Down-Regulation of Inhibitor of Apoptosis Proteins by Deguelin Selectively Induces Apoptosis in Breast Cancer Cells

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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 are absent or have very low levels of expression in normal adult tissues. We found that inhibiting AKT activity with 10 to 100 nM 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. It is noteworthy that 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 down-regulation 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. Because normal cells have very low levels of p-AKT, XIAP, survivin, and pre-existing caspase activity, deguelin had little effect on those cells. In addition, 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 chemo sensitization and could represent a new therapeutic agent for treatment of breast cancer.

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- 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 either by activation of molecules upstream of apoptosis signaling or by inhibition of antiapoptotic 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 antiapoptotic 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 IAP (Igney and Krammer, 2002; 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 antiapoptotic 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 IAP (Igney and Krammer, 2002; 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 antiapoptotic 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 IAP (Igney and Krammer, 2002; 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 antiapoptotic 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 IAP (Igney and Krammer, 2002; 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 antiapoptotic factors, such as the inhibitor of apoptosis (IAP) proteins may confer this insensitivity to apoptosis induction by caspase 3 activation in tumor cells.

ABBREVIATIONS:
IAP, inhibitor of apoptosis; BIR, baculoviral IAP repeat; XIAP, X-linked mammalian inhibitor of apoptosis protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; MAPK, mitogen-activated protein kinase; PARP, poly(ADP-ribose) polymerase; SV-40, simian virus-40; Ac, N-acetyl; AFC, amino-4-trifluoromethyl coumarin; PCR, polymerase chain reaction; RT, reverse transcription/transcriptase.
cetaxel, alone or in combination. Control cultures received 0.1% without various concentrations of deguelin, doxorubicin, and dox- were plated in 96-well plates and then cultured in medium with or deguelin on cell growth using a 3-(4,5-dimethylthiazol-2-yl)-2,5- MCF-10A cell lines were used to determine the inhibitory effect of human breast cancer and normal cells. results show that this differential apoptotic response was a epithelial cells and primary fibroblasts. Furthermore, our breast cancer cells but had little effect on normal mammary XIAP and induced apoptotic cell death selectively in human significantly down-regulated the levels of both survivin and treatment. Aventis (Bridgewater, NJ) and Sigma Chemical Co. (St. Louis, MO), therapy drugs docetaxel and doxorubicin were obtained from Sanofi tography-UV) was synthesized from commercially available rote- fixed Eagle's medium with 20% fetal bovine serum. Disease Center (Atlanta, GA), was maintained in Dulbecco's modi- ery slides (5 µm) 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), using the SpectraMax Plus spectrophotometer ( Molecular Devices, Sunnyvale, CA).

Colony Formation Assay. The cells were plated in six-well plates at a density of 1500 cells/well 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 Assay. Cells were plated in eight-well cham- ber slides (5 x 10^3 cells per well) for 24 h and then treated with different concentrations of deguelin. Forty-eight hours later, floating and adherent cells were collected and placed onto polylysine-coated glass slides using Shandon Cytospin 4 (Thermo Electron Corp., Waltham, MA). The presence of DNA fragmentation in the cells was examined using the DeadEnd Fluorometric TUNEL Sys- tem (Promega Corp., Madison, WI), according to the manufacturer’s instructions. In brief, 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 termin- al deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) reaction mixture for 1 h 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 concen- trations of deguelin alone or in combination with doxorubicin or docetaxel for 2 days, then collected for Western blot analysis using a protocol described previously (Yang et al., 2003). Primary antibodies were used to detect the levels of specific proteins: survivin (Santa Cruz Biotechnology, Santa Cruz, CA), XIAP, phosphor-serine 473 AKT, total AKT, 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., Danvers, MA), and 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'-TCCAC- TGCCCACTGTAGAAC-3'; reverse, 5'-TGCCCTCAAGCTTCCAC-3'. PCR primers for XIAP were: forward, 5'-CCGTCGGTGCTTTAGGT- TGTT-3'; reverse, 5'-TCTCTCCGGAATATAGATGCTTGA-3'. GAPDH-specific primers were: forward, 5'-TTGGATATCGT- GGAAGGAC- TCA-3'; reverse, 5'-TGTCATCATATGTTGGAGATT T-3'.

Luciferase Reporter Assay. Cells were plated in 24-well tissue culture plates at 70% confluence for 24 h and then cotransfected with 1.0 µg of a Survivin promoter-luciferase reporter plasmid (pLuc- cycl1.2; provided by Dr. Fengzhi Li, Roswell Park Cancer Institute, Buffalo, NY) and 20 ng of pRL-simian virus-40 internal control plasmid that expresses a Renilla reniformis luciferase gene (Promega) using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). The transfected cells were then treated with deguelin for 24 h. The cell lysates were collected for measuring luciferase activity using Dual Luciferase Assay System (Promega) and LUMIstar Galaxy (BMG Labtech, Winooski, VT). The relative luciferase activity for each sample was calculated as a ratio of firefly and R. reniformis lucif- erase activity. The level of R. reniformis luciferase activity, which was expressed from the cotransfected 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.

Materials and Methods

Cell Lines and Materials. Human breast cancer cell lines SK- BR-3 and MCF-7 and normal immortalized human mammary epithelial cell line MCF 10A were obtained from the American Type Culture Collection (Manassas, VA) and cultured in medium accord- ing to their recommendations. The primary normal human dermal fibroblast cell line HDF, purchased from Emory University Skin Disease Center (Atlanta, GA), was maintained in Dulbecco’s modified Eagle’s medium with 20% fetal bovine serum.

The deguelin (97%-98% purity; high-performance liquid chroma- tography-UV) was synthesized from commercially available rote- none, as described previously (Gerhauer et al., 1997). The chemo- therapy drugs docetaxel and doxorubicin were obtained from Sanofi Aventis (Bridgewater, NJ) and Sigma Chemical Co. (St. Louis, MO), respectively.

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. Cells (8 x 10^3/well) were plated in 96-well plates and then cultured in medium with or without various concentrations of deguelin, doxorubicin, and do- cetaxel, alone or in combination. Control cultures received 0.1% dimethyl sulfoxide, the solvent for the deguelin stock solution.

In this study, we demonstrate that deguelin, a natural product isolated from plants in the Mundulea sericea family, significantly down-regulated the levels of both survivin and XIAP and induced apoptotic cell death selectively in human breast cancer cells but had 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.
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). Control groups with specific caspase inhibitors, including caspase 3 inhibitor (N-benzyloxycarbonyl-DEVD-aldehyde; BD PharMingen) and caspase 9 inhibitor (N-benzyloxycarbonyl-LEHD-aldehyde; Alexis Biochemicals, San Diego, CA), were done to ensure specificity.

Transfection. Cells were cultured in 96-well plates for 24 h and then transfected with 0.2 μg of a plasmid containing the survivin (pcDNA.3-survivin, provided by Dr. D. C. Altieri, University of Massachusetts Medical School, Worcester, MA), or XIAP gene (pcDNA.3-XIAP 6x-myc, obtained from Dr. Robert Korneluk, Children's Hospital of Eastern Ontario, Ottawa, ON, Canada), or a control empty plasmid (pcDNA3) with LipofectAMINE 2000 (Invitrogen). Twenty-four hours 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 Inhibited the Phosphatidylinositol 3-Kinase/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 (Murillo et al., 2002; Chun et al., 2003; Lee, 2004). Increasing evidence indicates that the phosphatidylinositol 3-kinase/AKT pathway regulates the cell survival pathway and the IAP family of proteins (Dan et al., 2004; Asanuma et al., 2005; Belyanskaya et al., 2005). 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 deguelin markedly inhibited the levels of p-AKT, whereas the levels of total AKT protein were not affected significantly in MCF-7 cells (Fig. 1A). However, treatment with 100 nM deguelin sig-
significantly reduced the levels of both p-AKT and total AKT proteins, suggesting that 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 deguelin slightly reduced the level of total AKT protein (Fig. 1A). In addition, the MAPK activity was also down-regulated in breast cancer cells after treatment with 0.1 nM (MCF-7) or 100 nM (SK-BR-3) deguelin (Fig. 1A).

We further examined the effects of down-regulation of AKT and MAPK activities on the levels of survivin and XIAP using Western blot analysis. Treatment of MCF-7 cells with deguelin concentrations of ≥10 nM 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 more than 10 nM (MCF-7) or 100 nM (SK-BR-3) 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 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 because treatment of MCF-7 cells with 1 to 10 nM deguelin markedly reduced the survivin promoter activity (Fig. 1C).

**Down-Regulation of Survivin and XIAP by Deguelin**

**Induced Apoptosis and Lowered Cell Growth in Breast Cancer Cells 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 approximately 15 to 20% growth inhibition could be achieved using a concentration of deguelin as low as 10 nM in MCF-7 and SK-BR-3 cell lines detected by MTT assay (Fig. 2A). Growth inhibition of 50% 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 little effect on cell survival (Fig. 2A). Likewise, results of colony formation assay also demonstrated that normal MCF-10A and HDF cells were much more resistant to deguelin treatment compared with breast cancer cell lines (Fig. 2B).

To determine whether 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. 2C). Treatment with 100 nM deguelin 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 down-regulation 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. 3C). Therefore, our results strongly support the notion that the down-regulation of IAP proteins was the critical factor for achieving deguelin-induced apoptosis in breast cancer cells.

**Deguelin Induced Apoptosis in Breast Cancer Cells without Significant Caspase Activation.** In a previous study (Yang et al., 2003), we showed that the upstream apoptotic signals are already activated in many cancer cells because of the presence of abnormalities. Because the treatment of human breast cancer cells with deguelin concentrations at ≥10 nM 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.

Because caspase activation is an important step toward 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 with the normal MCF-10A cells, the SK-BR-3 breast cancer cells displayed higher levels of caspase 3-like and caspase 9 activity (Fig. 4A). 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. 4A). When we compared caspase 3-like activity in normal and cancer cells after deguelin treatment, we found that although a relatively high concentration of deguelin (1000 nM) slightly increased the level of caspase 3 activity in SK-BR-3 cells, the activity in MCF 10A cells remained low (Fig. 4B). In cells that were treated with 100 nM deguelin for 2 days, approximately 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, compared with deguelin treatment alone (Fig. 4C).

**Down-Regulation of IAPs Released 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 a 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 was sufficient to cleave cellular protein substrates, leading to an increase in cleaved PARP.

Deguelin Inhibits IAP Proteins Inducing Cancer Cell-Apoptosis

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 after 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 ± S.D. 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 was 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).
apoptotic phenotype (Fig. 5A). Although both 100 nM deguelin and 100 nM doxorubicin induced 50% the apoptotic cell death in SK-BR-3 cells, only doxorubicin induced very high levels of active caspase 3 and cleaved PARP. However, our study seems to indicate that deguelin-induced apoptosis was 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. In addition, activated caspase 3 fragments were not detected in normal cells (Fig. 5B). 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 Up-Regulation 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 up-regulation of survivin expression (Ikeguchi et al., 2002; Ling et al., 2004; Peng et al., 2005, Ling, 2004). 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 is possible that the combination of down-regulating survivin and XIAP levels with deguelin, along with the ability of the chemotherapy drug to activate caspases, might pro-

**Fig. 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 or 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; **, P < 0.01 (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).
duce a stronger apoptotic signal, sensitizing tumor cells to chemotherapy.

First, we examined whether deguelin could prevent drug-induced up-regulation 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, whereas 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 up-regulation 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 up-regulation 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 approximately 2.5-

Fig. 4. Examination of caspase activity in breast cancer cells after 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 compared with 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, treatment with 1 to 100 nM deguelin did not induce significant increases in caspase 3 (in SK-BR-3 cells) or caspase 7 (in MCF-7 cells) activity. Deguelin treatment did not activate caspase activity in those cells. 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 (LD50). As shown, caspase 3 activity was not increased after deguelin treatment at the LD50 dosage, whereas treating the cells with LD50 of docetaxel or doxorubicin induced approximately 4- to 7-fold increases in caspase activity. The numbers in the figure represent the mean values of three repeat samples.
fold increases in the level of survivin mRNA in SK-BR-3 cells compared with control cells. Consistent with Western blot results for levels of survivin protein, the combination of docetaxel with as little as 1 nM deguelin markedly prevented docetaxel-induced up-regulation of survivin gene expression (Fig. 6B).

Next, we examined the effect of blocking drug-induced survivin up-regulation with deguelin on both the apoptotic response and chemosensitivity of human breast cancer cells. We chose to use 1 and 10 nM 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. 7A). Fourteen days after treating SK-BR-3 breast cancer cells with doxorubicin in the presence of 1 or 10 nM deguelin, we observed a 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. 7A). In contrast, results from the colony formation assay obtained from normal HDF and MCF-10A cell lines after 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 the percentage of apoptosis cells by 12% compared with that detected in the SK-BR-3 cells treated with doxorubicin alone (Fig. 7B).

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 activate caspases 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 doxetaxel 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 provided two contributing effects that were 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 enhanced apoptotic responses, specifically in breast cancer cells. Our in vitro findings indicated that deguelin was 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 toward the successful identification of peptide antagonists or small molecule inhibitors for those proteins (Altieri, 2003; Schimmer et al., 2004; Fesik, 2005). In this study, we report that a small molecule derived from natural products, deguelin, selectively induced apoptosis in human breast cancer but not in normal mammary epithelial and fibroblast cells. We found that relatively low concentrations of deguelin ($\geq$10 nM) 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 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 to 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 (Murillo et al., 2002; Hail and Lotan, 2004). In this study, we examined the mechanisms by which deguelin induces apoptosis in human breast cancer cells. Our results indicate that down-regulation 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 was found in the tumor cells. A significantly 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. Because 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 down-regulation 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 (Fry, 2001; Hutchinson et al., 2001; Fang and Richardson, 2005).

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 down-regulation 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). Because the levels of both basal caspase activity and IAPs are very low in normal cells, deguelin treatment alone is unlikely to provide a sufficiently strong signal to induce apoptotic cell death in those cells. Therefore, the observed property of cancer-cell-selective apoptosis of deguelin may be due in part 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.

A. Western Blot analysis

B. Real time RT-PCR

Fig. 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, whereas XIAP level remained unaffected. Deguelin treatment blocked the drug-induced up-regulation 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 cotreated with as little as 1 nM deguelin. The relative level of survivin mRNA was calculated as the average ratio between the quantity of survivin and GAPDH PCR products in three repeat samples.
We and others have observed that human cancer cells further up-regulate 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 (to varying degrees) the sensitivity of the cells to these agents. 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 with deguelin treatment. Because 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 up-regulation of survivin. We believe that deguelin inhibition of survivin and XIAP levels may have led to higher levels of caspase 3 and 9 activity observed in the cancer cells after 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 down-regulating 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. It is noteworthy that deguelin seems to have advantages as a therapeutic agent for breast cancer because of its abilities to selectively induce the cell death of breast cancer cells and to be administered orally at relatively high dosages (Udeani et al., 2001;

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**A. Colony Formation Assay**

![Colony Formation Assay](image)

**B. TUNEL Assay**

![TUNEL Assay](image)

**C. Caspase Activity Assay**

![Caspase Activity Assay](image)

**Fig. 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 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.
Lee et al., 2005). Based on our in vitro mechanistic and synergistic results, deguelin seems to represent a promising and novel addition to the arsenal of agents capable of combating breast cancer.

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References


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