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Hypolipidaemic mechanisms of action of CM108 (a flavone derivative) in hyperlipidaemic rats

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Abstract

In the present study, the molecular mechanisms by which CM108, a flavone derivative, improves lipid profiles were investigated further. Hyperlipidaemia was induced by oral administration of high cholesterol and fat. After 4 weeks of treatment, the lipid levels in the serum, liver and faeces were measured and the liver genes involved in lipid metabolism were analysed to explore the molecular mechanisms of lowering lipids. CM108 modulated lipid profiles, including elevating the level of high-density lipoprotein cholesterol (HDL-C; 40%) and reducing serum levels of triglyceride (10%), total cholesterol (10%) and low-density lipoprotein cholesterol (26%). Levels of triglyceride and total cholesterol in the liver were reduced by 18% and 24%, respectively. Increased HDL-C level was attributed to the synergic effects of CM108 in increasing levels of ATP-binding cassette transporter (ABC)A1, apolipoprotein AI and apolipoprotein AII in the liver. Intriguingly, CM108 induced genes, including fatty acid transport protein, acyl-CoA synthetase and lipoprotein lipase that are important for more efficient fatty acid β-oxidation, thereby reducing serum and liver triglyceride levels. In addition, induction of ABCG5, ABCG8 and cholesterol 7α-hydroxylase contributed to cholesterol metabolism, leading to decreases in serum and liver cholesterol levels. Thus, the genes involved in lipid metabolism were systemically modulated by CM108, which contributed to the improvement of lipid profiles in hyperlipidaemic rats.

Introduction

The modulation of orphan nuclear receptors and their targeted genes provides alternative therapeutic strategies for the treatment of metabolic syndrome. Intense efforts are currently being directed into investigation of the biological roles and mechanisms of action of liver X receptors (LXR) and peroxisome proliferator-activated receptors (PPAR; Repa & Mangelsdorf 2002; Raalte et al 2004), which serve as sensors that regulate lipid homeostasis. More importantly, cross-talk between these receptors produces more effective functions in regulating metabolic homeostasis through modulating their target gene network. Coadministration to mice of the LXRα-agonist T0901317 and the PPAR-α agonists fenofibrate or WY14643 successfully attenuated the elevation of circulating triglycerides (TG) induced by LXR-α activation (Beyer et al 2004). PPARα and PPARγ hold great promise in the treatment of metabolic syndrome and associated cardiovascular disease. Ragaglitazar, a dual PPARα/γ agonist, has been shown to increase high-density lipoprotein (HDL), lower TG and increase insulin sensitivity in patients with type 2 diabetes (Chakrabarti et al 2003; Ebdrup et al 2003). Therefore, therapeutic agents that can simultaneously activate these nuclear receptors are urgently needed. More attention has been attached to bioactive components and their derivatives from plant substances in recent years. In our previous studies, we showed that CM108 (Figure 1), a derivative of flavone, can effectively increase HDL levels and lower TG levels in hyperlipidaemic rats (Guo et al 2006). However, the molecular mechanisms by which CM108 facilitated these functions in-vivo are unknown. To gain insight into the causes of lipid modulation in rats treated with CM108, we here conduct further experiments to investigate the molecular mechanisms by analysing the mRNA levels of genes for proteins involved in lipid homeostasis.

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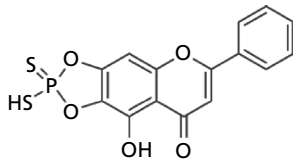

Figure 1 Chemical structure of CM108 (9-hydroxy-2-mercapto-6-phenyl-2-thioxo-1,3,5-trioxa-2λ^5-phospha-cyclopenta[*b*]naphthalen-8-one).

Materials and Methods

Materials

CM108 was synthesized by Comman Pharmaceutical Co. Ltd (Shanghai, China). Gemfibrozil was purchased from Sigma (St Louis, MI, USA). GW3965 [3-(3-(2-chloro-3-trifluoromethylbenzyl-2,2-diphenylethylamino)proproxy)phenylacetic acid] was prepared following standard chemical syntheses (Collins et al 2002). CM108, gemfibrozil and GW3965 were suspended in 1% carboxymethylcellulose (Sigma) aqueous solution, and a uniform suspension was obtained by ultrasonication.

Animals

Fifty male Sprague–Dawley rats (260 ± 20 g) were purchased from Shanghai Experimental Animal Center, China. The rats had free access to standard pelleted non-purified diet (Purina Lab Chow No. 5001) and were housed in an environmentally controlled room at 22–25°C, relative humidity 60 ± 10% and with a 12 h light cycle (08:00–20:00). Food and tap water were provided ad libitum.

Blood was always drawn after an overnight fast. Blood samples were taken before the experiments and the rats grouped so that the average lipid levels of each group were similar. After 1 week of acclimatization, the rats were divided into two groups: 10 rats (normal diet controls; NC) had free access to a normal diet; 40 rats were fed with a high-fat/high-cholesterol diet (a normal diet supplemented with 1% cholesterol, 0.5% choline bitartrate and 5% olive oil). This dietary regimen was continued throughout the experiment. The experiment was approved by the local animal ethics committee and performed based on international accepted guidelines for care and use of laboratory animals.

Drug and treatment

The rats on the high-fat/high-cholesterol diet were divided into four subgroups of 10 rats. One subgroup were given CM108 orally, 100 mg kg^{-1} day^{-1}; one subgroup were given gemfibrozil, 100 mg kg^{-1} day^{-1}; one subgroup were given GW3965, 100 mg kg^{-1} day^{-1}; the remaining group were the high-fat/high-cholesterol diet controls (FC), which were given vehicle. The NC animals were also treated with vehicle. During the last 3 days of the experimental period, samples of faeces were collected from each rat and stored at −20°C. After continuous daily treatment for 4 weeks, the animals were fasted for 6 h, then blood was harvested for lipid measurements and liver tissue for analysis of mRNA expression levels.

Measurement of lipids

Blood samples were kept at room temperature for 1 h, then the serum was separated by centrifugation at 1500 *g* for 15 min. The levels of serum total cholesterol (TC), HDL-cholesterol (HDL-C) and TG were measured by enzymatic methods using commercial kits (Roche, Indianapolis, IN, USA) and an automatic analyser (7170, Hitachi, Tokyo, Japan). Serum low-density lipoprotein cholesterol (LDL-C) levels were calculated by the method of Friedewald et al (1972). Liver TC and TG were determined by the method of Folch et al (1957). Faeces were extracted by the method of Tokunaga et al (1986), and the concentration of bile acids in the extracted solutions was determined enzymatically by the method of Mashige et al (1981). TC concentration was measured by the method of Folch et al (1957).

Analysis of mRNA expression levels

Total RNA was extracted from four frozen liver samples from the NC, FC, CM108, gemfibrozil and GW3965 groups, respectively. Real-time quantitative PCR was used to determine the relative levels of mRNA for ATP-binding cassette transporter (ABC)A1, ABCG5, ABCG8, apolipoprotein (Apo) AI, ApoAII, lipoprotein lipase (LPL), cholesterol 7α-hydroxylase (CYP7A1), fatty acid transport protein (FATP), acyl-CoA synthetase (ACS), sterol regulatory element binding protein 1c (SREBP-1c) and fatty acid synthase (FAS). RT-PCR was performed according to the manufacturer's instructions (TaqMan Gold real-time PCR protocol and TaqMan Universal PCR Master Mix; Applied Biosystems, Foster City, CA, USA). Sequence-specific amplification was detected as an increasing fluorescence signal of the reporter dye 6-carboxyfluorescein during the amplification cycle. Amplification of the mRNA for the human 23-kDa highly basic protein (HBP), also called ribosomal protein L13a, was performed in the same reaction on all samples tested, as an internal control for variations in RNA amounts. Random primes and random probes for each reaction were used as negative controls. Levels of the different mRNAs were subsequently normalized to HBP mRNA levels. Values presented are expression relative to that of the NC rats.

Statistical analysis

One-way analysis of variance with post-hoc multiple comparison (Student–Newman–Kewls test) was used to compare the differences among or between groups. All values were expressed as mean ± s.d. *P* values less than 0.05 were considered significant.

Results

Effects of drugs on lipids in the serum, liver and faeces

We first investigated the effects of CM108 on the lipid profiles in hyperlipidaemic rats fed a high-fat/high-cholesterol

diet. At the end of the experiment, mean weight had increased by 15%, with no differences between the groups (data not shown).

Table 1 shows the concentrations of lipids in the serum and liver in the five treatment groups. The high-fat/high-cholesterol diet resulted in significant increases ($P<0.01$) in serum lipids, including TC (28%), TG (25%) and LDL-C (54%), and liver lipids, including TC (105%) and TG (42%), and resulted in a significant decrease ($P<0.01$) in HDL-C (17%) compared with the NC group.

We used gemfibrozil (PPARα agonist) and GW3965 (LXRα agonist) as control compounds to evaluate the hypolipidaemic action of CM108. Significant increases in serum HDL-C levels were seen in rats treated with CM108 (40%), gemfibrozil (31%) and GW3965 (21%) compared with FC rats (all $P<0.01$; Table 1). In particular, CM108 and gemfibrozil treatment produced similar increases in HDL-C, whereas GW3965 treatment caused no change in HDL-C levels compared with NC rats. There were significant decreases in serum TG level with CM108 (10%) and gemfibrozil (17%) (both $P<0.01$), whereas there was no significant decrease with GW3965, the value being similar to that in FC rats (Table 1). CM108 and GW3965 produced significant decreases in serum TC (10%) and LDL-C (26%) levels compared with FC rats ($P<0.01$), whereas gemfibrozil had no significant effects on these lipids (Table 1).

CM108 and GW3965 caused significant decreases (24% and 28%, respectively) in liver TC levels compared with that in FC rats ($P<0.01$), whereas there was no significant decrease with gemfibrozil (Table 1). Concentrations of TC and bile acids in the faeces of rats treated with CM108 or GW3965 were significantly higher than in the FC rats ($P<0.01$) (Table 1). Furthermore, CM108 and gemfibrozil treatment caused significant decreases in liver TG levels compared with FC rats (18% and 23%; $P<0.01$), whereas GW3965 had no effect on liver TG levels (Table 1).

Effects of drugs on mRNA expression levels

To account for the differences in lipid profiles between rats treated with CM108, gemfibrozil and GW3965, changes of the expression of genes for enzymes involved in lipid metabolism were measured by quantitative real-time PCR. Levels of expression relative to that in NC rats are shown in Figure 2. The high-fat/high-cholesterol diet did not cause significant changes in the expression of any of the hepatic genes measured compared with levels in NC rats. In the liver, both CM108 and GW3965 induced ABCA1, ABCG5, ABCG8, CYP7A1, SREBP1c, FAS (Figure 2A), ApoAI, ApoAII, LPL, FATP and ACS expression to a similar extent (Figure 2B) ($P<0.01$). Expression of LPL mRNA was induced by GW3965 to a lower extent (1.61 fold) than that induced by CM108 or gemfibrozil (2.53 and 2.41 fold, respectively). Slight signals of gene expression were detected in the NC rats.

Discussion

In our previous study, we showed that CM108 increased HDL-C and lowered TG in hyperlipidaemic rats but had no influence on serum TC and TG levels (Guo et al 2006). In the current study, which used a different animal model from our previous study, Sprague–Dawley rats maintained on a high-cholesterol diet whilst being given daily CM108 were used to examine the effects of CM108 serum and liver lipid levels. This animal model was designed to provide more insight into the action of CM108. More importantly, analysis of the expression of various genes for proteins involved in lipid metabolism in the liver was used to decipher molecular mechanisms behind the hypolipidaemic activities of CM108. Gemfibrozil (a PPARα agonist) and GW3965 (an LXRα agonist) were used as positive controls, since CM108 may activate PPARα and LXRα simultaneously (Guo et al 2006). The doses of CM108, gemfibrozil and GW3965 (100 mg kg^{-1} day^{-1})

Table 1 Effects of CM108, gemfibrozil and GW3965 on serum and liver lipids

	NC rats	FC rats	CM108	Gemfibrozil	GW3965
Serum lipids (mg dL^{-1})					
Total cholesterol	86.59 ± 2.81	110.63 ± 7.29[a]	99.55 ± 8.51[b]	107.61 ± 7.15	94.77 ± 7.86[b]
Triglycerides	57.47 ± 2.68	71.67 ± 4.24[a]	64.52 ± 4.28[b,c,d]	59.56 ± 4.33[b]	69.49 ± 4.41
HDL cholesterol	27.41 ± 2.36	22.84 ± 2.86[a]	31.98 ± 2.55[b,d]	29.96 ± 3.16[b]	27.56 ± 4.12[b]
LDL cholesterol	47.69 ± 2.31	73.66 ± 7.58[a]	54.67 ± 9.57[b,c]	65.74 ± 8.13	53.31 ± 7.84[b]
Hepatic lipids (mg g^{-1})					
Total cholesterol	4.61 ± 0.57	9.46 ± 1.76[a]	7.23 ± 1.51[b,c]	8.81 ± 1.26	6.78 ± 1.42[b]
Triglycerides	41.47 ± 3.37	58.70 ± 5.16[a]	48.19 ± 5.37[b,d]	45.22 ± 6.31[b]	60.20 ± 5.23
Faecal lipids (mmol rat^{-1} day^{-1})					
Total cholesterol	0.73 ± 0.048	0.85 ± 0.055	1.09 ± 0.073[b,c]	0.89 ± 0.067[b]	1.12 ± 0.083[b]
Bile acids	0.31 ± 0.039	0.37 ± 0.045	0.56 ± 0.051[b,c]	0.39 ± 0.076	0.58 ± 0.049[b]

Values are mean ± s.d.

[a] $P<0.01$ FC vs NC group; [b] $P<0.01$ CM108, gemfibrozil or GW3965 groups vs FC group; [c] $P<0.01$ CM108 group vs gemfibrozil group; [d] $P<0.01$ CM108 group vs GW3965 group.

FC, high-fat/high-cholesterol diet controls; NC, normal diet controls.

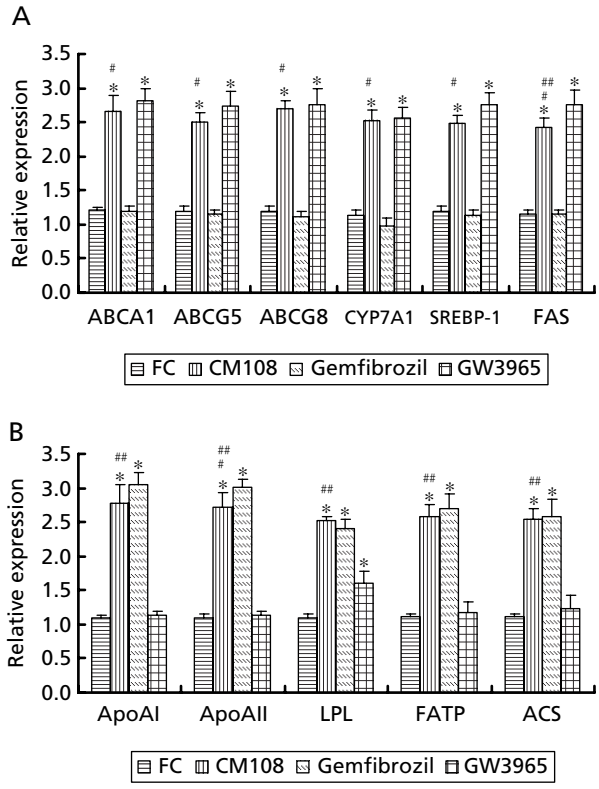

Figure 2 mRNA expression levels in the livers of rats given a high-fat/high-cholesterol diet, treated with CM108, gemfibrozil or GW3965. Data are mean ± s.d. $^{*}P < 0.01$ CM108, gemfibrozil and GW3965 groups vs FC group (control rats fed a high-fat/high-cholesterol diet); $^{\#}P < 0.01$ CM108 group vs gemfibrozil group; $^{\#\#}P < 0.01$ CM108 group vs GW3965 groups.

ABC, ATP-binding cassette transporter; CYP7A1, cholesterol 7α-hydroxylase; SREBP1c, sterol regulatory element binding protein 1c; FAS, fatty acid synthase; Apo, apolipoprotein; LPL, lipoprotein lipase; FATP, fatty acid transport protein; ACS, acyl-CoA synthetase.

have been shown to improve lipid profiles in rats (Nagao et al 1998; Miao et al 2004; Guo et al 2006).

In the current studies, a significant rise in HDL-C (40%, $P < 0.01$) was observed in rats treated with CM108, which was greater than the increases with gemfibrozil and GW3965 (31% and 21%, respectively). The results might be attributed to simultaneous up-regulation of ABCA1, apoAI and apoAII induced by CM108 (Figure 2A and 2B), whereas only ABCA1 was induced by GW3965, and only apoAI and apoAII were induced by gemfibrozil (Figure 2A and 2B). ApoAI and apoAII are the major apolipoproteins of the HDL fraction and participate in HDL particle formation (Attie er al 2001). ABCA1 has an essential role in the formation of HDL in hepatocytes and in the maintenance of HDL levels in plasma. ABCA1 facilitates transport of phospholipids to apoAI, which then promotes the efflux of free cholesterol from cells to generate nascent HDL particles. Our results suggest that the synergistic effects of CM108 on ABCA1, apoAI and apoAII resulted in a larger increase in HDL-C than that caused by gemfibrozil or GW3965.

However, we also observed increased expression of SREBP1c and FAS in rats treated with CM108 or GW3965 (Figure 2A). Although LXRs are attractive targets because they regulate the expression of genes involved in cholesterol metabolism and homeostasis, the promising results obtained with LXR agonists are countered by one significant liability: LXR activation leads to increased fatty acid synthesis and accumulation of TG (Repa et al 2000; Schultz et al 2000). These effects are caused by LXR-mediated induction of SREBP1c, a sterol-responsive transcription factor, which, in turn, induces some of the key enzymes involved in fatty acid synthesis, such as FAS (Horton et al 2002). In the present studies, CM108 and GW3965 induced SREBP1c and FAS (by about 2.4–3.0-fold) (Figure 2A), similar to the previous report by Miao et al (2004). LXRα activation by CM108 and GW3965 contributed to changes in the expression of SREBP1c and FAS, although no significant increases in serum or liver TG were observed (Table 1). However, a 10% decrease in serum TG and an 18% decrease in liver TG were observed with CM108, whereas GW3965 had no such effects. Induction of FATP, ACS and LPL (over 2.5-fold) (Figure 2B) by CM108 as a PPARα agonist could partially account for these differences in serum and liver TG concentrations.

FATP and ACS are both thought to play crucial roles in fatty acid metabolism (Schaffer & Lodish 1994). In the liver, FATP has been shown to act as a fatty acid transport protein, responsible in part for the increased fatty acid import necessary to sustain increased β-oxidation, while ACS prevented efflux of the newly imported fatty acids by their esterification with coenzyme A (Martin et al 1997). Meanwhile, induction of LPL expression will increase lipolysis in the vascular bed of the liver (Martin et al 1997), generating more fatty acids, which are then taken up by the cells, thanks to the higher level of FATP expression. Efflux of the imported fatty acids was prevented by induced ACS level, which, in addition, primed them for subsequent metabolism. ACS was of great assistance to FATP in increasing fatty acid import. In the current studies, gemfibrozil also induced FATP, ACS and LPL (about 2.5-fold), resulting in 23% and 17% decreases in liver and serum TG, respectively (Table 1). In fact, the expression levels of FATP, ACS and LPL in the liver were all induced after fibrate treatment (Schoonjans et al 1995, 1996; Martin et al 1997). The levels of liver and serum TG were even lower than those in rats treated with CM108. Taken together, these results suggest that CM108 induced the genes for proteins important for supplying the cells with the extra fatty acids, including FATP, ACS and LPL, and co-induction of these genes had the potential to prime the cells for more efficient β-oxidation. The anabolism of fatty acids caused by LXRα-mediated induction of SREBP1c might be offset by the catabolism of fatty acids in the liver caused by PPARα-mediated β-oxidation.

Induction of fatty acid synthesis and hypertriglyceridaemia by LXR agonists is controversial. LXR agonists induce TG accumulation in the liver (Grefhorst et al 2002), and increases in serum TG have also been reported (Schultz et al 2000). A hypothesis was therefore put forward that the time-dependent normalization of TGs in the blood could partially be explained by the sequestration of fatty acid in the liver (Miao et al 2004). We tested this hypothesis in rats treated with

CM108 and GW3965. GW3965, a selective LXR modulator, raises HDL-C without inducing hepatic steatosis and hypertriglyceridaemia (Miao et al 2004). The results from our analysis of gene expression provide new evidence to support the hypothesis in the present studies. The sequestration of fatty acid in the liver might be attributed to the more efficient β-oxidation caused by co-induction of FATP, ACS and LPL in the liver after CM108 treatment.

CM108 and GW3965 treatment also caused significant decreases in the serum and liver levels of TC and serum LDL-C (Table 1), whereas gemfibrozil treatment caused relatively slight decreases. The molecular mechanisms would be complicated and could be explained as follows. Firstly, HDL acted as a transporter to carry cholesterol from peripheral cells to the liver. Secondly, recent evidence indicates that hepatic LPL could serve as a 'bridging factor' to facilitate the uptake of HDL (and LDL) for ultimate cholesterol catabolism and secretion (Rinninger et al 1998; Merkel et al 2002). Thirdly, increased HDL carries cholesterol from peripheral cells to the liver, where it can be secreted by ABCG5 and ABCG8 into bile as free sterol (Graf et al 2002) or it could be catabolized by CYP7A1 and other enzymes to bile acids that are secreted into bile (Lehmann et al 1997). In the present studies, HDL-C levels were elevated by CM108, gemfibrozil and GW3965. Moreover, LPL levels were induced by CM108 and gemfibrozil to a similar extent (about 2.5-fold). ABCG5, ABCG8 and CYP7A1 were all induced to a similar extent (2.5–3.0-fold) by CM108 and GW3965. The combined potency of all these changes contributed to the cholesterol-lowering effects of CM018. This cholesterol-lowering effect was verified by the significantly higher concentrations of TC and bile acids in the faeces of rats treated with CM108 or GW3965 compared with those in the FC rats (Table 1). It can be concluded that CM108 at 100 mgkg^{-1} day^{-1} can produce similar effects on cholesterol metabolism to those of GW3965 at the same dose.

Conclusion

CM108 modulated lipid profiles, including increasing HDL-C, reducing TG and TC in the serum and liver, and reducing LDL-C. The molecular mechanisms by which CM108 improved lipid profiles can be attributed to its synergistic effects on ABCA1, ApoAI and ApoAII genes in the liver. CM108 induced genes important for more efficient fatty acid β-oxidation, including FATP, ACS and LPL, which offset the fatty acid synthesis caused potentially by LXRα-mediated induction of SREBP1c and subsequent FAS. In addition, co-induction of ABCG5, ABCG8 and CYP7A1 contributed to cholesterol metabolism, leading to decreases in serum and liver cholesterol levels. More importantly, interactions of PPARα/LXRα-targeted genes modulated in rats treated with CM108 produced more beneficial effects in maintaining lipid homeostasis than in rats treated with gemfibrozil or GW3965.

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