Original Article

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Screening for microbial potentiators of neutral lipid degradation in CHO-K1 cells

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SUMMARY A cell-based assay was conducted to screen microbial culture broths for potentiators of neutral lipid degradation in Chinese Hamster Ovary K1 cells. A total of 5,363 microbial cultures from fungi and actinomycetes were screened in this assay. Brefeldin A (**1**) from fungal cultures was found to promote the degradation of triacylglycerol (TG) with an EC_{50} of 2.6 μM. Beauveriolides I (**2**), III (**3**), beauverolides A (**4**), B (**5**), and K (**6**) from fungal cultures showed potentiating effect on cholesteryl ester (CE) degradation with EC_{50}s ranging from 0.02 to 0.13 μM. Among these compounds, **2** and **6** exhibited the strongest activities (EC_{50}, 0.02 μM). From actinomycete cultures, oxohygrolidin (**7**) (EC_{50} for TG and CE, > 1.7 and 0.8 μM, respectively) and hygrolidin (**8**) (EC_{50} for TG and CE, 0.08 and 0.004 μM, respectively) promoted degradation of CE more preferably than TG.

Keywords Neutral lipid degradation, screening, microbial potentiator, brefeldin A, beauveriolide, hygrolidin

1. Introduction

The synthesis and degradation of lipids are finely controlled not only to prevent intracellular accumulation of lipids but to provide energy supply and structural components of cell membranes. Lipid is stored in the final form of neutral lipids, such as triacylglycerol (TG) for long-chain fatty acids and cholesteryl ester (CE) for cholesterol (*1*). However, multiple factors, such as increased consumption of high-fat diet and lack of physical exercise, can determine the lipid storage capacity. In the end, excessive accumulation of stored TG and CE causes metabolic diseases, namely obesity, fatty liver, and atherosclerosis (*2*). Our laboratory has a long history of searching for and discovering various compounds involved in neutral lipid synthesis from microbial resources using mammalian cells (*3-5*). Biaryl dihydronaphthopyranone atropisomers produced by a fungus, dinapinones A1 (**9**) (DPA1) and A2 (**10**) (DPA2) (Figure 1), were originally discovered as inhibitors of neutral lipid accumulation. DPA2 showed potent inhibition of neutral lipid accumulation, whereas DPA1 showed almost no activity. Intriguingly, a 1:1 mixture of DPA1 and DPA2 was found to exhibit the most potent inhibition of neutral lipid accumulation (*6,7*). Further study of the mechanism of action revealed that, instead of inhibiting the synthesis of neutral lipids, DPA promoted neutral lipid degradation along with inducing autophagy (*8*).

Inhibitors of lipid synthesis primarily work as a preventive effect to stop or retard the progress of metabolic diseases in most cases. A number of inhibitors have been reported and some are practically used as preventive drugs. As a typical example, statins, which are hydroxymethylglutaryl co-enzyme A (HMG-CoA) reductase inhibitors, block hepatic cholesterol synthesis to lower the serum low-density lipoprotein (LDL) level and to retard the progression to atherosclerosis (*9*). Conversely, potentiators of accumulated neutral lipid degradation are rarely discovered. Such potentiators function as treatment drugs rather than preventive drugs for metabolic diseases involved in neutral lipid accumulation in the human body. Although discovering such potentiators seems more difficult and challenging than discovering inhibitors, our finding of dinapinones prompted us to establish an assay system and perform screening of potentiators from microbial cultures. The results are described in the present study.

2. Materials and Methods

2.1. General

NMR analyses were performed using a 400 MHz

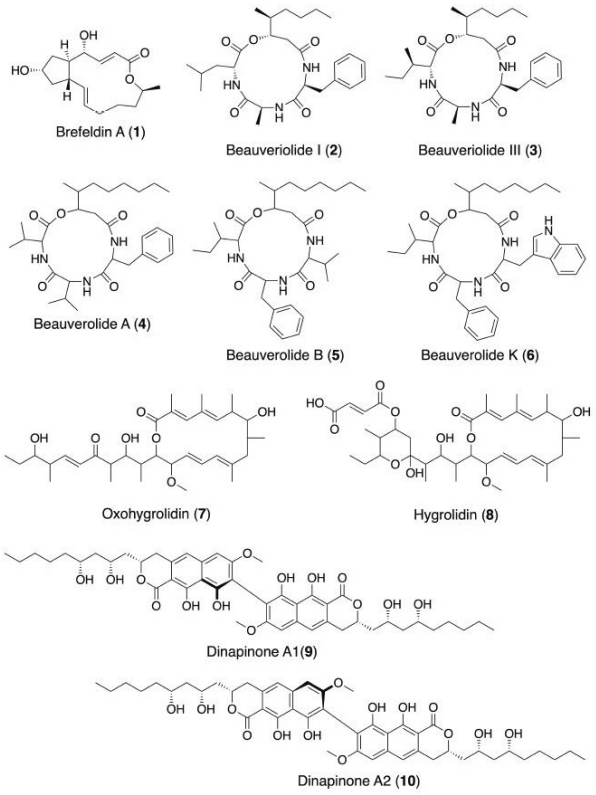

Figure 1. Structures of active compounds.

spectrometer (Agilent Technologies, Santa Clara, CA, USA) and 600 MHz spectrometer (Bruker, Karlsruhe, Germany). MS analyses were performed using an AccuTOF LC-plus JMS-T100LP system (JEOL, Tokyo, Japan) and Waters Xevo G2-XS Qtof Acquity UPLC H-class plus (Waters Corporation, Milford, MA, USA).

2.2. Materials

[1-^{14}C]Oleic acid (1.85 GBq/mmol) and [1-^{14}C] oleoyl-CoA (1.85 GBq/mmol) were purchased from PerkinElmer (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Capricorn Scientific (Ebsdorfergrund, Germany). Ham's F-12 medium was purchased from Nacalai Tesque (Kyoto, Japan). Plastic microplates (48-well) were purchased from Corning (Corning, NY, USA). Agar was purchased from Shimizu Food (Shizuoka, Japan). $CoCl_2 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$, $Fe_2(SO_4)_3 \cdot nH_2O$, glucose, KH_2PO_4, K_2HPO_4, $Mg_3(PO_4)_2 \cdot 8H_2O$, $MgCl_2 \cdot 4H_2O$, polypeptone, soluble starch, sucrose, $ZnSO_4 \cdot 7H_2O$, bovine serum albumin (BSA) (fatty acid free), and penicillin (10,000 U/mL) and streptomycin (10,000 mg/mL) solution were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). $CaCO_3$, $MgSO_4 \cdot 7H_2O$, and KBr were purchased from Kanto Chemical (Tokyo, Japan). Peptone was purchased from Life Technologies Corporation (Detroit, MI, USA). Ehrlich meat extract was purchased from Pharmaceutical Industrial (Tokyo, Japan). Solulys was purchased from Oriental Yeast (Tokyo, Japan). Yeast extract was purchased from Becton Dickinson (Sparks, MD, USA). EDTA was purchased from Dojindo (Kumamoto, Japan). Brefeldin A, beauveriolides I and III, and dinapinones (A1, A2, and DPA) were available from our compound library (*10-12*). Other beauverolides A, B, and K and hygrolidins were isolated from our culture broths.

2.3. Purification of active compounds from culture broths

Beauverolides (**4**-**6**): Fungal BF-0452 strain was seeded in medium containing 2.0% glucose, 0.2% yeast extract, 0.5% polypeptone, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% agar, and tap water, pH 6.0 for 3 days at 27°C in a rotary shaker. The seed culture was then inoculated into production medium containing 2.0% sucrose, 1.0% glucose, 0.5% solulys, 0.5% Ehrlich meat extract, 0.1% KH_2PO_4, 0.3% $CaCO_3$, 0.05% $Mg_3(PO_4)_2 \cdot 8H_2O$, 0.1% agar, 1.0% trace metal solution (1 mg/mL of each $FeSO_4 \cdot 7H_2O$, $MgCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, and $CoCl_2 \cdot 2H_2O$ in water), and tap water, pH 6.0. Fermentation was performed at 27°C for 14 days under static condition. The culture broth (1.8 L) was extracted with ethanol (1.8 L). Ethanol extracts were concentrated to remove ethanol, and the aqueous solution was extracted with ethyl acetate (3.6 L), yielding crude materials (227 mg). The extracts were then dissolved in a small volume of methanol, applied to an ODS column (11 g, i.d. 15 × 150 mm, 100-200 mesh, Fuji Silysia Chemical Ltd., Aichi, Japan) and eluted stepwise with 40%, 60%, 80%, and 100% CH_3CN solvents (2 fractions per 150 mL each). The 80%-2 CH_3CN eluents containing active materials were evaporated *in vacuo* to yield brownish materials (18 mg). The materials were purified by high performance liquid chromatography (HPLC) under the following conditions: column, Develosil C30 UG5 (Nomura Chemical Co., Ltd., Aichi, Japan) (i.d. 20 × 250 mm); eluent, 90% CH_3CN; flow, 6.0 mL/min; detection, UV at 210 nm. Compounds **4**-**6** were eluted as a peak with retention times of 16.8 min, 18.8 min, and 18.1 min, respectively. Each peak fraction was collected and concentrated to yield **4** (0.6 mg), **5** (3.2 mg), and **6** (1.1 mg) as white powders.

Hygrolidins (**7**-**8**): Actinomycete KM68-21 strain was grown in medium containing 1.0% soluble starch, 0.4% yeast extract, 0.2% peptone, and tap water, pH unadjusted at 27°C for 7 days in a rotary shaker. The culture was inoculated into new medium with additional 0.1% $CaCO_3$, 0.004% $Fe_2(SO_4)_3 \cdot nH_2O$ and 0.01% KBr at 27°C for 7 days in a rotary shaker. Culture broth (3 L) was centrifuged (3,000 rpm, 10 min) and the mycelia was collected and then extracted with 70% ethanol (3 L). Ethanol extracts were filtered to remove mycelia and then concentrated to remove ethanol. The remaining

aqueous solution was extracted with ethyl acetate (1.8 L). The organic layer was collected and dried under pressure to produce a brownish crude extract (388 mg). This crude extract was dissolved in a small amount of methanol, applied to an ODS column (17.5 g, i.d. 30 × 130 mm, 100-200 mesh, Fuji Silysia Chemical Ltd.), and eluted stepwise with 0%, 20%, 40%, 60%, 80%, and 100% CH_3CN solvents (120 mL each). The 80% and 100% CH_3CN eluents containing active materials were evaporated *in vacuo* to yield pale brown materials (38 mg and 44 mg, respectively). The materials from the 80% fraction were subjected to HPLC under the following conditions: column, Capcell pak C18 (Osaka Soda Co., Ltd., Osaka, Japan) (i.d. 4.6 × 250 mm); eluent, 20 minutes linear gradient from 75%-95% CH_3CN; flow, 1.0 mL/min; detection, UV at 210 nm. Compounds **7** and **8** were eluted as a peak with retention times of 10.0 min and 16.4 min, respectively. Each peak fraction was collected and concentrated to yield **7** (6.3 mg) and **8** (2.0 mg) as white powders. The 100% fraction was subjected to HPLC (column, Capcell pak C18 (i.d. 4.6 × 250 mm); eluent, 88% CH_3CN; flow, 1.0 mL/min; detection, UV at 210 nm). Under these HPLC conditions, **8** was eluted as a peak with retention time of 10.0 min, collected, and concentrated to yield **8** (4.6 mg) as a white powder.

2.4. Identification of active compounds

Compounds **1-3** were identified from our database using Ultra-Fast Liquid Chromatography (UFLC) (Prominence, Shimadzu, Kyoto, Japan) retention time and UV spectrum. Other compounds **4-8** were identified from the spectral data, including ^{1}H-NMR, UV and/or MS data, and the search results of Scifinder® and/or Dictionary of Natural Products (Supplementary Figures S1-S3, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=127*). These data were also identical with published data (*13-17*). The structures of active compounds are shown in Figure 1.

2.5. Cell culture

Chinese Hamster Ovary K1 cells (CHO-K1 cells) were maintained at 37°C and 5.0% CO_2 in Ham's F-12 medium supplemented with 10% heat-inactivated FBS, penicillin (100 units/mL), and streptomycin (100 mg/mL) according to a previously described method (*18*).

2.6. Assay of neutral lipid degradation in CHO-K1 cells

Assays for CE and TG degradation using CHO-K1 cells were performed according to established methods with some modifications (*8*). Cells (1.25×10^5 cells) were seeded in a 48-well plastic microplate in 250 µL of maintained medium (described above) and allowed to recover overnight in 37°C and 5.0% CO_2; 5.0 µL of [^{14}C]oleic acid (1 nmol, 1.85 kBq in 10% ethanol/phosphate-buffered saline (PBS) solution) was then added to each well of the cell culture. The cells were incubated for 24 hours at 37°C and 5.0% CO_2 to allow for accumulation of [^{14}C]TG and [^{14}C]CE within the cells. Medium were then removed and cells were washed twice with 250 µL of Buffer A (150 mM NaCl and 50 mM Tris-HCl, pH 7.4) containing 2.0 mg/mL BSA (fatty acid free) and then washed once with 250 µL of Buffer B (Buffer A without BSA) to remove the remaining [^{14}C]oleic acid. Fresh medium (250 µL) was then added along with 2.5 µL of sample and control (in methanol). After 12-hour incubation in 37°C and 5.0% CO_2, cells were washed twice with 250 µL of PBS. The cells were then lysed using 250 µL of 10 mM Tris-HCl (pH 7.5) containing 0.1% (w/v) SDS, and neutral lipids were extracted following the method of Bligh and Dyer (*19*). [^{14}C]TG and [^{14}C]CE were separated on a TLC plate (silica gel F254, 0.5-mm thick, Merck KGaA, Darmstadt, Germany) and then analyzed with a bioimaging analyzer (FLA7000; Fujifilm, Tokyo, Japan). Neutral lipid degradation activity (%) was defined as ([^{14}C]TG or [^{14}C]CE of sample/[^{14}C]TG or [^{14}C]CE of control) × 100. The EC_{50} value was defined as the sample concentration causing 50% degradation of neutral lipids (TG or CE).

2.7. Assay of neutral lipid synthesis in CHO-K1 cells

Assays for TG and CE synthesis using CHO-K1 cells were conducted according to established methods with some modifications (*8*). The IC_{50} value was defined as the sample concentration causing 50% inhibition of neutral lipid synthesis.

2.8. Sterol *O*-acyltransferase (SOAT) enzyme assay using microsomes prepared from CHO-K1 cells

SOAT assay using microsomes prepared from CHO-K1 cells was conducted using our established method (*20*). The IC_{50} value was defined as the sample concentration causing 50% inhibition of SOAT.

3. Results

3.1. Establishment of cell-based lipid degradation assay for screening

A cell-based screening method was conducted based on our neutral lipid degradation assay (*8*). In the previous study on DPA, three mammalian cell lines, CHO-K1, HeLa, and HepG2 cells, were tested for neutral lipid (TG and CE) accumulation. As a result, CHO-K1 cells were found to have the highest ability to incorporate [^{14}C]oleic acid into both CE and TG. Accordingly, we chose this cell line in our screening system. CHO-K1 cells were incubated with [^{14}C]oleic acid for 24 hours to

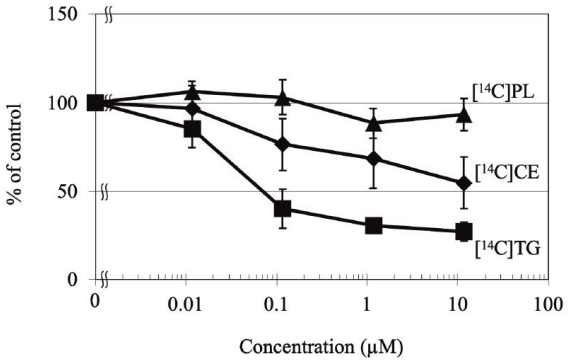

Figure 2. Effect of DPA (1:1 mixture of 9 and 10) on neutral lipid degradation. CHO-K1 cells were incubated for 24 hours with [^{14}C] oleic acid to accumulate [^{14}C]TG and [^{14}C]CE. After removing free [^{14}C]oleic acid, cells were treated with DPA 0, 0.12, 1.2, or 12 μM for 12 hours. Cells were then lysed. Cellular [^{14}C]TG (■), [^{14}C]CE (♦), and [^{14}C]PL (▲) were separated on TLC and quantified using an image analyzer. The results obtained were plotted as % of control (without drugs). Values represent means ± SD (n = 3).

accumulate [^{14}C]TG and [^{14}C]CE. After free [^{14}C]oleic acid was removed, cells were treated with a sample and control (in methanol) (time 0) and incubated for 12 hours. Accumulated [^{14}C]TG and [^{14}C]CE were naturally degraded after 12-hour incubation in the control to 63% and 60% from time 0, respectively. In the screening for lipid degradation potentiators, the amounts of [^{14}C]TG and [^{14}C]CE that remained following 12 hours of incubation were regarded as 100% (control value). Hit samples should promote degradation of [^{14}C]TG and/or [^{14}C]CE to less than 60% of the control with no morphological abnormalities in CHO-K1 cells under microscopy and no effect on phospholipid level ([^{14}C]phospholipid (PL)). EC_{50} values are defined as the effective concentration of a sample causing 50% promotion of each neutral lipid degradation.

First, the effects of DPA (1:1 mixture of **9** and **10**), DPA1 (**9**), and DPA2 (**10**) were confirmed in the assay. As shown in Figure 2, DPA strongly enhanced the TG degradation with an EC_{50} of 0.07 μM, whereas CE degradation was moderately enhanced with an EC_{50} of > 12 μM (approximately 20 μM). Almost no effect on PL degradation implied that DPA affected only neutral lipid degradation without any cytotoxic effect on CHO-K1 cells. DPA2 (**10**) showed a similar effect on the TG degradation with higher EC_{50} of 1.0 μM (TG) and weak effect on CE degradation (72% of control at 12 μM) (Table 3). DPA1 (**9**) itself showed no effect on neutral lipid degradation. These findings are fundamentally consistent with our previous study (*8*). In this screening, DPA was used as a positive control compound.

3.2. Screening result for neutral lipid degradation potentiators from microbial sources

A total of 5,363 microbial culture broths were screened for neutral lipid degradation potentiators in CHO-K1 cells with only 0.35% of them categorized as hit samples (Table 1). The hit rate from fungal cultures (hit rate 0.59%) was 7-fold higher compared to actinomycete cultures (0.08%). Out of 19 active culture broths (Table 2), 14 fungal broths promoted degradation of [^{14}C]TG. From these broths, brefeldin A (**1**) (*21*) was identified as an active compound by database analyses of the retention time and the UV spectrum using UFLC. From one of the fungal cultures, known beauveriolide I (**2**) (*22*) was identified as a potentiator of [^{14}C]CE degradation. Structurally related beauverolides A, B, and K (**4**-**6**) (*13,14*) were also isolated from the culture broth of soil isolate fungus BF-0452. Two broths from actinomycetes contain hygrolidins that promoted degradation of [^{14}C]CE more preferably than [^{14}C]TG. Known oxohygrolidin (**7**) (*17*) and hygrolidin (**8**) (*16*) were isolated from the culture broth of marine-derived actinomycete KM68-21.

Table 1. Screening result of microbial potentiators of neutral lipid degradation

Origin	Sample number	Hit sample (rate %)		Hit per-origin (rate %)
		[^{14}C]TG	[^{14}C]CE	
Fungi	2,837	14 (0.49%)	3 (0.10%)	17 (0.59%)
Actinomycetes	2,526	0 (0.00%)	2 (0.08%)	2 (0.08%)
Total	5,363	14 (0.26%)	5 (0.09%)	19 (0.35%)

Table 2. Hit strain and microbial potentiators of neutral lipid degradation

Hit strain	Active compounds	Potentiator
Fungal strain		
BF-0398, BF-0460, BF-0487, BF-0546, BF-0562, BF-0586, BF-0589, BF-0626, BF-0763, BF-0808, BF-0899, BF-0938, BF-0939 and BF-0973.	**1**	TG degradation
BF-0450	**2**	CE degradation
BF-0452 and BF-0453	**4, 5** and **6**	CE degradation
Actinomycetes strain		
KM59-1	**7**	CE degradation
KM68-21	**7** and **8**	CE degradation

3.3. Effect of microbial potentiators on neutral lipid degradation in CHO-K1 cells

A total of eight known compounds (**1**-**8**, Figure 1) were obtained as potentiators of neutral lipid degradation in the screening system. The enhancing activity of neutral lipid (TG and CE) degradation in CHO-K1 cells by these compounds is summarized in Table 3. Brefeldin A (**1**), a lactone-containing fungal metabolite, promoted [^{14}C]TG degradation with an EC_{50} of 2.6 μM, but had no effect on CE degradation. Thus, **1** is a potentiator

Table 3. Effect of microbial potentiators on neutral lipid degradation and synthesis

Compound	Cell-based lipid degradation		Cell-based lipid synthesis		Enzyme-based CE synthesis (SOAT)
	EC_{50} (μM)[a]		IC_{50} (μM)[a]		IC_{50} (μM)[a]
	[^{14}C]TG	[^{14}C]CE	[^{14}C]TG	[^{14}C]CE	[^{14}C]CE
Brefeldin A (**1**)	2.6	>34	1.6	>34	>34
Beauveriolide I (**2**)	>2.0	0.02	>2.0	0.01	0.05
Beauveriolide III (**3**)	>2.0	0.13	>0.2	0.04	0.19
Beauverolide A (**4**)	>1.8	0.10	>1.8	0.70	0.03
Beauverolide B (**5**)	>1.8	0.06	>1.8	0.02	0.03
Beauverolide K (**6**)	>1.5	0.02	>1.5	0.01	0.04
Oxohygrolidin (**7**)	>1.7	0.80	>1.7	0.63	0.30
Hygrolidin (**8**)	0.08	0.004	>0.14	0.0002	0.33
Dinapinone A1 (**9**)	>12	>12	>12[b]	>12[b]	>12
Dinapinone A2 (**10**)	1.0	>12	0.65[b]	>12[b]	>12
Dinapinone A	0.07	>12	0.054[b]	0.18[b]	>12

[a]Data are expressed as means (n ≥ 3). [b]from reference (*8*).

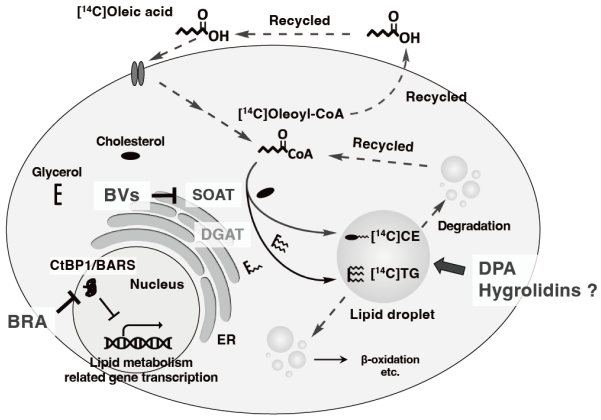

Figure 3 Graphical summary of microbial potentiators of neutral lipid degradation. CE; cholesteryl ester, TG; triacylglycerol, SOAT; sterol *O*-acyltransferase, DGAT; diacylglycerol acyltransferase, ER; endoplasmic reticulum, CtBP1/BARS; C-terminal-binding protein/brefeldin A-induced ADP-ribosylated substrate, BRA; brefeldin A, BVs; beauveriolides, DPA; dinapinone A.

selective for TG degradation.

Five beauveriolides and beauverolides including beauveriolide III (**3**) (*11,12*), **2**-**6** were found to exhibit strong [^{14}C]CE degradation activity. Compounds **2** and **6** showed the strongest with EC_{50} of 0.02 μM, followed by **5**, **4**, and **3** with EC_{50}s of 0.06 μM, 0.10 μM, and 0.13 μM, respectively. Sixteen-membered macrolide **8** exhibited strong potentiation of both [^{14}C]CE (EC_{50}, 0.004 μM) and [^{14}C]TG (EC_{50}, 0.08 μM) degradation, whereas the other macrolide **7** only potentiated [^{14}C]CE degradation with a higher EC_{50} of 0.08 μM.

4. Discussion

In this lipid degradation assay, CHO-K1 cells were incubated with [^{14}C]oleic acid overnight to incorporate a high amount of [^{14}C]oleic acid into [^{14}C]TG and [^{14}C]CE accumulated in lipid droplets (*8*). After removal of free [^{14}C]oleic acid, the accumulated [^{14}C]TG and [^{14}C]CE still remained at approximately 60% after further 12-hour incubation. This finding indicates that the CHO-K1 cell line is suitable for use in neutral lipid degradation assays.

Fungal brefeldin A (**1**) was identified as a potentiator of [^{14}C]TG degradation in our screening assay. Bartz *et al.* reported similar findings; in CHO-K2 cells pretreated with [^{3}H]oleic acid, **1** (7 μM) promoted 63% breakdown of stored [^{3}H]neutral lipids after 12-hour incubation (*23*). Brefeldin A (**1**) is well known to interfere with protein transport by preventing accumulation of coatomer proteins on the Golgi apparatus, which facilitates vesicle fusion from the endoplasmic reticulum (*24,25*). Accordingly, other studies reported that **1** induced accumulation of neutral lipid (*26-28*). However, Bartz *et al.* proposed that the lipid degradation potentiator effect of **1** is not linked to Golgi apparatus disruption and that, instead, **1** inactivates C-terminal-binding protein/brefeldin A-induced ADP-ribosylated substrate (CtBP1/BARS) by stimulating its mono-ADP-ribosylation. CtBP1/BARS works as a gene transcriptional co-repressor in nucleus. Retained CtBP1/BARS function leads to the up-regulation of genes that regulate lipid storage (*23*).

Fungal beauveriolides I (**2**) and III (**3**) are well known as inhibitors of SOAT enzymes involved in CE synthesis (*29*). In the present screening program, beauveriolides and beauverolides (**2**-**6**) were isolated as potentiators of CE degradation. To investigate CE synthesis (by SOAT activity), **2** to **6** inhibited the synthesis with analogous IC_{50}s (Table 3, middle column) to EC_{50}s in the present cell-based degradation assay (Table 3, left column). In a SOAT enzyme assay using cells microsomes, **2** to **6** also exhibited potent SOAT inhibition. These data indicated that [^{14}C]CE is degraded to free cholesterol and [^{14}C]oleic acid (also provided from [^{14}C]TG degradation), which are reused to produce [^{14}C]CE *via* SOAT during the degradation assay. Beauveriolides and beauverolides may block the

[^{14}C]CE recycling by inhibiting SOAT activity, leading to ostensible potentiation of [^{14}C]CE degradation. A variety of microbial SOAT inhibitors have been reported (*3-5,18*), but most of them were not discovered in the present screening system.

We evaluated both fungal and actinomycete culture broths in the present screening system and found that fungi were more preferable than actinomyces as a resource for this screening (Table 1). The sole actinomycete-derived potentiators that we found are hygrolidins, which are members of two diene-containing 16-membered macrolides with diverse chains. Compound **8** with a fumarate-linking tetrahydropyran in the side ring was found to be the strongest potentiator of both CE and TG degradation. Compound **7** with an alkenyl side chain exhibited markedly weaker activity compared to **8**. This group of macrolides are well known as V-ATPase inhibitors that damage the function of lysosomes (*30,31*). The mechanism of lipid droplet degradation in mammalian cells remains elusive; certain proteins located on lipid droplets (such as perilipines) regulate the incorporation of lipase into lipid droplets, and autophagy (lipophagy) is involved in lipid droplet degradation (*32-35*). Thus, lysosomes are not directly involved in lipid droplet degradation. Indeed, **7** and **8**, V-ATPase inhibitors, did not inhibit lipid droplet degradation, but conversely potentiated the degradation. The mechanism by which hygrolidins potentiate neutral lipid breakdown requires further investigation. In addition, both compounds exhibited weak SOAT inhibitory activities in the enzyme-based assay (Table 3), which may partially contribute to the potentiating effect of lipid degradation in our assay.

In conclusion, we conducted an assay of lipid droplet degradation using CHO-K1 cells and screened for potentiators from microbial cultures. All potentiators identified are known compounds with known mechanism of actions (Figure 3). These compounds will help to unveil the incompletely explored mechanism of lipid droplet degradation in mammalian cells.

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