

Downregulation of Inhibitor of Apoptosis Proteins by Deguelin Selectively Induces Apoptosis in Breast Cancer Cells

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Abbreviations: IAP: Inhibitor of Apoptosis, **PARP:** poly (ADP-ribose) polymerase

ABSTRACT

The identification of differentially regulated apoptotic signals in normal and tumor cells allows the development of cancer cell-selective therapies. Increasing evidence shows that the inhibitor of apoptosis (IAP) proteins survivin and XIAP are highly expressed in tumor cells, but absent or have very low levels of expression in normal adult tissues. We found that inhibiting AKT activity with 10 to 100 nM of deguelin, a small molecule derived from natural products, markedly reduced the levels of both survivin and XIAP, inducing apoptosis in human breast cancer cells but not in normal cells. Interestingly, we detected an elevated level of cleaved poly (ADP-ribose) polymerase, a signature of caspase activation, without a significant increase in caspase activity in deguelin-treated cancer cells. Our results suggest that severe downregulation of the IAPs by deguelin releases their inhibitory activity over pre-existing active caspases present in cancer cells, inducing apoptosis without the need for further caspase activation. Since normal cells have very low levels of p-AKT, XIAP, survivin, and pre-existing caspase activity, deguelin had little effect on those cells. Additionally, we found that combining deguelin with chemotherapy drugs enhanced drug-induced apoptosis selectively in human tumor cells, which suggests that deguelin has great potential for chemosensitization and could represent a new therapeutic agent for treatment of breast cancer.

INTRODUCTION

The development of mechanisms that give resistance to apoptosis confers both a high survival ability and a low drug sensitivity to human cancer cells (Deveraux and Reed, 1999; Reed, 1999). To develop cancer-specific therapeutic approaches, it is important to identify the molecular targets in the apoptotic pathway that are differentially regulated in normal and tumor cells. It is well known that a balance of pro-apoptotic and anti-apoptotic factors determines whether a cell survives or undergoes apoptosis (Igney and Krammer, 2002; Yang et al., 2003). In tumor cells, apoptosis can be induced by either activation of molecules upstream of apoptosis signaling or by inhibition of anti-apoptotic factors (Mesri et al., 2001; Reed, 2001). A previous study of ours shows that although direct activation of caspase 3 is able to induce apoptosis, normal cells still seem to be more sensitive to caspase 3-induced activation of apoptosis than human tumor cells are (Yang et al., 2003). The presence in cancer cells, but not normal cells, of high levels of anti-apoptotic factors, such as the inhibitor of apoptosis (IAP) proteins may confer this insensitivity to apoptosis induction by caspase 3 activation in tumor cells. Members of the IAP family of proteins contain one or more conserved regions termed baculoviral IAP repeat (BIR) N-terminal domains and a C-terminal RING domain (Deveraux and Reed, 1999; Reed, 1999). The BIR domain of these IAP binds to active caspases to block their activity, while the RING domain acts as an ubiquitin ligase to facilitate proteasomal degradation of caspases (Huang et al., 2001; Liston et al., 1997). It has been shown that a member of the IAP family of proteins, XIAP, blocks the active sites of both caspase-3 and -7 using its proximal link region of BIR2 and it inhibits active caspase 9 through a BIR3 domain (Huang et al., 2001). Downregulation of the level or function of another IAP protein, survivin, results in activation of

caspase-9 (Mesri et al., 2001). A recent study further shows that survivin is even able to associate with XIAP, promoting increased XIAP stability and synergistic inhibition of apoptosis (Dohi et al., 2004).

IAP upregulation is commonly observed in human tumors, and the level of IAP expression correlates with poor clinical outcome, as well as resistance to chemotherapy drugs and ionizing radiation (Altieri, 2003; Asanuma et al., 2000; Holcik et al., 2001; Salz et al., 2005; Tanaka et al., 2000). XIAP is expressed at a low level in normal cells and tissues, but it has a high level of expression in many human tumor cells (Holcik et al., 2001, Liston, 1997 #120; Liston et al., 1997). Unlike the other IAPs, survivin is expressed broadly in fetal tissues but is undetectable in most differentiated normal adult tissues. However, there is a high level of survivin in most tumor types, including over 70% of human breast tissues (Tanaka et al., 2000; Yang et al., 2003). It has been shown that polyphenylureas are able to inhibit XIAP activity and directly induce apoptosis in many types of tumor cell lines plus in tumor xenografts in mice, while displaying little toxicity to the normal tissues (Schimmer et al., 2004). Our previous study using adenoviral vectors expressing either a dominant negative survivin (survivinT34A) and/or the XIAP associated factor 1 (XAF1) gene, demonstrated that the vectors could also be used to selectively induce apoptotic cell death in cancer cell lines, with little effect on normal cell lines (Yang et al., 2003). Therefore, we believe that identification of any therapeutic agents that are capable of inhibiting the function or levels of survivin and XIAP should provide additional novel and more specifically targeted agents for use in cancer treatment.

In this study, we demonstrate that deguelin, a natural product isolated from plants in the *Mundulea sericea* family, significantly downregulated the levels of both survivin and XIAP and induced apoptotic cell death selectively in human breast cancer cells, while having little effect on

normal mammary epithelial cells and primary fibroblasts. Furthermore, our results show that this differential apoptotic response was a consequence of differences in apoptotic signaling between human breast cancer and normal cells.

MATERIALS AND METHODS

Cell lines and materials Human breast cancer cell lines SK-BR-3 and MCF-7, and a normal immortalized human mammary epithelial cell line MCF 10A were obtained from the American Type Culture Collection (ATCC) and cultured in the medium as recommended by ATCC (Manassas, VA). Primary normal human dermal fibroblast cell line HDF, purchased from Emory University Skin Disease Center (Atlanta, GA) HDF, was maintained in DMEM medium with 20% FBS.

The deguelin (97%–98% purity, HPLC-UV) was synthesized from commercially available rotenone, as previously described (Gerhauser et al., 1997). Chemotherapy drug docetaxel was obtained from Aventis Pharma (Bridgewater, NJ) and doxorubicin was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell growth inhibition assay The MCF-7, SK-BR-3 and MCF-10A cell lines were used to determine the inhibitory effect of deguelin on cell growth using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 8×10^3 cells /well were plated in 96-well plates and then cultured in medium with or without various concentrations of deguelin, doxorubicin, and docetaxel, alone or in combination. Control cultures received 0.1% DMSO, the solvent for the deguelin stock solution. Three days after the treatment, the percentage of viable cells in each well was examined by MTT assay (Sigma Chemical Co., St. Louis, MO), using the Spectra Max Plus Spectrophotometer (Molecular Devices, Sunnyvale, CA).

Colony formation assay The cells were plated in six-well plates at a density of 1500 cells/well for overnight and then were treated with the indicated concentrations of drugs for 14 days. The cells were fixed with methanol and stained with crystal violet. The number of colonies in each well was scored by counting of the cell colonies containing 50 or greater of cells.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

Cells were plated in 8-well chamber slides (5×10^3 cells per well) for 24 hrs, and then treated with different concentrations of deguelin. 48 hrs later, floating and adherent cells were collected and placed onto polylysine-coated glass slides using Shandon Cytospin® 4 (Thermo Electron Corp., MA). The presence of DNA fragmentation in the cells was examined using DeadEnd™ Fluorometric TUNEL System (Promega Corp., Madison, WI), according to the manufacturer's instruction. Briefly, slides were air dried and fixed in 4% paraformaldehyde for 20 min at 4 °C. After washing with PBS, the slides were treated with 0.1% Triton X-100 for 5 min at room temperature. The slides were then incubated with 50 µL of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction mixture for 1 hr at 37 °C in the dark. After mounted with cover slips using Vectashield mounting medium with propidium Iodide (BD PharMingen, San Diego, CA), the cells were analyzed under an Zeiss AxioPlan fluorescent microscope (Carl Zeiss, Inc., Thornwood, NY).

Western blot analysis Cells were treated with various concentrations of deguelin alone or in combination with doxorubicin or docetaxel for 2 days, then collected for Western blot analysis using an established protocol in our lab (Yang et al., 2003). Primary antibodies were used for detecting the levels of specific proteins: survivin (Santa Cruz Biotechnology, Santa Cruz, CA), XIAP, phosphor-serine 473 AKT, total ATK, p44/p42 mitogen-activated protein kinase

(MAPK), total MAPK, poly(ADP-ribose) polymerase (PARP) (both total and cleaved forms), and caspase 3 (Cell Signaling Technology Inc. Beverly, MA), β -actin (Sigma Chemical Co.).

Real-Time RT PCR. Total RNAs were isolated and amplified with an Omniscript RT kit (QIAGEN Inc, Valencia, CA). Real-Time PCR was performed on an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). The primer pairs for detecting the expression of survivin gene were forward: 5'-TCCACTGCCCCACTGAGAAC-3' and reverse: 5'-TGGCTCCCAGCCTTCCA-3'. PCR primers for XIAP were forward: 5'-CCGTGCGGTGCTTTAGTTGT-3' and reverse: 5'-TTCCTCGGG-TATATGGT-GTCTGAT-3'. GAPDH-specific primers were forward: 5'-TTGGTATCGT-GGAAGGACTCA-3' and reverse: 5'-TGTCATCATATTTGGCAGGTT T-3'.

Luciferase Reporter Assay:

Cells were plated in 24-well tissue culture plates at 70% confluence for 24 hrs and then co-transfected with 1.0 μ g of a Survivin promoter-luciferase reporter plasmid (pluc cyc1.2, provided by Dr. Fengzhi Li, Roswell Park Cancer Institute, Buffalo, NY) and 20 ng of pRL-SV-40 internal control plasmid that expresses a renilla luciferase gene (Promega) using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). The transfected cells were then treated with deguelin for 24 hrs. The cell lysates were collected for measuring luciferase activity using Dual Luciferase Assay System (Promega) and Lumistar galaxy (BMG, Winooski, VM). The relative luciferase activity for each sample was calculated as a ratio of firefly and renilla luciferase activity. The level of renilla luciferase activity, which was expressed from the co-transfected pRL-SV-40 plasmid, was used as an internal control for differences in transfection efficiency among the treatment groups.

Caspase activity assay. Caspase 3-like activity, which is generated by caspases 3, 7, and 10, was detected in cell lysates after various treatments using an Ac-DEVD-AFC substrate; and

caspase 9 activity was examined using an Ac-LEHD-AFC substrate (CALBIOCHEM, San Diego, CA). Measurements were made using a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA). Control groups with specific caspase inhibitors, including caspase 3 inhibitor (Z-DEVD-CHO, BD PharMingen) and caspase 9 inhibitor (Z-LEHD-CHO, Alexis Biochemicals, San Diego, CA), were done to ensure specificity.

Transfection. Cells were cultured in 96 well plates for 24 hrs, and then transfected with 0.2 μ g of a plasmid containing the survivin (pcDNA.3-survivin, provided by Dr. DC Altieri, University of Massachusetts Medical School, MA), or XIAP gene (pcDNA.3-XIAP 6x-myc, obtained from Dr. Robert Korneluk, Children's Hospital of Eastern Ontario, Canada), or a control empty plasmid (pcDNA3) with Lipofectamine™ 2000 (Invitrogen). 24 hrs after transfection, the cells were treated with different concentrations of deguelin for 3 days. Viable cells were quantitated by MTT assay. For colony formation assay, transfected cells were cultured with deguelin for 14 days and the number of colonies was examined as described previously.

RESULTS

Deguelin treatment significantly inhibits the PI3K/AKT pathway and expression levels of both survivin and XIAP.

Although previous studies have shown that deguelin inhibits AKT activity and induces apoptosis in human cancer cells, the mechanism for this deguelin-induced apoptotic cell death has yet to be elucidated (Chun et al., 2003; Lee, 2004; Murillo et al., 2002). Increasing evidence indicates that the PI3K/AKT pathway regulates the cell survival pathway and the IAP family of proteins (Asanuma et al., 2005; Belyanskaya et al., 2005; Dan et al., 2004). To understand the molecular events linking AKT inhibition to apoptosis induction by deguelin, we examined the effects of deguelin treatment on breast cancer cells. Treatment of the cells with 1 to 10 nM of

deguelin markedly inhibited the levels of p-AKT while the levels of total AKT protein were not affect significantly in MCF-7 cells (Fig. 1A). However, 100 nM of deguelin treatment significantly reduced the levels of both p-AKT and total AKT proteins, suggesting a different mechanism of deguelin-induced AKT inhibition is involved at high drug concentrations. Similar results were found in SK-BR-3 cells except that 10 nM of deguelin slightly reduced the level of total AKT protein (Fig. 1 A). Additionally, the MAPK activity was also downregulated in breast cancer cells following 0.1 nM (MCF-7) or 100nM (SK-BR-3) of deguelin treatment (Fig. 1A).

We further examined the effects that downregulation of AKT and MAPK activities had on the levels of survivin and XIAP using Western blot analysis. Treatment of MCF-7 cells with deguelin concentrations of 10 nM or above completely inhibited survivin expression (Fig. 1A). Even a deguelin concentration as low as 1 nM significantly inhibited the level of survivin in both MCF-7 and SK-BR-3 cells. A marked inhibition of levels of XIAP was also detected in breast cancer cells treated with over 10 nM (MCF-7) or 100 nM (SK-BR-3) of deguelin (Fig. 1A).

To study the mechanism of these decreases in survivin and XIAP protein levels induced by deguelin, we examined the levels of survivin and XIAP gene expression using Real-time RT PCR. We found that treatment of cancer cells with 100 nM of deguelin markedly inhibited the levels of both survivin and XIAP mRNAs (Fig. 1B). We further found that inhibition of survivin gene transcription resulted in a decreased level of survivin mRNA since treatment of MCF-7 cells with 1 to 10 nM of deguelin markedly reduced the survivin promoter activity (Fig. 1C).

Downregulation of survivin and XIAP by deguelin induces apoptosis and lowers cell growth in breast cancer, but not in normal cells.

To date, the effects of deguelin on human breast cancer cells have not been examined. We examined the effects of deguelin on the growth and survival of breast cancer and normal mammary epithelial (MCF-10A) and primary fibroblast (HDF) cell lines. Our results showed that about 15-20 % growth inhibition could be achieved using as low a concentration as 10 nM of deguelin in MCF-7 and SK-BR-3 cell lines detected by MTT assay (Fig. 2 A). 50% of growth inhibition was found in both cell lines after 100 nM deguelin treatment by MTT and colony formation assays (Fig. 2 A and B). Results from the clonogenic assay further confirmed the long-term effect of the drug on breast cancer cells.

In marked contrast, treatment of normal human mammary epithelial MCF-10A cells within the range of 1 to 100 nM deguelin for 3 days had a little effect on cell survival (Fig. 2A). Similarly, results of colony formation assay also demonstrated that normal MCF-10A and HDF cells were much more resistant to deguelin treatment as compared to breast cancer cell lines (Fig. 2 B).

To determine if the growth inhibition observed in breast cancer cells is the result of apoptosis, we employed a Fluorometric TUNEL System to determine the percentage of apoptotic cells. Our results revealed a dose-dependent apoptotic cell death in deguelin-treated cancer cells (Fig. 2 C). As shown, 100 nM of deguelin treatment induced 31.4% and 41.6% of apoptotic cells in SK-BR-3 and MCF-7 breast cancer cells, respectively (Fig. 2C).

To further demonstrate that downregulation of survivin and XIAP is one of the key factors responsible for deguelin-induced apoptosis, we examined the effects of overexpression of the survivin or XIAP gene on induction of apoptosis by deguelin. Our results from MTT and colony formation assays revealed that overexpression of either the survivin or XIAP gene in MCF-7 cells by transfecting the corresponding plasmids significantly blocked deguelin-induced

apoptotic cell death (Fig. 3 A and B). The protective effect of overexpressing survivin or XIAP gene on deguelin-induced apoptosis was further demonstrated by TUNEL assay (Fig. 3 C). Therefore, our results strongly support the notion that the downregulation of IAP proteins is the critical factor for achieving deguelin-induced apoptosis in breast cancer cells.

Deguelin induces apoptosis in breast cancer cells without significant caspase activation.

In a previous study, we showed that the upstream apoptotic signals are already activated in many cancer cells due to the presence of abnormalities (Yang et al., 2003). Since the treatment of human breast cancer cells with deguelin concentrations at 10 nM or above markedly blocked survivin expression and inhibited the level of XIAP, we speculated that effective apoptotic cell death can be induced in cancer cells having activated apoptotic signals once the level or function of anti-apoptotic factors, such as survivin and XIAP, become severely compromised.

Since caspase activation is an important step towards the induction of apoptosis, we searched for activity of caspase 3-like and caspase 9 in breast cancer cell lines. When we examined the basal levels of caspase 3-like, caspase 7, and caspase 9 activities in breast cancer and normal cell lines, we found that the levels of caspase activity were activated to different degrees in the cancer cells. Compared to the normal MCF-10A cells, the SK-BR-3 breast cancer cells displayed higher levels of caspase 3-like and caspase 9 activity (Fig. 4 A). Although the MCF-7 breast cancer cell line did not express the caspase 3 gene, it had a moderate level of caspase 7 and a higher level of caspase 9 activity (Fig. 4 A). When we compared caspase 3-like activity in normal and cancer cells after deguelin treatment, we found that while a relatively high concentration of deguelin (1000 nM) increased the level of caspase 3 activity in SK-BR-3 cells slightly, the activity in MCF 10A cells remained low (Fig. 4B). In cells that were treated with 100 nM deguelin for 2 days, about 50% became apoptotic, even though there was no detectable

increase in caspase 3-like or caspase 7 activity in either the SK-BR-3 or MCF-7 cells (Fig. 4B). In contrast, treating tumor cells with chemotherapy drugs, such as docetaxel or doxorubicin, at a dosage producing 50% apoptotic cell death, induced 4 to 7-fold higher levels of caspase 3-like or caspase 7 activity in the SK-BR-3 or MCF-7 cell lines, as compared with deguelin treatment alone (Fig. 4 C).

Downregulation of IAPs releases their inhibitory effects on pre-existing active caspases present in cancer cells, resulting in cancer-cell specific apoptosis.

To determine whether the levels of active caspases in deguelin-treated cancer cells were sufficient to induce caspase-mediated apoptotic response downstream, we examined deguelin-treated SK-BR-3 cells for the level of cleaved PARP, a substrate for caspase 3 and caspase 7 (Slee et al., 2001). Consistent with the results of the caspase 3 activity assay, we detected a basal level of active caspase 3 bands (17 and 19 KDa) in SK-BR-3 cells. Treatment with 1 or 100 nM deguelin only slightly increased the levels of cleaved caspase 3 (Fig. 5A). However, a marked increase in the level of cleaved caspase 3 was seen in the cells treated with doxorubicin. Interestingly, intermediate levels of cleaved PARP were detected in 100 nM deguelin-treated cells, suggesting that even the lower level of active caspase activity is sufficient to cleave cellular protein substrates, leading to an apoptotic phenotype (Fig. 5A). Although both 100 nM deguelin and 100 nM doxorubicin induce 50% the apoptotic cell death in SK-BR-3 cells, only doxorubicin induced very high levels of active caspase 3 and cleaved PARP. However, from our study, it appears that deguelin-induced apoptosis is mediated, at least in part, by a caspase-dependent mechanism.

To determine the mechanism of differential apoptotic responses in breast cancer and normal cells, we further examined changes in cell signal pathways in MCF-10A and HDF cells.

Unlike breast cancer cells, those normal cells did not exhibit an activated AKT signal and had very low levels of survivin expression. Additionally, activated caspase 3 fragments were not detected in normal cells (Fig. 5 B). Treatment of the MCF-10A cells with 10 and 100 nM deguelin did not induce changes in the levels of survivin, cleaved caspase 3 and PARP (p85) fragments, indicating that the deguelin treatment did not activate caspase activity and initiates the apoptotic cascade in those normal cells (Fig. 5B).

Deguelin treatment counteracts chemotherapy drug-induced upregulation of survivin expression and thus increases the sensitivity of cancer cells to docetaxel and doxorubicin.

The ability of deguelin to block both survivin and XIAP expression provides a great opportunity for sensitizing human cancer cells to chemotherapy. Previous studies including ours revealed that treatment of human cancer cells with many of the drugs used in chemotherapy today induced upregulation of survivin expression (Ikeguchi et al., 2002; Ling et al., 2004; Peng et al., 2005, Ling, 2004 #356). However, the undesirable effect of increasing the level of survivin has been shown to prevent the downstream apoptotic response and decrease drug sensitivity (Tamm et al., 1998). Therefore, we wanted to determine whether the combination of deguelin with either docetaxel or doxorubicin, two commonly used chemotherapy drugs for breast cancer, could help to increase the apoptotic response in human tumor cells. We reasoned that treatment of human cancer cells with those drugs should activate upstream apoptotic signals and increase the level of active caspases. However, a higher level of survivin produced by these drug-treated tumor cells would contribute toward a reduction of the apoptotic response. It appeared to be possible that the combination of downregulating survivin and XIAP levels with deguelin, alone with chemotherapy drug's ability to activate caspases, might produce a stronger apoptotic signal, sensitizing tumor cells to chemotherapy.

First, we examined whether deguelin could prevent drug-induced upregulation of survivin expression. In both SK-BR-3 and MCF-7 cell lines, we found that docetaxel or doxorubicin increased the level of survivin expression, while the level of XIAP remained unaffected (Fig. 6A). However, when given in combination with 10 or 100 nM deguelin, both docetaxel- and doxorubicin-induced survivin upregulation was significantly inhibited in the cells (Fig. 6A). Although chemotherapy drug treatment alone did not affect the level of XIAP, the combined treatments did decrease XIAP expression (Fig. 6A). Furthermore, we detected a higher level of cleaved PARP (p85) in the SK-BR-3 cells exposed to the combination treatment than was in cells receiving docetaxel or doxorubicin alone (Fig. 6A), suggesting that there was a higher level of activated caspase activity for the combination. In MCF-7 cells, strong inhibition of survivin and XIAP levels and a higher level of cleaved PARP were observed in cells treated by the combination of docetaxel with 100 nM deguelin (Fig. 6A).

To determine the mechanism by which deguelin blocks the upregulation of survivin in chemotherapeutic drug-treated cancer cells, we examined changes in the level of survivin gene expression by Real time RT-PCR (Fig. 6B). We found that docetaxel treatment alone induced about 2.5-fold increases in the level of survivin mRNA in SK-BR-3 cells as compared to control cells. Consistent with Western blot results for levels of survivin protein, the combination of docetaxel with as little as 1 nM of deguelin markedly prevented docetaxel-induced upregulation of survivin gene expression (Fig. 6B).

Next, we examined what the effect would be of blocking drug-induced survivin upregulation with deguelin on both the apoptotic response and chemosensitivity of human breast cancer cells. We chose to use 1 and 10 nM of deguelin, which on its own would not have a significant effect on the induction of tumor cell apoptosis but showed inhibition of p-AKT and

survivin. When used in combination, we found that even these levels of deguelin markedly enhanced the effects of chemotherapeutic drugs on breast cancer cells (Fig. 7 A). 14 days after treating SK-BR-3 breast cancer cells with doxorubicin in the presence of 1 or 10 nM deguelin, we observed decreased number of cell colonies compared with that of doxorubicin treated cells (Fig. 7A). The combination of docetaxel with deguelin significantly reduced the numbers of cell colonies in MCF-7 cells (Fig. 7 A). In contrast, results of colony formation assay obtained from normal HDF and MCF-10A cell lines following the combined treatment of 1 or 10 nM deguelin treatment with docetaxel or doxorubicin showed that deguelin did not significantly alter the drug sensitivity in normal cell lines (Fig. 7A). Furthermore, results from the TUNEL assay revealed that the combination of deguelin with doxorubicin increased 12% higher level of apoptosis than that detected in the cells treated with doxorubicin alone in SK-BR-3 cells (Fig. 7 B).

To determine the apoptotic signals required for increasing cell death in the combination treatment, we studied changes in the level of caspase activity. Treatment of either SK-BR-3 cells with doxorubicin or MCF-7 cells with docetaxel did activated caspase 3, 7 and 9 (Fig. 7C). Addition of deguelin to these drugs significantly enhanced caspase activity of the cancer cells relative to what was elicited by either doxorubicin or docetaxel alone ($P < 0.01$), even though deguelin alone did not significantly increase the levels of caspase activity. However, it is highly possible that the drug combination provides two contributing effects that are able to act in a synergistic manner: induction of caspase activity by the chemotherapy drugs and release of the inhibitory effects of survivin and XIAP by deguelin that subsequently enhances apoptotic responses specifically in the breast cancer cells. Our *in vitro* findings indicated that deguelin is an effective enhancer of chemosensitivity in drug-treated breast cancer cells.

DISCUSSION

The importance of IAPs in regulating the apoptotic response and as potential molecular targets for cancer therapy has begun to attract a great attention towards the successful identification of peptide antagonists or small molecule inhibitors for those proteins (Altieri, 2003; Fesik, 2005; Schimmer et al., 2004). In this study, we report that a small molecule derived from natural products, deguelin, selectively induces apoptosis in human breast cancer but not in normal mammary epithelial and fibroblast cells. We found that relatively low concentrations of deguelin (10 nM or above) significantly inhibited the levels of both the survivin and XIAP proteins as well as their gene expression in breast cancer cells. Previous studies have shown that deguelin induces apoptosis in human premalignant and malignant bronchial epithelial (HBE) and non-small cell lung cancer cell lines by inhibition of AKT (Chun et al., 2003; Lee, 2004). A recent study further demonstrates that deguelin suppressed AKT activation *in vivo* and that it was able to reduce tobacco-induced lung tumorigenesis (Lee et al., 2005). However, the downstream effectors of AKT that are implicated in the induction of apoptosis induction by deguelin have not been defined. Therefore, we chose to conduct the present study to investigate the mechanism of action of deguelin in human breast cancer cells as well as assess its potential to combine with chemotherapeutic drugs that are presently in use.

It has been reported that induction of expression of Bax, p53 and p21, or inhibition of mitochondrial bioenergetics may be associated with deguelin-induced apoptosis (Hail and Lotan, 2004; Murillo et al., 2002). In this study, we examined the mechanisms by which deguelin induces apoptosis in human breast cancer cells. Our results indicate that downregulation of both survivin and XIAP by deguelin contributes to the induction of apoptosis as well as the selectivity in induction of cell death in the breast cancer cells. Although treatment of the breast cancer cells

with 10 nM deguelin markedly reduced p-AKT and survivin, only 15 to 20% of growth inhibition were found in the tumor cells. A significant higher level of cell growth inhibition was detected when the cells were treated with 100 nM deguelin, which reduced the levels of survivin and XIAP. Since this drug concentration also reduced the level of total AKT, other changes in cell signaling pathways may enhance the apoptotic response.

Unlike previously observed in tumor cells after exposure to many chemotherapy drugs, treatment with deguelin did not markedly increase their levels of caspase activity. The presence of cleaved PARP in deguelin-treated tumor cells suggests that active caspases do play some roles in the apoptotic response. Significant downregulation of both survivin and XIAP levels after deguelin treatment may release them from inhibitory hold on the high basal levels of already activated caspases present in human tumor cells (Yang et al., 2003), allowing sufficient induction of a caspase-dependent downstream apoptotic response without further increases in caspase activity. In addition, it has been shown that survivin serves as a mitotic regulator involved in regulating mitosis and cytokinesis (Li et al., 1998). Severe inhibition of survivin by deguelin may prevent the progression of the cell cycle through the M phase, resulting in apoptosis. Consistent with this notion, we have observed the deguelin treatment blocked the cell cycle in the G2/M phase (data not shown). A strong apoptotic response relative to moderate levels of cleaved caspase 3 and PARP may also be possible result from inhibiting activities of AKT and MAPK on other cellular pathways and factors (Fang and Richardson, 2005; Fry, 2001; Hutchinson et al., 2001).

The mechanism of action for deguelin-induced apoptosis may also explain its selectivity in inducing apoptosis. We have shown that cancer cells displayed higher basal levels of caspase 3 and 9 activity than was detectable in normal MCF 10A cells. Those tumor cells also express

high levels of survivin and XIAP, which counteract the high basal caspase activity and prevent apoptosis of the cells. A marked downregulation of survivin and XIAP in tumor cells would change the balance between the levels of active caspases and IAP proteins, allowing the active caspases to execute apoptosis of the cancer cells (Yang et al., 2003). Since the levels of both basal caspase activity and IAPs are very low in normal cells, deguelin treatment alone is unlikely to provide a strong enough signal to induce apoptotic cell death in those cells. Therefore, the observed property of cancer-cell-selective apoptosis of deguelin may be in part due to its inhibition of the higher levels of survivin and XIAP in cancer cells that already have a high level of pre-existing activated caspases.

We and others have observed that human cancer cells further upregulate survivin expression after chemotherapy drug treatment (Ikeguchi et al., 2002; Ling et al., 2004; Peng et al., 2005). These increases may prevent the downstream apoptotic response and decrease the cells' sensitivity to these agents to varying degrees. Therefore, it is likely that much higher levels of caspase activity are needed in cancer cells treated by chemotherapy drugs to achieve a similar level of apoptotic cell death compared to deguelin treatment. Since deguelin is able to inhibit both survivin and XIAP, we examined whether a combination of deguelin with chemotherapy drugs could serve to enhance the apoptotic response in these cancer cells. Indeed, we found that the combination of low concentrations of deguelin (1 or 10 nM) with either docetaxel or doxorubicin increased apoptotic cell death in both SK-BR-3 and MCF-7 breast cancer cell lines. As could be expected, deguelin completely blocked the docetaxel- and doxorubicin-induced upregulation of survivin. We believe that deguelin's inhibition of survivin and XIAP levels may have led to higher levels of caspase 3 and 9 activity observed in the cancer cells following the

combination treatment, thus significantly enhancing the effects of the chemotherapy drug component.

In this study, we have demonstrated that deguelin induces apoptotic cell death in human breast cancer cells predominantly by downregulating cell survival signal pathways, including AKT, MAPK and the IAP family of proteins. Our results also showed that deguelin alone or in combination with other drugs have great potential in the development of new therapeutic approaches for breast cancer. Importantly, deguelin appears to have advantages as a therapeutic agent for breast cancer due to its ability to selectively induce the cell death of breast cancer cells and to be administered orally at relatively high dosages (Lee et al., 2005; Udeani et al., 2001). Based on our *in vitro* mechanistic and synergistic results, deguelin appears to represent a promising and novel addition to the arsenal of agents capable of combating breast cancer.

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Foot note

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FIGURE LEGENDS

Figure 1. Deguelin inhibits the levels of expression of both survivin and XIAP genes in human breast cancer cells.

A. Western blot analysis showed that treating cells with 1 to 100 nM deguelin for 2 days markedly inhibited AKT activity and the level of total AKT protein was reduced by deguelin at concentrations of 100 nM or more. Higher concentrations of deguelin also decreased MAPK activity. The levels of two IAP proteins, survivin and XIAP, were significantly reduced by deguelin. **B.** Deguelin treatment inhibited the levels of survivin and XIAP gene expression, as detected by Real-time RT PCR. Numbers represent the relative levels of gene expression, calculated as the ratio of the quantity of either survivin or XIAP and the GAPDH PCR products. Similar results were obtained in two repeat studies. **C.** Dual luciferase activity assay showed that 1 to 10 nM deguelin significantly inhibited survivin promoter activity. The relative luciferase activity was the mean value of three repeat samples calculated as a ratio of firefly and renilla luciferase activity.

Figure 2. Deguelin inhibits cell proliferation by inducing apoptosis in breast cancer cells.

A. Comparison of the effects of deguelin on human breast cancer and normal cell lines following deguelin treatment for 3 days. Results are expressed relative to the cell density of untreated cells. Independent experiments were repeated three times. Each value indicates the mean \pm SD of four to eight samples. *, $p < 0.05$; **, $P < 0.01$ (Student's t-test).

B. Colony formation assay. Cells were treated with or without 100 nM deguelin for 14 days. Cell colonies were examined after crystal violet staining. Bars represent the mean number of colonies of triplicate wells from two independent experiments; ***, $P < 0.001$ (Student's t-test).

C. TUNEL assay. The presence of apoptotic cells were examined by TUNEL assay 2 days after deguelin treatment. Cells showing yellow-green nuclei are apoptotic cells with DNA fragmentation. Propidium iodide (red) was used as a counterstain for cell nuclei. The percentage of apoptosis is calculated as a ratio of the number of TUNEL-positive cells and the total number of cells in each field. Similar results were obtained from repeat experiments. **, $P < 0.01$, ***, $P < 0.001$ (Student's t-test).

Figure 3. Overexpression of survivin or XIAP gene counteracts deguelin-induced growth inhibition and apoptosis in cancer cells.

A. MTT Assay. MCF-7 cells were transfected with pcDNA3 plasmids containing either survivin or XIAP gene, and then treated with 100 nM deguelin for 3 days. The transfection efficiency for MCF-7 cell line is usually around 30% detected by transfecting a plasmid expressing a GFP gene. After the treatment, the remaining viable cells were quantitated by MTT assay. Overexpression of survivin or XIAP gene significantly reduced deguelin-induced cell death. A stronger inhibitory effect was observed in the XIAP gene-transfected groups (survivin-transfected group: $P < 0.05$, XIAP-transfected groups: $P < 0.01$, Student's t-test).

B. Colony formation assay. MCF-7 cells were transfected with pcDNA3 control, survivin or XIAP expressing plasmids. Transfected cells were treated with 100 nM deguelin or 0.1% dimethyl sulfoxide (control) for 14 days. Bars represent the mean number of cell colonies of triplicate wells from two independent experiments; *, $p < 0.05$ (Student's t-test).

C. TUNEL assay. The percentage of apoptosis was examined 2 days after deguelin treatment. Overexpression of survivin or XIAP gene significantly blocked deguelin-induced apoptotic cell death. **, $p < 0.01$ (Student's t-test).

Figure 4. Examination of caspase activity in breast cancer cells following deguelin or chemotherapy drug treatment.

A. Examination of the basal level of caspase activity showed that normal MCF 10A cells have much lower levels of caspase 3 and caspase 9 activities, as compared to the breast cancer cell lines. MCF-7 cells lack caspase 3 gene expression but a moderate level of caspase 7 activity was detectable using the same substrate for caspase 3-like activity (Ac-DEVD-AFC). A high level of basal caspase 9 activity was also found in MCF-7 cells. **B.** Activities of caspase 3 (in SK-BR-3 cells) and caspase 7 (in MCF-7 cells) were slightly elevated in deguelin-treated cells, with the greatest level of activity detected with 1000 nM deguelin treatment. **C.** Comparison of caspase 3 activity in SK-BR-3 and MCF-7 cancer cells after treatment with deguelin or chemotherapy drugs. Cells were treated with deguelin (100 nM), docetaxel (SK-BR-3, 25 nM; MCF-7, 50 nM) or doxorubicin (100 nM for both cell lines), using dosages that kill 50% of the cancer cells (LD_{50}). As shown, caspase 3 activity was not increased after deguelin treatment at the LD_{50} dosage while treating the cells with LD_{50} of docetaxel or doxorubicin induced about four to seven-fold increases in caspase activity. The numbers in the figure represent the mean values of three repeat samples.

Figure 5. Examination of the apoptotic signals involved in deguelin-induced apoptosis.

A. A basal level of cleaved caspase 3 fragments (p17 and p19) was detected in SK-BR-3 cells by Western blot analysis. Treatment with 1 to 100 nM deguelin slightly increased the level of active caspase 3 fragments. A significant increase in cleaved PARP (p85) was detected in 100 nM deguelin-treated cells. SK-BR-3 cells treated with 100 nM doxorubicin showed a very high level of active caspase 3 fragments, and the level of cleaved PARP (p85) was higher than in deguelin treated cells. **B.** Unlike breast cancer cells, normal MCF-10A cells and HDF cells had no

detectable AKT activity and level of survivin expression. Basal level and deguelin-induced active caspase 3 and PARP fragments was not found in these normal cells.

Figure 6. Examination of the combined effects of deguelin and chemotherapy drugs on expression levels of survivin and XIAP.

Cells were treated with doxorubicin (100 nM) or docetaxel (SK-BR-3, 25 nM; MCF-7, 50 nM) alone or in combination with various concentrations of deguelin for 2 days. **A.** Western blot analysis: Doxorubicin or docetaxel treatment markedly increased the level of survivin in the cancer cells, while XIAP level remained unaffected. Deguelin treatment blocked the drug-induced upregulation of survivin protein and increased the amount of cleaved PARP fragments (p85). **B.** Real-time RT-PCR analysis showed that docetaxel treatment of SK-BR-3 cells increased the level of survivin mRNA that could be inhibited if the cells were co-treated with as low as 1 nM of deguelin. The relative level of survivin mRNA is calculated as the average ratio between the quantity of survivin and GAPDH PCR products in three repeat samples.

Figure 7. Combination effects of deguelin with chemotherapy drugs on normal and breast cancer cell lines.

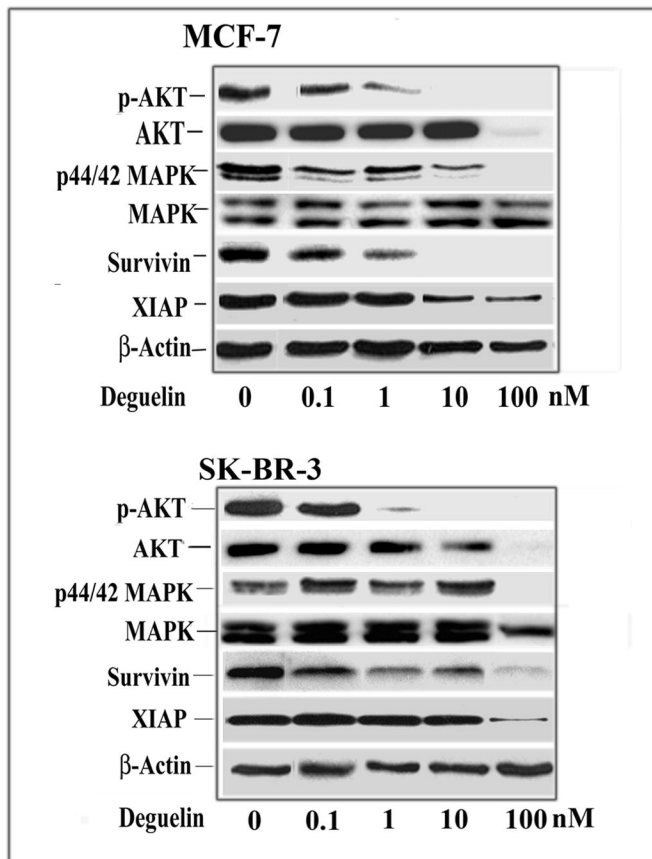
A. Colony formation assay. Cells were treated with 100 nM doxorubicin (SK-BR-3, HDF and MCF-10A) or 25 nM docetaxel (MCF-7, HDF and MCF-10A), in the absence or presence of 1 or 10 nM deguelin for 10 to 14 days. Cell colonies were detected by crystal violet staining. Bars represent the mean number of colonies of triplicate wells from two independent experiments; **, $P < 0.01$ (Student's t-test).

B. TUNEL assay. Combined treatment of 100 nM deguelin with 100 nM doxorubicin for 2 days significantly increased the percentage of TUNEL positive cells in SK-BR-3 cell line. Results showing are the mean values of three repeat samples. **, $P < 0.01$ (Student's t-test).

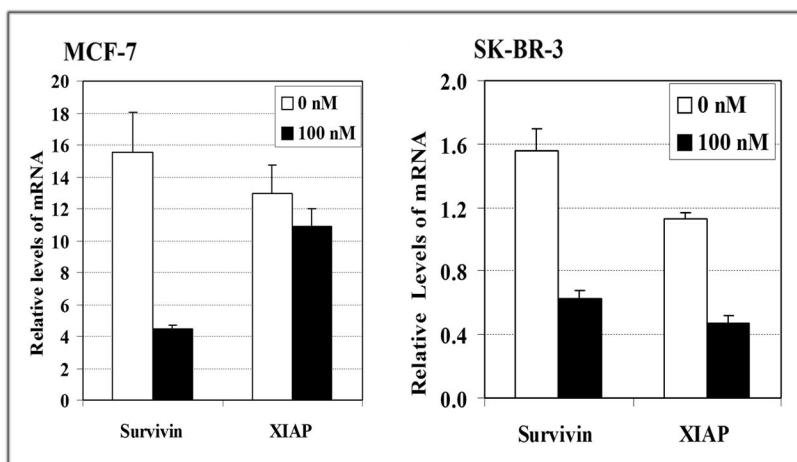
C. Caspase activity assay. The combination of deguelin with either docetaxel or doxorubicin significantly increased the level of caspase activity ($P < 0.01$, student's t-test). The numbers in the figure are the mean of three repeat sample values.

Figure 1. Deguelin inhibits the levels of expression of both survivin and XIAP genes in human breast cancer cells.

A. Western Blot Analysis



B. Real time RT-PCR



C. Dual luciferase assay of survivin promoter activity

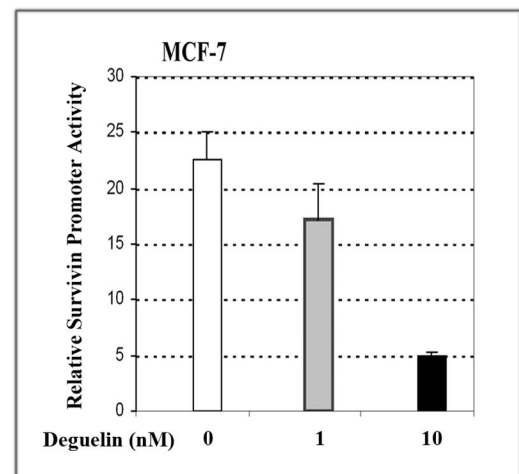
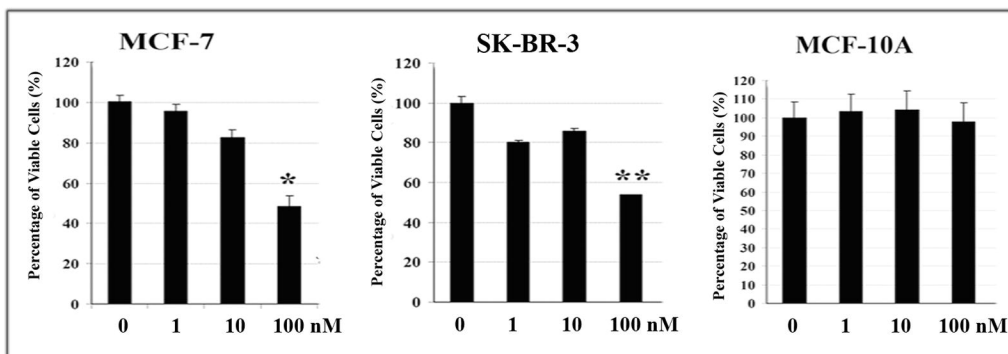
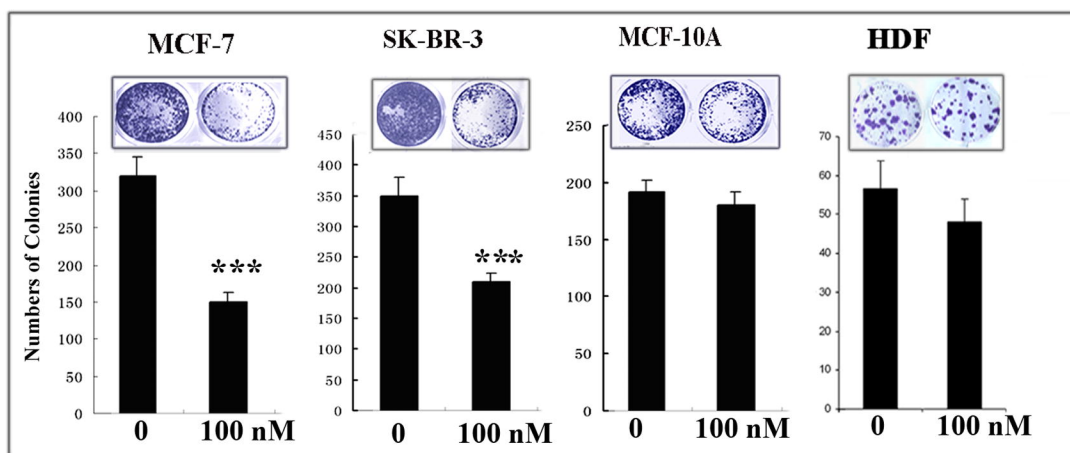


Figure 2. Deguelin inhibits growth of breast cancer cells by inducing apoptosis

A. MTT Cell Proliferation Assay



B. Colony Formation Assay



C. TUNEL Assay

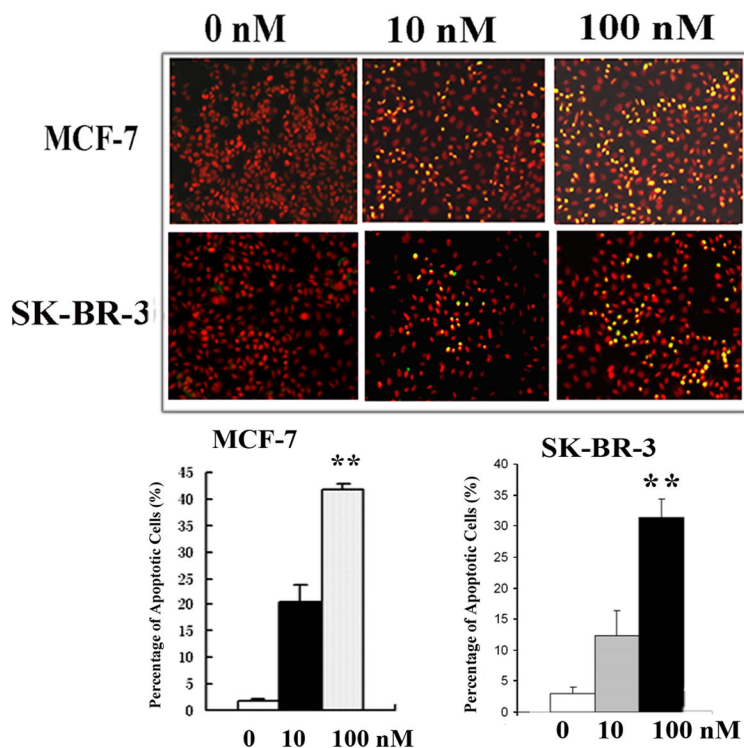
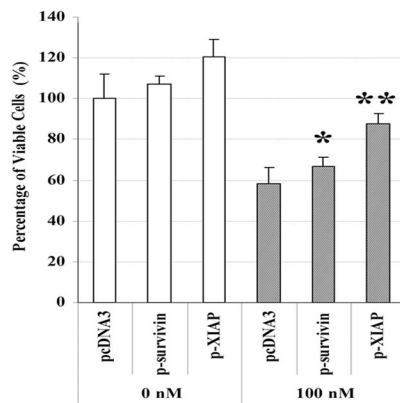
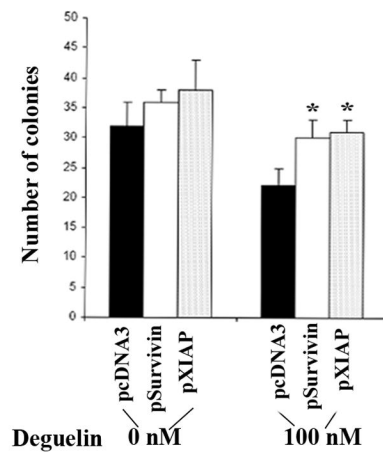


Figure 3. Overexpression of survivin or XIAP gene counteracts deguelin-induced growth inhibition and apoptosis in cancer cells.

A. MTT Assay



B. Colony Formation Assay



C. TUNEL Assay

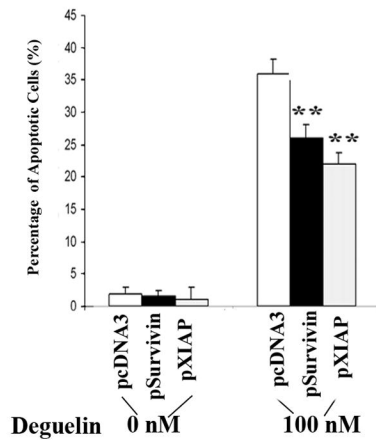


Figure 4. Examination of caspase activity in breast cancer cells following deguelin or chemotherapy drug treatment.

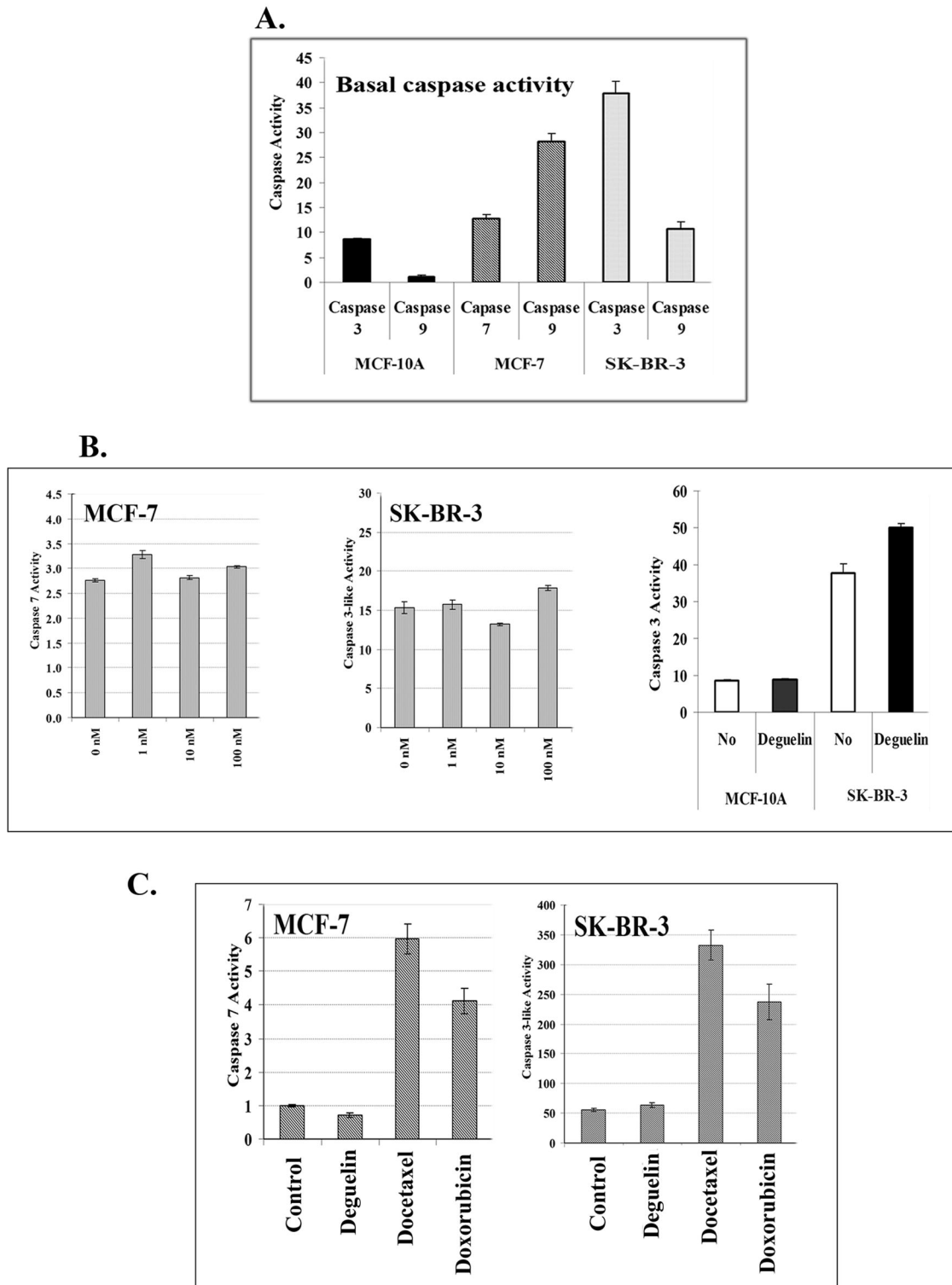
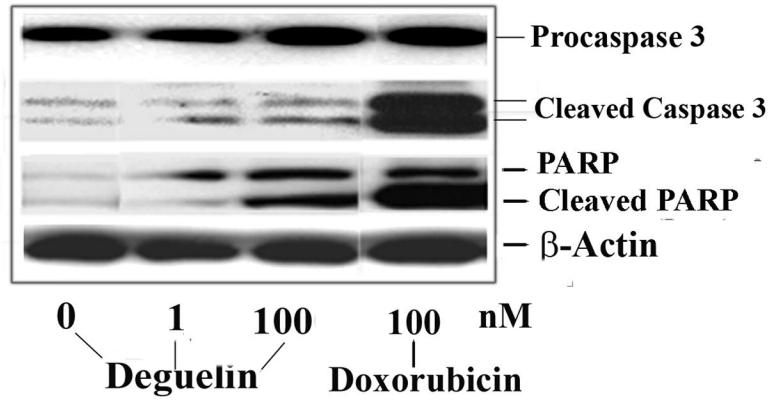


Figure 5 Examination of apoptotic signals in deguelin-induced apoptosis

A.

SK-BR-3



B.

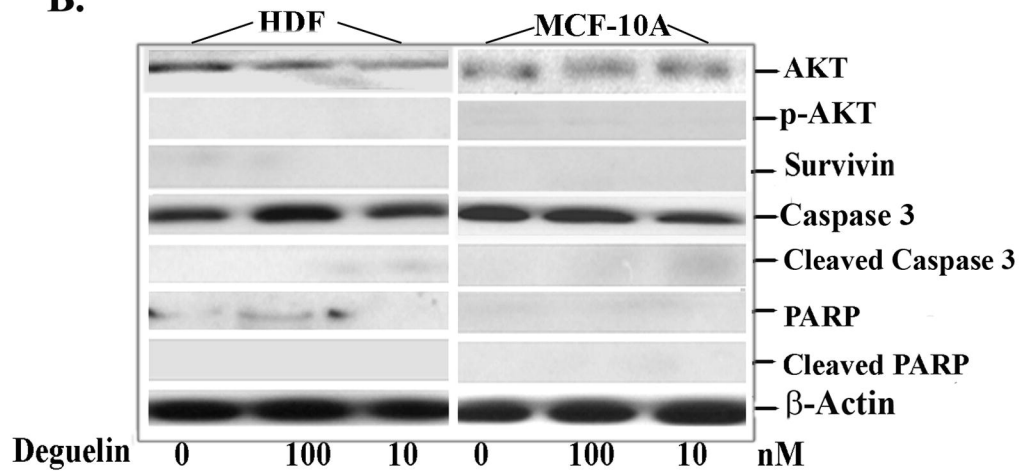
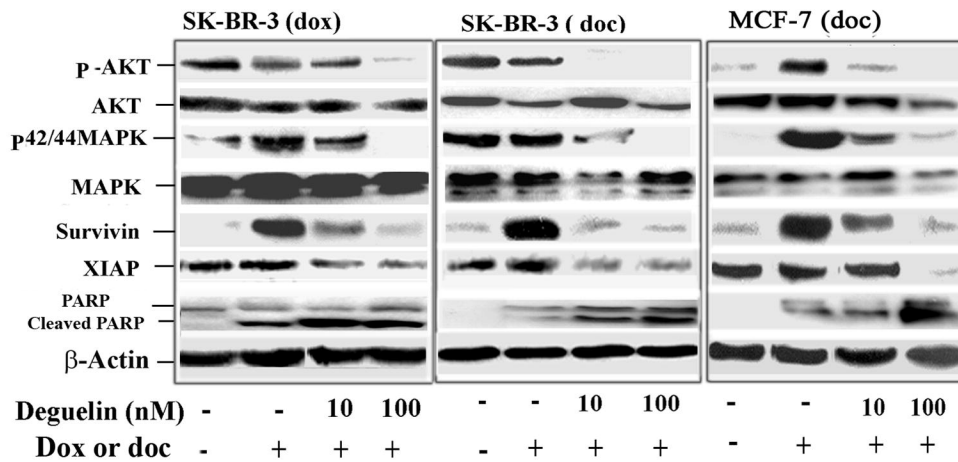


Figure 6. Examination of the combined effects of deguelin and chemotherapy drugs on expression levels of survivin and XIAP

A. Western Blot analysis



B. Real time RT-PCR

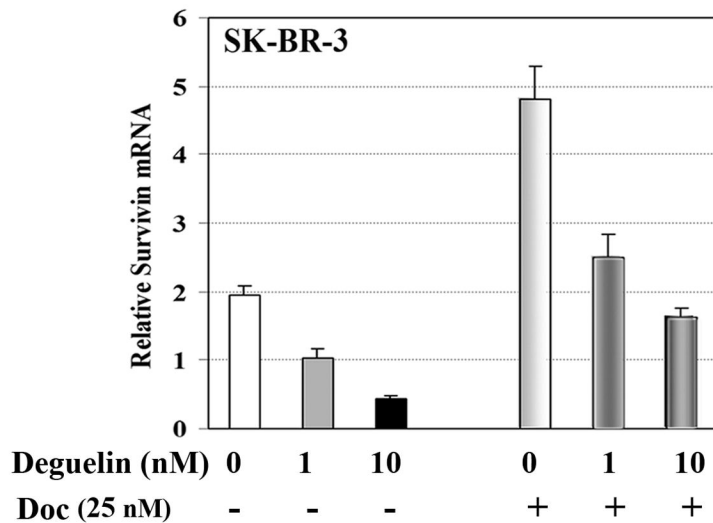
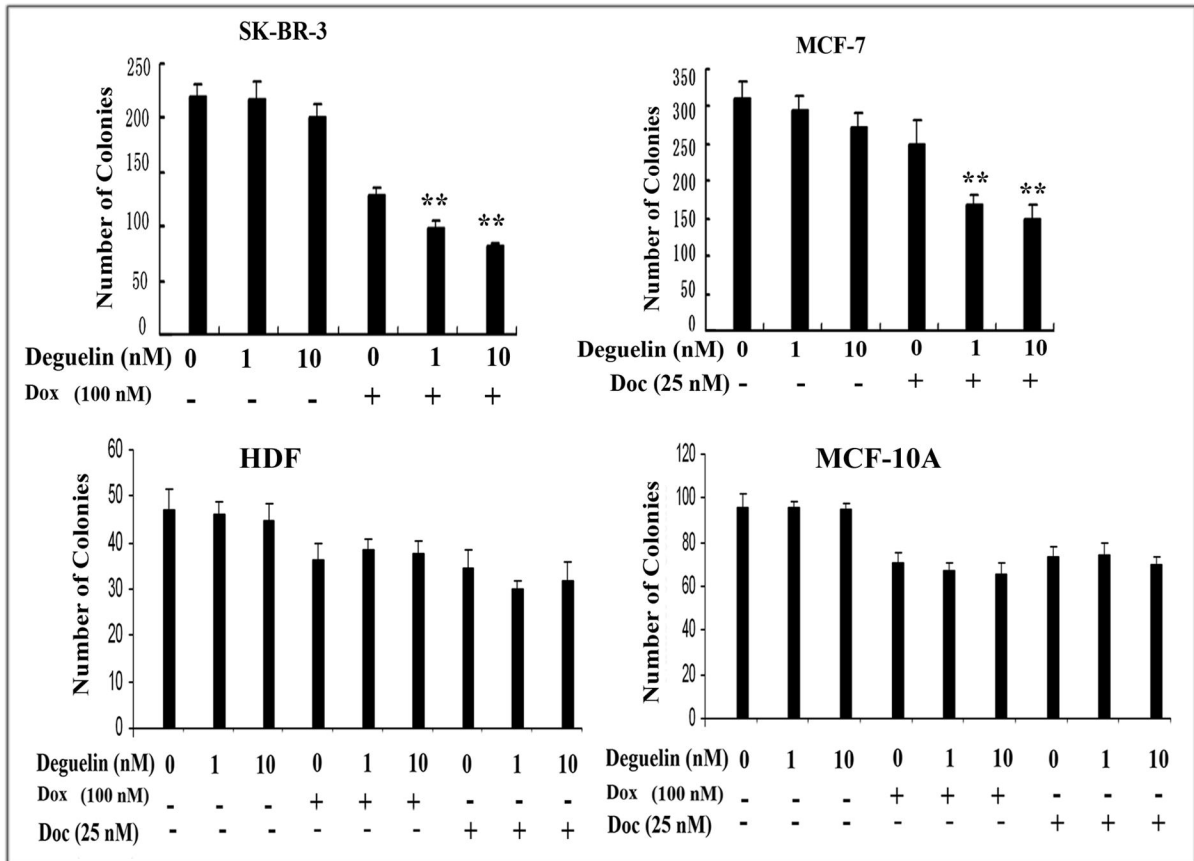
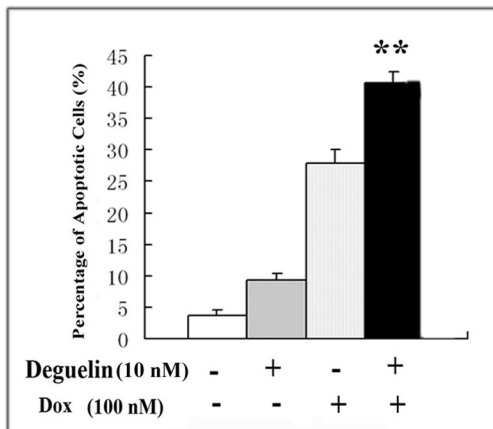


Figure 7. Combination effects of Deguelin with chemotherapy drugs on normal and breast cancer cell lines

A. Colony Formation Assay



B. TUNEL Assay



C. Caspase Activity Assay

