

Apoptosis of K562 leukemia cells by Abnobaviscum F[®], a European mistletoe extract

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Abstract. Evidence suggests that mistletoe extract has the potential to be used as an anticancer agent. Abnobaviscum F[®] is a European mistletoe extract from the host tree *Fraxinus*. We investigated the effect of Abnobaviscum F on the growth and survival of different leukemia cell lines. Abnobaviscum F treatment strongly reduced survival and induced apoptosis of K562 (human myeloid leukemia), RPMI-8226 (human plasmacytoma) and L1210 (murine lymphocytic leukemia) cells in culture. Using K562 cells to further investigate the mechanism of action of Abnobaviscum F, we showed that Abnobaviscum F-induced cell death was associated with the activation of caspase-9, JNK-1/2 and p38 MAPK, as well as with the downregulation of Mcl-1, and inhibition of ERK-1/2 and PKB phosphorylation. Moreover, Abnobaviscum F treatment led to both a reduction of cellular glutathione (GSH) and the induction of ER stress (GRP78 and CHOP induction and eIF-2 α phosphorylation). By contrast, Abnobaviscum F did not impact the expression of the DR4 and DR5 death receptors. The Abnobaviscum F-induced apoptosis of K562 cells was blocked by pretreatment with either GSH, z-VAD-fmk or SP600125. Our results, therefore, show that Abnobaviscum F induces apoptosis of K562 cells through the activation of the intrinsic caspase pathway, the phosphorylation of JNK-1, the reduction of cellular GSH, and the induction of ER stress.

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder driven by a translocation of chromosomes 9 and 22,

which encodes the oncogenic breakpoint cluster region-Abelson (Bcr-Abl) tyrosine kinase (1). Approximately 5,000 new patients are affected by CML each year in the US (2). Although considerable advance has been made in the treatment of CML by the development of imatinib mesylate (STI-571), a tyrosine kinase inhibitor which targets the Bcr-Abl kinase (3), evidence suggests that up to a third of CML patients require alternate therapy due to intolerance or resistance to STI-571 and/or progression of the advanced disease (4). Thus, further research is needed to better understand and overcome drug intolerance and resistance mechanisms as well as to identify new drugs or substances with better therapeutic efficacy against CML.

Mistletoe extract has been used as complementary therapy for several types of human solid tumors (5). All industrially produced mistletoe extracts are made from the semi-parasitic plant mistletoe *Viscum album* Loranthaceae, also called European mistletoe. Mistletoe extract contains several biologically active components, such as lectins, viscotoxins, flavonoides, and phytosterols (6,7). Accumulating evidence indicates that mistletoe extract or its components has anti-tumor effects by stimulating the immune system (8), reducing tumor mass (9), and inhibiting growth and/or inducing apoptosis of cancer cells (9-11). Results from numerous *in vitro* and *in vivo* studies suggest possible mechanisms underlying the antitumor effect of mistletoe extract or its components, including inhibition of cell cycle (11), reactive oxygen species (ROS) and loss of the mitochondrial membrane potential (12,13), activation of caspases (14-16), degradation of cytoskeletal proteins (16), inhibition of protein synthesis (17), and altered expression and/or activity of the family of B-cell lymphoma-2 (Bcl-2), telomerase, protein kinase B (PKB), and the family of mitogen-activated protein kinase (MAPK) (18-20).

Abnobaviscum F[®] is a standardized preparation of aqueous European mistletoe extract from the host tree *Fraxinus*. It has been shown that Abnobaviscum F inhibits growth of various established cancer cell lines and primary mammary carcinoma cells isolated from surgical resections (21). The actions of Abnobaviscum F on hematological malignancies, however, remain unknown. In this study, we investigated the effect of Abnobaviscum F on the growth and survival of K562 (human CML), RPMI-8226 (human plasmacytoma), and L1210 (murine lymphocytic leukemia) cells.

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Materials and methods

Materials. RPMI, FBS, and penicillin-streptomycin were purchased from WelGene (Daegu, Korea). ECL western detection reagents were obtained from Thermo Scientific (Waltham, MA, USA). Bradford reagent was purchased from Bio-Rad (Hercules, CA, USA). N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk), SP600125, and protease inhibitor cocktail (100X) were bought from Calbiochem (Madison, WI, USA). SB203580 was purchased from Biomol (Plymouth Meeting, PA, USA). An antibody of caspase-9 was purchased from Stressgen (Ann Arbor, MI, USA). Myeloid cell leukemia-1 (Mcl-1), protein kinase C- δ (PKC- δ), glucose-regulated protein 78 (GRP78), and activating transcription factor 6 (ATF6) was purchased from Santa Cruz Biotechnology (Delaware, CA, USA). An antibody of poly (ADP-ribose) polymerase (PARP) was purchased from Roche (Basel, Switzerland). Antibodies of phospho-extracellular signal regulated kinase-1/2 (p-ERK-1/2), total-ERK (T-ERK-1/2), phospho-c-Jun N-terminal kinase-1/2 (p-JNK-1/2), T-JNK-1/2, p-p38 MAPK, T-p38 MAPK, p-PKB, T-PKB, phospho-eukaryotic translation initiation factor 2 α (p-eIF-2 α) were purchased from Epitomics (Burlingame, CA, USA). An antibody of T-eIF-2 α was purchased from Cell Signaling Tech (Beverly, MA, USA). Other reagents, including anti-actin mouse monoclonal antibody and glutathione (GSH), were purchased from Sigma (St. Louis, MO, USA).

Cell culture. K562, RPMI-8226 (ATCC; Manassas, VA, USA), and L1210 (KCLB; Seoul, Korea) cells were grown at 37°C in a humidified condition of 95% air and 5% CO₂ in RPMI-1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

Cell count assay. K562, RPMI-8226, or L1210 cells were seeded in 24-well plates and treated without or with different concentrations of Abnobaiviscum F for 24 h. The number of surviving cells, which cannot be stained with trypan blue dye, were counted using standard light microscopy.

Measurement of DNA fragmentation. K562, RPMI-8226, or L1210 cells were seeded in a 60-mm dish and treated with Abnobaiviscum F at the indicated concentrations or times. The cells were harvested, washed, and lysed in a buffer [50 mM Tris (pH 8.0), 0.5% sarkosyl, 0.5 mg/ml proteinase K, and 1 mM EDTA] at 55°C for 3 h, followed by the addition of RNase A (0.5 μ g/ml) and incubation at 55°C for 18 h. The lysates were centrifuged at 10,000 \times g for 20 min. Genomic DNA was extracted with equal volume of neutral phenol-chloroform-isoamyl alcohol mixture (25:24:1), and analyzed by electrophoresis on a 1.8% agarose gel. The DNA was visualized and photographed under UV illumination after staining with ethidium bromide.

Preparation of whole cell lysates. Treated cells were washed with PBS and exposed to cell lysis buffer [50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.25% sodium deoxycholate, 1% Triton X-100, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, proteinase inhibitor cocktail (1X)]. The cell lysates were collected in a 1.5 ml tube and

centrifuged for 20 min at 4°C at 12,000 rpm. The supernatant was saved and protein concentrations were determined with Bradford reagent.

Western blotting. Proteins (50 μ g) were separated by SDS-PAGE (10%) and transferred onto nitrocellulose membranes (Millipore; Billerica, MA, USA). The membranes were washed with TBS (10 mM Tris, 150 mM NaCl) supplemented with 0.05% (vol/vol) Tween-20 (TBST) followed by blocking with TBST containing 5% (wt/vol) non-fat dried milk. The membranes were incubated overnight with antibodies specific for the protein of interest at 4°C. The membranes were exposed to secondary antibodies coupled to horseradish peroxidase at room temperature for 2 h. The membranes were washed, and immunoreactivities were detected by ECL reagents.

Reverse transcription-polymerase chain reaction (RT-PCR). Total-RNA was isolated using the RNazol-B (Tel-Test). Total-RNA (3 μ g) was reverse transcribed using a random hexadeoxynucleotide primer and reverse transcriptase. Single stranded cDNA was amplified by PCR with the following primers: Mcl-1 sense, 5'-ATCTCTCGGTACCTTCGGGAG-3' and anti-sense, 5'-ACCAGCTCCTACTCCAGCAAC-3'; death receptor-4 (DR4) sense, 5'-CTGAGCAACGCAGACTCGCTGTCCAC-3' and anti-sense, 5'-AAGGACACGGCAGAGCCTGTGCCAT-3'; DR5 sense, 5'-AGCCGCTCATGAGGAAGTTGG-3' and anti-sense, 5'-GGCAAGTCTCTCTCCCAGCGTCTC-3'; C/EBP homologous protein (CHOP) sense, 5'-GTCCCTAGCTTGCTGACAGA' and anti-sense, 5'-TGGAGAGCGAGGGCTTTG-3'; actin sense, 5'-GGTGAAGGTGGGTGTGAACG-3' and anti-sense, 5'-GGTAGGAACACGGAAGGCCA-3'. The PCR conditions applied were: Mcl-1, 25 cycles of denaturation at 95°C for 45 sec, annealing at 56°C for 45 sec, and extension at 72°C for 45 sec; DR4 and DR5, 35 cycles of denaturation at 95°C for 30 sec, annealing at 63°C for 30 sec, and extension at 72°C for 30 sec; CHOP, 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min, and extension at 72°C for 30 sec; actin, 25 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 30 sec.

Measurement of GSH content. Cellular GSH content was measured using a GSH Assay kit (Calbiochem). Briefly, K562 cells were grown in a 60-mm dish and treated with Abnobaiviscum F for 0.5 or 2 h. At each time point, the culture medium was removed and cells were sonicated in 5% metaphosphoric acid. The homogenate was centrifuged at 3,000 \times g for 10 min, and its supernatant was used for GSH measurement according to the manufacturer's instructions.

Statistical analysis. Cell count analysis was performed in triplicates and repeated three times. Data are expressed as mean \pm standard error. Significance ($p < 0.05$) was determined by one-way ANOVA.

Results

Abnobaiviscum F reduces survival and induces apoptosis of leukemic cells. Initially, we investigated whether Abnobaiviscum F affects the survival of K562, RPMI-8226,

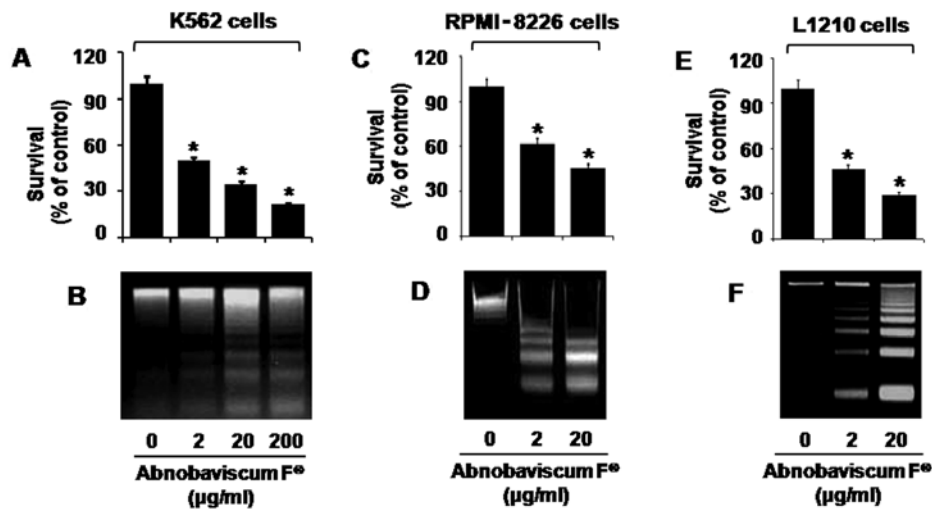


Figure 1. Effect of Abnobaviscum F[®] on the growth of various leukemic cells. (A, C and E) K562, RPMI-8226, or L1210 cells, respectively, were treated with the indicated concentrations of Abnobaviscum F for 24 h. The number of surviving cells was counted under microscope and normalized as percentage of drug-free control. Data are mean \pm SE of three independent experiments. * $p < 0.05$ compared to the value in the absence of Abnobaviscum F. (B, D and F) K562, RPMI-8226, or L1210 cells, respectively, were treated with Abnobaviscum F at the indicated doses for 24 h. Extra-nuclear fragmented DNA from the conditioned cells was extracted and analyzed on a 1.7% agarose gel. The image is representative of three independent experiments.

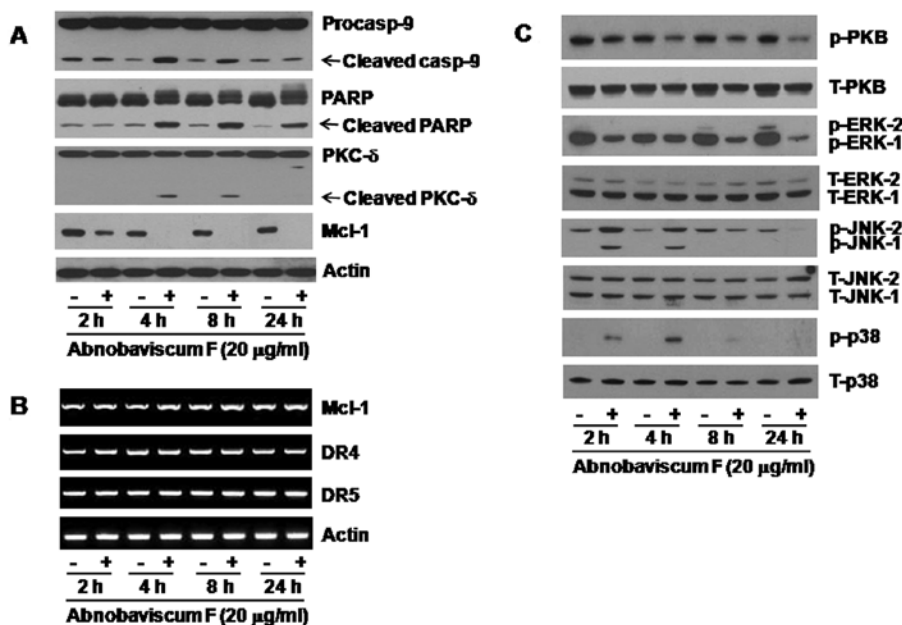


Figure 2. Effects of Abnobaviscum F[®] on the expression and/or activation of caspase-9, PARP, PKC- δ , Mcl-1, DRs, PKB, and MAPKs in K562 cells. (A-C) K562 cells were treated with Abnobaviscum F for the indicated times. At each time, whole cell lysates and total RNA were prepared and analyzed by (A and C) western blotting and (B) RT-PCR analysis, respectively. The image is representative of three independent experiments.

or L1210 cells by cell count analysis. Treatment with Abnobaviscum F for 24 h reduced survival of K562 (Fig. 1A), RPMI-8226 (Fig. 1C), or L1210 (Fig. 1E) cells in a concentration-dependent manner. We next analyzed whether Abnobaviscum F induces apoptosis of K562, RPMI-8226, or L1210 cells by measuring nuclear DNA fragmentation, a strong apoptotic index. Treatment with Abnobaviscum F for 24 h similarly induced apoptosis of K562 (Fig. 1B), RPMI-8226 (Fig. 1D), or L1210 (Fig. 1F) cells in a concentration-dependent manner. The 20 μ g/ml dose of Abnobaviscum F presented the strongest anti-survival and pro-apoptotic effects and was therefore used in further studies.

Abnobaviscum F induces activation of the intrinsic caspase pathway and the downregulation of Mcl-1 in K562 cells. To understand the mechanisms underlying Abnobaviscum F-induced apoptosis, we next treated K562 cells with Abnobaviscum F for the indicated times and measured expression of growth-related proteins. In Fig. 2A, Abnobaviscum F treatment, particularly at 4 h, decreased the expression of procaspase-9 but increased that of cleaved (active) caspase-9. High levels of cleaved PARP and PKC- δ were also observed at 4 h. Moreover, there was a substantial reduction of Mcl-1 at 2 h, followed by a complete loss at 4 h. However, mRNA levels of Mcl-1 and DR4 and DR5 were not

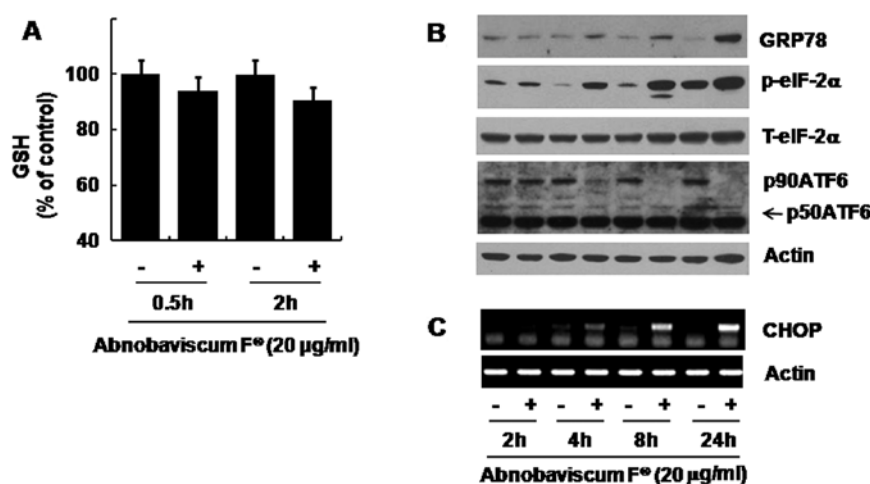


Figure 3. Effects of Abnobaviscum F® on cellular GSH level and ER stress in K562 cells. (A) K562 cells were treated with Abnobaviscum F for 0.5 or 2 h. Cellular GSH content remained in the conditioned cells was determined by a GSH assay. Data are mean \pm SE of three independent experiments. Cellular GSH content was normalized as percentage of Abnobaviscum F-free control. (B and C) K562 cells were treated with Abnobaviscum F for the indicated times. At each time, whole cell lysates and total RNA were prepared and analyzed by (B) western blotting or (C) RT-PCR. B and C are representative of three independent experiments.

changed in K562 cells treated with Abnobaviscum F at any time point measured (Fig. 2B).

Abnobaviscum F modulates expression levels and/or activities of signaling proteins in K562 cells. Treatment with Abnobaviscum F time-dependently reduced the levels of both phosphorylated PKB and total PKB (Fig. 2C). Abnobaviscum F treatment also reduced the levels of phosphorylated ERK-1/2 but did not affect expression of total ERK-1/2. Of note, Abnobaviscum F treatment at 2 or 4 h stimulated phosphorylation of JNK-1/2 and p38 MAPK. Expression levels of total JNK-1/2 and p38 MAPK remained unchanged by Abnobaviscum F treatment for all the times tested.

Abnobaviscum F decreases cellular GSH content but induces ER stress in K562 cells. We next determined whether Abnobaviscum F induces oxidative stress (by measuring cellular GSH levels) in K562 cells. In Fig. 3A, early treatments of Abnobaviscum F decreased cellular GSH levels. We also analyzed whether Abnobaviscum F triggers ER stress (by measuring expressions of ER stress-related proteins) in K562 cells. In Fig. 3B, treatment with Abnobaviscum F time-dependently increased the expression of GRP78 and phosphorylation of eIF-2α. However, levels of p90ATF6 were largely decreased at 4 h, followed by complete loss at 8 h. Although only mildly increased, p50ATF6 was also detected at 24 h. As shown in Fig. 3C, there was also a time-dependent induction of CHOP mRNA following treatment with Abnobaviscum F.

Role of GSH reduction, activation of caspases, and JNK-1 phosphorylation in Abnobaviscum F-induced apoptosis in K562 cells. We next evaluated the role of GSH depletion, activation of caspases, and phosphorylation of JNK-1/2 or p38 MAPK in the Abnobaviscum F-induced apoptosis of K562 cells. Abnobaviscum F-induced apoptosis of K562 cells was blocked by pretreatment with either GSH (a reducing agent; Fig. 4A), z-VAD-fmk (a pan-caspase inhibitor; Fig. 4C), or SP600125 (an inhibitor of JNK-1/2) but not SB203580 (a p38 MAPK inhibitor;

Fig. 4E). Pretreatment with GSH also blocked the activation of caspase-9, cleavage of PARP and PKC-δ, and phosphorylation of JNK-1 by Abnobaviscum F (Fig. 4B). The caspase inhibitor was also able to suppress the ability of Abnobaviscum F to cleave PARP and PKC-δ and to downregulate total PKB (Fig. 4D). SP600125, which only repressed phosphorylation of JNK-1, effectively inhibited the Abnobaviscum F-induced activation of caspase-9 and cleavage of PARP and PKC-δ.

Discussion

Herein we demonstrated for the first time that Abnobaviscum F, an aqueous European mistletoe extract from *Fraxinus*, induces apoptosis of K562, RPMI-8226 and L1210 leukemic cells. Moreover, our data suggest that at least in K562 cells Abnobaviscum F induces apoptosis through the modulation of cellular GSH levels, the regulation of expression levels and activities of a number of intracellular cell survival signaling proteins, and via the induction of ER stress.

Previous studies have suggested a similar antileukemic effect of mistletoe extract or its components. For instance, Iscador®, a fermented European mistletoe extract from oak, inhibits growth of Molt 4 human T-lymphoblastoid leukemic cells (22), and mistletoe lectins or agglutinins suppress growth and induce apoptosis of U937 human myeloleukemic cells (12,15,20) and HL-60 human acute lymphoblastic leukemia cells (14). In the present study, we demonstrated that Abnobaviscum F reduces survival and induces apoptosis of K562 cells. Apoptosis is closely related to two pathways; the intrinsic (mitochondrial) and the extrinsic (DR) pathways and several proteins are involved in both these pathways to mediate apoptosis. Central to both apoptosis pathways are the caspases, a group of the essential proteases required for the execution of cell death by apoptotic stimuli (23). In resting cells, caspases are synthesized as zymogens (inactive precursors), but upon exposure to apoptotic stimuli, they become processed via partial proteolytic cleavage and activated in cells. Active caspases then cleave many target proteins, including PARP, PKC-δ, and other vital proteins (23,24).

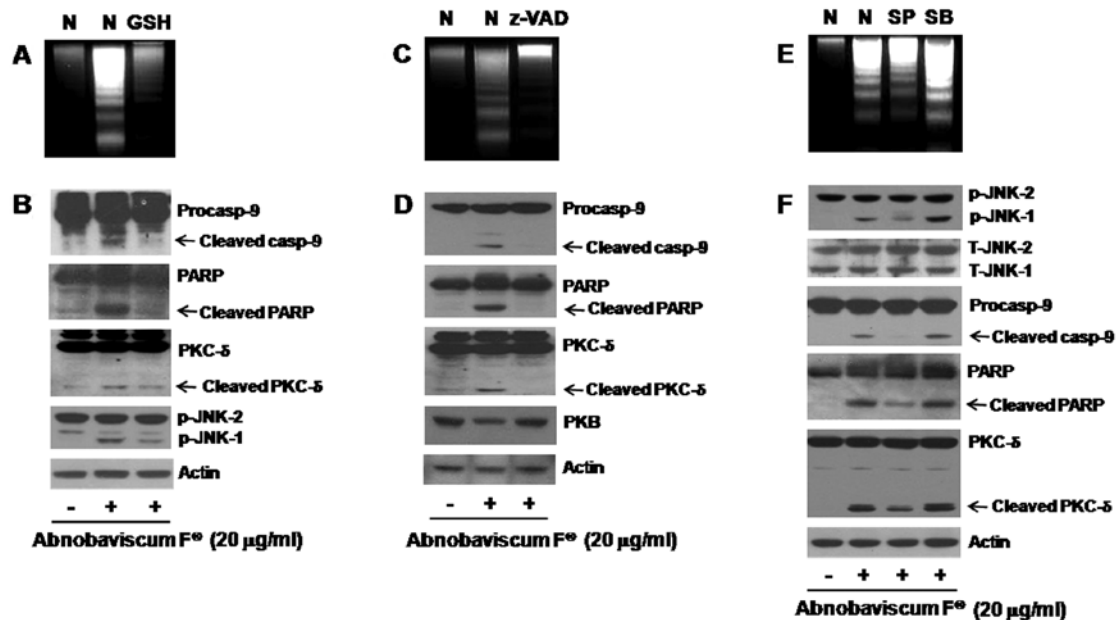


Figure 4. Effects of GSH, z-VAD-fmk, SP600125 or SB203580 on Abnobaviscum F[®]-induced apoptosis and cellular changes in K562 cells. (A, C and E) K562 cells were pretreated with 10 mM GSH (a reducing agent), 100 μ M z-VAD-fmk (a pan-caspase inhibitor), 25 μ M SP (SP600125; a JNK-1/2 inhibitor) or 25 μ M SB (SB203580; a p38 MAPK inhibitor) for 1 h and then treated with Abnobaviscum F in the absence or presence of each agent or inhibitor for an additional 24 h. Extra-nuclear fragmented DNA from the conditioned cells was extracted and analyzed on a 1.7% agarose gel. (B, D and F) K562 cells were pretreated with GSH, z-VAD-fmk, SP600125 or SB203580 for 1 h and then treated with Abnobaviscum F in the absence or presence of each agent for an additional 4 h. Whole cell lysates were prepared and analyzed by western blotting. The image is representative of three independent experiments.

It has previously been demonstrated that Iscador-induced apoptosis of human lung and breast cancer cells is mediated via the mitochondrial intrinsic caspase pathway (11). The present findings that Abnobaviscum F induces activation of caspase-9 (Fig. 2A) without affecting the expression of DRs (Fig. 2B) and z-VAD-fmk, a pan-caspase inhibitor, blocks Abnobaviscum F-induced apoptosis (Fig. 4C) in K562 cells also suggest that activation of caspases through the intrinsic pathway is important for Abnobaviscum F-induced apoptosis of K562 cells. Mcl-1 is an anti-apoptotic protein that is involved in apoptosis initiation and caspase activation by regulating the mitochondrial membrane integrity (25,26). There is further evidence suggesting Mcl-1 as a critical survival factor for multiple myeloma (27). Considering that Abnobaviscum F rapidly reduces expression of Mcl-1 (2 h) at the protein but not mRNA levels, which precedes processing and activation of caspase-9 (4 h) in K562 cells (Fig. 2A and B), the present study suggests that Abnobaviscum F targets Mcl-1 at post-transcriptional stage and loss of Mcl-1 may contribute to activation of the intrinsic caspase pathway and/or apoptosis of K562 cells exposed to Abnobaviscum F.

PKB is a survival protein kinase (28), and a high expression and/or activity of PKB often correlates strongly with the growth of cancer cells (29,30). Moreover, PKB dephosphorylation has been shown to play a role in the apoptosis of human head and neck cancer cells induced by mistletoe lectin (18). Herein Abnobaviscum F treatment led to PKB dephosphorylation in K562 cells (Fig. 2C), and to the caspase-dependent reduction of total PKB protein (Fig. 5D). ERK-1/2 are frequently activated and have a pro-survival function in AML and their pharmacological inhibitors are promising agents in the treatment of AML (31,32). On the contrary, JNK-1/2 and p38 MAPK are often acti-

vated in cells under stressful conditions (33), and their activities are linked to apoptotic death of K562 or B lymphoma cells by anticancer drugs and/or agents (34,35). Previous reports showed that mistletoe lectin-II-induced apoptosis of U937 cells is mediated by activating JNK-1/2 (19) or p38 MAPK (20). However, our results show that Abnobaviscum F-induced apoptosis of K562 cells is associated with the ability of Abnobaviscum F to inhibit PKB and ERK-1/2 but activate JNK-1 (Figs. 2C and 4E).

Oxidative stress is also involved in apoptosis induction (36). Both ROS-dependent mechanisms of mistletoe lectin-induced apoptosis (12,13) and ROS-independent mechanisms of *Viscum album* agglutinin-I-induced apoptosis (16) have been proposed. In our experimental conditions, we showed that oxidative stress, associated with a reduction of cellular GSH, is critical for the killing effect of Abnobaviscum F on K562 cells (Figs. 3A and 4A). Our data also suggest a crosstalk between reduction of cellular GSH and several other cellular events triggered by Abnobaviscum F, in which Abnobaviscum F may primarily lower cellular GSH that leads to activation of JNK-1, JNK-1 mediates the activation of caspases, and caspases induce the proteolysis of PARP, PKC- δ , and PKB (Fig. 4B, D, and F).

ER stress appears to be a common process in cancer cell death by anticancer drugs and/or agents (37,38). Cells undergoing ER stress are characterized by upregulation of molecular chaperones (e.g., GRP78) (39) and transcription factors (e.g., CHOP) (40), phosphorylation of eIF-2 α and inhibition of global translation (41), and reduction of p90ATF6 (42). In line with these studies, our present findings of Abnobaviscum F-mediated upregulation of GRP78 and CHOP, enhancement of eIF-2 α phosphorylation, and reduction of p90ATF6 in K562 cells (Fig. 3B and C) indicate that Abnobaviscum F induces ER stress, which may contribute to apoptosis.

Cancer cells differ in their cell of origin and the molecular alterations causing them to transform. Thus, drugs or agents that inhibit growth and induce apoptosis in different cancer cells may represent better anticancer strategies. In this context, the present study demonstrates the ability of Abnobaviscum F to inhibit growth and induce apoptosis in not only K562 (Fig. 1B) but also RPMI-8226 (Fig. 1D) and L1210 (Fig. 1F) leukemic cells. Collectively, the findings presented herein may shed light on the possibility of applying Abnobaviscum F to the treatment of human leukemia, as a single and/or combinatorial regimen with other antileukemic therapies.

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