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Sanguinarine-Induced Apoptosis: Generation of ROS, Down-Regulation of Bcl-2, c-FLIP, and Synergy With TRAIL

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Abstract Sanguinarine is a benzophenanthridine alkaloid derived from the root of *Sanguinaria canadensis* and other poppy-fumaria species, possessing potent antibacterial, antifungal, and anti-inflammatory activities. In this study, we investigated the underling mechanisms by which sanguinarine induce apoptosis in human breast cancer MDA-231 cells. Treatment of MDA-231 cells with sanguinarine induced remarkable apoptosis accompanying the generation of ROS. Consistently, sanguinarine-induced apoptosis was mediated by the increased reproductive cell death. Pretreatment with NAC or GSH attenuated sanguinarine-induced apoptosis, suggesting the involvement of ROS in this cell death. During sanguinarin-induced apoptosis, protein levels of pro-caspase-3, Bcl-2, cIAP2, XIAP, and c-FLIPs were reduced. Sanguinarine-mediated apoptosis was substantially blocked by ectopic expression of Bcl-2 and cFLIPs. Additionally, we found that sub-lethal doses of sanguinarine remarkably sensitized breast cancer cells to TRAIL-mediated apoptosis, but the cell death induced by sanguinarine and TRAIL in combination was not blocked by overexpression of Bcl-2 or Akt. Therefore, combinatory treatment of sanguinarine and TRAIL may overcome the resistance of breast cancer cells due to overexpression of Akt or Bcl-2. J. Cell. Biochem. 104: 895–907, 2008.

Key words: sanguinarine; TRAIL; apoptosis; reactive oxygen species; Bcl-2; c-FLIP

Breast cancer is a serious health problem, being the highest cause of cancer deaths in women. Despite the advent of new therapeutic strategies and agents, there are still significant numbers of women who die of breast cancer, accompanied by the progression into metastasis [Seltzer, 2000]. While metastatic breast cancer is initially responsive to hormonal therapy, many patients relapse. In this setting, chemotherapy is generally prescribed after failure of endocrine therapy. Although doxorubicin and paclitaxel are generally considered as the most active agent in advanced breast cancer [Paridaens et al., 2000], these treatments are frequently discontinued because of intolerable toxicity and/or the development of drug resistance [Levine et al., 1998; Paridaens et al., 2000]. Thus, there is an urgent need for novel therapeutic approaches for metastatic breast cancers that are resistant to conventional therapy. To overcome the resistance to anticancer drugs, current cancer therapy is trying

Abbreviations used: ROS, reactive oxygen species; TRAIL, tumor necrosis factor (TNF)-α-related apoptosis-inducing ligand; cIAP, cellular inhibitor of apoptosis protein; FLIP, FLICE inhibitory protein; PLC-γ1, phospholipase C-γ1; PARP, poly(ADP-ribose) polymerase; XIAP, X-linked inhibitor of apoptosis protein; z-VAD-fmk, Benzyloxy carbony-Val-Ala-Asp-fluoromethyl ketone; NAC, N-acetylcysteine; GSH, glutathione; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; SG, sanguinarine; CC, chelerythrine chloride; pNA, *p*-nitroanilide; QPs2, OxPhos complex II subunit; p, pcDNA 3.1 vector; B2, Bcl-2; F, c-FLIPs.

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to combine a cytotoxic agent, or a chemopreventive agent with various biologically active agents.

TRAIL has been shown to induce apoptosis in a wide variety of cancer cells including breast cancer cells, whereas most normal human cell types are resistant to TRAIL-induced cell death [Pan et al., 1997; Sheridan et al., 1997]. The action of TRAIL can be enhanced by pretreatment with chemotherapeutic drugs or chemopreventive agents resulting in a synergistic apoptotic response in many tumor cells [Butler et al., 2006]. Thus, the use of the sensitizers to enhance the activity of TRAIL is a promising approach to selectively induce apoptosis in breast cancer cells with minimal toxicity to normal cells.

Natural alkaloid sanguinarine (13-methyl [1, 3] benzodioxolo [5,6-c]-1,3-dioxolo [4,5-i] phenanthridium; Fig. 1A), derived from the root of *Sanguinaria canadensis*, exhibits antiviral, anti-inflammatory [Lenfeld et al., 1981] and tumor-targeting activity [Ahmad et al., 2000; Larsson et al., 2006]. Sanguinarine has been

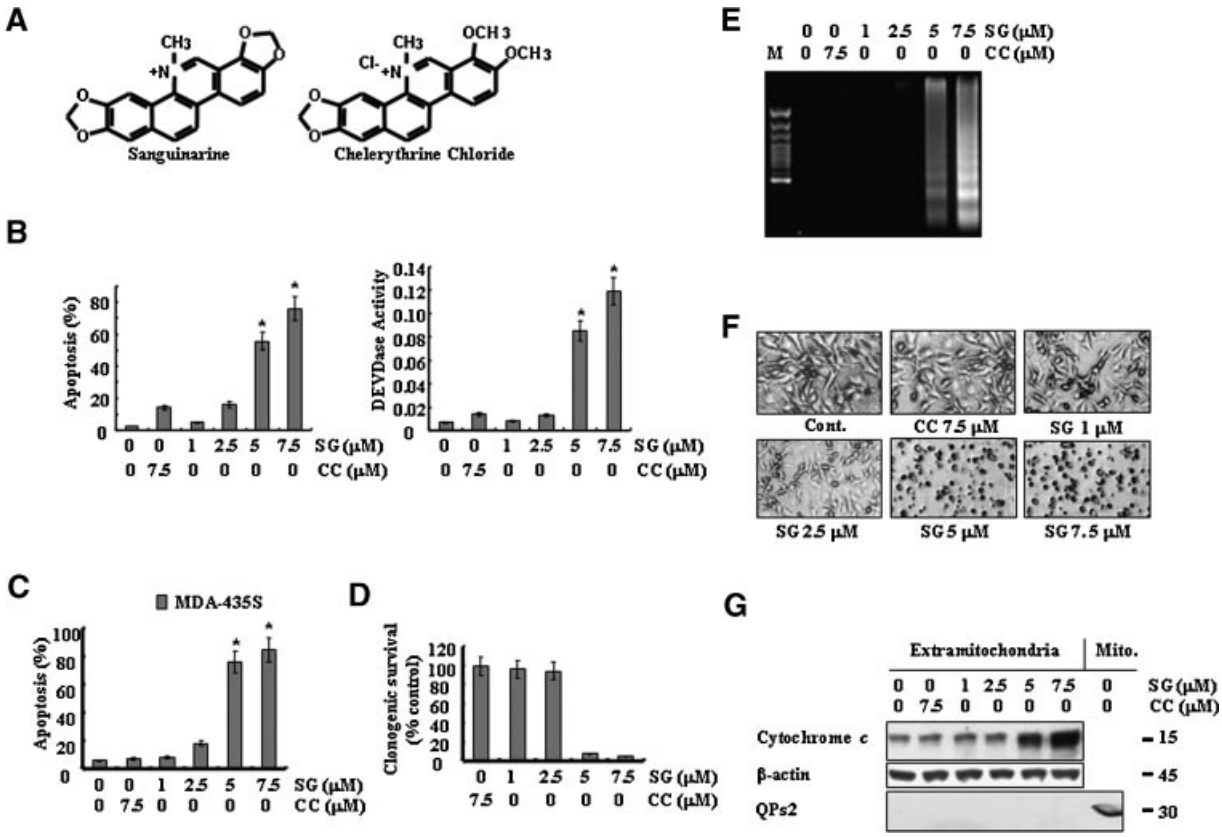

Fig. 1. Sanguinarine induces apoptosis in MDA MB 231 cells. **A**: The structures of SG and CC. **B**: Flow cytometric analysis of apoptotic cells. Cells were treated for 24 h with SG or CC, and then evaluated for DNA content after propidium iodide staining. The fraction of apoptotic cells is shown as indicated. Enzymatic activities of caspase-3 were determined by incubation of 30 μg of total protein with 200 μM chromogenic substrate (DEVD-pNA) in a 100 μl assay buffer for 1 h at 37°C. The release of chromophore pNA was monitored spectrophotometrically (405 nm). * Indicates $P < 0.05$ versus control. **C**: Quantitation of apoptosis by fluorescence-activated cell sorting analysis. Sub-G1 fraction was measured in MDA-435S cells treated with SG or CC for 24 h. * Indicates $P < 0.05$ versus control. **D**: MDA-231 cells were incubated with the indicated concentration of SG for 24 h, after which the cells were trypsinized and plated in a clonogenic survival assay, and their growth compared with cells that had not been treated with SG and whose growth was considered to be 100 %. **E**: Fragmentations of genomic DNA in MDA MB 231 cells treated for 24 h with SG or CC. Fragmented DNA was extracted and analyzed on 2% agarose gel. **F**: Cells were treated for 24 h with the indicated concentrations of SG or CC. After 24 h, morphological change was visualized using a light microscopy. **G**: Cytosolic extracts were prepared as described in Materials and Methods Section. Thirty micrograms of cytosolic protein was resolved on 12% SDS–PAGE and then transferred to nitrocellulose, and probed with specific anti-cytochrome *c* antibody or with anti-β-actin to serve as control for the loading of protein level. To show there is no mitochondrial contamination in the cytosolic preparation, we carried out Western blotting analysis using antibody against QPs2 that was expressed in mitochondria. Mitochondrial fraction derived from non-treated cells was used as a positive control. Data are mean values obtained from three independent experiments and bars represent standard deviation.

reported to induce apoptosis in various human cancer cells [Ahmad et al., 2000; Adhami et al., 2003, 2004] and its treatment at similar dose did not show any toxic effect on normal cells [Ahmad et al., 2000].

In this study, we investigated the underlying mechanisms of sanguinarine-induced apoptosis and the sensitizing effect of sanguinarine on TRAIL-induced in breast cancer cells. Here, we show for the first time that the combinatory treatment with sanguinarine and TRAIL effectively induce apoptosis in breast cancer cells overexpressing Bcl-2 or Akt, offering a potential therapeutic strategy against malignant breast cancer cells.

MATERIALS AND METHODS

Cell Lines and Culture

Human breast cancer cells MDA-231 and MDA-435S were obtained from the American Type Culture Collection (Manassas, VA). The culture media used throughout these experiments were Dulbecco's modified Eagle's medium, containing 10% heat-inactivated fetal bovine serum, 20 mM HEPES buffer, 100 μg/ml streptomycin and 100 μg/ml penicillin.

Drugs and Materials

Sanguinarine chloride hydrate was purchased from Sigma Chemical Co. (St. Louis, MO). A 10 mM solution was prepared in methanol (≥99.9%; MERCK), stored as smaller concentrated (1 mM) aliquots at −20°C, and then diluted as needed in cell culture medium. Sanguinarine was directly added to cell culture dishes at the indicated concentrations. Etoposide, oxaliplatin were purchased from Sigma Chemical Co. Anti-cIAP-1, anti-cIAP-2, anti-Bcl-2, anti-Bcl-xL, anti-FLIP, and anti-PLC-γ1 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-Akt, anti-Akt, β-actin, cleaved caspase-3 and anti-PARP antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-OxPhos Complex II subunit antibody was purchase from Molecular Probes (Eugene, OR). Anti-XIAP, anti-Bax and anti-cytochrome *c* antibodies were purchased from BD Biosciences PharMingen (San Jose, CA). Antibodies against the following proteins were purchased from the indicated suppliers: Procaspase-3 from Santa Cruz Biotechnology, Inc. z-VAD-fmk was purchased from Biomol (Plymouth Meeting, PA). Chelerythrine chloride was purchased from Sigma Chemical Co.

Western Blotting Analysis

Cellular lysates were prepared by suspending 0.8×10^6 cells in 100 μl of lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM MOPS, 100 μM phenylmethylsulfonyl fluoride and 20 μM leupeptin, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 min. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). Detection of specific proteins was carried out with an ECL Western blotting kit according to the manufacturer's instructions.

c-FLIPs Constructs and Transfection

The human c-FLIPs cDNA fragment was digested from pCA-FLAG-hFLIPs. pCA-FLAG-hFLIPs was kindly provided by Dr. Park SI (Korea Centers for Disease Control and Prevention, Seoul, Korea). c-FLIPs cDNA fragment was digested with *Kpn*I and *Xho*I and subcloned into the pcDNA 3.1 vector (Invitrogen, Carlsbad, CA), termed pcDNA 3.1-c-FLIPs. The MDA-231 cells were transfected in a stable manner with the pcDNA 3.1-c-FLIPs plasmid, or control plasmid pcDNA 3.1 vector using LipofectAMINE as prescribed by the manufacturer (Invitrogen). After 48 h of incubation, transfected cells were selected in primary cell culture medium containing 700 μg/ml G418 (Invitrogen). After 2 or 3 weeks, to rule out the possibility of clonal differences between the generated stable cell lines, the pooled MDA-231/pcDNA 3.1 and MDA-231/c-FLIPs clones were tested for c-FLIPs expression by immunoblotting and were used in this study.

Measurement of Reactive Oxygen Species (ROS)

The intracellular accumulation of ROS was determined using the fluorescent probes H_2DCFDA. H_2DCFDA was commonly used to measure H_2O_2 [LeBel et al., 1992]. Cells were collected by centrifugation, 1 h before treatment with the cytotoxic agents, resuspended in DMEM medium without red phenol, and loaded with 5 μM H_2DCFDA. The fluorescence was measured at the desired time intervals by flow cytometry. Control cells were subjected to the same manipulation, except for treatment with the sanguinarine.

Cell Counts and Flow Cytometric Analysis

Cell counts were performed using a hemocytometer. Approximately 0.8×10^6 MDA-231 cells were suspended in 100 μl PBS, and 200 μl of 95% ethanol was added while vortexing. The cells were incubated at 4°C for 1 h, washed with PBS, and resuspended in 250 μl of 1.12% sodium citrate buffer (pH 8.4) together with 12.5 μg RNase. Incubation was continued at 37°C for 30 min. The cellular DNA was then stained by applying 250 μl propidium iodide (50 μg/ml) for 30 min at room temperature. The stained cells were analyzed by a FACScan flow cytometer for relative DNA content based on red fluorescence.

DNA Fragmentation Assay

After treatment with sanguinarine and chelerythrine, MDA-231 cells were lysed in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA and 0.5% Triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 10,000*g* for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol: chloroform:isoamyl alcohol mixture (25:24:1) and analyzed electrophoretically on 2% agarose gels containing 0.1 μg/ml ethidium bromide.

DEVDase Activity Assay

To evaluate caspase-3 activity, cell lysates were prepared after their respective treatment with various drugs. Assays were performed in 96-well microtiter plates by incubating 20 μg cell lysates in 100 μl reaction buffer [1% NP-40, 20 mM Tris–HCl (pH 7.5), 137 mM NaCl, and 10% glycerol] containing the caspase-3 substrate (DEVD-pNA) at 5 μM. Lysates were incubated at 37°C for 2 h. Thereafter, the absorbance at 405 nm was measured with a spectrophotometer.

Analysis of Mitochondrial Cytochrome *c* Release

Cells (0.8×10^6) were harvested, washed once with ice-cold PBS and gently lysed for 2 min in 80 μl ice-cold lysis buffer (250 mM sucrose, 1 mM EDTA, 20 mM Tris–HCl pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM $MgCl_2$, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, 2 μg/ml aprotinin). Lysates were centrifuged at 12,000*g* at 4°C for 10 min to obtain the supernatants (cytosolic extracts free of mitochondria) and the pellets (fraction that contains mitochondria). The resulting cytosolic fractions were used for Western blot analysis with an anti-cytochrome *c* antibody.

Clonogenic Assay

MDA-231 cells were seeded into six-well plates at 2,000 per well in a final volume of 2 ml of medium containing appropriate drug concentrations. Triplicate cultures were used for each drug concentration and time tested. At the end of the drug exposures, the drug-containing medium was replaced with fresh media. All cultures were incubated for an additional 7 days. At this point, the medium was aspirated, and the dishes were washed once with PBS, fixed with 100% methanol for 30 min, and stained with a filtered solution of 0.5% (w/v) crystal violet (Sigma Chemical Co.) for 30 min. The wells were then washed with PBS and dried at room temperature. The colonies, defined as groups of $\geq$50 cells, were scored manually with the inverted microscope. Clonogenic survival was expressed as the percentage of colonies formed in sanguinarine-treated cells with respect to vehicle-treated cells.

Statistical Analysis

Three or more separate experiments were performed. Statistical analysis was done by paired Student's *t*-test. A P-value < 0.05 was considered to have pronounced difference between experimental and control groups.

RESULTS

Sanguinarine Induces Apoptosis in MDA-231 Cells

Human breast cancer MDA-231 cells were treated with sanguinarine (1–7.5 μM). We first analyzed the occurrence of apoptosis in MDA-231 cells using flow cytometric analysis to detect hypodiploid cell populations. As shown in Figure 1B, treatment of MDA-231 cells with sanguinarine resulted in a markedly increased accumulation of sub-G1 phase cells in a dose-dependent manner. We next analyzed whether treatment with sanguinarine caused the activation of caspase-3, a key executioner of apoptosis. Exposure of MDA-231 cells to sanguinarine strongly stimulated DEVDase activity. To determine whether sanguinarine can induce apoptosis in another breast cancer cell line, we tested sanguinarine on MDA-435S cell line. The effects of sanguinarine on MDA-435S cells were similar to those of MDA-231

cells (Fig. 1C). Effect of sanguinarine treatment on the reproductive potential of MDA-231 cells was assessed using the clonogenic assay. When 2,000 MDA-231 cells were seeded for testing, we found that 5 μM or higher concentration of sanguinarine treatment significantly low numbers of colonies survived compared to the number of colonies treated with 2.5 μM sanguinarine (Fig. 1D). Although 40% of MDA-231 cells were attached on culture plate after 5 μM sanguinarine treatment for 24 h, they are attached to the plate and lived 2–3 days, and died out 7 days after treatment. These results indicate that 5 μM or higher concentration of sanguinarine may increase reproductive cell death. Furthermore, we found the typical ladder pattern of internucleosomal fragmentation, which is a hallmark of apoptosis, in sanguinarine-treated cells (5–7.5 μM). In contrast, treatment with chelerythrine chloride, a sanguinarine inactive homologue with minor structural difference (Fig. 1A), did not induce internucleosomal fragmentation (Fig. 1E). In addition, treatment with 5 and 7.5 μM sanguinarine, but not chelerythrine chloride exhibited progressive morphological changes of typical apoptosis, including cell shrinkage, rounding, and detachment of the cells from the plate, as observed with light microscopy (Fig. 1F). There has been accumulating evidence that mitochondria play an essential role in apoptosis by releasing apoptogenic effectors such as cytochrome *c* [Le Bras et al., 2006]. When we performed Western blotting analysis using cytosolic fractions to examine the release of mitochondrial cytochrome *c* in sanguinarine-treated MDA-231 cells, sanguinarine treatment remarkably induced dose-dependent release of cytochrome *c* into the cytoplasm (Fig. 1G).

Sanguinarine Induces Caspase-Dependent Apoptosis

We next examined whether caspase-3 plays a critical role in sanguinarine-induced apoptosis. Not only sanguinarine induced the procaspase-3 degradation with formation of the 17 and 19-kDa active subunits and produced the cleavage product of PARP, a well-known substrate of caspase (Fig. 2A), but also it increased DEVDase activity (Fig. 2A). As shown in Figure 2A, sanguinarine-induced apoptosis was completely prevented by pretreatment with a general and potent inhibitor of caspases, z-VAD-fmk, as determined by FACS analysis. We also found that z-VAD-fmk prevented all these caspase-related events such as cleavage of procasapse-3 and PARP and increase of DEVDase activity (Fig. 2A).

Generation of ROS in Sanguinarine-Induced Apoptosis

Many antineoplastic agents eliminate tumor cells by inducing apoptosis and oxidative stress-mediated cellular changes are frequently induced in cells exposed to cytotoxic drugs [Fleury et al., 2002; Wen et al., 2002]. Therefore, we examined whether sanguinarine affects the cellular levels of ROS by measuring the changes in the fluorescence using H_2DCFDA and DHE. As shown in Figure 2B, treatment with 5 μM sanguinarine remarkably increased the H_2DCFDA and DHE-derived fluorescence, which was attenuated by pretreatment with 10 mM NAC or 10 mM GSH. After exposure of the cells to NAC or GSH plus sanguinarine for 24 h, there were remarkable inhibition of DEVDase activation and reduction of PARP cleavage (Fig. 2C). Treatment with sanguinarine in MDA-231 cells led to down-regulation of Bcl-2 and c-FLIPs. Pretreatment with NAC or GSH significantly attenuated the down-regulation of Bcl-2 and c-FLIPs (Fig. 2C). These data suggest that sanguinarine-mediated degradation of Bcl-2 and c-FLIPs might be associated with sanguinarine-induced ROS generation. As shown in Figure 2C, sanguinarine treatment resulted in a markedly increased accumulation of cells in the sub-G1 phase, which was remarkably attenuated by pretreatment with NAC or GSH (Fig. 2C). In addition, NAC or GSH pretreatment remarkably blocked sanguinarine-induced release of cytochrome *c* (Fig. 2D). These data clearly indicate that sanguinarine-induced cytochrome *c* release and caspase activation is preceded by ROS generation.

Ectopic Expression of Bcl-2 Attenuates Sanguinarine-Induced Apoptosis

To investigate the underlying mechanisms involved in sanguinarine-induced apoptosis, we analyzed the changes in the expression levels with various anti-apoptotic proteins. While protein levels of Bcl-xL were not altered in response to sanguinarine, Bcl-2 protein levels were remarkably reduced (Fig. 3A). In addition, protein levels of c-IAP2, XIAP and c-FLIPs were also remarkably decreased (Fig. 3A). Furthermore, sanguinarine induced

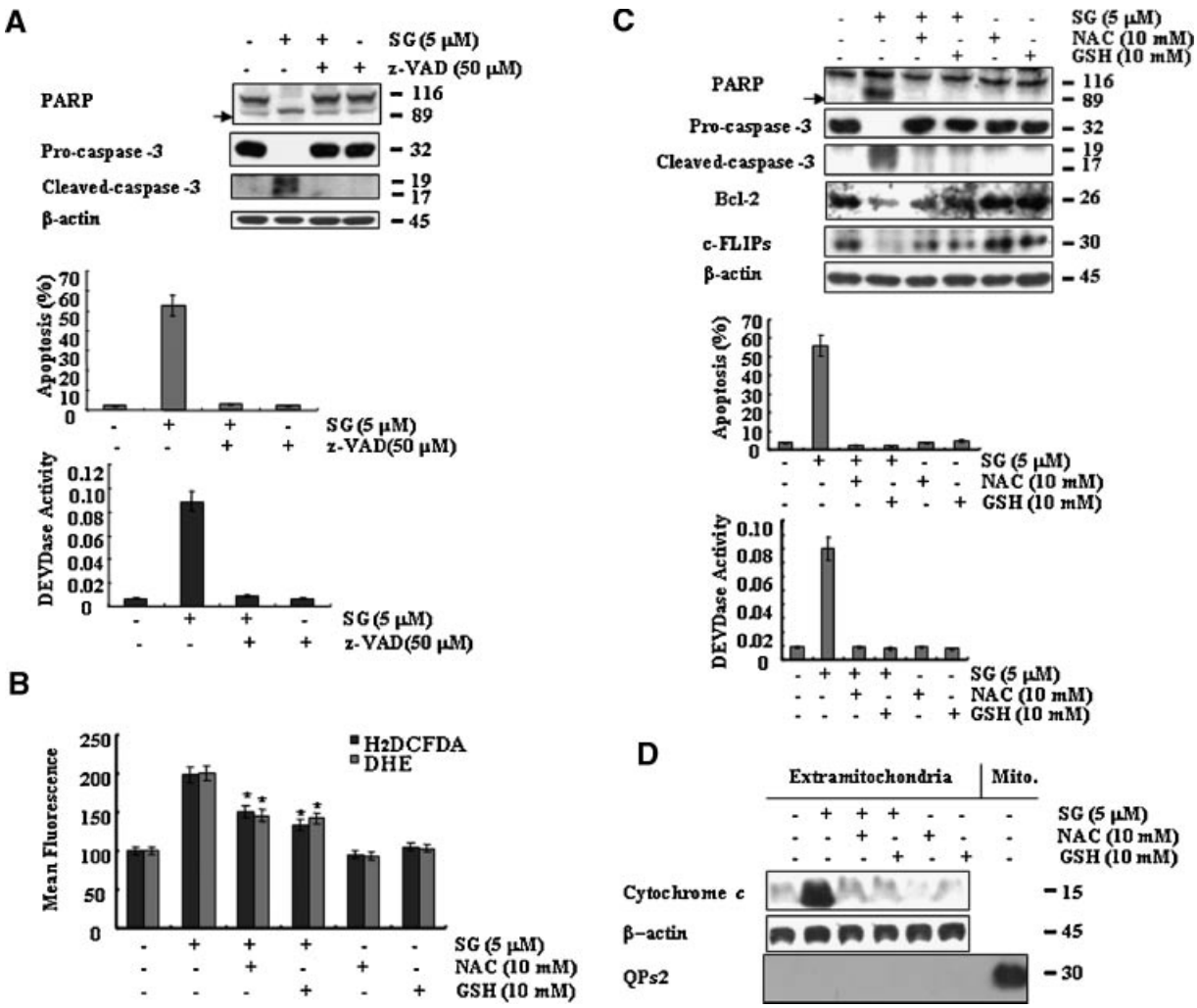

Fig. 2. Sanguinarine induces apoptosis via activation of caspase and generation of ROS. **A**: Effects of z-VAD-fmk on sanguinarine-induced apoptosis. Cells were incubated with z-VAD-fmk or solvent for 1 h before treatment with SG. Equal amounts of cell lysates (50 μg) were resolved by SDS–PAGE and analyzed Western blotting using anti-pro-caspase-3, anti-cleaved caspase-3, and anti-PARP antibodies. Anti-β-actin antibody was served as a control for the loading of protein level. The proteolytic cleavage of PARP is indicated by an arrow. The fraction of apoptotic cells and DEVDase activity were determined as described in Figure 1. **B**: Determination of the intracellular content of peroxide. Fluorescence of MDA-231 cells loaded H_2DCFDA and DHE and then pretreated with or without NAC (10 mM) or GSH (10 mM) for 30 min and further stimulated with SG (5 μM) was measured by flow cytometry. * Indicates $P < 0.05$ versus SG-treated cells. **C**: Equal amounts of cell lysates (50 μg) were resolved by SDS–PAGE and analyzed Western blotting using anti-pro-caspase-3, anti-PARP, anti-Bcl-2, anti-c-FLIPs and anti-β-actin antibodies. The proteolytic cleavage of PARP is indicated by an arrow. Cells were treated as above and then the fraction of apoptotic cells and DEVDase activity were determined as described in Figure 1. **D**: Cytochrome *c* release was determined as described in Figure 1. To show there is no mitochondrial contamination in the cytosolic preparation, we carried out Western blotting analysis using antibody against QPs2. Mitochondrial fraction derived from non-treated cells was used as a positive control. Data are mean values from three independent experiments and bars represent standard deviations.

proteolytic cleavage of Bax (Fig. 3A). In order to evaluate the functional role played by Bcl-2 in preventing sanguinarine-induced apoptosis, the cells stably overexpressing Bcl-2 was established. Excluding the possibility of clonal variation between the generated stable cell lines, pooled MDA-231/pcDNA 3.1 and MDA-231/Bcl-2 cells were used in this study (Fig. 3B). As shown in Figure 3C, treatment with 5 μM sanguinarine for 18 h in MDA-231/pcDNA 3.1 cells resulted in a markedly increased accumulation of sub-G1 phase cells. In contrast, the accumulation of sub-G1 phase induced by sanguinarine was inhibited by Bcl-2 overexpression. MDA-231/pcDNA 3.1 cells displayed a 9.2-fold increase in DEVDase activity

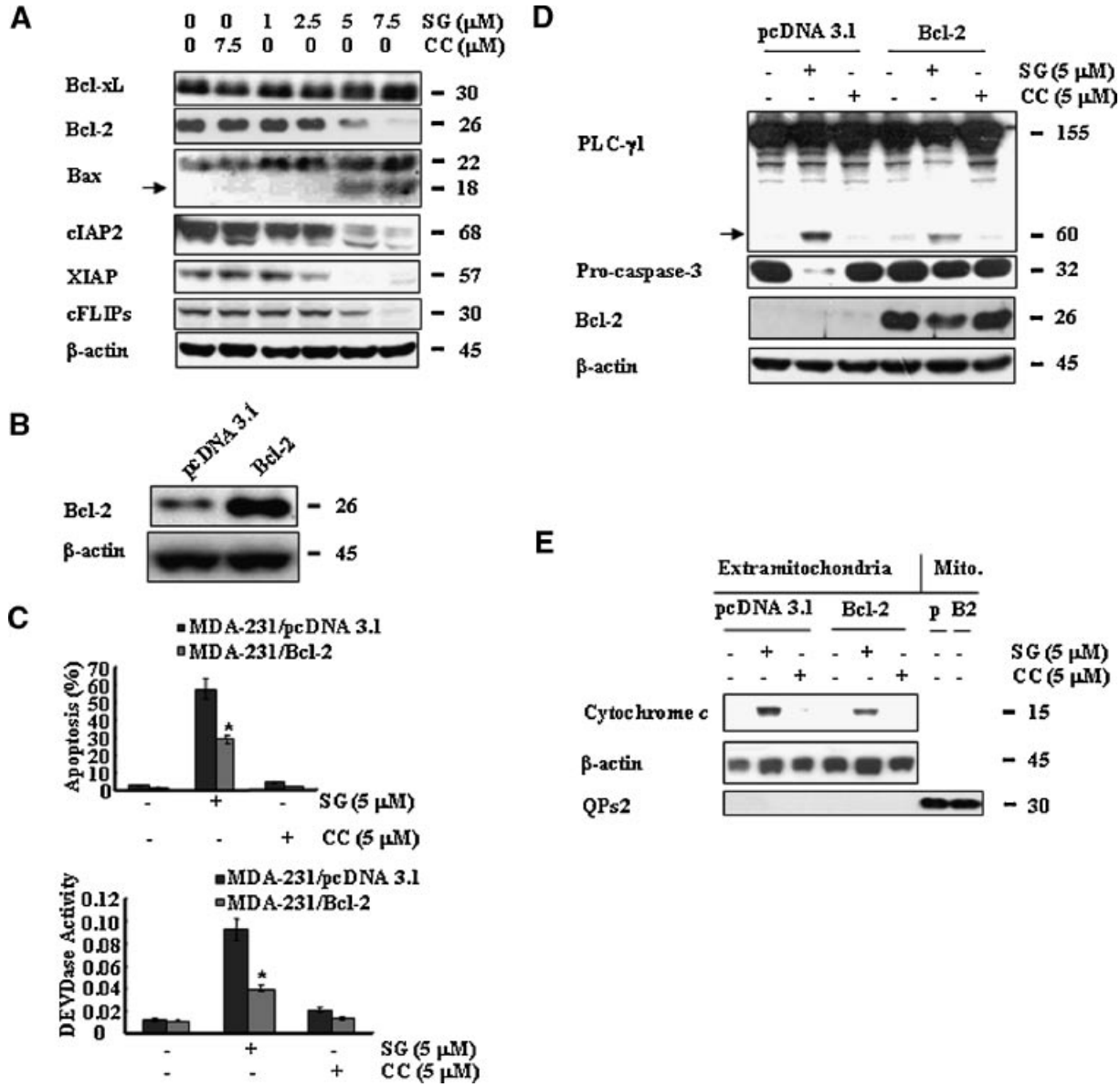

Fig. 3. Ectopic expression of Bcl-2 reduced sanguinarine-induced apoptosis. **A**: Cells were treated for 24 h with the indicated concentrations of SG or CC and harvested in lysis buffer and equal amounts of cell lysates (50 μg) were resolved by SDS–PAGE and analyzed Western blotting using anti-Bcl-xL , -Bcl-2, -Bax, -cIAP2, -XIAP, and c-FLIPs, or with anti-β-actin antibody to serve as control for the loading of protein level. The proteolytic cleavage of Bax is indicated by arrow. **B**: Immunoblot analysis of cell lysates (50 μg) from control pcDNA 3.1 vector (p) or Bcl-2 (B2) transfected cells with anti-Bcl-2 antibody. The blot was stripped of the bound antibody and reprobed with anti-β-actin antibody to confirm equal loading protein level. **C**: MDA-231/pcDNA 3.1 and MDA-231/Bcl-2 cells were treated for 18 h with SG or CC and then the fraction of apoptotic cells and DEVDase activity were determined as described in Figure 1. * Indicates $P < 0.05$ versus SG-treated pcDNA 3.1 vector cells. **D**: Cells were treated as above, equal amounts of cell lysates (50 μg) were resolved by SDS–PAGE and analyzed Western blotting using anti-pro-caspase-3, -PLC-γ1 and –Bcl-2. Anti-β-actin antibody was served as control for the loading of protein level. The proteolytic cleavage of PLC-γ1 is indicated by an arrow. **E**: Cytochrome *c* release was determined as described in Figure 1. To show there is no mitochondrial contamination in the cytosolic preparation, we carried out Western blotting analysis using antibody against QPs2. Mitochondrial fraction derived from non-treated cells were used as a positive control. Data are mean values obtained from three independent experiments and bars represent standard deviation.

after exposure to sanguinarine, compared with control. However, MDA-231/Bcl-2 cells showed slight increase by 3.4-fold (Fig. 3C). As shown in Figure 3D, protein levels of pro-caspase-3 decreased in MDA-231/pcDNA 3.1 cells exposed to 5 μM sanguinarine for 18 h, but its levels were rarely altered in sanguinarine-treated MDA-231/Bcl-2 cells. Subsequent Western blotting

analysis demonstrated that the proteolytic cleavage of PLC-γ1 in MDA-231/pcDNA 3.1 cells was more prominent than in MDA-231/Bcl-2 cells when exposed to sanguinarine. The introduced Bcl-2 protein was slightly decreased by sanguinarine treatment in MDA-231/Bcl-2 cells (Fig. 3D). Moreover, sanguinarine-induced release of cytochrome *c* from the mitochondria into the cytoplasm was also slightly blocked by Bcl-2 overexpression (Fig. 3E). Taken together, these results indicate that sanguinarine-induced down-regulation of Bcl-2 may be important for sanguinarine-induced apoptosis.

c-FLIPs Overexpression Prevents Sanguinarine-Induced Apoptosis in MDA-231 Cells

As shown in Figure 3A, protein levels of c-FLIPs were decreased by treatment with sanguinarine in a dose-dependent manner, indicating that its down-regulation might be associated with sanguinarine-induced apoptosis in MDA-231 cells. In order to evaluate the functional role played by c-FLIPs in inducing apoptosis caused by sanguinarine, c-FLIPs overexpressing cells were established (Fig. 4A). As shown in Figure 4B, overexpression of c-FLIPs remarkably inhibited sanguinarine-induced apoptosis as determined by accumulation of sub-G1 phase. In addition, overexpression of c-FLIPs inhibited the activation of DEVDase, the degradation of pro-caspase-3, and the proteolytic cleavage of Bax (Fig. 4C). The ectopic expressed c-FLIPs protein was slightly decreased by sanguinarine treatment in MDA-231/c-FLIPs cells (Fig. 4C). Furthermore, the overexpression of c-FLIPs in MDA-231 cells slightly blocked sanguinarine-induced release of cytochrome *c* from the mitochondria into the cytoplasm (Fig. 4D), suggesting that sanguinarine-induced down-regulation of c-FLIPs also contribute to the progression of apoptosis.

Sanguinarine Sensitizes Breast Cancer Cells to TRAIL-Mediated Apoptosis

Many breast cancer cells are resistant to the apoptotic effects of TRAIL [Keane et al., 1999]. We next examined the possibility that sanguinarine might sensitize TRAIL-resistant MDA-231 cells to TRAIL-mediated apoptosis. MDA-231 cells were treated with sanguinarine alone (2 μM), TRAIL alone (30 ng/ml), or a combination of sanguinarine and TRAIL. We found that subtoxic concentration of sanguinarine (2 μM) remarkably sensitizes breast cancer cells to TRAIL-mediated apoptosis. A typical ladder pattern of internucleosomal fragmentation, increase in the cell populations with hypodiploid DNA, increase in DEVDase activity, and cleavage of procaspase-3 and PLC-γ1 were observed only in MDA-231 cells cotreated with sanguinarine and TRAIL for 24 h (Fig. 5A–C). Furthermore, protein levels of cIAP1, XIAP and cFLIPs proteins were remarkably reduced by the combined treatment with sanguinarine and TRAIL (Fig. 5C). Pretreatment with z-VAD-fmk effectively inhibited sanguinarine-induced apoptotic events, including the accumulation of sub-G1 cell population, DEVDase activity and proteolytic cleavage of pro-caspase-3 and PARP (Fig. 5D), indicating the critical role in sanguinarine-sensitized TRAIL-induced apoptosis. To determine whether combinatory treatment with sanguinarine plus TRAIL can induce apoptosis in another breast cancer cell line, MDA-435S cells were co-treated with sanguinarine and TRAIL for 24 h. The effects of sanguinarine plus TRAIL on MDA-435S cells were similar to the results of MDA-231 cells (Fig. 5E). To investigate whether sanguinarine is able to sensitize the breast cancer cells to conventional chemotherapeutic drugs, MDA-231 cells were treated with etoposide, and oxaliplain for 24 h. As shown in Figure 5F, combinatory treatment with sanguinarine plus several chemotherapeutic agents enhances apoptosis in MDA-231 cells.

Apoptosis Induced by Sanguinarine Plus TRAIL is not Blocked by Akt or Bcl-2-Overexpression

Several compelling evidences support that Akt and Bcl-2 play a key role in conferring breast cancer cells resistance to chemotherapy [Clark et al., 2002; Johnstone et al., 2002; Knuefermann et al., 2003; Kim et al., 2005]. Thus, development of the strategies to bypass Akt- or Bcl-2-mediated chemoresistance is an important issue in the therapies of breast cancer. To examine whether Akt or Bcl-2 overexpression in breast cancer cells affects sanguinarine-stimulated TRAIL-induced apoptosis, we established the MDA-231 sublines stably overexpressing constitutively active (DA) Akt or Bcl-2 (Fig. 6A). In order to rule out the possibility clonal variations, we used pooled MDA-231/Bcl-2 and MDA-231/Akt-DA cells. MDA-231/Bcl-2 cells were treated with sanguinarine (2 μM) and TRAIL alone (30 ng/ml), or combination with

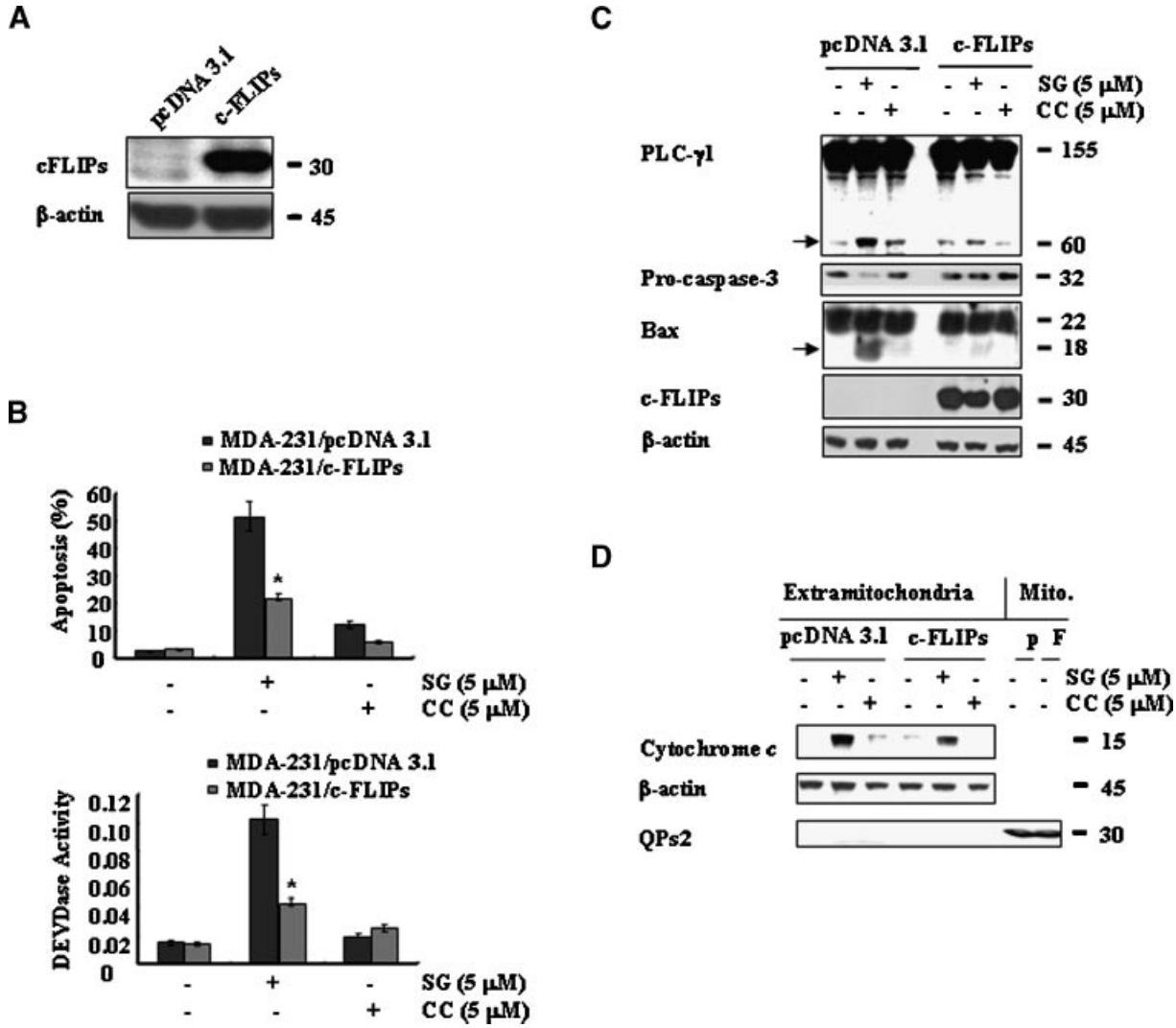

Fig. 4. Effect of c-FLIP on sanguinarine-induced apoptosis. **A**: Immunoblot analysis of cell lysate (50 μg) from control pcDNA 3.1 or c-FLIPs transfected cells with anti-FLIP antibody. The blot was stripped of the bound antibody and reprobed with anti-β-actin antibody to confirm equal loading protein level. **B**: MDA-231/pcDNA 3.1 and MDA-231/c-FLIPs cells were treated for 18 h with SG or CC and then the fraction of apoptotic cells and caspase activity were determined as described in Figure 1. * Indicates $P < 0.05$ versus SG-treated pcDNA 3.1 vector cells. **C**: Cells were treated as above, equal amounts of cell lysates (50 μg) were resolved by SDS–PAGE and analyzed Western blotting using anti-pro-caspase-3, -PLC-γ1, -Bax, and c-FLIPs or with anti-β-actin antibody to serve as control for the loading of protein level. The proteolytic cleavage of PLC-γ1 is indicated by an arrow. **D**: Cytochrome *c* release was determined as described in Figure 1. To show there is no mitochondrial contamination in the cytosolic preparation, we carried out Western blotting analysis using antibody against QPs2. Mitochondrial fraction derived from non-treated cells were used as a positive control. Data are mean values obtained from three independent experiments and bars represent standard deviation.

sanguinarine and TRAIL for 24 h. Interestingly, the apoptosis induced by the combined treatment with sanguinarine and TRAIL similarly occurred in both Akt- or Bcl-2 overexpressing cells as in control cells (Fig. 6B,C). These results suggest that the combined regimen with sanguinarine and TRAIL may be effective for the treatment of Akt- or Bcl-2-overexpressing breast cancer cells, which are resistant to various chemotherapies.

DISCUSSION

Sanguinarine has been reported to exert a variety of biological effects including antimicrobial, antioxidant and anti-inflammatory properties [Firatli et al., 1994; Colombo and Bosisio, 1996; Faddeeva and Beliaeva, 1997; Kataoka et al., 2000; Chau et al., 2005; Oehme et al., 2005]. Recently, sanguinarine was reported to selectively eliminate cancer cells

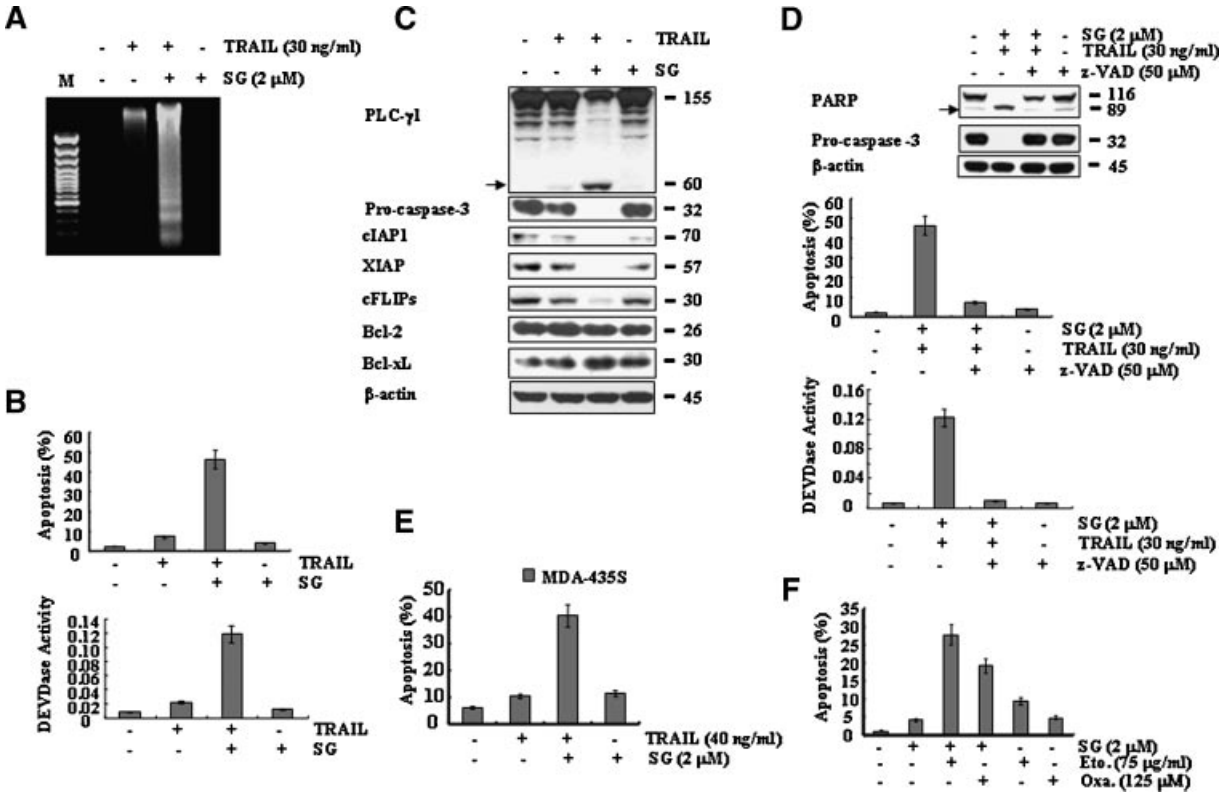

Fig. 5. Sanguinarine sensitizes MDA-231 cells to TRAIL-induced apoptosis. **A**: Fragmentations of genomic DNA in MDA-231 cells were cotreated with SG and TRAIL for 24 h. Fragmented DNA was extracted and analyzed on 2% agarose gel. **B**: Flow cytometric analysis and DEVDase activity were determined as described in Figure 1B. **C**: Cells were treated and harvested as above. Western blotting analysis was performed as above using anti-pro-caspase-3, -PLC-γ1, -c-IAP1, -XIAP, -c-FLIPs, -Bcl-2 and -Bcl-xL or with anti-β-actin antibody to serve as control for the loading of protein level. The proteolytic cleavage of PLC-γ1 is indicated by arrow. **D**: Effects of z-VAD-fmk on SG and TRAIL-induced apoptosis. Cells were incubated with z-VAD-fmk or solvent for 1 h before co-treatment with SG and TRAIL. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for procaspase-3 and PARP. The proteolytic cleavage of PARP is indicated by an arrow. Flow cytometric analysis of apoptotic cells and DEVDase activity were determined as described in Figure 1. Data are mean values obtained from three independent experiments and bars represent standard deviation. **E**: Quantitation of apoptosis by fluorescence-activated cell sorting analysis. Sub-G1 fraction was measured in MDA-435S cells treated with 2 μM sanguinarine alone, 40 ng/ml TRAIL alone, or sanguinarine plus TRAIL for 24 h. **F**: Quantitation of apoptosis by fluorescence-activated cell sorting analysis. Sub-G1 fraction was measured in MDA-231 cells treated with 2 μM sanguinarine alone, etoposide (Eto) 75 μg/ml, and oxaliplatin (Oxa) 125 μM in presence or absence of sanguinarine for 24 h.

without affecting the normal cells, suggesting its potential to be used as an anticancer drug [Colombo and Bosisio, 1996]. However, the molecular mechanisms involved in sanguinarine-induced apoptosis are poorly understood. In this study, we show that sanguinarine treatment induces significant apoptosis in MDA-231 cells. In this process, ROS generation and the decrease in the protein levels of Bcl-2, c-IAP2, XIAP and c-FLIPs, and activation of caspases were noted. Recently, it was reported that induction of superoxide radicals by sanguinarine is indispensable for its ability to trigger apoptosis in LNCaP cells [Ahsan et al., 2007]. We also found that sanguinarine treatment induced ROS generation and pretreatment with GSH or NAC efficiently inhibited sanguinarine-induced apoptosis, suggesting that ROS generation is critical for the induction of apoptosis by sanguinarine in human breast cancer cells.

Increased activity of anti-apoptotic proteins, such as Bcl-2, c-FLIPs, and Akt, may result in aggressively growing and therapy-resistant tumors [Clark et al., 2002; Johnstone et al., 2002; Knuefermann et al., 2003; Longley et al., 2006]. In particular, Bcl-2 is an attractive target for the development of ant-cancer drugs because of high levels of Bcl-2 in various human cancer cells [Gutierrez-Puente et al., 2002; Westphal and Kalthoff, 2003]. It has

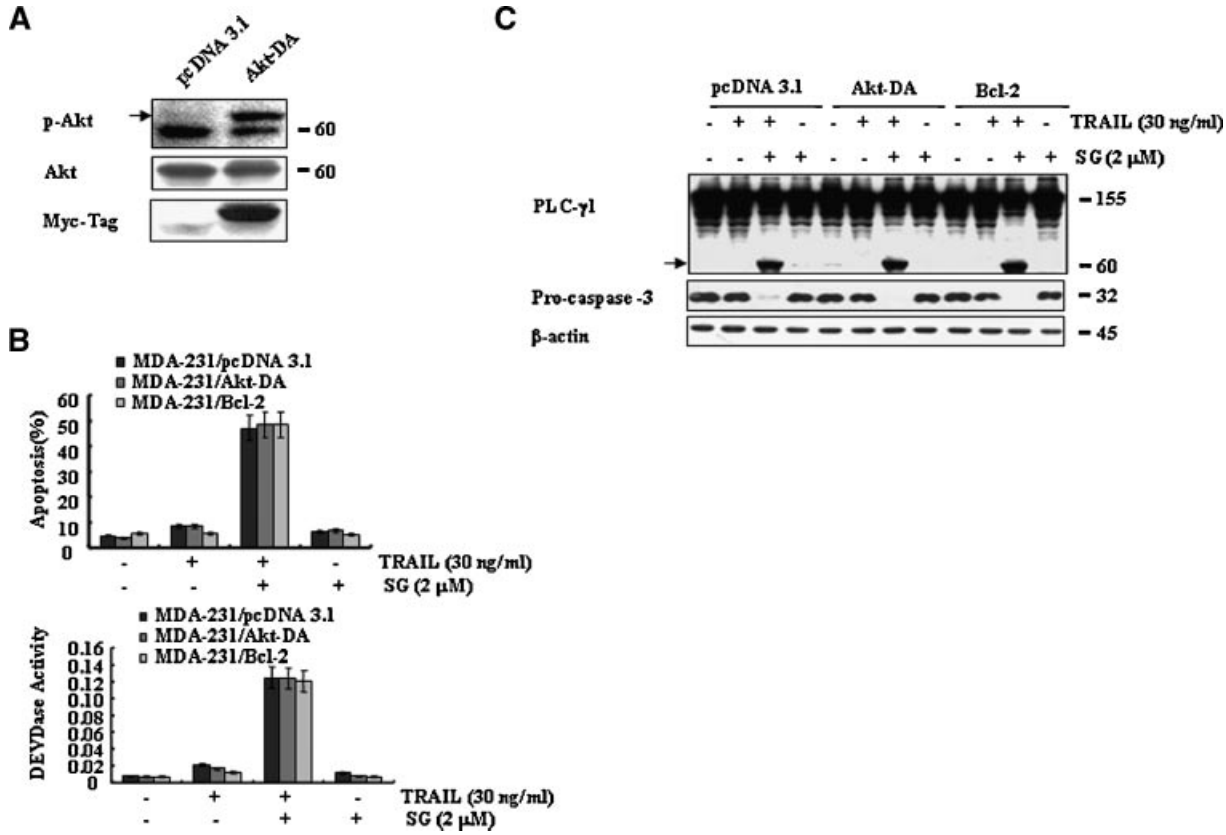

Fig. 6. Sanguinarine plus TRAIL induces apoptotic cell death in Akt and Bcl-2 overexpressing MDA-231 cells. **A**: Whole cell lysates obtained from MDA-231 cells stably transfected with a myr-Akt-expression vector or the empty vector was subjected to SDS-PAGE, trasnferred to membranes, and immunoblotted using anti-Akt, anti-phospho-Akt and anti-Myc antibodies as indicated. **B**: Cells were treated with SG plus TRAIL for 24 h and then flow cytometric analysis of apoptotic cells and DEVDase activity were determined as described in Figure 1. Data are mean values from three independent experiments and bars represent standard deviations. **C**: Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for procaspase-3 and PLC-γ1. Anti-β-actin antibody was served as control for the loading of protein level. The proteolytic cleavage of PLC-γ1 is indicated by an arrow.

previously been reported that sanguinarine-induced apoptosis was mediated by down-regulation of Bcl-2 [Huh et al., 2006]. In agreement with the previous observation, we found that sanguinarine induced remarkable down-regulation of Bcl-2 during apoptosis and Bcl-2 overexpression attenuated sanguinarine-induced apoptosis, suggesting that Bcl-2 down-regulation is directly associated with this cell death and sanguinarine is potentially useful against breast cancer.

c-FLIPs is known to be an inhibitory protein of death receptor-mediated apoptosis via inhibition of caspase-8 activation as well as mitochondria-mediated apoptosis induced by chemotherapeutic drugs in cancer cells [Matta et al., 2002; Jonsson et al., 2003; Natoni et al., 2005; Longley et al., 2006]. In recently published reports, chemotherapeutic agents including taxol and celecoxib induce apoptosis by down-regulating c-FLIP expression [Sun and Chao, 2005; Day et al., 2006; Liu et al., 2006]. In our study, sanguinarine treatment induced remarkable decrease of c-FLIPs protein levels and overexpression of c-FLIPs protects cells from sanguinarine-induced apoptosis, suggesting that down-regulation of c-FLIPs also contributes to sanguinarine-induced apoptosis. In addition, ectopic expression of constitutive active Akt prevented sanguinarine-induced apoptosis.

Recently, several papers demonstrated that combining TRAIL with chemotherapeutic or certain signaling inhibitors resulted in robust enhancement of apoptosis in drug resistant cancer cells. In the present study, we demonstrate for the first tine that apoptosis induced by sanguinarine plus TRAIL is not blocked by

exogenously overexpressed Bcl-2 or constitutive active Akt in human breast cancer cells. These results raise the possibility that a strategy using sanguinarine plus TRAIL or conventional chemotherapeutic drugs may be effective against drug-resistant breast cancer cells overexpressing Bcl-2 or Akt. To our knowledge, this is the first systematic study showing the therapeutic potential of sanguinarine in breast cancer cells.

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