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Report

The Hypolipidemic Activity of 1-*N*-3-Methylphthalimido-butan-3-semicarbazone in Rodents

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1-*N*-(3-Methylphthalimido)butan-3-one semicarbazone demonstrated potent hypolipidemic activity in normal rats and mice and hyperlipidemic diet-induced mice. The compound decreased tissue lipid levels and increased the fecal excretion of cholesterol and triglycerides. After 2 weeks of administration, serum lipoprotein levels were modulated so that very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) cholesterol concentrations were reduced and high-density lipoprotein (HDL) cholesterol concentrations were elevated to levels unprecedented by the cyclic imide derivatives previously tested. The VLDL triglyceride content was also reduced. Hepatic *in vitro* enzymatic studies demonstrated that the compound suppressed the activity of enzymes in the early synthesis of fatty acids and cholesterol and the regulatory enzymes for the de novo synthesis of triglycerides.

KEY WORDS: cyclic imides; semicarbazone; low-density lipoprotein (LDL) cholesterol; high-density lipoprotein (HDL) cholesterol; hypolipidemic activity.

INTRODUCTION

Previous investigation of phthalimide derivatives which have been substituted in the N atom of the cyclic imide ring has shown that 1-*N*-3-methylphthalimido-butan-3-semicarbazone possesses potent hypolipidemic activity in rodents (1). Whereas a large number of phthalimide (2), saccharin (3), and naphthalimide (4) derivatives have serum lipid lowering effects, the derivatives did not modulate the lipid content of the serum lipoprotein fractions favorably, i.e., reduction of low-density lipoprotein (LDL) cholesterol and elevation of high-density lipoprotein (HDL) cholesterol content. The latter ratio of lipoprotein cholesterol content supposedly promotes clearance of cholesterol from aorta plaques and protects against myocardial infarction in man. Reported herein is the investigation of 1-*N*-3-methylphthalimidobutan-3-semicarbazone, which appears to achieve this desired ratio of LDL and HDL cholesterol levels in rats.

METHODS

Source of the Compound

The 1-*N*-3-methylphthalimido-butan-3-one analogue (I) was synthesized by the method of Irai *et al.* (5). 1-*N*-3-Methylphthalimido-butan-3-one (8.39 mmol) was added to a sodium acetate/ethanol/H_2O solution containing a 10% excess of an equimolar amount of semicarbazide hydrochloride. The reaction mixture was refluxed until it appeared cloudy, then allowed to stand for 2 hr, and 40 ml of water was added. The precipitate was filtered and recrystallized from chloroform, which afforded 1.69 g (70%); m.p. = 215–217°C. Calculated for $C_{14}H_{16}N_4O_3$: C, 58.4%; H, 5.6% N, 19.4%. Found: C, 58.22%; H, 5.5%; N, 19.5%.

Hypolipidemic Screens in Normal Rodents

The test compounds were suspended in an aqueous 1% carboxymethylcellulose solution, homogenized, and administered to CF_1 male mice (~25 g) at 20 mg/kg/day intraperitoneally for 16 days. On days 9 and 16, blood was obtained by tail vein bleeding and the serum separated by centrifugation for 3 min. Sprague Dawley male rats (~250 g) were administered orally the test compound by intubation needle at 10 mg/kg/day. Blood was collected on days 9 and 14. The serum cholesterol levels were determined by a modification of the Liebermann–Burchard reaction (6). Serum was also analyzed for triglyceride content as determined by a commercial kit [BioDynamics/bmc single vial, triglycerides colormetric method 348201]. Food and water were available ad libitum for animals in experiments.

Testing in Hyperlipidemic Mice

CF_1 male mice (~25 g) were placed on a commercial diet (7) which produced a "hyperlipidemic" state. After the serum cholesterol and triglyceride levels were observed to be elevated, the mice were administered the test compound at 10 mg/kg/day intraperitoneally for an additional 12-day pe-

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riod while continuing the diet. Serum cholesterol and triglyceride levels were measured as previously described.

Animals Weights and Food Intake

Food consumption was determined daily as g food/rat/day for control rats and those treated orally with the test compound at 10 mg/kg/day. Body weights were obtained during the experiments and expressed as a percentage of the animal's weight on day 0. After dosing for 14 days with compounds, selected organs were excised, trimmed of fat, and weighed.

Enzymatic Studies

In vitro enzymatic studies were determined using 10% homogenates in 0.25 *M* sucrose and 0.001 *M* EDTA of CF_1 male mouse liver with 50–200 μmol of the compound. The enzyme activities were determined by following literature procedures (7): acetyl coenzyme A synthetase (8), adenosine triphosphate-dependent citrate lyase (9), mitochondrial citrate exchange (10,11), cholesterol-7α-hydroxylase (12), 3-hydroxy-3-methylglutaryl coenzyme A reductase (13,14), acetyl coenzyme A carboxylase activity (15), cholesterol ester hydrolase (16), *sn*-glycerol-3-phosphate acyl transferase activity (17), phosphatidylate phosphohydrolase activity (18), acyl CoA cholesterol acyl transferase (19), and heparin-activated hepatic lipoprotein lipase (20). Protein was determined for the cell extract appropriate for each enzyme assay by the Lowry *et al.* (21) technique.

Liver, Small Intestine, and Fecal Lipid Extraction

In Sprague Dawley male rats that had been administered the compound at 10 mg/kg/day for 14 days, the liver, small intestine, and fecal materials (24-hr collection) were removed, extracted (22,23), and analyzed for cholesterol levels (6), triglyceride levels, neutral lipid content (24), and phospholipid content (25).

Serum Lipoprotein Fractionation

Sprague Dawley male rats (~300 g) were administered the test compound at 10 mg/kg/day orally. Blood was collected from the abdominal aorta vein and lipoprotein fractions were obtained by the method of Hatch and Lees (26) and Havel *et al.* (27) as modified for the rat (28). Each of the fractions was analyzed for cholesterol (6), triglyceride, neutral lipids (24), phospholipids (25), and protein levels (21).

Cholesterol and Cholic Acid Absorption/Reabsorption from Gut

The effects of the drugs on *in situ* intestinal absorption of cholesterol or cholic acid were determined by the method of Douluisio *et al.* (29). Sprague Dawley male rats were anesthesized with phenobarbital and ketamine. The duodenum intestinal loop was isolated. The drug (0.2 ml, 10 mg/kg) with 200 mg% cholesterol or cholic acid was introduced into the lumen and the ends of the loop were tied off. Samples (50 μl) were removed from the lumen of the intestine every 15 min for the next 3 hr and analyzed for cholesterol (6) or cholic acid (33) content. The disappearance over time of cholesterol or cholic acid from inside the loop was plotted (Fig. 1).

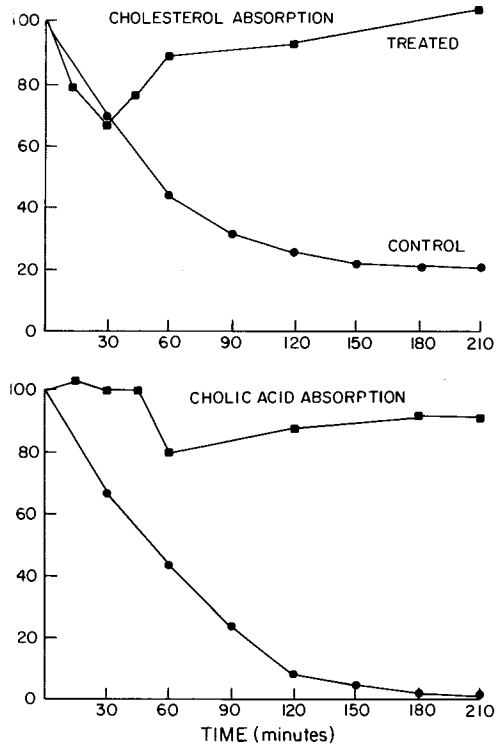

Fig. 1. The effects of 1-*N*-3-methyl-phthalimido-butan-3-semicarbazones on the percentage of cholesterol or cholic acid absorption/reabsorption from the rat gut over time. (●——●) Control, 1% CMC. (■——■) Treated with 1-*N*-3-methyl-butan-3-semicarbazone (10 mg/kg/orally).

RESULTS

1-*N*-3-Methylphthalimido-butan-3-semicarbazone was

Table I. The Effects of 1-*N*-3-Methylphthalimido-butan-3-semicarbazone Levels of Normal and Hyperlipidemic Diet-Induced CF_1 Mice at 20 mg/kg/day i.p. (N = 6)

	Percentage of control (X ± SD)		
	Serum cholesterol		Serum triglyceride
Normal diet	Day 9	Day 16	Day 14
Compound I	71 ± 5*	66 ± 7*	57 ± 6[g]
1% CMC	100 ± 6[a]	100 ± 5[b]	100 ± 7[c]
Hyperlipidemic diet induced	Day 12		Day 12
Compound I	83 ± 6*		62 ± 5*
1% CMC	100 ± 6[d]		100 ± 7[e]

[a] 125 mg%.
[b] 127 mg%.
[c] 137 mg%.
[d] 354 mg%.
[e] 376 mg%.
* $P < 0.001$.

Table II. The Effects of 1-*N*-3 Methylphthalimido-Butan-3-Semicarbazone on Sprague Dawley Rat Serum Lipid Levels After Oral Administration at 10 mg/kg/day ($N = 6$)

	Percentage of control ($X \pm$ SD)							
	Serum cholesterol		Serum triglyceride		Food consumption (g/day rat)	Total body weight		
	Day 7	Day 14	Day 7	Day 14		Day 0	Day 7	Day 14
Compound I	71 ± 5*	63 ± 4*	71 ± 6*	72 ± 6*	22.0	100	104.2	115.5
1% CMC	100 ± 6[a]	100 ± 6[b]	100 ± 9[c]	100 ± 7[d]	20.8	100	115.7	128.3

[a] 73 mg%.
[b] 78 mg%.
[c] 110 mg%.
[d] 112 mg%.
* $P < 0.001$.

effective in lowering serum cholesterol levels 29% on day 9 and 34% on day 16 in normal mice. Serum triglyceride levels were reduced 43% on day 16 in normal mice (Table I). In hyperlipidemic-induced mice, serum cholesterol levels were elevated 354 mg% and serum triglyceride levels were elevated 376 mg%. Compound I, when administered for 12 days, lowered the induced states of cholesterol 17% and triglycerides 38%, respectively (Table I).

When I was tested in Sprague Dawley male rats, it was observed that after 14 days the serum cholesterol content was reduced 37% and the triglyceride content was lowered 28% (Table II). The agent lowered the total body weight by 13% after 14 days of administration but had no effect on the daily food consumption. The total lipid levels of the liver, small intestine, and aorta were reduced by I treatment, i.e., cholesterol and triglyceride were reduced in all these tissues (Table III). The fecal cholesterol and triglyceride contents were elevated after drug treatment. The serum lipoprotein levels, after 14 days, showed that I treatment lowered cholesterol content in the very low-density lipoprotein (VLDL) (56%) and LDL (42%), with a 55% increase in the HDL cholesterol content (Table IV). The VLDL triglyceride content was reduced 39% by I treatment, with an increase in the chylomicron, LDL, and HDL triglyceride content. The phospholipid content was increased in the chylomicron and LDL fraction. When compound I was tested *in vitro* in hepatic homogenates at 100 μM, mitochondrial citrate exchange was reduced 27%, ATP citrate lyase activity was inhibited 18%, and *sn*-glycerol-3-phosphate acyl transferase activity was reduced 28%. Actyl CoA carboxylase and acetyl CoA synthetase activities were essentially unaffected by compound I. HMG CoA reductase activity was elevated 51% at 50 μM and 54% at 100 μM concentrations. Cholesterol ester hydrolase activity was elevated in a concentration-dependent manner, with 50 μM causing a 44% increase and 100 μM a 60% increase. Phosphatidylate phosphohydro-

Table III. The Effects of Compound I on Sprague Dawley Rat Lipids in Liver, Small Intestine, Aorta, and Feces After 14 Days of Oral Administration at 10 mg/kg/day ($N = 6$)

	Percentage of control ($X \pm$ SD)					
	mg lipid extracted	Cholesterol	Triglyceride	Neutral lipids	Phospholipids	Proteins
Liver						
Control	100 ± 5[a]	100 ± 5[b]	100 ± 7[c]	100 ± 6[d]	100 ± 7[e]	100 ± 7[f]
I	84 ± 6	86 ± 4*	86 ± 5	117 ± 8	61 ± 6*	110 ± 6
Small intestine						
Control	100 ± 5[g]	100 ± 4[h]	100 ± 7[i]	100 ± 7[j]	100 ± 8[k]	100 ± 6[l]
I	93 ± 6	88 ± 5	83 ± 6	91 ± 7	172 ± 7*	95 ± 5
Aorta						
Control	100 ± 5[m]	100 ± 4[n]	100 ± 5[o]	100 ± 6[p]	100 ± 5[q]	100 ± 5[r]
I	85 ± 4	90 ± 6	73 ± 4*	98 ± 5	145 ± 7	77 ± 6*
Feces						
Control	100 ± 6[s]	100 ± 7[t]	100 ± 6[u]	100 ± 6[v]	100 ± 5[w]	100 ± 6[x]
I	124 ± 6*	113 ± 5	182 ± 8*	95 ± 7	27 ± 3	82 ± 5

[a] 50.5 g 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] 12.02 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] 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 of lipid/g wet tissue. [t] 2.84 mg cholesterol/g wet tissue. [u] 1.86 mg triglyceride/g wet tissue. [v] 3.39 mg neutral lipids/g wet tissue. [w] 5.70 mg phospholipid/g wet tissue. [x] 6.99 mg protein/g wet tissue. * $P < 0.001$.

Table IV. The Effects of Compound I on Serum Lipoprotein Lipids of Sprague Dawley Rat After Oral Administration for 14 Days at 10 mg/kg/day Orally (N = 6)

	Percentage of control ($X \pm$ SD)				
	Cholesterol	Triglyceride	Neutral lipids	Phospholipids	Proteins
Chylomicrons					
Control	100 ± 6[a]	100 ± 7[b]	100 ± 7[c]	100 ± 8[d]	100 ± 7[e]
I	101 ± 7	146 ± 8*	75 ± 5*	164 ± 7*	94 ± 5
VLDL					
Control	100 ± 4[f]	100 ± 6[g]	100 ± 7[h]	100 ± 7[i]	100 ± 6[j]
I	44 ± 5*	61 ± 5*	97 ± 7	68 ± 8*	94 ± 5
LDL					
Control	100 ± 6[k]	100 ± 6[l]	100 ± 7[m]	100 ± 6[n]	100 ± 5[o]
I	58 ± 4*	124 ± 7*	60 ± 6*	133 ± 5*	82 ± 4
HDL					
Control	100 ± 6[p]	100 ± 7[q]	100 ± 6[r]	100 ± 7[s]	100 ± 6[t]
I	155 ± 7*	276 ± 8*	76 ± 7*	89 ± 6	95 ± 5

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

lase (IC_{50}, 97 μM) acyl CoA cholesterol acyl transferase (IC_{50}, 53 μM) and heparin-induced lipoprotein lipase (IC_{50}, 44 μM) activities were suppressed by I in a concentration-dependent manner. *In situ* loop studies (Fig. 1) showed that the semicarbazone inhibited reabsorption of cholesterol by 100% and of cholic acid by 90% after 210 min.

DISCUSSION

A number of cyclic imides have previously demonstrated potent hypolipidemic activity in rodents, effectively lowering both serum cholesterol and serum triglycerides greater than 35% after 16 days of administration (1–7). If an agent is expected to be effective in hyperlipidemic patients, then it should reduce high LDL cholesterol levels and elevate low HDL cholesterol levels in atherosclerotic patients.

The modulation of the ratio in this direction is important since apo B-containing lipoproteins, i.e., LDL, VLDL, and intermediate-density lipoproteins (IDL), are responsible for conducting cholesterol to the peripheral tissues including the plaques (30), whereas HDL is responsible for conducting cholesterol from the peripheral tissue to the liver for the purpose of clearance from the body. Unfortunately, the commercially available agents used in the clinic today to treat atherolosclerosis and hypolipidemic states are not effective in elevating HDL cholesterol and lowering LDL cholesterol levels, i.e., the magnitude of change is relatively small. Clofibrate elevated HDL cholesterol 4–16%, probucal has no effect (31) in man, and lovastatin afforded only a moderate elevation (27%) of HDL cholesterol in rats (unpublished results). In man elevating HDL cholesterol and reducing LDL cholesterol supposedly protect against myocardial infarction (32).

The previously tested cyclic imides were not very effective in elevating rat HDL cholesterol levels after 2 weeks of drug administration, although they generally did lower the LDL and VLDL cholesterol content, with reductions of VLDL triglycerides (1–4). The 1-*N*-3-methylphthalimidobutan-3-semicarbazone derivative appears to afford an increase in rat HDL cholesterol content at 10 mg/kg/day after 14 days of drug administration. Only one other cyclic imide, *o*-(*N*-phthalimido)acetophenone, elevated HDL cholesterol 40% but only after 8 weeks of drug administration at 20 mg/kg/day in rats (8). In contrast, the semicarbazone afforded a 55% increase after 2 weeks at 10 mg/kg/day orally. The semicarbazone agent did not remove lipids from the rat serum compartment and place them in the body tissue; rather the lipids were removed by the fecal route from the body. The aorta, small intestine, and liver demonstrated reduced lipid levels after treatment at 10 mg/kg/day, orally. These data suggest that modulation of the HDL and LDL ratio caused lipid removal from these tissues and clearance by the liver at a dose lower than required for other cyclic imides.

The fact that the semicarbazone inhibits acyl CoA acyl transferase activity with an elevation of cholesterol ester hydrolase activity suggests that the agent could inhibit cholesterol deposition in peripheral tissue. Other cyclic imide derivatives have been shown not to be inhibitors of HMG CoA reductase activity, nor was I. Rather these cyclic imide agents like I inhibit enzymes involved in the early synthesis of cytoplasmic acetyl CoA and the rate-limiting enzyme for de novo synthesis of triglycerides. Thus, the semicarbazone cyclic imides demonstrate promise as a potential hypolipidemic agent because of a favorable modulation of lipid deposition.

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