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Novel triacsin C analogs as potential antivirals against rotavirus infections

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ABSTRACT

Recently our group has demonstrated that cellular triglyceride (TG) levels play an important role in rotavirus replication. In this study, we further examined the roles of the key enzymes for TG synthesis (lipogenesis) in the replication of rotaviruses by using inhibitors of fatty acid synthase, long chain fatty acid acyl-CoA synthetase (ACSL), and diacylglycerol acyltransferase and acyl-CoA:cholesterol acyltransferase in association with lipid droplets of which TG is a major component. Triacsin C, a natural ACSL inhibitor from *Streptomyces aureofaciens*, was found to be highly effective against rotavirus replication. Thus, novel triacsin C analogs were synthesized and evaluated for their efficacies against the replication of rotaviruses in cells. Many of the analogs significantly reduced rotavirus replication, and one analog (**1e**) was highly effective at a nanomolar concentration range (ED_{50} 0.1 μM) with a high therapeutic index in cell culture. Our results suggest a crucial role of lipid metabolism in rotavirus replication, and triacsin C and/or its analogs as potential therapeutic options for rotavirus infections.

1. Introduction

Rotaviruses are non-enveloped, icosahedral viruses with an 11-segment double-stranded RNA genome [1]. The capsid of rotavirus is composed of the outer capsid proteins, VP4 and VP7, and the major inner capsid protein, VP6. Rotaviruses are divided into 7 (A to G) antigenically distinct serogroups based on VP6. Among them, group A rotaviruses are the leading cause of severe gastroenteritis in infants and children worldwide, associated with over 500,000 deaths in children younger than 5 years of age each year, although attenuated live vaccines are available [1–3]. Majority of rotavirus infection-associated mortality occurs in the developing countries. Nonetheless, nearly 1 in 80 children is hospitalized with rotavirus gastroenteritis by 5 years of age in the US [1–3]. Since there are no specific antiviral agents for rotavirus infection, the treatment options for rotavirus infection are limited to providing oral rehydration solution to restore and maintain hydration until the infection resolves [4]. However, the development of rotavirus antivirals to reduce the severity of diseases and duration of rotavirus-related hospitalization has been impeded by limited information on the therapeutic targets for rotavirus infections.

Previously it was shown that disruption of lipid rafts and/or lipid droplets decrease infectious rotaviruses by inhibition of rotavirus morphogenesis [5,6]. Lipid droplets are cellular organelles for storage of neutral fats such as TG and cholesterol ester, and play a crucial role in regulating cellular lipid levels. Lipid rafts are microdomains in cell membrane enriched in cholesterol, glycosphingolipids, and proteins. These structures are found important for infectious virus particle formation of human hepatitis C virus (HCV) [7,8] and dengue virus [9], suggesting the importance of lipid homeostasis in virus replication. Recently our group has demonstrated that rotavirus replication induced an increase in the TG levels in cells, and suppression of increase in TG levels by the farnesoid X receptor (FXR) agonists significantly inhibited rotavirus replication [10].

Lipogenesis is the process of producing fats from acetyl-CoA, which is then stored as an energy source. During lipogenesis, fatty acid (FA) is synthesized (de novo synthesis) from acetyl-CoA, and subsequently esterified with glycerol to form TG. Numerous enzymes participate in lipogenesis, which include fatty acid synthase (FASN), long chain fatty acid acyl-CoA synthetase (ACSL) 1–6, and diacylglycerol acyltransferase (DGAT) 1/2 and Acyl-CoA:cholesterol acyltransferase (ACAT)1/2 [11–15]. Lipolysis is the reverse pathway of lipogenesis to generate acetyl-CoA and energy from TG in which process multiple enzymes including lipases are involved. In this study, we found that the commercially available inhibitors for FASN, ACSL, DGAT and ACAT significantly reduced the replication of rotaviruses *in vitro*. Since triacsin C, a fungal metabolite from *Streptomyces aureofaciens*, is an ACSL inhibitor and showed the most potent

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inhibition on rotavirus replication, we synthesized its analogs and examined their antiviral effects against rotavirus. The synthetic sequence is straightforward and efficient (Schemes 1 and 2), which would provide flexibility for further optimization. Among the triacsin C and its analogs, **1e** is the most potent against rotavirus replication with the effective dose that reduce 50% of the virus replication (ED_{50}) of 0.1 μM. Our results suggest that lipogenic enzymes may represent potential therapeutic targets for rotavirus infection, and triacsin C analogs may be useful as therapeutic options for rotavirus infection.

2. Biochemical studies

The effects of commercially available inhibitors for lipogenesis as well as newly synthesized triacsin C analogs (described below) were examined against in rotavirus replication using SA11 rotavirus strain in MA104 cells. Various chemical inhibitors including FASN inhibitors (cerulenin and C75), ACSL inhibitors (triacsin C and troglitazone), DGAT inhibitors (A922500 and betulinic acid), and ACAT inhibitors (CI-976, hexadecylamino-*p*-amino benzoic acid [PHB]) (Fig. 1) were obtained from Sigma–Aldrich and Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

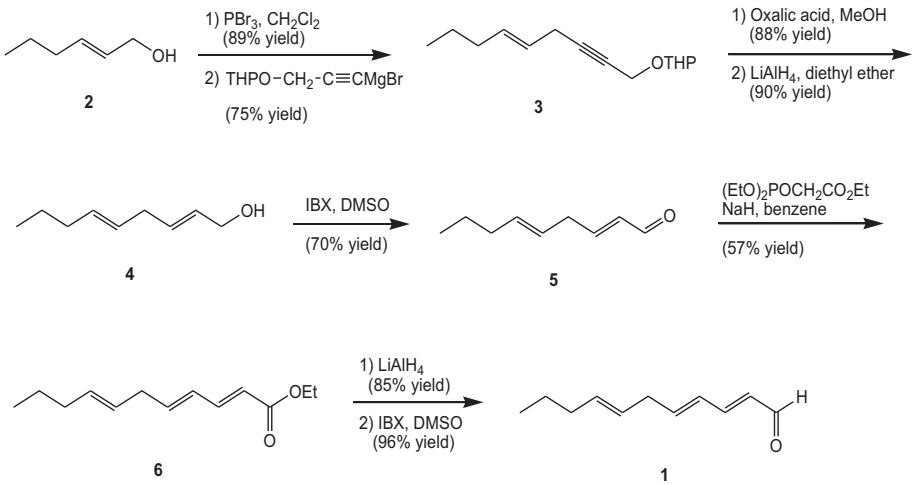
3. Chemistry

Various triascin C analogs were synthesized from (*E*,*E*,*E*)-2,4,7-undecatrienal (**1**), which was prepared by a modification of the reported procedure [16,17] (Scheme 1). Hence, bromination of (*E*)-2-hexen-1-ol (**2**) with phosphorus tribromide in dichloromethane followed by a displacement reaction with 3-(tetrahydropyranyloxy) propynyl magnesium bromide and a catalytic amount of copper cyanide afforded enyne **3**. Removal of the THP protecting group of **3** with oxalic acid in refluxing methanol followed by selective reduction of the propargylic alcohol function with lithium aluminum hydride in diethyl ether gave (*E*,*E*)-2,5-nonadien-1-ol (**4**). Oxidation of the hydroxyl function of **4** with *o*-iodoxybenzoic acid (IBX) in DMSO provided aldehyde **5**. A two-carbon extension utilizing the Horner–Wadsworth–Emmons protocol was carried out by the treatment of aldehyde **5** with triethyl phosphonacetate and sodium hydride in benzene furnished all-*trans* ester **6** in 57% yield. When THF was used as solvent, under similar reaction conditions only (*E*,*E*,*E*)-2,4,6-undecatrienoate (31% yield) was isolated, and **6** was not found. Reduction of ester **6** with lithium aluminum hydride followed by oxidation with IBX and DMSO afforded trienal **1** in 82% overall yield (from ester **6**).

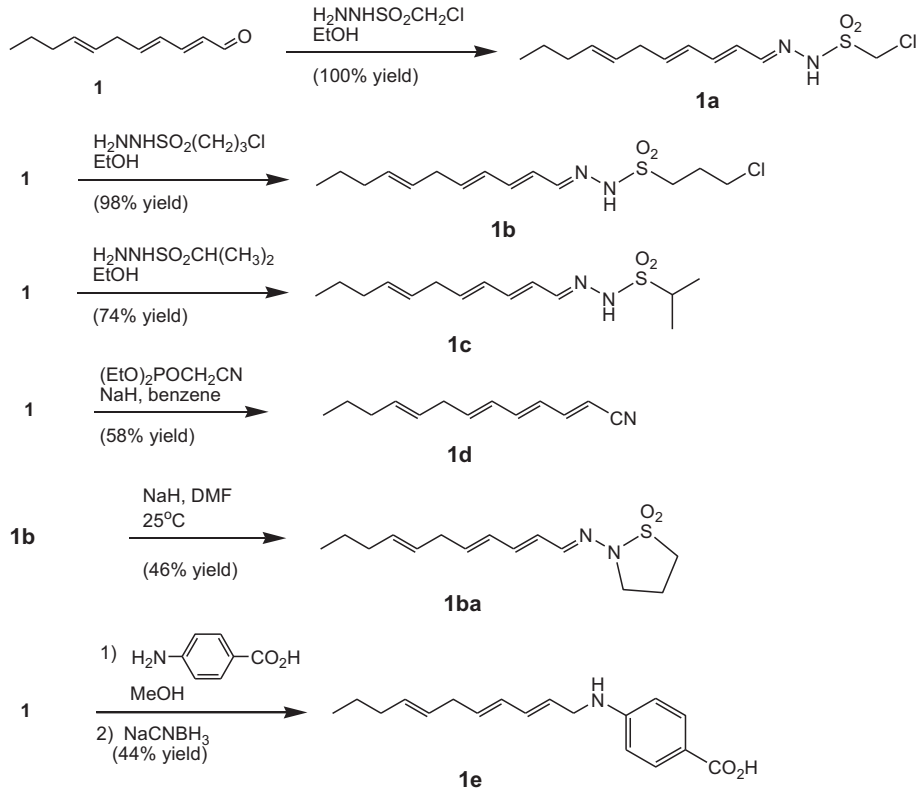
Triacsin C analogs including hydrazone derivatives **1a**–**1c** and **1ba**, cyano analog **1d**, and aminobenzoic acid **1e** were synthesized for bio-evaluation. Hydrazone formation of **1** with chloromethanesulfonohydrazide, 3-chloropropane-1-sulfonohydrazide, and propane-2-sulfonohydrazide separately in ethanol at room temperature gave hydrazones **1a**, **1b**, and **1c**, respectively, in good to excellent yields (Scheme 2). Intramolecular cyclization of **1b** with sodium hydride in *N*,*N*-dimethylformamide (DMF) at 25 °C gave **1ba**. It should be noted the aforementioned hydrazones are stable under silica gel column chromatographic conditions. Cyano compound **1d** was obtained from a Horner–Wadsworth–Emmons olefination of aldehyde **1** with diethyl cyanomethylphosphonate and sodium hydride [18]. Reductive amination of aldehyde **1** with 4-aminobenzoic acid in methanol followed by sodium cyanoborohydride produced **1e**. Chloromethanesulfonohydrazide, 3-chloropropane-1-sulfonohydrazide, and propane-2-sulfonohydrazide were prepared by the treatment of chloromethanesulfonyl chloride, 3-chloropropane-1-sulfonyl chloride, and propane-2-sulfonyl chloride, respectively, with 40% hydrazine (in aqueous solution) in THF at 25 °C for 2 h. After aqueous workup, extraction with diethyl ether, and concentration, the crude hydrazine intermediates were used in subsequent steps without purification. It should be noted that the synthesis of triacsin C from aldehyde **1** has been reported by Tanaka et al. [17] in less than 0.5% yield from a three-step sequence of reactions. **1e** not only has a greater bioactivity than triacsin C, its chemical yield is 44% prepared from aldehyde **1** in one step.

4. Results and discussion

All inhibitors, cerulenin, C75, triacsin C, troglitazone, A922500, betulinic acid, CI-976 and PHB, significantly reduced the replication of SA11 rotavirus in cells with ED_{50} values of 0.2–28.5 μM (Table 1). The ED_{50} values of cerulenin, C75, A922500, betulinic acid and PHB were similar at 11.3–28.5 μM, while troglitazone and CI-976 had lower ED_{50} values of 5.8 μM and 4.3 μM, respectively (Table 1). The TD_{50} of the inhibitors in MA104 cells varied at 8.5–85.4 μM (Table 1). Among the tested commercial inhibitors, triacsin C was the most effective against rotavirus replication with ED_{50} of 0.2 μM. The *in vitro* therapeutic index of triacsin C was 248. Similar ED_{50} values of the inhibitors were observed against Wa rotavirus strain, and SA11 rotavirus strain was used for further studies. These results



Scheme 1. Preparation of 2,4,7-undecatrienal (**1**).



Scheme 2. Syntheses of triacsin C analogs.

indicate that disruption of lipogenesis induced by rotavirus replication at any step significantly inhibited the virus replication.

Triacsin C is an analog of polyunsaturated FA, having an 11-carbon alkenyl chain with a *N*-hydroxytriazene moiety at the terminus, and competitively inhibits ACSL 1, 3, and 4 [21]. ACSL is a family of enzymes that converts long chain FA to acyl-CoA, which serves as a substrate at each cascading step in the synthesis of various lipid molecules including TG and cholesteryl ester. In

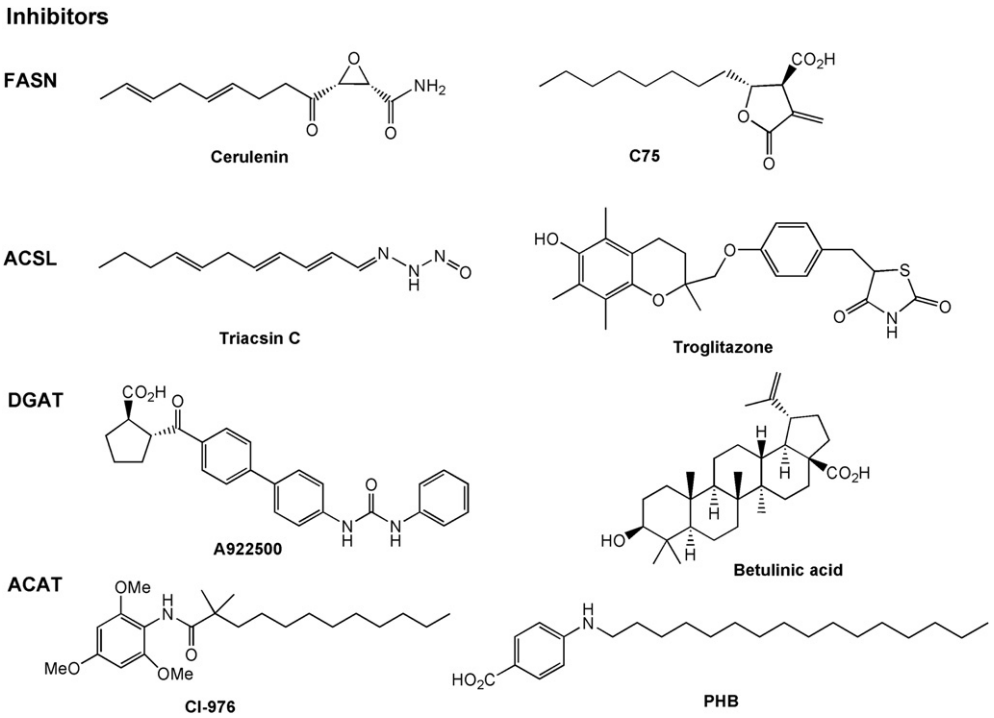


Fig. 1. Structures of inhibitors specific for FASN, ACSL, DGAT and ACAT.

Table 1
The effects of the inhibitors of lipogenic enzymes and triacsin C analogs in the replication of rotavirus.

Class	Inhibitors	Rotavirus (SA11)	
		ED_{50} (μM)[a]	TD_{50} (μM)
FASN	Cerulenin	28.5	56.7
	C75	21.2	28.5
ACSL	Triacsin C	0.2	49.6
	Troglitazone	5.8	18.5
DGAT	A922500	23.2	85.4
	Betulinic acid	22.5	27.8
ACAT	CI-976	4.3	8.5
	PHB	11.3	75.5
Triacsin C analogs	**1a**	8.7	74.0
	1b	11.5	86.3
	1c	9.8	90.2
	1d	21.3	78.8
	1ba	2.2	86.4
	1e	0.1	28.5

[a] Each ED_{50} and TD_{50} value was the average of at least 3 independent tests.

mammals, five isoforms of ACSLs are identified (ACSL 1, 3–6) [12]. In lipid droplet-enriched fractions from huh-7 cells, ACSL 3 is the most abundant isoform and ACSL 4 is a minor constituent [19]. The role of ACSL3 seems to be important in the synthesis of neutral lipids in lipid droplets, suggested by the finding that the addition of oleic acid as a source of FA in the cells led to the accumulation of lipid droplets in association with increased levels of ACSL 3 in lipid droplets [20].

By inhibiting ACSL, triacsin C consequently blocks the synthesis of TG, diglycerides, and cholesterol esters [22]. Troglitazone, a member of the thiazolidinediones family, is an anti-diabetic and anti-inflammatory drug. It is also known to inhibit ACSL4 (but not other ACSL isoforms), and decreased the size of lipid droplets in cells [23]. In our study, both triacsin C and troglitazone significantly reduced the replication of rotavirus. Furthermore, addition of triacsin C directly to the medium of cell culture showed potent antivirus effects against rotavirus replication, suggesting that triacsin C readily penetrates the cells.

Since triacsin C strongly inhibited rotavirus replication, we synthesized various analogs of triacsin C, and examined their effects in rotavirus replication and toxicity in MA104 cells. The synthetic analogs effective against rotaviruses are shown in Table 1 with ED_{50} and TD_{50} values, and therapeutic indexes. These analogs have a conserved 11-carbon chain with various functionalities at the alkylidene-terminal replacing 1,2,3-triazene of triacsin C. The presence of various sulfonylhydrazones and cyano functions (**1a**–**1ba**) at the alkylidene-terminal end decreased the antiviral effects compared to triazene moiety. However, **1e** that contains aminobenzoic acid at the alkylidene-terminal end is highly effective against rotavirus replication with lower ED_{50} (0.1 μM) and higher *in vitro* therapeutic index (285), compared to those of triacsin C. Antirotaviral effects of triacsin C, **1ba**, and **1e** were also confirmed with Western blot analysis and IFA (Fig. 2). The pre-treatment of SA11 rotavirus with triacsin C (100 μM) or **1e** (100 μM) did not affect the viral titer, indicating that the antiviral effects of triacsin C and **1e** are not the result of direct viral neutralization or virucidal effect on rotavirus. The suppression of an increase in lipid droplets by triacsin C, **1ba** and **1e** was dose-dependent in Huh-7 cells and positively correlated ($r^2 = 0.89$ and 0.87 for **1ba** and **1e**, respectively) with reduction of rotavirus replication in MA104 cells (Fig. 3). The results suggest that the inhibition of virus replication by these compounds is associated with the suppression of lipid accumulation in the cells triggered by viral infection.

In this study, we demonstrated that rotavirus replication is closely associated with lipid metabolism, and inhibition of lipogenic enzymes significantly decreased rotavirus replication. We have also synthesized novel triacsin C analogs by modification of the alkylidene-terminal end for evaluation of anti-rotavirus activity, and found one of the novel analogs, **1e**, was highly effective against rotavirus replication. The facile and high-yielding synthesis of the analogs would provide flexibility for further optimization. Based on these findings, the lipogenic enzymes may serve as potential therapeutic targets for rotavirus infection and triacsin C analogs as potential therapeutic options for rotavirus infections.

5. Experimental

5.1. General

NMR spectra were obtained from a 400-MHz spectrometer (Varian Inc.), in $CDCl_3$, unless otherwise indicated, and reported in ppm. Mass spectra were taken from an API 2000-triple quadrupole ESI-MS/MS mass spectrometer (from Applied Biosystems). Chemicals and solvents including chloromethanesulfonyl chloride, 3-chloropropane-1-sulfonyl chloride, and propane-2-sulfonyl chloride were purchased from Fisher Scientific and Aldrich Chemical Co. A modification of the reported procedure [16,17] was used to prepare (*E*,*E*,*E*)-2,4,7-undecatrienal (**1**), and spectral data of all synthetic intermediates including ^{13}C NMR data (which were not reported in previous publications [16,17]) are included herein.

5.2. Representative synthesis

5.2.1. (*E*)-1-(2-Tetrahydropyranyl)oxy-nona-5-en-2-yne (**3**)

To a solution of 10.0 g (0.1 mol) of *E*-2-hexen-1-ol (**2**) in 100 mL of dichloromethane at −10 °C under argon, was added 13.5 g (0.05 mol) of PBr_3. After stirring at 25 °C for 3 h, the reaction solution was washed with a saturated aqueous solution of $NaHCO_3$ (50 mL), water, and brine, dried ($MgSO_4$), concentrated to give 14.5 g (89% yield) of *E*-1-bromo-2-hexene, which was used in the following step without purification. ^1H NMR δ 5.8–5.65 (m, 2H, =CH), 3.96 (d, $J = 7$ Hz, 2H), 2.05 (q, $J = 7$ Hz, 2H), 1.41 (sextet, $J = 7$ Hz, 2H), 0.91 (t, $J = 7$ Hz, 3H); ^{13}C NMR δ 136.7, 126.7, 34.3, 33.9, 22.2, 13.8; MS (electrospray) *m*/*z* 165.1 and 163.1 ($M + H^+$; 100%; bromine isotopes).

To a solution of 15 g (0.18 mol) of dihydropyrane and 2 mg of *p*-toluenesulfonic acid was added 10 g (0.18 mol) of 2-propyn-1-ol. After stirring at 25 °C for 3 h, the reaction solution was diluted with 100 mL of diethyl ether and washed with 10% aqueous $NaHCO_3$, water, and brine, dried ($MgSO_4$), concentrated to give 20.5 g (82% yield) of 2-(2-propynyloxy)-tetrahydropyrane. ^1H NMR δ 4.83 (t, $J = 3.6$ Hz, 1H, CHO), 4.29 (dd, $J = 16$, 3 Hz, 1H, CH_2O), 4.26 (dd, $J = 16$, 3 Hz, 1H, CH_2O), 3.88–3.82 (m, 1H, CHO), 3.58–3.52 (m, 1H, CHO), 2.42 (d, $J = 3$ Hz, 1H, ≡CH), 1.90–1.50 (m, 6H); ^{13}C NMR δ 97.0, 79.9, 74.2, 62.1, 54.1, 30.4, 25.5, 19.1; MS (electrospray; negative mode) *m*/*z* 139.0 ($M - H^-$, 100%). MS (electrospray; positive mode) *m*/*z* 163.1 ($M + Na^+$; 100%).

To a solution of 18 mL (18 mmol) of 1 M ethylmagnesium bromide in THF under argon at 0 °C, were added 1.7 g (12 mmol) of 2-(2-propynyloxy)-tetrahydropyrane and 4 mg of CuCN. After stirring for 0.5 h, 2.0 g (12 mmol) of *E*-1-bromo-2-hexene was added, and the resulting solution was stirred at 25 °C for 0.5 h, neutralized with 0.1 N HCl, and extracted with diethyl ether three times. The combined ether extract was washed with water and brine, dried ($MgSO_4$), concentrated, and column chromatographed on silica gel using a mixture of hexane and diethyl ether (20:1) to give 2.0 g (75% yield) of compound **3**: ^1H NMR δ 5.66 (dt, $J = 16$, 7 Hz, 1H, =CH), 5.40 (dt, $J = 16$, 5.6 Hz, 1H, =CH), 4.83 (t, $J = 3$ Hz, 1H, CHO), 4.33 (dt, $J = 16$, 2.4 Hz, 1H, CH_2O), 4.24 (dd, $J = 16$, 2.4 Hz, 1H, CH_2O), 3.89–3.82 (m, 1H, CHO), 3.57–3.51 (m,, CHO), 2.95 (d, $J = 3.6$ Hz,

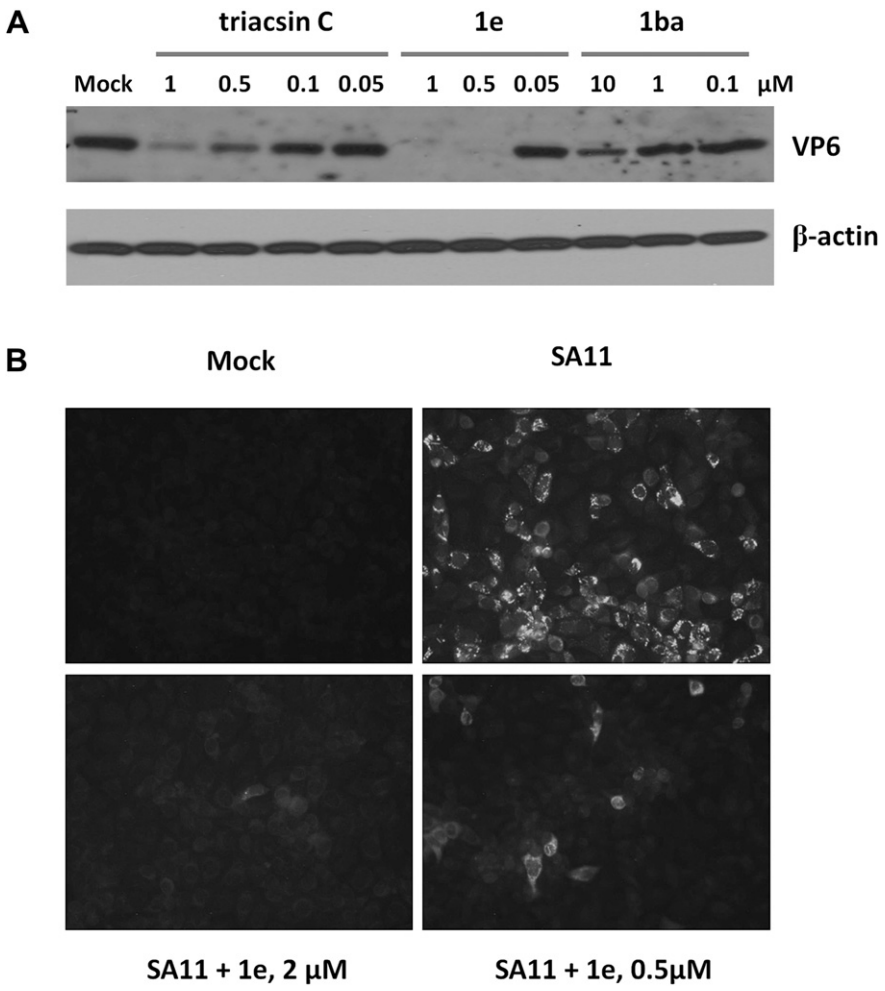


Fig. 2. The effects of triacsin C, and its analogs in rotavirus (SA11) replication determined by Western blot analysis (A) and IFA (B). SA11 rotavirus was inoculated to the cells at a MOI of 2, and cells were incubated with or without each inhibitor (triacsin C, **1e** or **1ba**). Cell lysates were collected at 12 h post infection (A) and virus infected cells were fixed at 12 h post infection (B) at the indicated concentrations.

2H), 2.00 (q, $J = 7$ Hz, 2H), 1.90–1.50 (m, 6H), 1.39 (sextet, $J = 7$ Hz, 2H), 0.90 (t, $J = 7$ Hz, 3H); ^{13}C NMR δ 132.5, 124.1, 96.9, 84.4, 77.6, 62.2, 54.9, 34.6, 30.5, 25.6, 22.6, 22.3, 19.3, 13.9; MS (electrospray) m/z 245.2 ($M + Na^+$; 100%).

*5.2.2. (E,E)-2,5-Nanodien-1-ol (**4**)*

A solution of 7.2 g (32 mmol) of compound **3** in 60 mL of 2% oxalic acid in methanol was heated under reflux for 2 h, and most of the methanol was removed under a rotary evaporator. The residue

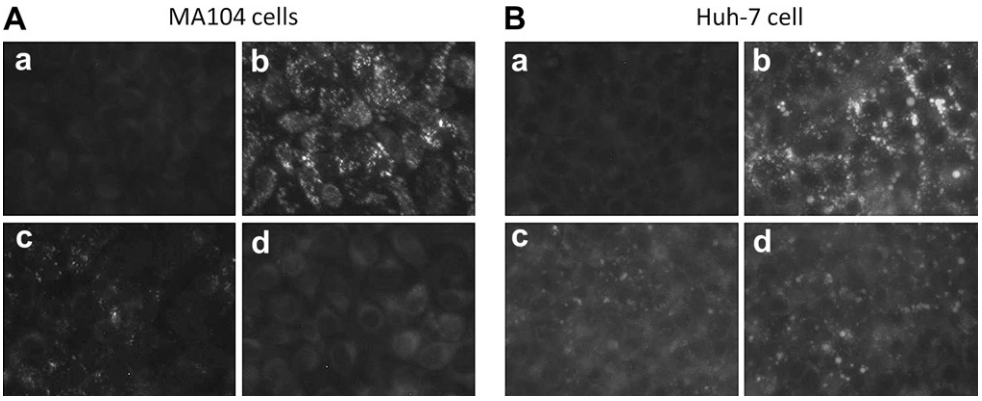


Fig. 3. Lipid droplets monitored with NBD-cholesterol in rotavirus (SA11) infected MA104 cells (A) or uninfected Huh-7 cells (B). A. Lipid droplets in MA104 cells infected with SA11 rotavirus with a MOI of 10. Panels, a and b: cells incubated with mock-medium (a) or SA11 rotavirus (b) for 10 h. Panels, c and d: SA11 rotavirus infected cells incubated in the presence of triacsin C (1 μM) (c) or **1e** (1 μM) (d) for 10 h. B. The development of lipid droplets in Huh-7 cells incubated with mock-medium, triacsin C, or **1e**. Panel a: 1 day old cells; b: 4 day old cells; c and d: 4 day old cells incubated in the presence of triacsin C (1 μM) (c) or **1e** (1 μM) (d).

was diluted with diethyl ether (100 mL), washed with water and brine, dried ($MgSO_4$), concentrated, and column chromatographed on silica gel using a gradient mixture of hexane and diethyl ether as eluant to give 3.94 g (88% yield) of *E*-5-nonen-2-yn-1-ol. ^{1}H NMR δ 5.67 (dt, $J = 16$, 7 Hz, 1H, =CH), 5.39 (dt, $J = 16$, 5.6 Hz, 1H, =CH), 4.29 (dt, $J = 6$, 2 Hz, 2H, CH_2O), 2.96 (d, $J = 2$ Hz, 2H), 2.00 (q, $J = 7$ Hz, 2H), 1.39 (sextet, $J = 7$ Hz, 2H), 0.90 (t, $J = 7$ Hz, 3H); ^{13}C NMR δ 132.6, 123.9, 84.4, 80.0, 51.7, 34.6, 22.6, 22.2, 13.9; MS (electrospray) *m*/*z* 161.1 ($M + Na^+$; 100%).

To a solution of 5.5 g (40 mmol) of *E*-5-nonen-2-yn-1-ol in 50 mL of diethyl ether under argon was added 1.5 g (39 mmol) of $LiAlH_4$ in portions. After stirring at 25 °C for 24 h, the reaction mixture was quenched with 50 mL of 1 N NaOH, and extracted with diethyl ether three times (50 mL each). The combined ether extracts were washed with water and brine, dried ($MgSO_4$), concentrated to give 5.0 g (90% yield) of compound **4**, which was used in the next step without further purification. ^{1}H NMR δ 5.75–5.6 (m, 2H, =CH), 5.50–5.35 (m, 2H, =CH), 4.12 (t, $J = 4$ Hz, 2H, CH_2O), 2.75 (t, $J = 5$ Hz, 2H), 1.99 (q, $J = 7$ Hz, 2H), 1.38 (sextet, $J = 7$ Hz, 2H), 0.89 (t, $J = 7$ Hz, 3H); ^{13}C NMR δ 131.9 (2 C), 129.6, 127.8, 63.9, 35.4, 34.9, 22.8, 22.2, 13.9; MS (electrospray) *m*/*z* 163.1 ($M + Na^+$; 100%).

5.2.3. (E,E)-2,5-Nonadienal (**5**)

To a solution of 5.0 g (36 mmol) of compound **4** in 13 mL of DMSO under argon at 25 °C was added 12.5 g (45 mmol) of *o*-iodoxybenzoic acid (IBX), and the solution was stirred for 4 h, diluted with diethyl ether (100 mL), and washed with water twice (50 mL each). The aqueous layers were extracted with diethyl ether, and the combined ether layers were washed with brine, dried ($MgSO_4$), concentrated, and column chromatographed on silica gel using hexane and diethyl ether (20:1) as eluant to give 3.45 g (70% yield) of aldehyde **5**. ^{1}H NMR δ 9.53 (d, $J = 7$ Hz, 1H, CHO), 6.85 (dt, $J = 16$, 7 Hz, 1H, =CH), 6.13 (dd, $J = 16$, 8 Hz, 1H, =CH), 5.55 (dt, $J = 15$, 5 Hz, 1H, =CH), 5.43 (dt, $J = 15$, 5 Hz, 1H, =CH), 3.03 (t, $J = 7$ Hz, 2H, CH_2), 2.01 (q, $J = 7$ Hz, 2H), 1.39 (sextet, $J = 7$ Hz, 2H), 0.91 (t, $J = 7$ Hz, 3H); ^{13}C NMR δ 194.2, 157.3, 134.4, 133.2, 124.6, 35.8, 34.8, 22.6, 13.8; MS (electrospray) *m*/*z* 161.2 ($M + Na^+$; 100%).

5.2.4. Ethyl (E,E,E)-2,4,7-Undecatrienoate (**6**)

To a mixture of 0.35 g (8.7 mmol) of sodium hydride (60% in oil; pre-washed with diethyl ether twice to remove mineral oil) in 20 mL of benzene under argon was added 1.95 g (8.7 mmol) of triethyl phosphonacetate, and the solution was stirred at 25 °C for 2 h. To it, a solution of 1.0 g (7.2 mmol) of compound **5** in 2 mL of benzene was added via cannula under argon. After stirring for 0.5 h, the reaction solution was neutralized with 0.1 N HCl, and extracted twice with diethyl ether. The combined extract was washed with water and brine, dried ($MgSO_4$), concentrated, and column chromatographed on silica gel using hexane and diethyl ether (30:1) as eluant to give 0.64 g (57% yield based on recovered starting material) of compound **6** and 0.25 g (25% recovery) of compound aldehyde **5**. ^{1}H NMR δ 7.27 (dd, $J = 16$, 10 Hz, 1H, =CH), 6.22–6.09 (m, 2H, =CH), 5.80 (d, $J = 16$ Hz, 1H, =CH), 5.52–5.37 (m, 2H, =CH), 4.20 (q, $J = 7$ Hz, 2H), 2.86 (t, $J = 6$ Hz, 2H, CH_2), 1.99 (q, $J = 7$ Hz, 2H), 1.39 (sextet, $J = 7$ Hz, 2H), 1.30 (t, $J = 7$ Hz, 3H), 0.90 (t, $J = 7$ Hz, 3H); ^{13}C NMR δ 167.5, 145.0, 142.8, 132.9, 128.8, 126.5, 119.8, 60.4, 36.1, 34.9, 22.7, 14.5, 13.9; MS (electrospray) *m*/*z* 231.0 and 163.1 ($M + Na^+$; 100%), 209.2 ($M + H^+$).

5.2.5. (E,E,E)-2,4,7-Undecatrienal (**1**)

To a solution of 0.63 g (3.0 mmol) of compound **6** in 20 mL of diethyl ether under argon at 0 °C was added 0.12 g (3.0 mmol) of $LiAlH_4$, and the solution was stirred for 1 h, diluted with water carefully, and extracted with diethyl ether twice. The combined extracts were washed with water and brine, dried ($MgSO_4$), and concentrated to give 0.43 g (85% yield) of (*E*,*E*,*E*)-2,4,7-undecan-1-ol. ^{1}H NMR δ 6.23 (dd, $J = 15$, 11 Hz, 1H, =CH), 6.05 (dd, $J = 15$, 11 Hz, 1H, =CH), 5.78–5.67 (m, 2H, =CH), 5.47–5.37 (m, 2H, =CH), 4.16 (q, $J = 6$ Hz, 2H, CHO), 2.78 (t, $J = 6$ Hz, 2H, CH_2), 1.98 (q, $J = 7$ Hz, 2H), 1.37 (sextet, $J = 7$ Hz, 2H), 0.89 (t, $J = 7$ Hz, 3H); ^{13}C NMR δ 134.1 (2 C), 132.1, 130.1 (2 C), 129.9, 127.7, 63.7, 35.8, 34.9, 22.8, 13.9; MS (electrospray) *m*/*z* 205.1 ($M + K^+$; 100%), 189.1 ($M + Na^+$).

To a solution of 0.43 g (2.6 mmol) of (*E*,*E*,*E*)-2,4,7-undecan-1-ol in 3 mL of DMSO under argon was added 0.91 g (3.3 mmol) of IBX, and the solution was stirred at 25 °C for 4 h, diluted with dichloromethane (50 mL), and washed with water (50 mL). The aqueous layer was extracted with dichloromethane, and the combined organic layers were washed with brine (20 mL), dried ($MgSO_4$), concentrated, and column chromatographed on silica gel using hexane and diethyl ether (20:1) as eluant to give 0.41 g (96% yield) of aldehyde **1**. ^{1}H NMR δ 9.55 (d, $J = 8$ Hz, 1H, CHO), 7.10 (dd, $J = 15$, 9 Hz, 1H, =CH), 6.37–6.24 (m, 2H, =CH), 6.10 (dd, $J = 15$, 7.6 Hz, 1H, =CH), 5.55–5.38 (m, 2H, =CH), 2.91 (t, $J = 9.6$ Hz, 2H, CH_2), 2.01 (q, $J = 7$ Hz, 2H), 1.40 (sextet, $J = 7$ Hz, 2H), 0.91 (t, $J = 7$ Hz, 3H); ^{13}C NMR δ 194.1, 152.8, 145.4, 133.4, 130.6, 129.0, 126.0, 36.3, 34.9, 22.7, 13.9; MS (electrospray) *m*/*z* 187.1 ($M + Na^+$, 100%).

5.2.6. (E)-Chloro-N′-((2E,4E,7E)-undeca-2,4,7-trienylidene) methanesulfonohydrazide (**1a**)

A solution of 20 mg (0.12 mmol) of (*E*,*E*,*E*)-2,4,7-undecatrienal (**1**) and 35 mg (0.24 mmol) of chloromethanesulfonohydrazide in 1 mL of ethanol was stirred under argon at 25 °C for 2 h, and ethanol was removed under a rotary evaporator. The residue was diluted with 20 mL of diethyl ether, washed with water (15 mL) three times, and brine (15 mL). The organic layer was dried ($MgSO_4$), concentrated, and column chromatographed on silica gel using a 1:1 mixture of hexane and diethyl ether as eluent to give 35 mg (100% yield) of **1a**: ^{1}H NMR ($CDCl_3$) δ 7.91 (s, 1H, NH), 7.55 (d, $J = 10$ Hz, 1H, N=CH), 6.56 (dd, $J = 15$, 10 Hz, 1H), 6.27–6.15 (m, 2H), 5.97 (dt, $J = 12$, 7 Hz, 1H), 5.47–5.38 (m, 2H), 4.70 (s, 2H, CH_2Cl), 2.85 (t, $J = 7$ Hz, 2H), 1.99 (q, $J = 7$ Hz, 2H), 1.39 (sextet, $J = 7$ Hz, 2H), 0.90 (t, $J = 7$ Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 152.0, 142.3, 140.0, 132.8, 129.7, 126.7, 125.3, 53.0, 36.1, 34.9, 22.7, 13.9; MS (electrospray) *m*/*z* 313.0 ($M + Na^+$; 100%).

5.2.7. (E)-3-Chloro-N′-((2E,4E,7E)-undeca-2,4,7-trienylidene) propane-1-sulfonohydrazide (**1b**)

A similar procedure to that described above was followed. From 20 mg (0.12 mmol) of aldehyde **1** and 42 mg (0.24 mmol) of 40% hydrazine, 38 mg (98% yield) of **1b** was obtained after column chromatographic purification. ^{1}H NMR ($CDCl_3$) δ 8.08 (s, 1H, NH), 7.49 (d, $J = 10$ Hz, 1H, N=CH), 6.52 (dd, $J = 15$, 10 Hz, 1H), 6.27–6.14 (m, 2H), 5.94 (dt, $J = 12$, 7 Hz, 1H), 5.49–5.38 (m, 2H), 3.68 (t, $J = 7$ Hz, 2H, CH_2Cl), 3.43 (dd, $J = 7$, 3 Hz, 2H, CH_2S), 2.84 (t, $J = 7$ Hz, 2H), 2.31 (pentet, $J = 7$ Hz, 2H), 1.99 (q, $J = 7$ Hz, 2H), 1.37 (sextet, $J = 7$ Hz, 2H), 0.89 (t, $J = 7$ Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 150.4, 141.3, 139.2, 132.7, 129.8, 126.9, 125.7, 48.8, 42.8, 36.0, 34.9, 26.7, 22.7, 13.9; MS (electrospray) *m*/*z* 341.0 ($M + Na^+$; 100%).

5.2.8. (E)-N′-[(2E,4E,7E)-Undeca-2,4,7-trienylidene]propane-2-sulfonohydrazide (**1c**)

Following a similar reaction sequence as that described for **1a**, from 25 mg (0.15 mmol) of aldehyde **1** and 42 mg (0.31 mmol) of propane-2-sulfonohydrazide, gave 32 mg (74% yield) of **1c**. ^{1}H NMR ($CDCl_3$) δ 7.90 (s, 1H, NH), 7.48 (d, $J = 10$ Hz, 1H, N=CH), 6.50 (dd, $J = 15$, 10 Hz, 1H), 6.24 (dd, $J = 15$, 10 Hz, 1H), 6.16 (dd, $J = 14$, 10 Hz,

1H), 5.92 (dt, $J = 15$, 7 Hz, 1H), 5.46–5.37 (m, 2H), 3.53 (heptet, $J = 7$ Hz, 1H, CH), 2.83 (t, $J = 7$ Hz, 2H), 1.99 (q, $J = 7$ Hz, 2H), 1.41 (d, $J = 7$ Hz, 6H), 1.38 (sextet, $J = 7$ Hz, 2H), 0.89 (t, $J = 7$ Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 149.4, 140.6, 138.8, 132.6, 129.9, 126.9, 126.0, 52.4, 36.0, 34.9, 22.7, 16.4, 13.9; MS (electrospray) m/z 307.1 ($M + Na^+$; 100%).

5.2.9. (2E,4E,6E,9E)-Trideca-2,4,6,9-tetraenenitrile (**1d**)

To a mixture of 15 mg (0.36 mmol) of sodium hydride (washed twice with distilled anhydrous diethyl ether) in 1 mL of benzene under argon was added 64 mg (0.36 mmol) of diethyl cyanomethylphosphonate, and the solution was stirred at 25 °C for 2 h. To it, a solution of 40 mg (0.24 mmol) of aldehyde **1** in 1 mL of benzene was added via syringe. The resulting solution was stirred for 1 h, diluted with water (10 mL), neutralized with 0.1 N HCl solution, and extracted with diethyl ether three times (20 mL each). The combined extract was washed with brine, dried ($MgSO_4$), concentrated, and column chromatographed on silica gel using a 30:1 mixture of hexane and diethyl ether as eluant to give 26 mg (58% yield) of **1d**. ^{1}H NMR ($CDCl_3$) δ 7.00 (dd, $J = 15$, 10 Hz, 1H), 6.52 (dd, $J = 15$, 10 Hz, 1H), 6.22–6.05 (m, 2H), 6.00 (dt, $J = 15$, 7 Hz, 1H), 5.46–5.38 (m, 2H), 5.28 (d, $J = 15$ Hz, 1H), 2.86 (t, $J = 7$ Hz, 2H), 1.99 (q, $J = 7$ Hz, 2H), 1.39 (sextet, $J = 7$ Hz, 2H), 0.90 (t, $J = 7$ Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 150.6, 142.0, 140.7, 132.8, 129.7, 127.5, 126.7, 195.0, 97.2, 36.1, 34.9, 22.7, 13.9; MS (electrospray) m/z 210.1 ($M + Na^+$; 100%).

5.2.10. 2-[(2E,4E,7E)-Undeca-2,4,7-trienylideneamino]-1,1-dioxo-1-isothiazolidine (**1ba**)

A solution of 15 mg (47 μmol) of **1b** and 2.0 mg (50 μmol) of sodium hydride in 0.5 mL of DMF (distilled) under argon was stirred at 25 °C for 3 days. The resulting solution was neutralized with diluted aqueous HCl solution, and extracted with diethyl ether twice (25 mL each). The combined extract was washed with water three times (20 mL each), and brine (20 mL), dried ($MgSO_4$), concentrated, and column chromatographed on silica gel using a 1:1 mixture of hexane and diethyl ether as eluant to give 6 mg (46% yield) of **1ba**. ^{1}H NMR ($CDCl_3$) δ 7.54 (d, $J = 10$ Hz, 1H, N=CH), 6.54 (dd, $J = 15$, 10 Hz, 1H), 6.32 (dd, $J = 15$, 10 Hz, 1H), 6.18 (dd, $J = 15$, 10 Hz, 1H), 5.91 (dt, $J = 15$, 7 Hz, 1H), 5.46–5.40 (m, 2H), 3.59 (t, $J = 7$ Hz, 2H), 3.22 (t, $J = 7$ Hz, 2H), 2.84 (t, $J = 7$ Hz, 2H), 2.49 (pentet, $J = 7$ Hz, 2H), 1.99 (q, $J = 7$ Hz, 2H), 1.38 (sextet, $J = 7$ Hz, 2H), 0.89 (t, $J = 7$ Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 151.5, 140.8, 138.6 (2C), 132.6, 130.1, 126.8, 46.3, 45.3, 36.1, 34.9, 30.6, 22.8, 18.0, 13.9; MS (electrospray) m/z 305.2 ($M + Na^+$; 100%).

5.2.11. 4-((2E,4E,7E)-Undeca-2,4,7-trienylamino)benzoic acid (**1e**)

A solution of 30 mg (0.18 mmol) of aldehyde **1** and 24 mg (0.18 mmol) of 4-aminobenzoic acid in 5 mL of methanol was stirred under argon at 25 °C for 1 h. To it, 10 μL of acetic acid and 30 mg (0.48 mmol) of sodium cyanoborohydride were added sequentially. After stirring at 25 °C for 12 h, the reaction solution was diluted with aqueous ammonium chloride, and extracted with diethyl ether twice (25 mL each). The combined extract was washed with brine, dried ($MgSO_4$), concentrated and column chromatographed on silica gel using a gradient mixture of hexane and ethyl acetate as eluant to give 23 mg (44% yield) of **1e**. ^{1}H NMR ($CDCl_3$) δ 7.93 (d, $J = 8$ Hz, 2H, Ar), 6.58 (d, $J = 8$ Hz, 2H, Ar), 6.25 (dd, $J = 15$, 10 Hz, 1H), 6.06 (dd, $J = 15$, 10 Hz, 1H), 5.75–5.64 (m, 2H), 5.45–5.40 (m, 2H), 3.86 (d, $J = 7$ Hz, 2H, CH_2H), 2.78 (t, $J = 7$ Hz, 2H), 1.98 (q, $J = 7$ Hz, 2H), 1.38 (sextet, $J = 7$ Hz, 2H), 0.90 (t, $J = 7$ Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 171.8, 152.6, 134.0, 132.9, 132.5, 132.1, 129.7, 127.6, 127.1, 117.6, 111.9, 45.3, 35.8, 34.9, 22.8, 13.9; MS (electrospray) m/z 308.1 ($M + Na^+$; 100%).

6. Biochemical studies

6.1. Cells, antisera and reagents

The MA104 cell line was maintained in minimum essential medium (MEM) containing 5% fetal bovine serum and antibiotics (penicillin and streptomycin). Antibodies specific to rotavirus VP6 or β-actin were obtained from Santa Cruz Biotechnology Inc. Rotavirus strains including Wa (human G1) and SA11 (primate G3) were obtained from American Type Culture Collection (ATCC, Manassas, VA).

6.2. Nonspecific cytotoxic effects

Confluent MA104 grown in 24-well plates were treated with various concentrations of each compound for 24 h. Cell cytotoxicity was measured by a CytoTox 96® non-radioactive cytotoxicity assay kit (Promega, Madison, WI), and TD_{50} value of each compound was determined using the method of Litchfield and Wilcoxon [24].

6.3. Rotavirus infection and treatments in cell culture

Fully confluent monolayered MA104 cells grown in 6- or 12-well plates were inoculated with Wa or SA11 rotavirus at high (2) or low (0.01) multiplicity of infection (MOI) for 1 h. Following washing step with phosphate buffered saline (PBS), MEM containing each compound or DMSO (0.1%) was added to each well. In addition, trypsin (Sigma–Aldrich) was included at 1 μg/mL in medium for rotavirus replication. The concentration of each compound in the cells were less than 50 μM for cerulenin, C75, triacsin C, various triacsin C analogs, A922500 and PHB, and less than 20 μM for betulinic acid, and CI-976. As a control, nitazoxanide was used at 0.5–5 μM. Virus replication was monitored at 12, 24 and 48 h post infection by immunofluorescent assay (IFA) or Western blot analysis with antibody against VP6, and the 50% tissue culture infectious dose ($TCID_{50}$)/mL was also determined. The ED_{50} was calculated based on $TCID_{50}$ titers at 24 h post infection (with 0.05 MOI) for each compound using the method of Litchfield and Wilcoxon [24].

6.4. Pre-treatment of viruses with triacsin C or **1e**

To investigate if triacsin C or its analog **1e** has virucidal effects on rotavirus, SA11 rotavirus of high titer ($>10^9$ $TCID_{50}$/mL) was pre-incubated with PBS (or DMSO), triacsin C (200 μM) or **1e** (200 μM) at 37 °C for 2 h. Then the mixture was diluted up to 100 times for cell inoculation. Virus infected cells were incubated with medium containing trypsin for up to 24 or 48 h, and the virus replication was monitored by the titration of progeny viruses using the $TCID_{50}$ method.

6.5. Determination of viral replication using IFA, Western blot analysis and $TCID_{50}$ method

Virus replication in cell culture was assessed by the IFA or Western blot analysis at 12 or 24 h post infection before the extensive cytopathic effects (CPE) are induced by viral infection. Viral replication was also titrated with the $TCID_{50}$ method at 24 and 48 h post infection after cells were lysed with repeated freezing and thawing. *IFA*: cells were fixed with 100% methanol at room temperature for 30 min. Then, monoclonal antibody specific for rotavirus VP6 was applied to the cells followed by goat anti-mouse IgG conjugated fluorescein isothiocyanate (FITC). The stained cells were observed under a fluorescence microscope. *Western blot analysis*: The expression levels of VP6 in the presence of each compound were also assessed by Western blot analysis. The cells were lysed at 12 h post infection, and the cell lysates were prepared

in SDS-PAGE sample buffer containing 1% β-mercaptoethanol, and sonicated for 20 s. The proteins were resolved in a 10% Novex Tris-Bis gel (Invitrogen) and transferred to a nitrocellulose membrane. The membranes were probed with monoclonal antibody specific for rotavirus VP6, and the binding of the antibodies was detected with peroxidase-conjugated goat anti-mouse IgG. In addition, separate membranes were probed with antiserum specific for β-actin, and appropriate peroxidase-conjugated secondary antibodies were used. Following incubation with a chemiluminescent substrate (Pierce Biotechnology, Rockford, IL), signals were detected on X-ray film. *$TCID_{50}$ method*: A standard $TCID_{50}$ method with the 10-fold dilutions of each sample was used for virus titration in MA104 cells according to the Reed and Muench method [25].

6.6. Lipid staining of cells in the absence or presence of rotavirus infection

Confluent MA104 cells in 96-well plates were infected with mock-medium or SA11 rotavirus at an MOI of 10. Virus infected cells were treated with various concentrations of mock (DMSO, 0.1%), cerulenin, C75, triacsin C, **1e**, A922500, betulinic acid, CI-976 or PHB, and incubated with medium with NBD-cholesterol (1 μg/mL) and trypsin (1 μg/mL). NBD-cholesterol rapidly distributes in the cells and targets lipid droplets by binding specifically to lipid droplet-specific protein with high affinity [26]. At 10 h after virus infection, cells were fixed with 4% formaldehyde for 10 min, followed by washing with PBS twice. Then the fluorescence signals from NBD-cholesterol in the cells were observed under a fluorescent microscope.

In human liver carcinoma Huh-7 cells, lipid droplets develop spontaneously in cell culture [27]. In separate experiments, confluent Huh-7 cells in opaque 96-well plates were treated with various concentrations of mock (DMSO, 0.1%), cerulenin, C75, triacsin C, **1e**, A922500, betulinic acid, CI-976, or PHB and incubated for 72 h. After 72 h, the medium containing NBD-cholesterol (1 μg/mL) was replaced and cells were further incubated for 12 h. Then cells were fixed with 4% formaldehyde for 10 min, and washed with PBS. The fluorescence signals of the cells were observed under a fluorescent microscope.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2012.02.010. ^{1}H and ^{13}C NMR spectra of compounds **1a** – **1e** are included in the Supplementary data.

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