Investigation of 3,5-Isoxazolidinediones as Hypolipidemic Agents in Rodents

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A series of 2-benzoyl-4,4-dialkyl-3,5-isoxazolidinediones proved to have potent hypolipidemic activity, lowering both serum cholesterol and triglyceride levels at 10 or 20 mg/kg/day, IP and orally in rodents. 2-(3,4,5-Trimethoxybenzoyl)-4,4-diethyl-3,5-isoxazolidinedione (*4*) afforded the best hypolipidemic activity lowering normolipidemic CF_1 mouse serum cholesterol levels 49% and serum triglyceride levels 34% at 20 mg/kg/day, IP. Compound *4* was selected as a typical derivative of the chemical class for further detailed studies. Serum cholesterol levels in normolipidemic Sprague Dawley male rats were reduced 45% after 8 weeks at 10 and 20 mg/kg/day of compound, orally. Serum triglyceride levels were reduced 38–49% at 10 and 20 mg/kg/day, orally. *In vitro* liver enzyme activities studies in normolipidemic CF_1 mice showed the compound inhibited mitochondrial citrate exchange, acetyl CoA synthetase, HMG CoA reductase, acyl CoA cholesterol acyl transferase, acetyl CoA carboxylase, *sn*-glycerol-3-phosphate acyl transferase, phosphatidylate phosphohydrolase and heparin-induced lipoprotein lipase activities with increases in the activities of cholesterol ester hydrolase and ATP-dependent citrate lyase. Similar enzyme activities were inhibited *in vivo* except HMG CoA reductase activity was not inhibited in rat liver or small intestinal mucosa after 8 weeks drug administration. Cholesterol levels were reduced in tissues after 8 weeks administration of compound *4* in normolipidemic rats. Bile cholesterol and triglyceride levels were elevated after two weeks administration to rats at 20 mg/kg/day. Serum lipoprotein levels in normolipidemic and hyperlipidemic rats showed the cholesterol levels in VLDL and LDL fractions after 4, 6 and 8 weeks at 10 and 20 mg/kg/day were reduced whereas HDL-cholesterol levels were significantly elevated. Studies demonstrated that ^{3}H-cholesterol and ^{14}C-palmitic acid incorporation into lipids of the lipoprotein fraction was reduced by the drug but ^{32}P-incorporation was generally elevated. The agent demonstrated no observable toxicity in rats after 8 weeks administration, orally. The acute toxicity study in normolipidemic mice at 20, 40 and 100 mg/kg/day, IP, demonstrated no observable harmful effects of the drug.

INTRODUCTION

Previously a number of cyclic imides (e.g., succinimides[1], dilantin[2], phenobarbital[3], terephthalic acid[4], 2-pyrrolidinones[5,6], 4-pyrimidine carboxylic acids[7], furoic acid[8], and 3 and 4 phenylpiperidine-2,6-diones[9]) have been shown to have potent hypolipidemic activity in rodents at the relatively low dose of 20 mg/kg/day. Substitution on the nitrogen atom within the ring structure of many of these derivatives afforded compounds that not only lowered both serum cholesterol and triglycerides but in addition elevated HDL cholesterol levels and lowered LDL or VLDL cholesterol levels within a 14-day period in rats.

Derivatives of 3,5-isoxazolidinedione have previously been synthesized[10,11] and are known to have anticoagulant activity[12] and to reduce lens aldolase reductase activity[13] in diabetes retinopathy.

Subsequently, we have shown that 3,5-isoxazolidinediones possess hypolipidemic activity, lowering both serum cholesterol and triglyceride levels in rats and mice[13]. It appeared appropriate to test the 2-benzoyl-3,5-isoxazolidine diones for hypolipidemic activity in rodents.

METHODS AND PROCEDURES

General Procedure

Melting points are uncorrected. IR spectra were recorded either on a Perkins Elmer 1600 FTIR or on a Beckman Acculab 10 spectrophotometer. ^{1}H NMR (400 MHz), and ^{13}C NMR (100MHz) spectra were recorded on a Varian XL-400 spectrometer. ^{1}H and ^{13}C NMR chemical shifts are reported relative to internal tetramethylsilane. Mass spectra were determined on an AEI-902 mass spectrometer at the Research Triangle Institute of Mass Spectrometry, Research Triangle Park, NC. Elemental analyses were performed by Desert Analytics, Tucson, AZ. Compounds were analyzed for C, H and N and were found to be with ± 0.4% of their theoretical values.

2,6-Dimethoxybenzohydroxamic acid was prepared by the method of Konaji [14]. The remaining benzohydroxamic acids were prepared by the general method of Izydore *et al.* [15].

2-Benzoyl-4,4-dialkyl-3,5-isoxazolidinediones (4-12). To a flask equipped with a dropping funnel, condenser, and drying tube was added a mixture consisting of the benzohydroxamic acid (10 mmol) and pyridine (5 mL) in dichloromethane (100 mL). The mixture was cooled in an ice bath, and the dialkylmalonyl chloride (10 mmol) was added dropwise over a 15 min period with stirring. The reaction mixture was stirred at room temperature for one hr after which time the hydroxamic acid had dissolved. The solution was extracted four times with 50 mL portions of water, four times with 50 mL portions of 5% HCl, and two times with 50 mL portions of 5% Na_2CO_3. The dichloromethane solution was dried ($MgSO_4$) and evaporated under reduced pressure to give the 2-benzoyl-3,5-isoxazolidinedione. The relatively pure product was purified by recrystallization from either abs. EtOH or by flash chromatography on silica

Pharmacological Methods

Radioisotopes were obtained from New England Nuclear. Biochemical reagents and co-factors were purchased from Sigma Chemical Co. Sprague Dawley male rats were obtained from Charles River Laboratory. CF_1 mice were obtained from Jackson Laboratory. Animals were maintained

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in light cycles of 12 h at 22°C. Rats were maintained in individual wire cages and mice were housed three/plastic cage. Food and water were *ad libitum*. The following pharmacological and biochemical assays have been outlined previously in detail[16–18].

Normolipidemic Studies

For structure activity studies, CF_1 male mice (~30g) were administered 2-benzoyl-4,4-dialkyl-3,5-isoxazolidinediones prepared in 1% carboxymethylcellulose (1% CMC) and administered IP at 20 mg/kg/day. Blood samples were obtained on days 9 and 16 between 7:30 and 8:30 a.m. Daily dosing of the agents was between 9:00 and 10:00 a.m.

Sprague Dawley male rats (~230 g) were administered orally 2-(3,4,5-trimethoxybenzoyl)-4,4-diethyl-3,5-isoxazolidinedione *4* at 10 or 20 mg/kg/day for eight weeks. Weekly blood samples were obtained by tail vein bleeding. The following parameters were determined: serum cholesterol levels [16–18], serum triglyceride levels [Bio-Dynamic/bmc Triglyceride Kit], BUN, glucose, LDH, CP kinase, bilirubin (direct and indirect), albumin, total protein, SGPT, creatinine [Sigma Clinical Chemistry Kit], uric acid, cholic acid, hematocrit, differential blood count and platelet estimates.

Hyperlipidemic Rodents

CF_1 male mice (~30g) and Sprague Dawley male rats (~300 g) were placed on a commercial diet (U.S. Biochemical Corporation Basal Atherogenic Diet) which contains butterfat (400 g), celufil (60 g), cholesterol (53 g), choline dihydrogen citrate (4 g), wesson oil (40 g), sodium cholate (20 g), sucrose (223 g), vitamin-free cassein (200 g) and total vitamin supplement for two weeks [16–18]. After the serum cholesterol and triglyceride levels were shown to be elevated i.e., after 14 days for mice and 28 days for rats, administration of compound *4* at 20 mg/kg/day was commenced and continued for the next four weeks orally in rats and two weeks IP in mice. The mice and rats were maintained on the Basal Atherogenic Diet throughout drug administration.

Animal Weight, Organ Weight, and Food Consumption

Control and treated normolipidemic and hyperlipidemic Sprague Dawley male rat (~240g) weights were obtained and expressed as a percentage of the initial body weight (week zero). Food consumption (gm/day/rat) was noted for weeks 6, 7 and 8 for control and treated rats. After eight weeks of drug administration, the animals were sacrificed and individual organ weights were obtained for control and treated rats [16–18].

Plasma Hydrolysis of Compound *4*

Blood was collected from the abdominal vein of normolipidemic Sprague Dawley male rats (~300g). The plasma was obtained by centrifuging 3400 × 3 min. The plasma (0.5 ml) was incubated with 0.5 ml drug *4* (1 mg/ml) for 6 hr. The level of compound *4* was monitored at 261 nm and the product 14 was read at 300 nm.

GI Mucosal Hydrolysis of Compound *4*

The gastric secretions (pH 2.0) from normolipidemic Sprague Dawley rats' (~300g) stomachs were collected by rinsing the gastric cavity with 15 ml PBS, pH 2.2. Compound *4*, 5 ml (1mg/ml) was incubated with the gastric secretions for 6 hr resulting in the hydrolysis of the amide bonds yielding 4,4-diethyl-3,5-isoxazolidinedione (*13*) and 3,4,5-trimethoxybenzoic acid (*14*). In order to confirm these structures, Compounds *13* [11] and *14* as well as 3,4,5 trimethoxybenzohydroxamic acid (*15*) [14, 15] along with diethylmalonic acid (*16*) were synthesized by known procedures. The gastric hydrolytic products had the same physical and chemical characteristics as the newly-synthesized Compounds *13* and *14*. These two compounds along with *15* and *16* were then tested for hypolipidemic activity. There was no evidence *15* and *16* were present in the plasma and gastric hydrolysate.

Enzymatic Studies

In vivo enzymatic studies were performed using 10% homogenates of liver or small intestinal mucosa from normolipidemic Sprague Dawley male rats (~280g) obtained after administering the agent for 8 weeks at a dose of 10 mg/kg/day, orally[16–18]. The liver and small intestinal mucosa homogenates from the *in vivo* studies were prepared in 0.25 mM sucrose + 0.001M (ethylenedinitrilo) tetraacetic acid, pH 7.2. *In vitro* studies were performed with 10% homogenates of CF_1 mouse (~28g) livers. Acetyl coenzyme A synthetase and adenosine triphosphate dependent citrate lyase activities were determined spectrophotometrically at 540 nm as the hydroxylamate of acetyl coenzyme A formed after 20 min at 37°C. Cholesterol-7α-hydroxylase activity was determined using [1,2-^{3}H] cholesterol (60 mCi/mmol), and acyl CoA cholesterol acyl transferase activity was determined using [1-^{14}C]oleic acid (56.7 mCi/mmol). Cholesterol synthesis was measured using [1-^{14}C] acetyl CoA (62 mCi/mmol) and a post-mitochondrial supernatant (9000 g × 20 min) which was incubated for 60 min at 37°C. The digitonide derivative of cholesterol was isolated and counted. For acetyl coenzyme A carboxylase activity, the enzyme had to be polymerized for 30 min at 37°C and then the assay mixture containing sodium ^{14}C-bicarbonate (41.0 mCi/mmol) was added and incubated for 30 min at 37°C with test drugs. *sn*-Glycerol-3-phosphate acyl transferase activity was determined with *sn*-glycerol-3-phosphate [L-2-^{3}H(N)] (7.1 Ci/mmol) with the microsomal fraction of liver homogenates. The reaction was terminated after 60 min and the lipids were extracted with chloroform/methanol (2:1) containing 1% HCl and counted. Phosphatidylate phosphohydrolase activity was measured as inorganic phosphate released after 60 min. The released inorganic phosphate after color development with ascorbic acid and ammonium molybdate was quantitated at 820 nm. Proteolytic activity was determined with BAEE as a substrate. The hydrolytic product was measured at 253 nm.

Tissue Lipid Levels

Normolipidemic Sprague Dawley male rats (~230 g) which were treated orally for eight weeks with compound *4* at 10 mg/kg/day, were sacrificed and tissue samples of the liver, small intestinal mucosa and aorta were removed. A 24

hr fecal sample was also obtained. A 10% homogenate in 0.25 M sucrose + 0.001 M EDTA, pH 7.2, was prepared for each tissue. An aliquot (2 ml) of the homogenate was extracted[16–18] and the number of mg of lipid extracted was weighed. The lipid residue was taken up in methylene chloride and the levels of cholesterol, triglycerides, neutral lipids and phospholipids were determined. Protein content of the whole homogenate was determined[16–18].

Serum Lipoprotein Fractions

Normolipidemic Sprague Dawley male rats (~230g) treated for eight weeks with compound *4* at 10 or 20 mg/kg/day orally as well as hyperlipidemic male rats (~360g) treated for four weeks with compound *4* at 10 mg/kg/day, orally were anesthetized with ether and blood (~10 ml) was collected from the abdominal vein. Serum was separated from whole blood by centrifugation at 3500 rpm. Aliquots of the serum were separated into chylomicrons, VLDL, HDL and LDL by ultracentrifugation as modified for normal rats [16–18]. Each of the fractions was analyzed for cholesterol, triglyceride, neutral lipids, phopholipid and protein levels.

Rate of Lipoprotein Synthesis

Normolipidemic Sprague Dawley rats (~300 g) were administered with compound *4* at 20 mg/kg/day for 14 days. On day 13, 20 μCi of L-[4,5-^{3}H(N)]-leucine (58.5 Ci/mmol), 1,2-^{3}H-cholesterol (40.7 mCi/mmol), ^{14}C-palmitic acid (57 mCi/mmol) or ^{32}P (H_2PO_3) buffered (2 μCi) was injected I.V. in the tail vein in isotonic phosphate saline buffer, pH 7.4 [16–18]. Sixteen hours later, the animals were anesthetized with ether and blood was collected from the abdominal vein. The serum lipoprotein fractions were separated by ultracentrifuge techniques, dialyzed and aliquots analyzed for radioactive content. The radioactivity was expressed as dpm/mL of serum lipoproteins isolated. The lipoprotein fractions which were analyzed for lipid classes are outlined above for comparison with the radio-precursor uptake into each fraction.

Apoprotein Analysis

After treatment of normolipidemic Sprague Dawley male rats orally for 8 weeks or hyperlipidemic rats (~360g) treated 4 weeks at 10 mg/kg/day, blood was collected and the serum lipoprotein fractions separated [16–18]. The lipoprotein fraction (5 ml) was dialyzed against 0.2 M NaCl, pH 7.3 (18–24 h) using Spectropor tubing with a molecular weight cut-off of 3500. The lipoprotein fractions were delipidated by extracting with equal volumes of Et_2O: EtOH (3:2). The aqueous layer was harvested by centrifugation (3500 g × 10 min). The organic residues were allowed to evaporate off the final aqueous residue. The aqueous layer were removed by rotovap for 10 min at 45°C. The residue was redissolved in 0.1 M NaCl pH 7.3 (1 mL). After analysis of the protein, the volume was adjusted to 1 mg protein/mL. An aliquot (5–50 mg) was loaded onto a 5–15% SDS-PAGE running gel with a 3% acrylamide stacking gel on top which had previously been polymerized. Rat serum albumin (10 mg) was used as standard. The gel was run at constant current of 20 amps for 4–4.5 h (negative electrode-(−) cathode on top, and the positive electrode-(+) anode on bottom). The gel was fixed in 10% TCA incubated from 30 to 60 min and stained in 0.5% coomassie blue in 20% EtOH deluted 25 fold with 7.5% acetic acid. The gel was destained with 10% acetic acid, 5% MeOH and 10% glycerol with 10 to 60 min per rinse.

Mouse Toxicity

For the mouse sub-acute toxicity study, normolipidemic CF_1 male mice (~28 g) were dosed at 20, 40 and 100 mg/kg/day IP for 7 days with compound *4*. The food consumption was determined daily and water was *ad libitum*. The animals were maintained in 12 h light and dark cycles at 22°C.

Clinical Chemistry

At the time of sacrifice, the major organs were excised, trimmed of fat and weighed. Blood was obtained from the carotid and centrifuged at 3500 g × 10 min to obtain the serum. Chemical or enzymatic assays were performed with Sigma Chemical kits: urea nitrogen (BUN, NO. 640), alanine amino transferase (SGPT. No. 505), alkaline phosphatase (AP. No. 104), glucose (No. 510), lactic dehydrogenase (LDH, No. 500), creatine phosphokinase (CP-kinase, No. 661), and total and direct bilirubin (No. 605). Serum triglycerides were determined with a diagnostic kit from Boehringer Mannheim. Serum cholesterol, albumin and total protein, cholic acid and uric acid levels were determined [16–18].

Blood Collection and Parameters

Blood was obtained from the carotid, a drop was placed on glass slides and fixed in Wright's stain. Differential white blood cell counts, platelet counts and hematocrits were obtained for each mouse group sacrificed at the specified times [19].

Histology

Normolipidemic CF_1 male mice (~28g) after treatment for 7 days were killed by carbon dioxide asphyxiation. After all vital signs had ceased, a midline incision was made from the lower jaw to the inguinal area. Thymus, spleen, liver, and kidney were excised and weighed. Representative tissue samples were fixed in 10% buffered formalin, trimmed and sectioned at 6μ in thickness and stained with hematoxylin and eosin.

Female Fertility

Normolipidemic CF_1 female mice (~30 g) were administered with compound *4* at 20, 40 or 100 mg/kg/day for three weeks. While continuing dosing the females were exposed to males (2:1) for another three weeks. The males were rotated every 7 days to eliminate infertility. After three weeks the males were removed. The % pregnancy, number of live births, deaths and birth weights were noted. Three weeks after birth the pups weight, % survival and sex were noted for each group.

Mean Survival Time

LD_{50} acute toxicity was determined in normolipidemic CF_1 male mice (28 g) IP. Commencing with doses of 5 mg/kg

and continuing to 1g/kg as a single dose on day 1. The number of deaths in each group were noted over the next 14 days. The number deaths were analyzed by the probit method.

RESULTS

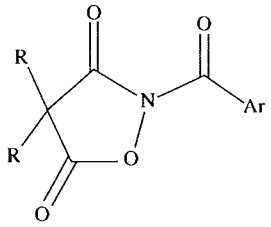
Chemistry

The 2-benzoyl-4,4-dialkyl-3,5-isoxazolidinediones (*3*) were synthesized by reacting dialkylmalonyl chlorides (*1*) with benzohydroxamic acids (*2*) in the presence of pyridine. Compounds *4-12* were prepared by this method. Compound *8* had been synthesized previously in lower yield by carrying out the reaction in the absence of solvent followed by treatment with water to crystallize *8* [20]. Attempts to synthesize 2-(4-nitrobenzoyl)-4,4-diethyl-3,5-isoxazolidinedione (*3*, R = Et, Ar = 4-NO_2-Ph) were unsuccessful. The reactions to produce this product apparently proceeded as evidenced by the disappearance of the diacid chloride starting material. However, the product was apparently unstable to the aqueous work-up conditions. Isolated as a by-product in the synthesis of *4* was the dibenzohydroxamic acid, N-(3,4,5-trimethoxybenzoyl)-3,4,5-trimethoxybenzamide [15]. Tables 1 and 2 give the physical and spectral properties of compounds *4–12*.

Products *4–12* were observed to be somewhat unstable in water. The instability of the products to hot water was demonstrated during purification of the products by recrystallization. Recrystallization from abs. EtOH led to good percent recoveries and high purities of *4–12* but recrystallization from 95% EtOH resulted in poor recoveries accompanied by extensive decomposition. It was also observed that *8* was much less stable than the other 3,5-isoxazolidinediones on storage in screw-capped vials under nitrogen, turning to an oily substance over several months, while the substituted benzoyl derivatives were stable under the same conditions. In a separate set of experiments, compound *4* was found to be 97% decomposed when heated under reflux in EtOH/water (50:50) for 15 min. However, when it was incubated at 21°C for 21.5 hrs. in phosphate buffered saline pH 7.2 the compound was less than 1% decomposed as shown by HPLC analysis on a silica column, indicating that it was stable for biological experiments.

The nature of the substituents appears to have an effect on the stabilities of the 3,5-isoxazolidinediones. When heated in refluxing MeOH under nitrogen for 12 hrs., the stabilities of the 2-benzoyl-3,5-isoxazolidinediones varied with the nature of the substitutents on the benzoyl group and decrease in the order: methoxy>methyl>chloro. It was observed that at position 4 of the isoxazolidine ring diethyl substitution led to higher stability than the di-*n*-butyl substitution. Analysis of the decomposition mixture by HPLC on a silica column using hexane/ethyl acetate solvent mixtures as the mobile phase showed the presence of the appropriate methyl benzoate as the major decomposition product in each instance. These results along with the observation of the apparent high instability of the 4-nitrobenzoyl derivative indicate that compounds having electron-donating substituents on the benzoyl group are more stable than those having electron-withdrawing substituents and that the stabilities decrease as the electron-donating abilities of the substituent(s) decrease. Moreover, since *4* is more stable than *10* the *n*-butyl groups of *10* destabilize the molecule. The *n*-butyl groups are apparently ineffective in sterically blocking the carbonyl groups in the molecule from nucleophilic attack. However, the general increase in stability of compounds having substituted benzoyl substituents, irrespective of the electronic

Table I. 2-Benzoyl-4,4-dialkyl-3,5-isoxazolidinediones Synthesized



Compound	R	Ar	Yield, %	MP °C	IR, C=O (cm^{-1})[e]
4	Et	3,4,5-$(MeO)_3$-Ph	82	109–110[a,b]	1802, 1758, 1698
5	Et	3,4-$(MeO)_2$-Ph	74	99–101[a]	1805, 1752, 1693
6	Et	4-MeO-Ph	64	118–119[a]	1810, 1742, 1685
7	Et	4-Me-Ph	49	92–93[a]	1803, 1750, 1698
8	Et	Ph	76	87–88[a]	1817, 1762, 1714
9	Et	4-Cl-Ph	15	96–97[c]	1811, 1745, 1698
10	n-Bu	3,4,5-$(MeO)_3$-Ph	18	107–108[a,d]	1812, 1756, 1699
11	Et	2,6-$(MeO)_2$-Ph	71	134–135[a]	1826, 1766, 1726
12	Et	2-MeO-Ph	80	87–88[a]	1810, 1745, 1695

[a] Purified by recrystallization from abs. EtOH.
[b] Purified by flash chromatography on silica using hexane/EtOAc (50:50).
[c] Purified by recrystallization from MeOH.
[d] Purified by flash chromatography on silica using hexane (100%) followed by recrystallization from abs. EtOH.
[e] Recorded as Nujol mulls.

Table II. ^{1}H NMR, ^{13}C NMR, and Mass Spectral Data of 2-Aroyl-4,4-dialkyl-3,5-isoxazolidinediones

Compound	^{1}H NMR, δ[a,b]	^{13}C NMR, δ[a,b]	MS, m/z, found (calcd)
4	1.03 (t, 6), 1.98 (q, 4), 3.91 (s, 6), 3.95 (s, 3), 7.11 (s, 2)	9.07, 28.81, 54.17, 56.33, 60.98, 108.17, 125.45, 143.61, 152.84, 161.50, 170.29, 170.72	351.1319 (351.1318), 195 (100%)
5	1.02 (t, 6), 1.97 (q, 4), 3.94 (s, 3), 3.98 (s, 3), 6.94–7.75 (complex m, 3)	9.07, 28.99, 54.18, 56.04, 56.16, 110.09, 112.66, 122.88, 125.68, 148.80, 154.42, 161.44, 170.50, 170.90	321.1208 (321.1212), 165 (100%)
6	1.01 (t, 6), 1.95 (q, 4), 3.89 (s, 3), 6.98 (d, 2), 7.83 (d, 2)	9.05, 28.97, 54.19, 113.76, 122.87, 132.93, 161.33, 164.60, 170.41, 170.92	291.1106 (291.1102), 135 (100%)
7	1.00 (t, 6), 1.95 (q, 4), 2.43 (s, 3), 7.29 (d, 2), 7.72 (d, 2)	9.02, 21.80, 28.95, 54.15, 128.19, 129.07, 130.35, 145.33, 161.87, 170.04, 170.70	275.1163 (275.1157), 119 (100%)
8	1.00 (t, 6), 1.87 (q, 4), 7.46 (m, 5)[c]		
9	1.04 (t, 6), 1.99 (q, 4), 7.51 (m, 2), 7.79 (m, 2)	9.05, 29.99, 54.14, 128.77, 129.37, 131.14, 140.67, 160.90, 170.01, 170.36	295.0615 (295.0612), 139 (100%)
10	0.90 (t, 6), 1.34 (m, 8), 1.93 (m, 4), 3.85 (s, 3), 3.89 (s, 3), 7.16 (s, 2)	13.84, 23.07, 27.27, 35.96, 53.22, 56.63, 60.77, 108.86, 126.99, (142.30, 153.92, 162.05, 170.86, 171.94)	407.1946 (407.1944), 195 (100%)
11	0.98 (t, 6), 1.89 (q, 4), 3.80 (s, 6), 6.59 (d, 2), 7.39 (t, 1)	8.80, 28.00, 54.33, 55.91, 103.91, 122.20, 132.66, 150.41, 157.61, 167.50, 170.19	321.1211 (321.1212), 165 (100%)
12	1.00 (t, 6), 1.92 (q, 4), 3.83 (s, 3), 7.46 (overlapping t, 2), 7.53 (overlapping t, 2)	8.87, 28.91, 54.41, 55.63, 111.12, 120.87, 122.21, 129.63, 133.61, 157.63, 158.85, 167.63, 170.20	291.1104 (291.1106), 135 (100%)

[a] Chemical shifts are relative to internal TMS.
[b] $CDCl_3$ was the solvent.
[c] Recorded at 60 MHz.

effect of the substituents, over that of *8* appears to be caused by the steric effects of the substituents.

The benzoyl derivatives *4–12* gave three IR carbonyl absorptions at 1803–1817, 1742–1762 and 1685–1714 cm^{-1}. The ^{13}C NMR spectra showed the carbonyl carbons of the benzoyl substituents at δ160.9–162.1 and the ring carbonyl carbons at δ170.0–170.8 and δ170.4–171.9, respectively.

Pharmacology

The lipid lowering effects of 2-benzoyl-3,5-isoxazolidinediones in normolipidemic CF_1 mice at 20 mg/kg/day, IP showed that trimethoxyphenyl substituted (*4*), the unsubstituted phenyl (*8*), 4-chlorophenyl (*9*) and the 4-methylphenyl (*7*) derivatives resulted in the best lowering of serum cholesterol from 47 to 55% (Table 3). Serum triglyceride levels were not suppressed by the same magnitude. Compound *10* caused 40% reduction in triglycerides whereas *4* and *6* caused 34 and 37% reductions, respectively, on day 16. Considering both screens for hypolipidemic activity, compound *4* appeared to have the best overall activity. In hyperlipidemic CF_1 male mice where serum cholesterol was elevated from 125 mg/dL to 358 mg/dL and serum triglycerides were elevated from 137 mg/dL to 367 mg/dL at 20 mg/kg/day IP, compound *4* reduced elevated serum cholesterol levels 60% and serum triglyceride levels 49% after 14 days dosing (Table 3).

3,4,5-Trimethoxybenzoyl-4,4-dialkyl-3,5-isoxazolidinedione (*4*) significantly suppressed serum cholesterol and triglycerides in normolipidemic Sprague Dawley male rats at 10 and 20 mg/kg/day orally (Table 4). At 10/mg/kg/day compound *4* was as effective as the higher dose by eight weeks in lowering serum cholesterol levels by 45%. The 20 mg/kg/day dose was more effective than 10 mg/kg/day in lowering serum triglyceride levels in rats after 8 weeks. The lowest level of triglycerides afforded by the agent was at two weeks with 57% reduction of control levels. The hematocrit and daily food consumption (18.11 g/day) were not significantly changed from the control (18.79 g/day) over the eight week period. The total body weight was decreased 38% of the weight increase demonstrated by the control rats over week 8.

Incubation of Compound *4* with rat plasma for 6 hr demonstrated that Compound *4* was stable with no hydrolysis. The half life of Compound *4* in gastric secretion was 69.3 hr. Incubation with the rat gastric secretion led to Compounds *13* and *14*, which maintained good hypolipidemic activity in both lipid screens. For normal intestinal absorption times in rats, it would appear that the parent drug, *4* is the major chemical species reaching the blood compartment and is responsible for the lipid lowering effects. Compound 4 may be a pro-drug which affords two active derivatives upon hydrolysis *in vivo* at later times, i.e. 6 hr.

In vitro hepatic enzyme activities from normolipidemic CF_1 mice were examined (Table 5). Mitochondrial citrate exchange, acetyl CoA synthetase, HMG CoA reductase, acyl CoA cholesterol acyl transferase, acetyl CoA carboxylase, *sn*-glycerol-3-phosphate acyl transferase, phosphatidylate phosphohydrolase and heparin-induced lipoprotein lipase were inhibited, generally, in a concentration dependent manner. Neutral cholesterol ester hydroxylase and ATP-dependent citrate lyase activities were stimulated in activity. Normolipidemic rat liver and small intestinal mucosa en-

Table III. The Effects of 3,5-Isoxazolidinediones on Serum Cholesterol and Triglyceride Levels of CF_1 Normolipidemic Male Mice at 20 mg/kg/day, IP

N = 6 Compound #	R	Ar	Percent of Control (X ± SD) Serum cholesterol day 9	Serum cholesterol day 16	Serum Triglycerides day 16
4	Et	3,4,5-$(MeO)_3$-Ph	82 ± 6	51 ± 4*	66 ± 5*
5	Et	3,4-$(MeO)_2$-Ph	81 ± 6	77 ± 6*	83 ± 7
6	Et	4-MeO-Ph	89 ± 7	62 ± 6*	63 ± 6*
7	Et	4-Me-Ph	72 ± 6*	46 ± 4*	87 ± 8
8	Et	Ph	77 ± 6*	53 ± 5*	63 ± 7*
9	Et	4-Cl-Ph	64 ± 5*	45 ± 4*	76 ± 6*
10	n-Bu	3,4,5-$(MeO)_3$-Ph	79 ± 7	66 ± 5*	60 ± 6*
11	Et	2,6-$(MeO)_2$-Ph	79 ± 5	68 ± 6*	77 ± 5*
12	Et	2-(MeO)-Ph	94 ± 6	87 ± 6	78 ± 6*
13 4,4-diethyl-3,5-isoxazolidinedione			64 ± 6*	52 ± 5*	61 ± 6*
14 3,4,5-trimethoxybenzoic acid			88 ± 8	74 ± 5*	84 ± 6*
15 3,4,5-trimethoxybenzohydroxamic acid			88 ± 7	58 ± 6*	72 ± 5*
16 Diethylmalonic acid			66 ± 5*	53 ± 4*	62 ± 6*
Clofibrate 150 mg/kg/day			88 ± 7	87 ± 5	75 ± 7*
1% carboxymethylcelluose			100 ± 6[a]	100 ± 5[b]	100 ± 6[c]

[a] 118 mg/dL.
[b] 122 mg/dL.
[c] 137 mg/dL.
* $P \leq 0.001$ Student's *t* test or Dunnett's test.

The Effects of Compound *4* on Serum Cholesterol and Triglyceride Levels of Hyperlipidemic CF_1 Male Mice at 20 mg/kg/day IP for 14 days

N = 6	Percent of Control Serum Cholesterol	Serum Triglyceride
Control-Diet	100 ± 7[a]	100 ± 6[b]
Treated with Compound *4*	40 ± 4*	51 ± 5*

[a] 358 mg/dL.
[b] 367 mg/dL.
* $p \leq 0.001$ Student's "t" test.

zyme activities were examined at eight weeks after dosing at 10 mg/kg/day (Table 6). Hepatic acetyl CoA synthetase, acyl CoA cholesterol acyl transferase, cholesterol-7α-hydroxylase, *sn*-glycerol-3-phosphate acyl transferase and phosphatidylate phosphohydrolase activities were reduced significantly. Neutral cholesterol ester hydrolase and acetyl CoA carboxylase activities were elevated after drug treatment in the small intestine mucosa whereas ATP-dependent citrate lyase, acyl CoA cholesterol acyl transferase, acetyl CoA carboxylase and *sn*-glycerol-3-phosphate acyl transferase activities were reduced by the agent. Neutral cholesterol ester hydrolase activity was elevated in the small intestinal mucosa. Free cathepsin proteolytic activity was suppressed by both agents, and acid phosphatase activity was elevated in the mucosa after 8 weeks of drug administration.

When the tissue lipids were examined after two weeks administration of compound *4* at 10 mg/kg/day to normolipidemic rats, liver triglycerides were reduced 17% and phospholipids were elevated 33% (Table 7). Small intestine mucosa cholesterol was reduced 27% and phospholipids 70%. Aorta triglycerides were reduced 18% and neutral lipids 20%. Fecal total lipids were reduced 23%; triglycerides were reduced 75% and phospholipids 67%. After two weeks at 20 mg/kg/day drug administration, liver total lipids, cholesterol, triglyceride and neutral lipids were reduced. Total lipids and phospholipids were elevated in the small intestinal mucosa. Aorta cholesterol was reduced 22% but triglycerides and phospholipids were elevated significantly.

At eight weeks treatment, only marginal changes occurred in the lipid content of the liver and small intestinal mucosa. Phospholipids were elevated in both tissues and triglycerides were reduced in the small intestine mucosa. Aorta total lipids, cholesterol, triglycerides and neutral lipids were reduced by the agent after 8 weeks. Fecal lipids were also reduced, e.g. cholesterol, triglycerides and phospholipids.

When the bile from normolipidemic rats treated two weeks at 20 mg/kg/day, orally was examined, bile flow was reduced 16% whereas bile cholesterol content was elevated 82% and triglyceride levels was elevated 138%.

When the serum lipoprotein fractions from normolipidemic rats were examined at 2, 4, 6 and 8 weeks (Table 8),

Table IV. The Effects of Compound *4* on Serum Cholesterol and Serum Triglyceride Levels of Normolipidemic Sprague Dawley Rats over 8 Weeks at 10 and 20 mg/kg/day, Orally

	Percent of Control (X ± SD)					
	Serum Cholesterol			Serum Triglycerides		
(*N* = 6) Weeks	Control	10 mg/kg/Day	20 mg/kg/Day	Control	10 mg/kg/Day	20 mg/kg/Day
0	100 ± 6[a]	100 ± 6	100 ± 6	100 ± 7[b]	100 ± 7	100 ± 7
1	100 ± 6	—	77 ± 6*	100 ± 6	89 ± 8	64 ± 6*
2	100 ± 7	78 ± 7*	61 ± 6*	100 ± 8	74 ± 7*	43 ± 4*
4	100 ± 5	72 ± 6*	69 ± 5*	100 ± 8	82 ± 6*	67 ± 6*
6	100 ± 6	66 ± 6*	50 ± 5*	100 ± 7	64 ± 6	63 ± 5[a]
7	100 ± 7	56 ± 5*	—	100 ± 6	—	61 ± 4*
8	100 ± 5	55 ± 4*	55 ± 6*	100 ± 7	62 ± 5*	51 ± 6*

[a] 75 mg/dL.
[b] 120 mg/dL.
— not determined.
* $P \leq 0.001$ Student's *t* test or Dunnett's test.

The Effects of Compound *4* on Serum Cholesterol and Serum Triglycide Levels of Hyperlipidemic Sprague Dawley Rats Over 4 Weeks at 10 mg/kg/day Orally

	Percent of Control	
N = 6	Serum Cholesterol	Serum Triglyceride
Control	100 ± 6[a]	100 ± 8
Treated with Compound *4*		
Week 2	83 ± 5	97 ± 6
Week 4	37 ± 4*	14 ± 3*

[a] 387 mg/dL.
[b] 389 mg/dL.
* $p \leq 0.001$ Student's "t" test.

cholesterol levels were reduced in the chylomicron fraction early; i.e. the 2nd and 4th weeks, but cholesterol level rebounded on the 8th week. VLDL cholesterol levels were the lowest on week 6 and again recovered on the 8th week. LDL cholesterol levels were reduced, e.g. 10 mg/kg/day caused 72% reduction on week 4 and rebounded on week 8 to a 48% reduction. At the 20 mg/kg/day dose of the agent, VLDL cholesterol content was reduced 76% on week 6. HDL cholesterol levels were elevated on weeks 2–8 at 10 and 20 mg/kg/day with the maximum on week 4. Triglyceride content was reduced in the VLDL fraction after 4 weeks at 10 mg/kg/day. The higher dose of 20 mg/kg/day did not improve the reduction of triglycerides in the VLDL. Triglyceride content in the LDL fraction was reduced 50% at 20 mg/kg/day on week 8. HDL triglyceride levels were reduced in the 6th week at 10 mg/kg/day and week 8 for 20 mg/kg/day. Neutral lipids were reduced on the 2nd week at 10 mg/kg/day but not at 20 mg/kg/day. VLDL neutral lipids were reduced maximum by 10 and 20 mg/kg/day on week 4. LDL neutral lipids were reduced at both doses on weeks 4 and 6. HDL neutral lipids were elevated at both doses on week 6. Phospholipids in the chylomicron fractions were reduced on week 4. VLDL phospholipids were reduced only in weeks 2 and 4 by both doses and LDL phospholipids were reduced 45% at 10 mg/kg/day. HDL phospholipids were reduced on weeks 4 and 6 but elevated on week 8.

Protein content in the chylomicron was unchanged. Protein content in VLDL and LDL was reduced in the 4th week, but it returned to normal on week 6 and was elevated by the 8 weeks at 20 mg/kg/day. Protein content of the HDL fraction was not seriously affected by either dose or at any week.

When incorporation of precursors of lipoprotein synthesis was examined after two weeks of drug administration at 20 mg/kg/day, orally in normolipidemic rats, ^{3}H-cholesterol incorporation into chylomicron VLDL and LDL after two weeks administration was reduced but incorporation was elevated 35% in the HDL fraction (Table 9). ^{14}C-Palmitic acid incorporation into every lipoprotein fraction was reduced after treatment for two weeks. ^{32}P-Incorporation into the chylomicron, VLDL and LDL fractions was elevated and into the HDL fraction was decreased. ^{3}H-Leucine incorporation into the chylomicrons and VLDL was increased and decreased in the LDL fraction.

In hyperlipidemic Sprague Dawley male rats, the serum cholesterol levels were elevated from 79 to 387 mg/dL and triglycerides 125 to 389 mg/dL after four weeks (Table 4). Drug treatment at 10 mg/kg/day, orally lowered serum cholesterol to 322 mg/dL on week 2 and 142 mg/dL on week 4 of treatment. Serum triglycerides after drug treatment were not affected on week 2 but lowered to 54 mg/dL on week 4 on treatment. When the serum lipoprotein lipids were examined from the hyperlipidemic rats treated at 10 mg/kg/day, orally

Table V. The *In Vitro* Effects of Compound *4* on the Activity of Liver Enzymes Involved in Lipid Metabolism from Normolipidemic CF_1 Mice

Enzyme Assay (N = 6)	Percent of Control (X ± SD) Compound *4*			
	Control	25 uM	50 uM	100 uM
Mitochondrial Citrate Exchange	100 ± 7[a]	44 ± 4*	27 ± 3*	23 ± 2*
ATP Dependent Citrate Lyase	100 ± 6[b]	99 ± 5	130 ± 6	165 ± 6
Acetyl CoA Synthetase	100 ± 5[c]	87 ± 4	82 ± 5	63 ± 4*
HMG CoA Reductase	100 ± 8[d]	59 ± 5	55 ± 4*	40 ± 3*
Cholesterol-7α-Hydroxylase	100 ± 6[e]	97 ± 4	102 ± 5	97 ± 5
Acyl CoA Cholesterol Acyl Transferase	100 ± 5[f]	51 ± 4*	48 ± 3*	54 ± 4*
Cholesterol Ester Hydrolase (Neutral)	100 ± 7[g]	120 ± 5*	131 ± 6*	135 ± 6*
Acetyl CoA Carboxylase	100 ± 6[h]	41 ± 3*	50 ± 4*	57 ± 5*
sn-Glycerol-3-Phosphase Acyl Transferase	100 ± 6[i]	20 ± 4*	19 ± 3*	12 ± 3*
Phosphatidylate Phosphohydroxylase	100 ± 5[j]	26 ± 4*	11 ± 3*	8 ± 2*
Heparin-Induced Lipoprotein Lipase	100 ± 6[k]	61 ± 4*	57 ± 4*	49 ± 3*

[a] 30.8 % exchange of mitochondria citrate.
[b] 30.5 mg citrate hydrolyzed/gm wet tissue/20 min.
[c] 28.5 mg acetyl CoA formed/gm wet tissue/20 min.
[d] 384,900 dpm cholesterol formed/gm wet tissue/60 min.
[e] 4808 dpm/mg microsomal protein/20 min.
[f] 224,000 dpm/mg microsomal protein/30 min.
[g] 56,436 dpm/gm wet tissue/h.
[h] 32.010 dpm/gm wet tissue/30 min.
[i] 537,800 dpm/gm wet tissue/20 min.
[j] 16.7 ug/iP/gm wet tissue/15 min.
[k] 278,583 dpm/gm wet tissue/h.
* $P \leq 0.001$ Student's or Dunnett's test.

Table VI. The Effects of Compound *4* on Normolipidemic Sprague Dawley Rat Enzyme Activities after 8 Weeks Administration Orally at 10 mg/kg/day

N = 8 Enzyme Assay	Percent of Control (X ± SD)			
	Liver		Small Intestine	
	Control	Treated	Control	Treated
ATP Dependent Citrate Lyase	100 ± 7[a]	103 ± 6	100 ± 6[l]	66 ± 6*
Acetyl CoA Synthetase	100 ± 8[b]	32 ± 5*	100 ± 5[m]	102 ± 5
HMG CoA Reductase	100 ± 8[c]	128 ± 7	100 ± 8[n]	107 ± 7
Acyl CoA Cholesterol Acyl Transferase	100 ± 7[d]	72 ± 6*	100 ± 7[o]	6 ± 3*
Cholesterol-7α-hydroxylase	100 ± 7[e]	74 ± 5*	100 ± 6[p]	82 ± 6*
Cholesterol Ester Hydrolase	100 ± 8[f]	170 ± 9*	100 ± 7[q]	169 ± 7
Acetyl CoA Carboxylase	100 ± 6[g]	157 ± 8*	100 ± 7[r]	78 ± 6*
sn-Glycerol-3-Phosphate Acyl-Transferase	100 ± 6[h]	78 ± 6*	100 ± 7[s]	53 ± 5*
Phosphatidylate Phosphohydrolase	100 ± 6[i]	78 ± 5*	100 ± 6[t]	106 ± 6
Proteolytic Cathespin	100 ± 5[j]	8 ± 2*	100 ± 5[u]	56 ± 6*
Acid Phosphatase	100 ± 4[k]	105 ± 6	100 ± 5[v]	130 ± 7

[a] 9.2 mg citrate hydrolyzed/g wet tissue.
[b] 10.0 mg acetyl CoA formed/g wet tissue.
[c] 103,020 dpm/g wet tissue.
[d] 86,640 dpm/g wet tissue.
[e] 289,450 dpm/g wet tissue.
[f] 22,443 dpm/g wet tissue.
[g] 43,000 dpm/g wet tissue.
[h] 87,620 dpm/g wet tissue.
[i] 11 mg Pi released/g wet tissue.
[j] 36.6% free.
[k] 42.2% free.
[l] 9.17 mg citrate hydrolyzed/g wet tissue.
[m] 5.27 mg acetyl CoA formed/g wet tissue.
[n] 113,322 dpm/g wet tissue.
[o] 64,819 dpm/g wet tissue.
[p] 259,099 dpm/g wet tissue.
[q] 259,099 dpm/g wet tissue.
[r] 54,892 dpm/g wet tissue.
[s] 73,219 dpm/g wet tissue.
[t] 114 mg Pi released/g wet tissue.
[u] 43.3% free.
[v] 35.2% free.
* $P \leq 0.001$ Student's or Dunnett's test.

Table VII. The Effects on Tissue Lipid Levels of Normolipidemic Sprague Dawley Rats after Oral Administration of Compound *4* at 10 or 20 mg/kg/day for 2 or 8 weeks

	Percent of Control (X ± SD)					
Tissue (N = 6)	Total Lipid (mg)	Cholesterol	Triglyceride	Neutral Lipids	Phospholipids	Proteins
Liver:						
Compound *4* 10 mg/kg @2 wk	100 ± 7	94 ± 6	83 ± 5	106 ± 7	133 ± 9*	106 ± 6
Compound *4* 20 mg/kg @2 wk	81 ± 5*	67 ± 6*	68 ± 8*	81 ± 5*	107 ± 8	96 ± 6
Compound *4* 10 mg/kg @8 wk	94 ± 5	91 ± 6	92 ± 4	114 ± 7	156 ± 5	96 ± 6
1% CMC Control 20 mg/kg	100 ± 6[a]	100 ± 5[b]	100 ± 7[c]	100 ± 6[d]	100 ± 6[e]	100 ± 5[f]
Small Intestines:						
Compound *4* 10 mg/kg @2 wk	112 ± 6	73 ± 4*	93 ± 7	92 ± 6	30 ± 5*	100 ± 6
Compound *4* 20 mg/kg @2 wk	134 ± 8*	88 ± 5	111 ± 7	111 ± 7	219 ± 8*	91 ± 7
Compound *4* 10 mg/kg @8 wk	105 ± 6	93 ± 8	63 ± 6*	84 ± 6	292 ± 8*	95 ± 7
1% CMC Control	100 ± 7[g]	100 ± 6[h]	100 ± 7[i]	100 ± 6[j]	100 ± 8[k]	100 ± 6[l]
Aorta:						
Compound *4* 10 mg/kg @2 wk	98 ± 4	91 ± 6	82 ± 6	80 ± 5	105 ± 7	96 ± 5
Compound *4* 20 mg/kg @2 wk	96 ± 5	78 ± 6*	203 ± 6*	96 ± 5	130 ± 7*	83 ± 5*
Compound *4* 10 mg/kg @8 wk	65 ± 6*	73 ± 6*	73 ± 7*	71 ± 6*	94 ± 3	97 ± 6
1% CMC Control	100 ± 6[m]	100 ± 5[n]	100 ± 6[o]	100 ± 5[p]	100 ± 6[q]	100 ± 4[r]
Feces:						
Compound *4* 10 mg/kg @2 wk	77 ± 5	98 ± 6	25 ± 4*	106 ± 6	33 ± 3*	72 ± 4*
Compound *4* 20 mg/kg @2 wk	60 ± 5*	96 ± 7	72 ± 5*	99 ± 6	167 ± 6*	118 ± 7
Compound *4* 10 mg/kg @8 wk	87 ± 7	43 ± 5*	37 ± 6*	104 ± 6	49 ± 5	92 ± 6
1% CMC Control	100 ± 7[s]	100 ± 6[t]	100 ± 6[u]	100 ± 5[v]	100 ± 7[w]	100 ± 7[x]
Bile: (N = 6)	Bile Flow	Cholesterol	Triglyceride	Neutral Lipids	Phospholipids	Proteins
Control	100 ± 6[y]	100 ± 7[z]	100 ± 7[aa]	100 ± 8[bb]	100 ± 7[cc]	100 ± 7[dd]
Treated 20 mg/kg/wk @2 wk	84 ± 5	182 ± 9*	238 ± 6	106 ± 8	107 ± 6	109 ± 5

[a] 50.0 mg lipid/g wet tissue.
[b] 9.18 mg cholesterol/g wet tissue.
[c] 6.37 mg triglyceride/g wet tissue.
[d] 15.70 mg neutral lipid/g wet tissue.
[e] 27.19 mg phospholipid/g wet tissue.
[f] 4.5 mg protein/g wet tissue.
[g] 68.20 mg lipid/g wet tissue.
[h] 12.02 mg cholesterol/g wet tissue.
[i] 11.20 mg triglyceride/g wet tissue.
[j] 16.98 mg neutral lipid/g wet tissue.
[k] 20.06 mg phospholipid/g wet tissue.
[l] 42.0 mg protein/g wet tissue.
[m] 67.5 mg lipid/g wet tissue.
[n] 5.77 mg cholesterol/g wet tissue.
[o] 9.85 mg triglyceride/g wet tissue.
* $P \leq 0.001$ Student's or Dunnett's test.
[p] 15.28 mg neutral lipid/g wet tissue.
[q] 28.8 mg phospholipid/g wet tissue.
[r] 11.71 mg protein/g wet tissue.
[s] 11.58 mg lipid/g wet tissue.
[t] 2.84 cholesterol/g wet tissue.
[u] 1.85 mg triglyceride/g wet tissue.
[v] 3.39 mg neutral lipid/g wet tissue.
[w] 5.70 mg phospholipid/g wet tissue.
[x] 6.99 mg protein/g wet tissue.
[y] 0.79 mL/hr.
[z] 118 mg %.
[aa] 5 mg/mL.
[bb] 1.70 mg/mL.
[cc] 1.75 mg/mL.
[dd] 3.34 mg %.

for 4 weeks, cholesterol was moderately reduced in the chylomicron and markedly reduced in the LDL fraction (Table 10). HDL cholesterol levels were elevated in treated animals. Triglyceride content was marginally reduced in the VLDL fraction and significantly reduced in the LDL fraction. Neutral lipids were reduced in the VLDL and LDL fractions. Phospholipids were elevated into the chylomicron and VLDL fractions but reduced significantly in the LDL and marginally reduced in HDL. Protein content of the VLDL and LDL fractions was reduced significantly. PAGE electrophoresis studies of LDL and HDL from treated rats for 8 weeks (Fig. 1) showed no additional apoproteins in the LDL fraction except apo B. On the other hand, the HDL fraction of treated hyperlipidemic rats showed higher densities in the areas of apo E, apo AI and the apo C's.

When the distribution of ^{3}H-cholesterol was examined in the tissues from normolipidemic rats after IV administration, large concentrations were found in the lung, liver, spleen, kidney, thymus, small intestine and the chyme (Table 11). ^{14}C-Palmitic acid distribution was reduced in most organs except the stomach and large intestine. ^{32}P distribution was also reduced in most tissue except the lung, liver, thymus and chyme where elevations were observed. However, when ^{14}C-cholesterol was administered orally to CF_1 mice treated with compound 4 at 20 mg/kg/day for 2 weeks (Table 12), the kidney, spleen, liver and chyme showed elevations

Table VIII. The Effects of Compound *4* on Normolipidemic Sprague Dawley Male Rat Serum Lipoproteins Lipid Content After Oral Administration

	Percent of Control (X ± SD)				
	Cholesterol	Triglyceride	Neutral Lipids	Phospholipids	Protein
Two weeks					
Chylomicrons					
Control	100 ± 7	100 ± 6	100 ± 7	100 ± 8	100 ± 6
Compound *4* 10 mg/kg/day	69 ± 5	89 ± 5	68 ± 6*	293 ± 9*	92 ± 5
Compound *4* 20 mg/kg day	23 ± 4	93 ± 6	114 ± 7	99 ± 7	90 ± 5
VLDL					
Control	100 ± 6	100 ± 5	100 ± 8	100 ± 7	100 ± 5
Compound *4* 10 mg/kg/day	78 ± 7*	140 ± 6*	120 ± 6	65 ± 6*	102 ± 6
Compound *4* 20 mg/kg/day	119 ± 8	46 ± 4*	76 ± 6*	38 ± 4*	96 ± 3
LDL					
Control	100 ± 6	100 ± 7	100 ± 6	100 ± 7	100 ± 5
Compound *4* 10 mg/kg/day	63 ± 5*	115 ± 6	79 ± 5*	55 ± 8*	80 ± 6
Compound *4* 20 mg/kg/day	89 ± 4	98 ± 7	98 ± 6	217 ± 3*	104 ± 6
HDL					
Control	100 ± 5	100 ± 6	100 ± 7	100 ± 6	100 ± 5
Compound *4* 10 mg/kg/day	221 ± 7*	141 ± 6*	86 ± 6	115 ± 7	85 ± 6
Compound *4* 20 mg/kg/day	447 ± 7*	81 ± 7	93 ± 5	80 ± 5	118 ± 6
4 weeks					
Chylomicrons					
Control	100 ± 7	100 ± 6	100 ± 7	100 ± 7	100 ± 5
Compound *4* 10 mg/kg/day	46 ± 3*	121 ± 7	139 ± 8*	40 ± 5*	97 ± 6
Compound *4* 20 mg/kg/day	36 ± 4*	129 ± 6*	141 ± 6*	67 ± 6*	82 ± 5
VLDL					
Control	100 ± 6	100 ± 5	100 ± 7	100 ± 6	100 ± 6
Compound *4* 10 mg/kg/day	90 ± 7	64 ± 5*	28 ± 3*	75 ± 6*	73 ± 4*
Compound *4* 20 mg/kg/day	34 ± 3*	87 ± 6	39 ± 4*	79 ± 7*	40 ± 3*
LDL					
Control	100 ± 6	100 ± 7	100 ± 6	100 ± 8	100 ± 6
Compound *4* 10 mg/kg/day	28 ± 3*	62 ± 5*	65 ± 5*	83 ± 9	40 ± 5*
Compound *4* 20 mg/kg/day	71 ± 6*	78 ± 4*	71 ± 6*	93 ± 7	55 ± 7*
HDL					
Control	100 ± 5	100 ± 6	100 ± 7	100 ± 7	100 ± 7
Compound *4* 10 mg/kg/day	1180 ± 16*	78 ± 5*	106 ± 8	33 ± 5*	120 ± 6
Compound *4* 20 mg/kg/day	913 ± 8*	93 ± 6	127 ± 7	46 ± 5*	83 ± 7
6 weeks					
Chylomicrons					
Control	100 ± 7	100 ± 6	100 ± 7	100 ± 8	100 ± 6
Compound *4* 10 mg/kg/day	70 ± 6*	88 ± 6	131 ± 6*	125 ± 9*	90 ± 5
Compound *4* 20 mg/kg/day	68 ± 5*	110 ± 7	95 ± 6	63 ± 7	80 ± 4
VLDL					
Control	100 ± 6	100 ± 5	100 ± 5	100 ± 7	100 ± 5
Compound *4* 10 mg/kg/day	41 ± 4*	78 ± 4*	70 ± 5*	155 ± 6*	94 ± 5
Compound *4* 20 mg/kg/day	24 ± 3*	111 ± 6	85 ± 6	135 ± 8*	96 ± 6
LDL					
Control	100 ± 6	100 ± 7	100 ± 6	100 ± 8	100 ± 7
Compound *4* 10 mg/kg/day	60 ± 5*	91 ± 7	69 ± 5*	139 ± 9*	73 ± 6*
Compound *4* 20 mg/kg/day	59 ± 6*	100 ± 6	85 ± 6	122 ± 8	99 ± 3
HDL					
Control	100 ± 5	100 ± 7	100 ± 8	100 ± 7	100 ± 6
Compound *4* 10 mg/kg/day	250 ± 6*	67 ± 6*	141 ± 9*	99 ± 8	102 ± 7
Compound *4* 20 mg/kg/day	376 ± 8*	76 ± 6*	186 ± 8*	62 ± 7*	109 ± 6
8 weeks					
Chylomicrons					
Control	100 ± 7	100 ± 7	100 ± 8	100 ± 7	100 ± 6
Compound *4* 10 mg/kg/day	78 ± 6*	106 ± 7	100 ± 7	98 ± 8	101 ± 6
Compound *4* 20 mg/kg/day	89 ± 7	77 ± 5*	95 ± 6	114 ± 8	102 ± 5
VLDL					
Control	100 ± 7	100 ± 7	100 ± 6	100 ± 8	100 ± 5
Compound *4* 10 mg/kg/day	63 ± 5*	73 ± 6*	99 ± 8	119 ± 7	90 ± 6
Compound *4* 20 mg/kg/day	34 ± 4*	92 ± 5	106 ± 7	100 ± 6	165 ± 7*

Table VIII. Continued

	Percent of Control (X ± SD)				
	Cholesterol	Triglyceride	Neutral Lipids	Phospholipids	Protein
LDL					
Control	100 ± 6	100 ± 7	100 ± 5	100 ± 8	100 ± 7
Compound *4* 10 mg/kg/day	52 ± 5*	67 ± 6*	98 ± 5	117 ± 7	106 ± 7
Compound *4* 20 mg/kg/day	89 ± 3	50 ± 4*	92 ± 4	121 ± 8	288 ± 8*
HDL					
Control	100 ± 5	100 ± 6	100 ± 5	100 ± 7	100 ± 7
Compound *4* 10 mg/kg/day	199 ± 7*	91 ± 6	91 ± 7	122 ± 8	98 ± 6
Compound *4* 20 mg/kg/day	166 ± 7*	62 ± 4*	104 ± 6	227 ± 9*	98 ± 6

[a] 337 ug cholesterol/mL serum.
[b] 420 ug triglyceride/mL serum.
[c] 67 ug neutral lipid/mL serum.
[d] 149 ug phospholipid/mL serum.
[e] 184 ug protein/mL serum.
[f] 190 ug cholesterol/mL serum.
[g] 22 ug triglyceride/mL serum.
[h] 98 ug neutral lipid/mL serum.
[i] 26 ug phospholipid/mL serum.
[j] 50 ug protein/mL serum.
[k] 210 ug cholesterol/mL serum.
[l] 10 ug triglyceride/mL serum.
[m] 45 ug neutral lipid/mL serum.
[n] 41 ug phospholipid/mL serum.
[o] 122 ug protein/mL serum.
[p] 544 ug cholesterol/mL serum.
[q] 27 ug triglyceride/mL serum.
[r] 620 ug neutral lipid/mL serum.
[s] 153 ug phospholipid/mL serum.
[t] 657 ug protein/mL serum.
* $P \leq 0.001$.

but the other tissue generally showed reductions of ^{14}C-cholesterol content.

The normolipidemic rat body weight and weight of the major organs were not different from the control's after 8 weeks administration of drug. The clinical chemistry studies showed no alteration which suggested drug toxicity (Table 13). BUN, CP-kinase, cholesterol and triglyceride values were reduced significantly. The hematocrit, platelet estimate and differential white blood cell count were not altered by drug treatment. The organ weights were all within the range of the control weights. Histology of sections of rat liver, kidney and spleen showed no morphological differences from the control rats. The LD_{50} value of compound *4*, IP as a single dose in CF_1 mice was > 500 mg/kg. Furthermore, sub-acute toxicity studies at 20, 40 and 100 mg/kg/day in normolipidemic CF_1 mice for 7 days demonstrated no significant effects. Small changes in organ weights were expressed as percentage of the total body weight were noted (Table 14), e.g. brain and stomach. Small intestine and reproductive organs decreased at the 100 mg/kg dosage, whereas lungs, spleen, liver, and large intestine weights were elevated.

Compound *4* had no effect on female mouse fertility except at 100 mg/kg/day there was a slight reduction [17%] which was not statistically different from the control value (Table 15). The number of fetuses/litter were elevated at 20 mg/kg/day but reduced at 100 mg/kg/day. The body weight of the pups after three weeks was elevated for all treatment groups compared to the control.

DISCUSSION

The 2-benzoyl-4,4-dialkyl-3,5-isoxazolidinediones were effective hypolipidemic agents, lowering serum cholesterol in normolipidemic CF_1 mice between 13–55% on day 16 at 20/mg/kg/day, IP. Serum triglycerides levels were not reduced as significantly, i.e., only 17–40% by compounds *4-12*. 2-(3,4,5-Trimethoxybenzoyl)-4,4-diethyl-3,5-isoxazolidinedione was effective in rats at 10 and 20 mg/kg/day

Table IX. The Incorporation of Radiolabelled Precursors into the Serum Lipoprotein Fraction of Normolipidemic Sprague Dawley Rats After 2 Weeks Administration of Compound *4* at 20 mg/kg/day, Orally

	Percent of Control (X ± SD)							
	^{3}H-Cholesterol		^{14}C-Palmitic Acid		^{32}P-Phospholipid		^{3}H-Leucine	
N = 6	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Chylomicron	100 ± 6[a]	56 ± 5*	100 ± 7[e]	30 ± 4*	100 ± 7[i]	130 ± 6*	100 ± 7[m]	154 ± 6*
VLDL	100 ± 6[b]	78 ± 6*	100 ± 5[f]	10 ± 2*	100 ± 8[j]	152 ± 5*	100 ± 5[n]	192 ± 8*
LDL	100 ± 6[c]	45 ± 5*	100 ± 6[g]	16 ± 3*	100 ± 5[k]	153 ± 7*	100 ± 4[o]	70 ± 5*
HDL	100 ± 4[d]	135 ± 7*	100 ± 5[h]	38 ± 5*	100 ± 4[l]	88 ± 5	100 ± 4[p]	112 ± 6

[a] 2091 dpm/mL.
[b] 434 dpm/mL.
[c] 403 dpm/mL.
[d] 424 dpm/mL.
[e] 764 dpm/mL.
[f] 386 dpm/mL.
[g] 110 dpm/mL.
[h] 379 dpm/mL.
[i] 211 dpm/mL.
[j] 424 dpm/mL.
[k] 140 dpm/mL.
[l] 362 dpm/mL.
[m] 11492 dpm/mL.
[n] 4722 dpm/mL.
[o] 2909 dpm/mL.
[p] 3866 dpm/mL.
* $P \leq 0.001$ Student's "t" or Dunnett's test.

Table X. Lipid Levels of Serum Lipoproteins of Hyperlipidemic Sprague Dawley Rats Administered Compound *4* for 4 Weeks at 10 mg/kg/day, Orally

	Percent of Control (X ± SD)				
N = 6	Cholesterol	Triglyceride	Neutral Lipids	Phospholipids	Protein
Chylomicron					
Control	100 ± 5[a]	100 ± 5[b]	100 ± 6[c]	100 ± 7[d]	100 ± 5[e]
Treated	84 ± 4	87 ± 6	100 ± 7	145 ± 8*	84 ± 4
VLDL					
Control	100 ± 4[f]	100 ± 6[g]	100 ± 8[h]	100 ± 5[i]	100 ± 4[j]
Treated	98 ± 5	85 ± 7	53 ± 5*	159 ± 7*	37 ± 5*
LDL					
Control	100 ± 5[k]	100 ± 6[l]	100 ± 4[m]	100 ± 6[n]	100 ± 5[o]
Treated	19 ± 3*	78 ± 5*	35 ± 4*	57 ± 5*	61 ± 5*
HDL					
Control	100 ± 7[p]	100 ± 5[q]	100 ± 3[r]	100 ± 4[s]	100 ± 6[t]
Treated	185 ± 6*	88 ± 6	85 ± 5	83 ± 6	95 ± 7

[a] 371 mg/mL. [f] 773 mg/mL. [k] 836 mg/mL. [p] 4689 mg/mL.
[b] 79 mg/mL. [g] 142 mg/mL. [l] 6.2 mg/mL. [q] 16 mg/mL.
[c] 554 mg/mL. [h] 294 mg/mL. [m] 54 mg/mL. [r] 397 mg/mL.
[d] 231 mg/mL. [i] 78 mg/mL. [n] 231 mg/mL. [s] 184 mg/mL.
[e] 96 mg/mL. [j] 15 mg/mL. [o] 73 mg/mL. [t] 315 mg/mL.
* $P \leq 0.001$ Student's 't' or Dunnett's test.

orally, lowering serum cholesterol and triglyceride levels greater than 40% by the eighth week in normolipidemic rats. The inhibition of lipid synthesizing enzyme activities *in vivo* and *in vitro* suggests that the agent reduced the *de novo* synthesis of cholesterol and fatty acids in their early synthetic steps. HMG CoA reductase activity was suppressed *in vitro*, but not *in vivo* although this was observed in two different species. *In vitro* inhibition by compound *4* may be due to a metabolite of the drug generated in the rat. Additional studies have demonstrated that compound *4* reduces the activity of LDL receptor binding and degradation in human and rat hepatocytes and fibroblast[21]. This action of the drug should lead to a membrane LDL receptor mediated increase in HMG CoA reductase activity which also has been observed with cyclic imides, and boron derivatives[22–24].

The reduction of acyl CoA cholesterol acyl transferase activity, and the elevation of neutral cholesterol ester hydrolases activity observed *in vitro* and *in vivo* should lead to less cholesterol ester storage in tissues. As observed after *in vivo* administration of the drug, tissue lipids, e.g., total cholesterol, were indeed reduced. This would be of particular importance in aortic plaques when cholesterol ester accumulation dictates the growth of the plaques. Total cholesterol levels were reduced in the aorta tissue after treatment with *4* for two or eight weeks.

Table XI. The Incorporation of Radioactive Precursors into Major Tissues of Normolipidemic Sprague Dawley Male Rats After 2 Weeks Administration of Compound *4* at 20 mg/kg/day Orally

	Percent of control							
	^{3}H-Cholesterol		^{14}C-Palmitic acid		^{32}P-Phospholipid		Organ wt. (% total body wt.)	
(*N* = 6) Organ	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Brain	100	91	100	55	100	85	0.641	0.565
Lung	100	203	100	18	100	130	0.620	0.468
Heart	100	104	100	49	100	62	0.320	0.382
Liver	100	349	100	20	100	112	3.691	3.891
Spleen	100	460	100	25	100	85	0.203	0.234
Kidney	100	147	100	83	100	63	0.793	0.831
Thymus	100	176	100	47	100	122	0.160	0.167
Reproductive	100	86	100	106	100	94	2.928	3.000
Stomach	100	50	100	117	100	73	0.560	0.517
Small intestine	100	175	100	4	100	86	2.363	2.157
Large intestine	100	104	100	172	100	82	1.002	0.820
Chyme	100	200	100	13	100	203	—	—
Feces	100	118	100	107	100	68	—	—

Table XII. The Effects of Compound *4* on ^{14}C-Cholesterol Distribution in Normolipidemic CF_1 Male Mice at 20 mg/kg/day IP for 2 Weeks

	dpm/Total Organ	
(*N* = 6)	Control	Compound 4
Plasma	58922	47348 (80.36%)
Brain	81688	37270 (45.62%)
Liver	1569396	2628990 (167.52%)
Kidney	270241	315260 (116.65%)
Spleen	210789	230720 (109.45%)
Heart	86965	72380 (83.23%)
Lung	422392	333710 (79.00%)
Stomach	231324	122432 (52.93%)
Small Intestine	2033713	1441330 (70.87%)
Large Intestine	427295	293670 (68.72%)
Chyme	953527	1177290 (123.46%)
Feces	6916462	6908420 (99.88%)
TOTAL	13423904	13608820

Figure 1. PAGE Electrophoresis of HDL from Sprague Dawley Rats: Lane 1, Control 1% CMC; Lane 2, Compound 4 - 10mg/kg/day for 6 weeks; Lane 3, Compound 4 - 20mg/kg/day for 6 weeks.

Lipid modulation by the drug was also observed in the serum lipoprotein fractions. The agent appears to suppress the incorpation of cholesterol and palmitic acid into VLDL and LDL fraction in normolipidemic rats. Cholesterol incorporation into the HDL fraction also was increased. This was also evident in the chemical lipid analysis of the lipoprotein fractions. It was interesting that the maximum elevation of HDL cholesterol levels occurred on week 4 at both doses employed and then decreased at 6 and 8 weeks. Modulation of lipoproteins by drug therapy is important since in hyperlipidemic states, LDL cholesterol levels are elevated and HDL cholesterol levels are reduced [25]. Modulation of lipids in the opposite direction achieves two things. First, less

Table XIII. The Effects of Compound *4* on Clinical Chemistry Values, Hematopoietic Parameters and Organ Weights After 8 Weeks Administration to Normolipidemic Sprague Dawley Rats at 10 mg/kg/day, Orally

	Percent of Control (X ± SD)		Organ Weight (g) (Percent of Total Body Weight)		
(*N* = 8) Clinical Assay	Control	Treated Compound 4	Organ	Control	Treated Compound 4
Total protein	100	85	Brain	0.561	0.565
BUN	100	69	Heart	0.399	0.382
Glucose	100	116	Lung	0.523	0.468
SGPT	100	99	Liver	3.691	3.891
LDH	100	107	Spleen	0.204	0.234
Creatine phosphatase	100	102	Adrenal	0.032	0.016
Uric acid	100	96	Kidney	0.793	0.831
Bilirubin (Direct)	100	89	Stomach	0.560	0.517
Bilirubin (Indirect)	100	90	Small Intestine	2.363	2.157
iPi	100	104	Large Intestine	1.002	0.820
CP Kinase	100	63			
Cholic acid	100	97			
Cholesterol	100	56			
Triglyceride	100	62			
Albumin	100	100			
Hematocrit	100	96			
Platelet Estimate	100	99			
Differential WBC Count					
Lympocytes	72.75	72.62			
PMNs	23.20	24.04			
Monocytes	1.56	1.22			
Eosinophils	1.04	0.43			
Basophils	1.39	1.30			

Table XIV. Sub-acute Toxicity of Compound *4* When Administered 7 days IP to Normolipidemic CF_1 Male Mice

	Control	20 mg/kg/day	50 mg/kg/day	100 mg/kg/day
Body Wt. Increase (g)	+2.9	+2.4	+0.6	0
Survival	100	100	100	100
Food Consumption (g/day)	100	89	94	88
Organ Weight (% body wt.)				
Brain	1.63	1.03	0.95	0.92
Heart	0.41	0.44	0.68	0.46
Lungs	0.74	0.81	0.82	0.92
Spleen	0.33	0.48	0.68	0.65
Liver	6.48	6.11	6.47	7.39
Kidney	1.31	1.25	1.71	1.41
Stomach	3.77	2.94	2.59	3.07
Small Intestine	9.51	7.58	5.27	5.82
Large Intestine	4.67	4.63	4.63	6.85
Reproductive Organs	1.52	1.47	2.72	1.31
Clinical Assays		Percent of Control		
SGPT (28.3 IU/mL)	100	105	92	97
BUN (29 IU/mL)	100	93	90	101
Bilirubin	100	32	65	76
Glucose (170.2 mg %)	100	83	83	116
LDH (Sigma Unit/mL)	100	—	93	106
Creatine Phosphokinase (Sigma Unit/mL)	100	—	101	104
Cholesterol (mg %)	100	—	98	116
Triglycerides (mg %)	100	—	60	74
Protein (g %)	100	—	125	136
Hematocrit (%)	45.1	47.8	43.9	44.9
Platelet estimate ($\times 10^4$)	19.1	19.1	19.2	19.1

lipids are deposited in peripheral tissues by VLDL and LDL and secondly, more cholesterol is conducted back to the liver for clearance and excretion in the bile as observed after *in vivo* treated with the compound *4* in normolipidemic rats. The same type of phenomena was observed in hyperlipidemic diet induced rats. The drug lowered both serum lipid levels after 4 weeks. Cholesterol levels were significantly reduced in the hyperlipidemic VLDL fraction (81%) and HDL fraction was elevated 85% by the drug. Obviously this was not as high as in normal rats but the modulation was in the correct direction. Examination of the apoprotein-LDL and HDL-fractions by PAGE from the normal 8 week treated rats showed that no other apoprotein except apo B was found in the LDL fraction. In the HDL fractions, high magnitudes of density of apo E and apo AI were observed after treatment for eight weeks with compound *4* (Fig. 1). Since HDL cholesterol uptake in the liver is mediated by apo E and apo AI receptors, this may explain why higher levels of intravenously-administered ^{14}C-cholesterol uptake into the liver occurred in the treated rats as well as the high levels of cholesterol (82%) observed in the bile at 2 weeks.

These studies would suggest that 3,5-isoxazolidinediones have potential use as hypolipidemic agents. Their effects on lipid metabolism and serum lipoproteins indicate that they are affecting parameters which should lower cholesterol deposition in the arterial wall while causing excretion of cholesterol in the bile/feces.

The eight week studies in normolipidemic rats and the sub-acute toxicity study in mice suggest no deleterious effects of compound *4* at the doses employed based on histological examination of tissue, hematopoeitic parameters, and clinical chemistry values.

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