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Identification and Characterization of a Novel Δ6/Δ5 Fatty Acid Desaturase Inhibitor As a Potential Anti-Inflammatory Agent

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ABSTRACT. The anti-inflammatory properties of essential fatty acid deficiency or n-3 polyunsaturated fatty acid supplementation have been attributed to a reduced content of arachidonic acid (AA; 20:4 n-6). An alternative, logical approach to depleting AA would be to decrease endogenous synthesis of AA by selectively inhibiting the Δ5 and/or the Δ6 fatty acid desaturase. High-throughput radioassays were developed for quantifying Δ5, Δ6, and Δ9 desaturase activities *in vitro* and *in vivo*. CP-24879 (*p*-isopentoxyaniline), an aniline derivative, was identified as a mixed Δ5/Δ6 desaturase inhibitor during the screening of chemical and natural product libraries. In mouse mastocytoma ABMC-7 cells cultured chronically with CP-24879, there was a concentration-dependent inhibition of desaturase activity that correlated with the degree of depletion of AA and decreased production of leukotriene C_4 (LTC_4). Production of LTC_4 was restored by stimulating the cells in the presence of exogenous AA, indicating that endogenous AA was limiting as substrate. In the livers of mice treated chronically with the maximally tolerated dose of CP-24879 (3 mg/kg, t.i.d.), combined Δ5/Δ6 desaturase activities were inhibited approximately 80% and AA was depleted nearly 50%. These results suggest that Δ5 and/or Δ6 desaturase inhibitors have the potential to manifest an anti-inflammatory response by decreasing the level of AA and the ensuing production of eicosanoids. BIOCHEM PHARMACOL **55**;7:1045–1058, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. Δ5 desaturase; Δ6 desaturase; fatty acid desaturase inhibitor; arachidonic acid; linoleic acid; essential fatty acids; polyunsaturated fatty acids; eicosanoids

EFAD¶ and supplementation with n-3 PUFAs have been used as dietary means to manifest an anti-inflammatory response in several animal models [1–5]. Compared to n-3 PUFA supplementation, EFAD is a more extreme dietary means to deplete AA (20:4 n-6). In addition, EFAD decreases inflammation more markedly and is disease-modifying [6–12]. In either case, the anti-inflammatory response is due, in part, to the depletion of AA and a corresponding decrease in the synthesis of pro-inflammatory eicosanoids [4, 5, 13–16]. The correlative factor associated with compromised macrophage or neutrophil function in EFAD appears to be partial depletion and accompanying unavailability of AA in key lipid pools [17–19]. Decreased synthesis and, thus, availability of AA might mitigate the inflammatory response by altering AA metabolism, for example, by decreasing eicosanoid levels and/or by altering AA-mediated cell signalling or the physicochemical properties of membranes [13–15, 20–26]. Inhibition of the synthesis of AA could possibly go beyond amelioration of symptoms currently provided by nonsteroidal anti-inflammatory drugs and, like EFAD, be disease-modifying.

Mammals can desaturate fatty acids at positions Δ5, Δ6, or Δ9 by the respective, discrete microsomal desaturase [27–35]. Stearic acid (18:0), a saturated fatty acid, is converted to a monounsaturated fatty acid, oleic acid (18:1 n-9), by the action of Δ9 desaturase (Fig. 1). The essential PUFA pathway in mammals converts LA to AA or α-LN to eicosapentaenoic acid (EPA; 20:5 n-3) by a sequential series of reactions: (1) Δ6 desaturation by the Δ6 desaturase, the rate-limiting step, (2) two-carbon elongation by four discrete enzymes, collectively referred to as elongase, and (3) Δ5 desaturation by the Δ5 desaturase (Fig. 1).

Depletion of AA by severe dietary intervention is probably not feasible in humans. Besides fish oil supplementation, a more acceptable way to decrease AA and accompanying pro-inflammatory prostaglandins and leukotrienes

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¶ *Abbreviations:* AA, arachidonic acid; DGLA, dihomo-γ-linolenic acid; EFAD, essential fatty acid-deficient (deficiency); FBS, fetal bovine serum; GLA, γ-linolenic acid; LA, linoleic acid; α-LN, α-linolenic acid; LTC_4, leukotriene C_4; OA, oleic acid; PUFA, polyunsaturated fatty acid; and t.i.d., L. *ter in die*, three times a day dosing.

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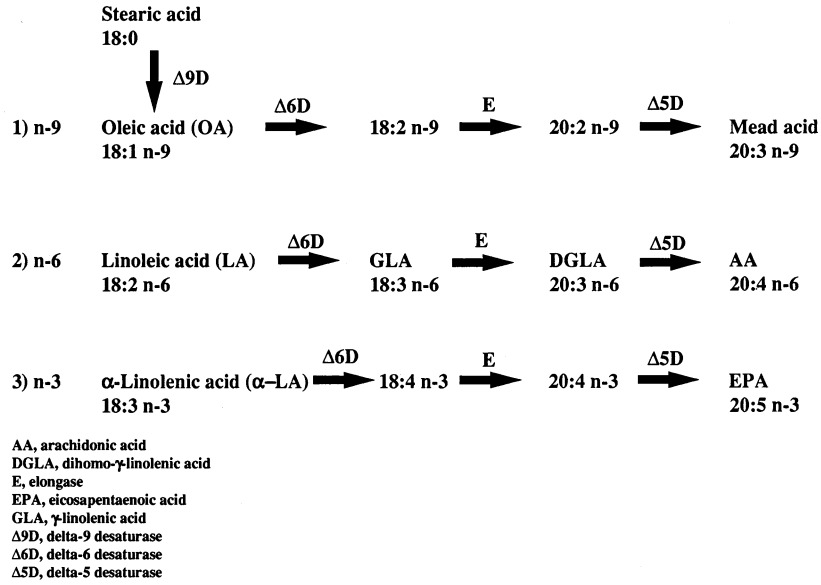


FIG. 1. Desaturation and elongation pathways of n-9, n-6, and n-3 polyunsaturated fatty acids in mammals.

might be to inhibit Δ5 and/or Δ6 desaturase activities chronically. Such intervention might result in the anti-inflammatory effects associated with EFAD, but without some of the accompanying untoward side-effects of EFAD [3] because LA would be present in the diet. Desaturase inhibitors have been described that are selective for the Δ6 desaturase (i.e. norflurazone [36, 37]), Δ5 desaturase (i.e. sesamin and related compounds [38–40]), or mixed Δ5/Δ6 desaturases (i.e. curcumin and related compounds [41–43], alkyl gallate derivatives [44], and cyclopropene fatty acids [45, 46]). None of these desaturase inhibitors was very potent in a rat liver microsomal assay, having, at best, IC_{50} values in the mid-high micromolar range. Sesamin and curcumin are not strictly desaturase inhibitors in that they also inhibit two-carbon elongation in primary rat hepatocytes [43]. Most importantly, sesamin, the only desaturase inhibitor to be evaluated *in vivo*, does not cause a decrease in the level of AA in liver or brain when administered to rats chronically at a very high level of dosing [40, 47].

It was hypothesized that chronic inhibition of the Δ5 and/or Δ6 desaturase would partially deplete AA in lipid pools and mimic the anti-inflammatory properties of EFAD or n-3 PUFA supplementation. Liver is the targeted organ for desaturase inhibition because AA is synthesized primarily in the liver and then is mobilized to inflammatory cells via blood lipoproteins. The objectives of this study were to identify novel, potent Δ5, Δ6, or mixed Δ5/Δ6 desaturase inhibitors and then evaluate whether chronic desaturase inhibition causes depletion of AA *in vitro* and *in vivo*. Described herein are methods developed for the identification and characterization of desaturase inhibitors. CP-24879 (*p*-isopentoxyaniline), an aniline derivative, was identified in the Monsanto/Searle library of compounds as a mixed Δ5/Δ6 desaturase inhibitor. In mouse mastocytoma ABMC-7 cells treated chronically with CP-24879, the degree of desaturase inhibition correlated with the depletion of AA and the decreased production of LTC_4. In mice, chronic dosing with CP-24879 inhibited desaturase activity and, concomitantly, caused substantial depletion of AA in liver.

MATERIALS AND METHODS

Materials and Reagents

Authentic fatty acids used as standards in GC or added exogenously in cell culture were purchased from Nu-Chek Prep, Inc. The vehicle for i.p. dosing consisted of 0.5% methyl cellulose + 0.025% Tween-20 (polyoxyethylene-sorbital monolaurate), both purchased from the Sigma Chemical Co. Organic solvents were Optima grade from Fisher Scientific. Routine laboratory chemicals were purchased from Sigma or Fisher Scientific. [1-^{14}C]Fatty acids (sp. act. approximately 55 mCi/mmol) were purchased from American Radiolabeled Chemicals. Precoated silica gel TLC plates (20 × 20 cm LK5D plates, 150 Å pore diameter, 250 μm thick, 19 channels, 500 μm thick pre-absorbent strip) were purchased from Whatman. The plates were immersed for 15–20 sec in a 10% $AgNO_3$ solution in water, after which they were drained and then air-dried for a minimum of 2 days. During storage, the plates were kept in the dark. Prior to use, the plates were activated for 1 hr at 110°. Sample preparation of lipids/fatty acids was done in glass test tubes having teflon-lined caps.

Preparation of Rat Liver Microsomes

Male Sprague–Dawley rats (150–175 g) were deprived of food for 3 days and then refed an EFAD diet for 2 days in order to induce Δ9 desaturase activity [48]. The rats were killed, after which their livers were removed and placed on ice. The livers were diced with scissors and then homogenized with a Polytron (PTA 10TS probe) in a 2× volume

of homogenization buffer (150 mM of KCl, 250 mM of sucrose, 50 mM of Tris–HCl, pH 7.5, 5 mM of EDTA, 1.5 mM of reduced glutathione) at 4°. The homogenate was centrifuged at 1500 *g* for 20 min at 4°. The supernatant was filtered through gauze and centrifuged twice more at 10,000 *g* for 20 min each at 4°. The supernatant was saved and centrifuged one final time at 100,000 *g* for 60 min at 4°. The supernatant was discarded, and the microsomal pellet was resuspended in homogenization buffer to a protein concentration of 10 mg/mL, as determined by the Bradford protein assay [49]. The microsomal preparation was divided into aliquots and stored at −80°.

Cells and Culture Conditions

ABMC-7 (mouse mastocytoma) cells were utilized in a secondary *in vitro* screening assay to evaluate potency, selectivity, and overt toxicity of the desaturase inhibitors identified in the liver microsomal assay. The cells were cultured in RPMI-1640 medium supplemented with 10% FBS at 37° in a humidified incubator maintained at 5% CO_2. Upon reaching stationary phase growth (approximately 2×10^6 cells/mL), the cells were split (1:25).

ABMC-7 cells were adapted to grow in HL-1, a serum-free medium (Hycor, Ventrex Division). The cells were cultured initially in HL-1 + 10% FBS. After every second passage, the serum concentration was decreased by a factor of two until the cells adapted to grow in HL-1 minus serum. The n-6 PUFAs were repleted by culturing the cells in HL-1 supplemented with 10 μM of LA (10 mg/mL of stock in ethanol) + 15 μM of BSA (fatty acid-free; 1 mg/mL of stock in PBS) as a fatty acid carrier (referred to as HL-1 + LA medium). ABMC-7 cells cultured in HL-1 + LA were used to evaluate the effects of chronic desaturase inhibition by CP-24879. The cells were seeded at a starting density of 10^4/mL in T flasks (75 cm^2), each containing 20 mL of HL-1 + LA. Saline (control) or CP-24879 (6 mM of stock of HCl salt in saline) was added to each of two T flasks, providing duplicate flasks of 0, 100 nM, 300 nM, 1 μM, 3 μM, or 10 μM of CP-24879. The cells were cultured until they reached a density of approximately 2×10^6/mL (4 days), after which they were assayed for viability (trypan blue exclusion), Δ5/Δ6 desaturase activities, and fatty acid composition. Maximum LTC_4 production is obtained in ABMC-7 cells that are actively dividing (unpublished results). After 4 days of culture, the cells were split 1:20 into fresh HL-1 + LA ± CP-24879 (1×10^5/mL) and then cultured for an additional day (4×10^5/mL), at which time they were actively dividing. The cells were washed in saline and resuspended in HL-1 ± 30 μM of AA (30 mM of stock concentration in ethanol). Duplicate cell samples from each flask were stimulated to synthesize LTC_4 by adding the calcium ionophore A-23187 (40 μM), for 20 min at 37°. Following stimulation, the sample tubes were placed in ice. The cells were pelleted by centrifugation, and the supernatants were aspirated and stored at −20°. The level of LTC_4 in each supernatant was quantified by ELISA (Cayman Chemical Co.).

Desaturase Assays

RAT LIVER MICROSOMAL ASSAY. All three desaturase activities, Δ5, Δ6, and Δ9, were assayed simultaneously in a single sample. Assay of Δ9 desaturase activity was included as a control in order to evaluate inhibitor selectivity. The assay conditions were optimized in a 48-well microtiter plate format to achieve a relatively high-throughput rate for screening. Into each well, the following, in order, were added: 1) 150 μL of buffer/cofactors (250 mM of sucrose, 150 mM of KCl, 40 mM of NaF, 100 mM of sodium phosphate, pH 7.4, 1.3 mM of ATP, 1.5 mM of reduced glutathione, 0.06 mM of reduced coenzyme A, 0.33 mM of nicotinamide, 1 mg/mL of $MgCl_2 \cdot 5H_2O$, and 0.67 mg/mL of NADH), 2) 50 μL of rat liver microsomes (approximately 0.5 mg total protein), 3) 2.2 μL of test compound (DMSO stock; 1% final DMSO concentration), and 4) 20 μL of [^{14}C]fatty acid substrates (mixture of [^{14}C]18:0, [^{14}C]18:3 n-3, and [^{14}C]20:3 n-6 in the buffer/cofactor solution). The mixture of [^{14}C]fatty acid substrates was made by adding 0.5 μL (0.05 μCi) of each [^{14}C]fatty acid to 18.5 μL of the buffer/cofactor solution (20 μL total volume).

The test compounds were preincubated with the microsomes for 10 min at room temperature with intermittent swirling. The desaturase reaction was started by the addition of the 20-μL mixture of the [^{14}C]fatty acid substrates. The plates were placed on a rotating shaker inside a 37° oven for 1 hr. The samples were then saponified by adding 200 μL of 2.5 N KOH in methanol:H_2O (4:1) to each well. The plates were wrapped in three layers of plastic wrap to prevent evaporation and then placed in a shaking incubator set at 65° for 4 hr. Following saponification, the free fatty acids were protonated by the addition of 280 μL of formic acid to each well (final pH ≤ 3). Hexane (700 μL) was added to each well, and the protonated fatty acids were extracted into the hexane phase by thorough mixing. Two hundred microliters was drawn from the hexane layer of each well and spotted onto the pre-absorbent loading strip of the $AgNO_3$-TLC plates. Desaturase activity was quantified following argentation-TLC (see below).

CELL ASSAY. An ABMC-7 cell-based desaturase assay was performed in a 48-well microtiter plate format to maximize sample throughput. Cells (2×10^5) in 0.5 mL of medium (RPMI-1640 + 10% FBS) were added to each well followed by 5 μL of the test compound (DMSO stock solution; 1% final DMSO concentration). The cells were preincubated with inhibitor for 1 hr at 37°, after which the medium was aspirated. Fresh medium (0.5 mL RPMI-1640 + 1% FBS) and inhibitor were added. A 10-fold lower amount of FBS was used to limit the binding of serum proteins to the [^{14}C]fatty acid substrates. Lastly, 2.5 μL (10 μM) of the

[^{14}C]fatty acid substrate (ethanol stock; 0.5% final ethanol concentration) was added.

The cells were incubated overnight at 37°, after which the medium was aspirated and the cells were washed with 0.5 mL of PBS. The PBS was aspirated, and cellular lipids were saponified directly by adding 200 μL of 2.5 M of KOH in methanol:H_2O (4:1) to each well. Then the samples were processed as described for the rat liver microsomal assay. Desaturase activity was quantified following argentation-TLC (see below).

Argentation-TLC

Argentation-TLC was performed in a solvent system consisting of chloroform:methanol:acetic acid:water (90:8:1:0.8). Following chromatography, the plates were removed, air-dried, and exposed to x-ray film (Kodak, X-OMAT AR) overnight. Inhibition of Δ5, Δ6, or Δ9 desaturase activity was determined qualitatively by autoradiography. Quantification of desaturase activities was determined directly from the $AgNO_3$-TLC plates by electronic autoradiography using an Instant Imager (Packard) or a PhosphorImager (Molecular Dynamics). The IC_{50} values were calculated by linear regression using the straight line portions of the concentration–response curves.

Fatty Acid Composition Analysis

GC using electron capture detection was used to quantify the fatty acid composition of ABMC-7 cells or mouse liver tissue. ABMC-7 cells (2×10^6 cells/sample) were washed twice in PBS. Lipids in the cell pellet were saponified (60°, 1 hr) directly in 200 μL of 2.5 N KOH in methanol:water (4:1) that was spiked with 2.5 μg of heneicosanoic acid (21:0; 0.1 mg/mL stock in hexane), the internal standard.

Lipids from frozen liver tissue were prepared according to a modified Bligh and Dyer [50] procedure. Briefly, liver tissue (100 mg) was added to 4 mL of chloroform:methanol:water (1:2:0.3). Liver tissue was homogenized with a handheld homogenizer (Tissue Tearor, Biospec Products, Inc.) at room temperature. The homogenate was centrifuged at 1000 *g* for 5 min at room temperature. The supernatant was decanted and saved. To the tissue sediment, 2.3 mL of chloroform:methanol:water (1:2:0.8) was added. The tissue was vortexed vigorously and then centrifuged at 1000 *g* for 5 min at room temperature. The supernatant was decanted and pooled with the first supernatant. The pooled supernatants were diluted with 1.8 mL of chloroform and then 1.8 mL of water, followed by gentle, but thorough mixing. The chloroform and methanol/water phases were separated by centrifugation at 1000 *g* for 5 min at room temperature. The chloroform layer was removed, transferred to a new tube, and spiked with 0.5 mg of 21:0. The lipids were saponified (60°, 1 hr) by the addition of 1 mL of 2.5 N KOH in methanol:water (4:1).

Following saponification, fatty acids were protonated by the addition of 2 mL of formic acid and then extracted into hexane by the addition of 2 mL of water + 6 mL of hexane followed by thorough mixing. The hexane layer was removed and transferred to a new tube containing a small amount of Na_2SO_4 to remove any residual water. A portion of the hexane layer (1/10 volume) was transferred to another new tube and evaporated to dryness under nitrogen gas. For electron capture detection, the fatty acids were derivatized to pentafluorobenzyl esters by the addition of 10 μL of diisopropyl ethylamine (Sigma) + 20 μL of 35% pentafluorobenzylbromide in acetonitrile (Pierce Chemical Co.) in tightly sealed tubes. The samples were incubated for 15 min in a 50° water bath, after which the contents were evaporated under nitrogen gas. Hexane (1 mL) was added, and the samples were washed twice with 1 mL of water. The hexane layer was removed and evaporated under nitrogen gas. The sample residue was resolubilized in 1 mL of hexane and then transferred to a GC autosampler vial. Derivatized fatty acids were separated and identified using a Hewlett–Packard model 5880 gas chromatograph equipped with an SP-2380™ fused silica capillary column (30 m, 0.32 mm i.d., 0.20 μm film thickness; Supelco), an electron capture detector, and an HP-5880A terminal integrator. Fatty acid pentafluorobenzyl esters were identified by comigration with authentic pentafluorobenzyl ester standards.

Dietary Paradigms

Female Balb/C mice were purchased as weanlings (3 weeks old) and maintained on a normal rodent chow diet (Teklad 2215 [W] rodent diet 8640, Harlan) or an EFAD diet (5803C, low essential fatty acid P.D., Purina Test Diets) for a minimum of 8 weeks. Water was provided *ad lib.* throughout. A state of EFAD was confirmed by fatty acid composition analysis of liver tissue; the ratio of Mead acid (20:3 n-9)/AA (20:4 n-6) was approximately 5 at the end of the study (see Table 5), much higher than the defined minimum value of 0.4 for EFAD [3]. Approximately 0.5 mg of AA is consumed per day in the standard chow diet by a 20 g mouse (Lab Diet™, The Richmond Standard™, Animal Diet Reference Guide, PMI Feeds, Inc.). This level of AA may be high enough to circumvent depletion of AA during chronic inhibition of desaturase activity. To eliminate this possibility, mice initially fed a chow diet or EFAD diet were switched to a corn oil diet after initiating dosing with CP-24879. The AIN-76-based corn oil diet (DYETS, Inc.) contains LA and OA (18:1 n-9) as the principal unsaturated fatty acids, whereas AA is absent (Table 1). The high level of LA in the corn oil diet provides substrate for the *in vivo* synthesis of AA which, in the presence of a Δ5 and/or 6 desaturase inhibitor, would be blocked.

Pharmacokinetics

CP-24879 (HCl salt) was prepared as a 20 mM stock solution in saline (pH 4). Swiss–Webster mice (25 g) were injected i.v. in the tail vein (0.2 mL), resulting in a bolus dose of approximately 33 mg/kg (0.9 mg or 4 μmol/mouse). Four mice were included per time point. Time points were

TABLE 1. Fatty acid composition of the chow, corn oil, and EFAD diets

	Fatty acid composition (μg/mg diet)		
Fatty acid	**Chow diet**	**Corn oil diet**	**EFAD diet**
16:0	6.42 ± 0.98	4.46 ± 0.49	0.45 ± 0.05
16:1 n-7	1.14 ± 0.19	0.09 ± 0.01	0.06 ± 0.00
18:0	1.83 ± 0.31	0.69 ± 0.09	0.16 ± 0.01
18:1 n-9	6.65 ± 1.00	7.55 ± 0.81	0.4 ± 0.04
18:2 n-6	10.03 ± 1.53	17.22 ± 1.94	0.04 ± 0.01
18:3 n-3	1.41 ± 0.23	0.32 ± 0.05	ND*
20:4 n-6	0.05 ± 0.01	ND	ND
20:5 n-3	0.39 ± 0.07	ND	ND
22:6 n-3	0.29 ± 0.06	ND	ND
Total fatty acids	28.19 ± 4.36	30.34 ± 3.38	1.10 ± 0.09

Values are means ± SEM, $N = 3$ replicate samples.
*ND, not detectable.

taken at 0 (saline controls), 1, 2, 5, 10, 15, 30, 60, 90, 120, 180, 240, and 360 min following injection of CP-24879. At the end of each time point, the mice in each group were anesthetized in CO_2/O_2 (80/20), and blood was collected by retro-orbital eye bleeds. Approximately 0.5 mL blood/mouse was collected into a tube containing 1 U of heparin. The heparinized blood samples were kept on ice until the end of the experiment, after which plasma was prepared by centrifuging the samples at 1500 *g* for 20 min and withdrawing the supernatant. The amount of CP-24879 present in each plasma sample was determined by GC–MS.

Samples were prepared for GC–MS by adding 0.1 mL of mouse plasma to a 3-mL glass vial containing 1 mL of water. Pentoxyaniline (0.1 mL of a 1 μg/mL stock in methanol), the straight-chain isomer of CP-24879, was added to each sample as the internal, surrogate standard. Sodium hydroxide (0.2 mL of a 25% aqueous stock solution) was added per sample to remove any bound CP-24879. CP-24879 was extracted by adding the above solution to a 6-mL C_8 solid phase extraction column that was conditioned previously with 2 vol. of methanol followed by 2 vol. of water. The columns were washed with 1 mL of water and air-dried. CP-24879 and pentyloxyaniline were eluted from the column with 2 mL of methanol. Quantitative analyses were carried out on a Hewlett–Packard 5989A Engine GC–MS system comprised of a model 5980 gas chromatograph and a model 7673 autosampler. One microliter of sample was injected using the splitless injection technique at 225° onto a J&W DB5 capillary column (0.32 mm × 30 m) with helium carrier gas at a head pressure of 5 psi. The GC column temperature was held at 100° for 4 min and then increased at 10°/min to 250°. The column effluent was directed into the ion source of the mass spectrometer that was maintained at 200°. The quadrupole mass spectrometer was operated in the electron impact ionization mode at 70 eV using selected ion monitoring of the following mass-to-charge (m/z) ratios:

m/z 109.1 Fragment ion of CP-24879

and pentyloxyaniline (loss of C_5H_{10})

m/z 179.1 Molecular ion of CP-24879

and pentyloxyaniline

Standard pharmacokinetic equations were utilized to calculate clearance (CL), volume of distribution (V), and half-life ($T_{1/2}$) [51]:

$$CL = \text{dose/area under the curve (AUC)}$$

$$k \text{ (rate constant of elimination)} = 0.693/T_{1/2}$$

$$V = CL/k$$

$$T_{1/2} = 0.693(V/CL)$$

In Vivo *Assay of* Δ6 *and* Δ5 *Desaturase Activities*

An appropriate amount of [1-^{14}C]18:2 n-6 (ethanolic solution) was evaporated to dryness under nitrogen and immediately dissolved in 18.2 mM of Na_2CO_3 (10-fold molar excess) to a specific activity of 100 μCi/mL. Mice ($N = 5$/group) were injected i.p. with 0.1 mL (10 μCi) of [^{14}C]18:2 n-6. After 24 hr, the mice were killed by CO_2 inhalation. Their livers were removed quickly, frozen on dry ice, and then stored at −70°. Total liver lipids were extracted from 100–200 mg liver tissue in chloroform: methanol:water and then saponified in 2.5 N of KOH in methanol:water as described above. Following evaporation of the chloroform layer under nitrogen gas, the fatty acids were transmethylated by the addition of 2 mL of methanolic-HCl (3–5% HCl, made by mixing 0.1 vol. of acetyl chloride with 1 vol. of cold methanol). Following transmethylation (65–70°, 2 hr), 2 mL of water and 6 mL of hexane were added to each tube. The contents were mixed vigorously, and the phases were separated by centrifugation at 1000 *g* for 5 min at room temperature. The hexane layer was removed, transferred to a new tube, and dried under nitrogen gas. The fatty acid-methyl esters (approximately 10^5 dpm) were solubilized in 0.3 mL of acetonitrile and then transferred to an HPLC autosampler vial. [^{14}C]Fatty acid substrate and [^{14}C]fatty acid products were resolved and quantified using a Waters HPLC system (Machery-Nagel C_{18} reverse phase column) connected in-line with a FLO-ONE Beta (Radiomatic) radioactivity detector. Chromatography conditions were as follows: 70–90% acetonitrile gradient for 65 min followed by 90% acetonitrile for 10 min; flow rate = 1 mL/min. [^{14}C]Fatty acids were identified by co-migration with authentic methylated [^{14}C]fatty acid standards. Combined Δ6 desaturase/elongase/Δ5 desaturase activities were calculated as the percent conversion of substrate ([^{14}C]18:2 n-6) to products ([^{14}C]18:3 n-6 + [^{14}C]20:3 n-6 + [^{14}C]20:4 n-6).

Statistics

Comparisons between control and treatment groups were done as two-sample *t*-tests. If there was a value below the

detection limit, the true value was assumed to be at the detection limit and was used to calculate the mean. Only values above the detection limit were used to calculate the SEM. This approach yields an underestimate of the difference between groups and a realistic estimate of the SEM. As such, it is conservative. No adjustment was made for multiple comparisons because it was preferred that α (type I error rate) be set on the individual comparisons.

RESULTS

Rat Liver Microsomal Assay

A microtiter plate assay was developed for relatively high-throughput screening of Δ5 and/or Δ6 desaturase inhibitors. Assay of Δ9 desaturase activity was included as a control to exclude the possibility of non-specific, indirect inhibition of Δ5 and/or Δ6 desaturase activity. The assay utilized hepatic microsomes from rats that were fasted for 3 days and then refed an EFAD diet for 2 days. In agreement with previous findings [48, 52, 53], this fasting and refeeding paradigm stimulated both Δ9 and Δ6 desaturase activities (approximately 3- and 4-fold, respectively), whereas Δ5 desaturase activity was unaffected (results not shown). α-LN rather than LA was used as the substrate for the Δ6 desaturase [54]. A comparison of the two fatty acid substrates showed that Δ6 desaturase activity was approximately 3-fold higher with α-LN as opposed to LA as the substrate (data not shown). By adding all three [^{14}C]fatty acid substrates to a single microtiter dish well, the single-step conversion to the respective desaturated [^{14}C]product was resolved by argentation-TLC. Elongation of the [^{14}C]fatty acid product did not occur because malonyl-CoA, the substrate for two-carbon elongation [32], was omitted from the buffer. All three desaturase activities were thus quantified in a single lane of a TLC plate (Fig. 2A). Optimal conditions for the desaturase assay were as follows: 0.5 mg of protein, 5 μM (0.1 μCi) of [^{14}C]fatty acid substrate, 37°, 1-hr incubation. The buffer concentration was kept high (100 mM of phosphate buffer) in order to provide better buffering capacity when assaying natural product extracts. DMSO (≤ 10%) and ethanol (≤ 2%) had no effect on the three desaturase activities.

Intact Cell Assay

A cell-based assay using mouse mastocytoma ABMC-7 cells was developed in which desaturase activities were measured by the conversion of [^{14}C]fatty acid substrates to their respective [^{14}C]fatty acid product(s) (Fig. 2, B–D). In contrast to the liver microsomal assay, the separate measurement of Δ6 desaturase activity could not be performed in these cells (Fig. 2B) because of the rapid coupling of Δ6 desaturation to subsequent elongation and Δ5 desaturation [29]. This was not an issue with Δ9 desaturase or Δ5 desaturase activity measurements because the products, OA or AA, respectively, do not readily undergo further elongation and desaturation (Fig. 2, C and D).

Identification of CP-24879 As a Mixed Δ6/Δ5 Desaturase Inhibitor

The liver microsomal and ABMC-7 cell assays identified CP-24879 (*p*-isopentoxyaniline) as a mixed Δ6/Δ5 desaturase inhibitor (Table 2). In both assays, CP-24879 inhibited Δ6 desaturase activity and, to a lesser extent, Δ5 desaturase activity (Fig. 2). No inhibition of Δ9 desaturase activity was observed at 200 μM, the highest concentration tested in the microsomal assay (Fig. 2A). Only minimal inhibition, at best, was observed at 67 μM, the highest concentration tested in the ABMC-7 cell assay (Fig. 2D). Compared with results in the microsomal assay, CP-24879 was a more potent and selective Δ6 desaturase inhibitor in the ABMC-7 cell assay (Table 2). In both control and CP-24879-treated ABMC-7 cells, the cellular uptake of [^{14}C]LA or [^{14}C]DGLA was nearly 100% (data not shown), and the recovery of [^{14}C]fatty acid substrate and corresponding [^{14}C]fatty acid product(s) was also similar at every concentration (Fig. 2, B–D), demonstrating indirectly that CP-24879 was not acutely toxic.

Effects of CP-24879 in ABMC-7 Cells

ABMC-7 cells were chosen for evaluating the effects of chronic desaturase inhibition by CP-24879 because they have relatively high Δ5, Δ6, and Δ9 desaturase activities and produce a copious amount of LTC_4 following stimulation with calcium ionophore. It was hypothesized that chronic exposure of ABMC-7 cells to increasing concentrations of CP-24879 would correlate with increased inhibition of Δ6/Δ5 desaturase activities, depletion of AA, and decreased LTC_4 production. One major caveat was that AA was present at a concentration of 15 μM in the standard growth medium, RPMI-1640 + 10% FBS, a concentration that was sufficiently high to circumvent depletion of AA by chronic desaturase inhibition (results not shown). To overcome the presence of AA in serum-containing media, ABMC-7 cells were adapted to grow in HL-1, a defined, serum-free medium. Except for LA (2 μM), HL-1 medium does not contain detectable levels of other PUFAs. ABMC-7 cells cultured in HL-1 without serum became EFAD; the cells accumulated Mead acid (approximately 0.8 μg/10^6 cells), but were depleted of LA and AA (< 0.05 μg/10^6 cells). To selectively replete n-6 PUFAs, particularly AA, in ABMC-7 cells, the HL-1 medium was supplemented with additional LA (10 μM) and fatty acid-free BSA (1 mg/mL; 15 μM) as a fatty acid carrier. The rationale was to assess whether desaturase inhibition would cause depletion of AA and decreased LTC_4 production if the source of cellular AA was solely from precursor LA.

When ABMC-7 cells were cultured for 2 weeks in HL-1 supplemented with LA, n-6 PUFAs were repleted to approximately the same levels as in cells cultured in RPMI-1640 + 10% FBS. AA increased from an undetectable level (< 0.05 μg/10^6 cells) to approximately 1.4 μg/10^6 cells,

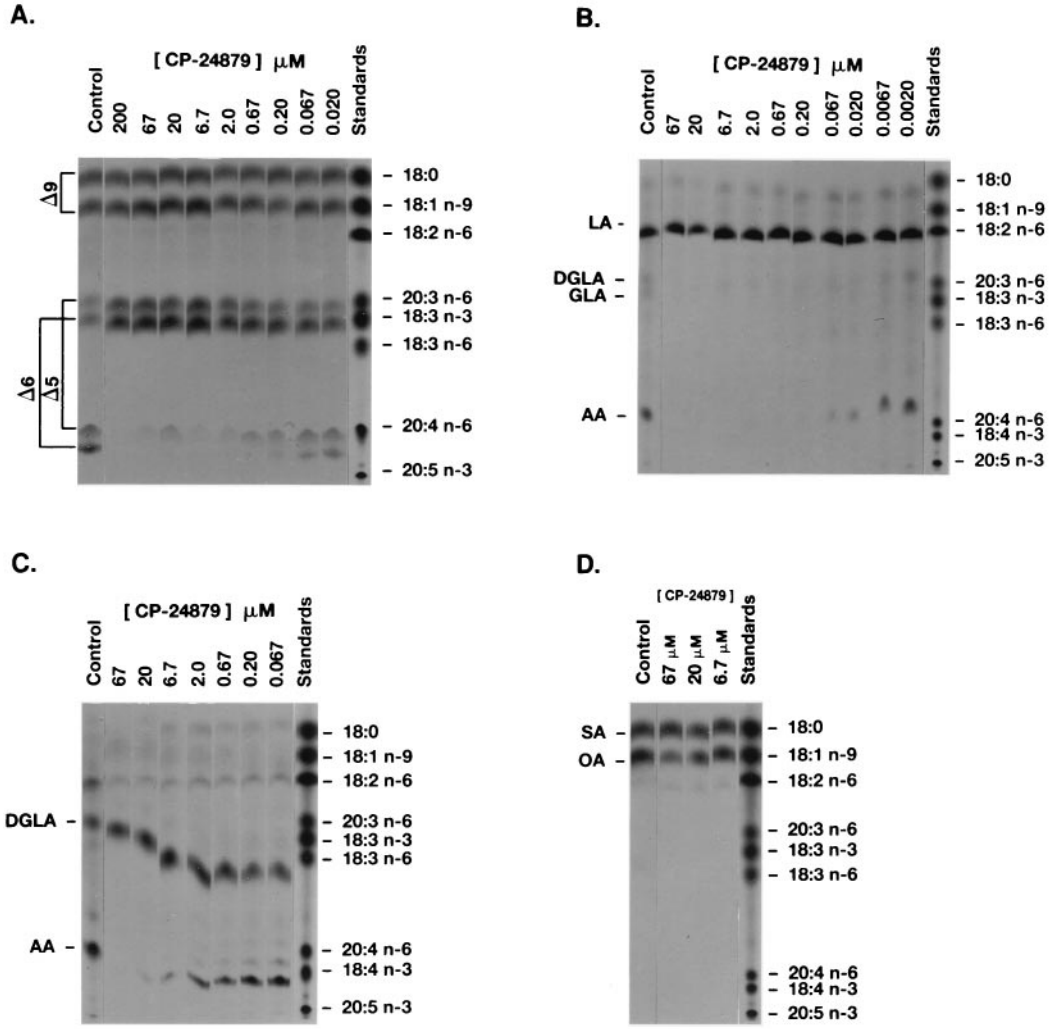


FIG. 2. Concentration-dependent inhibition of Δ5 and Δ6 desaturase activities by CP-24879 in the rat liver microsomal assay (A) and ABMC-7 cell assay (B–D). In the rat liver microsomal assay (A), all three desaturase activities were quantified by measuring separately the conversion of the respective [^{14}C]fatty acid substrate to its respective [^{14}C]fatty acid product in a single lane following argentation-TLC.

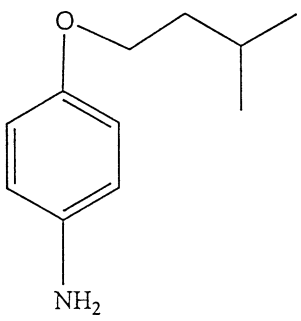
[1-^{14}C]Substrate	Desaturase	[1-^{14}C]Product
18:0(stearic acid,SA)	Δ9	18:1 n-9
18:3 n-3	Δ6	18:4 n-3
20:3 n-6	Δ5	20:4 n-6

In ABMC-7 cells (B–D), desaturation is coupled to elongation; hence, measurement of Δ6 desaturase activity could not be performed independently of Δ5 desaturase activity. Instead, combined Δ6 desaturase/elongase/Δ5 desaturase activities were measured (B), while Δ5 and Δ9 desaturase activities were measured separately (C and D, respectively).

[1-^{14}C]Substrate	Desaturase	[1-^{14}C]Product(s)
18:0	Δ9	18:1 n-9
18:2 n-6	Δ6 + Δ5	18:3 n-6 + 20:3 n-6+20:4 n-6
20:3 n-6	Δ5	20:4 n-6

With both assays, inhibition of Δ5, Δ6, or Δ9 desaturase activity was determined qualitatively by autoradiography. Quantification of desaturase activities [% conversion of [^{14}C]fatty acid substrate to [^{14}C]fatty acid product(s)] was determined directly from the $AgNO_3$-TLC plates by electronic autoradiography (see Materials and Methods for details). Each sample was run in duplicate on different TLC plates. Results did not vary by more than 10% per sample duplicate. A representative autoradiogram is shown for each TLC plate.

TABLE 2. CP-24879: Structure and desaturase inhibition profile in the rat liver microsomal and ABMC-7 cell assays

CP-24879	Desaturase	*In vitro* IC_{50} (μM)	
		Liver microsomes	ABMC-7 cells
	Δ5	3.4	0.67
	Δ6	0.56	0.015
	Δ9	>200	>67
	Ratio (IC_{50}) Δ5/Δ6	6	45

The IC_{50} values were calculated from a representative concentration–response curve ($N = 4$) in which percent conversion of the [^{14}C]fatty acid substrate to its respective [^{14}C]fatty acid product(s) was quantified directly by β or phosphorescent imaging (see legend of Fig. 2).

while the level of LA increased from an undetectable level (< 0.05 μg/10^6 cells) to approximately 2.6 μg/10^6 cells. The level of Mead acid (20:3 n-9) decreased from approximately 0.9 μg/10^6 cells to 0.4 μg/10^6 cells. Following repletion of n-6 PUFAs, ABMC-7 cells were exposed to increasing concentrations of CP-24879 (0, 100 nM, 300 nM, 1 μM, 3 μM, or 10 μM) in order to evaluate whether a concentration-dependent relationship existed between desaturase inhibition and depletion of AA and decreased LTC_4 production. Following incubation of ABMC-7 cells in HL-1 + LA ± CP-24879 for 4 days (approximately eight replication cycles), the cultures were analyzed for cell viability, desaturase activity, fatty acid composition, and LTC_4 production. Cell viability, measured by trypan blue exclusion, was not affected by CP-24879, even at 10 μM, the highest concentration tested (viability ≥ 98%). CP-24879 inhibited Δ6 + Δ5 desaturase activities in a concentration-dependent manner (Fig. 3A). Complete inhibition was observed at the highest concentration tested (10 μM), whereas at the lowest concentration tested (0.1 μM) there was virtually no inhibition of the conversion of [^{14}C]LA to [^{14}C]AA (Fig. 3A). Correspondingly, there was a concentration-dependent depletion of AA (Fig. 3B) and decrease in LTC_4 production (Fig. 3C). In contrast, when inhibitor-treated cells were stimulated with calcium ionophore in the presence of exogenous AA (30 μM), the production of LTC_4 was equal to or higher than in untreated cells (≥ 30 ng/mL). These results indicated that 1) depletion of AA, the immediate substrate for the 5-lipoxygenase, was solely responsible for the decreased production of LTC_4, and 2) CP-24879 did not inhibit either 5-lipoxygenase or LTC_4 synthase activity.

Pharmacokinetic Properties of CP-24879

Pharmacokinetic experiments with CP-24879 were performed in mice in order to calculate the half-life of the molecule in plasma and, thence, the dosing regimen required to inhibit Δ6 and Δ5 desaturase activities chronically in the liver. Circulating plasma concentrations of CP-24879 were determined by extraction of the compound from plasma and quantification by GC–MS (Fig. 4). GC–MS analysis of whole blood spiked with CP-24879 showed that only approximately 50% of the compound was free in plasma; the remaining 50% was bound to blood components (data not shown). The volume of distribution (V = 1.9 mL/g) was nearly twice the weight of the mouse, indicating that CP-24879 was readily distributed to the peripheral tissues. CP-24879 was cleared quite rapidly (CL = 0.56 mL/min) and had a relatively short half-life ($T_{1/2}$ = 59 min). The relatively short half-life of CP-24879 suggested that t.i.d. dosing would be required to sustain a plasma level that would be sufficient to chronically inhibit combined Δ6 and Δ5 desaturase activities.

Effect of CP-24879 *on Desaturase Activity and Fatty Acid Composition* In Vivo

It was hypothesized that chronic dosing of CP-24879 would inhibit liver Δ6 and Δ5 desaturase activities and, subsequently, cause depletion of AA in liver tissue. This hypothesis was evaluated using two dietary paradigms: (1) an AA depletion paradigm. Chow-fed mice were dosed with CP-24879 and then switched to a corn oil diet. Desaturase inhibition should cause depletion of AA; and (2) an AA repletion paradigm. EFAD mice were dosed with CP-24879 and then switched to a corn oil diet. Desaturase inhibition should prevent repletion of AA.

The maximally tolerated dose of CP-24879 was determined empirically by injecting CP-24879 (i.p., t.i.d.) into chow-fed mice at doses of 30, 10, 3, or 1 mg/kg for 3 days. Dosings of 30 or 10 mg/kg were toxic by 24 hr. In contrast, after 3 days, no overt toxicity was observed with the 3 or 1 mg/kg dosings.

The effects of chronic administration of CP-24879 on Δ6/Δ5 desaturase activity and the fatty acid composition of

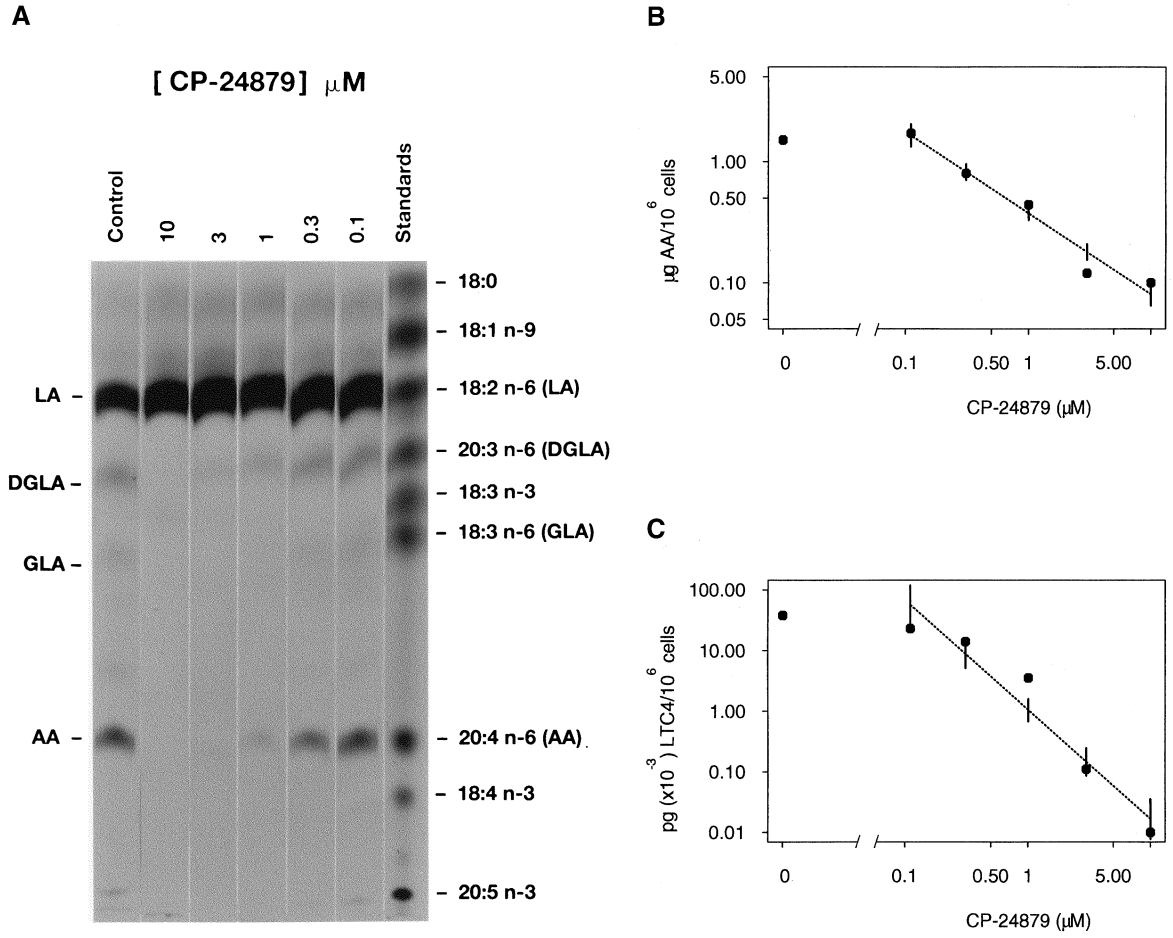


FIG. 3. Effect of CP-24879 on desaturase activity, arachidonic acid levels, and LTC_4 production in ABMC-7 cells. ABMC-7 cells were cultured in a defined, serum-free medium (HL-1) that was supplemented with 10 μM LA (HL-1 + LA) as the only source for the synthesis of AA. Cells were cultured in duplicate T flasks for 4 days in HL-1 + LA containing increasing concentrations of CP-24879, after which they were analyzed for desaturase activity (A), cellular content of AA (B), and LTC_4 production (C). Combined Δ6 + Δ5 desaturase activities (A) were quantified by measuring the conversion of [^{14}C]fatty acid substrate to the combined [^{14}C]fatty acid products:

[1-^{14}C]Substrate	Desaturase	[1-^{14}C]Products
18:2 n-6	Δ6 + Δ5	18:3 n-6 + 20:3 n-6 + 20:4 n-6

LTC_4 production was determined by stimulating the cells with the calcium ionophore A-23187, and then measuring the level of LTC_4 in the supernatants by standard ELISA (in triplicate) in the absence or presence of exogenous AA (see Materials and Methods for details). A representative autoradiogram is shown for desaturase inhibition. The levels of AA and LTC_4 are the mean values of duplicate samples. The variation in the response increased with the response, but was not more than 10–15% of the mean value. Hence, the mean data values were transformed to logarithms and a straight line regression was fit to the log dose. The fitted regression line and the standard error of the fitted line are shown.

mouse liver were evaluated by injecting CP-24879 at the maximally tolerated dose (3 mg/kg, i.p., t.i.d.) into chow-fed mice for 6 days and into EFAD mice for 4 days. Twenty-four hours prior to terminating the experiment, 10 μCi of [^{14}C]LA was injected i.p. into the mice, after which time the mice were killed and liver samples were prepared for quantification of desaturase activity and fatty acid composition.

Compared with chow-fed mice injected with saline, there was an 80% inhibition of the combined Δ6 + Δ5 desaturase activities in the livers of chow-fed mice injected with CP-24879 and then switched to a corn oil diet (Fig. 5; Table 3). Concomitantly, AA dropped by 46% in the livers of mice injected with CP-24879 (Table 4). The drop in AA was also reflected in the higher ratio of LA/AA in the livers of mice injected with CP-24879 versus saline (4.70 vs 2.00, respectively; Table 4).

Within 4 days of feeding EFAD mice the corn oil diet, the levels of LA, DGLA, and AA increased markedly, while the levels of OA and Mead acid (20:3 n-9) decreased (Table 5), demonstrating the rapidity of reversal of the EFAD state. In mice treated with CP-24879, there was

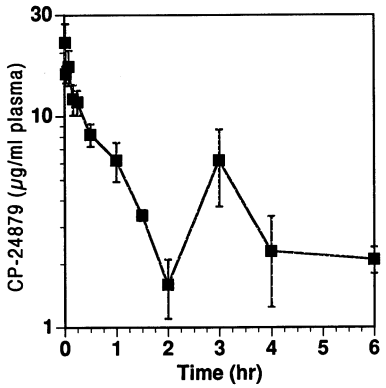


FIG. 4. Pharmacokinetics of CP-24879 in the mouse. CP-24879 was administered as a 33 mg/kg bolus dose into the tail vein of male Swiss–Webster mice. Blood was extracted at 0 (saline controls), 1, 2, 5, 10, 15, 30, 60, 90, 120, 180, 240, and 360 min. CP-24879 was extracted from plasma and quantified by GC–MS (see Materials and Methods for details). Results are expressed as μg CP-24879/mL plasma (mean ± SEM, N = 4/time point).

approximately 80% inhibition of combined Δ6 + Δ5 desaturase activities (Fig. 5; Table 3) with only partial repletion of AA (Table 5). Repletion of LA was complete, leading to an LA/AA ratio nearly 2-fold higher in mice injected with CP-24879 versus mice injected with saline (2.46 vs 1.40, respectively; Table 5).

With both chow-fed and EFAD mice, the ratio of stearic acid (18:0)/OA was lower in the livers of mice injected with CP-24879 versus saline (0.72 vs 0.90 and 0.26 vs 0.46, respectively; Tables 4 and 5). This was probably due to induction of Δ9 desaturase activity in mice dosed with CP-24879.

TABLE 3. Desaturase activity in livers of chow-fed and EFAD mice injected with saline or CP-24879 and then fed a corn oil diet

	% Conversion ([^{14}C]LA → [^{14}C]AA)		% Inhibition	
Treatment	Chow-fed	EFAD	Chow-fed	EFAD
Saline	39.6 ± 1.6	30.2 ± 1.8		
CP-24879	8.1 ± 1.4*	5.1 ± NA*†	80	83

Chow-fed and EFAD Balb/C mice were dosed with CP-24879 (3 mg/kg, i.p., t.i.d.). After the first injection, chow-fed or EFAD mice were switched to a corn oil diet for 6 or 4 days, respectively, as a source of linoleic acid. Twenty-four hours prior to terminating the experiment, 10 μCi of [^{14}C]LA was injected i.p. into the mice, after which time they were killed, and liver samples were prepared for quantification of desaturase activity. Combined Δ6 + Δ5 desaturase activities are expressed as the percent conversion of [^{14}C]LA to [^{14}C]AA (mean ± SEM; $N = 5$/group).

*$P < 0.001$.

†NA: standard error of the mean is not available.

DISCUSSION

The development of high-throughput liver microsomal and cell assays to measure desaturase activity provided the tools necessary to facilely screen and identify selective Δ6 and/or Δ5 desaturase inhibitors. CP-24879 (*p*-isopentoxyaniline)

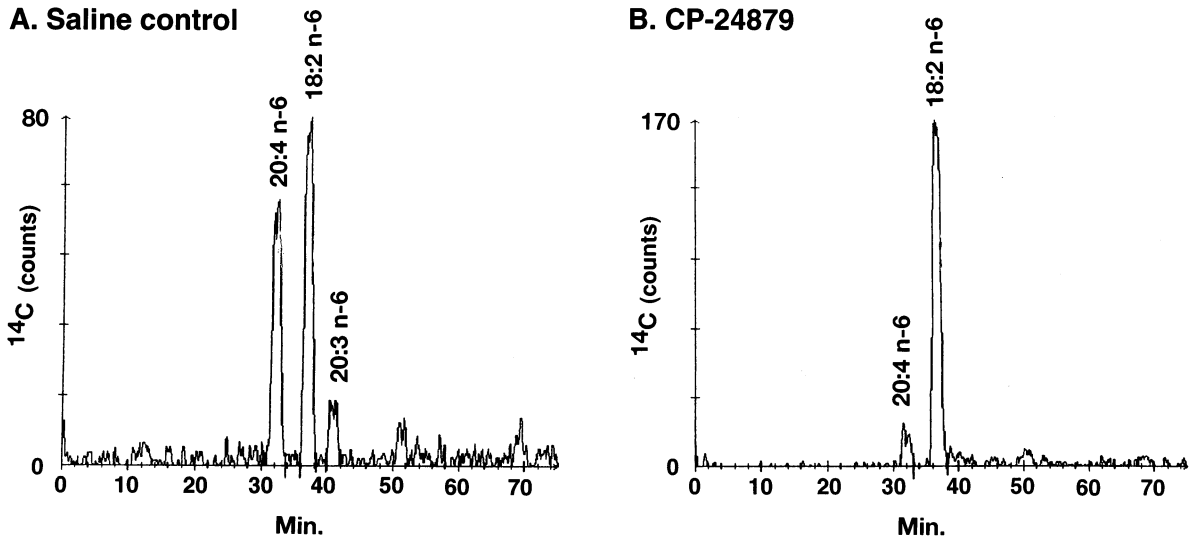


FIG. 5. Inhibition of combined Δ6 and Δ5 desaturase activities by CP-24879 in mouse liver. Chow-fed or EFAD mice were injected with the maximally tolerated dose of CP-24879 (3 mg/kg, i.p., t.i.d.) and, after the first dose, were switched to a corn oil diet. After 6 days of dosing, the mice were injected i.p. with 10 μCi of [^{14}C]LA to measure combined Δ6 desaturase/elongase/Δ5 desaturase activities (conversion of [^{14}C]LA to [^{14}C]AA). After 24 hr, the mice were killed, and their livers were removed. Lipids were extracted from liver tissue and saponified. Liberated fatty acids were transmethylated, and [^{14}C]LA and its metabolic products, [^{14}C]GLA, [^{14}C]DGLA, and [^{14}C]AA, were resolved and quantified by radiometric reverse phase-HPLC. Representative tracings are shown for saline-injected (A) and CP-24879-injected (B) mice. In the livers of saline-injected mice, [^{14}C]GLA (27 min elution time) was not detected following the injection of [^{14}C]LA. As anticipated, inhibition of combined Δ6 and Δ5 desaturase activities by CP-24879 caused [^{14}C]LA to accumulate to a level approximately 2-fold higher in the livers of CP-24879-injected mice than in the livers of saline-injected controls.

TABLE 4. Fatty acid composition of livers from chow-fed mice injected with saline or CP-24879 and then fed a corn oil diet

	Fatty acid composition (μg/mg liver tissue)	
Fatty acid	Saline	CP-24879
16:0	15.88 ± 0.64	16.40 ± 1.57
16:1 n-7	1.86 ± 0.05	1.38 ± 0.27
18:0	6.10 ± 0.14	5.70 ± 0.51
18:1 n-9 (OA)	6.74 ± 0.22	9.02 ± 1.69
18:2 n-6 (LA)	9.92 ± 0.38	11.72 ± 1.99
20:3 n-9 (Mead acid)	0.2 ± NA*	0.2 ± NA*
20:3 n-6 (DGLA)	0.80 ± 0.03	0.2 ± NA*†
20:4 n-6 (AA)	4.96 ± 0.13	2.66 ± 0.29†
Total fatty acids	53.34 ± 1.31	52.78 ± 5.26
	Ratio	
18:2 n-6/20:4 n-6	2.00 ± 0.08	4.70 ± 1.19
18:0/18:1 n-9	0.90 ± 0.32	0.72 ± 0.14

Chow-fed and EFAD Balb/C mice were dosed with CP-24879 (3 mg/kg, i.p., t.i.d.). After the first injection the mice were fed a corn oil diet as a source of linoleic acid. In the chow-fed mice switched to a corn oil diet, depletion of AA in the livers was evaluated after 6 days of dosing. Fatty acid composition is expressed as micrograms of fatty acid per milligram of liver tissue (mean ± SEM; $N = 5$/group).

*NA: standard error of the mean is not available.

†$P < 0.01$.

was the first compound identified and partially characterized as a mixed, Δ6 > Δ5, desaturase inhibitor in the Monsanto/Searle chemical and natural product libraries (Table 2). CP-24879 could be classified as a selective desaturase inhibitor because partial inhibition of the Δ9 desaturase, a non-target enzyme, first occurred at a concentration > 1000-fold higher than the IC_{50} value for inhibition of Δ6 or Δ5 desaturase activity in ABMC-7 cells. There was a discrepancy, however, between the IC_{50} values in the rat liver microsomal assay versus the ABMC-7 cell assay; the IC_{50} values were considerably lower in the ABMC-7 cell assay versus the rat liver microsomal assay. Possible explanations are that (1) the cells concentrated the compound, especially after an overnight incubation, (2) the compounds were metabolized by the ABMC-7 cells to a more potent inhibitor, or (3) the preparation of microsomes distorted the overall organization of the electron transport-desaturase components in the endoplasmic reticulum.

The relative lack of inhibition of Δ9 desaturase activity by CP-24879 in the rat liver microsomal assay and the ABMC-7 cell assay suggested that CP-24879 inhibited the Δ6 and Δ5 desaturases specifically rather than inhibiting common pathways or enzymes common to other desaturases, viz. electron transport through cytochrome b_5 and cytochrome b_5 reductase or acylation of CoA by acyl-CoA synthetase [55]. Unequivocal proof of direct or indirect inhibition of the Δ6 and Δ5 desaturases, however, will have to wait until these enzyme systems are reconstituted reproducibly *in vitro*.

This study provides the first evidence *in vitro* and *in vivo* that chronic inhibition of Δ6/Δ5 desaturase activities can cause depletion of AA and ensuing decreases in eicosanoid production. In ABMC-7 cells that were incubated in the

TABLE 5. Fatty acid composition of livers from EFAD mice dosed with saline or CP-24879 and then fed a corn oil diet

	Fatty acid composition (μg/mg liver tissue)		
Fatty acid	EFAD control	Saline	CP-24879
16:0	11.40 ± 0.40	12.46 ± 0.60	10.58 ± 1.03
16:1 n-7	4.50 ± 0.12*	2.96 ± 0.18	2.98 ± 0.50
18:0	4.47 ± 0.22	4.96 ± 0.22	3.90 ± 0.70
18:1 n-9 (OA)	16.87 ± 0.55†	11.30 ± 0.62	15.44 ± 1.29†
18:2 n-6 (LA)	0.2 ± NA*‡	6.64 ± 0.23	4.70 ± 1.30
20:3 n-9 (Mead acid)	4.87 ± 0.07*	0.54 ± 0.03	1.16 ± 0.17
20:3 n-6 (DGLA)	0.2 ± NA*‡	1.12 ± 0.07	0.38 ± NA‡§
20:4 n-6 (AA)	0.97 ± 0.07*	4.84 ± 0.16	2.04 ± 0.67§
Total fatty acids	55.07 ± 0.54	51.18 ± 0.85	47.38 ± 4.57
		Ratio	
18:2 n-6/20:4 n-6	0.21 ± 0.01*	1.40 ± 0.05	2.46 ± 0.10§
18:0/18:1 n-9	0.26 ± 0.03*	0.46 ± 0.04	0.26 ± 0.06*
20:3 n-9/20:4 n-6	5.00 ± 0.38*	0.11 ± 0.03	0.72 ± 0.15§

Chow-fed and EFAD Balb/C mice were dosed with CP-24879 (3 mg/kg, i.p., t.i.d.). After the first injection, the mice were fed a corn oil diet as a source of linoleic acid. Repletion of AA in the livers of EFAD mice switched to a corn oil diet was evaluated after 4 days of dosing. An EFAD control group ($N = 3$) is included for additional comparison. Mice in this group were maintained on the EFAD diet and were not injected with saline or CP-24879. Statistical comparisons were made between the EFAD and saline groups and between the saline and CP-24879 groups. Fatty acid composition is expressed as micrograms of fatty acid per milligram of liver tissue (mean ± SEM; $N = 5$/group).

*$P < 0.001$.

†$P < 0.05$.

‡NA: standard error of the mean is not available.

§$P < 0.01$.

presence of CP-24879 for 4 days, there was a concentration-dependent inhibition of Δ6/Δ5 desaturase activities that correlated with the degree of depletion of AA and the degree of decreased LTC_4 production (Fig. 3). In chow-fed mice dosed with CP-24879 and then switched to a diet containing LA, but devoid of AA (i.e. corn oil diet), AA was depleted significantly after 6 days (Table 4). In a corollary *in vivo* study, repletion of AA was blocked in the livers of EFAD mice dosed with CP-24879 and then fed a corn oil diet for 4 days (Table 5). These results indicated that CP-24879 inhibited Δ6 + Δ5 desaturase activities *in vivo*, causing depletion of AA in the livers of chow-fed mice and preventing repletion of AA in the livers of EFAD mice. With both *in vivo* paradigms, there was an increase in OA in the livers of CP-24879-treated mice compared with the saline controls (Tables 4 and 5). The increase in OA suggested that when n-6 PUFA synthesis was inhibited, the liver compensated by synthesizing more OA from stearic acid (18:0) by up-regulating Δ9 desaturase activity. This type of regulation of Δ9 desaturase activity *in vivo* is analogous to induction of Δ9 desaturase activity following deprivation of n-6 PUFAs [56]. In chow-fed mice, LA increased in the CP-24879-treated group compared with the saline control group (Table 4). This increase in LA was due most likely to inhibition of the Δ6 desaturase, preventing its further metabolism to GLA, DGLA, and AA. Analogously, in EFAD mice, LA increased markedly in the CP-24879-treated group compared with the unmanipulated EFAD control group that was maintained on the EFAD diet (Fig. 3C). However, complete repletion of LA in the CP-24879-treated group did not occur compared with the saline control group (Fig. 3C) due, in part, to the maintenance of OA and possibly because CP-24879 inhibited other aspects of fatty acid metabolism (e.g. acylation or transacylation into or among lipid species). As expected, the level of Mead acid decreased considerably in both CP-24879- and saline-treated EFAD mice compared with the unmanipulated EFAD control group (Table 5); in both groups, the level of Mead acid was correlated inversely with the levels of LA + AA. These combined results suggested that there is high-level *de novo* synthesis of AA from LA and an accompanying rapid turnover of n-6 PUFAs, notably in AA pools that are used for eicosanoid synthesis [57–60].

Overt side-effects were observed with CP-24879 in mice when they were dosed more than 6 days at ≥ 9 mg/kg/day. The side-effects appeared to be CNS-related because there was lethargy accompanied by hypothermia and late-stage tremoring with body movement. Besides the overriding issue of toxicity, CP-24879 is a mixed Δ6/Δ5 desaturase inhibitor. Chronic inhibition of Δ6 desaturase may not be desirable because LA is not sufficient to ameliorate all of the symptoms of EFAD, and inadequate Δ6 desaturase activity has been correlated with chronic inflammatory diseases, viz. atopic eczema and diabetic neuropathy [61]. Instead, selective inhibition of the Δ5 desaturase may be the preferred target.

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