3β-Hydroxy-5,16-pregnadien-20-one exhibits both hypolipidemic and hypoglycemic activities

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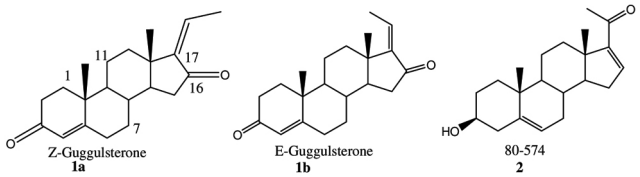
The increasing incidence of disorders due to metabolic syndrome, which include hyperlipidemias, diabetes and hypertension, has taken serious dimension world over, including in India, and has become a matter of deep concern. Drugs which can inhibit more than one disorder of this syndrome would add much therapeutic value to its management. 3β-Hydroxy-5,16-pregnadien-20-one (80-574, 2), selected based on the structural lead of the presence of a β-enone pharmacophore in/around ring D of *Guggulsterone* (1), the main active constituent of the phytopharmaceutical *Gugulip* developed at CDRI, Lucknow from the Ayurvedic herbal drug *Gum Guggul*, has been found to exhibit both hypolipidemic and hypoglycemic activities in the conventional experimental animal models used for studying these activities. In mode of hypolipidemic action studies in high fat diet-fed rats, compound 80-574 was found to enhance lipolysis of serum lipoproteins, enhance the uptake of LDL by hepatocyte membranes, indicating increased catabolism of LDL, and caused increased faecal excretion of bile acids indicating enhanced mobilization of cholesterol from fatty deposits. Compound 80-574 also exhibited antioxidant activity in *in vitro* and *in vivo* experimental models, and protected LDL against oxidation, indicating protection against atherogenesis. Compound 80-574 also exhibited moderate inhibition of cholesterol biosynthesis in liver slices. Treatment with 80-574 also improves glucose tolerance in diabetic rats. With these multiple sites of hypolipidemic action accompanied with its hypoglycemic activity, compound 80-574 appears a promising candidate for the treatment of dyslipidemias and diabetes and thus for management of metabolic syndrome. A synoptic view of the various functional activities exhibited by compound 80-574 is presented in this article.

Keywords: Diabetes, lipoproteins, lipid peroxides, triglycerides.

THERE is a growing realization of the multi-factorial causation of many of the non-communicable diseases, especially those causing metabolic dysfunction, which is focusing attention on discovering and developing drugs acting on multiple targets, an important shift from single-drug single-target paradigm[1]. *Guggulsterones* Z and E (GSs, **1a** and **1b**, Scheme 1), the main active constituents of *Gugulip*, a hypolipidemic drug developed at CDRI, Lucknow from the Ayurvedic drug *Gum Guggul*[2,3], are novel prototypes for hypolipidemic activity. In a study of the molecular mechanism of hypolipidemic action, Wu *et al.*[4] reported that *Guggulsterones* Z and E are antagonists of nuclear bile acid receptors (BAR), also called farnesoid X receptors (FXR), which would increase excretion of bile acids and cause increased mobilization of cholesterol from its deposits and metabolism to bile acids, indicating *Guggulsterones* to have a novel mode of hypolipidemic action. But GSs in addition to inhibition of FXR, also antagonized pregnane X receptors (PXR)/ steroid X receptors (SXR), which would increase cytochrome P-450 3A (CYP 3A) enzymes and thus could increase the propensity of interaction of GSs with other commonly used drugs. In order to overcome this limitation of this new class of hypolipidemic agents, structural analogues of GSs with β-enone pharmacophore were designed/selected and screened, of which 3β-hydroxy-5,16-pregnadien-20-one (80-574) was found to exhibit both hypolipidemic and hypoglycemic activities in most of the commonly used experimental animal models for testing these activities. In mechanism of action studies, compound 80-574 was found to result in increased faecal excretion of bile acids, increased uptake of LDL by hepatocytes, strong antioxidant activity in cell-based assays, inhibited the non-enzymatic generation of hydroxyl free radicals ($OH^{\bullet}$), and thus aided in preventing atherosclerotic activity.



Scheme 1. Hypolipidemic pregnanes.

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Wu *et al.*[4] found that compound 80-574 also antagonized BAR/FXR nuclear receptors. However, unlike *Guggulsterones*, its action was selective for bile acid receptor, and it had no action on PXR or SXR, which would provide safety against disturbance of Cyp enzymes cascade[4]. Thus compound 80-574 with both hypolipidemic and hypoglycemic actions with selective action on FXR nuclear receptors appears a better therapeutic candidate for control of metabolic syndrome over *Guggulsterones*, as described in this article.

Experimental results

Materials

Compound 80-574 (**2**) was prepared by basic hydrolysis of 16-dehydro-pregneloneacetate (16-DPA), obtained from diosgenin. 16-DPA (1.5 kg) was added to a mixture of KOH (375 g) in 500 ml of demineralized (DM) water and *iso*-propanol (~12 l) in a glass-lined MS reactor and refluxed for ~3 h (till TLC ensures completion of the reaction), the solvent removed by distillation, DM water (~10 l) added, pH adjusted to neutrality by acetic acid, the product filtered through centrifugation and washed thoroughly with DM water. The product was poured into the reactor with ethyl acetate (~12 l), 10% activated charcoal (60 g) added under stirring and refluxed for 2 h. After cooling, the slurry was filtered to remove charcoal. The process was repeated again. The filtered solution was concentrated in a rotatory evaporator at water bath temperature ~45°C till crystals start appearing. The crystallized product was collected and washed with cold ethyl acetate and dried. Yield 600 g; m.p. 210–112°C; ^{1}H NMR ($CDCl_3$): δ 6.8 (s, 1H, H-16), 5.35 (bs, 1H, H-6), 3.5 (m, 1H, H-3), 2.26 (s, 3H, CH_3CO), 1.0 (s, 3H, CH_3), 0.9 (s, 3H, CH_3).

Hypolipidemic activity of compound 80-574

Lipid lowering activity in triton-treated rats: The primary hypolipidemic activity of 80-574 was tested in triton-induced acute hyperlipidemia rat model[5,6], using gemfibrozil as the reference compound. Adult Charles Foster male rats (200–225 g) were divided into triton, triton plus compound 80-574 and triton plus gemfibrozil-treated groups with six animals in each group. Triton WR 1339 (Sigma Chemical Co, USA) was administered (400 mg/kg b.w.) i.p., while compound 80-574 or gemfibrozil macerated with 2% aqueous gum acacia suspension was fed orally (100 mg/kg) to rats fasted overnight. The next day (after ~18 h) animals were inoculated with 1 ml/kg b.w. heparin (10 mg/ml in normal saline) and after 15 min their blood was withdrawn from the retro-orbital plexus, plasma-separated and estimated for total cholesterol (TC)[7], phospholipids (PL)[8], triglycerides (TG)[9] and post-heparin lipolytic activity (PHLA)[10] following standard photometric methods.

The results provided in Table 1 show that treatment with compound 80-574 as with gemfibrozil caused a significant reversal of triton-induced rise in plasma level of TC (38 and 38%), PL (22 and 26%) and TG (26 and 26%) respectively. The effect of compound 80-574 and gemfibrozil on lipid lowering was also observed through reactivation of PHLA (35 and 45%). Thus there is a significant correlation between the ability of tissues to incorporate free fatty acids by hydrolysis of lipoproteins, triacylglycerol and the activity of enzyme lipoprotein lipase. Data in Table 1 suggest the reactivation of PHLA by treatment with compound 80-574, as with gemfibrozil.

Lipid lowering activity in normal rats: In order to assess the optimal effect of compound 80-574 on serum lipid profile, Charles Foster rats (200–225 g) were fed with compound 80-574 or gemfibrozil suspended in 2% gum acacia at a dose of 50 mg/kg p.o., once daily for 30 days; the results are given in Table 2.

Blood was withdrawn after 10, 20 and 30 days, serum separated and lipids estimated as described earlier[7–9]. It was found that lipid lowering effect of compound 80-574 was comparable to that of gemfibrozil (Table 2).

Lipid lowering activity in normal Rhesus monkeys: In a set of experimental *Rhesus* monkeys (3.9–5.4 kg, four in each group), compound 80-574 in gum acacia was orally administered daily for 90 days in doses of 62.5, 125 and 250 mg/kg. At the end of the experiment, animals were fasted overnight, their blood was withdrawn through vein puncture and serum separated. Serum TC and TG were estimated according to the described methods[7,9]. A portion of the serum was fractionated into β-lipoproteins, very low and low-density lipoproteins (VLDL and LDL) and high density lipoprotein (HDL) by poly-anionic precipitation methods of Burstein and Legman[11]. Total cholesterol content in HDL was estimated[5], and the levels of TC in VLDL and LDL calculated according to the Friedwald formula[12].

Table 3 shows that feeding with compound 80-574 caused significant dose-related decrease in serum total cholesterol, triglycerides, VLDL-cholesterol with a more marked lowering of LDL-cholesterol up to 125 mg/kg, beyond which there was no further significant effect; it appeared that maximum effect had been reached. The changes in HDL-cholesterol were not significant.

Hypolipidemic activity of compound 80-574 in high-fat diet (HFD)-fed animal models

Lipid lowering activity in HFD-fed rabbits: Hypercholesterolemia was induced in male albino rabbits (1.5–2.0 kg) kept on a stock diet by feeding cholesterol

Table 1. Effect of compound 80-574 on plasma lipids and PHLA in triton-induced hyperlipidemia in rats

Experimental schedule	Total cholesterol[a]	Phospholipids[a]	Triglycerides[a]	PHLA[b]
Control	85.6 ± 3.8	68.9 ± 2.0	82.3 ± 4.7	17.77 ± 1.08
Triton only	329.9 ± 6.1** (× 3.85F)	133.6 ± 6.3** (× 1.93F)	127.0 ± 11.0** (× 1.54F)	10.44 ± 0.06** (–41)
Triton + 80-574 (100 mg/kg)	203.2 ± 16.8** (–38)	104.0 ± 9.7* (–22)	93.4 ± 6.4** (–26)	14.14 ± 1.20** (+35)
Triton + Gemfibrozil (100 mg/kg)	203.0 ± 4.5** (–38)	98.5 ± 6.2** (–26)	94.0 ± 4.4** (–26)	15.13 ± 1.33** (+45)

Units: [a]mg/dl; [b]μmol FFA released/h/ml. Values are mean ± SD of six rats. *$P < 0.05$; **$P < 0.001$. Values in parentheses show % change from triton only.

Table 2. Hypolipidemic effect on 30 days administration of compound 80-574 in normal rats

Experimental schedule	Total cholesterol[a]	Phospholipids[a]	Triglycerides[a]
Control	86.66 ± 5.10	74.70 ± 2.47	95.01 ± 3.47
80–574 (50 mg/kg) 10 days	73.48 ± 2.89* (–15)	58.94 ± 3.72** (–21)	81.02 ± 4.70** (–14)
20 days	72.44 ± 2.87** (–17)	56.47 ± 2.37*** (–24)	75.28 ± 4.86** (–20)
30 days	58.99 ± 5.42*** (–32)	51.24 ± 1.32*** (–31)	60.97 ± 2.92*** (–36)
Gemfibrozil (50 mg/kg) 10 days	74.12 ± 3.35* (–14)	59.61 ± 2.19** (–20)	78.35 ± 3.72** (–18)
20 days	71.23 ± 4.54** (–18)	57.43 ± 3.0*** (–24)	72.48 ± 1.46** (–23)
30 days	57.25 ± 4.13*** (–34)	53.16 ± 2.13*** (–29)	64.08 ± 2.53*** (–32)

Units: [a]mg/dl. Values are mean ± SD of six rats. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to control. Values in parentheses show % decrease.

Table 3. Hypolipidemic effects of compound 80-574 on serum lipoprotein cholesterol in *Rhesus* monkeys

		80-574 administered		
Serum parameters	Control	62.5 mg/kg	125 mg/kg	250 mg/kg
Cholesterol	110.5 ± 9.0	83.3 ± 8.5 (–25)***	65.8 ± 2.2 (–40)***	63.30 ± 2.3 (–43)***
Triglycerides	80.0 ± 1.0	66.3 ± 6.9 (–17)*	60.0 ± 2.9 (–25)*	58.5 ± 3.8 (–27)***
VLDL-Cholesterol[a]	16.0 ± 0.2	13.2 ± 1.38 (–17)*	12.0 ± 0.58 (–25)***	11.70 ± 7.6 (–27)***
LDL-Cholesterol[a]	55.0 ± 8.8	24.3 ± 8.0 (–56)***	14.6 ± 0.9 (–73)***	13.1 ± 0.7 (–76)***
HDL-Cholesterol[a]	43.0 ± 0.5	45.0 ± 2.5	50.8 ± 0.5 (+15)[NS]	41.8 ± 0.9 (–3)*[NS]

Units: [a]mg/dl. Values are mean ± SD from four animals. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, Not significant. Values in parentheses show % change.

(0.5 g/kg, b.w.) dissolved in groundnut oil. The animals were divided into four groups of four rabbits each: (i) control group on stock diet; (ii) cholesterol-fed animals (0.5 g cholesterol/kg); (iii) cholesterol plus compound 80-574 (50 mg/kg)-fed animals; and (iv) cholesterol plus compound 80-574 (100 mg/kg)-fed animals. Treatment with compound 80-574 (at the above doses orally, once daily) along with cholesterol feeding was continued for 90 days.

Blood was withdrawn at 30, 60 and 90 days intervals and serum lipids estimated (Table 4). There was rise in the serum level of TC and TG in the cholesterol-fed animals related to the days of feeding with cholesterol. The level of serum TC of compound 80-574-treated animals showed time-related decrease at 30, 60 and 90 days, though the difference in lowering was not marked between doses of 50 and 100 mg/kg. TG increased significantly only after 60 days of cholesterol feeding, which also showed a dose and time-related decrease.

Effect of compound 80-574 on serum lipids and lipoproteins in HFD-fed rats: Dietary fat after digestion is absorbed through the gastrointestinal tract in the form of circulating chylomicrons and VLDL, part of which is metabolized to provide energy and the rest enters the liver and lymph for circulation and becomes available to adipose tissues. Various enzymes and carrier lipoproteins are involved in this process and therefore, it was considered of interest to study the effect of compound 80-574 on these parameters in the serum and some important tissues of HFD-fed rats. HFD was prepared by mixing cholesterol and coconut oil with normal chow diet in the proportion of 1 : 29 and 70% w/w and pelleted; this was fed to adult male Charles Foster rats (200–225 g) for 36 days. The animals were divided into three groups of six animals each. Group I served as control, while animals of groups II and III administered compound 80-574 as suspension in 2% gum acacia at doses of 50 and 100 mg/kg respectively, orally along with HFD once a day for

Table 4. Lipid lowering effect of compound 80-574 in hyperlipidemic male albino rabbits

Treatment	Cholesterol[a]			Triglycerides[a]		
Days	30	60	90	30	60	90
Cholesterol (0.5 g/kg)	269.66 ± 94.1	506.30 ± 92.83***	1337.5 ± 268***	69.0 ± 6.9	70.75 ± 14.7^{NS}	93.12 ± 7.81***
Cholesterol (0.5 g/kg) + 80-574 (50 mg/kg)	228 ± 73.81* (–15)	305.5 ± 54.61*** (–40)	672.75 ± 84.18*** (–50)	69.50 ± 8.58	67.00 ± 2.44^{NS} (–5)	56.5 ± 3.69*** (–39)
Cholesterol (0.5 g/kg) + 80-574 (100 mg/kg)	190.5 ± 47.1* (–29)	309.25 ± 110.0*** (–39)	633.65 ± 346.0*** (–53)	71.0 ± 18^{NS}	54.00 ± 11.4** (–24)	44.15 ± 2.74*** (–53)

Units: [a]mg/dl. Values are mean ± SD of four rabbits.*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. NS, Not significant. Cholesterol + 80-574 treated groups were compared with cholesterol-fed group. Values in parentheses show % decrease.

Table 5. Effect of compound 80-574 on lipids of HFD-fed rats

Serum parameters	Control	HFD	HFD + 80-574 (50 mg/kg)	HFD + 80-574 (100 mg/kg)
Triglycerides[a]	48.75 ± 5.1	137.5 ± 8.5*** (+2.82F)	61.5 ± 10.5*** (–55)	53.0 ± 7.5*** (–61)
Total cholesterol[a]	73.2 ± 5.8	293.2 ± 16.6*** (× 4.0F)	165.0 ± 26.7*** (–44)	106.4 ± 4.6**** (–64)
VLDL-cholesterol[a]	9.75 ± 1.2	27.20 ± 1.7*** (× 2.28F)	12.3 ± 2.1*** (–55)	10.60 ± 1.5*** (–61)
LDL-cholesterol[a]	27.5 ± 6.3	189.25 ± 18.0*** (× 6.8F)	123.87 ± 14.00*** (–35)	48.64 ± 5.1*** (–74)
HDL-cholesterol[a]	37.25 ± 5.0	39.75 ± 2.8*** (× 1.06F)	37.57 ± 2.8^{NS} (–5)	47.20 ± 3.7* (+19)

HFD, High fat diet; units: [a]mg/dl. Values are mean ± SD of six rats. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, Not significant cholesterol-fed group was compared with control and HFD plus drug-treated group. *F*, Fold. Values in parentheses below drug-treated group show % reversal compared with HFD-fed group.

another 30 days. Blood was withdrawn from fasted animals and serum separated. A portion of the serum was fractioned into VLDL, LDL and HDL by the method of Burstein and Legman[11]. TC in serum lipoproteins and serum TG were measured spectrophotometrically[7,9]. The TC content in VLDL and LDL was deduced using the Friedwald formula[12].

Table 5 indicates that the animals fed with HFD for 36 days showed marked increase in their serum level of TG, TC, VLDL-TC and LDL-TC by 2.82, 4.00, 2.28 and 6.88-fold respectively. Treatment with compound 80-574 at the doses of 50 and 100 mg/kg caused a significant dose-related lipid lowering. At 100 mg/kg dose, serum levels of TG, TC, VLDL-TC, LDL-TC were lowered by 61%, 64%, 61% and 74% respectively, in the above animals. Some beneficial effect on HDL-TC was also observed at 100 mg/kg dose.

Effect of long-term (60 days) administration of compound 80-574 on serum lipids and lipoproteins of HFD-fed rats: It was considered of interest to study the effect of the treatment with compound 80-574 on the status of serum and tissue lipids and lipoproteins of HFD-fed animals. For this, another set of rats was administered compound 80-574 simultaneously with cholesterol for 60 days, using gemfibrozil as reference standard. At the end of the experiment, blood was withdrawn from retro-orbital plexus for biochemical analysis. The animals were sacrificed and their liver, adipose tissue and heart excised promptly, rinsed with cold KCl and kept at –70°C till analysis. Faeces was collected the duration of the experiment (60 days) from all groups of animals and analysed for bile acids content.

Plasma lipoproteins were fractionated by poly-anionic precipitation method of Burstein and Legman[11]; plasma was incubated in the presence of heparin (0.0125% w/v) and $MnCl_2$ (12% w/v) at 40°C for 60 min. The resulting precipitate of VLDL complex was separated by centrifugation.

From VLDL free supernatant, LDL was precipitated by adding dextran sulphate (0.001% w/v) and $MnCl_2$ (20% w/v) and LDL complex recovered by centrifugation. The supernatant obtained after isolation of VLDL and LDL was further precipitated for HDL by adding dextran sulphate (0.65% w/v) and $MnCl_2$ (20% w/v). These lipoprotein solutions were dialysed against 0.1 M NaCl before analysis. The levels of TC, PL and TG in the lipoproteins were estimated as mentioned earlier[7–9]. Total proteins in serum and apolipoprotein content in lipoproteins were estimated spectrophotometrically by the methods of Lowry *et al.*[13], and Radding and Steinberg[14] respectively. Plasma LCAT activity was measured by the method of Nagasaki and Akanuna[15]. Lipoprotein lipase activity in tissue homogenate was assayed by the method of Wing and Robins[10]. Tissues were delipidated with a mixture of chloroform : methanol (2 : 1 v/v) and the resulting lipid extracts were used for estimation of TC, PL and TG as above.

The results showed that feeding rats with cholesterol produced marked hyperlipidemia with increased levels of plasma TC, PL and TG (Table 6), which was also reflected in the levels of plasma lipoproteins causing

Table 6. Effect of 60 days administration of compound 80-574 on serum lipoproteins and lipid profile of HFD-fed rats

Serum parameters		Control	HFD-fed	HFD and drugs-fed	
				80-574 (100 mg/kg)	Gemfibrozil (100 mg/kg)
Serum	TC[a]	86.49 ± 3.12	197.46 ± 7.89*** (× 2.28F)	117.34 ± 6.21*** (–41)	128.92 ± 6.21*** (–35)
	PL[a]	79.48 ± 2.64	160.37 ± 9.97*** (× 2.02F)	104.92 ± 4.81*** (–35)	102.92 ± 5.34*** (–36)
	TG[a]	93.41 ± 5.12	142.46 ± 4.92*** (+53)	117.64 ± 7.37** (–17)	100.18 ± 3.33*** (–30)
	Protein[b]	5.83 ± 0.24	7.21 ± 0.50** (+24)	6.18 ± 0.31* (–14)	6.08 ± 0.37** (–16)
VLDL	TC[a]	8.32 ± 0.41	32.43 ± 2.12***(× 3.9F)	21.09 ± 1.64*** (–35)	21.00 ± 1.80*** (–34)
	PL[a]	12.87 ± 0.31	30.18 ± 1.24*** (× 2.34F)	20.14 ± 1.66*** (–33)	20.00 ± 0.31*** (–34)
	TG[a]	40.69 ± 4.0	82.77 ± 5.12*** (× 2.03F)	38.69 ± 2.44*** (–53)	31.94 ± 3.00*** (–61)
	Apo VLDL[a]	6.30 ± 0.50	12.12 ± 1.80*** (+92)	8.73 ± 0.21*** (–28)	7.75 ± 0.52*** (–36)
LDL	TC[a]	13.23 ± 0.88	64.16 ± 5.72*** (× 4.85F)	34.91 ± 2.61*** (–46)	44.30 ± 2.88*** (–31)
	PL[a]	12.14 ± 0.47	43.36 ± 3.36*** (× 3.57F)	24.12 ± 1.64*** (–44)	29.73 ± 1.64*** (–31)
	TG[a]	8.12 ± 0.17	36.12 ± 2.68*** (× 4.51F)	20.12 ± 1.60*** (–45)	25.00 ± 2.00*** (–32)
	Apo LD[a]	17.56 ± 1.00	28.62 ± 1.88*** (+63)	18.33 ± 1.14*** (–36)	16.30 ± 1.08*** (–43)
HDL	TC[a]	45.38 ± 3.71	38.14 ± 2.81* (–16)	44.22 ± 2.64* (+16)	44.19 ± 3.64* (+16)
	PL[a]	32.41 ± 2.61	28.81 ± 2.14* (–11)	32.25 ± 3.00* (+12)	33.00 ± 2.21* (+14)
	TG[a]	15.14 ± 1.10	13.13 ± 0.84* (–13)	14.96 ± 0.92* (+14)	15.00 ± 0.82* (+14)
	Apo HDL[a]	168.20 ± 13.50	120.35 ± 14.41*** (–28)	138.23 ± 10.21* (+15)	148.24 ± 10.00** (+23)
Plasma LCAT activity[c]		62.30 ± 3.24	40.23 ± 2.81*** (–35)	50.31 ± 4.12*** (+25)	51.23 ± 3.21** (+27)
Plasma PHLA		15.96 ± 0.86	11.23 ± 0.23*** (–30)	13.02 ± 0.41* (+16)	14.01 ± 0.62*** (+25)

Units: [a]mg/dL, [b]g/dl, [c]μmol FFA/h/l. Values are mean ± SD of six rats. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. HFD-fed group was compared with control and HFD + drug-treated group. F, Fold. Values in parentheses below drug-treated groups are either % reversal compared with HFD-fed group.

hyper-β-lipoproteinemia in VLDL and LDL. These alterations in lipid metabolism are a result of the inhibition of LCAT enzyme and decreased levels of lipids and *apo*-lipoprotein component of HDL, indicating the dysfunction of this lipoprotein in the above situation[16]. Treatment with compound 80-574 and gemfibrozil restored lipid and protein components of HDL and stimulated the plasma LCAT enzyme which is a key enzyme involved with the synthesis and lipid regulatory role of HDL. With recovery of this vital lipoprotein, the hyper-β-lipoproteinemia regressed significantly as the lipid and protein components of VLDL and LDL were significantly reversed towards their normal levels. The lipid lowering activity of compound 80-574 was again comparable to that of gemfibrozil (Table 6).

Effect on tissue lipids and lipoproteins of HFD fed rats: (i) Effect on hepatic lipids: It was observed that feeding with HFD caused marked accumulation of TC, PL and TG by 67%, 42% and 95% respectively, accompanied with a decrease of total lipolytic activity (LPL) by 56% in liver of hyperlipidemic rats (Table 7). Treatment with compound 80-574 decreased the levels of TC, PL and TG by 27%, 28% and 25% respectively, accompanied by stimulation of LPL by 46%, similar to the action of gemfibrozil. In HFD-fed animals due to an excess of exogenous fat load on the body, various lipases are suppressed because of a higher level of free fatty acids (FFA) in animals and a significant reduction of PHLA. However, this was reactivated by treatment with compound 80-574 or gemfibrozil (Tables 7 and 8).

Apart from the role of compound 80-574 and gemfibrozil in the reactivation of lipolytic enzymes resulting in lipid lowering in hyperlipidemic animals, some other mechanisms such as inhibition of cholesterol biosynthesis and a faster catabolism of LDL-cholesterol may also contribute to regulation of cholesterol homeostasis in hyperlipidemic animals. Therefore, the effect of compound 80-574 on hepatic cholesterol biosynthesis and receptor-mediated catabolism of human LDL was also studied in rats.

(ii) Hepatic cholesterol biosynthesis inhibition: The hepatic rate of cholesterol biosynthesis was investigated using [1-^{14}C] sodium acetate according to the method of Nityanand and Kapoor[17]. Briefly, rat liver homogenate (0.2 ml) containing appropriate amounts of reactants; nicotinamide, NAD, EDTA, $MgCl_2$ and [1-^{14}C] sodium acetate in 0.2 ml volumes of each added in 0.1 M PO_4^- buffer, pH 7.4, to a final volume of 3.00 ml. The reaction mixture was incubated at 37°C for 90 min in a metabolic shaker and then centrifuged. The resulting sediment was hydrolysed with 5 ml aqueous KOH at 37°C for 4 h and then extracted with petroleum ether and dried. This unsaponifiable fraction containing sterols, dissolved in a mixture of ethanol and acetone, added with ethanolic

Table 7. Effect of compound 80-574 on hepatic lipids in HFD-fed rats

Experimental test		Control group	HFD-fed	HFD + 80-574 (100 mg/kg)	HFD + gemfibrozil (100 mg/kg)
Liver	TC[a]	6.00 ± 0.90	10.02 ± 0.6*** (+67)	7.32 ± 0.23*** (–27)	7.02 ± 0.14*** (–30)
	PL[a]	20.98 ± 1.50	29.79 ± 2.0*** (+42)	21.41 ± 1.2*** (–28)	19.90 ± 1.0*** (–33)
	TG[a]	9.90 ± 0.24	19.36 ± 1.07*** (+95)	14.5 ± 0.71* (–25)	14.22 ± 0.9*** (–27)
	Total protein[a]	110.0 ± 8.30	170.0 ± 4.0*** (+54)	142.8 ± 8.2* (–16)	138.00 ± 9.2** (–19)
	Lipolytic activity[b]	125.70 ± 9.37	55.05 ± 3.72*** (–56)	80.23 ± 12.00*** (+46)	83.95 ± 5.02*** (+52)
Hepatic cholesterol biosynthesis[c]		3270 ± 140.12	1400 ± 88.21*** (–57)	1100 ± 52.92** (–21)	1061 ± 42.27*** (–24)
Receptor mediated catabolism of liver LDL[c]		19200 ± 870	5700 ± 300*** (–70)	18,100 ± 618*** (+216)	16,000 ± 780*** (+181)

Units: [a]mg/g; [b]µmol FFA/h/mg protein; [c]Counts/mg protein. Values are mean ± SD of six rats. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Hyperlipidemic animals were compared with control and drug-treated animals. Values in parentheses below drug-treated groups show % change compared to HFD-fed group.

Table 8. Effect of compound 80-574 on lipid turnover in adipose and heart tissues of HFD-fed rats

Experimental schedule		Control	HFD	HFD + 80-574 (100 mg/kg)	HFD + gemfibrozil (100 mg/kg)
Adipose tissues	TC[a]	2.46 ± 0.25	4.24 ± 0.75*** (+72)	3.5 ± 0.12** (–17)	3.15 ± 0.40*** (–26)
	PL[a]	3.50 ± 0.15	6.0 ± 0.32*** (+71)	5.0 ± 0.21** (–17)	4.65 ± 0.40*** (–23)
	TG[a]	442.0 ± 23.0	874.0 ± 45.0*** (+97)	630.0 ± 30.0*** (–28)	602.0 ± 30.0*** (–31)
	Total proteins[a]	5.25 ± 0.12	8.35 ± 0.32*** (+59)	6.77 ± 0.12** (–19)	6.25 ± 0.36*** (–25)
	Lipolytic activity[b]	82.14 ± 4.32	43.49 ± 1.79*** (–47)	71.1 ± 2.60*** (+63)	78.83 ± 2.17*** (+81)
Heart	TC[a]	3.92 ± 0.50	5.2 ± 0.30*** (+32)	4.41 ± 0.03* (–15)	3.96 ± 0.10*** (–24)
	PL[a]	9.80 ± 0.40	13.44 ± 0.15*** (+37)	11.72 ± 0.14* (–13)	11.25 ± 0.37** (–16)
	TG[a]	7.0 ± 0.50	9.6 ± 0.40*** (+26)	8.0 ± 0.17** (–17)	7.50 ± 0.12** (–22)
	Total proteins[a]	120.00 ± 10.0	152.0 ± 8.00*** (+26)	135.0 ± 6.0* (–11)	130.0 ± 12.0* (–14)
	Lipolytic activity[b]	142.30 ± 10.0	89.49 ± 4.00*** (–37)	98.11 ± 5.40[NS] (+10)	102.7 ± 7.33* (+15)

Units: [a]mg/g; [b]µmol FFA/h/mg protein. Values are mean ± SD of six rats. *$P < 0.05$; **$P < 0.01$;***$P < 0.001$. NS, Not significant. Hyperlipidemic animals were compared with control and drug-treated animals. Values in parentheses below drug-treated groups show % change compared to HFD-fed group.

digitonin, was incubated at 4°C for 24 h. The precipitated complex of cholesterol-digitonin was recovered by centrifugation. The complex was dissolved in a mixture of ethyl acetate and acetic acid added into liquid scintillation fluid; the radioactivity was read in α-radioactivity counter. The results are expressed as cpm/mg liver protein.

The hepatic LDL-receptor binding assay was performed according to the method of Singh *et al.*[18]. Briefly, rats were sacrificed, their liver excised promptly and homogenized (10% w/v) in buffer (150 mM NaCl, 1 mM $CaCl_2$ and 10 mM Tris-HCl; pH 7.5). The receptor-rich membrane from liver homogenate was isolated according to the method of Kovanen *et al.*[19]. Human LDL was isolated from the fresh serum of normal healthy donors by fractional poly-anionic precipitation method[11]. LDL was purified, characterized by PAGE and radiolabelled with ^{125}I according to the method of Howard *et al.*[20]. The specific radioactivity of ^{125}I-LDL was $10–15 \times 10^7$ cpm/mg LDL proteins. The reaction mixture containing membrane protein (100 µg/ml), different concentrations of human ^{125}I-LDL (20–250 µg/ml), bovine serum albumin (BSA), 100 mM NaCl, 0.5 mM $CaCl_2$, 50 mM Tris-HCl (pH 7.5) was incubated at 4°C for 60 min and centrifuged. The resultant pellet was washed and counted for ^{125}I-LDL for measuring the bound LDL to liver membrane in LKB WALLAC-1275 Mini-gamma counter. The specific binding (high affinity binding) was calculated by subtracting the non-specific binding, i.e. binding in the presence of unlabelled LDL to the total binding, i.e. binding in the absence of unlabelled LDL.

Table 7 shows that due to increase in the level of exogenous cholesterol, the hepatic cholesterol biosynthesis is suppressed by 57%, likely via feedback regulation pathway. Treatment with compound 80-574 or gemfibrozil further inhibited the synthesis of cholesterol by 21% and 24% respectively, via feedback regulation pathway demonstrating its own cholesterol biosynthesis inhibition activity[21]. Exogenous fat load and hyperlipidemia alter the integrity of LDL receptors and membrane function as the hepatic catabolism of this cholesterol carrying lipoprotein was suppressed by 70% in cholesterol-fed animals was also recovered significantly by 216% after treatment with compound 80-574 (Table 7).

Treatment with compound 80-574 or gemfibrozil may also increase the utilization of cholesterol for the synthesis

of hormones, and bile acids and in the formation of biomembranes. Such action of the drugs should be reflected by the stimulation of receptor-mediated catabolism of LDL according to further requirement of cholesterol, and this was observed as described above.

(iii) Effect of 80-574 on adipose and heart tissues: It is evident from Table 8 that feeding with HFD caused increase in the level of TC, PL and TG in adipose tissues by 72%, 71% and 97%, as well as in heart by 32%, 37%, and 37% respectively, following suppression of LPL activity in adipose tissue (47%) and heart (37%) in hyperlipidemic animals. Treatment with compound 80-574 reversed the levels of TC, PL and TG in adipose and heart tissues with a significant reactivation of LPL activity in adipose tissue by 63% in treated animals. The effect of gemfibrozil (100 mg/kg) was similar in the above models.

(iv) Effect of compound 80-574 on faecal excretion of bile acids in rats: Cholesterol performs multiple functions in the system. It is utilized for the synthesis of steroidal hormones, bile acids and maintenance of membrane cytoskeletons. Bile acids in turn act as an emulsifier to make globules of fatty acids, cholesterol, phospholipids and lipoproteins in the form of chylomicrons for further systemic circulation. A part of bile acids in the plasma is reabsorbed/recycled and processed through the nuclear receptors in liver or excreted in the faeces[22], which would accelerate cholesterol mobilization from reserve sites or atherosclerotic plaques in the liver. As a part of the study of the mechanism of cholesterol-lowering effect of compound 80-574, the excretion of cholic and deoxycholic acids in rat feces was determined by the method of Mosback *et al*[23]. It was observed that in animals fed with cholesterol-rich diet (HFD), the faecal excretion of cholic and deoxycholic acids was reduced by 42% and 57% respectively, but with co-administration of compound 80-574, the bile acids excretion in faeces was markedly enhanced (27% and 73% respectively; Table 9). The effect of gemfibrozil on the increase of bile acids excretion was only marginal, pointing to possible differences in the mechanism of hypolipidemic action.

(v) Effect of compound 80-574 on stimulation of LDL receptor binding on liver membrane in rats: The increased excretion of bile acids and other related changes in lipid profile would increase the mobilization of cholesterol and related products from deposits, which would stimulate the turnover of LDL and other lipoproteins. This work was therefore aimed to study the changes in LDL receptor binding affinity on liver cell membranes, and LDL synthesis through the binding of ^{125}I-LDL and also by increase in RNA synthesis required for receptor protein in HFD-fed animals, with and without treatment with compound 80-574 in rat liver membrane. The results are given in Tables 10–12 and Figure 1.

The effect of drug treatment on hepatic LDL receptors of rats was studied using ^{125}I-LDL according to the method of Singh *et al.*[18]. Male adult Charles Foster rats (200–225 g) were divided into two groups of six animals each, and compound 80-574 (100 mg/kg) was fed orally to one group for 7 days and the second group served as control. On the eighth day blood was taken from the retro-orbital plexus of overnight fasted rats. The rats were then sacrificed and their liver membranes were isolated according to the method of Kovanen *et al.*[19]. Human serum for LDL was prepared and radiolabelled with ^{125}I according to the method of Howard *et al.*[20]. The reaction mixture contained membrane protein (100 μg), different concentrations of ^{125}I-LDL and BSA 20 mg/ml in 50 mM Tris HCl buffer pH 7.5, added with 100 mM NaCl, 0.5 mM $CaCl_2$ in the presence or absence of unlabelled LDL (1 mg/ml) to a final volume of 1 ml. The tubes were incubated at 37°C for 60 min, centrifuged, washed with buffer and then studied for LDL receptors binding.

After incubation, the reaction mixture was centrifuged. The resultant precipitate was washed and counted for ^{125}I incorporation on LDL on radioactive counter. Different experiments with saturable concentration of ^{125}I-LDL and variable concentrations of unlabelled LDL (0.1–1.0 mg/ml) were performed to assess the total, specific and non-specific binding and these values were used for Scatchard analysis as given in Figure 1 and Table 10.

The bound LDL/mg proteins are plotted on the *X*-axis and bound/free LDL on the *Y*-axis. The slope is calculated by the least square procedure by fitting the simple linear regression model of Scatchard[24] and Howard *et al.*[20].

Liver membranes were delipidated for the estimation of TC, PL and TG as mentioned earlier[7–9]. Total lipid was estimated according to the method of Folch *et al.*[25]. Marked stimulation of LDL binding to liver membrane by compound 80-574 indicates increased binding sites which recognize ^{125}I-LDL. The significant increase in LDL binding and decrease in serum and membrane lipids suggest the rapid catabolism of LDL through stimulation of its binding activity (Table 11)[26].

The Scatchard plot analysis of ^{125}I-LDL binding depicted in Figure 1 reveals that under the influence of compound 80-574, the lipoprotein receptors of liver membrane bind more LDL particles as shown by increase in binding maxima (B_{max}) by 78%. However, the high affinity binding (K_d) almost remained the same (6%). The effect of compound 80-574 was therefore studied on the biosynthesis of protein and nucleic acid in rat liver *in vivo* and *in vitro*. Rats were fed compound 80-574 at the dose of 100 mg/kg for 7 days. At the end of the experiment their liver was excised. Liver homogenate was used for *in vitro* uptake studies of ^{14}C-L-leucine, ^{3}H-uridine and ^{3}H-thymidine as described by Singh and Kapoor[26]. Enhanced LDL receptor proteins were evident (Table 12) from increased protein synthesis as observed with

Table 9. Effect of compound 80-574 and gemfibrozil on faecal excretion of bile acids in HFD-fed rats

Bile acids	Control	HFD-fed	HFD + 80-574 (100 mg/kg)	HFD + gemfibrozil (100 mg/kg)
Cholic acid[a]	81.47 ± 4.87	47.62 ± 3.14*** (–42)	60.34 ± 4.12*** (+27)	54.42 ± 4.0* (+14)
Deoxycholic acid[a]	53.66 ± 3.00	23.31 ± 1.66*** (–57)	40.41 ± 2.84*** (+73)	34.40 ± 2.33*** (+48)

Units: [a]μg/g faeces. Values are mean ± SD of six rats. $^{**}P < 0.01$; $^{***}P < 0.001$. Hyperlipidemic animals were compared with control and drug-treated animals. Values in parentheses below drug-treated groups show % change compared to HFD fed group.

Table 10. Effect of compound 80-574 on the binding of ^{125}I-LDL on liver membrane

Experimental schedule	Control	80-574 (100 mg/kg)	% Increase
^{125}I-LDL binding at saturation			
cpm × 10^{-4}/m g protein	11.26 ± 1.12	21.03 ± 2.20*	87
ηg ^{125}I-LDL protein/mg receptor protein	1024 ± 102	1912 ± 200*	86
Binding maxima			
B_{max}; μg/mg protein	1.34 ± 0.034	2.38 ± 0.103*	78
High affinity binding (K_d)	217 ± 10	231 ± 4.00^{NS}	6.45

Values are mean ± SD of six rats. $^{*}P < 0.001$; NS, Not significant compared to control.

Table 11. Effect of compound 80-574 on liver membrane lipids

Lipids	Control	80-574 (100 mg/kg)	% Decrease
Total lipids[a]	1125 ± 30	900 ± 25**	20
Cholesterol[a]	98 ± 3.5	82.1 ± 3.9*	16
Phospholipids[a]	749 ± 55	637 ± 49*	15
Triglycerides[a]	85.0 ± 4.9	65.5 ± 5.5**	23

Unit: [a]μg/mg. Values are mean ± SD of six rats. $^{*}P < 0.05$; $^{**}P < 0.01$ compared to control.

increased incorporation of ^{14}C-L-leucine *in vitro* in compound 80-574-treated liver homogenate.

The results showed that compound 80-574 caused increase in the synthesis of nucleic acids and proteins enhanced the number of functional LDL receptors that bind more LDL particles as shown by increased B_{max} and K_d values.

(vi) Antioxidant action: Reactive oxygen species (ROS) have been implicated in the pathogenesis of a variety of metabolic diseases, including hyperlipidemias and atherosclerosis. Polyunsaturated fatty acids within cell membranes and lipoproteins are particularly susceptible to oxidative attack (lipid peroxidation), often as a result of metal ion-dependent $OH^{\bullet}$ radical formation. LDL can cross the vascular endothelial barrier and remain in the sub-endothelial space where free radicals from inflammatory cells/macrophages, smooth muscle cells or endothelial cells and $OH^{\bullet}$ radicals oxidize LDL[27,28]. Macrophages and smooth muscle cells are equipped with scavenger receptors which capture oxidized LDL, but not unmodified LDL. Oxidized LDL is then intracellularly decomposed into its molecular components[29] and the resulting intracellular-free cholesterol is acylated by ACAT[30]. Accumulation of cholesteryl esters in macrophages and smooth muscle cells leads to the formation of foam cells which accumulate and constitute the fatty streak, the initial lesion of atherosclerosis. Cholesterol ester uptake process in macrophages or smooth cells occurs without any regulation, while in normal cells it occurs under tight control[31]. Oxidized LDL is also cytotoxic to vascular cells and ruptures atherosclerotic plaques, causing vasoconstriction and local thrombosis, resulting in partial or total arterial obstruction[32]. As LDL oxidation is mainly caused by $OH^{\bullet}$ radicals, antioxidant property of compound 80-574 was studied on oxidation of LDL as well as generation of the $OH^{\bullet}$ radicals *in vitro* (Tables 12 and 13). It was found that in HFD-fed rats, the circulatory lipoproteins of VLDL and LDL appeared with increased oxidation. Table 13 shows that feeding HFD to rats causes lipid peroxidation in circulatory lipoproteins, predominantly in VLDL and LDL due to large amounts of polyunsaturated fatty acids (PUFA) in them. These lipoproteins were shown to contain more lipid peroxidized products, which were measured as thiobarbituric acid reactive substances (TBARS). However, HDL which is well known for its own antioxidative properties was almost not affected[33]. Lipid peroxide levels were estimated as TBARS according to the method of Ohkawa and Ohishi[34]. Table 13 shows that serum as well as serum VLDL, LDL and HDL isolated from HFD-fed rats have increased levels of lipid peroxides by 82%, 60%, 72% and 12% respectively.

Treatment with compound 80-574 at the dose of 100 mg/kg significantly inhibited peroxidation in serum, which in turn appeared with low levels of TBARS in blood lipoproteins, namely serum, VLDL and LDL by 24%, 18% and 30% respectively. The effect of gemfibrozil as an antioxidant was insignificant. Compound 80-574 seemed to have stronger antioxidant action than

Table 12. *In vitro* uptake of radioactive precursor in rat liver treated with compound 80-574

Treatment	^{14}C-L-Leucine[a]	^{3}H-Uridinea	^{3}H-Thymidine[a]
Control	8.41 ± 0.668	2.44 ± 0.098	2.05 ± 0.166
80-574 (100 mg/kg)	10.0 ± 0.473* (+19)	2.86 ± 0.037* (+17)	2.94 ± 0.477** (+43)

Units: [a]dpm × 10^{-5}/g liver. Values are mean ± SD of six rats in each group. *P < 0.01, **P < 0.001 compared to control. Values in parentheses show % increase.

Table 13. Effect of compound 80-574 on TBARS in serum lipoprotein of HFD-fed hyperlipidemic rats

Lipid peroxide	Control	HFD	HFD + 80-574 (100 mg/kg)	HFD + gemfibrozil (100 mg/kg)
Serum	675 ± 82	1228 ± 130** (+82)	931.5 ± 51** (–24)	1161 ± 74^{NS} (–5)
VLDL	237 ± 25	379 ± 20** (+60)	310 ± 15* (–18)	346 ± 10^{NS} (–9)
LDL	351 ± 17	604 ± 24* (+72)	421 ± 20** (–30)	568 ± 62^{NS} (–6)
HDL	123 ± 13	138 ± 17^{NS} (+12)	125 ± 10^{NS} (–9)	134 ± 13^{NS} (–3)

Units: [a]nmol MDA/mg protein. Values are mean ± SD of six rats. *P < 0.01; **P < 0.001. NS, Not significant. HFD-fed group was compared with control and HFD-fed plus drug-treated with HFD-fed group. Values in parentheses below drug-treated groups show % reversal compared with HFD-fed rats.

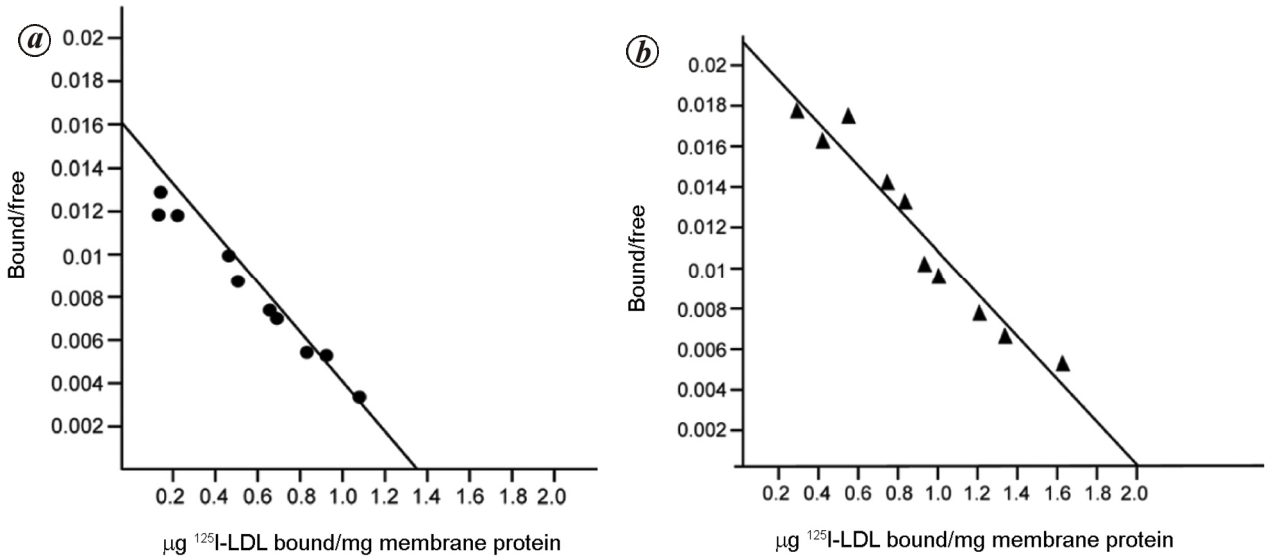

Figure 1. Scatchard plot for specific binding data of ^{125}I-LDL to (***a***) normal rat and (***b***) compound 80/574-treated rat liver membranes.

Guggulsterones[35,36]. The results in Table 13 show that compound 80-574 exhibits more than twofold antioxidant activity compared to *Guggulsterones*.

The antioxidant activity of compound 80-574 was further evaluated *in vitro*. The $OH^{\bullet}$ radicals were generated in a system of Cu^{+2}, Na-ascorbate and H_2O_2 in the absence/presence of compound 80-574 (5–20 μmol/ml) and the effect of these radicals on fragmentation of deoxyribose was studied (Table 14)[35]. It inhibited the LDL oxidation in a concentration-dependent manner and was found to cause 36%, 76% and 91% lowering at 5, 10 and 20 μM respectively.

In another set of experiments, LDL and $CuCl_2{\cdot}2H_2O$ in the absence or presence of different concentrations of compound 80-574 were incubated at 37°C for 16 h, according to the method of Singh *et al.*[36]. The amount of lipid peroxide formed was assessed as TBARS[34]. The data showed that compound 80-574 potentially scavenged the $OH^{\bullet}$ radicals and caused a dose-related inhibition of the oxidation of human LDL by $CuCl_2$ oxidation. Prevention of peroxidative changes in LDL lipids by compound 80-574 may play a role in scavenging the free radicals from fatty acid hydroperoxides so as to inhibit the chain of peroxidation. In support, it was observed that compound 80-574 could inhibit the generation of $OH^{\bullet}$ radicals by Cu^{+2}-induced non-enzymatic systems *in vitro*.

(vii) Platelet aggregation inhibition by compound 80-574: Blood platelets play an important role in the development of atherosclerosis[37]. Platelets are fragile cellular

Table 14. *In vitro* antioxidant activity of compound 80-574

Concentration (μM)	Inhibition of $OH^{\bullet}$[a]	Inhibition of LDL oxidation[b]
None	90.35 ± 5.80	44.75 ± 10.27
5.00	65.00 ± 5.35* (–28)	28.70 ± 3.98** (–36)
10.00	52.75 ± 4.20** (–42)	10.55 ± 0.56** (–76)
20.00	23.20 ± 1.80** (–74)	4.00 ± 0.50** (–91)

Units: [a]nmol MDA, [b]nmol MDA/mg protein. Values are mean ± SD of four separate observations. *P < 0.01; **P < 0.001 compared to the system in which drug was not added. Values in parentheses show % inhibition.

Table 15. Inhibition of platelet aggregation by compound 80-574

Experimental schedule	Final concentration in plasma	Aggregation %	Inhibition of aggregation %
Control vehicle	–	100	0
80-574	2×10^{-4} M	70	30
	5×10^{-4} M	0	100
Clofibrate	1×10^{-4} M	70	30
	2×10^{-4} M	0	100

structures, and self-sealing mechanism of the circulatory system is brought into action whenever platelets encounter obstructions such as hardened arteries and reactive metabolites of unsaturated fatty acids such as arachidonic acid and products of cyclo-oxygenase pathways in platelets such as thromboxane A_2 (TXA_2)[38,39]. The aggregation of platelets is also initiated by lipoprotein-a, homo-cysteine, thrombin, collagen and fibrinogen, the risk factors of atherosclerotic vascular disease[40]. The effect of compound 80-574 on activation of platelet aggregation was therefore studied by the common test mentioned in the literature[41].

Compound 80-574 completely inhibited ADP, adrenaline or serotonin-induced platelet aggregation, comparable to clofibrate (Table 15).

(viii) Hypoglycemic activity of compound 80-574 in streptozotocin-induced hyperglycemic rats: Hyperglycemia was produced in rats by streptozotocin treatment[42]. Streptozotocin (50 mg/kg i.p.)-treated animals showing blood glucose levels between 300 and 350 mg/dl were selected[43] and administered compound 80-574 or tolbutamide (100 mg/kg, b.w.). Blood samples were collected at intervals as described in Table 16 and blood glucose levels were estimated immediately.

The results showed lowering in blood glucose levels of compound 80-574 similar to that with tolbutamide. The lowering started within 1 h to a maximum of 67% at 8 h with compound 80-574, compared to the highest of 40% with tolbutamide at 6 h (Table 16). Compound 80-574 does seem to have a promising hypoglycemic activity.

(ix) Effect of compound 80-574 on progesterone and androgen receptors: As compound 80-574 is of pregnane structural class, it was evaluated *in vitro* for its progesterone and androgen receptors affinity and *in vivo* for progestational activity by Clauber-McPhail assay[44]. *In vitro* estimation of relative affinity (potency) ratio of compound 80-574 for cytoplasmic RBA of progesterone receptors present in human breast tumour cells (MCF-7) was studied along with standard 16α-ethyl–21-hydroxy-19-norpregna-4-ene-3, 20-dione (Org-2058) in three concentrations with ratios 1 : 2 : 4. Compound 80-574 was found to have no or only negligible binding affinity for cytoplasmic progesterone receptors from MCF-7 cells. Similarly, relative affinity of the test compound for cytoplasmic androgen receptors present in human breast tumour cells compared with 5α-dihydrotestosterone (DHT) was estimated at three different concentrations in the ratio 1 : 2 : 4 (ref. 45). Compound 80-574 showed no or negative binding affinity for cytoplasmic androgen receptors from MCF-7 cells.

For *in vivo* studies, compound 80-574 was administered to estrogen primed ovariectomized immature rabbits at 100 mg/kg dose by oral route. Histological evaluation of the uteri of rabbits treated with compound 80-574 showed no progestational activity at this dose[46].

Discussion

Currently statins and fibrates are the two most important classes of therapeutic agents for the treatment of hyperlipidemic conditions. Statins bring reduction in serum cholesterol level through inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyses the conversion of hydroxymethyl-glutarate to mevalonate, a rate-limiting step in the cholesterol biosynthesis. Fibrates lower triglycerides by activation of

Table 16. Effect of compound 80-574 and tolbutamide on blood glucose level of streptozotocin-treated diabetic rats

	Blood glucose[a]									
Treatment (h)	0	1	2	3	4	5	6	7	8	24
Control streptozotocin (50 mg/kg, i.p.)	301 ± 7	314 ± 11	318 ± 12	328 ± 10	328 ± 15	333 ± 21	347 ± 19	355 ± 24	340 ± 22	349 ± 12
Streptozotocin (50mg/kg, i. p.) + 80-574 (100 mg/kg p.o.)	274 ± 9	243 ± 10* (11)	231 ± 14* (16)	203 ± 20*** (26)	183 ± 14*** (33)	157 ± 21*** (43)	133 ± 16*** (51)	102 ± 9*** (63)	91 ± 8*** (67)	128 ± 18*** (52)
Streptozotocin (50 mg/kg, i.p.) + tolbutamide (100 mg/kg p.o.)	292 ± 10	218 ± 14*** (25)	207 ± 17*** (29)	202 ± 19*** (31)	195 ± 23*** (33)	183 ± 23*** (37)	174 ± 23*** (40)	192 ± 29*** (34)	242 ± 18* (17)	332 ± 26

Units: [a]mg/dl. Values are mean ± SD of six rats. *$P < 0.05$; ***$P < 0.001$. Values in parentheses show % lowering from 0 h value.

nuclear receptor PPARα. Statins cause adverse effects such as hepatic toxicity and myopathy (defined as the occurrence of muscle symptoms and almost a tenfold elevation of serum creatine kinase activity), whereas fibrates are associated with rhabdomyolysis[47,48]. HMG-CoA reductase inhibitors block mevalonate production, an early stage in cholesterol biosynthesis. As mevalonate is a common precursor for all iso-prenoids[49], long-term blockade of mevalonate would lead to a decrease in the synthesis of some other essential non-steroid metabolites also, which may contribute to the side effects observed with HMG-CoA reductase inhibitors[50–54]. Therefore, hypolipidemics acting by a different mechanism of action than fibrates or statins would greatly add to the value of therapeutics in this area. *Guggulsterone* (**1a** and **1b**), which act mainly as modulators of bile acid nuclear receptors, offer this possibility. However, GSs do not have a selective action on bile acid nuclear receptors, and inhibit other nuclear receptors too, such as the PXR/SXR[4,54,55], which thus increases the propensity of drug–drug interaction, and becomes a limitation for the use of GS in metabolic syndrome. However, the unique sub-structural features of GS, especially the enone structure around ring D of pregnane, offered a good structural platform for the design of new hypolipidemics. This study was thus directed to designing and screening the lipid lowering activity of analogues built around the structural feature of GS, and compound 80-574 was one such molecule identified and studied extensively. In primary broad biological screening it was observed that compound 80-574, in addition to its hypolipidemic activity, also exhibited promising hypoglycemic activity in streptozotocin-induced hyperglycemic rats, which added a new dimension to this molecule, making it more suitable for treatment and control of metabolic syndrome.

Compound 80-574 caused significant lowering of blood lipids in both triton-induced hyperlipidemic rats and in normal rats. Feeding compound 80-574 to normal rats or monkeys for a prolonged period (60–90 days) caused a significant lowering of plasma lipids, indicating that compound 80-574 may interfere with the absorption of exogenous (dietary) fat in the body. This compound was found to lower significantly the lipid levels seen in cholesterol-fed hyperlipidemic rabbits, rats and monkeys, suggesting that the lipid-lowering action of compound 80-574 may be mediated through other mechanisms as well.

In hyperlipidemic animals compound 80-574 could also increase the level of HDL by increasing the activity of LCAT, which plays a key role in lipoprotein metabolism[16]. Increase in the receptor-mediated catabolism of LDL was shown to be mediated through enhancement of formation of new LDL receptor proteins by increasing the biosynthesis of proteins and nucleic acids (Figure 1 and Table 12). Compound 80-574 also caused a significant increase in plasma PHLA and tissue lipoprotein lipase activity, which would accelerate the catabolism of lipoproteins. The observed increased synthesis of receptor proteins and decrease in the rate of hepatic cholesterol biosynthesis may regulate the level of cholesterol in the body. Faster catabolism of lipoprotein lipids by the drug-mediated activation of lipoprotein lipases affects the availability of these lipids for utilization. Excess cholesterol is used for faster synthesis of bile acids in the liver as well as their disposal through faeces. All this appeared with normalization in lipid and protein components of hyperlipidemic VLDL and LDL in the above models. Furthermore, it has been found that compound 80-574 decreases the level of lipid peroxidation in body tissues of the treated animals. This is because compound 80-574 has potent antioxidant property and thereby inhibits the generation of oxygen free radicals[52]. Oxidatively modified LDL is known to play an important role in the initiation and progression of atherosclerosis[53,54]. Alteration in

the oxidation states of metal ions, mainly of iron and copper, may initiate the reaction of oxygen free radicals-induced peroxidative damage in the body[56]. As compound 80-574 inhibited the oxidative changes in lipoprotein lipids and protected against the metal-induced oxidative changes in LDL, it would stimulate regression of atherogenic conditions in patients. Though the lipid lowering actions of compound 80-574 were similar to those of gemfibrozil in terms of the above-mentioned biochemical parameters and the ability to inhibit cholesterol biosynthesis in the liver, its high antioxidant activity and thus the ability to prevent atherogenesis, and selective inhibitory action on nuclear receptors BAR/FXR resulting in increased faecal excretion of bile acids leading to the mobilization of cholesterol from fatty deposits are important for hypolidemic agents.

Compound 80-574 thus seems to have multiple sites of action for hypolipidemic activity, which include interference with lipid absorption processes, enhanced mobilization of cholesterol from its deposits, and modulation of bile acid receptor resulting in increased excretion of bile acids in faeces, antioxidant activity as well as stimulation of catabolic activity of lipids and lipoproteins. The structures of compound 80-574, and in retrospect of *Guggulsterone* **1a** and **1b**, add an interesting dimension to structure–activity relationships of this class of compounds. These compounds are closely related to progesterone and second generation progestogens. Progesterone also has significant lipid lowering activity. It is interesting that introduction of a conjugated enone sub-structure in or around ring D, totally aborts the progestogenic activity, while greatly enhancing the lipid-lowering properties and introducing the propensity for hypoglycemic activity, an important shift from single-drug single target paradigm[1]. This structural ramification seems to add many interesting facets to metabolomic activity of this class of compounds.

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