Inhibition of Cytochrome P450 Mediated Enzyme Activity by Alkylphosphocholines

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The inhibitory potency of four alkylphospholipids: rac-1-O-phosphocholine-2-hydroxy-octadecane (rac-2-OH), rac-1-O-phosphocholine-2-O-acetyl-octadecane (rac-2-O-acetyl), rac-1-O-phosphocholine-2-amino-octadecane (rac-2-NH_2) and rac-1-O-phosphocholine-2-N-acetyl-octadecane (rac-2-N-acetyl), on the cytochrome P450-dependent monooxygenase activity has been evaluated. The IC_{50} values of the alkylphosphocholines with 7-ethoxycoumarin as substrate in liver microsomal fractions of PB-treated rats and with a reconstituted CYP2B1: NADPH-P450-reductase system are in the range of 3.2–5.0 μM and 2.8–3.5 μM, respectively. Lineweaver-Burk plots with the inhibitors in concentrations that were found to cause roughly a 50% inhibition and with 7-ethoxycoumarin as substrate revealed for all four alkylphospholipids a competitive inhibition type. The degree of the competitive inhibition is quantified by the K_i values. With liver microsomal fractions of PB-treated rats, the K_i values of rac-2-OH (K_i = 1.36 μM) and rac-2-O-acetyl (K_i = 1.33 μM) differs slightly from those of rac-2-NH_2 (K_i = 2.2 μM) and rac-2-N-acetyl (K_i = 2.2 μM), but with the reconstituted CYP2B1: NADPH-P450-reductase system all K_i values are in the small range of 1.8 – 2.6 μM, indicating that the short substituted group at the 2-position (OH; O-acetyl; NH_2; N-acetyl) of the long chain octadecanol part of the phosphodiesters exhibit no essential role on the strong inhibitory potency of these alkylphosphocholines on the 7-ethoxycoumarin-O-deethylase activity.

Key words: Alkylphosphocholines, Competitive Inhibitors of Cytochrome P450 Activity, Single Chain Lipids

Introduction

The cytochrome P450-dependent monooxygenase system consists of a NADPH-cytochrome P450-reductase (reductase) and a family of cytochrome P450 forms, which are tightly anchored with their membrane-binding domains into the lipid bilayer of the endoplasmatic reticulum (ER) of liver cells (Guengerich, 1995; Gonzales, 1992).

The major effect of the double chain phospholipid matrix on the P450-dependent enzyme activity is to facilitate the formation of the binary P450:reductase complex, enhancing the specific P450-activity of reconstituted P450:reductase systems by optimizing the concentration of the binary enzyme complex (Müller-Enoch *et al.*, 1984; Müller-Enoch, 1993). Therefore we were surprised that single chain phospholipids like lysolecithin act as competitive inhibitors for P450 substrates, suggesting that these molecules can operate as a new class of P450 inhibitors (Müller-Enoch *et al.*, 2001).

We were able to show that the natural monochain lipids L-α-lysophosphatidyl-choline, L-α-lysophosphatidyl-inositol and the free fatty acids arachidonic acid and oleic acid as well as the monoacylglycerols 1-monooleoyl-glycerol and 2-monopalmitoyl-glycerol exhibit K_i values in the range of 1.5–9.5 μM, indicating their strong competitive inhibition of the 7-ethoxycoumarin-O-deethylase activity in a reconstituted CYP2B1: NADPH-cytochrome P450-reductase system.

In the present investigation we will evaluate the inhibitory effects of unnatural alkylphosphocholines (APCs) on the cytochrome P450 enzyme activity. This group of phosphocholine-diesters is very similar in structural elements with lysophospholipids, in having hydrophilic amphoteric head groups and lipophilic long chain tails (octadecanol, C_{18}). APCs represent a novel class of drugs with promising antitumor activity *in vitro* and *in vivo* (Matzke *et al.*, 2001; Massing and Eibl, 1994; Unger *et al.*, 1988, 1989). They were derived from the

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antineoplastic alkyllysophospholipids that are related to natural lysophospholipids. We will show that four newly synthetized racemic APC compounds (Massing and Eibl, 1994) (Fig. 1) potently inhibit the 7-ethoxycoumarin-O-deethylase activity in rat liver microsomes and in an isolated reconstituted CYP2B1:NADPH-P450-reductase system allowing to further characterize the structural features in the inhibition process.

Materials and Methods

Materials

7-Ethoxycoumarin and 7-hydroxycoumarin were purchased from EGA-Chemie KG (Steinheim, Germany) and recrystallized from hot water. The alkylphosphocholines: rac-1-O-phosphocholine-2-hydroxy-octadecane, rac-1-O-phosphocholine-2-O-acetyl-octadecane, rac-1-O-phosphocholine-2-amino-octadecane-hydrochlorid and rac-1-O-phosphocholine-2-N-acetyl-octadecane were synthesized by U. Massing as described by Massing and Eibl (1994). All other chemicals and biochemicals used were of highest purity available and obtained from E. Merck (Darmstadt, Germany), Boehringer Mannheim (Mannheim, Germany) and Sigma Chemie (Deisenhofen Germany).

Enzyme preparations

Liver microsomal fractions were prepared out of male Sprague-Dawley rats (200–250 g). These rats were pretreated with sodium phenobarbital (PB) by addition of 0.1% (w/v) PB to the drinking water for 6 d. Livers were removed and placed in ice-cold 1.15% KCl. The final microsomal fraction was suspended in 10 mmol/l Tris(hydroxymethyl)-aminomethane-acetate buffer (pH 7.4) containing 1 mmol/l EDTA and 20% (v/v) glycerol. These microsomal fractions had a specific content of 27 nmol P450/ml. The cytochrome P450 from CYP2B1 was purified to electrophoretic homogeneity out of the microsomal preparations described above using procedures described by Guengerich and Martin (1980). The SDS-PAGE-pure rat liver CYP2B1 had a specific content of 11 nmol CYP2B1/ml. The NADPH-cytochrome P450-reductase was purchased from Calbiochem-Novabiochem GmbH (Schwalbach, Germany) and had a specific activity of 44 u/mg protein and a specific content of 23.8 nmol reductase/ml.

General assays

Protein concentrations were estimated using the method of Lowry *et al.* (1951). The contents of CYP2B1 and cytochrome P450 in the rat liver microsomes were determined by the method of Omura and Sato (1964). The activity of the reductase was determined by the method of Yasukochi and Masters (1976).

Alkylphosphocholine solutions

All alkylphospholipids were dissolved in ethanol or microdispersed by sonication in the buffer (0.1 mol/l) N-2-hydroxylethylpiperazine-N′-2-ethanesulfonic acid potassium salt (potassium HEPES, pH 7.6) or in water in a concentration range of 1–3 mg/ml solvent.

Reconstitution of the CYP2B1:NADPH-cytochrome P450-reductase monooxygenase system

The purified CYP2B1 was mixed with the NADPH-cytochrome P450-reductase (reductase) in a 1:2 molar ratio at concentrations of 5.5 and 11 μM, respectively, in a final volume of 20–60 μl for 2 h at 25 °C in 0.1 M Tris-HCl buffer (pH 7.6) containing 20% glycerol as described by Müller-Enoch *et al.* (1984, 2001). Following this preincubation of the two enzymes, aliquots of these preformed CYP2B1:reductase complexes were used for measuring their enzyme activity with 7-ethoxycoumarin as substrate.

Enzyme activity measurements

The NADPH-cytochrome P450-reductase activity was determined according to Yasukochi and Masters (1976) and expressed as NADH-cytochrome c-reductase activity at 30 °C in 0.3 M potassium phosphate buffer, pH 7.7.

The 7-ethoxycoumarin-O-deethylase activity of rat liver microsomal fractions and of reconstituted CYP2B1:reductase systems were assayed using the continuously fluorometric test described by Ullrich and Weber (1972). The test system contained in a total volume of 600 μl: 0.1 mmol/l 7-ethoxycoumarin; 3.3 mmol/l $MgCl_2$; 0.1 mol/l potassium HEPES buffer, pH 7.6, and either 8 μl of a dilution of the microsomal rat liver preparations, containing 72 pmol P450, or 5 μl (27.5 pmol CYP2B1 and 55 pmol reductase) of the reconstituted CYP2B1:reductase complex, and 1–4 μl portions of the dispersed or solved alkylphospholipids, in a quartz

cuvette kept for 3 min at 37 °C constant in a sample holder of the spectrophotometer. All reactions were started by the addition of 0.17 mmol/l NADPH. The formation of 7-hydroxycoumarin was monitored fluorometrically (λE = 365 nm; λF = 460 nm) as a function of time. To calibrate each assay 10 μl of a 0.1 mmol/l solution of 7-hydroxycoumarin was added twice at the end of each experiment.

Results

Evaluation of the inhibitory potency of alkylphosphocholines on cytochrome P450-dependent monooxygenase activities

Four racemic alkylphosphocholines: 1-O-phosphocholine-2-hydroxy-octadecane (rac-2-OH), 1-O-phosphocholine-2-O-acetyl-octadecane (rac-2-O-acetyl), 1-O-phosphocholine-2-amino-octadecane (rac-2-NH_2) and 1-O-phosphocholine-2-N-acetyl-octadecane (rac-2-N-acetyl) (Fig. 1), were dissolved in water or ethanol and examined for their inhibitory effect on 7-ethoxycoumarin-O-deethylation by rat liver microsomes with high CYP2B1 levels (PB-treated rats) and with a reconstituted system of isolated CYP2B1 and NADPH-cytochrome P450-reductase (preformed complex).

The IC_{50} values, shown in Table I ranging from 2.2–5.0 μM for all alkylphosphocholines, reflect a high inhibitory potency, comparable with the drug ketoconazole (IC_{50} = 3–40 μM), one of the best known inhibitors of cytochrome P450-dependent enzyme activities (Newton *et al.*, 1995; Draper *et al.*, 1997; Haehner *et al.*, 2004). The slightly higher IC_{50} values of rac-2-NH_2 and its acetyl derivative rac-2-N-acetyl in rat liver microsomes might reflect an involvement of other P450 forms in 7-ethoxycoumarin-O-deethylation activity inhibition. This possibility is supported by the results with the isolated CYP2B1:reductase system, showing lower IC_{50} values in a shorter IC_{50} value range of 2.2–3.5 μM.

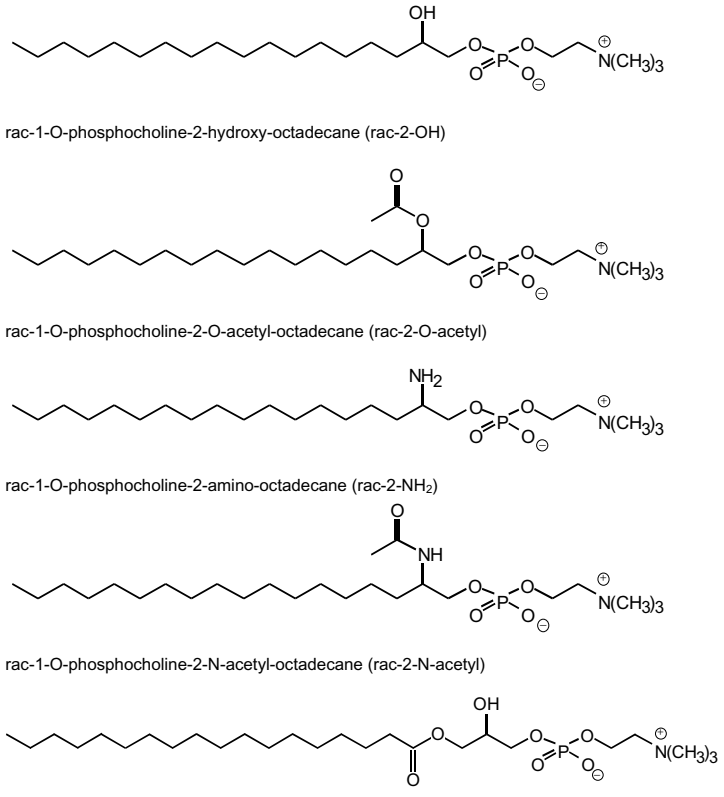

Fig. 1. Structures and abbreviations of synthetic alkylphosphocholines used in this paper and lysolecithin.

Table I. IC_{50} and K_i values determined for alkylphosphocholines with 7-ethoxycoumarin as substrate in liver microsomal fractions of phenobarbital-treated rats, and with a reconstituted system of CYP2B1:NADPH-P450-reductase.

Alkylphosphocholines	Microsomes of PB-treated rats		Reconstituted system of CYP2B1:reductase	
	IC_{50} values [μM]	K_i values [μM]	IC_{50} values [μM]	K_i values [μM]
rac-2-OH	3.2	1.36	2.8	1.98
rac-2-O-acetyl	3.3	1.33	2.7	2.00
rac-2-NH_2	4.5	2.20	2.2	1.81
rac-2-N-acetyl	5.0	2.23	3.5	2.65

rac-2-OH = rac-1-O-phosphocholine-2-hydroxy-octadecane,
rac-2-O-acetyl = rac-1-O-phosphocholine-2-O-acetyl-octadecane,
rac-2-NH_2 = rac-1-O-phosphocholine-2-amino-octadecane,
rac-2-N-acetyl = rac-1-O-phosphocholine-2-N-acetyl-octadecane.

Inhibition of 7-ethoxycoumarin-O-deethylation activity in rat liver microsomes by alkylphosphocholines

Fig. 2 shows a double reciprocal (Lineweaver-Burk) plot of substrate concentration versus 7-ethoxycoumarin-O-deethylation activity ($1/v$) by liver microsomal fractions of phenobarbital treated rats. The K_m for 7-ethoxycoumarin-O-deethylation without inhibitor was estimated to be 120 μM. The maximal enzyme activity, v_{max}, is determined from the ordinate intercept, v_{max} = 6.7 nmol 7-hydroxycoumarin × min^{-1} × nmol $P450^{-1}$ (●—●). In case of adding a fixed concentration of 6.77 μM rac-2-N-acetyl and different 7-ethoxycoumarin concentrations, (▲—▲), v_{max} did not change but the apparent K_m value was higher (K_m = 444 μM). The corresponding K_i value was calculated to 2.23 μM. In case of adding 6.75 μM rac-2-O-acetyl to varying substrate concentrations (■—■),

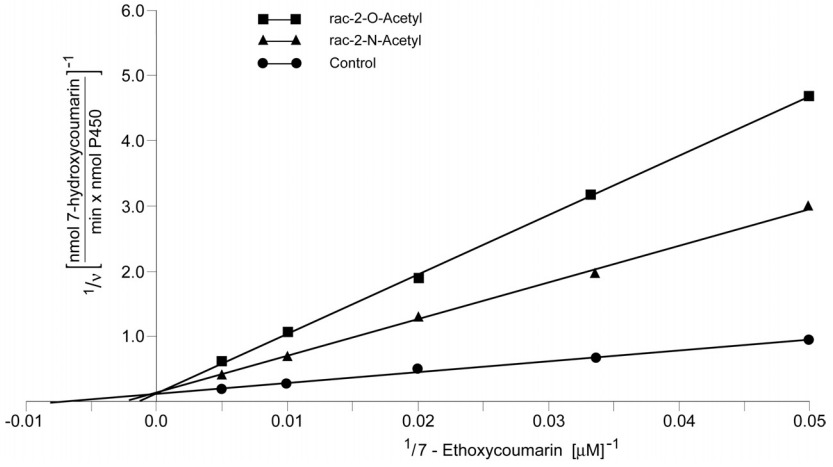

Fig. 2. Lineweaver-Burk plots demonstrating the inhibitory effect of alkylphosphocholines on the 7-ethoxycoumarin-O-deethylation activity. Liver microsomal fractions of phenobarbital-treated rats equivalent to 72 pmol P450 were incubated under standard conditions (see Materials and Methods) for 10 min with variing 7-ethoxycoumarin-concentrations (20–200 μM) and fixed rac-2-N-acetyl or rac-2-O-acetyl concentrations, and the rates of 7-hydroxy-coumarin formation were determined by fluorescence spectroscopy. The apparent K_m and v_{max} values without alkyl-phosphocholines were estimated to 120 μM and 6.67 nmol 7-hydroxycoumarin × min^{-1} × nmol $P450^{-1}$, respectively (●—●). The apparent K_m values in the presence of rac-2-N-acetyl (▲—▲) and rac-2-O-acetyl (■—■) were determined to 444 μM and 667 μM, respectively. The v_{max} values were the same as without inhibitors. The corresponding inhibitor constants K_i with [I] = 6.77 μM rac-2-N-acetyl and [I] = 6.75 μM rac-2-O-acetyl were calculated to K_i = 2.23 μM and K_i = 1.33 μM, respectively.

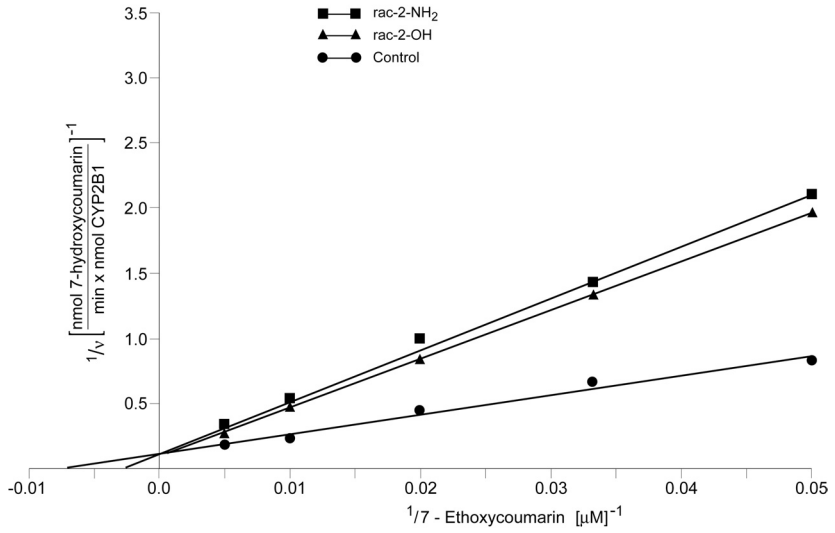

Fig. 3. Lineweaver-Burk plots in the absence and presence of fixed concentrations of rac-2-OH and rac-2-NH_2. Aliquots of reconstituted isolated CYP2B1:reductase preparations (1:2 molar ratio), equivalent to 27.5 pmol CYP2B1 and 55 pmol reductase, were incubated (see Materials and Methods) for 10 min with 3.67 μM rac-2-OH or 3.42 μM rac-2-NH_2, and varying concentrations of 7-ethoxycoumarin (20–200 μM). The rate of 7-hydroxycoumarin production was monitored. The apparent K_m and v_{max} values without inhibitor (●—●) were estimated to 140 μM and 10 nmol 7-hydroxycoumarin × min^{-1} × nmol $CYP2B1^{-1}$, respectively. The apparent K_m values in the presence of 3.67 μM rac-2-OH (▲—▲) or 3.42 μM rac-2-NH_2 (■—■) were determined to 400 μM and 405 μM, respectively. The v_{max} values were the same as without inhibitors. The inhibitor constants K_i with [I] = 3.67 μM rac-2-OH and [I] = 3.42 μM rac-2-NH_2 were calculated to K_i = 1.98 μM and K_i = 1.81 μM, respectively.

v_{max} was unchanged, the apparent K_m value was calculated to 667 μM. Therefore both alkylphosphocholines cause a strong competitive inhibition of the substrate 7-ethoxycoumarin in rat liver microsomal fractions. The same type of inhibition was estimated for the two other alkylphosphocholines, rac-2-OH and rac-2-NH_2, and the K_i values were calculated to K_i = 1.36 μM and K_i = 2.2 μM, respectively, as summarized in Table I.

Competitive inhibition of 7-ethoxycoumarin-O-deethylase activity in isolated and reconstituted CYP2B1:NADPH-cytochrome P450-reductase systems by alkylphosphocholines

In Fig. 3 Lineweaver-Burk representations of the inhibitors rac-2-NH_2 and rac-2-OH, and without an inhibitor, are given for a reconstituted CYP2B1:NADPH-cytochrome P450-reductase system with 7-ethoxycoumarin as substrate. The maximal enzyme activity, v_{max}, was calculated to 10 nmol 7-hydroxycoumarin × min^{-1} × mol $CYP2B1^{-1}$ without inhibitor (●—●) and the K_m value to 140 μM 7-ethoxycoumarin, respectively. The K_i value for rac-2-NH_2 was calculated from the apparent K_m = 405 μM and an inhibitor concentration [I] = 3.42 μM to K_i = 1.81 μM (■—■). In the same manner the K_i value for rac-2-OH was calculated from the apparent K_m = 400 μM and [I] = 3.67 μM to K_i = 1.98 μM (▲—▲). The maximal enzyme activity, v_{max}, did not change by adding the inhibitors used. This indicates a competitive inhibition for the alkylphosphocholines rac-2-NH_2 and rac-2-OH. The same type of inhibition was determined for rac-2-N-acetyl and rac-2-O-acetyl having K_i values of 2.65 μM and 2.0 μM, respectively, as stated in Table I.

Discussion

Recently we published a systematic study, in which the inhibitory effects of long single chain (C_{12}–C_{20}) lipid molecules on the cytochrome P450-dependent monooxygenase system were kinetically analyzed (Müller-Enoch *et al.*, 2001). Saturated free fatty acids from lauric acid (C_{12}) up to stearic acid (C_{18}) are referred to be substrates for several cytochrome P450 forms (Imaoka *et al.*, 1990; Aoyama *et al.*, 1990) and, as revealed in our study, show K_i values in the range of 50–100 μM

for 7-ethoxycoumarin-O-deethylase activity. The unsaturated fatty acid arachidonic acid is converted into ω, and ω-1 hydroxylated products as well as into epoxyeicosatrienoic acids by several cytochrome P450 forms, including CYP2B1 used in this study (Capdevila *et al.*, 1990; Falck *et al.*, 1990). Unsaturated fatty acids have a higher degree of inhibition than saturated fatty acids with the same chain length (*e.g.* oleic acid $K_i = 1.5\,\mu$M and stearic acid $K_i = 120\,\mu$M). In case of the monoglycerol esters of 1-monooleoyl- and 1-monostearylglycerol the differences in K_i values, which are 1.1 μM and 15.5 μM, respectively, are not so large compared with the corresponding free fatty acids. Lysophosphatidyl-choline, containing primarily palmitic and stearic acids in α-position produced a K_i value of 3.0 μM in a soluble or a vesicular reconstituted CYP2B1:reductase system with 7-ethoxycoumarin as substrate (Müller-Enoch *et al.*, 2001).

These high inhibition effects of free fatty acids as well as of monoacyl-glycerol-esters and lysophospholipids such as lysophosphatidyl-choline on the cytochrome P450-dependent monooxygenase system are important, because double chain phospholipids like lecithin do not have an inhibitory effect on this enzyme system (Müller-Enoch *et al.*, 1984; Müller-Enoch, 1993).

The K_i values of the alkylphospholipids (K_i = 2.0–2.6 μM) revealed in this investigation, indicate, that the substituent at position C-2 (OH; O-acetyl; NH_2; N-acetyl) of the long chain octadecanol part of the phosphodiesters does not exert an essential influence on the inhibitory potency of these molecules. The inhibitory effects are high for all alkylphosphocholines and comparable to those of L-α-lysophosphatidyl-choline (K_i = 3.0 μM), 2-monopalmitoyl-glycerol (K_i = 3.5 μM), and L-α-lysophosphatidyl-inositol (K_i = 1.5 μM), which all have a long lipophilic monochain (C_{18}) ester function, but different hydrophilic head groups. This suggests that the long lipophilic part of the molecule is essential for the inhibitory effect and the amphoteric or hydrophilic part is necessary for the solubility of the molecules to be available for the enzyme as single molecules.

Single and double chain lipid molecules markedly differ in their biophysical properties. Whereas the critical micelle concentration (CMC) of double chain lipids is very small due to the large hydrophobic interaction produced by the two hydrocarbon chains, *e.g.* CMC = $0{,}5 \cdot 10^{-9}$ M for L-α-dipalmitoylphosphatidyl-choline (Smith and Tanford, 1972), the CMC of single chain lipids is several decimal exponents (10^6) larger than for double chain lipids, *e.g.* CMC = $0.5 \cdot 10^{-3}$ M for lysopalmitoylphosphatidyl-choline (Hamori and Michaels, 1971), which means that there is a factor of one million in CMC-difference. A consequence of the water solubility is, that double chain lipids are forced to form liposomal aggregates and bilayer membranes at low lipid concentrations, whereas the single chain lipids are still dissolved as single molecules in the aqueous solution and consequently reach the lipophilic pocket of the active center of the cytochrome P450-enzymes, competing with the substrate 7-ethoxycoumarin for the catalytic center. Therefore, monochain lipids with hydrophilic head groups are sufficiently soluble to act as substrates or inhibitors for cytochrome P450-dependent monooxygenase systems.

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