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Plumbagin Induces Apoptosis in Her2-Overexpressing Breast Cancer Cells through the Mitochondrial-Mediated Pathway

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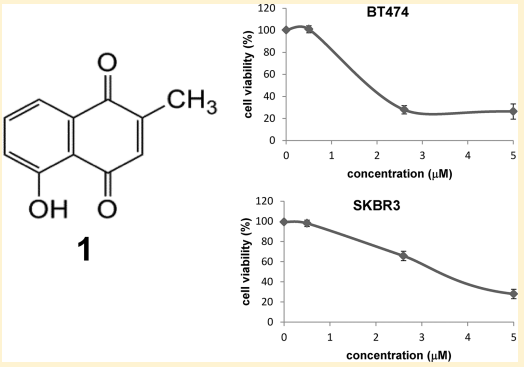
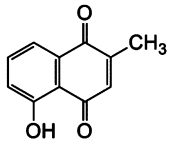
ABSTRACT: Breast cancer is the leading cause of death-related cancers in women. Approximately 30% of breast cancers overexpress the Her2 oncogene, which is associated with a poor prognosis and increased resistance to chemotherapy. Plumbagin (**1**), a constituent of species in the plant genera *Drosera* and *Plumbago*, displays antineoplastic activity toward various cancers. The present study was aimed at determining the anticancer potential of **1** toward Her2-overexpressing breast cancer cells and defining the mode of cell death induced in these cells. The results showed that **1** exhibited high antiproliferative activity toward the Her2-overexpressing cell lines SKBR3 and BT474. The antiproliferative activity of **1** was associated with apoptosis-mediated cell death, as revealed by caspase activation and an increase in the sub-G1 fraction of the cell cycle. Compound **1** increased the levels of the proapoptotic Bcl-2 family of proteins and decreased the level of the antiapoptotic Bcl-2 protein in SKBR3 and BT474 cells. Thus, these findings indicate that **1** induces apoptosis in Her2-overexpressing breast cancers through the mitochondrial-mediated pathway and suggest its potential for further investigation for the treatment of Her2-overexpressing breast cancer.



Breast cancer is the leading malignancy diagnosed in women and the second leading cause of cancer-related deaths.[1] Amplification and overexpression of certain oncogenes has been correlated with breast cancers. One of the most well-characterized oncogenes is Her2, which encodes a 185 kDa transmembrane tyrosine kinase receptor belonging to the epidermal growth factor receptor family. The Her2 gene is amplified and as a result overexpressed in ~30% of breast cancer occurrences and is associated with a poor prognosis, increased resistance to chemotherapy, and enhanced metastatic potential.[2] Several agents have been developed for the treatment of Her2-overexpressing breast cancers that target the Her2 receptor. These include monoclonal antibodies and small-molecule tyrosine kinase inhibitors, with the most well-known being trastuzumab (Herceptin) and lapatinib (Tykerb).[3] However, not all Her2-overexpressing breast cancers respond equally to treatment with trastuzumab, and some develop resistance to this drug, which limits its clinical efficacy.[4] These findings warrant the search for new agents to improve the therapeutic outcome of patients with Her2-overexpressing breast cancers.

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) (**1**) is a naphthoquinone present in plants of the *Plumbago* and *Drosera* genera.[5,6] In Indian traditional medicine, the roots of *Plumbago zeylandica* L. (Plumbaginaceae) have been used for over 2500 years as an antiatherogenic, cardiotonic, hepatoprotective, and neuroprotective agent. The main constituent, **1**, is an active compound exhibiting various reported pharmacological and biological activities including antimicrobial, hypolipidemic, antiatherosclerotic, and anticarcinogenic effects.[7,8] The antineoplastic potential of **1** has been demonstrated in various cancer models both in vivo and in vitro. Compound **1** has been shown to induce cell death in cancer cells through the induction of apoptotic pathways. This has been demonstrated in many cancer cell models, including leukemia,[9−11] prostate,[12,13] melanoma,[14] lung,[15,16] cervical,[17,18] ovarian,[19,20] and colon.[21]



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Research in relation to the anticancer potential of **1** toward breast cancer has been described previously. Compound **1** was found to induce apoptosis and autophagy in two breast cancer cell lines, MCF-7 (Her2-negative/ER-positive) and MDA-MB-231 (Her2/ER-negative).[22,23] The present investigation is the first research regarding the antiproliferative activity of **1** toward Her2-overexpressing breast cancer cells and in defining the mode of cell death induced in these cells by **1**.

RESULTS AND DISCUSSION

To determine the inhibitory effects of plumbagin (**1**) on the proliferation of Her2-overexpressing breast cancer cells, SKBR3

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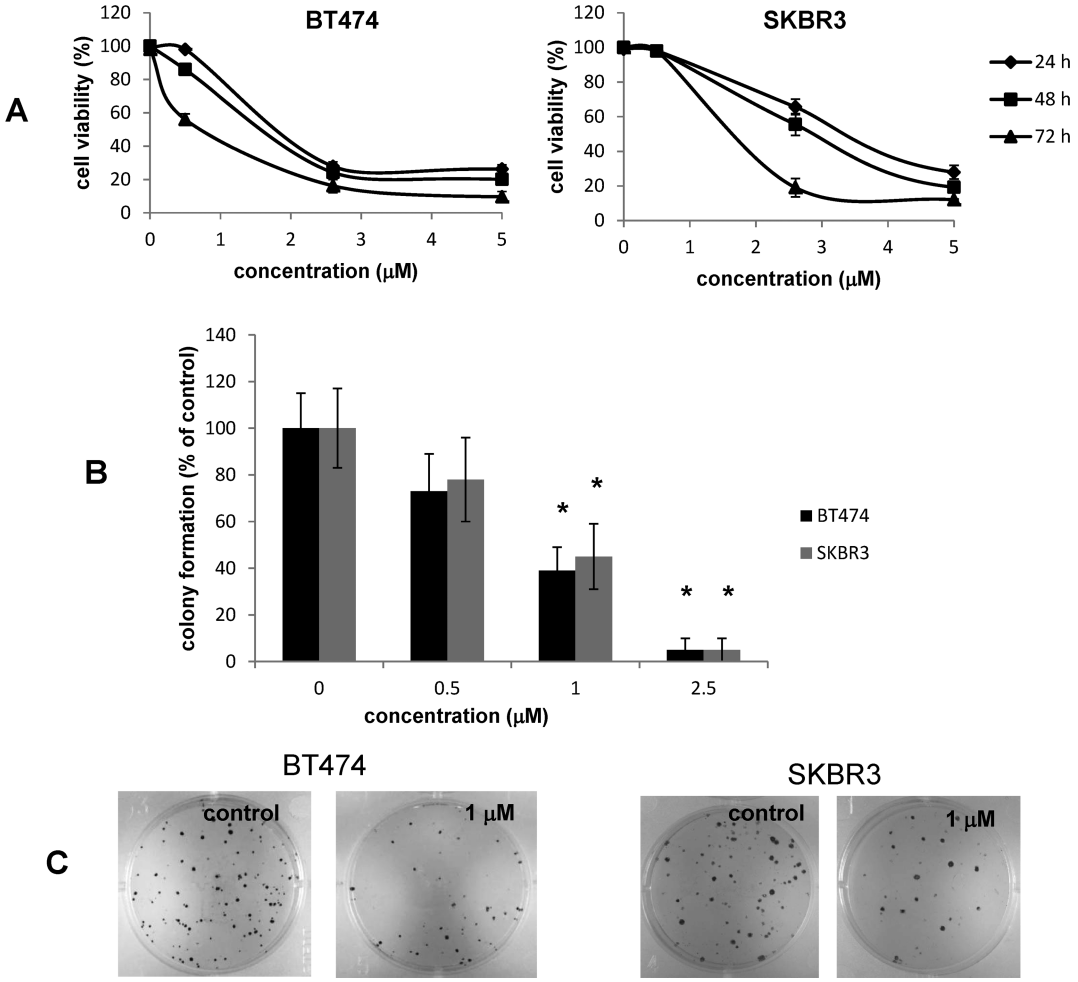

Figure 1. Effects of plumbagin (**1**) on the viability of SKBR3 and BT474 cells and colony formation. (A) Cytotoxic activity of **1**. BT474 and SKBR3 cells were treated with **1** (0−15 μM) for 24, 48, and 72 h, and cell survival was assessed with the MTT assay (n = 3). (B) Influence of **1** on the number of colony-forming SKBR3 and BT474 cells. Cells were treated with **1** (0−5 μM) for 6 h and allowed to grow for 16 days to form colonies. (C) Representative dishes of the colony-forming assay. Data were analyzed by one-way ANOVA with Tukey's posthoc tests [$p < 0.05$ (*) indicates differences between control and **1**-treated cells].

and BT474 cells were treated with increasing concentrations of **1**. As shown in Figure 1A, **1** induced a dose-dependent decrease in cell viability in both breast cancer cell lines. Higher activity was observed toward BT474 cells, with an IC_{50} value of 1.8 μM after a 24 h treatment with **1**. After 72 h, the antiproliferative activity of **1** increased ~2-fold with an IC_{50} value of 0.8 μM. For SKBR3 cells, a 50% inhibition of cell proliferation was detected at concentrations of 3.2 and 1.8 μM after exposure to **1** for 24 and 72 h, respectively. To examine the long-term effects of **1** on the proliferation of the breast cancer cells used, a clonogenic assay was performed. A dose-dependent inhibition in clonogenicity was observed on treatment of these cells with **1**. At a concentration of 1 μM, **1** inhibited ~40% and ~50% of clone formation in BT474 cells and SKBR3, respectively (Figure 1B, C). The clonogenic assay has been reported to correlate well with in vivo assays of tumorigenicity in nude mice,[24] indicating the high efficacy of **1** toward Her2-overexpressing breast cancer cells.

Cell cycle distribution was evaluated in SKBR3 cells treated with plumbagin (**1**). DNA fragmentation induced by **1** in SKBR3 cells was analyzed by determining the increase in the sub-G1 fraction of the cell cycle on treatment with **1**. As shown in Figure 2, **1** induced a concentration-dependent increase in the percentage of the sub-G1 fraction. The treatment of BT474 cells with **1** did not result in an increase in the sub-G1 fraction, most probably due to the fact that, being an aneuploid line, aneuploid populations of BT474 cells undergoing apoptosis did not appear in the sub-G1 peak.

Caspases play a central role in apoptosis execution. Their activation is essential for the occurrence of the typical hallmarks of apoptosis such as chromatin condensation and DNA fragmentation.[25] The involvement of caspase activation in **1**-mediated cell death in Her2-overexpressing cell lines was determined with the use of a carboxyfluorescein (FAM) derivative of valylalanylaspartic acid (VAD) fluoromethyl ketone (FMK), which is an inhibitor of caspase activity. FAM-VAD-FMK binds irreversibly to reactive cysteine residues on caspases (caspases 1 through 9) in the cell and inhibits their activity. This allows for the direct measurement of caspase activity. Caspase activity was measured after a 6 h incubation with plumbagin (**1**). As determined by fluorescence measurement, **1** induced a dose-dependent increase in caspase activity in BT474 and SKBR3 cells. A statistically significant increase in caspase activity was observed at a concentration of 10 μM, with a 5-fold and 4-fold increase in caspase activity observed in BT474 and SKBR3 cells, respectively (Figure 3).

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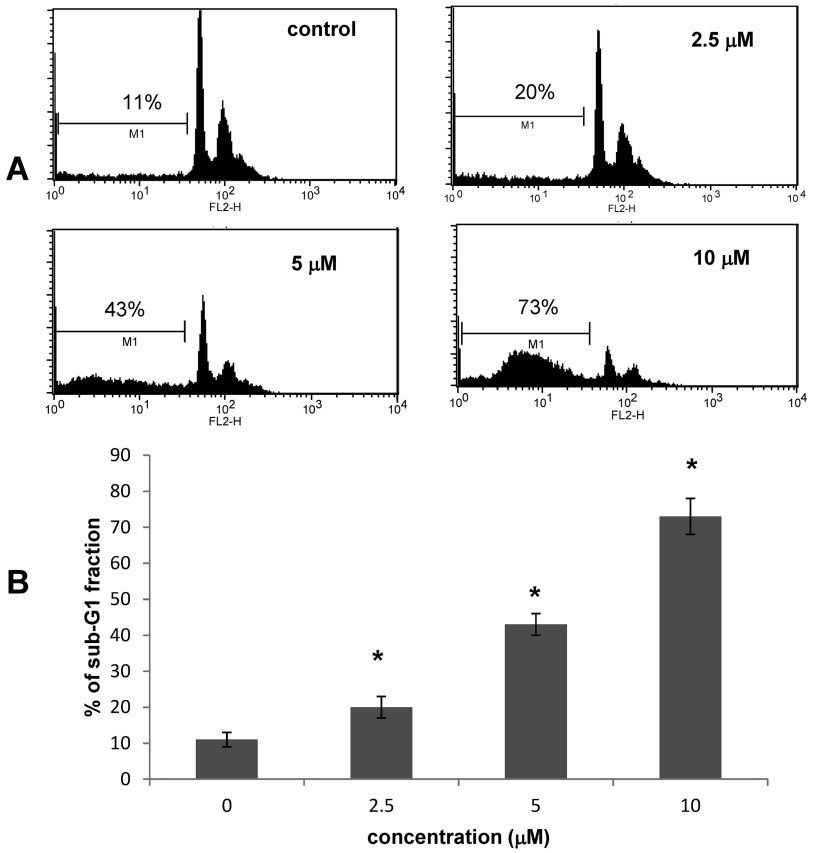

Figure 2. Induction of cells in the sub-G1 fraction of the cell cycle in SKBR3 cells by plumbagin (**1**). Cells were treated with **1** (0−10 μM) for 24 h, and cell cycle analysis was performed with flow cytometry. (A) Histograms representing PI fluorescence intensities. (B) Results are the mean of three independent experiments. Data were analyzed by one-way ANOVA with Tukey's posthoc tests [$p < 0.05$ (*) indicates differences between control and **1**-treated cells].

The Bcl-2 family of proteins play a central role in the regulation of the intrinsic (mitochondrial) apoptotic pathway. To determine their involvement in **1**-mediated cell death in Her2-overexpressing breast cancer cells, levels of the proapoptotic and antiapoptotic Bcl-2 proteins were analyzed in **1**-treated SKBR3 and BT474 cells. Western blot analysis showed an increase in the levels of Bax and Bak proteins in a concentration-dependent manner in both SKBR3 and BT474 cells. A decrease in the level of antiapoptotic Bcl-2 protein was observed in **1**-treated SKBR3 and BT474 cells (Figure 4). These results point to the involvement of the intrinsic pathway in **1**-mediated cell death in Her2-overexpressing breast cancer cells.

Plumbagin (**1**) is gaining increasing interest due to its promising anticancer potential, as a result of previous experimental work. In vivo studies indicated that **1** reduces the size of tumors by 70% in nude mice implanted with MDA-MB-231 tumor cell xenografts. The reduction of tumor growth was accompanied by no observed toxic side effects, as determined by examining tissue sections of lungs, livers, and kidneys.[22] Compound **1** was found to inhibit the migration and invasion of breast cancer cells by downregulating the expression of CXCR4, a chemokine receptor that has been associated with tumor proliferation, invasion, and metastasis. CXCR4 receptor downregulation was not mediated through its proteolytic degradation but through transcript downregulation. Moreover, the downregulation of CXCR4 led to the inhibition of migration and invasion induced by ligand CXL12 in breast

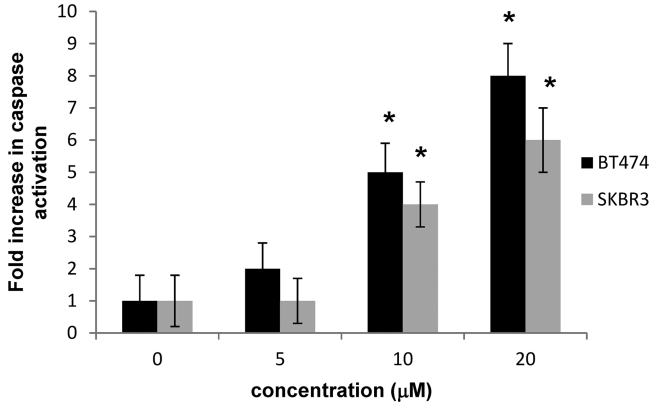

Figure 3. Induction of caspase activity in SKBR3 and BT474 cells by plumbagin (**1**). Cells were treated with **1** (0−20 μM) for 6 h, and enzyme activity was determined with a caspase inhibitor, FAM-VAD-FMK. Results are the mean of three independent experiments. Data were analyzed by one-way ANOVA with Tukey's posthoc tests [$p < 0.05$ (*) indicates differences between control and **1**-treated cells].

cancer cells.[26] Recent research shows that **1** inhibits breast cancer-stimulated osteoclastogenesis. This was demonstrated since **1** inhibits breast cancer-induced osteolytic metastasis through suppression of RANKL signaling.[27]

The proapoptotic activity of **1** toward breast cancer cells has been studied previously in two breast cancer cell lines, namely, MCF-7 and MDA-MB-231. Studies performed by Kuo et al.[22] revealed that the mode of cell death induced by **1** is

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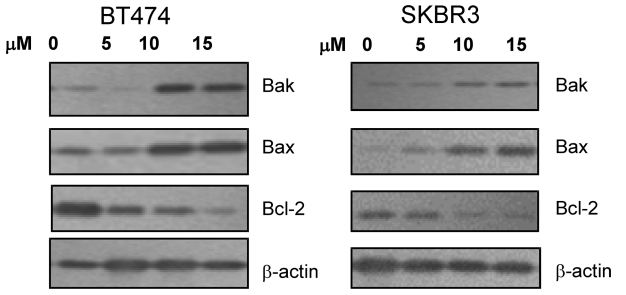

Figure 4. Induction of apoptosis in SKBR3 and BT474 cells by plumbagin (**1**) via the mitochondrial pathway. Cells were treated with **1** (0−20 μM) for 24 h, and the effects of **1** on the levels of Bax, Bak, and Bcl-2 proteins were analyzed with Western blotting using respective antibodies. The results are representative of three independent experiments.

predominately through autophagy. Results using the TUNEL assay showed that **1** induced only a small percentage of apoptotic cell death. These results were verified with the use of a pan-caspase inhibitor, which resulted in only a small decrease in breast cancer cell proliferation. Further studies revealed that treatment with **1** led to the appearance of autophagic vacuoles in MCF-7 and MDA-MB-231 cells. Furthermore, the use of an autophagy inhibitor, bafilomycin, blocked the induction of autophagy by **1** in MDA-MB-231 cells.[22] However, Ahmad et al.[23] reported that **1** inhibited the proliferation of MCF-7 and MDA-MB-231 cells through the induction of apoptotic cell death. A significant increase in **1**-induced apoptosis was determined with the use of two methods, namely, the histone/DNA ELISA and the annexin V-propidium iodide procedures. The induction of apoptosis in both breast cancer cell lines was accompanied by downregulation of the antiapoptotic Bcl-2 and caspase-3 and caspase-9 activation. The present results are in agreement with these findings, since **1** induced apoptosis-mediated cell death in BT474 and SKBR3 cells. Apoptosis induction was associated with caspase activation and increased proapoptotic Bax and Bak protein levels. Furthermore, the current data showed that **1** decreases the level of the antiapoptotic Bcl-2 protein, which is in agreement with the results of Ahmad et al.,[23] who demonstrated that **1** targets the NF-κB/Bcl-2 pathway in breast cancer cells. The antiapoptotic protein Bcl-2, expressed in 70% of breast cancers, can inhibit apoptosis induction and thus promote cancer proliferation.[28] Elevated Bcl-2 levels have been associated with decreased breast cancer sensitivity toward various chemotherapeutic agents.[29] Furthermore, increased levels of Bcl-2 have been associated with resistance to trastuzumab in Her2-overexpressing breast cancer cells. The pharmacological inhibition of Bcl-2 was shown to increase the sensitivity of cells with acquired resistance to trastuzumab treatment.[30] Therefore, the downregulation of Bcl-2 in Her2-overexpressing breast cancer cells indicates the promising anticancer potential of **1** in the treatment of this type of cancer.

A major barrier in effective cancer treatment is impaired apoptosis. Her2-overexpressing breast cancer cells, in particular, display increased resistance to apoptosis induced by chemotherapeutic agents. An obstacle in the clinical efficacy of Her2 receptor targeting agents is the acquired resistance of Her2-overexpressing cells toward these compounds. Compound **1** could be a promising agent in the treatment of Her2-overexpressing breast cancer due to its ability to induce apoptosis in these cells, particularly through Bcl-2 down-regulation. The potential of **1** in breast cancer treatment is also indicated by its selective activity toward breast cancer cells. Studies carried out by Ahmad et al.[23] showed that **1** inhibited the proliferation of breast cancer cells without any effects on normal breast epithelial cells.

In conclusion, the research presented herein shows that plumbagin (**1**) induced apoptosis in Her2-overexpressing breast cancer cell lines, with this activity associated with caspase activation and mitochondrial pathway induction. The activity of **1** toward Her2-overexpressing breast cancer cells supports the further investigation of this compound for its potential use as a therapeutic agent in breast cancer.

■ EXPERIMENTAL SECTION

Chemicals. Plumbagin (**1**) was obtained at >95% purity from Sigma-Aldrich (St. Louis, MO, USA). All cell culture materials and other chemicals, if not indicated otherwise, were purchased from the same company.

Cell Culture. The BT474 breast cancer cell line was purchased from Cell Lines Service (Eppelheim, Germany), and the SKBR3 cell line was obtained from the Department of Microbiology, Tumor and Cell Biology, Karolinska Institute (Stockholm, Sweden). BT474 cells were cultured in DMEM/F12 medium, and SKBR3 cells were cultured in DMEM medium. The medium was supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C in an incubator (Heraceus, HeraCell).

Cytotoxicity Assay. The viability of cells was determined using the MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Cells were treated with **1** (0−15 μM) for 24, 48, and 72 h. Analysis was performed according to a previously published procedure.[31]

Clonogenicity Assay. To determine long-term effects, cells seeded in six-well plates (10^3 cells/well) were treated with **1** (0−5 μM) for 6 h. The medium was discarded, and fresh medium was added to the wells, after which cells were allowed to grow for 16 days to form colonies, which were stained with crystal violet (0.5%).

Cell Cycle Analysis. To determine cell cycle distribution, BT474 and SKBR3 cells were seeded in six-well plates (5×10^5) and treated with plumbagin (**1**, 0−10 μM) for 24 h. Analysis was performed with flow cytometry according to a previously published procedure.[31]

Caspase Activity Determination. The activation of caspase activity by **1** (0−20 μM) was determined with the use of the FLICA apoptosis detection kit (Alexis, San Diego, CA, USA). Cells treated with plumbagin (**1**) for 6 h were collected and suspended in a buffer containing a carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspases. After a 1 h incubation at 37 °C under 5% CO_2, cells were washed with washing buffer and the fluorescence intensity of fluorescein was measured with a plate reader (Victor, 1420 multilabel counter). Caspase activity was determined as the amount of fluorescence emitted from the probes bound to the caspases.

Western Blot Analysis. BT474 and SKBR3 cells were treated with **1** (0−20 μM) for 24 h, after which cells were collected and lysed in a lysis buffer (20 mM Tris pH 7.5, 0.15 M NaCl, 1 mM EDTA, 1% NP-40, 1 mM DTT, 0.25 mM PMSF) for 30 min on ice. Lysates were centrifuged for 30 min at 4 °C, and the supernatants were collected. Equivalent amounts of protein were resolved by SDS-PAGE and transferred onto PVDF membranes. After blocking with TBS buffer (500 mM NaCl, 20 mM Tris-HCl (pH 7.4)) containing 5% nonfat dry milk for 1 h, the membranes were incubated overnight at 4 °C with specific primary antibodies: anti-β-actin (1:1000) (Sigma), anti-Bax, anti-Bak, and anti-Bcl-2 (1:250) (Santa Cruz, Heildeberg, Germany). Membranes were further incubated at room temperature for 1 h with HRP-conjugated secondary antibodies (Santa Cruz), and proteins were detected by chemiluminescence (ChemiDoc, Bio-Rad) with a HRP substrate (Pierce, Rockford, IL, USA).

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Statistical Analysis. Values are expressed as means ± SEM of at least three independent experiments. Differences between control and **1**-treated samples were analyzed by one-way ANOVA with Tukey's posthoc tests. A p value of <0.05 was considered as statistically significant in each experiment.

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Notes

The authors declare no competing financial interest.

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