared to reactions that were carried out in parallel with no inhibitor present. The inhibition constants K_I and K_I' were calculated from Lineweaver–Burk plots using the following relationship:

$$1/V = (\alpha K_m/V_{max}) \times (1/[S]) + (\alpha'/V_{max}),$$

where $\alpha = 1 + ([I]/K_I)$ and $\alpha' = 1 + ([I]/K_I')$. That the inhibition of *p*-nitrophenol oxidation in rabbit liver microsomes by farnesol was reversible was determined using a procedure described by Raner et al. [24]. In this assay, microsomes, farnesol, and NADPH were pre-incubated for 20 min at 37 °C, at which time 50 μL of this mixture was added to 950 μL of a solution containing *p*-nitrophenol and NADPH, bringing the final concentrations to 1.0 mg/mL protein, 1.0 mM NADPH, 5.0 μM farnesol, and 100 μM *p*-nitrophenol, in 100 mM phosphate buffer, pH 7.4. These activities were compared to those obtained in control experiments in which no farnesol was included in the pre-incubation. Inhibition studies involving farnesyl pyrophosphate, geranylgeraniol, and geranylgeranyl pyrophosphate were performed in an identical manner.

To monitor farnesol metabolism by cytochrome $P450_{2E1}$, 0.118 nmol of the recombinant enzyme was reconstituted with approximately 0.1 nmol of partially purified P450 reductase and 35 μg DLPC, and incubated for 5 min at room temperature. To this mixture was added 100 μM farnesol, 100 mM phosphate buffer, pH 7.4, and 1.0 mM NADPH (all final concentrations). Mixtures were incubated between 0 and 60 min at 37 °C, at which time 50 μM (final concentration) 6-phenylhexanol was added as an internal standard, and the reaction was quenched by addition of 3.0 mL ethyl acetate. Products were extracted twice and ethyl acetate was evaporated under vacuum. The resulting residue was dissolved in ethyl acetate and injected onto an HP5890 Series II GC (HP-1 Crosslinked Methyl Silicone Gum, 5 m × 0.53 mm × 2.65 μm) coupled with an HP 5971 Series Mass Selective Detector operating in the positive ion mode. The column temperature was set at 85 °C for 2 min, followed by increasing temperature at a rate of 10 °C/min until 230 °C was reached. Under these conditions the farnesol eluted at 13.2 min and the internal standard appeared at 11.9 min. Peak areas for farnesol were all normalized using the integrated area of the internal standard.

The same protocol was used for analysis of the reaction products from microsomal incubations with farnesol and NADPH. In these experiments, 1.0 mg of microsomal protein was incubated with 100 μM farnesol for up to 60 min at 37 °C. For the quantification of farnesol, the $m/z = 69$ peak was integrated, and the areas were normalized using the $m/z = 91$ peak areas of the internal standard. The individual ion peaks were selected based on their intensities. An additional control was carried out for the 30-min incubation with microsomes in which CO was continuously bubbled through the reaction mixture at a rate of 2.0 mL/min.

Results and discussion

In the presence of farnesol, the *p*-nitrophenol 3-hydroxylation activity of rabbit liver microsomes was reduced in a concentration-dependent manner (Fig. 1). The inhibition was mixed in nature with K_I and K_I' values of 90 and 115 μM, respectively. The inhibition appeared to be reversible as indicated by the fact that pre-incubation of liver microsomes with 80 μM farnesol and NADPH had no effect on the resulting *p*-nitrophenol oxidation activity (data not shown). The value obtained for K_m in these experiments (48 μM *p*-nitrophenol) was consistent with reported values in the literature for the rabbit liver $P450_{2E1}$ [19].

Several other P450-related activities in the rabbit liver microsomes were also examined with respect to inhibition by farnesol. These included taxol-6-hydroxylation, caffeine-N-demethylation, caffeine-8-hydroxylation, ethoxy coumarin O-de-ethylation (EROD), and diclofenac-4-hydroxylation. The kinetic parameters obtained for all of these assays, including K_I and K_I' are given in Table 1. Taxol-6-hydroxylation and caffeine-8-hydroxylation appeared to be unaffected by farnesol at concentrations up to 160 μM. This result was significant because it suggested that the observed inhibitory effect of farnesol on certain other P450 activities was not due to inhibition of the reductase enzyme or disruption of the P450–reductase complex via lipophilic interactions with farnesol. Of the six activities examined, the EROD and diclofenac-4-hydroxylation activities appeared to be most sensitive to farnesol, with K_I and K_I' values falling in the range of 11–44 μM. Caffeine-N-demethylation and *p*-nitrophenol 3-hydroxylation in the rabbit liver microsomes were much less sensitive to farnesol, with K_I and K_I' values of ~100 μM or higher. Kim et al. [25] have also examined the effects of farnesol on the activity of retinoic acid hydroxylase, a P450-catalyzed activity in rabbit liver, and found that farnesol did not affect the activity of this specific 450 enzyme, although analogs of farnesol were shown to inhibit the activity at low micromolar concentrations. The results from our study show that farnesol did, in fact, inhibit certain P450-related activities in the rabbit liver, the inhibition appeared to be reversible, and that the inhibition occurred at concentrations below 10 μM farnesol.

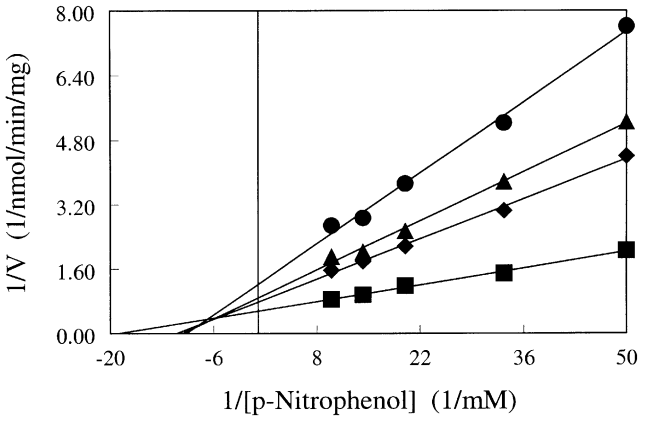

Fig. 1. Inhibition of rabbit liver microsome-catalyzed *p*-nitrophenol oxidation by farnesol. The concentration of farnesol in each of the experiments was (■) 0 μM, (◆) 45 μM, (▲) 90 μM, and (●) 180 μM.

Whether this inhibition has any physiological significance or not depends on the concentration of farnesol normally present in the liver cells. Although this value is very difficult to determine, due to rapid metabolism and/or sequestration by intracellular or extra cellular binding proteins, many of the observed biological effects of farnesol have been shown to occur in vitro at concentrations between 20 and 80 μM, suggesting this may represent a reasonable physiological range of concentrations. For example, FXR binding [10], the induction of the degradation of HMG-CoA reductase [9], inhibition of cholinephosphotransferase activity [26], and apoptosis [12] have all been linked to farnesol, and in each case, the effective concentration falls within this range in vitro.

Three related endogenous isoprenoid compounds were also examined with respect to their ability to inhibit certain P450 activities in the liver microsomes. The compounds tested included FPP, geranylgeraniol, and GGPP, and the activities included *p*-nitrophenol 3-hydroxylation, diclofenac-4-hydroxylation, and caffeine-N-demethylation. Essentially no inhibition of any of the P450-related activities examined was observed with any of the three compounds (Table 2). This was somewhat surprising given structural similarity of these compounds with farnesol. These results indicate that the alcohol function and the size of the molecule are important factors for binding to and inhibiting the P450s. It also illustrates the very specific nature of the interaction involving farnesol, which argues against a non-specific mode of inhibition such as the disruption of interactions between P450s and the reductase.

Inhibition experiments were also carried out using a reconstituted $P450_{2E1}$ system, and the results compared to those for the microsome-catalyzed hydroxylation of *p*-nitrophenol (Table 1). As with microsomes, the inhibition by farnesol was mixed in the reconstituted system,

Table 1
Kinetic parameters for various P450-catalyzed reactions and the effects of farnesol on activity[a]

Activity	K_m (mM)	V_{max} (nmol/min/mg)	K_I (μM)	K_I' (μM)
In rabbit liver microsomes				
p-Nitrophenol oxidation	0.048	1.8	90	115
Ethoxycoumarin O-de-ethylation	3.0×10^{-4}	0.070	11	44
Diclofenac-4-hydroxylation	0.205	2.0	21	21
Caffeine-N-demethylation	1.8	0.080	137	160
Caffeine-8-hydroxylation[b]	25	3.2	NI	NI
Taxol-6-hydroxylation[b,c]	0.016	ND	NI	NI
In reconstituted P450$_{2E1}$-Δ3-27 system				
p-Nitrophenol oxidation	0.042	2.5	66	64

[a] Values represent the average of three separate trials with a standard deviation of less than 10%.
[b] NI indicates no inhibition was observed in the 8-hydroxylation of caffeine and 6-hydroxylation of taxol.
[c] ND indicates that the actual V_{max} was not determined due to degradation of the standard 6-hydroxy taxol.

Table 2
Inhibition of different P450 activities in rabbit liver microsomes and a reconstituted P450$_{2E1}$ system by four different isoprenoids[a]

Activity	Farnesol (80 μM) % inhibition	Geranylgeraniol (80 μM) % inhibition	FPP (80 μM) % inhibition	GGPP (80 μM) % inhibition
In rabbit liver microsomes				
p-Nitrophenol hydroxylation (30 μM)	60 ± 5	0 ± 1	0 ± 2	10 ± 5
Diclofenac-4-hydroxylation (0.20 mM)	45 ± 2	2 ± 1	0 ± 1	1 ± 1
Caffeine-N-demethylation (2.0 mM)	35 ± 2	0 ± 1	0 ± 1	0 ± 1
In reconstituted P450$_{2E1}$-Δ3-27				
p-Nitrophenol hydroxylation (45 μM)	43 ± 3	2 ± 2	5 ± 3	3 ± 1

[a] Errors are based on the average deviation from the mean for three or more trials.

and the kinetic parameters were reasonably consistent with those observed for microsomal inhibition, although the inhibition constants determined in the reconstituted system were lower than those observed in the microsomes. One possible explanation for this is that since farnesol is very hydrophobic, it may be sequestered to some extent through interaction with the membranous material present in the microsomes. This would have the effect of lowering the free farnesol in solution, and consequently the K_I would increase. Regardless, the experiments show very clearly that farnesol acts as a P450$_{2E1}$ inhibitor both in microsomes and a reconstituted system with similar inhibition characteristics.

To determine whether the farnesol was just an inhibitor of rabbit liver microsomal P450s or a potential competing substrate, we used GC/MS analysis to monitor farnesol concentration. In the reconstituted P450$_{2E1}$ system there was no detectable decrease in the farnesol concentration with time, and no observable product peaks in the presence of NADPH that were not present in controls. This suggested that farnesol was not metabolized by rabbit liver P450$_{2E1}$. In microsomal incubations, however, farnesol metabolism was observed, and the metabolism was time- and NADPH-dependent (Fig. 2). After a 30-min incubation with 1 mg of microsomal protein, approximately 50% of the farnesol had been metabolized. The metabolism

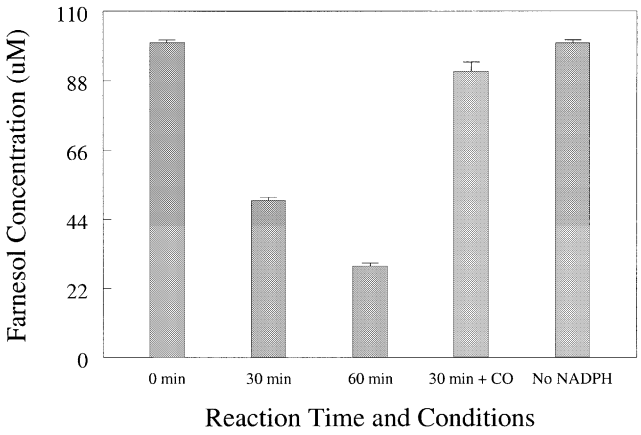

Fig. 2. Time-dependent metabolism of farnesol in rabbit liver microsomes in the presence of NADPH. Also shown are the results of identical reactions in the absence of NADPH after a 60-min incubation, and in the presence of CO after a 30-min incubation. Reactions were terminated by addition of ethyl acetate, and samples were analyzed by GC/MS and signals normalized using the internal standard 6-phenylhexanol.

could be inhibited by CO indicating the likely involvement of P450 isozymes. No additional peaks were detected in the ethyl acetate extracts from the reactions, indicating the products were most likely more hydrophilic in nature. Vaidya et al. [27] reported that

the squalene synthase inhibitor, zaragozic acid, when given to mice resulted in massive production of dicarboxylic acids presumably via metabolism of farnesol. To determine whether carboxylic acid products were formed in the reaction with microsomes, the aqueous phase from the initial ethyl acetate extraction was acidified and re-extracted with ethyl actetate. Once again, GC/MS analyses of the extracts from reactions carried out in the absence and presence of NADPH were unable to detect NADPH-dependent products. Although studies are currently underway to determine the identities of any metabolites formed in this reaction, we tentatively conclude that P450 enzymes convert farnesol to more hydrophilic non-carboxylic acid products in rabbit liver microsomes.

In summary, this study shows very clearly that farnesol, but not the related terpenoids geranylgeraniol, FPP, or GGPP, is an inhibitor of certain rabbit liver microsomal P450 enzymes. The inhibition appears to be reversible and non-competitive, and occurs at farnesol concentrations that may have physiological significance, particularly under conditions that can result in elevated farnesol production. It appears that farnesol is metabolized by constitutive rabbit liver microsomal P450 enzymes to more polar products that cannot be extracted into ethyl acetate even under acidic conditions. These findings raise the possibility that endogenously produced farnesol could play a role in the attenuation of cytochrome P450 activity under certain conditions, and that P450s may have a critical role in the metabolism of this biologically significant molecule.

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