Pharmaceutical Research, Vol. 3, No. 2, 1986

Research Article

The Hypolipidemic Activity of a Series of 2,3-Dihydrophthalazine-1,4-dione Derivatives in Rodents

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Received August 8, 1985; accepted October 20, 1985

A series of substituted 2,3-dihydrophthalazine-1,4-dione derivatives as well as the corresponding *N,N*-diaminophthalamides were prepared and were demonstrated to have potent hypolipidemic activity, lowering both serum triglyceride and cholesterol levels significantly at 20 mg/kg/day after 16 days of dosing in CF_1 male mice. The parent compound, 2,3-dihydrophthalazine-1,4-dione, lowered serum cholesterol 51% and serum triglyceride 43%. 2-(2-Carboxyethyl)-2,3-dihydrophthalazine-1,4-dione demonstrated the best hypocholesterolemic activity, with a 66% reduction after 16 days. The 2-(*p*-chlorophenyl) derivative demonstrated good activity (>40% reduction) in both screens, as did the 6-methyl-2,3-dihydrophthalazine-1,4-dione derivative. Of the amides, 4-methyl-*N,N*-diaminophthalamide demonstrated the best hypolipidemic activity, affording a greater than 40% reduction. 2,3-Dihydrophthalazine-1,4-dione was found to inhibit the enzyme activity of acetyl CoA synthetase, ATP-dependent citrate lyase, *sn*-glycerol-3-phosphate acyl transferase, phosphatidylate phosphohydrolase, and mitochondrial citrate exchange of liver. In mice after 16 days of dosing, there was a reduction of cholesterol, triglycerides, neutral lipids, and phospholipids in the liver. Cholesterol and neutral lipids were reduced in rat chylomicrons, very low-density lipoproteins, and low-density lipoproteins. The cholesterol content of the high-density lipoprotein fraction was slightly elevated, but reductions in the triglycerides and phospholipids were observed in this lipoprotein fraction. ^{3}H-Cholesterol distribution studies showed a lower concentration in the major organs and plasma, with a higher ^{3}H-cholesterol content in the stomach and large intestine.

KEY WORDS: 2,3-dihydrophthalazine-1,4-dione derivatives; cholesterol; hypolipidemic activity.

INTRODUCTION

A number of cyclic imides as well as their *N*-substituted derivatives have been investigated for hypolipidemic activity in mice and rats. The cyclic imides including phthalimide (1), saccharin (2), 1,8-naphthalimide (3), succinimide (1), and glutarimide (1) were found to be active in reducing serum cholesterol and triglyceride levels. Substitution with a nitrogen inside or outside the cyclic imide ring to afford indazolone (4) or 3-iminophthalimide (5), respectively, resulted in compounds which possessed significant hypolipidemic activity in rodents. The phthalazine-1,4-dione derivatives have a six-membered ring rather than the five-membered cyclic imide ring; furthermore, in this ring there are two nitrogen atoms rather than the one atom as in the cyclic imide structures. This heterocycle is structurally related to phthalimide, and for that reason we examined the potential of the 2,3-dihydrophthalazine-1,4-dione derivatives acting to reduce serum lipid levels. Those results are reported herein.

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MATERIALS AND METHODS

Chemistry

Alkylation of 2,3-dihydrophthalazine-1,4-dione (1) was reported earlier by Buu-Hoi *et al.* (6). According to this procedure, the dione (1) was treated with alkyl halide Rx (R = propyl and *n*-butyl) in the presence of aqueous sodium hydroxide for 10 days to isolate 2-propyl-4-propoxyphthalazine-1(2H)-one (7) and 2-butyl-4-butoxyphthalazine-1(2H)-one (9). Buu-Hoi *et al.* (6) have converted these *N,O*-dialkyl products into 2-propyl-2,3-dihydrophthalazine-1,4-dione (6) and 2-butyl-2,3-dihydrophthalazine-1,4-dione (8) by treating 7 and 9 with pyridinium hydrochloride. This prolonged reaction time with low yields and our need to generate various *N*-monoalkyl derivatives prompted us to adopt a different procedure of alkylation. Thus, the dione (1) when treated with 1 equivalent of sodium hydride and alkyl halide Rx (R = $-CH_3$ to C_5H_{11}) for 24 hr afforded 2-alkyl-2,3-dihydrophthalazine-1,4-dione in a good yield, with a trace amount of the *N,O*-dialkyl product. The reaction of dione (1) with 2.2 equivalents of sodium hydride and an excess of alkyl halide resulted in 2-alkyl-4-alkoxyphthalazin-1(2H)-one exclusively (55–75% yield). 2-Phenyl and several of its

0724-8741/86/0400-0093$05.00/0

substituted phenyl 2,3-dihydrophthalazine-1,4-diones (14–20) were prepared by refluxing a mixture of phthalic anhydride and substituted phenylhydrazine hydrochloride in absolute ethanol following the procedure of Biguard and Grammaticakis (7). The propionic acid (12) and the ketone (13) were obtained by Michael addition of 2,3-dihydrophthalazine-1,4-dione with methyl acrylate and methyl vinyl ketone, respectively. The 5- and 6-substituted ($-CH_3$, $-OCH_3$, $-Cl$)-2,3-dihydrophthalazine-1,4-diones were prepared by treating 3- and 4-substituted phthalic anhydrides with 1 mol of hydrazine hydrate. However, the reaction of these phthalic anhydrides with excess hydrazine hydrate resulted in the corresponding *N,N*-diaminophthalamides (28–33). The ester (27) was made by reacting 2,3-dihydrophthalazine-1,4-dione (1) with sodium hydride and ethyl bromoacetate.

Analytical Procedures

Melting points were determined using a Thomas–Hoover melting point apparatus and are uncorrected. NMR data were obtained using a JEOL-FX-60 spectrophotometer. Elemental analyses were conducted by MHW Laboratories, Phoenix, Arizona, and were within ±0.4% of theory.

2-Alkyl-2,3-dihydrophthalazine-1,4-diones: General Procedure (2, 4, 6, 8, and 10). To a suspension of sodium hydride (0.02 mol; 50% suspension in mineral oil) in anhydrous dimethylformamide (100 ml) was added 2,3-dihydrophthalazine-1,4-dione (0.02 mol), followed by the slow addition of suitable alkyl halide (0.03 mol). The resulting mixture was stirred under reflux for 24 hr. The solvent was evaporated under vacuum. The solid product was recrystallized from the solvent specified in Table I.

2-Alkyl-4-alkoxyphthalazin-1(2H)-ones: General Procedure (3, 5, 7, 9, and 11). To a suspension of sodium hydride (0.04 mol; 50% suspension in mineral oil) in anhydrous dimethylformamide (100 ml) was added 2,3-dihydrophthalazine-1,4-dione (0.02 mol), followed by the slow addition of alkyl halide (excess). The mixture was stirred under reflux for 24 hr. The solvent was evaporated, then the products (3 and 5) were purified by recrystallization and the others (7, 9, and 11) were purified over a silica gel column using chloroform:ethyl acetate (1:1).

Aryl-Substituted 2,3-Dihydrophthalazine-1,4-diones: General Procedure (12–17). A mixture of 3- or 4-substituted phthalic anhydride (0.02 mol) and hydrazine hydrate (0.02 mol) in dry ethanol (50 ml) was heated to reflux overnight. During the course of the reaction phthalic anhydride disappeared, and after completion of the reaction a white crystalline solid was deposited on the walls of the reaction flask. The solid was filtered and recrystallized from ethanol.

2-(2'-Carboxyethyl)-2,3-dihydrophthalazine-1,4-dione (18). Following the procedure of Berre *et al.* (8), 2,3-dihydrophthalazine-1,4-dione (8.1 g; 0.05 mol) was suspended in absolute ethanol (100 ml) containing a catalytic amount of sodium ethoxide. Ethyl acrylate (5.0 g; 0.05 mol) was added and the reaction mixture set to reflux for 42 hr. The hot reaction mixture was filtered and the solvent removed *in vacuo*. The product was dissolved in sodium hydroxide (0.07 *M;* 250 ml) and stirred overnight. Upon acidification (HCl), a white crystalline solid mass was formed which was recrystallized from water.

2-(3'-Oxobutyl)-2,3-dihydrophthalazine-1,4-dione (20). Utilizing the procedure of Feuer *et al.* (9), 2,3-dihydrophthalazine-1,4-dione (16.2 g; 0.1 mol) was suspended in 95% ethanol (200 ml). The suspension was heated to reflux, and methyl vinyl ketone (8.41 g; 0.12 mol) added. After refluxing for 41 hr, the remaining solid was filtered, and the filtrate was concentrated to obtain the product which was crystallized from ethanol.

2-Aryl-2,3-dihydrophthalazine-1,4-diones: General Procedure (21–27). A mixture of phthalic anhydride (0.02 mol) and phenyl hydrazine or the suitably substituted phenyl hydrazine hydrochloride (0.04 mol) in 95% acetic acid (70 ml) was heated to reflux for 24 hr in the presence of a catalytic amount of sodium acetate (0.4 g). The mixture was diluted with water, and the precipitate was collected and washed with water. The residue was thoroughly agitated with 2% sodium hydroxide and filtered. The filtrate was acidified with concentrated HCl. The precipitate was collected and recrystallized from appropriate solvent.

N,N'-Diaminophthalamides: General Procedure (28–33). A mixture of phthalic anhydride (0.01 mol) and hydrazine hydrate (excess) in absolute ethanol (25 ml) was refluxed for 24 hr. Solvent was evaporated and the product was recrystallized from ethanol.

Biological Studies

Radioisotopes were purchased from New England Nuclear. Reagents for enzyme assays were obtained from Sigma Chemical Company.

Antihyperlipidemic Screens in Normal Rodents

The test compounds were suspended in an aqueous 1% carboxymethyl cellulose solution, homogenized, and administered to CF_1 male mice (~25 g) intraperitoneally for 16 days or Sprague Dawley male rats (~350 g) orally by an intubation needle for 14 days. On days 9 and 14 or 16, blood was obtained by tail vein bleeding and the serum separated by centrifugation for 3 min. The serum cholesterol levels were determined by a modification of the Liebermann-Burchard reaction (14). Blood was also collected on day 14 or 16 and the triglyceride content was determined by a commercial kit (Bio-Dyamics/bm triglyceride kit).

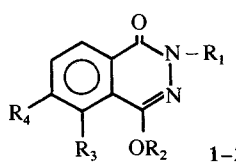
Testing in Hyperlipidemic Mice

CF_1 male mice (~25 g) were placed on a commercial diet (U.S. Biochemical Corp. basal atherogenic test diet) which produced a "hyperlipidemic" state (15). After the serum cholesterol and triglyceride levels were observed to be elevated, the mice were administered test drugs at 20 mg/kg/day intraperitoneally for an additional 14-day period. Serum cholesterol and triglyceride levels were measured at that time.

Animal Weights and Food Intake

Periodic animal weights were obtained during the experiments and expressed as a percentage of the animal's

Table I. Physical Characteristics of Substituted 2,3-Dihydrophthalazine-1,4-dione and Its Analogues



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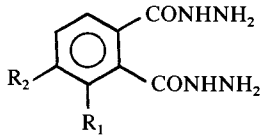

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Compound no.	R_1	R_2	R_3	R_4	MP (lit.), °C	Yield	Formula
1	H	H	H	H	—	—	—
2[a]	CH_3	H	H	H	132–136 (139–140)[g]	45	—
3[b]	CH_3	CH_3	H	H	93–96	78	$C_{10}H_{10}N_2O_2$
4[a]	C_2H_5	H	H	H	151–153	52	$C_{10}H_{10}N_2O_2$
5[b]	C_2H_5	C_2H_5	H	H	71–73	73	$C_{12}H_{14}N_2O_2$
6[c]	n-C_3H_7	H	H	H	160–162[h]	52	—
7	n-C_3H_7	n-C_3H_7[h]	H	H	—	70	—
8[d]	n-C_4H_9	H	H	H	95–96	48	—
9	n-C_4H_9	n-C_4H_9[h]	H	H	—	60	—
10[c]	n-C_5H_{11}	H	H	H	90–92	43	$C_{13}H_{16}N_2O_2$
11	n-C_5H_{11}	n-C_5H_{11}	H	H	—	56	$C_{18}H_{26}N_2O_2$
12[d]	H	H	Cl	H	>300	65	$C_5H_5N_2O_2Cl$
13[d]	H	H	H	Cl	>300	76	$C_8H_5N_2O_2Cl$
14[d]	H	H	CH_3	H	>300	83	$C_9H_8N_2O_2$
15[d]	H	H	H	CH_3	>300 (>300)[i]	75	$C_9H_8N_2O_2$
16[d]	H	H	OCH_3	H	236–240	68	$C_9H_8N_2O_3$
17[d]	H	H	H	OCH_3	290–291 (293)[j]	72	—
18	CH_2CH_2COOH	H	H	H	201–203 (206)[k]	26	$C_{11}H_{10}N_2O_4$
19[d]	CH_2COOEt	H	H	H	68–70	10	$C_{12}H_{12}N_2O_4$
20[d]	$CH_2CH_2COH_3$	H	H	H	152–153	80	$C_{12}H_{12}N_2O_3$
21[f]	C_6H_5	H	H	H	207–209 (212)[l]	58	—
22[d]	o-Cl C_6H_4	H	H	H	213–215	54	$C_{14}H_9N_2O_2Cl$
23[d]	m-Cl C_6H_4	H	H	H	229–232	60	$C_{14}H_9N_2O_2Cl$
24[d]	p-Cl C_6H_4	H	H	H	251–252	67	$C_{14}H_9N_2O_2Cl$
25[d]	o-CH_3 C_6H_4	H	H	H	203–205 (207)[m]	38	—
26[d]	m-CH_3 C_6H_4	H	H	H	218–220	52	$C_{15}H_{12}N_2O_2$
27[d]	p-CH_3 C_6H_4	H	H	H	222–224 (225)[m]	65	—
28[d]	H	Cl	—	—	>300	60	$C_8H_9N_4O_2Cl$
29[d]	Cl	H	—	—	>300	52	$C_8H_9N_4O_2Cl$
30[d]	H	CH_3	—	—	>300	78	$C_9H_{12}N_4O_2$
31[d]	CH_3	H	—	—	>300	62	$C_9H_{12}N_4O_2$
32[d]	OCH_3	H	—	—	244–247	75	$C_9H_{10}N_4O_3$
33[d]	H	OCH_3	—	—	296–298	60	$C_9H_{10}N_4O_3$

[a] Methanol. [b]Benzene. [c]Chloroform. [d]Ethanol. [e]H_2O. [f]Ethyl acetate. [g]Ref. 10. [h]Ref. 6. [i]Ref. 11. [j]Ref. 12. [k]Ref. 9. [l]Ref. 7. [m]Ref. 13.

weight on day 0. After dosing for 14 days with test drugs, selected organs were excised, trimmed of fat, and weighed. Food consumption was determined daily.

Toxicity Studies

The acute toxicity (LD_{50} value) (16) was determined in CF_1 male mice (~25 g) by administering test drug intraperitoneally from 100 mg to 2 g/kg as a single dose. The number of deaths was recorded over a 7-day period for each group.

Enzymatic Studies

In vitro enzymatic studies were determined using 10% homogenates of CF_1 male mouse liver with 50–200 μmol of test drug. *In vivo* enzymatic studies were determined using 10% liver homogenates [prepared in 0.25 *M* sucrose + 0.001 *M* (ethylenedinitrilo)-tetraacetic acid, pH 7.2] from CF_1 male mice obtained after administering the agents for 16 days at a dose ranging from 10 to 60 mg/kg/day intraperitoneally. The enzyme activities were determined by following literature procedures: acetyl coenzyme A synthetase (17), adenosine triphosphate-dependent citrate lyase (18), mitochondrial citrate exchange (19,20), cholesterol-7α-hydroxylase (21), 3-hydroxy-3-methylglutaryl coenzyme A (22,23), acetyl coenzyme A carboxylase activity (24), fatty acid synthetase activity (25), *sn*-glycerol-3-phosphate acyl transferase activity (26), phosphatidate phosphohydrolase activity (27), cholesterol acyl transferase (28), and heparin-activated hepatic lipoprotein lipase (29). Protein was determined for all enzyme assays by the technique of Lowry *et al.* (30).

Liver, Small Intestine, and Fecal Lipid Extraction

In CF_1 male mice that had been administered the agent for 16 days, the liver, small intestine, and fecal materials

(24-hr collection) were removed, extracted (31,32), and analyzed for cholesterol levels triglyceride levels (Bio-Dynamics/bmc triglyceride kit), neutral lipid content (33), and phospholipid content (34).

^{3}H-Cholesterol Distribution in Rats

Sprague Dawley rats (~300 g) were administered the agent for 14 days orally. On day 13, 10 μCi of ^{3}H-cholesterol was administered, orally by intubation needle to male rats, and according to the procedures described previously (4), some tissue samples were combusted in a Packard tissue oxidizer or plated on filter paper, dried and digested for 24 hr in hyamine hydroxide (New England Nuclear) at 40°C, and counted (Fisher Scintiverse in a Packard scintillation counter). Results are expressed as disintegrations per minute (dpm) per total organ.

Bile Cannulation Study

Sprague Dawley male rats (~300 g) were treated with test drugs at 20 mg/kg/day orally for 14 days. After anesthetizing the animal, the bile duct was cannulated as previously described (10). 1,2-^{3}H-Cholesterol (40.7 mCi/mmol) (10 μCi) was administered orally 18 hr prior to commencing the surgery. The bile was collected over the next 6 hr and the volume (ml) measured. Aliquots were counted (Fisher Scintiverse in a Packard scintillation counter) as well as analyzed for cholesterol content (14), triglyceride, and phospholipids (34).

Plasma Lipoprotein Fractions

Sprague Dawley male rats (~300 g) were administered test drugs for 14 days at 20 mg/kg/day orally. Blood was collected from the abdominal vein and lipoprotein fractions were obtained by the method of Hatch and Lees (35) and Havel *et al.* (36). Each of the fractions was analyzed for cholesterol (14), triglyceride (Bio-Dynamics/bmc triglyceride kit), neutral lipids (33), phospholipids (34), and protein levels (30).

RESULTS

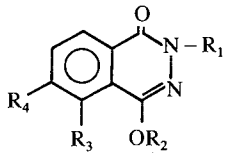
2,3-Dihydrophthalazine-1,4-dione proved to be a potent hypolipidemic agent, lowering both serum cholesterol and serum triglyceride levels significantly (Tables II and III). The compound was effective in affording a 51% reduction of the serum cholesterol level at 20 mg/kg/day and a 56% reduction of the serum triglyceride level at 40 mg/kg/day intraperitoneally after 16 days of dosing in mice. In Sprague Dawley male rats at 10 mg/kg/day orally, serum cholesterol levels were reduced 32% and serum triglycerides were lowered 36% after 14 days of dosing. The compound was also effective in induced hyperlipidemic states in mice, where cholesterol levels were elevated to 375 mg% and triglyceride devels were elevated to 367 mg/dl. The compound after 12 days of administration at 20 mg/kg lowered the cholesterol level to 221 mg%, a 41% reduction, and lowered serum triglycerides to 161 mg/dl, a 56% reduction. Thus the agent was effective in hyperlipidemic induced mice.

A series of substituted 2,3-dihydrophthalazine-1,4-diones demonstrated potent hypolipidemic activity in CF_1 male mice at 20 mg/kg/day ip (Table II) and was more active than clofibrate at the optimum dose of 150 mg/kg/day. The hypotriglyceridemic activity of the 2,3-dihydrophthalazine-1,4-dione nucleus was enhanced markedly by introducing the propionic acid side chain on one of the nitrogens, i.e., 2-(2′-carboxyethyl)-2,3-dihydrophthalazine-1,4-dione (**18**), which lowered the serum cholesterol by 66% and the triglyceride level by 40% after 16 days of dosing. Of all the *N*-alkyl analogues, 2-ethyl-2-3,dihydrophthalazine-1,4-dione (**4**) afforded the best activity, reducing both the lipid levels by greater than 45%. None of the *N,O*-dialkyl products (**3, 5, 7, 9,** or **11**) demonstrated improved activity compared to its monoalkyl analogue (**2, 4, 6, 8,** or **10**). Introduction of the phenyl ring on one of the nitrogens led to retention of the hypocholesterolemic activity compared to *N*-alkyl analogues; however, the ability to decrease the serum triglyceride levels was suppressed slightly after 16 days of dosing. 2-Phenyl-2,3-dihydrophthalazine-1,4-dione (**21**) was found to have a good activity, lowering the serum cholesterol by 42% and the triglyceride level by 35%. The influence of substituents such as methyl and chloride having similar lipophilic values and different steric effects were evaluated. Among the three possible positional isomers of each substituent, the paraisomers showed an improvement in hypotriglyceridemic activity. 2-(4′-Chlorophenyl)-2,3-dihydrophthalazine-1,4-dione (**24**) and 2-(4′-methylphenyl)-2,3-dihydrophthalazine-1,4-dione (**27**) demonstrated the ability to reduce triglyceride levels by 41 and 43%, respectively. The former compound (**24**) demonstrated the best hypolipidemic activity, i.e., a 46% reduction, among the 2-aryl-substituted diones (**21–27**). The effects of substituents on positions 5 and 6 of 2,3-dihydrophthalazine-1,4-diones were evaluated. Introduction of a CH_3 group at position 6 caused retention of the activity, while its other isomer suppressed the activity significantly. The chloride substituted in position 6 also resulted in good hypolipidemic activity. Nevertheless, the methoxy substitutions in position 5 or 6 led to less activity.

In an attempt to make the above 5- or 6-substituted 2,3-dihydrophthalazine-1,4-diones, the corresponding *N,N′*-diaminophthalamides (**28–33**) were isolated and tested for hypolipidemic activity. Of all these amides, 4-methyl-*N,N′*-diaminophthalamide (**30**) exhibited the best activity, lowering the serum cholesterol by 42% and the triglyceride level by 48%.

When regulatory enzymes of lipid metabolism from mouse liver were examined *in vitro,* mitochondrial citrate exchange was inhibited 24% at 200 μ*M* 2,3-dihydrophthalazine-1,4-dione (Table IV). ATP-dependent citrate lyase activity was significantly reduced at 50 and 100 μ*M,* whereas acetyl CoA synthetase activity was not inhibited significantly. The regulatory enzyme of the cholesterol pathway, HMG CoA reductase, was not significantly reduced at any of the concentrations tested of the compound. The enzyme responsible for conversion of cholesterol to bile acid, cholesterol-7α-hydroxylase, was suppressed by the agent by 24% at 50 μ*M*. More effective suppression by 2,3-dihydrophthalazine-1,4-dione (from 50 to 200 μ*M* concentration) was observed for acyl-CoA cholesterol acyl transferase activity. The activity for the initial enzyme in the fatty acid

Table II. Hypolipidemic Activity of Substituted 2,3-Dihydrophthalazine-1,4-diones at 20 mg/kg/day ip in CF_1 Male Mice



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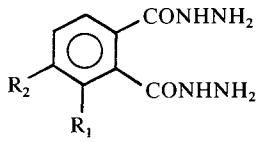

28–33

Compound no.	R_1	R_2	R_3	R_4	% control ($\bar{X}$ ± SD) Serum cholesterol Day 9	Serum cholesterol Day 16	Serum triglyceride, day 16
1	H	H	H	H	53 ± 4	49 ± 5	57 ± 4
2	CH_3	H	H	H	72 ± 5	77 ± 3	66 ± 4
3	CH_3	CH_3	H	H	84 ± 6	81 ± 5	64 ± 5
4	C_2H_5	H	H	H	71 ± 7	58 ± 5	59 ± 4
5	C_2H_5	C_2H_5	H	H	77 ± 5	76 ± 4	57 ± 3
6	C_3H_7	H	H	H	77 ± 6	68 ± 5	62 ± 2
7	C_3H_7	C_3H_7	H	H	68 ± 3	69 ± 5	69 ± 2
8	$n-C_4H_9$	H	H	H	59 ± 2	58 ± 3	73 ± 4
9	$n-C_4H_9$	C_4H_9	H	H	65 ± 4	61 ± 3	66 ± 3
10	$n-C_5H_{11}$	H	H	H	74 ± 3	71 ± 5	55 ± 4
11	$n-C_5H_{11}$	C_5H_{11}	H	H	86 ± 6	63 ± 4	59 ± 3
12	H	H	Cl	H	72 ± 5	69 ± 4	86 ± 5
13	H	H	H	Cl	72 ± 4	64 ± 5	65 ± 4
14	H	H	CH_3	H	80 ± 4	75 ± 3	73 ± 3
15	H	H	H	CH_3	66 ± 8	55 ± 4	54 ± 4
16	H	H	OCH_3	H	82 ± 5	78 ± 3	81 ± 4
17	H	H	H	OCH_3	77 ± 5	76 ± 4	69 ± 3
18	CH_2CH_2COOH	H	H	H	54 ± 6	34 ± 3	60 ± 4
19	CH_2COOEt	H	H	H	78 ± 6	69 ± 5	73 ± 4
20	$CH_2CH_2COCH_3$	H	H	H	72 ± 6	59 ± 3	70 ± 7
21	C_6H_5	H	H	H	59 ± 5	58 ± 4	65 ± 3
22	$o-Cl\ C_6H_4$	H	H	H	72 ± 5	67 ± 3	68 ± 4
23	$m-Cl\ C_6H_4$	H	H	H	73 ± 4	62 ± 3	69 ± 5
24	$p-Cl\ C_0H_4$	H	H	H	60 ± 3	54 ± 4	59 ± 5
25	$o-CH_3\ C_0H_4$	H	H	H	82 ± 7	66 ± 5	76 ± 6
26	$m-CH_3\ C_6H_4$	H	H	H	87 ± 6	74 ± 4	65 ± 3
27	$p-CH_3\ C_6H_4$	H	H	H	89 ± 5	73 ± 6	57 ± 2
28	H	Cl	—	—	68 ± 4	64 ± 5	62 ± 3
29	Cl	H	—	—	85 ± 6	73 ± 5	62 ± 2
30	H	CH_3	—	—	63 ± 5	58 ± 3	52 ± 4
31	CH_3	H	—	—	75 ± 6	75 ± 5	78 ± 3
32	H	OCH_3	—	—	54 ± 3	71 ± 4	65 ± 3
33	OCH_3	H	—	—	70 ± 4	55 ± 2	70 ± 7
Phthalimide					63 ± 8	57 ± 7	44 ± 8
Clofibrate, 150 mg/kg/day					88 ± 4	87 ± 5	75 ± 5
1% Carboxymethyl cellulose					100 ± 5	100 ± 6	100 ± 5

synthesis pathway, acetyl CoA carboxylase, was inhibited significantly by the agent at 100 and 200 μM, by 71 and 57%, respectively. Fatty acid synthetase activity was inhibited maximally by 19% at 100 and 200 μM of 2,3-dihydrophthalazine-1,4-dione. *sn*-Glycerol-3-phosphate acyl transferase activity was inhibited >50% by the agent at 50 and 100 μM. Phosphatidylate phosphohydrolase and heparin-induced hepatic lipoprotein lipase activity were not affected by 2,3-dihydrophthalazine-1,4-dione *in vitro*.

To confirm the effects of 2,3-dihydrophthalazine-1,4-dione *in vivo*, liver enzyme activities were evaluated after treatment with the agent from 10 to 60 mg/kg/day for 16 days in mice (Table V). Acetyl CoA synthetase activity was suppressed at 20 mg/kg/day by 30%. ATP-dependent citrate lyase activity was inhibited more markedly, by at least 50%, at all doses employed. Acetyl CoA carboxylase activity was inhibited 15% at 60 mg/kg/day. Fatty acid synthetase activity was inhibited at the lower doses of 10 and 20 mg/kg/day by 20%. *sn*-Glycerol-3-phosphate acyl transferase activity was inhibited in a dose-dependent manner, 60 mg/kg/day affording a 75% inhibition. Phosphatidylate phosphohydrolase activity was inhibited maximally at 20 mg/kg/day by 68%,

Table III. The Hypolipidemic Effects of 2,3-Dihydrophthalazine-1,4-dione Administered ip to CF_1 Male Mice and Orally to Sprague Dawley Rats

	% control ($\bar{X}$ ± SD)						
	CF_1 male mice			Sprague Dawley rats			
	Serum cholesterol		Serum triglyeride, day 16	Serum cholesterol		Serum triglyceride	
Compound (N = 6)	Day 9	Day 16		Day 7	Day 14	Day 7	Day 14
1% CMC	100 ± 6[a]	100 ± 7[b]	100 ± 6[c]	100 ± 8[d]	100 ± 7[e]	100 ± 6[f]	100 ± 7[g]
2,3-Dihydrophthalazine-1,4-dione							
10 mg/kg/day	88 ± 5	76 ± 7*	67 ± 5*	75 ± 5	68 ± 8*	66 ± 6*	64 ± 5*
20 mg/kg/day	53 ± 4*	49 ± 8*	57 ± 4*				
40 mg/kg/day	71 ± 7*	60 ± 6*	44 ± 3*				
60 mg/kg/day	76 ± 8*	55 ± 5*	49 ± 5*				
Clofibrate							
20 mg/kg/day	98 ± 7	97 ± 9	95 ± 7				
150 mg/kg/day				88 ± 5	87 ± 5		75 ± 6

[a] 118 mg/dl. [b]122 mg/dl. [c]137 mg/dl. [d]73 mg/dl. [e]78 mg/dl. [f]110 mg/dl. [g]112 mg/dl. *$P \leq 0.001$.

whereas 40 and 60 mg/kg/day resulted in a greater than 60% inhibition.

Reduction in lipid levels of liver after administration of the drug was more obvious in the mouse than the rat. The cholesterol content was reduced 44% at 10 mg/kg/day. Neutral lipids were reduced, with the lowest dose showing the maximum effect of 59% at 10 mg/kg/day. Triglyceride levels were reduced >40% at 20 and 40 mg/kg/day and phospholipids were reduced >30% at the same dose levels. After 2 weeks of administration of the drug, the rat livers demonstrated only an 18% reduction of cholesterol, a 14% reduction of neutral lipid, and a 25% reduction of phospholipids

Table IV. The *in Vitro* Effects of 2,3-Dihydrophthalazine-1,4-dione on CF_1 Mouse Liver Enzyme Activities

	Percentage control ($\bar{X}$ ± SD)			
		Drug concentration (μM)		
Enzyme assay (N = 6)	Control, 1% CMC	50	100	200
Mitochondrial citrate exchange	100 ± 5[a]	97 ± 5	90 ± 5	76 ± 7*
ATP-dependent citrate lyase	100 ± 7[b]	31 ± 3*	30 ± 6*	53 ± 6*
Acetyl CoA synthetase	100 ± 7[c]	89 ± 8	92 ± 8	81 ± 5
HMG CoA reductase	100 ± 6[d]	112 ± 9	91 ± 8	105 ± 9
Cholesterol-7α-hydroxylase	100 ± 6[e]	76 ± 7*	85 ± 6	92 ± 7
Acyl CoA cholesterol acyl transferase	100 ± 5[f]	32 ± 5*	46 ± 4*	59 ± 6*
Acetyl CoA carboxylase	100 ± 6[g]	103 ± 6*	29 ± 3*	43 ± 4*
Fatty acid synthetase	100 ± 8[h]	91 ± 2	81 ± 9	81 ± 9
sn-Glycerol-3-phosphate acyl transferase	100 ± 6[i]	45 ± 5*	48 ± 6*	57 ± 5*
Phosphatidylate phosphohydrolase	100 ± 6[j]	82 ± 6	81 ± 7	78 ± 5
Hepatic lipoprotein lipase	100 ± 7[k]	104 ± 6	107 ± 5	103 ± 6

[a] 30.8% exchange of mitochondrial citrate.
[b] 30.5 mg citrate hydrolyzed/g wet tissue/20 min.
[c] 28.5 mg acetyl CoA formed/g wet tissue/20 min.
[d] 384,900 dpm cholesterol formed/g wet tissue/60 min.
[e] 224,000 dpm/μg microsomal protein.
[f] 4808 dpm/mg microsomal protein/20 min.
[g] 32,010 dpm/g wet tissue/30 min.
[h] 37,656 dpm/g wet tissue/20 min.
[i] 537,800 dpm/g wet tissue/20 min.
[j] 16.7 μP_i/g wet tissue/15 min.
[k] 278,583 dpm/g wet tissue/hr.
* $P \leq 0.001$.

Table V. The *in Vivo* Effects of 2,3-Dihydrophthalazine-1,4-dione on CF_1 Mouse Liver Enzymes on Day 16 After Administration, 20 mg/kg/day[a]

Enzyme activity (N = 6)	Percentage control ($\bar{X}$ ± SD)				
	10 mg/kg	20 mg/kg	40 mg/kg	60 mg/kg	1% CMC
Acetyl CoA synthetase	98 ± 6	70 ± 7*	92 ± 6	81 ± 7	100 ± 7
ATP-dependent citrate lyase	48 ± 6*	40 ± 3*	27 ± 4*	37 ± 4*	100 ± 7
HMG CoA reductase	103 ± 8	128 ± 7	133 ± 7	104 ± 6	100 ± 6
Acetyl CoA carboxylase	94 ± 9	93 ± 4	90 ± 5	85 ± 6	100 ± 6
Fatty acid synthetase	72 ± 6*	78 ± 6*	99 ± 4	102 ± 7	100 ± 8
sn-Glycerol-3P acyl transferase	65 ± 3*	61 ± 7*	40 ± 2*	25 ± 5*	100 ± 6
Phosphatidylate phosphohydrolase	57 ± 7*	31 ± 5*	38 ± 3*	36 ± 4*	100 ± 6

[a] See Table II for control values.
* $P \leq 0.001$.

(Table VI). No reduction in the small intestine lipids was observed. In fact the triglyceride and neutral lipid content was elevated ~14–15%. The drug did not cause an increase in lipid excretion by the fecal route. The bile cannulation study demonstrated that drug treatment resulted in an 8% reduction of cholesterol, a 9% reduction of triglyceride, and a 31% reduction of phospholipids in the bile secretions.

The cholesterol content was reduced in the very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) fractions but not in the high-density lipoprotein (HDL) fraction. Triglycerides and neutral lipids were lowered by ~15% in the chylomicrons; however, these lipids were not lower in the VLDL fraction after drug treatment. The cholesterol distribution study showed that in all of the major organs, there was a reduction of the ^3H content, 24 hr after the administration of ^3H-cholesterol orally (Table VII). The plasma level of ^3H-cholesterol was reduced from 31,352 to 23,108 dpm/ml. The stomach of the treated rats had two times the concentration of ^3H-cholesterol as the stomach of the control animals. The small intestine tissue as well as the

Table VI. The Effects of 2,3-Dihydrophthalazine-1,4-dione on Rat Liver, Small Intestine, and Fecal Lipid Levels and Serum Lipoprotein Fractions in Rats Treated Orally at 10 mg/kg/day for 2 Weeks

(N = 6)	Percentage control ($\bar{X}$ ± SD)					
	mg lipid wt	Cholesterol	Triglyceride	Neutral lipids	Phospholipids	Protein
Tissue						
Liver						
Control	100 ± 7	100 ± 8[a]	100 ± 8[b]	100 ± 9[c]	100 ± 8[d]	100 ± 6[e]
Treated	89 ± 9	82 ± 8	98 ± 9	86 ± 6	75 ± 7	97 ± 8
Small intestine						
Control	100 ± 5	100 ± 6[f]	100 ± 7[g]	100 ± 6[h]	100 ± 8[i]	100 ± 8[j]
Treated	82 ± 9	106 ± 7	115 ± 10	114 ± 9	103 ± 8	103 ± 7
Feces						
Control	100 ± 5	100 ± 7[k]	100 ± 6[l]	100 ± 8[m]	100 ± 7[n]	100 ± 6[o]
Treated	108 ± 8	102 ± 7	84 ± 6	100 ± 10	93 ± 9	109 ± 8
Lipoprotein fraction						
Chylomicrons						
Control		100 ± 5[p]	100 ± 6[q]	100 ± 7[r]	100 ± 6[s]	100 ± 5[t]
Treated		82 ± 6	85 ± 7	84 ± 8	92 ± 7	102 ± 6
VLDL						
Control		100 ± 6[u]	100 ± 6[v]	100 ± 7[w]	100 ± 7[x]	100 ± 4[y]
Treated		30 ± 5*	134 ± 10	81 ± 3	91 ± 6	103 ± 7
LDL						
Control		100 ± 5[z]	100 ± 6[aa]	100 ± 7[bb]	100 ± 6[cc]	100 ± 5[dd]
Treated		35 ± 6*	94 ± 7	88 ± 6	125 ± 7	92 ± 8
HDL						
Control		100 ± 4[ee]	100 ± 5[ff]	100 ± 6[gg]	100 ± 6[hh]	100 ± 7[ii]
Treated		107 ± 3	42 ± 4*	86 ± 3	42 ± 6*	93 ± 7

[a] 24.03 mg cholesterol/g tissue. [b]44.11 mg neutral lipid/g tissue. [c]6.37 mg triglyceride/g tissue. [d]7.19 mg phospholipid (P)/g tissue. [e]4.5 mg protein/g wet tissue. [f]7.82 mg/g. [g]6.98 mg/g. [h]1.12 mg/g. [i]2.06 mg/g. [j]42 mg/g. [k]28.47 mg/g. [l]33.94 mg/g. [m]1.86 mg/g. [n]1.239 kg/g. [o]6.99 mg/g. [p]337 μg/ml. [q]67 μg/ml. [r]420 μg/ml. [s]149 μg/ml. [t]184 μg/ml. [u]190 μg/ml. [v]98 μg/ml. [w]22 μg/ml. [x]26 μg/ml. [y]50 μg/ml. [z]210 μg/ml. [aa]10 μg/ml. [bb]45 μg/ml. [cc]41 μg/ml. [dd]122 μg/ml. [ee]544 μg/ml. [ff]620 μg/ml. [gg]27 μg/ml. [hh]153 μg/ml. [ii]657 μg/ml. *$P \leq 0.001$.

Table VII. The Effects of 2,3-Dihydrophthalazine-1,4-dione on Organ Weights and ^{3}H-Cholesterol Distribution of Rats Treated for 14 days at 10 mg/kg/day

	Organ wt (g)		Control		Treated	
(N = 6)	Control	Treated	dpm total organ	% recovery	dpm total organ	% recovery
Brain	1.87	1.70	16,430.4	0.347	7,379.2	0.152
Heart	1.06	1.37	25,516.8	0.539	28,157.6	0.580
Lung	1.53	1.65	148,046.4	3.128	147,867.3	3.046
Liver	10.16	14.10	1,569,395.8	33.161	1,323,942.5	27.271
Spleen	0.81	1.10	134,740.8	2.847	167,149.5	3.443
Kidney	1.94	2.45	65,020.3	1.374	49,372.9	1.017
Stomach	2.10	2.32	6,662.3	0.141	118,844.6	2.448
Small intestine	8.09	9.42	2,033,713.2	42.972	1,972,490.3	40.630
Large intestine	3.23	4.43	167,240.6	3.534	492,321.5	10.141
Chyme	8.57	8.77	425,615.1	8.993	420,422.5	8.660
Feces	4.88	8.30	108,850.5	2.300	113,310.2	2.334
Plasma	—	—	31,382.8	0.663	23,108.6	0.476

chyme and feces had no increase in ^{3}H-cholesterol after drug administration. The LD_{50} of the compound was 700 mg/kg/day ip in CF_1 mice.

DISCUSSION

2,3-Dihydrophthalazine-1,4-dione proved to be an effective hypolipidemic agent in two species of rodents, as well as being active by two routes of administration, i.e., orally and intraperitoneally. In hyperlipidemic induced mice, 2,3-dihydrophthalazine-1,4-dione demonstrated similar percentages of reduction of elevated serum cholesterol and triglyceride levels compared to normal rodents.

This derivative was one of the few compounds that reduced serum triglycerides in mice as potently as previously reported for phthalimide (by 56%) (1). Key regulatory enzymes of lipid synthesis were inhibited by the 2,3-dihydrophthalazine-1,4-dione. By inhibition of mitochondrial citrate exchange and ATP-dependent citrate lyase and acetyl CoA synthetase activities, the available acetyl CoA in the cytoplasm is reduced. Acetyl CoA is needed for fatty acid, cholesterol, and triglyceride *de novo* synthesis. Suppression of the activities of regulatory enzymes of triglyceride synthesis, i.e., *sn*-glycerol-3-phosphate acyl transferase and phosphatidate phosphohydrolase, positively correlates with reduction of serum triglyceride levels. Similar results have been observed with cyclic imides (1–5) as well as other hypolipidemic agents (26).

The lipids that were removed from the plasma compartments were not deposited in the organs. This was shown by the lipid analysis of the liver as well as the cholesterol distribution study of the major organs. A high lipid content was not observed in the feces after treatment with 2,3-dihydrophthalazine-1,4-dione. This is different from the results with phthalimide, where a higher content of cholesterol was observed in the feces. The rat lipoprotein lipid content after drug treatment was encouraging, since the cholesterol content was reduced in the chylomicron, VLDL, and LDL fractions but not in the HDL fraction. Triglyceride and neutral lipid levels were marginally reduced in the chylomicron, LDL, and HDL fractions. Reduction of the cholesterol content of the VLDL and LDL fractions with elevation of the HDL fractions has been reported in humans to protect against cardiovascular problems related to atherosclerosis (37).

The structure–activity relationship studies demonstrated that substituted derivatives of 2,3-dihydrophthalazine-1,4-dione as well as select *N,N*-diaminophthalamides were equally as active as hypolipidemic agents as the parent drug. The fact that the cyclic imide ring can be modified to a six-membered ring containing two nitrogen atoms offers another class of agents to investigate for potential hypolipidemic activity.

ACKNOWLEDGMENTS

The authors thank Patricia Ann Day for her technical assistance. Research support for this project was from National Institutes of Health Grant HL 25680.

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