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Sulfuretin From Heartwood of *Rhus verniciflua* Triggers Apoptosis Through Activation of Fas, Caspase-8, and the Mitochondrial Death Pathway in HL-60 Human Leukemia Cells

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ABSTRACT

Sulfuretin, a flavonoid isolated from heartwood of *Rhus verniciflua*, has been reported to have anti-cancer activities but the underlying molecular mechanism was not clear. In this study, sulfuretin induced apoptosis by activating caspases-8, -9, and -3 as well as cleavage of poly(ADP-ribose) polymerase. Furthermore, treatment with sulfuretin caused mitochondrial dysfunctions, including the loss of mitochondrial membrane potential ($\Delta\Psi_m$), the release of cytochrome *c* to the cytosol, and the translocations of Bax and tBid. Sulfuretin also activated the extrinsic apoptosis pathway, that is, it increased the expressions of Fas and FasL, the activation of caspase-8, and the cleavage of Bid. Furthermore, blocking the FasL–Fas interaction with NOK-1 monoclonal antibody prevented the sulfuretin-induced apoptosis. The therapeutical effect of sulfuretin in leukemia is due to its potent apoptotic activity through the extrinsic pathway driven by a Fas-mediated caspase-8-dependent pathway. J. Cell. Biochem. 113: 2835–2844, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: SULFURETIN; *Rhus verniciflua*; APOPTOSIS; Fas; CASPASE-8; Bcl-2

Despite advances in diagnosis, treatment, and patient survival, hematologic cancers continue to present significant challenges. Leukemia, lymphoma, and myeloma constitute the fourth most common form of cancer, and though the precise cause of leukemia has not been determined, but it is known to be influenced by genetic and environmental factors. Leukemias, like other cancers, are caused by somatic mutations in DNA that activate oncogenes, inactivate tumor suppressor genes, and disrupt the regulations of cell death, differentiation, and division [Rossi and Gaidano, 2003].

Cancer development largely results from the uncontrolled growth of malignant cells, and the resulting imbalance between cell proliferation and cell death and the deregulation of apoptosis. This deregulation of apoptosis, which occurs in the vast majority of cancers, has become a major target for anti-cancer strategies. Apoptosis is a genetically controlled mechanism that plays an essential role in the regulation of cellular homeostasis [Kaufmann and Earnshaw, 2000], and compounds that block or suppress the proliferation of cancer cells by inducing apoptosis are viewed as potential anti-cancer agents.

Several compounds used in cancer chemotherapy, such as, paclitaxel, camptothecin, etoposide, and the vinca alkaloids, were derived from plant sources [Cragg and Newman, 2005], and many pro-apoptotic compounds derived from natural sources, are being actively investigated in terms of their therapeutic effects and modes of action against various cancers. *Rhus verniciflua* Stokes (RVS),

Kyung-Won Lee and Kyung-Sook Chung contributed equally to this work.

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commonly known as the lacquer tree, is indigenous to East Asia, and has been used in traditional herbal medicines to treat cancer in Korea and China since the 15th century. Recently, RVS extracts were found to have various biological activities, including anti-proliferative and apoptotic effects on human cancer cell lines, and to induce apoptosis via caspase-9 activation and inhibition of the PI3K/Akt pathway [Jang et al., 2005; Kim et al., 2008]. Furthermore, the anti-cancer effect of RVS extract has been demonstrated in several clinical studies. In particular, Lee et al. [2009a] reported that supplementary treatment with standardized RVS extract in metastatic colorectal cancer (36 patients) positively affected overall survival without inducing significant side effects. Furthermore, in a case study of a patient with advanced pulmonary adenocarcinoma, treatment with RVS extract for 1 month markedly reduced pleural effusion and mass sizes [Lee et al., 2009b]. In addition, patients with metastatic renal cell carcinoma and recurrent hepatocellular carcinoma were successfully treated using allergen-free RVS extract [Kim et al., 2010; Lee et al., 2010b]. However, despite the large amount of evidence available on the anti-cancer effects of RVS extract, little information is available on anti-proliferative effects of its active constituents. Much attention has been focused on flavonoids because of their potential selective abilities to induce cancer cell apoptosis. For example, a purified flavonoid fraction prepared from RVS was found to have anti-proliferative and apoptotic effects on human lymphoma, breast cancer, and osteosarcoma cell-lines, and on transformed hepatoma cells [Son et al., 2005], and butein, a flavonoid isolated from RVS, was found to suppress the clonogenic growth of a breast cancer cell line [Samoszuk et al., 2005].

Sulfuretin, a flavonoid of RVS, has a remarkable spectrum of biological activities, which range from anti-inflammatory [Jung et al., 2007; Shin et al., 2010], anti-platelet [Jeon et al., 2006], anti-mutagenic [Park et al., 2004], and anti-rheumatoid arthritis effects [Choi et al., 2003]. However, the mechanism underlying the apoptosis-inducing activity of sulfuretin has not been elucidated. Thus, the aim of the present study was to identify the mechanism responsible for the apoptosis induced by sulfuretin in human leukemia HL-60 and U937 cells.

MATERIALS AND METHODS

CHEMICALS

The sulfuretin used in this study was isolated from the heartwood of *R. verniciflua* (Anacardiaceae) and structural identity were determined spectroscopically (^{1}H and ^{13}NMR, IR, MS) as described previously [Choi et al., 2003]. The compound isolated was checked by HPLC and was found to be >98% pure. RPMI 1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tertazolium bromide (MTT), dimethyl sulfoxide (DMSO), RNase A, leupeptin, aprotinin, phenylmethylsulfonylfluoride (PMSF), 4′,6-diamidino-2-phenylindole-dihydrochloride (DAPI), Triton X-100, Nonidet P-40, protein A/G-Sepharose beads and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies for caspase-3 (E-8), caspase-9 (H-170), Bid (N-19), Bax (B-9), Bcl-2 (C-2), Bcl-xL (H-5), cellular FLICE-inhibitory protein L (c-FLIPL, H-150), c-FLIPS (G-11), Fas (B-10), FasL (C-178), poly(ADP-ribose) polymerase (PARP) (F-2), and β-actin (ACTBD11B7) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for FADD (1/FADD), caspase-8 (3-1-9), cytochrome *c* (7H8.2C12), and FITC-conjugated annexin V were purchased from BD Biosciences, Pharmingen (San Diego, CA). z-VAD-fmk and z-DEVD-fmk were purchased from A.G. Scientific Inc. (San Diego, CA). z-IETD-fmk and z-LEHD-fmk were obtained from Calbiochem (Bad Soden, Germany).

CELL CULTURE AND MTT ASSAY

HL-60 (human promyelocytic leukemia), U937 (human histocytic lymphoma), HeLa (human cervical carcinoma), A431 (human epridermoid carcinoma), P388 (murine lymphocytic leukemia), Chang (Human epithermal conjunctival cell line), Ect/E6E7 (normal human ectocervix), and L132 (human lung epithelial cell line) were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin sulfate (100 μg/ml) (Life Technologies). Cells were cultured at 37°C in an atmosphere of 5% CO_2 in the presence or absence of the sulfuretin.

The cytotoxicity was assessed using a MTT assay. Briefly, the cells (5×10^4) were seeded in each well containing 100 μl of the RPMI medium supplemented with 10% FBS in a 96-well plate. After 24 h, various concentrations of sulfuretin were added. After 48 h, 50 μl of MTT (5 mg/ml stock solution) was added and the plates were incubated for an additional 4 h. The medium was discarded and the formazan blue, which was formed in the cells, was dissolved with 100 μl DMSO. The optical density was measured at 540 nm.

DNA FRAGMENTATION ASSAY

DNA fragmentation was quantitated as previously reported [Lee et al., 2005]. In brief, cells were washed in PBS and resuspended in ice-cold lysis buffer (10 mM Tris–HCl pH 7.4, 1 mM EDTA, 0.2% Triton X-100). After incubating for 15 min at 4°C, cell lysates were centrifuged at 25,000×g for 20 min to separate low molecular weight DNA from intact chromatin. The lysate and supernatant were sonicated for 15 s and the level of DNA in each fraction was measured by a fluorometric method using DAPI. The amount of the fragmented DNA was calculated as the ratio of the amount of DNA in the supernatant to that in the lysate. Genomic DNA was prepared for gel electrophoresis as previously described [Lee et al., 2005]. Electrophoresis was performed in a 1.5% (w/v) agarose gel in 40 mM Tris–acetate buffer (pH 7.4) at 50 V for 1 h. The fragmented DNA was visualized by staining with ethidium bromide after electrophoresis.

QUANTIFICATION OF APOPTOSIS BY FLOW CYTOMETRY

Cells were harvested and washed with PBS, and hypodiploid cells were analyzed by flow cytometry. Briefly, 1×10^6 cells were washed with PBS, fixed in 70% ice-cold ethanol and kept in a freezer overnight. The fixed cells were washed twice in PBS and resuspended in 500 μl PBS containing 50 mg/ml PI and 100 μg/ml DNase-free RNase A. The cell suspension was incubated in the dark for 30 min at 37°C and analyzed by the fluorescence-activated cell sorting (FACS) cater-plus flow cytometry (Becton Dickinson Co, Heidelberg, Germany). Finally, the

extent of apoptosis was determined by measuring DNA content of the cells below the G_0/G_1 peak (sub-G_1). For annexin V and PI double staining, cells were suspended with 100 μl of binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and stained with 5 μl of FITC-conjugated annexin V and 5 μl of PI (50 μg/ml). The mixture was incubated for 15 min at room temperature in dark place and analyzed by the FACS cater-plus flow cytometry (Becton Dickinson Co).

CELL FRACTIONATION AND WESTERN BLOT ANALYSIS

Sulfuretin treated cells were washed with ice cold PBS and extracted by mitochondrial fractionation kit (Activemotif, CA). Cells were collected by centrifugation (600×*g*, 5 min, 4°C), washed twice with ice-cold PBS, and then centrifuged (600×*g*, 5 min, 4°C). The cell pellet obtained was resuspended in ice-cold cytosolic buffer for 15 min on ice. The cells were then homogenized with a glass dounce and a B-type pestle (80 strokes), homogenates were spun at 10,000×*g* for 20 min at 4°C, and the supernatant (cytosolic fraction) was removed whilst taking care to avoid the pellet. The resulting pellet (mitochondrial fraction) was resuspended in completed mitochondria buffer. For total cell protein extracts, sulfuretin treated cells were washed with ice cold PBS and extracted in protein lysis buffer (Intron, Seoul, Korea). Protein concentration was determined by Bradford assay. Protein samples of cell lysate were mixed with an equal volume of 5× SDS sample buffer, boiled for 4 min, and then separated by 10–15% SDS–PAGE gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membrane by electroblotting and nonspecific-binding sites were blocked by incubation in Tris-buffered saline (TBS) containing Tween-20 (0.1%) and 5% (w/v) dry milk. Immunoblot analyses were performed with the indicated antibodies. Bound primary antibodies were visualized with horseradish peroxidase-conjugated goat anti-rabbit-IgG, goat anti-mouse-IgG, and rabbit anti-goat IgG (Jackson Immunoresearch Laboratories, West Grove, PA) and ECL (Amersham, Freiburg, Germany). Bio-rad Quantity One® Software was used for the densitometric analysis.

ANALYSIS OF THE MITOCHONDRIAL MEMBRANE POTENTIAL ($\Delta\Psi_M$)

Dissipation of $\Delta\Psi_m$ occurs early during apoptosis and is detected using the 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$), membrane-permeable lipophilic cationic fluorochromes. The $DiOC_6$ uptake by charged mitochondria driven by the transmembrane potential is detected by the shift in color of fluorescence. However, depolarization is evidenced by a decrease in $DiOC_6$ uptake undergoing apoptosis [Koning et al., 1993]. Changes in $\Delta\Psi_m$ were monitored by flow cytometric analysis. Cells were incubated with 50 nM $DiOC_6$ for 30 min, washed twice with PBS, and analyzed by flow cytometric analysis (Becton Dickinson Co) with excitation and emission settings of 484 and 500 nm, respectively. To ensure that $DiOC_6$ uptake was specific for $\Delta\Psi_m$, we also treated cells with 100 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). CCCP was used as a reference depolarizing agent and cyclosporin A (CsA) was used as an inhibitor of mitochondrial permeability transition.

IMMUNOPRECIPITATION ANALYSIS

After harvesting and washing, pellets were lysed in EBC buffer (30 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 2.5 mM EGTA, 5 mM NaF, 0.1 mM Na_3VO_4, 1% Triton X-100, and protease inhibitor) for 15 min on ice. After centrifugation (10,000×*g*, 5 min), protein concentrations were determined. Equal amount of protein (100 μg) was incubated with anti-caspase 8 (C-20) for 12 h at 4°C, followed by incubation with 20 μl protein A/G-Sepharose beads for 1 h. The protein complex was washed four times with EBC buffer and released from the beads by boiling in 2× sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 2% glycerol, and 0.02% bromophenolblue) for 5 min. The reaction mixture was then resolved by a 12% SDS–polyacrylamide gels, transferred to nitrocellulose membrane and probed with anti-FasL, anti-Fas, anti-FADD, and anti-c-FLIPL antibodies. Immuno-positive bands were visualized by ECL kit (Amersham, NJ).

STATISTICAL ANALYSIS

Results are expressed as the means ± SD of triplicate experiments. Statistically significant values were compared using ANOVA and Dunnett's post-hoc test, and *P* values of less than 0.05 were considered statistically significant.

RESULTS

SULFURETIN INDUCED APOPTOSIS IN HUMAN LEUKEMIC HL-60 AND U937 CELLS

To determine the effect of sulfuretin on cell viability, MTT assays were performed on various cancer and normal cell lines (Table I). The effects of sulfuretin were assessed using IC_{50} values. Interestingly, sulfuretin was found to have significant cytotoxic effects on hematological cancer cell lines, such as HL-60, U937, and P388, but not on other cancer or normal cell lines (IC_{50} values were >150 μM). Since HL-60 and U937 cells were found to be markedly sensitive to sulfuretin, further experiments were performed to evaluate the effects of sulfuretin on apoptosis and to identify the molecular mechanism involved in HL-60 and U937 cells. To determine whether the cytotoxic effect of sulfuretin was due to its apoptogenic activity toward HL-60 and U937 cells, these cells were treated with sulfuretin (70 μM) and assayed using annexin V- and PI-double staning (Fig. 1B). As shown in Figure 1B, proportions of annexin V-positive cells (early apoptotic cells) were found to increase in a time-

TABLE I. Cytotoxic Effects of Sulfuretin on Cancer Cell Growth In Vitro

Cell line	$IC_{50}{}^{a}$ (μM)
HL-60	56.8
U937	76.3
HeLa	>150
A431	>150
P388	120.2
Chang	>150
Ect/E6E7	>150
L132	>150

[a] IC_{50} is defined as the concentration that results in a 50% decrease in the number of cells compared with that of the control cultures in the absence of an inhibitor. The values represent the mean of three independent experiments.

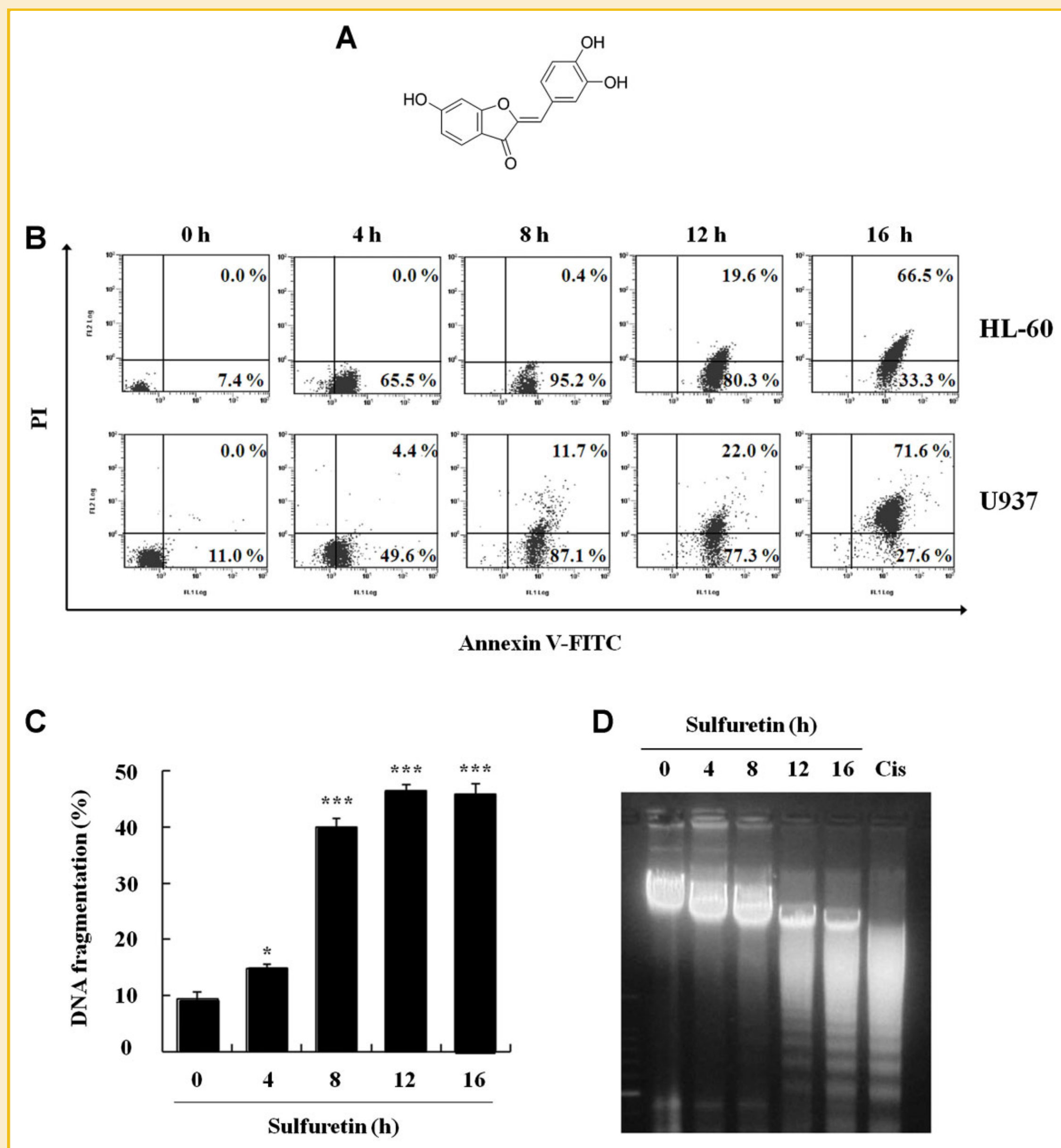

Fig. 1. Induction of apoptosis in human leukemia cells by sulfuretin. A: Chemical structure of sulfuretin. B: Cells were treated with sulfuretin for the indicated times in HL-60 and U937 cells (70 and 100 μM, respectively) and then stained with PI and FITC-conjugated annexin V, which specifically detects exposed phosphatidyl serine residues on cell surfaces. Fluorescence intensities are shown on a log scale. Results are representative of three separate experiments. C: Cells were treated with sulfuretin (70 μM) for the indicated times in HL-60 cells. The extent (%) of DNA fragmentation was determined fluorometrically using DAPI as described in Materials and Methods Section. The results presented are the means ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the non-treated control group. C: After HL-60 cells had been treated with sulfuretin (70 μM) for the indicated times, total genomic DNA was extracted and resolved on 1.5% agarose gels. Some cells were treated with 50 μM cisplatin (Cis), as a positive control. Apoptotic DNA fragmentation was visualized by ethidium bromide staining. Experiments were repeated three times and similar results were obtained (D).

dependent manner after treatment with sulfuretin in HL-60 and U937 cells. In addition, internucleosomal DNA fragmentation was analyzed by DAPI assays after treating cells with sulfuretin in HL-60 cells. DAPI assays revealed that sulfuretin induced DNA fragmentation in a time-dependent manner (Fig. 1C). To further characterize the end stage of apoptosis induced by sulfuretin, we examined whether sulfuretin induces a typical ladder pattern of internucleosomal DNA fragmentation in HL-60 cells. As shown in Figure 1D, the laddering pattern of internucleosomal DNA fragmentation was found to occur in a time-dependent manner after treating HL-60 cells with sulfuretin (70 μM). Furthermore, the distinctive morphological features of apoptosis, that is, cell shrinkage, highly condensed chromatin, and apoptotic bodies were also observed (data not shown). Taken together, these findings indicate that sulfuretin-induced cell death in HL-60 and U937 cells is due to apoptotic cell death.

CASPASE-8, -9, AND -3 WERE REQUIRED FOR SULFURETIN-INDUCED APOPTOSIS

To identify the mechanism involved in sulfuretin-induced apoptosis, we investigated the activations of caspases -8, -9, and -3, and the cleavage of PARP in HL-60 cells. As shown in Figure 2A, sulfuretin significantly and time dependently increased the activations of caspase-8, -9, and -3 and the cleavage of PARP (an endogenous substrate of caspase-3). Following treatment with sulfuretin (70 μM), the cleaved forms of caspase-8 and -3 were observed at 4 and 8 h, respectively, whereas caspase-9 was cleaved after 12 h of treatment. To further confirm the involvements of caspases in sulfuretin-induced apoptosis, various caspase inhibitors, namely, z-VAD-fmk (a broad caspase inhibitor), z-DEVD-fmk (a specific caspase-3 inhibitor), and z-IETD-fmk (a specific caspase-8 inhibitor) were added at concentrations that completely block the activations of their corresponding caspases. All three caspase inhibitors markedly attenuated sulfuretin-stimulated DNA fragmentation, whereas z-LEHD-fmk (a specific caspase-9 inhibitor) only partly prevented sulfuretin-induced apoptosis (Fig. 2B). These observations suggest that initiator caspase-8 and effector caspase-3 play major roles in sulfuretin-induced apoptosis in HL-60 cells, whereas caspase-9, an initiator caspase of the mitochondrial pathway, is also associated with this apoptosis.

SULFURETIN INDUCED MITOCHONDRIAL DYSFUNCTION: LOSS OF MITOCHONDRIAL MEMBRANE POTENTIAL ($\Delta\Psi_M$) AND MODULATION OF BCL-2 FAMILY PROTEINS

Mitochondria are organelles that control the intrinsic apoptotic pathway, and the key events of mitochondria-dependent apoptosis are $\Delta\Psi_m$ dissipation, cytochrome *c* release, and caspase-9 activation. In view of the partial involvement of caspase-9 in sulfuretin-induced apoptosis, we examined whether sulfuretin induces mitochondrial dysfunction, that is, loss of $\Delta\Psi_m$ and release of cytochrome *c*, upstream of caspase-9 activation in HL-60 cells. As shown in Figure 3A, treatment with 70 μM sulfuretin for 4 h or treatment with CCCP (the positive control) led to a significant reduction in $\Delta\Psi_m$, as measured by flow cytometry. In addition, levels of cytosolic cytochrome *c* were elevated by sulfuretin, whereas mitochondrial cytochrome *c* levels were diminished, suggesting the involvement of the mitochondrial pathway in sulfuretin-induced apoptosis (Fig. 3B).

To investigate the mechanism underlying sulfuretin-induced $\Delta\Psi_m$ changes in HL-60 cells, we examined the translocations of tBid and Bax into mitochondria after treating HL-60 cells with sulfuretin. As shown in Figure 3C, treatment with sulfuretin (70 μM) reduced the cytosolic levels of pro-apoptotic Bid and Bax, but increased their mitochondrial levels. In addition, the mitochondrial expressions of Bcl-2 and Bcl-xL (both mitochondrial anti-apoptotic proteins) were reduced by sulfuretin. These results suggest that sulfuretin reduces $\Delta\Psi_m$ by modulating the expression of pro- and anti-apoptotic Bcl-2 family proteins, and that this leads to the releases of cytochrome *c* to cytosol in HL-60 cells.

CASPASE-8 ACTIVATION WAS REQUIRED FOR SULFURETIN-INDUCED BID CLEAVAGE, CYTOCHROME *C* RELEASE, AND CASPASE-3 ACTIVATION

Caspase-8 triggers the activations of caspases or via Bid cleavage [Kumar, 2007]. Since the activation of caspase-3, the mitochondrial translocation of tBid, and releases of cytochrome *c* were observed following caspase-8 activation in sulfuretin-treated HL-60 cells, we

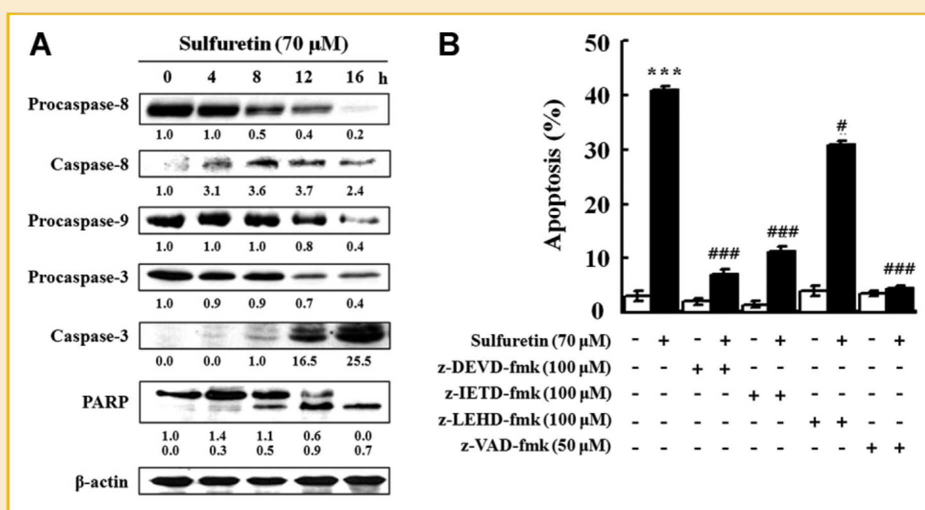

Fig. 2. Activations of caspases during the sulfuretin-induced apoptosis of HL-60 cells. A: Sulfuretin induced the activations of caspases -8, -9, and -3, and PARP cleavage. HL-60 cells were treated with sulfuretin (70 μM) for the indicated times, total cell lysates were then prepared, and protein expressions were determined by Western blotting, as described in Materials and Methods Section. Numbers below lanes show fold increases in protein expression levels. Experiments were repeated three times and similar results were obtained. Densitometric analysis was performed using Bio-rad Quantity One® Software and β-actin was used as an internal control. B: Inhibition of sulfuretin-induced apoptosis by caspase inhibitors. HL-60 cells were pretreated with z-VAD-fmk (50 μM), z-DEVD-fmk (100 μM), z-IETD-fmk (100 μM), or z-LEHD-fmk (100 μM) for 1 h and then exposed to sulfuretin (70 μM) for 16 h. Percentages of apoptotic cells were determined using DAPI assays as described in Materials and Methods Section. Data are the means ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus non-treated control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus sulfuretin-treated group.

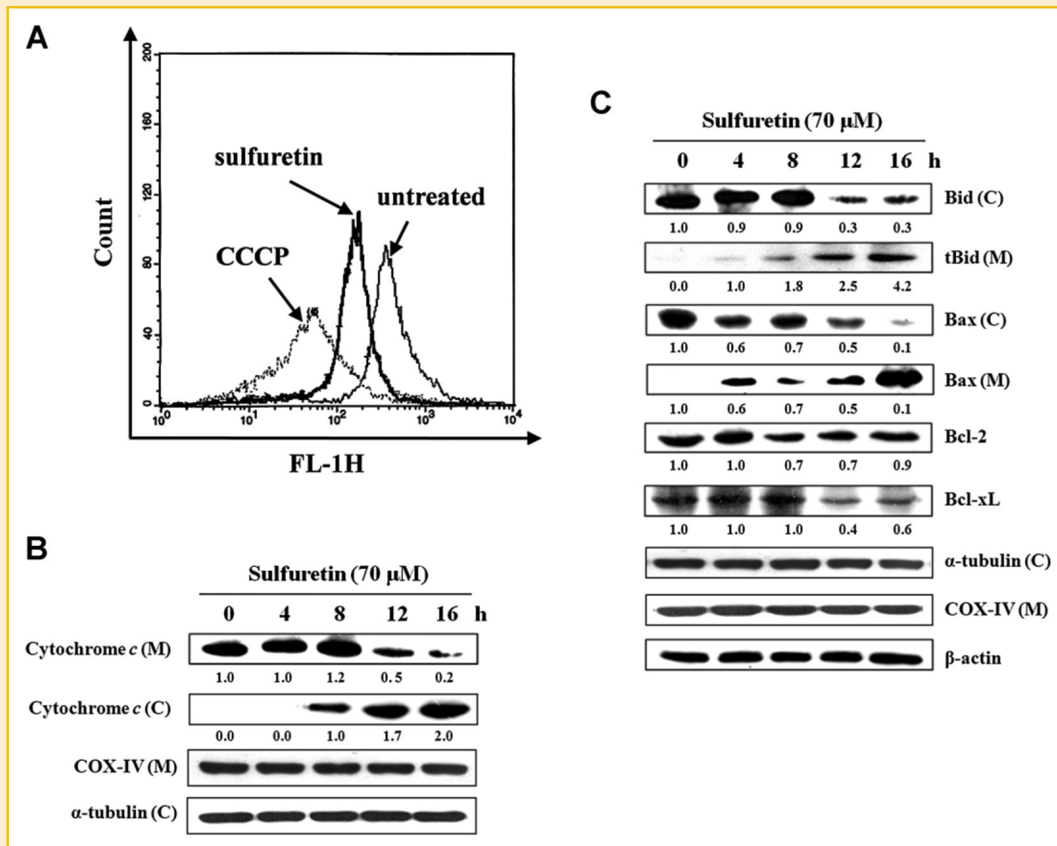

Fig. 3. Effects of sulfuretin on mitochondrial membrane potential ($\Delta\Psi_m$), the releases of cytochrome *c* and regulation of Bcl-2 family proteins in HL-60 cells. A: Cells were treated with or without 70 μM sulfuretin for 4 h; some cells were treated with CCCP (100 μM) as a positive control. $DiOC_6$ (50 nM) was then added to culture media for 30 min. $DiOC_6$ fluorescence intensities in cells were analyzed by flow cytometry. Experiments were repeated three times and similar results were obtained. B and C: Cells were harvested after being treated with sulfuretin 70 μM for the indicated times. Equal amounts of protein from mitochondrial (M) and cytosolic (C) fractions (prepared as described in Materials and Methods Section) were separated on 15% SDS–PAGE gels, and the expression of cytochrome *c* was determined by Western blotting. Experiments were repeated three times and similar results were obtained. Densitometric analysis was performed using Bio-rad Quantity One® software.

investigated whether caspase-8 acts upstream of caspase-3 activation, Bid cleavage, and the releases of cytochrome *c*. HL-60 cells were pretreated with z-IETD-fmk or z-VAD-fmk for 1 h and then treated with sulfuretin for 16 h. As shown in Figure 4, pretreatment with IETD-fmk or z-VAD-fmk completely inhibited sulfuretin-induced Bid cleavage, the releases of cytochrome *c*, and caspase-3 activation. These results suggest that caspase-8 plays a key role in the apoptosis induced by sulfuretin, and that it influences Bid cleavage, cytochrome *c* release, and caspase-3 activation.

SULFURETIN UP-REGULATED FAS AND ITS LIGAND AND INCREASED THE FORMATION OF DISC

To determine whether sulfuretin-triggered caspase-8 activation is mediated by the FasL/Fas death receptor pathway, we monitored the expressions of death receptor proteins and the formation of DISC after treating HL-60 and with sulfuretin (70 μM). Western blot analysis showed that sulfuretin began to increase the levels of Fas at 1 h and those of FasL at 4 h (Fig. 5A), and immunofluorescence analysis using flow cytometry confirmed the up-regulated expressions of Fas and FasL in sulfuretin-treated HL-60 cells (Supplementary Fig. 1). Sulfuretin, however, reduced levels of c-FLIP L and c-FLIP S, and c-FLIP is known to suppress the apoptosis induced by death receptors by interacting with caspase-8 and FADD [Irmler et al., 1997]. In addition, we immunoprecipitated Fas using a specific Fas antibody, and then monitored FasL, procaspase-8, FADD, and c-FLIP levels by Western blotting (Fig. 5B). Interactions between caspase-8 and Fas/FasL were observed after only 1 h of sulfuretin treatment, and subsequently, after treatment with sulfuretin (70 μM) for 4 h associations were observed between caspase-8 and FADD. Interestingly, c-FLIP completely dissociated from the DISC at 8 h. These findings suggest that sulfuretin stimulates DISC formation and the subsequent activation of procaspase-8.

CASPASE-8-MEDIATED APOPTOSIS BY SULFURETIN WAS DUE TO AN INTERACTION BETWEEN FASL AND FAS

To determine whether the sulfuretin-induced interaction between FasL and Fas at the cell surface mediates the induction of apoptosis by sulfuretin in HL-60 cells, we used NOK-1 mAb which interferes with the FasL to Fas interaction. Pretreatment with NOK-1 mAb

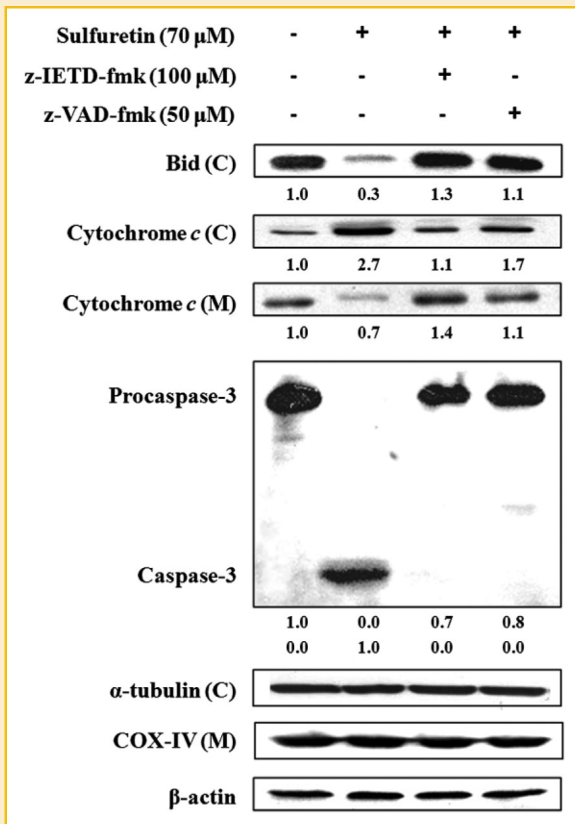

Fig. 4. The activation of caspase-8 was required for the sulfuretin-induced activation of mitochondrial apoptotic signaling in HL-60 cells. Cells were pretreated with z-IETD-fmk (100 µM) or z-VAD-fmk (50 µM) for 1 h and then treated with sulfuretin (70 µM) for 16 h. Cytosolic (C) and mitochondrial (M) fractions were prepared and caspase-3, Bid, and cytochrome *c* levels were analyzed by Western blotting. Densitometric analysis was performed using Bio-rad Quantity One® Software. Experiments were repeated three times and similar results were obtained.

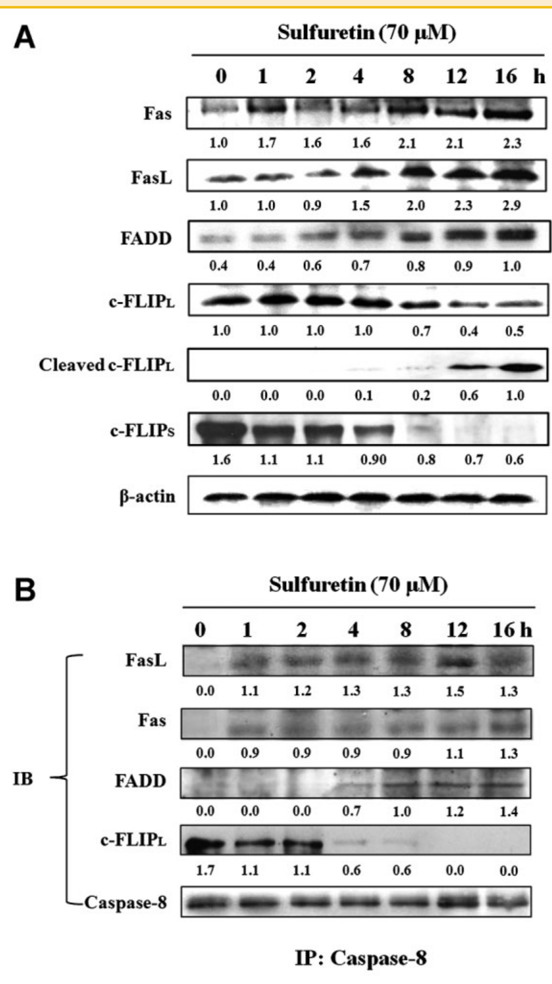

Fig. 5. Effects of sulfuretin on the expressions of death receptor proteins and on the formations of DISC complex in HL-60 cells. A: After being treated with sulfuretin (70 µM) for the indicated times, protein levels were determined by Western blotting. Experiments were repeated three times and similar results were obtained. Densitometric analysis was performed using Bio-rad Quantity One® Software. B: Lysates from non-treated control cells and sulfuretin (70 µM) treated cells were immunoprecipitated (IP) with anti-caspase-8 antibody, and the immune complexes obtained were analyzed by Western blotting (IB) using anti-FasL, anti-Fas, anti-FADD, and anti-c-FLIPL antibodies. Experiments were repeated three times and similar results were obtained. Densitometric analysis was performed using Bio-rad Quantity One® Software.

significantly inhibited sulfuretin-triggered apoptosis in HL-60 cells (Fig. 6A). Furthermore, sulfuretin-induced Bid cleavage, the releases of cytochrome *c* and caspase-3 activation in HL-60 cells were completely prevented by NOK-1 mAb (Fig. 6B). These results show that the FasL/Fas signaling pathway is essentially involved in sulfuretin-induced apoptosis via the cleavage of Bid, the releases of cytochrome *c* and caspase-3 activation.

DISCUSSION

A wide variety of naturally occurring substances have proven to be important sources of clinically useful anti-cancer agents [Cragg and Newman, 2005]. Furthermore, the aqueous extract of RVS has been shown experimentally to have anti-cancer properties against carcinomas of the breast and uterine cervix in vitro and to suppress tumor growth in a xenograft mouse model of A549 nonsmall cell lung cancer and Lewis lung cancer in vivo by inhibiting the proliferation and migratory activity of vascular endothelial growth factor (VEGF) [Choi et al., 2002, 2006]. Previously, a flavonoid fraction, consisting mainly of protocatechuic acid, fustin, fisetin, sulfuretin, and butein, was prepared from a crude acetone extract of RVS, and found to induce the apoptosis of osteosarcoma cells [Kook et al., 2007]. Furthermore, during our studies, we found that sulfuretin has more potent cytotoxic effects on various cancer cells than protocatechuic acid, fustin, fisetin, or butein, especially on hematologic cancer cells (data not shown). Accordingly, in the present study, we chose to examine sulfuretin and sought to elucidate the mechanism underlying its anti-cancer effect.

The caspases are a family of aspartate-specific cysteine proteases that play important roles in the apoptosis triggered by various

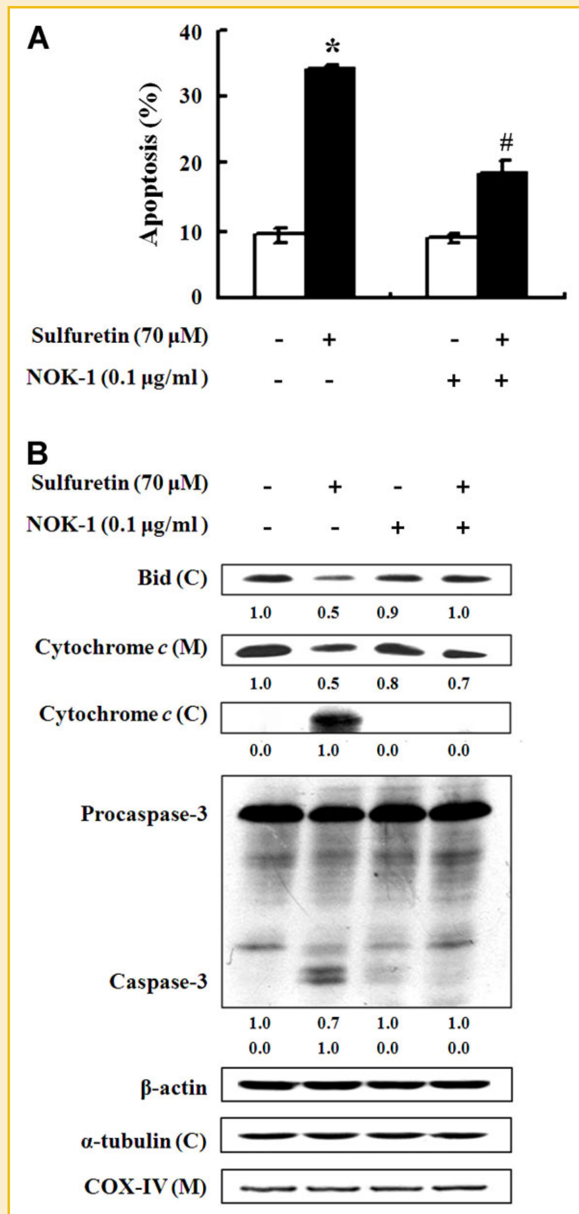

Fig. 6. Involvement of the FasL/Fas interaction in sulfuretin-induced apoptosis in HL-60 cells. A: Cells were pretreated with 0.1 μg/ml NOK-1 mAb for 1 h and then with sulfuretin (70 μM) for 16 h. Extents of apoptosis were determined using DAPI assays. Data are presented as the means ± SD of three independent experiments. $^{*}P < 0.05$ versus non-treated control group, $^{\#}P < 0.05$ versus sulfuretin-treated group. B: After pretreatment with 0.1 μg/ml NOK-1 mAb for 1 h, cells were treated with sulfuretin (70 μM) for 16 h. Cytosolic (C) and mitochondrial (M) fractions were prepared and caspase-3, Bid, and cytochrome *c* levels were determined by Western blotting. Experiments were repeated three times and similar results were obtained. Densitometric analysis was performed using Bio-rad Quantity One® Software.

pro-apoptotic signals [Lamkanfi et al., 2007]. In general, the activation of the caspase cascade requires both initiator caspases, such as, caspases-8, -9, and -10, and effector caspases, such as, caspase-3, -6, and -7 [Kumar, 2007]. In the present study, we found that sulfuretin activates caspase-3, -8, and -9, and increases the cleavage of their substrate PARP. In addition, z-VAD-fmk (a broad caspase inhibitor), z-DEVD-fmk (a caspase-3 inhibitor), and z-IETD-fmk (a caspase-8 inhibitor) completely inhibited sulfuretin-induced apoptosis, whereas z-LEHD-fmk (a caspase-9 inhibitor) mildly inhibited it. These findings suggest that sulfuretin induces caspase-dependent apoptosis in HL-60 cells and that caspase-9 activation is involved.

Mitochondrial membrane permeabilization is a crucial step in the intrinsic apoptotic pathway and often occurs concomitantly with a decrease in $\Delta\Psi_m$ due to a loss of integrity in the outer mitochondrial membrane. The activation of the intrinsic mitochondrial pathway leads to the release of several apoptogenic factors, such as, cytochrome *c*, Smac/DIABLO, and AIF [Kim et al., 2006]. In the present study, we observed that sulfuretin induces remarkable cytochrome *c* release from mitochondria to the cytosol and that it significantly reduces $\Delta\Psi_m$, indicating that sulfuretin causes a loss of outer mitochondrial membrane integrity.

Bcl-2 family proteins are critical regulators of the mitochondrial apoptotic pathway. In man, more than 20 members of this family, which includes proteins that suppress apoptosis (e.g., Bcl-2 and Bcl-xL) and proteins that promote apoptosis (e.g., Bax, Bak, and Bid), have been identified [Wang and Youle, 2009]. The anti-apoptotic members Bcl-2 and Bcl-xL inhibit cytochrome *c* release by preventing permeability transition and/or by stabilizing the barrier function of the outer mitochondrial membrane. Of the pro-apoptotic proteins in this family, the multidomain protein Bax and the BH3-only proteins, such as, Bid migrate into mitochondria when an apoptotic stimulus is sensed. Recent studies have shown that Bax translocation is not dependent on Bid, but that it can be enhanced by tBid [Wei et al., 2001; Zhai et al., 2005]. Furthermore, Bid can strongly interact with Bax via its BH3 domain to promote a Bax conformational change, and thus, facilitate the translocation and/or the insertion of conformational altered Bax into mitochondria, and subsequent cytochrome *c* release [Ott et al., 2009]. In addition, the direct binding of Bid to Bax appears to be critical for mitochondrial permeabilization, because in a previous study of mitochondria from Bax-deficient tumor cell lines, it was found that the Bid-induced release of cytochrome *c* was minimal when Bid was added alone, but was dramatically increased when Bid and Bax were present together [Ott et al., 2009]. In the present study, sulfuretin was found to reduce the levels of Bcl-2 and Bcl-xL and promote the translocations of Bax and tBid into mitochondria time-dependently (Fig. 3C). These results suggest that sulfuretin regulates mitochondrial outer membrane permeability via Bax and tBid, and that this leads to a loss of $\Delta\Psi_m$ and the release of cytochrome *c*.

As mentioned previously, there are two main caspase cascade pathways, that is, the extrinsic and intrinsic pathways. In the extrinsic pathway, the activations of initiator caspase-8 or caspase-10 are triggered by the ligations of death receptors, such as, Fas, DR3, DR4, and DR5, by the adaptor molecule FADD. Caspase-8 then activates the effector caspase-3 in two ways, either directly or via the tBid-mediated activation of the intrinsic pathway. In the intrinsic pathway, a variety of extracellular and intracellular death stimuli trigger the release of cytochrome *c* from mitochondria [Gogvadze et al., 2006]. Cytosolic cytochrome *c* then binds to Aparf-1, which promotes procaspase-9 activation in the presence of dATP or ATP,

and the resulting active caspase-9 activates effector caspases like caspases -3, -6, and -7. In the present study, we observed that sulfuretin induces the activation of the mitochondrial-dependent intrinsic pathway via caspase-9 activation. However, caspase-9 inhibition was only found to have a mildly inhibitory effect on sulfuretin-stimulated apoptosis. These findings suggest that activation of the intrinsic pathway alone is not sufficient to explain the induction of apoptosis by sulfuretin in HL-60 cells. In some experimental systems, anti-cancer drugs like doxorubicin, methotrexate, and bleomycin, have been found to induce the up-regulations of membrane Fas receptor and FasL, and thus, to activate a receptor/ligand paracrine mechanism that results in Fas-dependent apoptosis [Yoshimoto et al., 2005]. Furthermore, the caspase-8/Bid-dependent signal amplification loop has been found to be important for FasL/Fas-induced apoptosis [Opferman and Korsmeyer, 2003]. Caspase-8, which binds FADD, is activated by self-cleavage as a result of oligomerization, and subsequently, activates effector caspases like caspase-3 [Fulda, 2009]. Furthermore, Bid appears to play a key role in the crosstalk between the intrinsic mitochondrial pathway and the extrinsic death receptor pathway. In the present study, pretreatment with z-IETD-fmk abrogated sulfuretin-induced Bid cleavage, the releases of cytochrome *c* and caspase-3 activation (Fig. 4), which suggests that Bid is involved in the release of cytochrome *c* in a caspase-8 dependent manner.

In the present study, treatment of HL-60 and U937 cells with sulfuretin time-dependently increased the expressions of Fas and FasL (Fig. 5A and Supplementary Fig. 2A) and DISC formations from FasL, Fas, FADD, and procaspase-8 (Fig. 5B and Supplementary Fig. 2B), which suggests that sulfuretin-induced caspase-8 activation is probably due to the stimulation of death receptors. Thus, we hypothesized that the secretion and autocrine/paracrine engagement of FasL with Fas at the cell surface are associated with the induction of apoptosis. To test this possibility, cells were preincubated with NOK-1 mAb, which blocks the FasL/Fas interaction and inhibits Fas signaling [Bertram et al., 2006] before being treated with sulfuretin. It was found that preincubation with NOK-1 mAb blocked sulfuretin-induced DNA fragmentation, tBid translocation, cytochrome *c* release, and the cleavage of procaspase-3 in HL-60cells (Fig. 6), whereas NOK-1 mAb alone had no effect. Several studies have shown that TRAIL receptors, such as, DR4 (TRAIL R1) and DR5 (TRAIL R2), employ similar or at least intracellular signaling pathways that are closely related to those employed by Fas to induce apoptosis [Mahalingam et al., 2009]. Thus, we investigated whether TRAIL receptors are involved in the sulfuretin-induced apoptosis of HL-60 cells. We found that treatment with a TRAIL R1/Fc and TRAIL R2/Fc chimera cocktail (an inhibitor of TRAIL), failed to inhibit sulfuretin-induced apoptosis in HL-60 cells (data not shown), which indicated that sulfuretin-triggered HL-60 apoptosis is not due to the TRAIL-mediated DR4/5 death receptor pathway.

Many reports revealed that Fas-ligand gene expression could be induced by the transcription factors, such as FoxO3a, mammalian heat shock transcription factor-1 and PI3-kinase signaling [Suhara et al., 2002; Bouchier-Hayes et al., 2010; Zhang et al., 2011]. Among these regulators, it was well known that FoxO3a inhibited acute myeloid leukemia cell proliferation and regulated the extrinsic apoptotic pathway through enhancing the transcription of proapoptotic factors like Fas-ligand [Zhang et al., 2011]. In the absence of survival factors, it was found that FoxO3a was dephosphorylated and translocated to the nucleus, and then triggered cell death by a Fas-ligand-dependent mechanism [Brunet et al., 1999]. Based on these reports, we examined whether sulfuretin had an effect on de/phosphorylation status and nuclear translocation of FoxO3a in HL-60 cells. As shown in Supplementary Figure 3, sulfuretin (70 μM) treatment time-dependently reduced the levels of p-FoxO3 and elevated the translocations of FoxO3a into nucleus (Supplementary Fig. 3). These findings suggest that Fas-ligand expression induced by sulfuretin involved activation of FoxO3a transcription factor in HL-60 cells. Taken together, these results suggest that sulfuretin induces apoptosis via the selective induction of FasL/Fas, which activates caspase-8 followed by caspase-9 and -3. Furthermore, caspase-8-mediated caspase-9 activation is seemingly associated with the mitochondrial translocations of Bid and Bax, loss of mitochondrial permeability, the releases of cytochrome *c*. Since caspase-9 activation mediated by the FasL/Fas/caspase-8 pathway, is also involved in sulfuretin-induced apoptosis, it appears that active caspase-8 may stimulate the activation of effector caspase-3. Similarly, quercetin, a naturally occurring flavonoid abundant in fruits and vegetables, has also been shown to induce FasL in HL-60 cells, and thus, to induce apoptosis [Lee et al., 2010a]. These results further support the involvement of the FasL/Fas pathway in the sulfuretin-stimulated apoptosis of HL-60 cells.

In our in vivo study of a 3LL tumor cell-bearing CDF-1 mouse model, the administration of sulfuretin (50 mg/kg/day, p.o.) for 24 days was found to significantly inhibit tumor growth ($P < 0.05$) (Supplementary Fig. 4). Cisplain (1.5 mg/kg/6 day, i.p.) more potently inhibited tumor growth than sulfuretin. Taken together, our findings suggest that sulfuretin induces apoptotic cell death in HL-60 cells in vitro by activating caspase-8 via the FasL/Fas pathway, and thus, inhibiting tumor growth. Accordingly, sulfuretin appears to be a potential therapeutic agent for the treatment of human promyelocytic leukemia. The therapeutic potential of sulfuretin is further supported by several clinical case studies, in which treatment with standardized RVS extract was found to prevent tumor progression in patients with colorectal adenocarcinoma, metastatic renal cell carcinoma, and hepatocellular carcinoma [Lee et al., 2009b, 2010b; Kim et al., 2010].

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