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

Tyrone Woodard,¹ Manik L. Debnath,¹ Rupendra Simlot,² Robert A. Izidore,¹ Dwayne L. Daniels,¹ Oi T. Wong,² Hamby ElSourady,² and Iris H. Hall²,³

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A series of 2-benzoyl-4,4-diaryl-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, 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, mice showed the compound inhibited mitochondrial citrate exchange, acetyl CoA synthetase, HMG CoA reductase, acyl CoA cholesterol acyl transferase, acyl CoA carboxylase, sn-glycero-3-phosphate acyl transferase, phosphadiylate phosphohydrolase and hep shall 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 $^{13}$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-pyrolidinones[5,6], 4-pyridimidine carboxylic acids[7], furonic acids[8], and 3 and 4 phenyl piperidine-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-isoxazolidinone 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 Izidore et al.[15].

2-Benzoyl-4,4-diaryl-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$_2$CO$_3$. 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, mice were obtained from Jackson Laboratory. Animals were maintained.
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, CF₁ 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-dietethyl-3,5-isoxazolidinidine 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**

CF₁ 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), cellulose (60 g), cholesterol (53 g), choline dihydrogen citrate (4 g), wesson oil (40 g), sodium cholate (20 g), sucrose (223 g), vitamin-free casein (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-dietethyl-3,5-isoxazolidinedione (13) and 3,4,5-tri-methoxybenzoic acid (14). In order to confirm these structures, Compounds 13 [11] and 14 as well as 3,4,5 trimethoxybenzyhydroxamic 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 CF₁ 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-7a-hydroxylase activity was determined using [1,2-3H] cholesterol (60 mCi/mmol), and acyl CoA cholesterol acyl transferase activity was determined using [1-14C]oleic acid (56.7 mCi/mmol). Cholesterol synthesis was measured using [1,3-14C] acetyl CoA (62 mCi/mmol) and a post-mitochondrial supernatant (9000 g × 20 min) which was incubated for 60 min at 37°C. The digonitride 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 14C-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-2-3H(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