Differential Induction of Apoptosis of Leukemic Cells by Rhizochalin, Two Headed Sphingolipids from Sponge and Its Derivatives

Jun-O JIN,^{a,b} Valeria SHASTINA,^{a,b} Joo-In PARK,^{a,b} Jin-Yeong HAN,^{b,c} Tatyana MAKARIEVA,^d Sergey FEDOROV,^d Valery RASSKAZOV,^d Valentin STONIK,^{*,d} and Jong-Young KWAK^{*,a,b}

^a Department of Biochemistry, School of Medicine, Dong-A University; ^b Medical Research Center for Cancer Molecular Therapy, Dong-A University; ^c Department of Clinical Pathology, School of Medicine, Dong-A University; Busan 602–714, Korea; and ^d Pacific Institute of Bioorganic Chemistry, Far East Branch of the Russian Academy of Sciences; Vladivostok 690022, Russia. Received October 23, 2008; accepted March 3, 2009; published online March 13, 2009

Two-headed sphingolipids bear a certain similarity with sphingolipids in the structure and but differing from classical sphingolipids in α,ω -position of the basic groups. We analyzed the apoptotic effects of some two-headed sphingolipids including rhizochalin (Rhzh) and its derivatives isolated from sponge *Rhizochalina incrustata* on human leukemia HL-60 cells. Direct addition of Rhzh induced apoptosis of HL-60 cells. The aglycon of Rhzh, which has no galactosyl residue, showed a stronger ability to induce apoptotic activity than Rhzh. Rhzh congeners with acetate derivatives only weakly induced apoptosis. The usual mitochondrial membrane permeability changes and the decrease of protein levels of procaspases-8, -9, and -3 correlated with the apoptotic activity of Rhzs. These results suggest that derivatives of two-headed sphingolipids potentially induce apoptosis in mammalian cells when administered exogenously and this cell death was dependent on caspase activation pathways.

Key words rhizochalin; apoptosis; leukemia; two-headed sphingolipid; caspase

An attractive way to develop innovative in cancer chemoprevention strategies or chemotherapies is development of pharmaceutical agents that induce the death of tumor cells through apoptosis. Chemically-induced apoptosis can occur with or without the activation of caspases, a family of cysteine proteases which are constitutively expressed as inactive zymogens.^{1,2} There are two types of caspases, upstream caspases called initiator caspases (e.g., caspases-8, -9, and -10) and their downstream targets known as executioner caspases (e.g., caspases-3, -6, and -7). Two pathways of caspase activation during apoptosis have been described.³ The extrinsic pathway involves apoptosis mediated by death receptors, such as Fas or tumor necrosis factor receptors, which is dependent on the initiator caspase-8.⁴ In the intrinsic pathway, diverse pro-apoptotic signals provoke the translocation of cytochrome *c* from mitochondria to cytoplasm and caspase-9 activation, which cleaves and activates downstream caspases.

Sphingolipids are receiving an increased level of interest as anti-cancer agents because of their ability to regulate processes of cell growth, differentiation and apoptosis. Sphingolipids are found within the cell membranes of all living organisms and are known to play an important role in cellular regulation. Marine invertebrates are rich sources of sphingolipids, which are different in chemical structure and biological activity from ones in organisms that live on land. Marine sphingolipids demonstrate cytotoxic, antitumor, antimicrobial and antifungal activities. Unusual sphingolipid analogues may act on the metabolism of normal sphingolipids, or as agonists or antagonists interacting with sphingolipid recognition sites in regulatory processes.⁵

Two-headed sphingolipids from marine sponges are striking because of their rare α,ω -bifunctionalized structures and high biological activity. The first compound of this series was rhizochalin (Rhzh) (Fig. 1), isolated in 1989 by our group from the Madagascarian sponge *Rhizochalina incrustata*.⁶ The group of two-headed sphingolipids includes a limited number of compounds: Rhzh, Rhzh A, Rhzh C, Rhzh D and

rhizochalinin A from the marine sponge *Rhizochalina incrustata*,^{6–10} oceanapiside from *Oceanapia phillipensis*,¹¹ oceanalin A, Rhzh B, and rhizochalinin B from *Oceanapia* sp.,^{7,12} calyxoside from *Calyx* sp.,¹³ leucettamols A and B from *Leucetta leptorhaphis*¹⁴ and compound BRS1 from an unidentified sponge.¹⁵ Their structures have been established on the basis of spectroscopic data and chemical transformations. Previous investigation of the biological properties of two-headed sphingolipids revealed antibacterial activity of Rhzh against *Staphylococcus aureus* and cytotoxic activity against mouse Ehrlich carcinoma cells (IC₅₀, 10 μ g/ml).⁶ Another analogue of Rhzh, calyxoside is a selective DNA-damaging agent, but lacks inhibitory activity against topoisomerase I or II.¹³ Oceanalin A and oceanapiside exhibit significant antifungal activity against the pathogenic

Fig. 1. Chemical Structure of Two-Headed Sphingolipids

* To whom correspondence should be addressed. e-mail: jykwa@dau.ac.kr; stonik@piboc.dvo.ru

fluconazole-resistant yeast *Candida glabrata*.^{7,14}) Compound BRS1 was shown to inhibit protein kinase C.¹⁵)

Studies on the structure–activity relationships of natural products or synthesized analogs as cytotoxic agents provide insight on the mechanism of action of these toxins. The aim of the present work was to investigate the antileukemic activity of several compounds, including Rhz and its derivatives such as aglycon of Rhz (AglRhz), Rhz peracetate (AcRhz), peracetyl aglycon of Rhz (AcAglRhz), and aglycon of Rhz A (AglRhz A).

MATERIALS AND METHODS

Chemicals Annexin-V was obtained from BD Biosciences Clontech (Palo Alto, CA, U.S.A.). Anti-poly (ADP ribose) polymerase (PARP) and anti-Bid antibodies were purchased from Santa-Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antibodies against procaspases-3, -9, -8, and β -actin, were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). The inhibitors benzyloxycarbonyl-Asp(OMe)-Glu(O-Me)-Val-Asp(O-Me) fluoromethyl ketone (z-DEVD-fmk), benzyloxycarbonyl-Ile-Glu-Thr-Asp(OMe) fluoromethyl ketone (z-IETD-fmk), and benzyloxycarbonyl-Leu-Glu-His-Asp(OMe) fluoromethyl ketone (z-LEHD-fmk) were purchased from Calbiochem (San Diego, CA, U.S.A.). Unless stated otherwise, all other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Purification of Rhz or Its Analogs and Preparation of Its Derivatives The chemical structures of Rhz and its derivatives are shown in Fig. 1. The chemicals were extracted, purified, and prepared as described previously.^{7,9})

Cell Culture Human promyelocytic HL-60 cells and human acute monocytic leukemia THP-1 (THP-1) cells were obtained from the Korean Cell Line Bank (Seoul National University, Seoul, Korea) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin, and were incubated at 37 °C in a humidified atmosphere of 95% air/5% CO₂.

Neutral Red Release Assay Cytotoxicity was studied using the neutral red release assay according to Borenfreund and Puerner.¹⁶) The neutral red release assay is an *in vitro* viability test, based on the incorporation of neutral red stain into the lysosomes of viable cells. Briefly, 1×10^4 exponentially growing cells were seeded in 96-well plates with Rhz or Rhz derivatives for 24 h. Cells were washed with 200 μ l phosphate buffered saline (PBS), and filtered neutral red stock solution (4%, w/v, 0.9% NaCl) was diluted 1 : 60 in serum-free media and added to each well. After 4 h incubation, cells were washed twice and 150 μ l of lysing solution was added (50%, v/v, ethanol with 1% , v/v, acetic acid) to the wells. Absorbance at 540 nm was measured using a spectrophotometric microplate reader. All results were expressed as percentage of viable cells.

Cell Cycle Assay Rhz-treated cells were harvested, suspended in PBS, and fixed with ice-cold 70% ethanol for at least 3 h. Fixed cells were stained with propidium iodide (PI) at 37 °C for 45 min. DNA content of cells (10000 cells per experimental group) was analyzed by a fluorescent activated cell sorter (FACS) caliber flow cytometer (BD) using Lysis II and CELL-FIT programs (BD).

Apoptosis Assay The extent of apoptosis was evaluated by annexin V-fluorescein-isothiocyanate (FITC), binding using the apoptosis detection kit. Briefly, HL-60 cells were treated with different concentrations of Rhz or derivatives for 24 h. The concentration of the sphingolipid stock solutions was adjusted so that the final concentration of dimethylsulfoxide in the culture medium was 0.1%. In control cultures or cultures without sphingolipids, dimethylsulfoxide (0.1%) was added as a vehicle control. After incubation, cells were harvested, washed with PBS, centrifuged, and stained with annexin V-FITC and PI in binding buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.4/140 mM NaCl/2.5 mM CaCl₂) for 15 min in the dark. The samples were analyzed by flow cytometry using a FACScan flow cytometer. Data analysis was performed using Cell-Quest software (Becton-Dickinson, CA, U.S.A.).

Western Blot Analysis HL-60 cells treated with sphingolipids for 24 h were harvested, washed with ice-cold PBS, and treated with lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 1.5 mM MgCl₂, 1 mM ethylene glycol tetraacetic acid (EGTA), 10% glycerol, 1 mM NaF, and 1% Triton X-100 accompanied by protease inhibitors (1 mM phenylmethylsulfonylfluoride, 20 U/ml aprotinin). The concentration of protein in each lysate was determined using the Bio-Rad Protein Assay Reagent (Bio-Rad Lab., Richmond, CA, U.S.A.) following the manufacturer's protocol. Then, 25 μ g of proteins were separated by 12% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham Life Science, Inc., Piscataway, NJ, U.S.A.). The membranes were blocked with a blocking buffer (10 mM Tris HCl, 0.15 M NaCl, 0.1% NaN₃, and 5% skim milk) for 1 h at 25 °C and incubated with primary polyclonal antibodies directed against procaspases-3, -8, -9, PARP, Bid, or β -actin (1 : 1000 dilution) in a blocking buffer overnight at 4 °C. The membranes were incubated with the secondary antibodies for 1 h at 25 °C. The signals were detected using ECL chemiluminescence (Amersham, Buckinghamshire, U.K.) following the manufacturer's instructions. The integrated optical density for the protein band was calculated using a Kodak Imaging Program and Image-Pro-Plus (IPP) software (Eastman Kodak Company, New Haven, CT, U.S.A.).

Mitochondrial Permeability Assay Rhz-treated HL-60 cells (1×10^6 /ml) were exposed to 40 nM of 3,30-dihexyloxa-carbocyanine iodide [DiOC₆(3), Molecular probes] for 30 min at 37 °C and then washed with PBS. Stained cells were analyzed by flow cytometry.

Statistical Analysis Data are expressed as mean $\pm$ standard deviation (S.D.). Analyses were carried out using Student's *t*-tests. A *p* value of <0.05 was considered significant.

RESULTS

Cytotoxic Effects of Two-Headed Sphingolipids In the present study, we used HL-60 cells to test for anti-leukemic activity of five different compounds, the so called two-headed sphingolipids. These included Rhz and its derivatives AglRhz, AcRhz, AcAglRhz, and AglRhz A. To explore the effect of Rhz on leukemic cell viability, HL-60 cells were cultured with 10 μ M of Rhz or its derivatives for 24 h and cell viability was measured using the neutral red release assay. As

Fig. 2. Inhibition of Cell Proliferation and Induction of Apoptosis by Sphingolipids Rhz, AgIRhz, AcRhz, AcAgIRhz, and AgIRhz A in HL-60 Cells

(A) HL-60 cells were treated with $10 \mu\text{M}$ of each of the above compounds for 24 h. Neutral red solution was added to the washed cells. After 4 h incubation, the viability of cells was detected by measuring absorbance at 540 nm. (B) HL-60 cells were treated as in panel A and stained with PI. The cell cycle profile was measured using flow cytometry. (C) HL-60 cells were grown and treated with the indicated concentrations of Rhz or its derivatives. After 24 h, the cells were subjected to annexin V-FITC and PI staining and apoptosis was measured by flow cytometry. Results are shown as mean $\pm$ S.D. for three independent experiments. Representative experiments are shown. * $p < 0.05$ compared to untreated control.

shown in Fig. 2A, AgIRhz and AgIRhz A more potently inhibited cell viability than Rhz, AcRhz, or AcAgIRhz. To further compare the effect of Rhz on cell cycle progression, Rhz-treated HL-60 cells were subjected to the flow cytometric analysis of cell cycle distribution. The cytotoxic concentration of AgIRhz and AgIRhz A increased the sub-G₁ fraction (Fig. 2B).

Apoptotic Effect of Rhz and Derivatives on HL-60 Cells Next, we tested whether Rhz or its derivatives induce apoptosis of HL-60 cells using annexin-V/PI staining. The basic compound Rhz started to induce apoptosis in HL-60 cells at a concentration of $10 \mu\text{M}$ (33%, total apoptosis; 27% early apoptotic cells plus 6% late apoptotic and early necrotic cells). The proportion of apoptotic cells was increased in a dose-dependent manner up to $25 \mu\text{M}$ (86%, total apoptosis; 26% early apoptotic cells plus 60% late apoptotic and early necrotic cells) during a 24 h incubation (Fig. 2C). AcRhz had a similar effect on HL-60 cells, but induced apoptosis from $20 \mu\text{M}$ and increased the proportion of total apoptotic cells to 86% at $25 \mu\text{M}$ (29% early apoptotic cells plus 55% late apop-

totic and early necrotic cells). Another two derivatives, AgIRhz or AgIRhz A demonstrated analogous influence on HL-60 cells inducing, respectively, 93% and 62% total apoptotic cells at $5 \mu\text{M}$. The fifth compound, AcAgIRhz caused no apoptosis in HL-60 cells at the concentrations indicated during a 24 h incubation. We also tested these compounds on different leukemic cell lines. Rhz, AgIRhz and AgIRhz A induced *ca.* 55% total apoptosis in THP-1 cells at $5 \mu\text{M}$ and this was increased to *ca.* 80% at $20 \mu\text{M}$ (data not shown). A $20 \mu\text{M}$ concentration of AcRhz induced only 44% apoptosis of THP-1 cells. Moreover, AcAgIRhz under the same conditions had no apoptotic effect on THP-1 cells during 24 h of incubation.

Effect of Rhz or Its Derivatives on the Intracellular Level of Apoptotic Proteins in HL-60 Cells The relative activities for inducing apoptotic alterations were compared with the protein levels of procaspases in HL-60 cells treated with $10 \mu\text{M}$ of each of the five Rhzs. At the indicated conditions, the apoptotic rates induced by Rhz, AgIRhz, and AgIRhz A were, respectively, $31 \pm 5\%$, $87 \pm 7\%$, and $80 \pm 4\%$ (Fig. 2C). After treatment of HL-60 cells with AcRhz or AcAgIRhz, total apoptotic levels were only $10 \pm 3\%$ and $6 \pm 2\%$, respectively. Western blot analysis showed that the expression levels of procaspases-3, -8, -9, and Bid were decreased after treatment of HL-60 cells with $10 \mu\text{M}$ of Rhz, AgIRhz, and AgIRhz A, while the same concentrations of AcRhz and AcAgIRhz had no significant effects on intracellular levels of the procaspases and Bid proteins. Moreover, the changes in the levels of cleaved caspase-3, -8, and -9 were closely related to the changes in procaspases. Activation of caspase-3 leads to the cleavage of a number of proteins, including PARP. We observed that exposure of HL-60 cells to Rhz congeners elicited similar degradation of PARP and procaspase-3. The loss of detectable procaspases, Bid and PARP proteins was well correlated to the apoptotic rate in HL-60 cells. These results suggest that the procaspases may be converted into cleaved active forms after treatment of HL-60 cells with these sphingolipids.

For further detailed investigation of apoptosis in leukemic cells, we chose Rhz as the basic compound and AgIRhz as the strongest Rhz derivative. As shown in Fig. 3B, Rhz noticeably decreased procaspases-3, -8, -9, and Bid protein levels, and induced the cleavage of PARP in a dose-dependent manner. Among checked procaspases, the most significant changes were seen in the level of procaspase-8. At a $10 \mu\text{M}$ concentration of Rhz, protein levels of procaspase-8 almost completely vanished. AgIRhz also decreased protein levels of the main procaspases and Bid dose-dependently during incubation with 1, 1.5, and $2 \mu\text{M}$ concentrations (Fig. 3B). The apoptotic rate reached 60% of total cells at $1.5 \mu\text{M}$ of AgIRhz. At this concentration, procaspase-3, and -8 levels were dramatically decreased but the protein levels of procaspase-9 and Bid were not decreased as much as those of procaspases-8 and -3, although the decrease of procaspase-9 and Bid levels was significant. Therefore, these results indicate that apoptosis induced by Rhz and its derivative is mainly mediated through caspase-8 and caspase-3 activation pathways, although caspase-9 activation pathway is involved in Rhz-induced apoptosis.

Next, we tested the time-dependent effect of Rhz and AgIRhz on the apoptotic rate and levels of procaspases in

Fig. 3. Effects of the Sphingolipids Rhz, AgIRhz, AcRhz, AcAgIRhz, and AgIRhz A on Intracellular Levels of Apoptotic Proteins in HL-60 Cells

(A) HL-60 cells were treated with 10 μM of each compounds for 24 h. (B) HL-60 cells were treated with the indicated concentrations of Rhz and AgIRhz for 24 h. Apoptotic rates were determined using annexin-V/PI staining and the expression of caspase-associated proteins was detected by Western blot analysis. Expression of β -actin protein served as a loading control. In the lower panel, the graphs represent the densitometric analyses of immunoblots for procaspases, PARP, Bid, and β -actin. Protein levels were expressed in arbitrary units relative to untreated controls. Results are shown as mean $\pm$ S.D. for three independent experiments. * $p < 0.05$ compared to untreated control.

HL-60 cells. As shown in Fig. 4A, treatment of HL-60 cells with 1.5 μM of AgIRhz for 12 h resulted in a 35% apoptosis rate in total cells, while the apoptotic rate reached 20% of total cells at 20 μM of Rhz after 12 h in culture. At the same time, Rhz or AgIRhz treatment caused a time-dependent reduction in the levels of procaspases (Fig. 4B). In addition, a more pronounced decrease in the levels of procaspases-8 and -3 was also detected in AgIRhz-treated cells.

To determine if activation of caspase-like proteases is necessary for apoptosis induced by Rhz, caspase inhibitors were used to block intracellular proteases. HL-60 cells were treated for 24 h with z-DEVD-fmk, a caspase-3 inhibitor, alone or in combination with Rhz or AgIRhz. z-DEVD-fmk abrogated the effect of Rhz and AgIRhz on apoptosis (Fig. 5). z-IETD-fmk or z-LEHD-fmk, inhibitors of caspase-8 and caspase-9, respectively, significantly inhibited Rhz and AgIRhz effects on apoptosis, but the effect by z-LEHD-fmk was not as strongly as by caspase-3 and caspase-8 inhibitors. These results further support the ability of Rhz to activate the

caspase-dependent apoptotic cascade.

Disruption of Mitochondrial Transmembrane Potential ($\Delta\Psi\text{m}$) by Rhz and Effect of Antioxidants on Rhz-Induced apoptosis in HL-60 Cells Our results showed that the level of mitochondrial proteins Bid and procaspase-9 significantly decreased after treatment of HL-60 cells with Rhz or AgIRhz, which indicates that the mitochondrial pathway may be critical for initiating apoptotic death by these sphingolipids. To further elucidate the role of mitochondria in the induction of apoptosis in response to Rhz or AgIRhz, we first examined changes in mitochondrial membrane permeability using a DiOC₆(3) staining assay. As shown in Fig. 6A, after treatment with 20 μM of Rhz and 1.5 μM of AgIRhz the permeability of mitochondria extensively decreased (pretreatment; $93 \pm 5\%$ vs. post-treatment: $32 \pm 6\%$ and $45 \pm 5\%$, for Rhz and AgIRhz, respectively). Thus, Rhz- and AgIRhz-induced apoptosis could involve activation of a mitochondria pathway. It is well-known that mitochondria are sensitive to changes in cellular redox state, and reactive oxygen species

Fig. 4. Time-Dependent Effects of Rhz and AgIRhz on Apoptosis and Intracellular Levels of Apoptotic Proteins in HL-60 Cells

HL-60 cells were incubated with or without Rhz (20 μM) or AgIRhz (1.5 μM) for the indicated times. Apoptotic rates were determined using annexin-V & PI staining (A) and the expression of caspase-associated proteins was detected by Western blot analysis (B). * $p < 0.05$ compared to untreated control.

(ROS) can cause mitochondrial dysfunction. In order to investigate whether the production of ROS is key to sphingolipid-induced apoptosis, we tested the effect of several main ROS-inhibitors on Rhz-induced mitochondrial permeability changes. We pretreated HL-60 cells for 1 h with *N*-acetylcysteine (NAC), glutathione, or diphenylene iodonium (DPI). NAC and glutathione did not significantly inhibit Rhz- and AgIRhz-induced mitochondrial membrane permeability changes. In contrast, a low concentration of DPI, an inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, enhanced mitochondrial membrane permeability changes in the presence but not in the absence of Rhz or AgIRhz. These results are consistent with the changes we ob-

Fig. 5. Effects of Caspase-Inhibitors on Apoptosis of HL-60 Cells Induced by Rhz and AgIRhz

HL-60 cells were pretreated with or without 50 μM caspase inhibitors for 1 h, and then challenged with Rhz (20 μM), or AgIRhz (1.5 μM) for 24 h. Apoptotic rates were analyzed using annexin V-FITC and PI staining. * $p < 0.05$ compared to control without caspase inhibitors.

Fig. 6. Effects of Anti-oxidants on Rhz and AgIRhz-Induced Apoptosis in HL-60 Cells

HL-60 cells were pre-incubated with or without the indicated antioxidants for 1 h and further incubated in the presence of Rhz (20 μM) or AgIRhz (1.5 μM) for 24 h. (A) Mitochondria membrane permeability was measured using DiOC₆(3) staining. In the left panel, representatives are shown. In the right panel, results are shown as mean ± S.D. for three independent experiments. (B) Apoptosis was measured using annexin V-FITC and PI staining. The concentrations of the compounds were as follows: *N*-acetyl cysteine (NAC), 100 μM; glutathione (GSH), 20 μM; diphenylene iodonium (DPI), 5 μM; apocynin, 100 μM; superoxide dismutase (SOD), 10 units/ml. (C) HL-60 cells were pre-incubated with or without DPI for 1 h and further cultured in the presence or absence of Rhz (20 μM) for 24 h. Expression levels of procaspases were measured by Western blot. * $p < 0.05$ compared to Rhz or AgIRhz alone.

Fig. 7. Effects of Sphingomyelinase Inhibitors on Rhz-Induced Apoptosis of HL-60 Cells

(A) HL-60 cells were pre-incubated with or without desipramine (20 μ M) or 3-*O*-methyl-sphingomyelin (Methyl-SM) (20 μ M) for 1 h and further incubated in the presence of Rhz (20 μ M) or AgIRhz (1.5 μ M) for 24 h. Apoptosis was measured by annexin V-FITC and PI staining. (B) Cells were treated as in panel A and cell lysates were subjected to Western blotting for analysis of the expression of procaspases. * p <0.05 compared to Rhz or AgIRhz alone.

served in apoptotic rate. As shown in Fig. 6B, NAC, glutathione, and superoxide dismutase had no effect on Rhz-induced apoptosis. However, DPI increased the apoptotic rate although DPI alone had no cytotoxic effect on untreated cells. In contrast, a structurally distinct NADPH oxidase inhibitor, apocynin,¹⁷⁾ did not affect the apoptotic rate induced by Rhz in HL-60 cells. Moreover, the decrease in protein levels of procaspases by Rhz was augmented by DPI rather than abrogated. These results suggest that Rhz might cause non-oxidative rather than oxidative cytotoxicity to HL-60 cells.

Effects of Sphingomyelinase Inhibitors on Rhz-Induced Apoptosis The breakdown of complex sphingolipids results in the formation of ceramide. Acid sphingomyelinase (SMase) and/or neutral SMase contribute to ceramide generation. Desipramine and 3-*O*-methyl-sphingomyelin (methyl-SM), an acid and a neutral SMase inhibitor, respectively,¹⁸⁾ further increased apoptosis of HL-60 cells in the presence of Rhz or AgIRhz rather than prevented it (Fig. 7). The decrease in protein levels of procaspases-3 was also more prominent in HL-60 cells treated with desipramine or methyl-SM in the presence of Rhz than in cells treated with Rhz alone. However, the decrease in procaspase-9 level in the presence of Rhz was not enhanced by desipramine or methyl-SM. These results suggest that Rhz-induced apoptosis is related to SM metabolism, which affects the caspase-8 pathway.

DISCUSSION

The effect of exogenous sphingolipids on cancer cells *in*

vitro has not been reported. Using HL-60 cells, we confirmed that Rhz suppresses the growth of leukemic cells. An analysis of structure-activity relationship generated by mass spectrometry and nuclear magnetic resonance spectroscopy showed that Rhz consists of a C₂₈ linear carbon skeleton with amino groups at C-2 and C-27, one glycosylated and one unsubstituted hydroxyl groups at C-3 and C-26, and a ketone at C-18. This study showed that the different sensitivities of HL-60 cells to Rhz may be attributed to structural differences between Rhz congeners. Cell cycle analysis revealed that the aglycon type of Rhz induces apoptosis as evidenced by the appearance of a sub-G₁ fraction compared to Rhz and AcRhz. Moreover, dose- and time-dependence experiments showed similar results and the addition of AgIRhz and AgIRhz A at low concentrations decreased the levels of procaspase-8, -9, and -3 proteins, whereas AcRhz and AcAgIRhz did not significantly influence the level of proteins that drive apoptosis. These results indicate that AgIRhz is the most potent derivative of Rhz.

Rhz was formally classified as a glycolipid, but the galactosyl residue is not on the same hydroxyl as in cerebroside. The observed apoptotic activity of Rhz appeared to relate to the presence or absence of the galactosyl residues. The less apoptotic activity of the Rhz compared to that of AgIRhz may be due to the presence of the galactosyl residue in Rhz, while AgIRhz has the unsubstituted hydroxyl group in the same position. The D-galactose is the most common sugar encountered among the Rhzs from the sponge *R. inrustata*.⁷⁾ Hydrolysis of Rhz yields the aglycon derivatives. Results of the present study show that aglycon derivatives are more potent than Rhz in inducing apoptosis of HL-60 cells. Since aglycon derivatives of Rhz or Rhz A may be more permeable through HL-60 cell membranes than Rhz or acetate Rhz, the aglycon derivatives may be more potent for inducing apoptosis. AgIRhz A was produced by hydrolysis of Rhz A, the 2-ethyl-carbamate form of Rhz, and had a potency similar to that of the tested AgIRhz, suggesting that the addition of an ethyl-carbamate chain may not significantly affect the apoptosis-inducing effect of Rhz. It has been reported that the presence of acetyl groups usually increases cytotoxic potency.¹⁹⁾ Our study also indicated that the acetyl group in Rhz may play a significant role in Rhz's cytotoxicity and caspase activation since AcRhz and AcAgIRhz had less potent effects than Rhz and AgIRhz on cytotoxicity, cell cycle changes, caspase activation, and apoptosis.

The apoptosis induced by Rhz and its derivatives is consistent with an increase in the hydrolysis of the inactive procaspases observed in lysates from Rhz-treated cells and an increase in mitochondrial membrane permeability. There are two well-characterized apoptotic pathways in mammalian cells, referred to as the death receptor (extrinsic) and mitochondrial (intrinsic) pathways. In order to know which caspase activation pathway is associated with cell death, selective inhibitors of caspases were used. In this study, Rhz-induced apoptosis was almost completely abolished in z-DEVD-fmk (caspase-3) or z-IETD-fmk (caspase-8)-pretreated cells and partially inhibited by z-LEHD-fmk (caspase-9), which supports a caspase-dependent cell death mechanism. Another notable target of caspase-8 is Bid. Activated caspase-8 induces the cleavage of Bid to yield a truncated fragment that triggers mitochondrial membrane perme-

ability and cytochrome *c*-mediated caspase activation. This treatment also caused a decrease in the amount of the pro-form of Bid, which represents indirect evidence of protein truncation/activation. The observation that Bid was cleaved in response to Rhz suggests the possible activation of caspase-8, since Bid is a substrate of this caspase. We found that z-IETD-fmk was significantly more effective than z-LEHD-fmk in preventing apoptosis induced by Rhz treatment, and the decrease in protein levels of procaspases-8 and -3 was more prominent than the decrease in procaspase-9 levels. These results suggest that Rhz induces apoptosis in HL-60 cells, possibly *via* a mechanism that includes mitochondria and a caspase-dependent apoptotic cascade, and caspase-3 activation is mainly mediated through this intrinsic pathway.

Elevation of ROS levels can initiate apoptosis. It has been shown that ROS are generated in undifferentiated HL-60 cells through the activation of NADPH oxidase in response to various stimuli.²⁰⁾ Our study showed that antioxidants, including NAC, superoxide dismutase, and glutathione, failed to prevent apoptosis induced by Rhz. However, under DPI and Rhz or AglRhz co-treatment, DPI significantly potentiated apoptosis of HL-60 cells. In contrast, apocynin failed to augment Rhz-induced apoptosis. Actions of DPI in the absence of Rhz probably result from interactions with mitochondrial enzyme complexes because the addition of DPI with Rhz increased mitochondrial membrane permeability. Redox-regulated release of Ca^{++} can promote activation of caspase and induce apoptosis.²¹⁾ We found that the apoptosis induced by Rhz or Rhz plus DPI was not abrogated by EGTA or BAPTA-AM, chelators of Ca^{++} (data not shown). The generation of extracellular ROS was suppressed by DPI but this agent accelerated apoptosis in A23187-treated HL-60 cells.²²⁾ Other groups have also suggested that the ROS generated by NADPH oxidase plays an essential role in the survival of undifferentiated HL-60 cells.²³⁾ Others showed that high concentrations of DPI (50–100 μM) induced apoptosis of HL-60 cells by producing mitochondrial superoxide and decreasing the mitochondrial membrane potential.²⁴⁾ However, we observed that a relatively low concentration of DPI (5 μM) was not able to induce apoptosis in the absence of Rhz. It has been shown that ROS production can inactivate caspase through the oxidation of the cysteine residues at the enzyme's active sites.²⁵⁾ Since DPI can also inhibit a wide range of other flavin-containing enzymes, we speculate that NADPH oxidase can be ruled out as a possible DPI target. Overall, our study suggests that pro-oxidant activity may not be involved in Rhz-induced apoptosis.

Sphingolipid analogs may act by altering normal sphingolipid metabolism, or by interacting as agonists or antagonists with sphingolipid-binding sites in regulatory processes. Sphingosine appears to act in a similar fashion to ceramide, which is known to be a strong pro-apoptotic signaling mediator.²⁶⁾ On the other hand, sphingosine introduced into a cell is rapidly phosphorylated to sphingosine 1-phosphate, which exerts pro-survival effect on cells. Our study showed that inhibitors of SMase enhance Rhz-induced apoptosis of HL-60 cells. It was reported that desipramine causes the non-oxidative apoptotic damage to different types of human colon carcinoma cells through either a non-mitochondrial or a mitochondrial pathway.²⁷⁾ Activation of SMase results in formation of ceramide-enriched membrane platforms that trap and

cluster signaling proteins. Fas receptors become concentrated in the macrodomains.²⁸⁾ Therefore, we speculate that the change in membrane permeability due to glycosylated or acetylated Rhz analogs may not allow exogenous addition of Rhz to alter their intracellular levels.

We previously observed that Rhz exhibited antifungal activity against the fluconazole-resistant yeast, *Candida glabrata* but its derivatives, such as Rhz C and Rhz D, were not active under the same condition.^{7,11)} From these results, we concluded that the minimum Rhz structure necessary for apoptosis induction in HL-60 cells is one possessing a two-headed sphingosine. Moreover, our study suggests that the aglycon derivatives of Rhz or its analog AglRhzA induce apoptosis more effectively than intact Rhz or its peracetylated derivatives, which makes the former better analogs of two-headed sphingolipids than the intact toxin as anti-tumor agents. Glycosylation or acetylation of at least one of these hydroxy-groups significantly diminished pro-apoptotic activity of these compounds.

Acknowledgments This work was supported by the Korea Science and Engineering Foundation (KOSEF) Grant funded by the Korea government (MEST) (No. R13-2002-044-04001-0). Russian co-authors are grateful for support by RFBR Grant 05-04-48246, Grant of Support of the Leading Science School No. 6491.2006.4, Program of Presidium of RAS "Molecular and Cell Biology" No. 06-I-10-019, and FEB RAS Grant 06-III-A-05-122.

REFERENCES

- 1) Kroemer G., El-Deiry W. S., Golstein P., Peter M. E., Vaux D., Vandenabeele P., Zhivotovsky B., Blagosklonny M. V., Malorni W., Knight R. A., Piacentini M., Nagata S., Melino G., *Cell. Death Differ.*, **12**, 1463–1467 (2005).
- 2) Nuñez G., Benedict M. A., Hu Y., Inohara N., *Oncogene*, **17**, 3237–3245 (1998).
- 3) Boatright K. M., Salvesen G. S., *Curr. Opin. Cell Biol.*, **15**, 725–731 (2003).
- 4) Nagata S., *Cell*, **88**, 355–365 (1997).
- 5) Shier W. T., Shier A. C., *J. Toxicol.*, **19**, 189–246 (2000).
- 6) Makarieva T. N., Denisenko V. A., Stonik V. A., Milgrom Y. M., Rashkes Y. W., *Tetrahedron Lett.*, **30**, 6581–6584 (1989).
- 7) Makarieva T. N., Guzii A. G., Denisenko V. A., Dmitrenok P. S., Santalova E. A., Pokanevich E. V., Molinski T. F., Stonik V. A., *J. Nat. Prod.*, **68**, 255–257 (2005).
- 8) Makarieva T. N., Denisenko V. A., Dmitrenok P. S., Guzii A. G., Santalova E. A., Stonik V. A., MacMillan J. B., Molinski T. F., *Org. Lett.*, **7**, 2897–2900 (2005).
- 9) Makarieva T. N., Dmitrenok P. S., Zakharenko A. M., Denisenko V. A., Guzii A. G., Li R., Skepper C. K., Molinski T. F., Stonik V. A., *J. Nat. Prod.*, **70**, 1991–1998 (2007).
- 10) Makarieva T. N., Zakharenko A. M., Denisenko V. A., Dmitrenok P. S., Guzii A. G., Shubina L. K., Kapustina II., Fedorov S. N., *Chem. Nat. Comp.*, **43**, 468–469 (2007).
- 11) Nicholas G. M., Hong T. W., Molinski T. F., Lerch M. L., Cancilla M. T., Lebrilla C. B., *J. Nat. Prod.*, **62**, 1678–1681 (1999).
- 12) Makarieva T. N., Guzii A. G., Denisenko V. A., Dmitrenok P. S., Stonik V. A., *Izv. Akad. Nauk. Ser. Chim.*, **3**, 656–660 (2008).
- 13) Zhou B. N., Mattern M. P., Johnso R. K., Kingston D. G., *Tetrahedron*, **57**, 9549–9554 (2001).
- 14) Kong F. H., Faulkner D. J., *J. Org. Chem.*, **58**, 970–971 (1993).
- 15) Willis R. H., De Vries D. J., *Toxicon*, **35**, 1125–1129 (1997).
- 16) Borenfreund E., Puerner J. A., *Toxicol. Lett.*, **24**, 119–124 (1985).
- 17) Stolk J., Hiltermann T. J., Dijkman J. H., Verhoeven A. J., *Am. J. Respir. Cell Mol. Biol.*, **11**, 95–102 (1994).
- 18) Lister M. D., Ruan Z. S., Bittman R., *Biochim. Biophys. Acta*, **1256**, 25–30 (1995).

- 19) Mimaki Y., Yokosuka A., Kuroda M., Sashida Y., *Biol. Pharm. Bull.*, **24**, 1286—1289 (2001).
- 20) Muranaka S., Fujita H., Fujiwara T., Ogino T., Sato E. F., Akiyama J., Imada I., Inoue M., Utsumi K., *Antioxid. Redox Signal.*, **7**, 1367—1376 (2005).
- 21) Jacobson J., Duchon M. R., *J. Cell Sci.*, **115**, 1175—1188 (2002).
- 22) Kajitani N., Kobuchi H., Fujita H., Yano H., Fujiwara T., Yasuda T., Utsumi K., *Biosci. Biotechnol. Biochem.*, **71**, 2701—2711 (2007).
- 23) Dong J. M., Zhao S. G., Huang G. Y., Liu Q., *Free Radic. Res.*, **38**, 629—637 (2004).
- 24) Li N., Ragheb K., Lawler G., Sturgis J., Rajwa B., Melendez J. A., Robinson J. P., *Free Radic. Biol. Med.*, **34**, 465—477 (2003).
- 25) Chandra J., Samali A., Orrenius S., *Free Radic. Biol. Med.*, **29**, 323—333 (2000).
- 26) Hannun Y. A., Obeid L. M., *Trends Biochem. Sci.*, **20**, 73—77 (1995).
- 27) Arimochi H., Morita K., *Pharmacology*, **81**, 164—172 (2008).
- 28) Bollinger C. R., Teichgraber V., Gulbins E., *Biochim. Biophys. Acta*, **1746**, 284—294 (2005).