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Inhibition of Lipid Droplet Accumulation in Mouse Macrophages by Stemphone Derivatives

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Abstract From a study on the biological activity of fungal stemphones and their derivatives, five derivatives having an *O*-alkyl moiety at C-11 of stemphone C were found to inhibit lipid droplet accumulation in macrophages without any cytotoxic effect. Among the derivatives, those having *O*-isopropyl and *O*-isobutyl were the most potent inhibitors by blocking the synthesis of both cholesteryl ester (CE) and triacylglycerol (TG), the main constituents of lipid droplets in macrophages.

Keywords stemphone derivative, atherosclerosis, macrophage, lipid droplet accumulation, ACAT, DGAT

Introduction

Fungal stemphones B and C produced by *Aspergillus* sp. FKI-2136 were previously discovered as potentiators of imipenem activity against methicillin-resistant *Staphylococcus aureus* (MRSA) [1]. As described in our preceding paper [2], new stemphones D, E, F and G were discovered from the culture broth of strain FKI-2136, and additional 21 semisynthetic derivatives were prepared from stemphones C, E and G to investigate the structure-activity relationships. At the same time, we started exploring other biological activities of these stemphones, and discovered that certain stemphone derivatives (Fig. 1), which did not potentiate imipenem activity against MRSA, exhibited potent inhibition of lipid droplet accumulation in macrophages. This event occurs in the early stage of atherosclerogenesis; macrophages penetrate into the intima of the artery, efficiently take up modified low density lipoprotein, store cholesterol and fatty acid as a respective form of cholesteryl ester (CE) and triacylglycerol (TG) in the cytosolic lipid droplets, and are converted into foam cells, leading to the development of atherosclerosis in the arterial wall. Therefore, inhibition of lipid droplet accumulation in macrophages would be expected to retard the progression of atherosclerosis [3]. We reported several new inhibitors of microbial origin in this assay system [4]. In this study, the effects of stemphone derivatives on lipid droplet accumulation and the synthesis of cholesteryl ester (CE) and triacylglycerol (TG), the main components of lipid droplets, in macrophages are described.

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Materials and Methods

General Experimental Procedures

Fungal strain *Aspergillus* sp. FKI-2136 was used for production of stemphones B to G and cochlioquinone D, and 21 derivatives were semisynthetically prepared from stemphones C, E and G [1, 2].

Morphological Assay for Lipid Droplet Formation in Macrophages

An assay for lipid droplet formation in mouse macrophages was carried out according to a previously described method [3]. In brief, primary mouse peritoneal macrophages (2.0×10^6 cells/ml) in GIT medium (Nippon Seiyaku) were added to each well of a 96-well plastic microplate (Corning) and incubated in a humidified CO_2 (5.0% v/v) atmosphere at 37°C for 2 hours. The medium was then replaced immediately with 0.125 ml of medium A

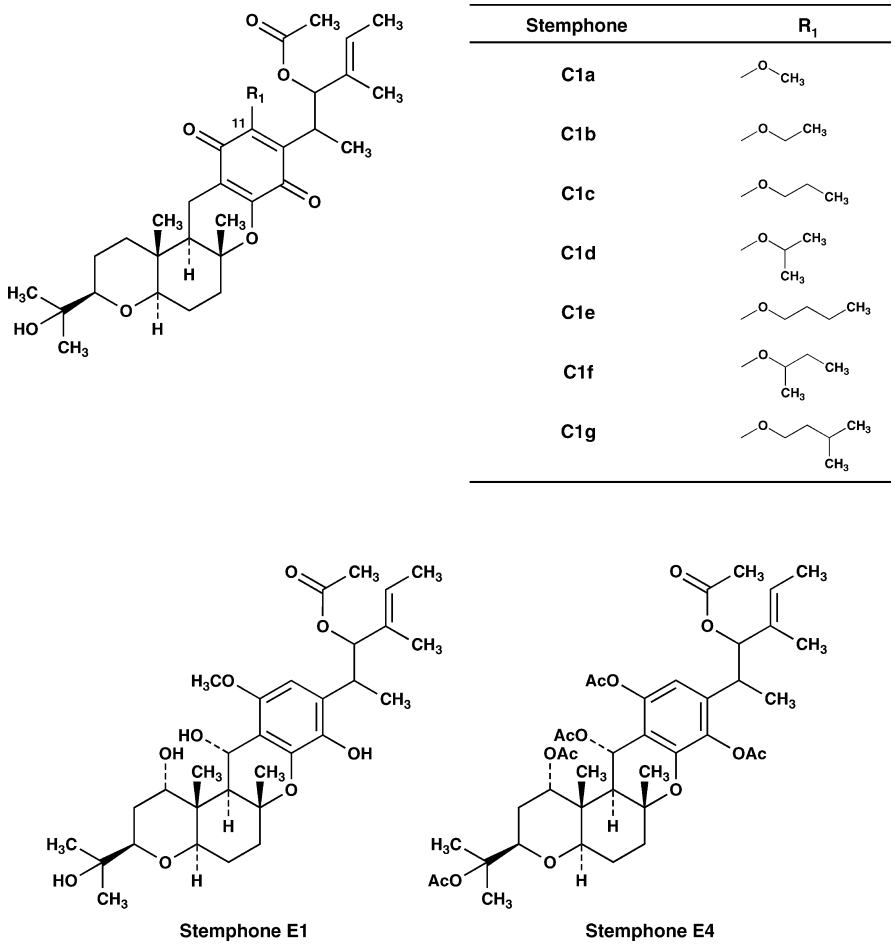

Fig. 1 Structures of semisynthetic stemphones inhibit lipid droplet accumulation in macrophages.

[Dulbecco's modified Eagle medium (Nissui Seiyaku) containing 8.0% (v/v) lipoprotein-deficient serum [5], penicillin (100 units/ml) and streptomycin (100 μg/ml)]. After another 2-hour preincubation, 1.25 μl of a sample (methanol solution) and 5.0 μl of liposomes (phosphatidylcholine 1.0 μmol, phosphatidylserine 1.0 μmol, dicetylphosphate 0.20 μmol and cholesterol 1.5 μmol suspended in 1.0 ml of 0.3 M glucose) were added to each well. After a 14-hour incubation, the cells were washed three times with PBS and then fixed by soaking in 10% formalin. Nuclei and intracellular neutral lipid droplets were then stained with hematoxylin (Sigma) and oil red O (Sigma), respectively, and the stained cells were observed by a light microscopy (Vanox-S model, Olympus).

Assay for Cytotoxicity

Cytotoxicity Detection Kit (Roche Diagnostics GmbH) was used to evaluate the cytotoxicity of stemphones to macrophages by the LDH assay [6].

Assay for [^{14}C]Neutral Lipid Synthesis by Macrophages

An assay for the synthesis of [^{14}C]CE and [^{14}C]TG by mouse macrophages was carried out according to a previously described method [3]. Mouse peritoneal macrophages (5.0×10^5 cells per 0.25 ml of medium A mentioned above) were cultured in a 48-well plastic microplate (Corning), and then 2.5 μl of a sample (MeOH solution) and 10 μl of liposomes together with 5.0 μl of [^{14}C]oleic acid (1.0 nmol, 1.85 kBq, 10% EtOH/PBS solution) were added to each culture. After a 14-hour incubation, the medium was removed, and the cells in each well were washed three times with PBS. The cells were lysed by adding 0.25 ml of PBS containing 0.1% (w/v) SDS, and the cellular lipids were extracted and separated on a TLC plate using a hexane - diethyl ether - AcOH (70 : 30 : 1) solvent system [7]. [^{14}C]Cholesteryl ester ([^{14}C]CE) and [^{14}C]triacylglycerol ([^{14}C]TG) were analyzed with a bio-imaging analyzer (BAS 2000, Fuji Film).

Assay for the Metabolism of Lysosomal [^{14}C]Cholesterol by Macrophages

The metabolism of lysosomal [^{14}C]cholesterol in mouse macrophages was measured as described previously [8]. Macrophages (5.0×10^5 cells) were incubated for 2 hours in a 48-well microplate, washed with Hank's balanced salt solution, and placed in 0.25 ml of medium A containing 10 μl of liposomes supplemented with [^{14}C]cholesterol (3.7 nmol, 200 nCi) and pregnenolone (added as a 2.5-μl MeOH solution to make a final concentration of 10 μM). After incubation for 12 hours, the medium was removed, and the cells were washed twice with buffer B (150 mM NaCl and 50 mM Tris-HCl, pH 7.4) containing BSA (2.0 mg/ml), then with buffer B without BSA, and subsequently incubated in 0.25 ml of medium A containing inhibitors (added as a 2.5-μl MeOH solution) for 5 hours. The cells were washed three times with PBS, and the cellular lipids were extracted twice with 1.0 ml of hexane-2-propanol (3 : 2). After reducing the organic solvent by evaporation, the total lipids were separated on a TLC plate and the radioactivity of [^{14}C]CE was measured according to the same method described above.

Assay for Acyl-CoA: Cholesterol Acyltransferase Activity in Mouse Macrophage Microsomes

Acyl-CoA: cholesterol acyltrasnferase (ACAT) activity was assayed according to an established method [8]. The reaction mixture contained 0.1 M potassium phosphate buffer (KPB) (pH 7.4), 300 μM bovine serum albumin (BSA), 30 μM cholesterol (added as a dispersion of cholesterol-Triton WR 1339, 30 : 1, w/w in 20 μl of 0.1 M KPB), 50 μg of mouse macrophage microsomal protein and a sample dissolved in 5.0 μl of MeOH in a total volume of 198 μl was preincubated at 37°C for 30 minutes. Then, the reaction was initiated by the addition of 2.0 μl of [^{14}C]oleoyl-CoA solution (0.4 nmol, 0.02 μCi, Amersham) and the mixture was incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 2.0 ml of $CHCl_3$-MeOH (2 : 1), and the lipids were extracted. After the total lipid extracts were separated on a TLC plate, the amount of [^{14}C]CE was determined with a bio-imaging analyzer.

Assay for Acyl-CoA: Diacylglycerol Acyltransferase Activity in Mouse Macrophage Microsomes

Acyl-CoA: diacylglycerol acyltransferase (DGAT) activity was measured according to a previous method with some modifications [9]. The reaction mixture contained 0.175 M Tris-HCl (pH 8.0), 50 μg microsomal protein, 14.5 μM BSA, 15 μM [1-^{14}C]palmitoyl-CoA (0.02 μCi, Amersham), 8.0 mM $MgCl_2$, 2.5 mM diisopropyl fluorophosphate and 0.075 mM 1,2-dioleoyl-*sn*-glycerol (Sigma) and each sample was dissolved in 5.0 μl MeOH in a total volume of 200 μl. The assay was initiated by the addition of mouse macrophage microsomal protein. After a 15-minute incubation at 23°C, the reaction was stopped by the addition of 1.2 ml of $CHCl_3$-MeOH (2 : 1) and the lipids were extracted. After the total lipid extracts were separated on a TLC plate, the amount of [^{14}C]TG was determined with a bio-imaging analyzer.

Results

Inhibition of Lipid Droplet Accumulation in Macrophages

In the control assay (no drug), mouse peritoneal macrophages accumulated a massive amount of lipid droplets in the cytosol as shown in Fig. 2A. Under the conditions, the effect of all the stemphones (6 natural and 21 semisynthetic) on lipid droplet accumulation was tested.

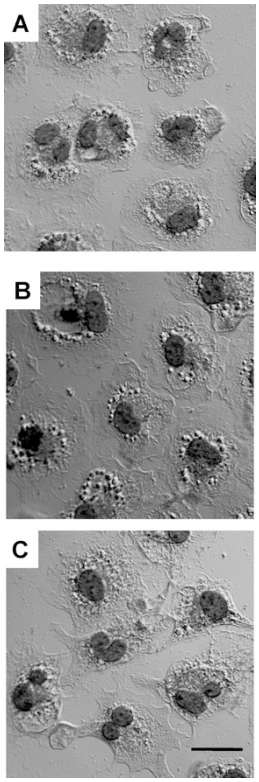

Fig. 2 Inhibition of lipid droplet accumulation in mouse macrophages by stemphone C1a.

Macrophage monolayers in a tissue culture chamber (LAB-TEK 8-chamber, Nunc) were incubated with 10 μl of liposome for 16 hours in the absence (A) or in the presence of 1.75 (B) or 17.5 μM (C) stemphone C1a. Lipid droplets were stained by oil red O. Bar represents 20 μm. Data are representative of three experiments performed.

In the presence of stemphones C1a to C1g, E1 and E4, the size and number of lipid droplets in macrophages obviously declined in a dose-dependent manner (1.5～50 μM). The typical results of stemphone C1a are shown in Fig. 2B and C. No morphological changes or cytotoxic effects on macrophages were observed even at the highest dose (50 μM). At the same time, the viability of macrophages was evaluated using the LDH assay, but stemphone C1a had almost no effect on the viability of macrophages (IC_{50} >20 μM). The other stemphones, which inhibited the lipid droplet accumulation, have almost no cytotoxicity against macrophages. Furthermore, stemphones C1h, G and G1 had almost no effect on lipid droplet accumulation in macrophages even at 20 μM. The other 9 stemphones caused morphological changes in macrophages, probably due to their cytotoxic effects. Taken together, among all the stemphones, 9 derivatives listed in Fig. 1 selectively inhibited lipid droplet accumulation in macrophages.

Inhibition of CE and TG Synthesis in Macrophages

In the control assay (no drug), about 40% of exogenously added [^{14}C]oleic acid was incorporated into the neutral lipids, [^{14}C]CE (about 25%) and [^{14}C]TG (about 5.0%), which are the main constituents of lipid droplets in macrophages [8]. Therefore the effect of stemphone derivatives, most of which inhibited lipid droplet accumulation on the synthesis of [^{14}C]CE and [^{14}C]TG, was studied, and the results are summarized in Table 1. Stemphones C1b to C1g showed potent inhibition of both [^{14}C]CE and [^{14}C]TG synthesis in a dose-dependent manner with IC_{50} values of 0.13～1.29 μM and 0.96～4.99 μM, respectively. Stemphones C1a, E1 and E4 were less potent, and stemphones C1h, G and G1 showed almost no inhibition of the synthesis. Among them, stemphone C1f was the most potent with IC_{50} values of 0.13 μM for [^{14}C]CE synthesis and 0.96 μM for [^{14}C]TG synthesis, followed by stemphone C1d. These data are consistent with those obtained in the morphological assay for lipid droplet accumulation in macrophages.

Inhibition of Postlysosomal Cholesterol Metabolism in Macrophages by Stemphone C1f

Since stemphone C1f is the most potent inhibitor of lipid droplet formation and the synthesis of CE and TG in macrophages, its target molecule in inhibition of CE synthesis in macrophages was first studied by investigating the effect of this compound on the postlysosomal process of cholesterol metabolism [8]. When macrophages were incubated with [^{14}C]cholesterol-supplemented liposomes in the presence of 10 μM pregnenolone, [^{14}C]CE formation was almost completely suppressed, and unesterified [^{14}C]cholesterol accumulated in the lysosomes of macrophages. After removal of pregnenolone by washing the cells with buffer, lysosomal [^{14}C]cholesterol metabolism to [^{14}C]CE was restarted in the presence or absence of stemphone C1f. As shown in Fig. 3, stemphone C1f inhibited [^{14}C]CE synthesis in a dose-dependent fashion with an IC_{50} of 0.10 μM, which is comparable to that of [^{14}C]CE synthesis from [^{14}C]oleic acid (Fig. 3 and

Table 1 Effect of stemphone derivatives on CE and TG synthesis in macrophages

Stemphone	IC_{50} (μM)	
	CE	TG
C1a	9.49	16.1
C1b	1.29	4.99
C1c	0.58	1.40
C1d	0.16	1.31
C1e	0.60	1.33
C1f	0.13	0.96
C1g	0.98	4.69
C1h	>20.0	>20.0
E1	8.11	11.8
E4	9.24	11.9
G	15.9	>20.0
G1	>20.0	>20.0

Results represent the mean values from three separate experiments.

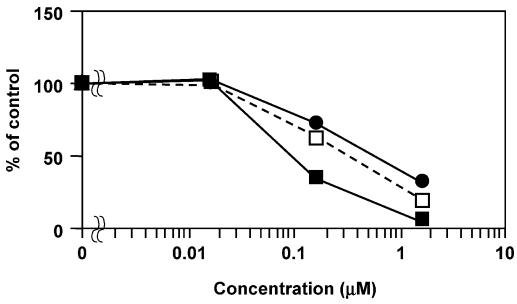

Fig. 3 Effect of stemphone C1f on [^{14}C]CE and [^{14}C]TG synthesis from [^{14}C]oleic acid and on [^{14}C]CE synthesis from lysosomal [^{14}C]cholesterol in intact macrophages.

The following two experiments are indicated; 1) The amounts of [^{14}C]CE (■) and [^{14}C]TG (●) synthesized from [^{14}C]oleic acid in mouse macrophages were determined as described in Materials and Methods. 2) The amounts of [^{14}C]CE (□) synthesized from lysosomal [^{14}C]cholesterol were determined as described in Materials and Methods. These results are plotted as a percent of control (without a drug). Results represent the mean values from three separate experiments.

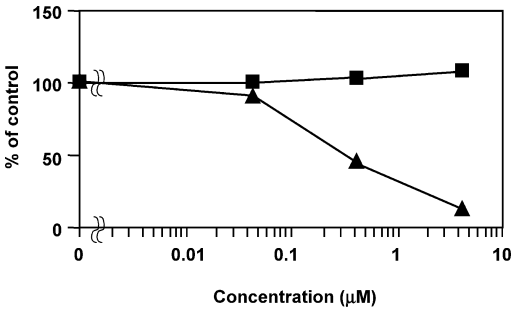

Fig. 4 Effect of stemphone C1f on ACAT and DGAT activity in microsomes of mouse macrophages.

ACAT activity (▲) and DGAT activity (■) in microsomes prepared from mouse macrophages were tested in the presence of stemphone C1f (0~4.5 μM) as described in Materials and Methods.

Table 1). These data indicated that stemphone C1f blocks the postlysosomal process of cholesterol metabolism.

Inhibition of ACAT Activity in Macrophage Microsomes by Stemphone C1f

The results using pregnenolone indicated that the inhibition site of stemphone C1f is between the point of cholesterol departure from lysosomes and the point of cholesterol esterification in the ER. Since epi-cochlioquinone, structurally related to stemphones, is known to inhibit ACAT activity [10], the effect of stemphone C1f on ACAT activity was studied. For this purpose, microsomes prepared from mouse macrophages were used as an enzyme source. Stemphone C1f was found to inhibit the ACAT activity with an IC_{50} value of 0.25 μM (Fig. 4).

Effect of Stemphone C1f on DGAT Activity in Macrophage Microsomes

As described above, [^{14}C]TG synthesis in macrophages was also inhibited by stemphone C1f. Therefore, we expected that another target of stemphone C1f in the TG biosynthesis pathway might be DGAT. However, stemphone C1f had almost no effect on DGAT activity even at 30 μM (Fig. 4).

Discussion

Fungal stemphones were originally discovered and semisynthetic derivatives were developed as potentiators of imipenem activity against MRSA [1, 2]. In this study, certain stemphone derivatives (Fig. 1) were found to inhibit lipid droplet accumulation in macrophages. Their activity profile is, however, quite different from that of imipenem potentiation activity: stemphone C1f and C1d exhibited the most potent inhibition of lipid droplet accumulation, but showed no potentiation of imipenem activity [2]. To the contrary, stemphones B, C and E showed the most potent potentiation of imipenem activity [2], but exhibited no inhibition of lipid droplet accumulation.

The presence of an *O*-alkyl residue at C-11 in stemphone is responsible for this inhibitory activity (Table 1). These stemphones included the quinone in their structures. The quinone has been known as a reactive group and might covalently bind to the relevant active site in the target molecule as a Michael acceptor, resulting in the inhibition of lipid droplet accumulation [11]. Furthermore, derivatives having a carbon length of C3 and C4, in particular, isobutyl (C1f) and isopropyl residues (C1d), show the best inhibition, suggesting that moderate hydrophobicity at this position is important for the activity.

Stemphone C1f is the most potent inhibitor of lipid droplet accumulation in macrophages by blocking the synthesis of both CE and TG. ACAT activity in microsomes of macrophages was inhibited by this compound (Fig. 4). Stemphone C1f also inhibited both ACAT1 and ACAT2 isozymes with similar potency in the cell-based assay (data not shown). Therefore, it is plausible that ACAT is one of the targets of stemphone C1f. However, the target of the compound in TG biosynthesis was not defined. DGAT, genetically related to ACAT [12], in macrophage microsomes was not inhibited by this compound. Stemphone C1f might have multiple inhibition sites responsible for the inhibition of lipid droplet accumulation. Further studies are necessary to demonstrate and understand its complete mode of action.

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