Boswellic acid acetate induces apoptosis through caspase-mediated pathways in myeloid leukemia cells

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Abstract

The mechanism of the cytotoxic effect of boswellic acid acetate, a 1:1 mixture of α-boswellic acid acetate and β-boswellic acid acetate, isolated from *Boswellia carterri* Birdw on myeloid leukemia cells was investigated in six human myeloid leukemia cell lines (NB4, SKNO-1, K562, U937, ML-1, and HL-60 cells). Morphologic and DNA fragmentation assays indicated that the cytotoxic effect of boswellic acid acetate was mediated by induction of apoptosis. More than 50% of the cells underwent apoptosis after treatment with 20 μg/mL boswellic acid for 24 hours. This apoptotic process was *p53* independent. The levels of apoptosis-related proteins Bcl-2, Bax, and Bcl-X_L were not modulated by boswellic acid acetate. Boswellic acid acetate induced Bid cleavage and decreased mitochondrial membrane potential without production of hydrogen peroxide. A general caspase inhibitor (Z-VAD-FMK) and a specific caspase-8 inhibitor II (Z-IETD-FMK) blocked boswellic acid acetate–induced apoptosis. The mRNAs of death receptors 4 and 5 (DR4 and DR5) were induced in leukemia cells undergoing apoptosis after boswellic acid acetate treatment. These data taken together suggest that boswellic acid acetate induces myeloid leukemia cell apoptosis through activation of caspase-8 by induced expression of DR4 and DR5, and that the activated caspase-8 either directly activates caspase-3 by cleavage or indirectly by cleaving Bid, which in turn decreases mitochondria membrane potential. [Mol Cancer Ther 2005;4(3):381–8]

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Introduction

Conventional chemotherapy of acute myelocytic leukemia (AML) with either cytarabine or daunorubicin given as a single agent induces complete remission in ~30% to 40% of patients and combination treatment with both agents induces complete remission in more than 50% of patients. However, long-term, disease-free survival occurs in only 25% to 50% of the patients who achieve complete remission (1–3). The majority of patients with AML still die of this disease. Thus, novel agents need to be developed for treatment of AML.

Boswellic acid and its acetates (Fig. 1) were isolated from the gummy exudates of *Boswellia serrata* and *Boswellia carterri* Birdw. Boswellic acid derivatives are inhibitors of topoisomerases and nonredox, noncompetitive, specific inhibitors of 5-lipoxygenase (4–6). Among the natural boswellic derivatives, it has been found that 3-*O*-acetyl-11-keto-β-boswellic acid was the most active inhibitor of 5-lipoxygenase in rat peritoneal polymorphonuclear leukocytes with an IC_{50} of 1.5 to 3 μmol/L (4, 7). Using pure topoisomerase assay it was found that both boswellic acid acetate and 3-*O*-acetyl-11-keto-β-boswellic acid were more potent topoisomerase I and IIα inhibitors than camptothecin and amsacrine or etoposide, respectively (6). Previously, we and other groups have found that boswellic acid acetate induced HL-60 cell differentiation at low concentrations and induced cytotoxic effects through apoptosis induction at higher doses (8–10). Recently, the apoptotic effects of boswellic acids have also been found in malignant glioma cells and colon cancer cells (11, 12). It seems that boswellic acids are potent apoptotic agents to cancer cells. In the present study, the mechanism(s) of apoptosis induction by boswellic acid acetate in six human myeloid leukemia cell lines (NB4, SKNO-1, K562, U937, ML-1, and HL-60 cells) was studied. The obtained data indicate that boswellic acid acetate induces apoptosis in the six cell lines tested regardless of the type of *p53* present (wild-type or mutant). It is also independent of reactive oxygen species (ROS) production. Boswellic acid acetate seems to induce apoptosis through a caspase-mediated pathway that is activated by the induction of the death receptors 4 (DR4) and 5 (DR5).

Materials and Methods

Reagents

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Boswellic acid acetate (a mixture of 1:1 α- and β-boswellic acid acetate, Fig. 1) was isolated from *B. carterii* Birdw as described before (8).

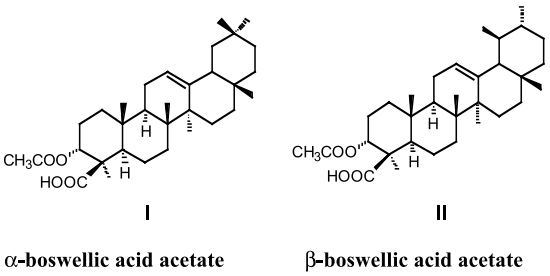

Figure 1. The chemical structure of boswellic acid acetate.

Cell Lines

NB4, HL-60, SKNO-1, ML-1, U937, and K562 cells were cultured in RPMI 1640 supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 1 mmol/L L-glutamine, and 10% heat-inactivated fetal bovine serum. HL-60/neo and HL-60/BCL2 cells stably transfected with empty vector or Bcl-2 expression vector, respectively, were provided by Dr. M. Cleary (13).

Cell Growth Inhibition

Leukemia cells were seeded at a density of 1×10^5 and incubated with various concentrations of boswellic acid acetate (0, 2.5, 5, 7.5, 10, 12.5, and 15 μg/mL). Total cell number in each group was counted and the drug concentration that inhibited half of the cell growth (IC_{50}) was calculated. The cell viability was calculated after staining with trypan blue exclusion.

Clonogenic Assay

One milliliter RPMI 1640 containing 0.5% agar, 15% fetal bovine serum, and different concentrations of boswellic acid acetate (0, 1, 2.5, 5, 7.5, 10, 12.5, and 15 μg/mL) were plated on the bottom of 35-mm tissue culture dishes as the bottom layer. U937 and K562 cells (1,000 cells) in the logarithmic phase of growth were seeded on the top of these dishes in 1 mL RPMI 1640 containing 0.3% agar and 15% fetal bovine serum. The dishes were cultured at 37°C in a humidified atmosphere containing 5% CO_2 for 7 days. Colonies (>50 cells) were counted using an inverted microscope at 30× magnification.

Quantitation of Apoptotic Cells

Apoptotic cells were determined by morphologic observation and DNA fragmentation analysis. For morphologic evaluation, cells were stained with acridine orange and ethidium bromide and assessed by fluorescence microscopy as described previously (14). For DNA fragmentation analysis, cells were harvested by centrifugation and the pellets were suspended in lysis buffer containing 15 mmol/L Tris-HCl (pH 8.0), 20 mmol/L EDTA, and 0.5% Triton X-100. After 30 minutes on ice, samples were centrifuged at 14,000 × *g* at 4°C for 30 minutes, and cellular DNA was extracted with a Puregene DNA isolation kit (Gentra System, Minneapolis, MN). Electrophoresis was done in 1% agarose gel in 40 mmol/L Tris-acetate buffer (pH 7.4), at 50 V. After electrophoresis, DNA was visualized by ethidium bromide staining.

Hydrogen Peroxide Production

Hydrogen peroxide production was quantitated using 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate as previously reported (15). Briefly, exponentially growing cells were seeded at 2×10^5 cells/mL and treated with boswellic acid acetate for various times followed by labeling with 0.5 μmol/L 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate for another 2 hours. After washing with PBS, 10,000 cells were analyzed by FACscan (Becton Dickinson, San Jose, CA) with excitation and emission settings of 495 and 525 nm, respectively. Arithmetic histogram statistics analysis was used to determine the mean of the oxidized 2′,7′-dichlorofluorescein peak in each sample.

Mitochondria Membrane Potential Assay

Mitochondrial membrane potential was measured by using a Mitochondrial Membrane Sensor Kit as described by the manufacturer (Clontech Laboratories, Inc., Palo Alto, CA). Briefly, after boswellic acid acetate treatment, cells were washed with serum-free medium after centrifuging at 350 × *g* and 4°C for 5 minutes, then the cell pellets were suspended in 1 mL diluted BD MitoSensor reagent per tube. The cells were then incubated at 37°C in a humidified, 5% CO_2 incubator for 15 to 20 minutes. One milliliter of incubation buffer was added to each tube and the cells were recentrifuged. The cell pellets were resuspended in incubation buffer (1 mL) and examined under a microscope using a band-pass filter. The MitoSensor reagent aggregates in the mitochondria of healthy cells, forming red fluorescence. When mitochondrial membrane potentials are altered, the MitoSensor reagent does not accumulate in the mitochondria and remains as monomers in the cytoplasm where it fluoresced green.

Western Blot Analysis

Protein extracts (50 μg) prepared with radioimmunoprecipitation assay lysis buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, 1 mmol/L phenylmethylsulfonylfluoride, 100 μmol/L leupeptin, and 2 μg/mL aprotinin (pH 8.0)] and were subjected to electrophoresis on 8% or 12% SDS-polyacrylamide gels. The separated proteins were then transferred to nitrocellulose membranes that were stained with 0.2% Ponceau S red to assure equal protein loading and transfer. After blocking with 5% nonfat milk, the membranes were incubated with a polyclonal antibody to poly(ADP-ribose) polymerase (Boehringer Mannheim, Indianapolis, IN) and monoclonal antibodies to *p53*, Bcl-2, Bcl-X_L, Bid, caspase-3, caspase-8 and actin (Oncogene Research Products, Cambridge, MA). Immunocomplexes were visualized by chemiluminescence (ECL kit, Amersham Biosciences Corp, Piscataway, NJ).

Northern Blot Analysis

Total RNA was isolated with PURESCRIPT (Gentra Systems, Minneapolis, MN) from 10^6 cells. Twenty micrograms RNA were size-fractionated on a 1.2% agarose-2.2 mol/L formaldehyde gel, transferred to a hybrid-N+ membrane (Amersham Biosciences Corp) in 20× SSC, and UV-cross-linked (Stratalinker, Stratagene, La Jolla, CA). *DR4* and *DR5* cDNAs (provided by Dr. Gen Sheng Wu, Department of Pathology, Wayne State University School

of Medicine, Detroit, MI) and glyceraldehyde-3-phosphate dehydrogenase (Ambion, Austin, TX) cDNAs were used for probing the membranes. The probes were labeled with [α^{32}P]dCTP by random priming to a specific activity of (0.5–1) $\times 10^9$ cpm/ng. The membranes were prehybridized for 4 hours at 42°C in 50% formamide, 6× saline-sodium phosphate-EDTA, 5× Denhardt's reagent, and 0.2 mL of 1 mg/mL SS DNA, then hybridized with the various radiolabeled probes and visualized by radioautography with Kodak BioMax film.

Statistics

Data were analyzed for statistical significance using the Student's *t* test (Microsoft Excel, Microsoft Corporation, Seattle, WA). Differences were considered significant at $P < 0.05$.

Results

Boswellic Acid Acetate Inhibits Cell Growth and Induces Cell Toxicity of Myeloid Leukemia Cell Lines through Apoptosis

The cell growth inhibition of boswellic acid acetate in leukemia cell lines was measured by direct cell counting and clonogenic assay. Boswellic acid acetate inhibited cell growth in a dose-dependent pattern with IC_{50}s of 5.8, 8.7, 7.3, 6.2, 9.8, and 6.6 μg/mL in NB4, SKNO-1, HL-60, U937, K562, and ML-1 cells at 4 days' treatment, respectively. Because NB4, ML-1, SKNO-1, and HL-60 cells only formed a few colonies in soft agar culture, the effect of boswellic acid acetate on clonogenic forming ability was only tested in U937 and K562 cells. Boswellic acid acetate inhibited the clonogenic formation of both cell lines after 14 days' culture with IC_{50}s 6.6 and 5.1 μg/mL, respectively. The cytotoxic effect of boswellic acid acetate in six myeloid leukemia cell lines was determined by trypan blue staining after 24 hours treatment. As shown in Fig. 2A, 20 μg/mL boswellic acid acetate markedly decreased cell viability after 24 hours of treatment in all of the lines except in K562 cells in which the decrease was only about 30%. Because boswellic acid acetate inhibited cell growth at lower concentrations (<10 μg/mL) without causing cytotoxicity (Fig. 2A), other actions of boswellic acid acetate such as differentiation induction as we reported before may involve in boswellic acid acetate–mediated cell growth inhibition in these cells (8). However, boswellic acid acetate is a potent cytotoxic agent for most leukemia cells at concentrations between 15 and 20 μg/mL (Fig. 2A). Based on the morphologic examination after staining with acridine orange and ethidium bromide, all of the tested cell lines underwent >50% apoptosis after 24 hours of treatment at 20 μg/mL of boswellic acid acetate (Fig. 2B) and apoptotic cells can be detected in HL-60 and NB4 cells after treatment as short as 8 hours at a concentration of 20 μg/mL (data not shown).

Boswellic Acid Acetate Induces Apoptosis through a *p53*-Independent Pathway

p53 was detected in NB4, SKNO-1, and ML-1 cells, but not in HL-60, U937, and K562 cells (Fig. 3A). p53 levels were not changed by boswellic acid acetate (15 μg/mL) treatment in NB4 and SKNO-1 cells, although it was somewhat decreased in ML-1 cells. Boswellic acid acetate treatment induced marked DNA fragmentation in NB4, HL-60, and U937 cells (Fig. 3B). NB4 cells that express mutant *p53*, and HL-60 cells, which are null for *p53*, are both highly sensitive to boswellic acid acetate–induced apoptosis, and SKNO-1 and ML-1 cells (which express wild-type *p53*) were less sensitive to boswellic acid acetate–induced DNA fragmentation. Thus, it seems that *p53* is not necessary in boswellic acid acetate–induced apoptosis.

Boswellic Acid Acetate Induces Proteolysis of Bid, but not Other Bcl-2 Family Members in Apoptotic Leukemia Cells

The effects of boswellic acid acetate on the levels of Bcl-2 protein family members, Bcl-2, Bcl-X_L, and Bax, were determined (Fig. 3A). Bcl-2 protein was detected in all of the cell lines except K562 and the Bcl-2 protein levels were not changed in any of the cell lines. HL-60/BCL2 cells with overexpression of Bcl-2 displayed only a slight

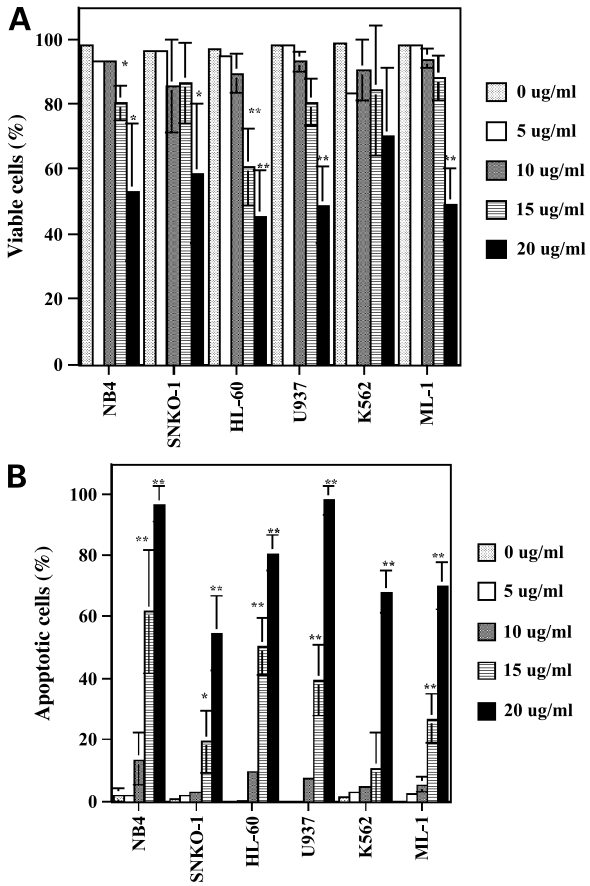

Figure 2. Boswellic acid acetate induces cytotoxicity and apoptosis in six myeloid leukemia cell lines. **A,** cytotoxicity. **B,** apoptosis. The cells were treated with the indicated concentrations of boswellic acid acetate for 24 h. The cell viability was determined by trypan blue staining and the apoptotic cells were determined by morphologic changes as described in materials and methods. *Columns,* mean of triple experiments; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$ (comparing with control group of each cell line).

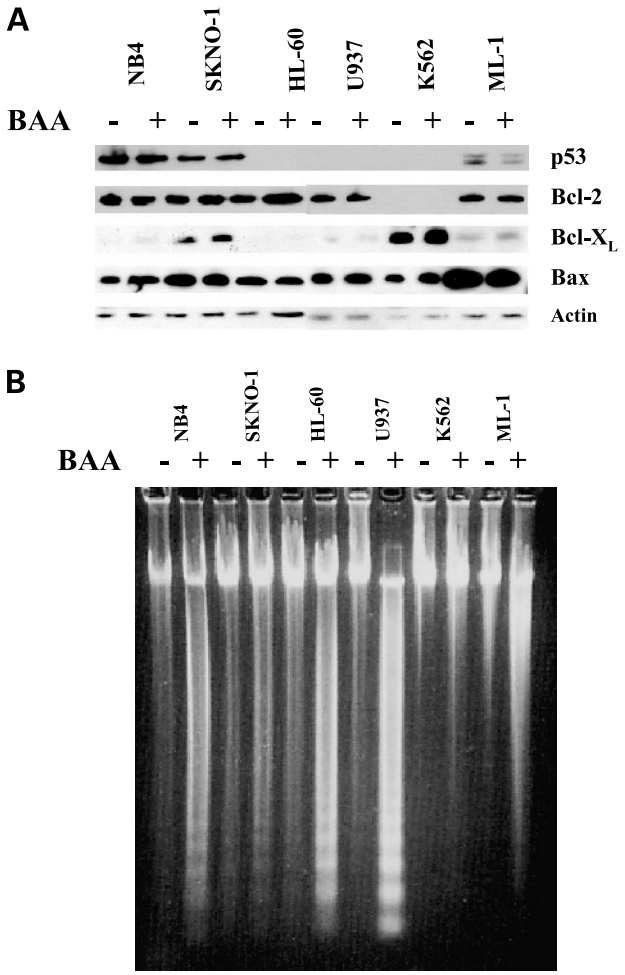

Figure 3. Boswellic acid acetate induces apoptosis of myeloid leukemia cells independent of p53, Bcl-2, Bcl-X_L or Bax. **A,** levels of p53, Bcl-2, Bcl-X_L, and Bax proteins measured by Western blot analysis. **B,** DNA fragmentation. The cells were treated with 15 μg/mL of boswellic acid acetate for 24 h. Western blot analysis was used to compare protein levels and the level of DNA fragmentation was determined as described in Materials and Methods. *BAA,* boswellic acid acetate.

decrease of boswellic acid acetate–induced apoptosis compared with cells transfected with vector alone (Fig. 4). Therefore, Bcl-2 does not seem to be involved in the effect of boswellic acid acetate on apoptosis induction. Bax protein was also detected in all of the cell lines and its level was not changed after boswellic acid acetate treatment. Bcl-X_L protein was detected in K562, SKNO-1, and ML-1 cells, and its level was increased in these lines after boswellic acid acetate treatment. Because the cell lines expressing Bcl-X_L were less sensitive to boswellic acid acetate than the cell lines without Bcl-X_L expression, it seems that Bcl-X_L partially blocked boswellic acid acetate–induced apoptosis in these leukemia cells. Additional studies were undertaken on NB4 and HL-60 cells, which were both highly sensitive to boswellic acid acetate–induced apoptosis (Figs. 2 and 3). It was found that Bid protein was proteolyzed after treatment of the cells with 20 μg/mL boswellic acid acetate, which induced apoptosis. However, Bid was not proteolyzed by 10 μg/mL boswellic acid acetate treatment, which did not induce apoptosis in either of these cell lines (Fig. 5).

Boswellic Acid Acetate Induces Apoptosis through Caspase-Dependent Pathways

Because Bid is a substrate of caspase-8 and was decreased after boswellic acid acetate treatment (Fig. 5), it is likely that caspase-8 will be activated by boswellic acid acetate. Activation of both caspase-8 and caspase-3 occur after the cleavage of their respective precursors (16, 17). The levels of caspase-8 and caspase-3 precursors were compared in HL-60 and NB4 cells with or without boswellic acid acetate treatment (Fig. 5). The levels of the precursors of both caspase-8 and caspase-3 were decreased, and the active cleavage fragment of caspase-8 was detected in apoptotic HL-60 and NB4 cells (Fig. 5). These findings suggest that caspase activation is involved in boswellic

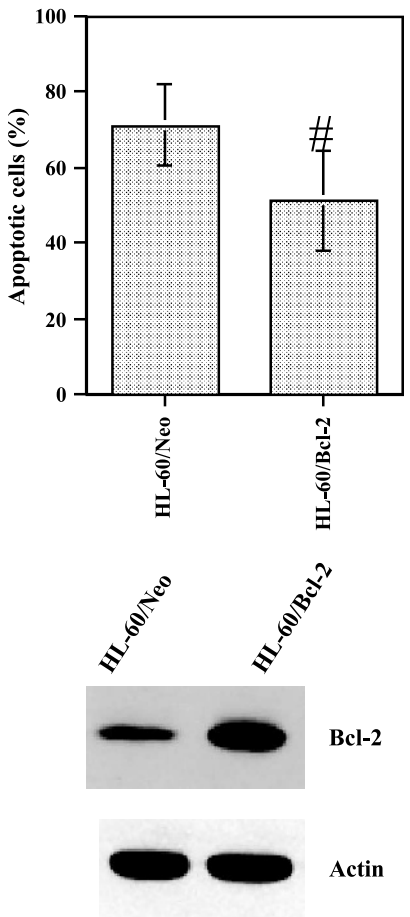

Figure 4. Bcl-2 overexpression in HL-60 cells only slightly decreases their sensitivity to boswellic acid acetate – induced apoptosis. **A,** apoptosis. The percentage of apoptotic cells were determined based on morphologic changes as described in Materials and Methods. **B,** Bcl-2 protein levels. Bcl-2 levels were determined by Western blot analysis. HL-60/Neo and HL-60/BCL2 cells were grown in the absence and presence of 20 μg/mL boswellic acid acetate for 24 h. #, $P > 0.1$ (comparing with HL-60/neo cells).

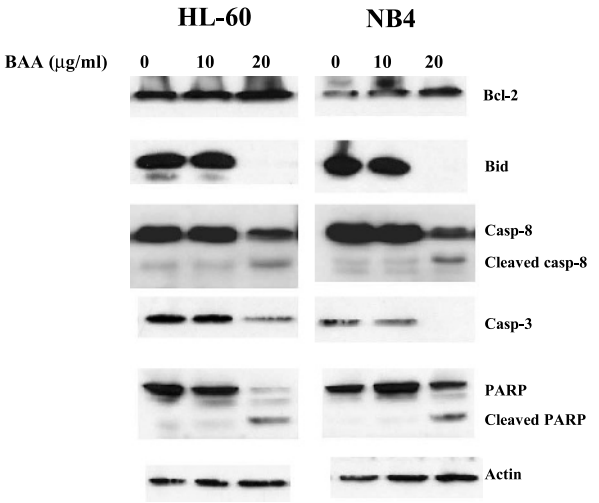

Figure 5. Boswellic acid acetate decreases pro-caspase-8, pro-caspase-3, and Bid levels in HL-60 and NB4 cells. Western blot analysis of caspase-8 (*Casp-8*), caspase-3 (*Casp-3*), and Bid in HL-60 and NB4 cells after treatment with 10 and 20 μg/mL boswellic acid acetate for 24 h as described in Materials and Methods. *PARP,* poly(ADP-ribose) polymerase.

acid acetate–induced apoptosis. Caspase-3 is known to be activated after cleavage of its precursor by caspase-8 or by releasing cytochrome *c* from mitochondria and to cleave poly(ADP-ribose) polymerase in the apoptosis process (15, 16, 18). Therefore, the level of poly(ADP-ribose) polymerase cleavage before and after boswellic acid acetate treatment was determined. Indeed, poly(ADP-ribose) polymerase was cleaved after boswellic acid acetate treatment (Fig. 5). Moreover, boswellic acid acetate–induced apoptosis in both cell lines was completely blocked by Z-VAD-FMK, a general caspase inhibitor (Fig. 6A). To further dissect the members of the caspase family that are involved in boswellic acid acetate–induced apoptosis, several specific caspase inhibitors were also used. As shown in Fig. 6B, a caspase-8 inhibitor (Z-IETD-FMK), but not a caspase-1 inhibitor (AC-YVAD-CMK), blocked boswellic acid acetate–induced apoptosis in NB4 cells (Fig. 6B). A caspase-9 inhibitor (Z-LEHD-FMK) had a weak blocking effect alone and had an additive effect with the caspase-8 inhibitor on blocking boswellic acid acetate–induced apoptosis in NB4 cells (Fig. 6B). These results suggest that caspase-8 activation and partial caspase-9 activation contribute to boswellic acid acetate–induced apoptosis.

Boswellic Acid Acetate Decreases Mitochondrial Membrane Potential

Bid belongs to a family of proteins involved in maintaining the potential of the mitochondrial membrane (19). Therefore, the effect of boswellic acid acetate treatment on mitochondrial membrane potential was determined by using fluorescence microscopy and the MitoSensor reagent. The MitoSensor reagent was taken up in mitochondria in control cells and exhibited intense red fluorescence. The MitoSensor reagent did not accumulate in the mitochondria and the dye remained in the monomeric form in the

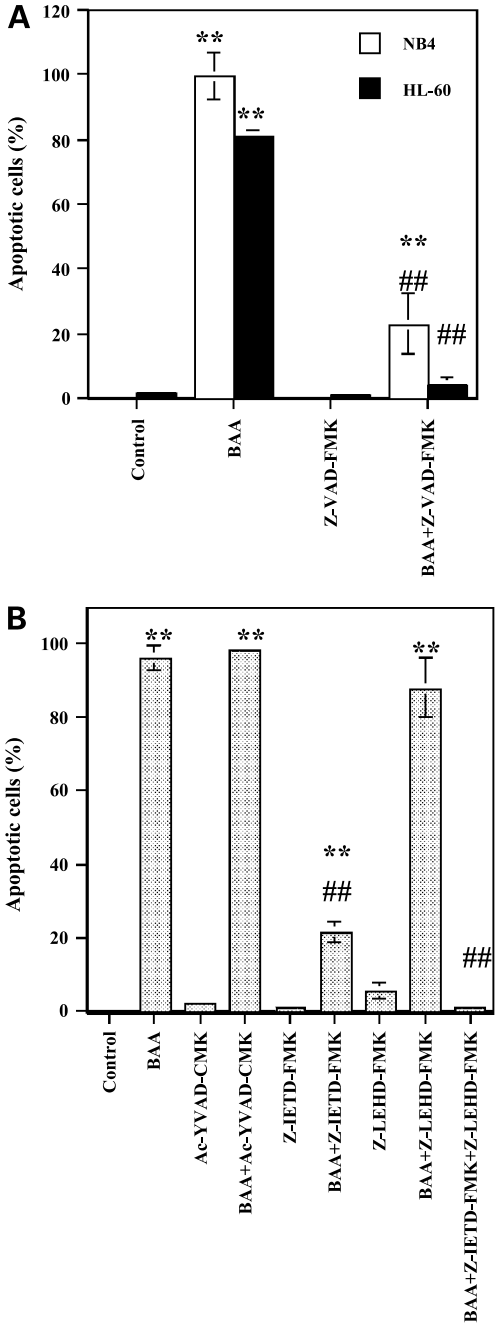

Figure 6. Caspase inhibitors Z-VAD-FMK and Z-IETD-FMK inhibit boswellic acid acetate–induced apoptosis in NB4 and HL-60 cells. **A,** effect of Z-VAD-FMK on boswellic acid acetate–induced apoptosis. NB4 and HL-60 cells were treated with boswellic acid acetate (20 μg/mL), Z-VAD-FMK (100 μm) alone or in combination for 24 h and the percentage of apoptotic cells was determined by morphologic changes as described in Materials and Methods. **B,** effect of AC-YVAD-CMK, Z-IETD-FMK, or Z-LEHD-FMK on boswellic acid acetate–induced apoptosis in NB4 cells. NB4 cells were treated with 20 μg/mL boswellic acid acetate, 100 μm AC-YVAD-CMK, Z-IETD-FMK, and Z-LEHD-FMK alone or in combination as indicated for 24 h and the percentage of apoptotic cells was determined by morphologic changes as described in Materials and Methods. **, $P < 0.01$ (comparing with control group); ##, $P < 0.01$ (comparing with boswellic acid acetate–treated group of each cell line).

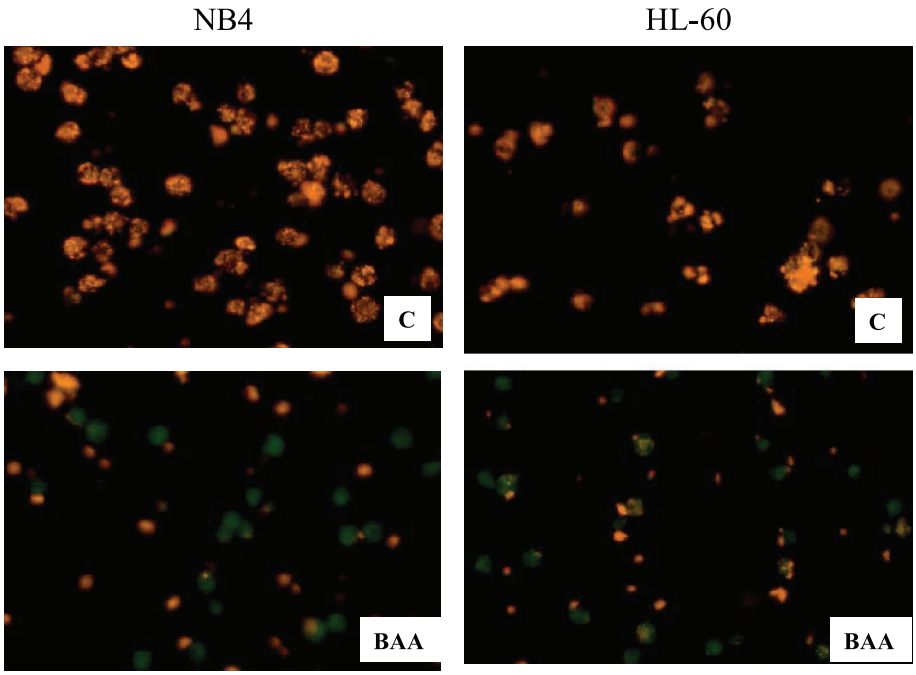

Figure 7. Boswellic acid acetate decreases the mitochondrial membrane potential in NB4 and HL-60 cells. The effect on mitochondrial membrane potential due to boswellic acid acetate (20 μg/mL) treatment for 24 h was determined by using the MitoSensor reagent as described in Materials and Methods.

cytoplasm where it fluoresced green in both NB4 and HL-60 cells treated with boswellic acid acetate for 24 hours (Fig. 7). Therefore, the mitochondrial membrane potential was decreased by boswellic acid acetate treatment.

Reactive Oxygen Species Production Is Not Involved in Boswellic Acid Acetate–Induced Apoptosis

Because ROS are known to decrease mitochondrial membrane potential, cellular hydrogen peroxide levels were determined by fluorescence-activated cell-sorting analysis of cells labeled with 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate. The oxidized 2′,7′-dichlorofluorescein mean peak was not increased in either HL-60 or NB4 cells after treatment with boswellic acid acetate (data not shown). Therefore, the hydrogen peroxide content was not increased by boswellic acid acetate treatment. Moreover, the ROS scavengers, *N*-acetylcystein and vitamin C, did not inhibit boswellic acid acetate–induced apoptosis (data not shown). These data indicate that boswellic acid acetate induces apoptosis through a ROS-independent pathway.

Boswellic Acid Acetate Increases the Levels of *DR4* and *DR5* mRNA in Apoptotic Cells

DR4 and *DR5* mRNA levels were measured by using Northern blot analysis. The mRNA levels of both *DR4* and *DR5* were weakly expressed in NB4 and HL-60 cells and found that the levels of *DR4* and *DR5* mRNA were increased when these cell lines were treated with 20 μg/mL but not with 10 μg/mL boswellic acid acetate for 24 hours (Fig. 8). Because the up-regulation of death receptors, mainly DR4, is correlated with apoptosis induction, it suggests that boswellic acid acetate induces apoptosis, at least in part, through a death receptor–mediated pathway.

Discussion

Defective control of apoptosis has recently been shown to play a central role in the pathogenesis of several diseases including leukemia (20). Chemotherapeutic agents and ionizing radiation can induce cancer cell death, at least partially, by the induction of apoptosis. The *p53* gene is an important regulator of apoptosis in AML blasts and recent studies suggest that the expression of certain conformational variants of *p53* due to mutation are associated with a poor prognosis (21–23). The *p53* protein is usually mutated in AML cells and thus agents that can induce apoptosis via

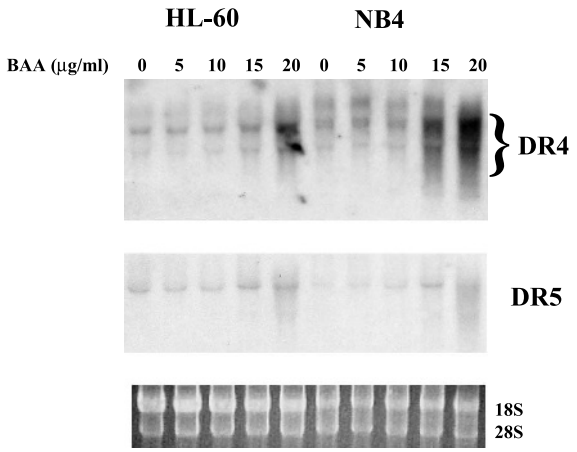

Figure 8. Boswellic acid acetate increases *DR4* and *DR5* mRNA levels in NB4 and HL-60 cells. Both NB4 and HL-60 cells were treated by boswellic acid acetate at indicated concentrations for 24 h and mRNA levels of *DR4* and *DR5* were detected by Northern blot analysis as described in Materials and Methods. The ethidium bromide staining of 28s and 18s RNA was used as internal control of RNA loading.

a *p53*-independent pathway should have broad beneficial therapeutic potential in AML patients. Because boswellic acid acetate promotes more apoptosis in NB4 cells, which express mutated *p53* (24), and in HL-60 cells, which are null for *p53* (25), than in ML-1 cells, which express wild-type *p53* (Fig. 3), it seems that *p53* is not involved in boswellic acid acetate–induced apoptosis. The antiapoptotic mediator, Bcl-2, has been found to be important for the therapeutic outcome of AML and an association existed between high Bcl-2 levels and decreased sensitivity to chemotherapeutic agent–induced apoptosis in native AML blasts (26–28). Because Bcl-2 functions as an apoptotic inhibitor, agents that can bypass Bcl-2 blockage might be developed into new effective agents for leukemia treatment. Boswellic acid acetate was more active in inducing apoptosis in NB4 and HL-60 cells, which express higher levels of Bcl-2 protein, than in K562 cells, which do not express Bcl-2 protein (Fig. 3), and the overexpression of Bcl-2 in HL-60 cells via transfection only weakly decreased boswellic acid acetate–induced apoptosis compared with cells transfected vector alone (Fig. 4). These findings suggest that Bcl-2–mediated antiapoptotic pathways do not play an important role in boswellic acid acetate–induced apoptosis. Because boswellic acid acetate induced apoptosis in all of the tested AML cell lines, it may provide another modality for AML therapy even with mutant *p53* and increased Bcl-2 levels.

It has been shown that many cells undergo a reduction of mitochondrial membrane potential before they exhibit signs of nuclear apoptosis, and the mitochondrial membrane potential has been found to be regulated by numerous effectors (29–31). Previously, we and other groups have found that As_2O_3 and other chemotherapeutic agents decreased mitochondrial membrane potential by producing hydrogen peroxide (15). However, although the mitochondrial membrane potential of both NB4 and HL-60 cells was decreased after boswellic acid acetate treatment (Fig. 7), the level of hydrogen peroxide was not increased in these cells (data not shown). These data suggest that boswellic acid acetate induces apoptosis through a ROS-independent new pathway.

The members of Bcl-2 family are important regulators of the mitochondrial membrane potential (32–35). The data shown in Fig. 3 suggest that Bcl-2, Bcl-X_L, and Bax might not be involved in the boswellic acid acetate–induced decrease in mitochondrial membrane potential because their levels were not changed in any of the cell lines tested. However, the level of Bid was decreased by boswellic acid acetate treatment at a dose that induced apoptosis (Fig. 5). It is known that the cleaved Bid product decreases mitochondrial membrane potential in apoptotic cells (36, 37); thus, it seems that boswellic acid acetate decreases mitochondrial membrane potential, at least in part, through a Bid-mediated pathway. The disruption of mitochondrial membrane integrity involves a loss of metabolic functions, the release of mitochondrial proteins into the cytosol, and the activation of caspase-3 (15, 31). Western blot analysis determined that precursors of both caspase-3 and caspase-8 were decreased in both NB4 and HL-60 cells after boswellic acid acetate treatment (Fig. 5). Furthermore, the general caspase inhibitor Z-VAD-FMK blocked boswellic acid acetate–induced apoptosis (Fig. 6A). It has been found that a decrease in mitochondrial membrane potential will release cytochrome *c*, which activates caspase-9, and leads to the activation of caspase-3 (18). Although Z-VAD-FMK, a general caspase inhibitor, blocked boswellic acid acetate–induced apoptosis significantly (Fig. 6A), Z-LEHD-FMK, a specific caspase-9 inhibitor, only had minimal blocking effect on boswellic acid acetate–induced apoptosis (Fig. 6B). Therefore, the mitochondrial-mediated pathway probably does not play an important role in boswellic acid acetate–induced apoptosis. Recently it has been found that caspase-8 activates caspase-3 by direct cleavage (16). The levels of pro-caspase-8 and caspase-8 substrates and pro-caspase-3 and Bid were decreased, and cleaved caspase-8 fragment was formed in NB4 and HL-60 cells after boswellic acid acetate treatment (Fig. 5). These data suggest that boswellic acid acetate treatment activates caspase-8 and that the activated caspase-8 decreased mitochondrial membrane potential by Bid cleavage or directly activated caspase-3 by cleavage of pro-caspase-3 (16, 36). Because Z-IETD-FMK, a specific caspase-8 inhibitor, blocked boswellic acid acetate–induced apoptosis significantly, caspase-8 is involved in the boswellic acid acetate effect, and the activation of caspase-8 will be an important event in the boswellic acid acetate–induced apoptosis process. These data are consistent with previous reports that showed boswellic acid derivatives induced apoptosis in colon and hepatoma cells through a caspase-8–mediated pathway (12, 38).

Tumor necrosis factor receptor 1 and an emerging family of membrane death receptors transduce apoptotic signals through caspase-8 activation (39–41). It has been found that apoptosis induction of cancer cells by several chemotherapeutic agents is also associated with the up-regulation of

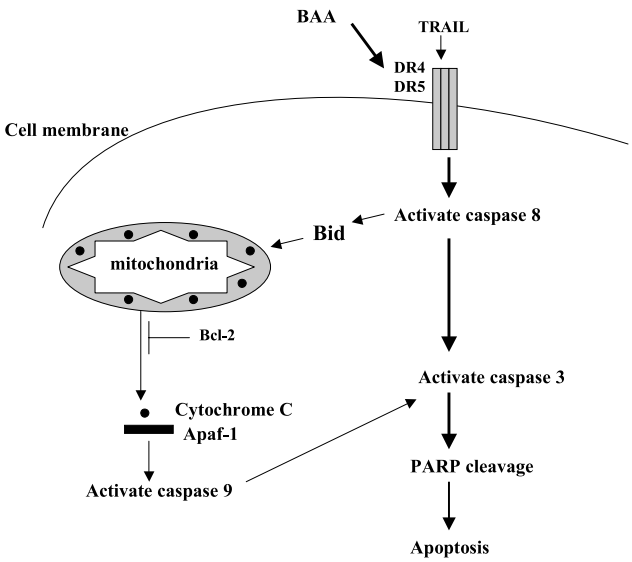

Figure 9. The hypothetical pathways of boswellic acid acetate–induced apoptosis in myeloid leukemia cells.

DR4 and DR5 (42–44). *DR4* and *DR5* mRNA were found to be increased in NB4 and HL-60 cells after boswellic acid acetate treatment, which correlated with apoptosis induction (Figs. 2 and 8). These data suggest that boswellic acid acetate may induce apoptosis by increasing the levels of death receptors and indirectly activating caspase-8 (Fig. 9).

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References

1. Appelbaum FR, Kopecky KJ. Long-term survival after chemotherapy for acute myeloid leukemia: the experience of the Southwest Oncology Group. Cancer 1997;80:2199–04.

2. Buechner T, Hiddemann W. Treatment strategies in acute myeloid leukemia (AML). A. First-line chemotherapy. Blut 1990;60:61–7.

3. Bloomfield CD, Shuma C, Regal L, et al. Long-term survival of patients with acute myeloid leukemia: a third follow-up of the Fourth International Workshop on Chromosomes in Leukemia. Cancer 1997;80:2191–8.

4. Safayhi H, Mack T, Sabieraj J, Anazodo MI, Subramanian LR, Ammon HP. Boswellic acids: novel, specific, nonredox inhibitors of 5-lipoxygenase. J Pharmacol Exp Ther 1992;261:1143–6.

5. Safayhi H, Sailer ER, Ammon HP. Mechanism of 5-lipoxygenase inhibition by acetyl-11-keto-β-boswellic acid. Mol Pharmacol 1995;47:1212–6.

6. Syrovets T, Buchele B, Gedig E, Slupsky JR, Simmet T. Acetyl-boswellic acids are novel catalytic inhibitors of human topoisomerases I and IIα. Mol Pharmacol 2000;58:71–81.

7. Schweizer S, von Brocke AF, Boden SE, Bayer E, Ammon HP, Safayhi H. Workup-dependent formation of 5-lipoxygenase inhibitory boswellic acid analogues. J Nat Prod 2000;63:1058–61.

8. Jing Y, Nakajo S, Xia L, et al. Boswellic acid acetate induces differentiation and apoptosis in leukemia cell lines. Leuk Res 1999;23:43–50.

9. Hoernlein RF, Orlikowsky T, Zehrer C, et al. Acetyl-11-keto-β-boswellic acid induces apoptosis in HL-60 and CCRF-CEM cells and inhibits topoisomerase I. J Pharmacol Exp Ther 1999;288:613–9.

10. Shao Y, Ho CT, Chin CK, Badmaev V, Ma W, Huang MT. Inhibitory activity of boswellic acids from boswellia serrata against human leukemia HL-60 cells in culture. Planta Med 1998;64:328–31.

11. Glaser T, Winter S, Groscurth P, et al. Boswellic acids and malignant glioma: induction of apoptosis but no modulation of drug sensitivity. Br J Cancer 1999;80:756–65.

12. Liu JJ, Nilsson A, Oredsson S, Badmaev V, Zhao WZ, Duan RD. Boswellic acids trigger apoptosis via a pathway dependent on caspase-8 activation but independent on fas/fas ligand interaction in colon cancer HT-29 cells. Carcinogenesis 2002;23:2087–93.

13. Naumovski L, Cleary ML. Bcl2 inhibits apoptosis associated with terminal differentiation of HL-60 myeloid leukemia cells. Blood 1994;83:2261–7.

14. Dai J, Weinberg RS, Waxman S, Jing Y. Malignant cells can be sensitized to undergo growth inhibition and apoptosis by arsenic trioxide through modulation of the glutathione redox system. Blood 1999;93:268–77.

15. Jing Y, Dai J, Chalmers-Redman RM, Tatton WG, Waxman S. Arsenic trioxide selectively induces acute promyelocytic leukemia cell apoptosis via a hydrogen peroxide-dependent pathway. Blood 1999; 94:2102–11.

16. Stennicke HR, Jurgensmeier JM, Shin H, et al. Pro-caspase-3 is a major physiologic target of caspase-8. J Biol Chem 1998;273:27084–90.

17. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol 1999;15:269–90.

18. Reed JC. Apoptosis-regulating proteins as targets for drug discovery. Trends Mol Med 2001;7:314–9.

19. Esposti MD. The roles of Bid. Apoptosis 2002;7:433–40.

20. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995;267:1456–62.

21. Sugimoto K, Toyoshima H, Sakai R, et al. Frequent mutations in the p53 gene in human myeloid leukemia cell lines. Blood 1992;79:2378–83.

22. Nakano Y, Naoe T, Kiyoi H, et al. Prognostic value of p53 gene mutations and the product expression in *de novo* acute myeloid leukemia. Eur J Haematol 2000;65:23–31.

23. Melo MB, Ahmad NN, Lima CS, et al. Mutations in the p53 gene in acute myeloid leukemia patients correlate with poor prognosis. Hematology 2002;7:13–9.

24. Calabresse C, Venturini L, Ronco G, et al. Selective induction of apoptosis in myeloid leukemic cell lines by monoacetone glucose-3 butyrate. Biochem Biophys Res Commun 1994;201:266–83.

25. Wu GS, El-Deiry WS. Apoptotic death of tumor cells correlates with chemosensitivity, independent of p53 or Bcl-2. Clin Cancer Res 1996;2:623–33.

26. Campos L, Rouault JP, Sabido O, et al. High expression of Bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. Blood 1993;81:3091–96.

27. Karakas T, Maurer U, Weidmann E, Miething CC, Hoelzer D, Bergmann L. High expression of Bcl-2 mRNA as a determinant of poor prognosis in acute myeloid leukemia. Ann Oncol 1998;9:159–65.

28. Tothova E, Fricova M, Stecova N, Kafkova A, Elbertova A. High expression of Bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. Neoplasma 2002;49:141–44.

29. Decaudin D, Marzo I, Brenner C, Kroemer G. Mitochondria in chemotherapy-induced apoptosis: a prospective novel target of cancer therapy [review]. Int J Oncol 1998;12:141–52.

30. Serafino A, Sinibaldi-Vallebona P, Lazzarino G, et al. Modifications of mitochondria in human tumor cells during anthracycline-induced apoptosis. Anticancer Res 2000;20:3383–94.

31. Hockenbery DM, Giedt CD, O'Neill JW, Manion MK, Banker DE. Mitochondria and apoptosis: new therapeutic targets. Adv Cancer Res. 2002;85:203–42.

32. Gross A, McDonnell JM, Korsmeyer SJ. Bcl-2 family members and the mitochondria in apoptosis. Genes Dev 1999;13:1899–911.

33. Adams JM, Huang DC, Puthalakath H, et al. Control of apoptosis in hematopoietic cells by the Bcl-2 family of proteins. Cold Spring Harb Symp Quant Biol 1999;64:351–8.

34. Vander Heiden MG, Thompson CB. Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? Nat Cell Biol 1999;1:E209–16.

35. Reed JC. Bcl-2 family proteins: regulators of apoptosis and chemoresistance in hematologic malignancies. Semin Hematol 1997;34:9–19.

36. Yin XM. Signal transduction mediated by Bid, a pro-death Bcl-2 family proteins, connects the death receptor and mitochondria apoptosis pathways. Cell Res 2000;10:161–7.

37. Crompton M. Bax, Bid and the permeabilization of the mitochondrial outer membrane in apoptosis. Curr Opin Cell Biol 2000;12:414–9.

38. Liu JJ, Nilsson A, Oredsson S, Badmaev V, Duan RD. Keto- and acetyl-keto-boswellic acids inhibit proliferation and induce apoptosis in hep g2 cells via a caspase-8 dependent pathway. Int J Mol Med 2002;10:501–5.

39. Gupta S. Molecular signaling in death receptor and mitochondrial pathways of apoptosis [review]. Int J Oncol 2003;22:15–20.

40. Sheikh MS, Fornace AJ Jr. Death and decoy receptors and p53-mediated apoptosis. Leukemia 2000;14:1509–13.

41. Singh A, Ni J, Aggarwal BB. Death domain receptors and their role in cell demise. J Interferon Cytokine Res 1998;18:439–50.

42. Higuchi H, Bronk SF, Takikawa Y, et al. The bile acid glycochenodeoxycholate induces trail-receptor 2/dr5 expression and apoptosis. J Biol Chem 2001;276:38610–8.

43. Nimmanapalli R, Perkins CL, Orlando M, O'Bryan E, Nguyen D, Bhalla KN. Pretreatment with paclitaxel enhances apo-2 ligand/tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis of prostate cancer cells by inducing death receptors 4 and 5 protein levels. Cancer Res 2001;61:759–63.

44. Sun SY, Yue P, Lotan R. Implication of multiple mechanisms in apoptosis induced by the synthetic retinoid CD437 in human prostate carcinoma cells. Oncogene 2000;19:4513–22.