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Short-chain 3-ketoceramides, strong apoptosis inducers against human leukemia HL-60 cells

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Abstract—Ceramides act as a second messenger of the apoptotic signaling process. The allylic alcohol portion comprising the C-3, C-4, and C-5 carbons is essential for this function. The suggestion has been made that this alcohol moiety is oxidized in mitochondria to a carbonyl moiety, with the generation of reactive oxygen species. However, there is no established precedent for the apoptotic performance of 3-ketoceramides thus presumed. In this work, we have synthesized three different types of short-chain 3-ketoceramides, that is, (2*S*,4*E*)-2-acetylamino-3-oxo-4-octadecen-1-ol (**A**), (2*S*,4*E*,6*E*)-2-acetylamino-3-oxo-4,6-octadecadien-1-ol (**B**), and (2*S*,4*E*)-2-acetylamino-1-methoxy-3-oxo-4-octadecene (**C**), and demonstrated that these 3-ketoceramides are capable of inducing effective apoptosis in human leukemia HL-60 cells. In particular, the two monoenoic compounds, **A** and **C**, are far more powerful than the corresponding alcoholic analogue, *N*-acetyl-D-*erythro*-sphingosine. Observations of DNA fragmentation, caspase-3 activation, and cytochrome *c* release from mitochondria provide substantiated evidence for mitochondrial apoptosis and the effects of exogenous glutathione on these phenomena are also discussed.

1. Introduction

Sphingolipids are ubiquitous membrane components of all eukaryotic cells and play a physiologically important role in bioorganisms.[1] The metabolites or precursors of sphingolipids, ceramides (*N*-acyl-D-*erythro*-sphingosines), are an important second messenger involved in mediating a variety of cell functions, especially, apoptosis.[2] Some biosubstances and environmental stresses, such as the anti-Fas antibody,[3] tumor necrosis factor, α[4] and ionizing radiation,[5] promote apoptotic ceramide generation by enhancing the mitochondrial sphingomyelinase-mediated hydrolysis of sphingomyelin (the so-called 'sphingomyelin cycle').[6] Exogenously added bacterial sphingomyelinase is capable of inducing apoptosis by increasing endogenous ceramide levels.[7] In addition, daunorubicin, an anti-cancer reagent, which activates neutral sphingomyelinase, or glucosylceramide-synthase inhibitors such as D-*erythro*-2-(*N*-myristoylamino)-1-phenyl-1-propanol, *N*-oreoylethanolamine, etc. could also increase endogenous ceramide levels.[8,9]

Some exogenous cell-permeable ceramides such as *N*-acetyl- (**C2-Cer**) and *N*-octanoyl-D-*erythro*-sphingosine (**C8-Cer**) induce apoptosis, even when exogenously added.[10] In particular, **C2-Cer** is widely used as a standard tool for the study of apoptosis. Related dihydroceramides, which lack the 4,5-*trans* C–C double bond, are biologically inactive.[10] Meanwhile, the mitochondrial oxidation of the C-3 hydroxy group of ceramides to produce the corresponding 3-ketoceramides together with the reactive oxygen species (ROS) has been proposed as the primary step of ceramide-induced apoptosis.[11] For the purpose of proving this assumption, Chun et al. synthesized 4,6-diene-ceramide which, compared to normal ceramide, is expected to be readily oxidized to the dienone analogue in mitochondria,[12] and Struckhoff et al. indicated that 4,6-diene-ceramides are more potent than ceramide in inducing apoptosis via the mitochondrial pathway in MCF-7 cells.[13] It is implied, accordingly, that the Michael addition of mitochondrial glutathione (GSH) to the dienone product would cause a decreased concentration of the ROS scavenger GSH, and then an increase in ROS concentration, which

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increase is responsible for apoptotic cell death. However, no detailed study has been reported so far, for not only the cell-death inducing ability of the 3-ketoceramide but also the formation of the 3-ketoceramide-GSH adduct.

In this work, we examined whether the allylic ketone part of 3-ketoceramides is critical for the cell death event in the human leukemia HL-60 cell. For this purpose, three new 3-ketoceramides with a short acyl-chain were prepared: (2*S*,4*E*)-2-acetylamino-3-oxo-4-octadecen-1-ol (**A**, Fig. 1), (2*S*,4*E*,6*E*)-2-acetylamino-3-oxo-4,6-octadecadien-1-ol (**B**), and (2*S*,4*E*)-2-acetylamino-1-methoxy-3-oxo-4-octadecene (**C**).

2. Syntheses

The short-chain 3-ketoceramide **A** was obtained through its precursor, (2*S*,4*E*)-3-ketosphingosine hydrochloride (prepared according to a previous method[14]). The N-acetylation of the HCl salt with acetic acid using WSC in the presence of *N*-hydroxybenzotriazole (HOBt) and Et_3N gave compound **A** in 57% yield. The diene analogue **B** was derived from N,O-protected dienone **1** (prepared according to a previous method[12]) by its O-deprotection with 10% HCl followed by N-acetylation according to the procedure mentioned above, giving the desired compound **B** in 34% yield (Scheme 1).

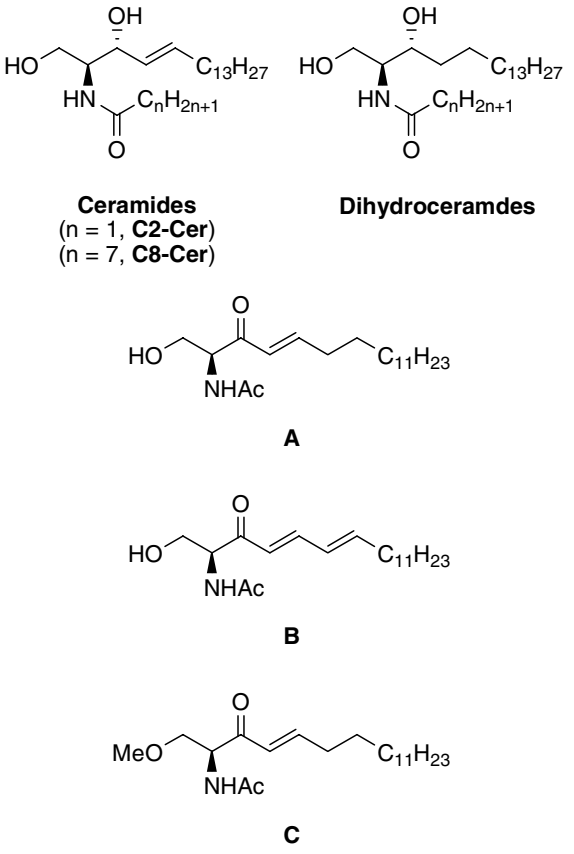
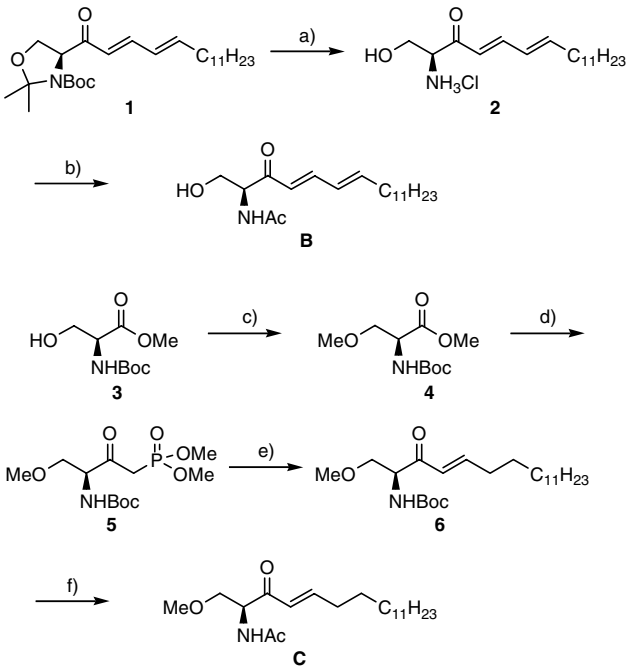


Figure 1. Structures of short-chain ceramides and 3-ketoceramides.



Scheme 1. Reagents and conditions: (a) 10% HCl, MeOH, reflux; (b) AcOH, WSC, HOBt, Et_3N, CH_2Cl_2, rt; (c) MeI, Ag_2O, acetone, reflux; (d) $LiCH_2PO(OMe)_2$, THF, −78 °C; (e) tetradecanal, Et_3N, LiCl, THF, rt; (f) TFA, rt, then Ac_2O, pyridine, rt.

The 1-methoxy analogue **C** was prepared following Scheme 1. (2*S*)-*N*-Boc-serine methyl ester **3**[15] was O-methylated with methyl iodide in the presence of silver oxide to provide the N,O-protected serine ester **4** in 61% yield. The obtained serine ester **4** was treated with excess lithium dimethyl methylphosphonate to be converted to the β-ketophosphonate **5** in 69% yield. The Horner–Wadsworth–Emmons (HWE) olefination of the phosphonate **5** with tetradecyl aldehyde using Et_3N as a base in the presence of anhydrous LiCl at −78 °C in THF afforded *N*-Boc-1-*O*-methyl-3-ketosphingosine **6** in 70% yield. After removal of the Boc protection with trifluoroacetic acid (TFA), the resulting amine was immediately acetylated to give compound **C** in 72% yield.

3. Biological evaluations and discussion

3.1. Cell death

Evaluation of the antileukemic property of the newly synthesized C2-3-ketoceramides (compounds **A**, **B**, and **C**) was made by MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay. HL-60 cells were treated for 6 h with several different concentrations (1–20 μM) of each analogue. As Figure 2 shows, the C2-3-ketoceramides were all high in activity compared with **C2-Cer**, a widely used apoptosis inducer. In particular, compounds **A** and **C** maintained high levels of activity even at a low concentration such as 1 μM, where about 70% of the cells were brought to death in 6 h. The fact that both **A** and its 1-methoxy derivative **C** exhibit nearly the same activity suggests that the hydroxy moiety at

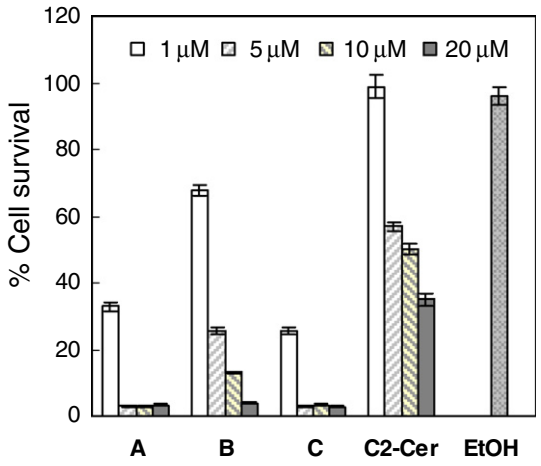


Figure 2. Antileukemic effects of short-chain ceramide and 3-ketoceramides against HL-60 cells. Cells were treated with the indicated concentrations of each compound for 6 h.

the 1-position plays no critical role against the antileukemic cell death event. The activity of the dienone **B** was intermediate between those of **A** and **C2-Cer**, and fairly close to that of the former rather than the latter.

3.2. DNA fragmentation

Next, we investigated whether C2-3-ketoceramides could induce DNA fragmentation, a typical phenomenon accompanying apoptosis. Figure 3 shows that DNA fragmentation with 5 μM of **A** and **C** was completed within just 2 h, which leads us to expect that compounds **A** and **C** will be promising, practical apoptosis inducers. However, **C2-Cer** was so much less active as to require four times the concentration and more than 4 h to accomplish the same extent of fragmentation.

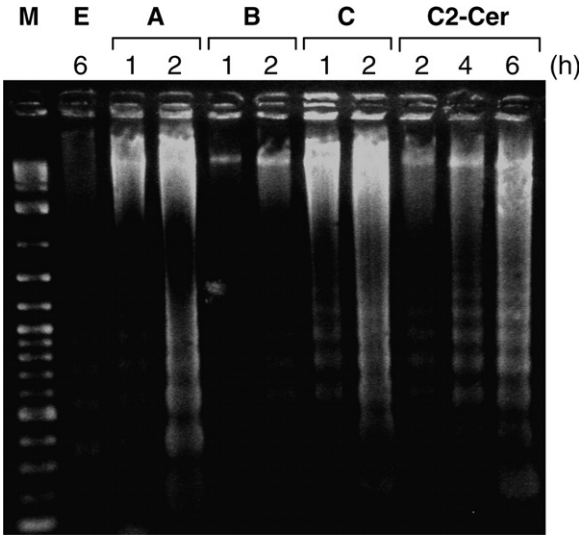


Figure 3. Agarose gel electrophoresis of DNA following treatment of HL-60 cells with short-chain ceramide and 3-ketoceramides. HL-60 cells (1×10^6 cells/mL) were exposed to C2-3-ketoceramides (5 μM) or **C2-Cer** (20 μM) for various periods. Each genomic DNA was subjected to agarose gel electrophoresis; M, marker; E, ethanol vehicle.

The dienone **B** caused no fragmentation even after 2 h. These results are consistent with those of the MTT assay mentioned above.

3.3. Caspase-3 activation

Caspases, a family of aspartate-specific cysteine proteases, play a crucial role in apoptotic cell death by cleaving a specific site of numerous cellular targets in the execution phase; in particular, caspase-3 could be activated by the proteolytic processing of pro-caspase-3 in response to exogenous apoptosis inducers. In order to elucidate the involvement of caspase-3 in the active form in the C2-3-ketoceramides-induced cell death process in the HL-60 cell, we attempted to analyze the amount of caspase-3 activated in response to exogenously added C2-3-ketoceramides by the Western blot analysis employing a polyclonal anti-caspase-3 antibody. The upper panels of Figure 4a display time courses of 2–6 h for the generation of active caspase-3 induced by the addition of the three C2-3-ketoceramides (5 μM) or **C2-Cer** (5 or 20 μM), indicating that all of the 3-ketoceramides can activate caspase-3 within 2 h and their activity exceeds that of **C2-Cer**; in particular, caspase-3 activation treated with compound **A** or **C** was completed in 2 h. Figure 5 shows time courses of 30–90 min for caspase-3 activation by 5 μM of the C2-3-ketoceramides. The activated caspase-3 appeared after 1 h treatment with compounds **A** and **C**. We also investigated whether poly(ADP-ribose) polymerase (PARP), a proteolytic target of the activated caspase-3, could be cleaved by the 3-ketoceramides. As the lower panels of Figure 4a indicate, any analogue can cleave PARP after a delay of the caspase-3 activation.

It has been generally accepted that ceramides induce apoptosis in HL-60 cells through a mitochondrial signaling pathway.[16] Although C2-3-ketoceramides activate caspase-3, there is the possibility that the executor caspase-3 could be directly activated by the initiator caspase-8 in such a way that the Fas-mediated apoptosis takes the non-mitochondrial pathway.[17] To determine whether C2-3-ketoceramide-induced apoptosis adopts the non-mitochondrial pathway, HL-60 cells were pretreated with the caspase-8-specific inhibitor Ac-IETD-cho or the broad caspase inhibitor *Z*-VAD-fmk for 30 min before addition of compound **A**. Caspase-3 activation and subsequent PARP cleavage were completely inhibited by *Z*-VAD-fmk, but not inhibited at all by Ac-IETD-cho (Fig. 4b). Although caspase-8 can also activate the mitochondrial pathway of apoptosis by cleaving Bid, this result demonstrates that the caspase-3 mediation by 3-ketoceramide **A** does not pass through caspase-8 activation.

3.4. Mitochondrial pathway

In order to determine the contribution of the mitochondrial pathway when HL-60 cells were treated with the C2-3-ketoceramides as well as **C2-Cer**, Western blot analysis was performed. The results are summarized in Figure 6a, which shows that the mitochondrial cytochrome *c* release from the mitochondria to the cytoplasm

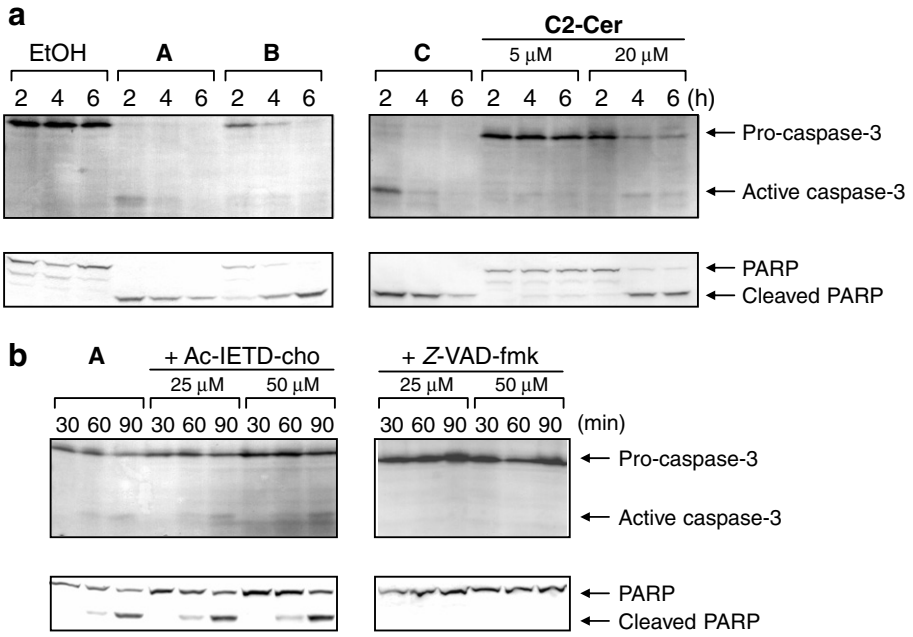


Figure 4. Activation of caspase-3 and PARP cleavage induced by C2-3-ketoceramides. (a) HL-60 cells were treated with C2-3-ketoceramides (5 μM) or **C2-Cer** (5 or 20 μM) for various periods (2–6 h); (b) HL-60 cells were preincubated with Ac-IETD-cho or *Z*-VAD-fmk for 30 min and then incubated with compound **A** (5 μM). Each cell extract was subjected to Western blot analysis using anti-caspase-3 or anti-PARP antibody.

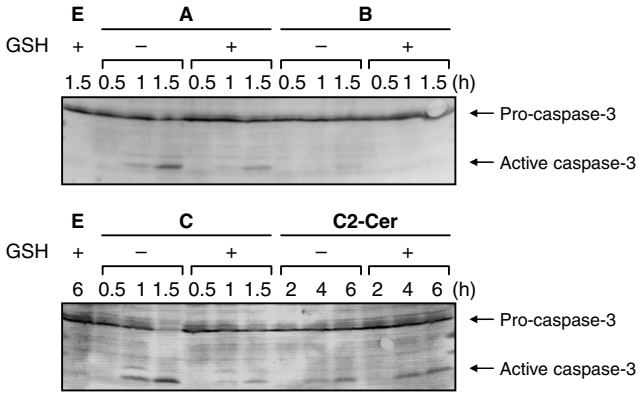


Figure 5. Inhibition effect of C2-3-ketoceramides-induced caspase-3 activation by extracellularly supplemented GSH. HL-60 cells were pretreated with GSH (10 mM) for 30 min and then incubated with C2-3-ketoceramides (5 μM) or **C2-Cer** (20 μM). Each cell extract was subjected to Western blot analysis using anti-caspase-3; E, ethanol vehicle.

takes place in a time-dependent manner, as with the result of caspase-3 activation. Thus, the immunoblot demonstrates also that the C2-3-ketoceramides can give rise to rapid mitochondrial apoptosis of HL-60 cells.

3.5. Exogenous GSH effect

Radin has recently proposed that the oxidation of the C-3 hydroxy group of ceramides by mitochondrial ubiquinone in complex III could produce the corresponding ketoceramides together with ROS.[11] The 3-ketoceramide thus formed would trap GSH through Michael addition. As a result of the GSH deletion, ROS would be allowed to increase, eventually leading to apoptosis. In fact, markedly enhanced apoptosis of HL-60 cells by extracellularly added C2-3-ketoceramides appears to support the above scenario. Furthermore, in order to understand the vital role of GSH in C2-3-ketoceramide-mediated apoptosis in more detail, we examined the influence of pretreatment of the cells with GSH on the dynamics of two successive processes, that is, the mitochondria-to-cytoplasm cytochrome *c* release and the caspase-3 activation. The results are displayed in Figures 5 and 6, which indicate that GSH pretreatment indeed was able to largely suppress both of those consecutive processes in the C2-3-ketoceramide-driven apoptosis. However, GSH cannot be a depressor for releasing mitochondrial cytochrome *c*, because the **C2-Cer**-driven apoptosis was not affected by GSH pretreatment at all, as anticipated also from the above scenario.

One possible explanation for these suppressions is as follows: unlike **C2-Cer** having no keto group, 3-ketoceramides undergo Michael addition with GSH during their migration to the mitochondria, and then, the Michael addition lowers the effective concentration of the 3-ketoceramides which triggers the cell death signaling. This explanation might have been supported by the result of Schultz et al. that 0.1375 mM GSH underwent Michael addition to simple α,β-enone compounds at rather high concentrations of the millimolar order in pH 7.4 aqueous buffered solution.[18] However, no remarkable data have been presented for the Michael addition to 3-ketoceramides themselves. Figure 7a shows a considerable decreasing change in 230 nm absorbance due to the enone **A** in the presence of GSH, strongly suggesting that enone **A** has the capability of reacting with GSH even under the conditions of UV measurements (0–2 mM GSH in pH 7.4 buffered solution at 37 °C). Thus, we could acquire chemical

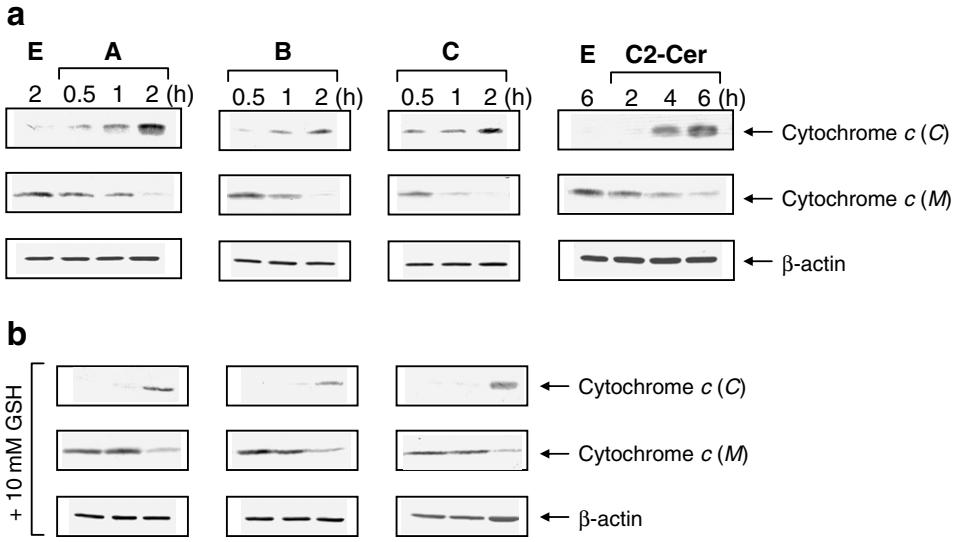


Figure 6. Cytochrome *c* release from mitochondria to the cytoplasm induced by C2-3-ketoceramides. (a) HL-60 cells were treated with C2-3-ketoceramides (5 μM) or **C2-Cer** (20 μM) for various periods, and both the cytosolic (*C*) and mitochondrial (*M*) fractions were subjected to Western blot analysis using anti-cytochrome *c* antibody; (b) the same experiments were carried out after pretreatment with 10 mM GSH for 30 min; E, ethanol vehicle.

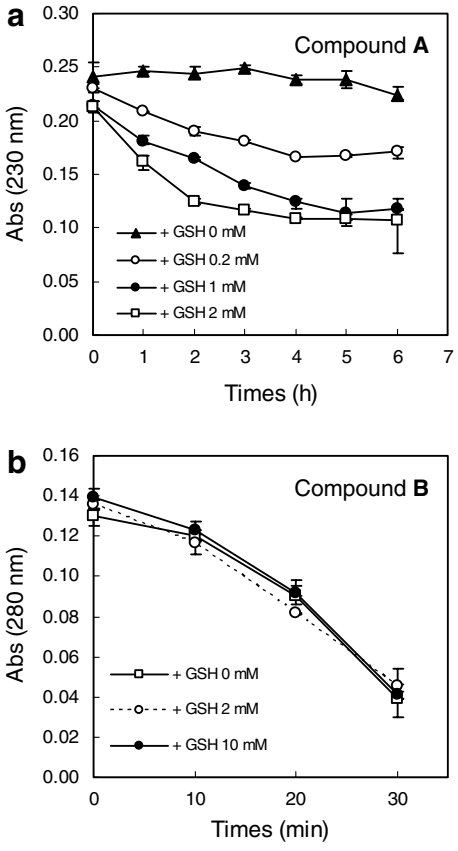


Figure 7. Effects of GSH on C2-3-ketoceramides. Compounds **A** (0.2 mM) and **B** (0.05 mM) were incubated with GSH (0–10 mM) in aqueous buffer solutions (pH 7.4) for the indicated times at 37 °C and each absorbance (a: 230 nm, b: 280 nm) was plotted.

evidence for the Michael reaction of the enone **A** and GSH in such concentrations that are close to the intracellular GSH concentration.

On the other hand, the observed diminution in the dienone **B** absorbance at 280 nm, the results being revealed also from Figure 7b, is not attributed to the Michael addition, but merely to a self-decomposition of the dienone substrate in buffer, because time-tracing of absorbance change is independent of the GSH concentration. It is evident, therefore, that the relatively low activity of compound **B** versus **A** can be favorably accounted for entirely by this instability.

Thus, we acquired definite, chemical evidence for the Michael addition of enone **A** and GSH in aqueous buffered solution. However, it is still unclear whether the anti-apoptotic effect of additional GSH on enone **A**-induced apoptosis is due to the reduction in effective 3-ketoceramide level by the Michael addition, because the anti-apoptotic effect of exogenous GSH was also observed in the case of dienone **B**. Further pursuing of the detailed mechanism of the 3-ketoceramide-induced apoptosis is currently in progress in this laboratory.

3.6. Summary

We demonstrated, for the first time, that 3-ketoceramides have a high apoptotic activity against HL-60 cells, and are now studying apoptosis by C2-3-ketoceramides against other cell lines. For example, compound **A** also shows a great activity over **C2-Cer** in HeLa cells (data not shown). Some synthetic methods for N,O-protected 3-ketosphingosines have been reported as utilizable precursors of D-*erythro*-sphingosine,[14,19] because, as compared with a normal ceramide (or sphingosine) having two chiral centers, 3-ketoceramide (or 3-ketosphingosine) having only one chiral center is easily prepared. 3-Ketoceramides may be a better motif than normal ceramides for developing new ceramide-like anti-cancer agents, accordingly.

4. Experimental

All materials obtained commercially (guaranteed reagent grade) were used without further purification. All solvents were freshly distilled under nitrogen before use; THF and CH_2Cl_2 were distilled from $LiAlH_4$ and CaH_2, respectively. Column chromatography was performed on silica gel. **C2-Cer** was prepared according to our previous report.[20] Protease inhibitors, *Z*-VAD-fmk and Ac-IETD-CHO, were purchased from the Peptide Institute (Osaka, Japan) and Sigma Chemical Co. (St. Louis, MO, USA), respectively. Polyclonal antibody for caspase-3 and monoclonal antibody for cytochrome *c* were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibodies for PARP and for β-actin were purchased from Trevigen (Gaithersburg, MD, USA) and Abcam (Cambridge, UK), respectively. Anti-rabbit and anti-mouse IgGs coupled to alkaline phosphatase were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

4.1. (2*S*,4*E*)-2-acetylamido-3-oxo-4-octadecen-1-ol (A)

To a suspension of acetic acid (14 mg, 0.234 mmol), WSC (45 mg, 0.234 mmol), and HOBt (36 mg, 0.234 mmol) in dry CH_2Cl_2 (10 mL), a solution of (2*S*,4*E*)-2-amino-3-oxo-4-octadecen-1-ol · HCl[14] (60 mg, 0.18 mmol) and Et_3N (27 mg, 0.27 mmol) in dry CH_2Cl_2 (2 mL) was added dropwise, and the mixture was stirred overnight at room temperature. The resulting mixture was washed with saturated brine, dried with Na_2SO_4, and then concentrated. The obtained residue was purified by column chromatography with hexane/EtOAc (1:3) and recrystallized from hexane to give compound **A** (35 mg, 57%) as a colorless powder; mp 64 °C; $[\alpha]_D^{20}$ +9.26 (*c* 1.05, $CHCl_3$); IR (KBr) 2918, 2851, 2350, 1697, 1682, 1568, 1470, 1379, 1302, 1267, 1161, 1067, 970, 719 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$); δ 0.88 (t, 3H, J = 6.8 Hz), 1.26 (br s, 20H), 1.44–1.52 (m, 2H), 2.08 (s, 3H), 2.26 (q, 2H, J = 6.8 Hz), 3.36 (br s, 1H), 3.8–3.88 (m, 1H), 3.93–4.01 (m, 1H), 4.91 (ddd, 1H, J = 3.6, 4.8, 6 Hz), 6.27 (d, 1H, J = 15.6 Hz), 6.8 (d, 1H, J = 6 Hz), 7.11 (dt, 1H, J = 15.6, 7.2 Hz); HRMS (FAB, direct) calcd for $C_{20}H_{38}NO_3$: $[M+H]^+$ 340.2852; found: 340.2852; Anal. Calcd: C, 70.75; H, 10.98; N, 4.13. Found: C, 70.45; H, 11.05; N, 4.13.

4.2. (2*S*,4*E*,6*E*)-2-amino-3-oxo-4,6-octadecadien-1-ol hydrochloride (2)

To a solution of **1**[12] (210 mg, 0.48 mmol), in MeOH (20 mL), 10% HCl (1.2 mL) was added, and the mixture was stirred for 1 h at reflux. After cooling, the resulting mixture was washed with hexane and then concentrated. The crude product was recrystallized from *i*-PrOH/Et_2O to give **2** (80 mg, 50%) as a colorless powder; mp 130 °C; $[\alpha]_D^{25}$ +7.35 (*c* 1, MeOH); IR (KBr) 3420, 2922, 2853, 2435, 1684, 1636, 1601, 1468, 1213, 1057, 1005, 721 cm^{-1}; ^1H NMR (400 MHz, CD_3OD); δ 0.88 (t, 3H, J = 6.8 Hz), 1.29 (br s, 16H), 1.42–1.52 (m, 2H), 2.24 (q, 2H, J = 6.8 Hz), 3.95 (dd, 1H, J = 4.9, 12.2 Hz), 4.02 (dd, 1H, J = 3.7, 12.2 Hz), 4.39–4.43 (m, 1H), 6.28–6.46 (m, 3H), 7.44 (dd, 1H, J = 10.2, 15.4 Hz); HRMS (FAB, direct) calcd for $C_{18}H_{34}NO_2$, $[M-Cl]^+$ 296.259; found: 296.2586.

4.3. (2*S*,4*E*,6*E*)-2-acetylamido-3-oxo-4,6-octadecadien-1-ol (B)

The reaction was carried out as described for **C2-keto-Cer**, using **2** (50 mg, 151 μmol) to give compound **B** as a powdery solid (22 mg, 44%). The specific rotation of **B** could not be measured accurately, because the compound was decomposed by irradiation with a sodium lamp in $CHCl_3$; mp 79 °C; IR (KBr) 3233, 2927, 2851, 1684, 1630, 1752, 1466, 1379, 1283, 1144, 1069, 995, 723 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$); δ 0.88 (t, 3H, J = 6.8 Hz), 1.26 (br s, 16H), 1.39–1.75 (m, 2H), 2.09 (s, 3H), 2.21 (q, 2H, J = 7.1 Hz), 3.4 (br s, 1H), 3.81–3.89 (m, 1H), 3.92–4.02 (m, 1H), 4.9 (ddd, 1H, J = 3.2, 4.6, 6.1 Hz), 6.22 (dd, 1H, J = 10.5, 15.1 Hz), 6.24 (d, 1H, J = 15.1 Hz), 6.32 (dt, 1H, J = 15.1, 6.8 Hz), 6.83 (d, 1H, J = 6.1 Hz), 7.44 (dd, 1H, J = 10.5, 15.1 Hz); HRMS (FAB, direct) calcd for $C_{20}H_{36}NO_3$, $[M+H]^+$ 338.2695; found: 338.2691; Anal. Calcd: C, 71.18; H, 10.45; N, 4.15. Found: C, 70.92; H, 10.51; N, 4.15.

4.4. (*S*)-(*N*-Boc-*O*-methyl)serine methyl ester (4)

(*S*)-*N*-Boc-serine methyl ester **3**[15] (2.3 g, 10.5 mmol) was dissolved in dry acetone (50 mL) containing methyl iodide (15 mL) and then Ag_2O (3.77 g, 16.3 mmol) was added. The mixture was refluxed and stirred overnight. The solids were removed by filtration and the solution was concentrated. The residue was purified by column chromatography with hexane/EtOAc (4:1) to give **4** (1.31 g, 54%) as colorless oil; $[\alpha]_D^{20}$ +12.2 (*c* 1.76, $CHCl_3$); IR (NaCl) 3447, 2980, 1749, 1720, 1506, 1456, 1367, 1167, 1119, 1063, 980, 874, 779 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$); δ 1.45 (s, 9H), 3.34 (s, 3H), 3.59 (dd, 1H, J = 3.2, 9.3 Hz), 3.77 (s, 3H) 3.8 (dd, 1H, J = 3.2, 9.3 Hz), 4.38–4.44 (m, 1H), 5.37 (d, 1H, J = 8 Hz); HRMS (FAB, direct) calcd for $C_{10}H_{20}NO_5$, $[M+H]^+$ 234.1341; found: 234.134.

4.5. (3*S*)-[3-(*tert*-butoxycarbonyl)amino-4-methoxy-2-oxo-butyl]phosphonic acid methyl ester (5)

To a solution of methylphosphonic acid dimethyl ester (6.14 g, 49.5 mmol) in dry THF (150 mL), *n*-BuLi (1.6 M in hexane, 30.9 mL, 49.5 mmol) was added dropwise at −78 °C under N_2. After the mixture was stirred for 30 min at −78 °C, a solution of **4** (3.85 g, 16.5 mmol) in dry THF (20 mL) was added dropwise. The mixture was stirred for 1 h at −78 °C and then warmed up to 0 °C. The reaction mixture was poured into ice-cooled 1 M HCl and extracted with EtOAc. After drying with Na_2SO_4, the solution was concentrated, and the residue was purified by column chromatography with $CHCl_3$/MeOH (20:1) to give **5** (1.18 g, 65%) as colorless oil; $[\alpha]_D^{20}$ +35.3 (*c* 1.8, $CHCl_3$); IR (NaCl) 3300, 2977, 1711, 1520, 1367, 1252, 1169, 1117, 1032, 866, 812 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$); δ 1.45 (s, 9H), 3.18 (dd, 1H, J = 14.8, 22 Hz), 3.35 (s, 3H), 3.38 (dd, 1H, J = 14.8, 22 Hz), 3.59 (dd, 1H, J = 4.4, 9.6 Hz), 3.78 (dd, 3H, J = 1.2, 2.8 Hz), 3.81 (dd, 3H, J = 1.2, 2.8 Hz), 3.85

(dd, 1H, J = 4.0, 9.6 Hz), 4.47–4.53 (m, 1H), 5.58 (d, 1H, J = 7.2 Hz); HRMS (FAB, direct) calcd for $C_{12}H_{25}NO_7P$, $[M+H]^+$ 326.1369; found: 326.1362.

4.6. (2*S*,4*E*)-2-[(*tert*-butoxycarbonyl)amino]-1-methoxy-3-oxo-4-octadecene (6)

To a solution of dimethyl methanephosphonate **3** (1.6 g, 4.92 mmol), tetradecanal (2.09 g, 9.84 mmol) and anhydrous LiCl (625 mg, 14.8 mmol), Et_3N (1.49 g, 14.8 mmol) was added dropwise at room temperature under N_2. The mixture was stirred overnight at room temperature. The reaction mixture was treated with 1 M HCl and extracted with EtOAc. The organic layer was dried with Na_2SO_4 and concentrated. The residue was purified by column chromatography with hexane/EtOAc (10:1) to give **6** (1.42 g, 70%) as a waxy solid; mp 39 °C; $[\alpha]_D^{20}$ +10.4 (c 1.84, $CHCl_3$); IR (NaCl) 3325, 2918, 2853, 1722, 1693, 1472, 1366, 1173, 1123, 1043, 1026, 978, 716 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$); δ 0.88 (t, 3H, J = 6.8 Hz), 1.26 (br s, 20H), 1.45 (br s, 11H), 2.23 (dq, 2H, J = 1.4, 7.1 Hz), 3.32 (s, 3H), 3.63 (dd, 1H, J = 4.4, 9.8 Hz), 3.75 (dd, 1H, J = 3.6, 9.8 Hz), 4.59–4.65 (m, 1H), 5.57 (d, 1H, J = 7.6 Hz), 6.28 (d, 1H, J = 15.6 Hz), 7.01 (dt, 1H, J = 15.6, 7.1 Hz); HRMS (FAB, direct) calcd for $C_{24}H_{46}NO_4$, $[M+H]^+$ 412.343; found: 412.3427.

4.7. (2*S*,4*E*)-2-acetylamido-1-methoxy-3-oxo-4-octadecene (C)

The carbamate **6** (200 mg, 0.486 mmol) was dissolved in TFA (2 mL). After stirring for 2 h at room temperature, the solvent was evaporated in vacuo. The resulting amine · TFA salt was suspended in dry CH_2Cl_2(5 mL). Acetic anhydride (149 mg, 3 equiv) and Et_3N (1 mL) were added dropwise to the suspension, and then the mixture was stirred for 2 h at room temperature. The reaction mixture was washed with 1 M HCl, and the organic layer was dried with Na_2SO_4 and concentrated. The residue was purified by column chromatography with $CHCl_3$/MeOH (20:1) and recrystallized from hexane to give compound **C** (124 mg, 72%) as a powdery solid; mp 79 °C; $[\alpha]_D^{20}$ +7.87 (c 1.06, $CHCl_3$); IR (KBr) 3321, 2922, 2849, 1699, 1632, 1529, 1468, 1379, 1188, 1121, 1086, 972, 723 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$); δ 0.88 (t, 3H, J = 6.8 Hz), 1.26 (br s, 20H), 1.42–1.52 (m, 2H), 2.06 (s, 3H), 2.25 (q, 2H, J = 6.8 Hz), 3.31 (s, 3H), 3.67 (dd, 1H, J = 4, 10 Hz), 3.77 (dd, 1H, J = 3.6, 10 Hz), 4.94 (ddd, 1H, J = 3.6, 4.0, 7.2 Hz), 6.26 (d, 1H, J = 15.6 Hz), 6.58 (d, 1H, J = 7.2 Hz), 7.04 (dt, 1H, J = 15.6, 7.2 Hz); HRMS (FAB, direct) calcd for $C_{21}H_{40}NO_3$, $[M+H]^+$ 354.3008; found: 354.3004; Anal. Calcd: C, 71.34; H, 11.12; N, 3.96. Found: C, 71.26; H, 11.14; N, 3.95.

4.8. Cell culture

Human leukemia HL-60 cells were grown in RPMI 1640 medium containing 10% heat-incubated fetal bovine serum. On the day of the experiment, cells were washed twice in serum-free RPMI 1640 and resuspended in the serum-free medium ($0.4–1 \times 10^6$ cells/mL). Compounds **A**, **B**, **C** and **C2-Cer** were dissolved in EtOH at 1–20 mM, and added to the cells as ethanolic solutions. The final concentration of EtOH was 0.1%. Control experiments were performed with EtOH (0.1%) as the vehicle.

4.9. MTT assay

The cell viability was assessed by the reduction of MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide]. MTT is a water-soluble tetrazolium salt that is reduced by metabolically viable cells to a colored, water insoluble formazan salt. 4×10^5 cells/100 μL were plated in 96-well dishes. All of the ketoceramide analogues and **C2-Cer** were diluted into each well at the indicated concentrations. Ten microliters of 5 mg/mL MTT was added to each well 2 h before the end of the culture, and reaction was stopped by adding 100 μL of 0.04 M HCl in isopropanol. Two optical densities of the mixture were measured at 570 and 650 nm. Final values were obtained by subtracting 650 nm from 570 nm. All data were the average of at least three separate determinations.

4.10. DNA fragmentation

HL-60 cells (2×10^6 cells/2 mL) were treated with C2-ketoceramides (5 μM) or **C2-Cer** (20 μM) for various periods. Cells were collected by centrifugation at 200*g* for 5 min at 4 °C. Cells were lysed in 100 μL of a lysis buffer [10 mM Tris–HCl (pH 7.4), 10 mM EDTA, and 0.5% Triton X-100]. Soluble cell lysates were collected by centrifugation at 14,000*g* for 5 min. Cell lysates were treated for 1 h at 37 °C with RNase A (0.2 mg/mL). Proteinase K (0.2 mg/mL) was added, and the sample was incubated at 50 °C for 30 min. 5 M NaCl (20 μL) and isopropanol (120 μL) were added and the sample was incubated at −20 °C for one night. DNA pellets were collected by centrifugation at 14,000*g* for 15 min and dissolved in 20 μL of a TE buffer [10 mM Tris–HCl (pH 7.4), 1 mM EDTA]. The DNA was then electrophoresed at 50 V through 2.0% agarose gel. The DNA bands were visualized under UV light after staining with ethidium bromide.

4.11. Immunoblot analysis for whole cell extraction

HL-60 cells (2×10^6 cells/2 mL) were treated with C2-3-ketoceramides (5 μM) or **C2-Cer** (5 or 20 μM) for various periods. Cells were collected by centrifugation at 200*g* for 5 min at 4 °C, washed twice with phosphate buffer (pH 7.4), and lysed in 100 mL of lysis buffer [62.5 mM Tris–HCl (pH 6.8), 6 M urea, 10% glycerol, 2% SDS, 0.00125% bromophenol blue, and 5% β-mercaptoethanol]. Cell lysates were boiled for 3 min and separated on 8% (for PARP) or 14% (for caspase-3) SDS–polyacrylamide gels, transferred to a polyvinylidene difluoride membrane, and probed with rabbit polyclonal anti-caspase-3 antibody or monoclonal anti-PARP antibody, followed by probing with goat anti-rabbit antibody (for Caspase-3) or anti-mouse antibody (for PARP) coupled to alkaline phosphatase.

4.12. Preparation of cytosolic and mitochondrial fractions and immunoblot analysis

We prepared cytosolic and mitochondrial fractions according to Lee's method.[21] HL-60 cells (6×10^6 cells/6 mL) were treated as described above, collected by centrifugation at 200*g* for 5 min at 4 °C, and washed twice with phosphate buffer (pH 7.4). The cell pellet was resuspended in ice-cold cell extraction buffer [20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM $mgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, PMSF 100 μM, including protease inhibitor cocktail], and then homogenized with a glass Dounce B-type pestle (120 strokes). Unbroken cells, pellet nuclei, and heavy membranes were removed by centrifuging the homogenates at 1000*g* for 5 min. The supernatant was centrifuged at 14,000*g* for 30 min to provide a pellet of mitochondria-enriched heavy membrane fraction, and the resulting supernatant was further centrifuged at 100,000*g* to obtain the cytosolic fraction. The mitochondria-rich fraction was washed once with a cell extraction buffer and then solubilized in a lysis buffer [150 mM NaCl, 50 mM Tris–HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, and 1 mM EGTA]. Cytosolic and mitochondrial fractions remained frozen in tubes at −80 °C until use. Western blot analysis was carried out by using mouse monoclonal anti-cytochrome *c* antibody, as described above.

4.13. UV spectrometric analysis

Freshly prepared before use were 0.1 M HEPES buffers (pH 7.4) containing 0.2, 2, and 10 mM of reduced GSH. The aliquots of the GSH solutions were taken up in 0.5 mL-Eppendorf tubes, incubated at 37 °C. The 3-ketoceramides were diluted into each tube at the indicated concentrations. The 3-ketoceramide-free GSH solutions were also incubated and used as the blank. After incubating at 37 °C for the indicated times, each absorbance at 230 nm (for compound **A**) or 280 nm (for compound **B**) was measured on a NanoDrop ND-1000 spectrophotometer.

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