Deguelin Inhibits Growth of Breast Cancer Cells by Modulating the Expression of Key Members of the Wnt Signaling Pathway

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Abstract An emphasis in early detection and more effective treatments has decreased the mortality rate of breast cancer. Despite this decrease, breast cancer continues to be the leading cause of death among women between 40 and 55 years of age and is the second overall cause of death among women. Hence, the aim of the present study was to assess the therapeutic efficacy of deguelin, a rotenoid isolated from several plant species, which has been reported to have chemopreventive and/or chemotherapeutic effects in skin, mammary, colon, and lung cancers. The effect of deguelin on cell proliferation was evaluated using four human breast carcinoma cell lines (MCF-7, BT474, T47D, and MDA-MB-231) by cell count and MTT. Moreover, apoptosis was evaluated by acridine/ethidium staining and DNA laddering. Gene expression changes following deguelin treatment in MDA-MB-231 cells was assessed through microarray analysis. Deguelin at 1 μmol/L was found to inhibit the growth of the breast cancer cell lines tested with a range of 37% to 87%. The highest inhibition was noted for the MDA-MB-231 cell line (MDA-MB-231>BT474>MCF7>T47D>MCF12F). An arrest at the S phase of the cell cycle and apoptosis were shown in the MDA-MB-231 cells treated with deguelin. The microarray profile indicated differential expression of two independent pathways, including clusters of apoptosis and Wnt/β-catenin signaling genes in cells as a result of deguelin treatment. These studies support the antiproliferative effects of deguelin in human breast cancer cells and, perhaps more importantly, illustrate novel actions by deguelin in the Wnt signaling pathway.

Despite advances in novel therapeutic agents, breast cancer remains the second leading cause of malignancy-related death in women in the United States. Part of the cause may be related to the fact that the most widely used drugs, including tamofixen and aromatase inhibitors, work by blocking the ability of estrogen to trigger abnormal cell growth. Yet, some 65,000 breast cancer cases or about one third are considered as estrogen receptor (ER)–negative tumors; thus, medications that work by blocking estrogens have little effect on the incidence of cancers that are not sensitive to the hormone. As a result, women with ER-negative breast tumors find themselves in an especially grave need for safe and efficacious therapy. The discovery of agents that regulate biological pathways unrelated to hormone receptor status may provide new avenues for the treatment of breast cancer.

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The canonical Wnt signaling pathway is highly conserved in evolution, widely used throughout development, and frequently hyperactive in cancer. This pathway involves numerous proteins that control the production and destruction of Wnt signaling molecules, their interactions with crucial receptors, and the actions of target cells exposed to Wnt ligands (1, 2). In the last decade, research in this pathway has continued to expand and as a result the known components in or associated with the canonical Wnt signaling pathway has grown considerably with >50 proteins currently known to be associated with the pathway (3, 4). A large part of the research has focused on β-catenin, a transcription factor of the canonical Wnt signaling pathway. In the absence of ligand, cytoplasmic β-catenin interacts with APC and Axin and serves as a substrate for the kinases CKI and GSK3β. Phosphorylated β-catenin is then ubiquitinated and destroyed by the proteosome (3). When Wnt ligand binds to a Frizzled family receptor and a coreceptor (LRP-5 or LRP-6), the APC/Axin/CK1/GSK3β destruction complex is inhibited, leading to the stabilization of β-catenin and its translocation to the nucleus where it interacts with T-cell factor/lymphoid enhancer factor (TCF/LEF) family transcription factors. In the absence of signal, TCF/LEF factors bind DNA at Wnt-responsive genes and interact with other factors (e.g., Groucho, histone deacetylase) to repress transcription. β-Catenin binding to TCF/LEF proteins provides a transcription activation domain, which leads to target gene expression and/or activation.

In human breast cancer development, the role of Wnt signaling has been reported to involve elevated levels of nuclear and/or cytoplasmic β-catenin, overexpression or downregulation of specific Wnt proteins, downregulation of WIF-1, and amplification of DVL-1. Using immunohistochemistry, Prasad et al. (5) found elevated levels of nuclear and/or cytoplasmic β-catenin in ~48% of breast carcinomas. This increased expression of β-catenin was shown to correlate with the expression of several of its target genes, including cyclin D1. Moreover, high levels of β-catenin have been reported to be associated with poor patient prognosis (6). Thus, the Wnt/β-catenin pathway is an attractive target for chemoprevention and treatment for breast cancer.

Deguelin, a rotenoid from *Mundulea sericea* within the Leguminosae family, has shown great potential as a chemopreventive and therapeutic agent against several types of cancers, including colon, lung, and breast (7–11). Previously, using human colon cancer cells, we showed that deguelin suppresses the growth of HT-29 colon cancer cells mediated by a G_1-S phase cell cycle arrest and by inducing apoptosis (10). Furthermore, using an azoxymethane-induced colon aberrant crypt foci model, deguelin was shown to be highly effective at suppressing the incidence of colonic aberrant crypt foci in a dose-dependent manner (11). Deguelin is currently being evaluated for its efficacy against lung cancer.

Deguelin has been shown to induce apoptosis in several cancer cell lines, including colon, human premalignant and malignant bronchial epithelial, and non–small cell lung cancer cell lines (10, 12, 13). In lung cancer cells, apoptosis has been shown to be mediated by changes in Akt (12, 13). In human breast cancer cell lines, deguelin induces apoptotic death by downregulating cell survival pathways, including Akt, mitogen-activated protein kinase, and survivin (8). Furthermore, deguelin was shown to enhance chemosensitization of breast cancer cells by inhibiting survivin expression (8). *In vitro*, deguelin has shown ability to increase radiation-induced Akt signaling and enhance radiosensitivity of MDA-MB-231 cells (14). Deguelin has also been reported to disrupt the function of heat shock protein 90 (Hsp90), a molecular chaperone needed by cancer cells for growth and survival (15). More recently, deguelin has been shown to suppress hypoxia-inducible factor-1 (HIF-1α)/Hsp90 interaction and expression in radioresistant lung cancer cells (16). In summary, the actions of deguelin seem to support its use as a potential chemotherapeutic agent for several types of cancers, including breast cancer.

In this study, we evaluated the effects of deguelin in MDA-MB-231 breast cancer cells in part by using microarray gene analyses. These data showed that, in addition to genes involved in cell cycle and apoptosis, several key members of the Wnt/β-catenin signaling pathway were also highly regulated by deguelin. Quantitative real-time PCR was then used to verify the microarray data at the mRNA level. We conclude that the deguelin-induced alternations of gene expression involving Wnt/β-catenin signaling may be exploited for devising chemopreventive and therapeutic strategies for breast cancer.

Materials and Methods

Cell lines and materials

Human breast cancer cell lines, MDA-MB-231, MCF-7, BT474, T47D, and a normal immortalized human mammary epithelial cell line, MCF12F, were obtained from the American Type Culture Collection and cultured in medium according to their recommendations. Deguelin (≥98% purity; high-performance liquid chromatography grade) was purchased from Sigma-Aldrich. Deguelin was dissolved in 100% ethanol for all the *in vitro* studies.

Cell growth inhibition studies

For determination of proliferation, breast cancer cells were seeded at a density of 2×10^4 per well onto 12-well cell culture plates and allowed to adhere for 24 h. After incubation with or without deguelin, cells were detached with trypsin and cell number was determined by Coulter counter. In addition, the MTT assay was conducted to confirm the actions of deguelin in cell proliferation. For this, cells were seeded in a 96-well plate at a density of 1×10^3 per well as indicated by the manufacturer (TACS MTT kit; Trevigen). After 24-h incubation, cells were treated with or without deguelin for various time points. Before the determination, 10 μL of MTT reagent were added to each well. After 2-h incubation, 100 μL of detergent were added and cells were incubated for an additional 2 h before measuring the absorbance (*A*) at 570 nm. The percentage of viable cells was calculated as follows: *A* of experimental group / *A* of control group) × 100.

Cell cycle analysis

Breast cancer cells were treated without or with 1.0 μmol/L deguelin for 24, 48, or 72 h, and then harvested with trypsin and washed with PBS. After the final wash, the cells were resuspended in 1.0 mL of PBS, 9 mL of ice-cold 70% ethanol were added, and the samples were stored at −20°C until staining. In preparation for staining, cells were washed three times with PBS and resuspended in 0.3 mL of citrate buffer [250 mmol/L sucrose, 40 mmol/L trisodium citrate, 0.05% v/v DMSO (pH 7.6)]. The samples were then stained with propidium iodide using the detergent-trypsin method as previously described (10).

Assessment of apoptosis

Induction of apoptosis was determined by acridine/ethidium staining (17). Cultured cells were centrifuged and suspended in PBS and the mixture of dyes was added. Fluorescent microscopy was used to identify nonviable cells with nuclei stained bright orange. Viable cells exclude ethidium bromide and stain bright green. A quantitative assessment was made by determining the percentage of apoptotic cells with nuclei that were highly condensed or fragmented. Next, apoptosis was evaluated by examining the characteristic pattern of DNA laddering generated in apoptotic cells using gel electrophoresis. Briefly, after 72 h of treatment with vehicle (ethanol) or deguelin dissolved in ethanol, cells were suspended in PBS and lysed in 0.5 mL of lysis buffer (0.6% SDS + 10 mmol/L EDTA). NaCl was added to a concentration of 1 mol/L and mixed by inversion. The mixture was left at 4°C for at least 12 h and then centrifuged. RNase A was added to the supernatant, incubated at 37°C for 30 min, and extracted with phenol/chloroform. The samples were electrophoresed through a 2% agarose gel containing 0.5 μg/mL ethidium bromide.

Western blot analyses

Treated and untreated cells were lysed in freshly prepared extraction buffer [20 mmol/L HEPES (pH 7.9), 400 mmol/L NaCl, 0.1% NP40, 10% glycerol, 1 mmol/L sodium vanadate, 1 mmol/L sodium flouride, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl flouride, 10 μg/mL aprotinin, 10 μg/mL leupeptin] for 45 min on ice. Lysates were centrifuged, the supernatant was collected, and protein concentration was determined using a modified Lowry method (Bio-Rad). Samples were separated (200 μg of protein per lane) on 7.5% to 10% polyacrylamide gels and transferred to a nitrocellulose membrane. The membranes were blocked in 5% milk, then incubated with primary antibody for 2 h at room temperature. The appropriate secondary antibodies were incubated with the membrane for 45 min at room temperature. The chemiluminescence reaction was done

using the ECL system from Amersham Pharmacia Biotech according to their protocol. Bands were analyzed by densitometry using the UnScan-It gel program (Silk Scientific, Inc.). The band intensities from the proteins of interest were compared with that of actin and relative intensity ratios were calculated. Western blot analyses of the proteins of interest were examined in three separate experiments.

RNA isolation and microarray analysis

MDA-MB-231 cells were treated with ethanol control or deguelin (1 μmol/L) for 24 h. At the 24-h time point, TRIzol (1 mL) was added to each culture flask, the cells were incubated, and insoluble material was removed by centrifugation at 10,000 rpm for 10 min at 4°C. Chloroform (0.2 mL) was added to each sample and incubated for phase separation. Samples were centrifuged at 10,000 rpm for 15 min at 4°C and the RNA (top aqueous phase) was isolated. RNA was precipitated by mixing with isopropanol (0.8 mL) and centrifuging at 10,000 rpm for 10 min at 4°C. The RNA pellet was washed with 75% ethanol, dried, and dissolved in Rnase-free water. Cleanup of the RNA was done using an RNeasy spin column (Qiagen). The control and deugelin-treated samples were hybridized according to the manufacturer's protocols as a pay per service provided by Mergen, Inc. For this experiments, we used the Mergen ExpressChip DNA Microarray System Human HO5, an oligo-based array representing 11,904 UniGene human genes. Hybridization of biotin-labeled cRNA with oligonucleotide microarray and detection was done according to the manufacturer's established protocols (Mergen Ltd).

In GeneSpring v7.2 (Agilent Technologies), cell files were preprocessed using Robust Multichip Average, genes were normalized to the mean expression of the control sample, and detection calls were used to filter for probes sets Present of Marginal in one fourth of the arrays. Fold change values for each experimental sample were exported to Excel for mean and SD calculations. Canonical pathways were analyzed through the use of the software package Ingenuity Pathway Analysis (IPA; Ingenuity Systems).[3] Genes from that dataset that met the fold change cutoff of 1.5 and were associated with a canonical pathway in the IPA knowledge base were considered for the analysis. Biological processes were analyzed with PANTHER. Genes that met the fold change cutoff of 2.0 and were associated with a biological process with a known Gene Ontology (GO) identification number were considered for analysis.

RNA extraction and real-time reverse transcriptase-PCR

Total RNA extraction and the reverse transcriptase (RT) reaction were done as described previously (18). RNA was further subjected to DNase I (Ambion) digestion and purification using an RNeasy Mini Kit (Qiagen) before the RT reaction. Real-time PCR was done with 2 μL diluted RT product in a MyiQ Real-time PCR Detection System (Bio-Rad) by using IQ SYBR Green PCR Supermix (Bio-Rad) according to the manufacturer's guidelines. The PCR cycling conditions used were as follows: 15 s at 95°C, 15 s at 60°C, and 20 s at 72°C for 40 cycles. Fold inductions were calculated using the formula $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct$ is $\Delta Ct_{(treatment)} - \Delta Ct_{(control)}$, ΔCt is $Ct_{(target\ gene)} - Ct_{(actin)}$, and Ct is the cycle at which the threshold is crossed. The gene-specific primer pairs (and product size) for real-time PCR were as follows: WIF1 gene forward 5′-AAGGTTGGCATGGAAGACAC-3′ and reverse 5′-TTAAGTGAAGGCGTGTGCTG-3′ (103 bp), WNT14 gene forward 5′-GGGTGTGAAGGTGATCAAGG-3′ and reverse 5′-CTTGTGCTTCAGATGCTTGC-3′ (133 bp), β-actin forward 5′-CTCTTCCAGCCTTCCT-TCCT-3′ and reverse 5′-AGCACTGTGTTGGCGTACAG-3′ (116 bp). PCR product quality was monitored using post-PCR melt curve analysis.

[3] http://www.ingenuity.com

Statistical analyses

All the experiments were done three times to ascertain reproducibility of the results and the data shown are representative of three experiments. Data were evaluated either by *t* test or ANOVA depending on the number of samples. Group means of treatment, at various doses or time points, was compared with mean values for control treatment through one-way ANOVA. χ^2 was used for nominal variables (e.g., percent growth inhibition).

Results

Deguelin inhibits growth of breast cancer cells in a dose-dependent manner

The effects of various concentrations of deguelin (0.1-10.0 μmol/L) on cell proliferation were examined on four breast cancer cell lines (MCF-7, MDA-MB-231, BT474, and T47D) and a commercially available immortalized breast epithelial cell line (MCF-12F). Whereas deguelin treatment showed no significant inhibition for up to 7 days in an early passage (passage 5) of MCF-12F cells, it mediated growth inhibition in a dose-dependent manner in several breast cancer cell lines, including the MDA-MB-231 and T47D (Fig. 1A). Moreover, using different cell lines, deguelin (1 μmol/L) treatment for 72 hours showed growth inhibition in breast cancer cell lines at a range of 37% to 87% (Fig. 1B). Because deguelin showed the highest growth suppression in the MDA-MB-231 cell line, which grows very aggressively and exhibits triple-negative characteristics (ER−, PR−, and Her2/neu−), time-dependent studies were completed for this cell line. Growth inhibition in the MDA-MB-231 cells treated with deguelin peaked by 72 hours (Fig. 1C). As such, studies that followed were continued for 24, 48, or 72 hours.

Cell cycle analysis

To determine whether inhibition of cell proliferation was associated with cell cycle arrest, breast cancer cells were treated for 24 and 72 hours with vehicle or deguelin (1 μmol/L) and analyzed by flow cytometry. Cell cycle analysis showed that deguelin-treated MCF-7 and BT474 cells were arrested in the G_1 phase of the cell cycle, whereas MDA-MB-231 cells were accumulated in the S phase (Table 1). In the MDA-MB-231 cells, exposure to deguelin triggered an arrest in the S phase of the cell cycle, where the amount of cells in the S phase significantly increased to 63% and 34% following 24 and 72 hours, respectively (Fig. 2A). MCF-7 and BT474 cells were found to accumulate in the G_1 phase following 72 hours of treatment with deguelin.

Induction of apoptosis by deguelin

In addition to cell cycle arrest, the overall cell growth inhibition induced by deguelin could be due to increased apoptosis. Two separate assays were used to investigate the induction of apoptosis in MDA-MB-231 cells by deguelin. First, induction of apoptosis as a result of treatment with deguelin was assessed by acridine orange/ethidium bromide staining. MDA-MB-231 cells treated with 1 μmol/L deguelin for 48 hours were stained and cell morphology was examined by fluorescent microscopy. Characteristic features of apoptosis such as orange color, fragmented nuclei, blebbing, and irregular cytoplasmic membranes were observed in MDA-MB-231 cells treated with deguelin, whereas live cells were green. Following 48 hours of deguelin treatment, 52% of the

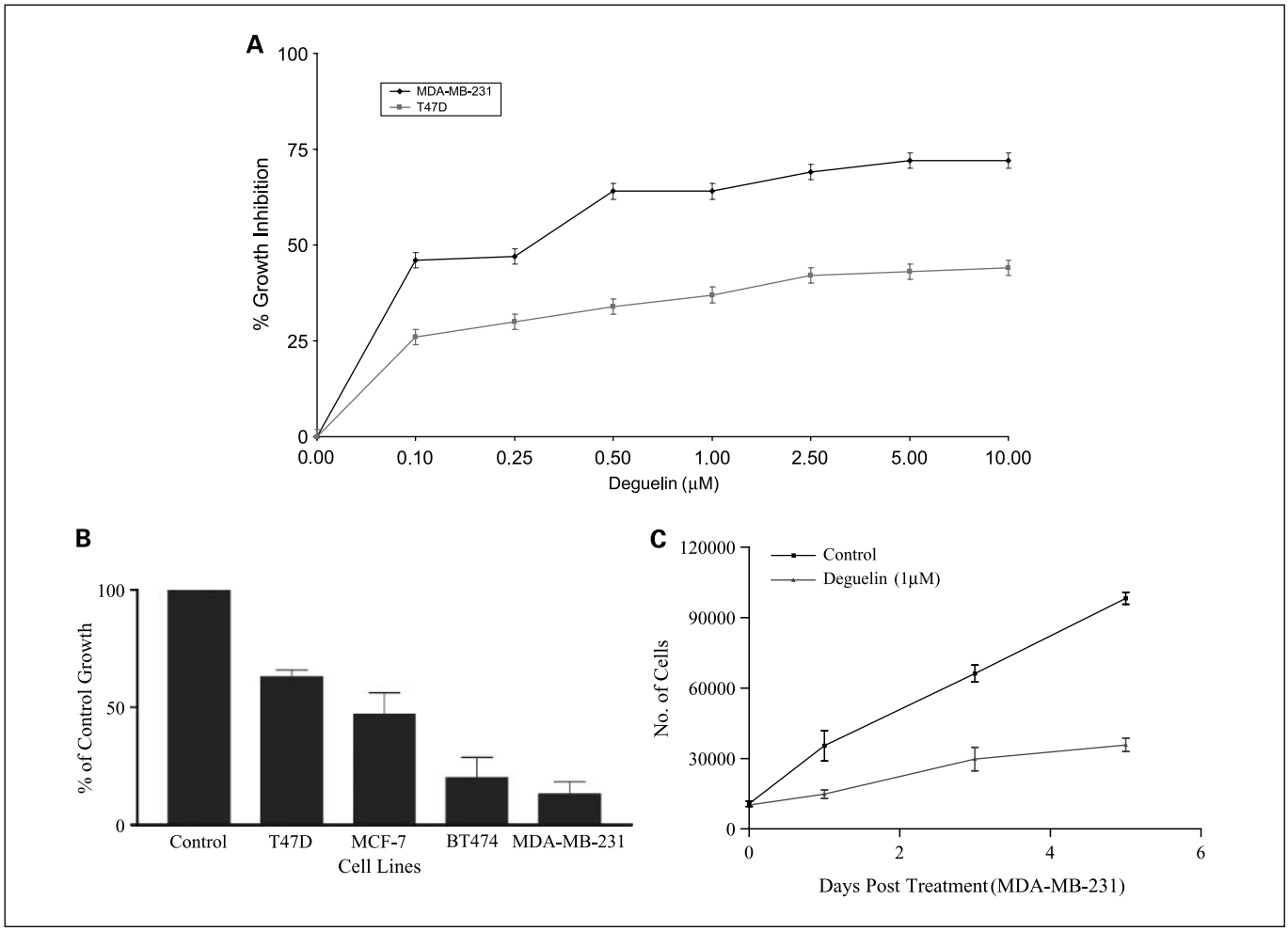

Fig. 1. Effects of deguelin on cell proliferation. *A*, dose-dependent effects of deguelin were examined in the MDA-MB-231 and T47D cell lines. For this, the MTT assay was used. *B*, the effects of deguelin on various human breast cancer cell lines were compared following 72 h posttreatment (1 μmol/L). *C*, a time course study was completed using MDA-MB-231 cells. Cells were exposed to deguelin (1 μmol/L; ▲) or vehicle (■) for 1, 3, and 5 d.

MDA-MB-231 cells were orange, 43% were observed to be blebbing, and 5% were green. The control cells, treated with ethanol, were 12% orange, 4% blebbing, and 84% green (data not shown). Second, agarose gel electrophoresis was used to show low-molecular-weight DNA ladder formation. Nuclear DNA fragmentation, a classic feature of apoptotic cell death, was clearly shown in MDA-MB-231 cells treated with 1 μmol/L of deguelin for 48 hours (Fig. 2B).

Table 1. Cell cycle analysis of breast cancer cells treated with deguelin (1 μmol/L) at 24- and 72-h time points

Cell line	Treatment/time (h)	%G_1	%S	%G_2	%Apoptotic
MCF-7	Cont-24	55.0	34.1	10.9	3
	Deg-24	54.1	30.5	15.4	5
	Cont-72	51.5	35.9	12.5	6
	Deg-72	86.7	0.5	12.8	6
BT474	Cont-24	76.8	14.5	8.7	8
	Deg-24	74.5	15.7	9.8	10
	Cont-72	61.2	26.7	12.1	6
	Deg-72	74.3	10.2	15.5	14
MDA-MB-231	Cont-24	52.8	33.6	13.7	13
	Deg-24	28.7	63.1	8.1	45
	Cont-72	73.1	19.0	7.9	7
	Deg-72	59.1	34.1	6.7	25

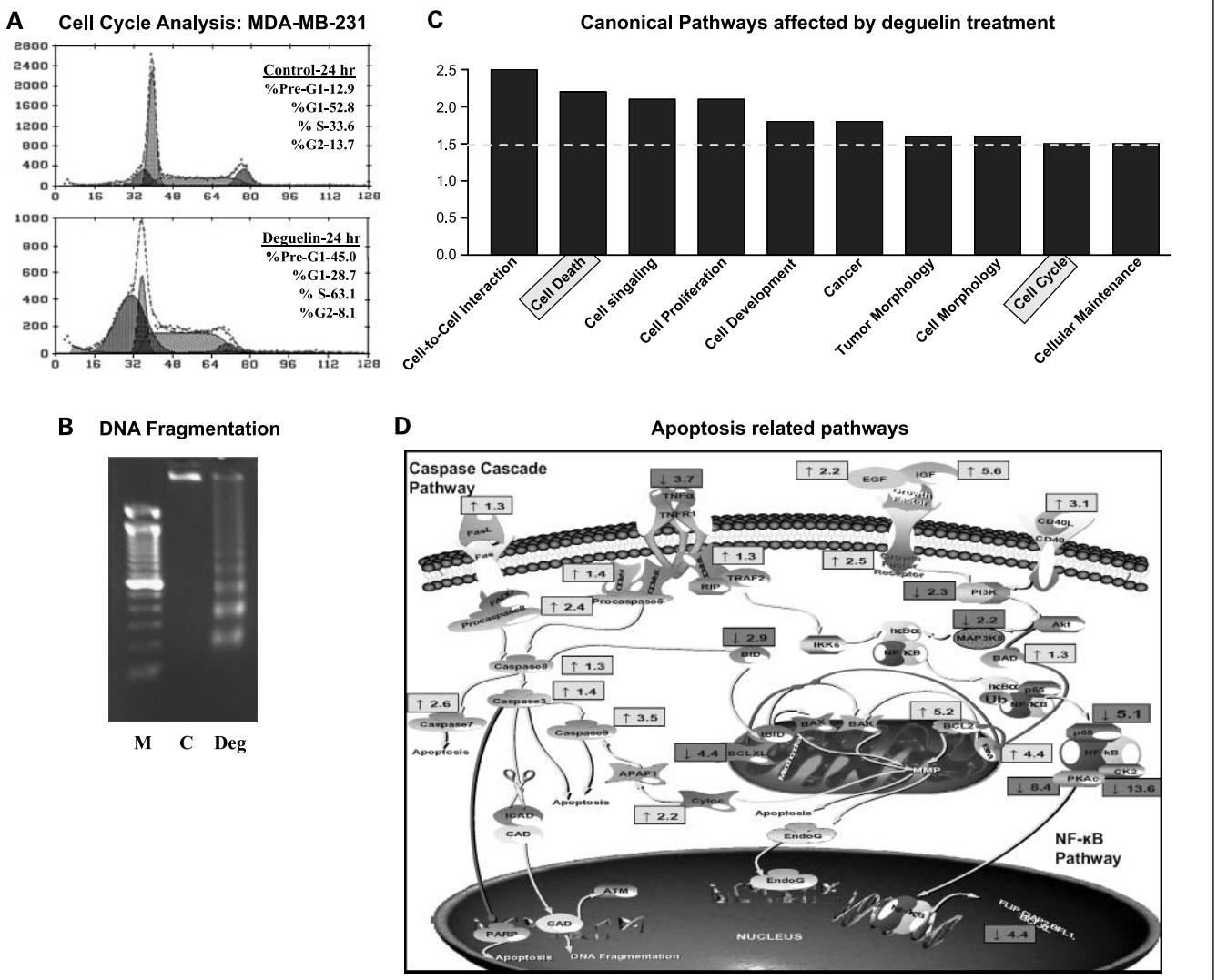

Fig. 2. Effects of deguelin on cell cycle, apoptosis, and key genes. *A*, effects of deguelin on MDA-MB-231 cell cycle distribution. Cell cycle analysis was done as described in Materials and Methods. *a*, control 24 h; *b*, deguelin, 24 h. *B*, DNA fragmentation in MDA-MB-231 cells treated with deguelin (1 μmol/L) for 48 h. DNA was isolated as described in Materials and Methods. *C*, control cells treated with ethanol; *Deg*, deguelin-treated cells. *C*, following the normalization of the microarray data, the IPA software was used to identify canonical pathways affected by deguelin treatment. *D*, the Protein ANalysis THrough Evolutionary Relationships (PANTHER) Classification System program was used to identify biological processes found to be significantly regulated by deguelin. The diagram highlights the genes found to be significantly regulated by deguelin.

Gene and protein expression changes in MDA-MB-231 cells induced by deguelin

The Mergen Ltd. DNA Microarray System Human HO5, an oligo-based array representing 11,904 UniGene human genes, was used to examine the alterations in MDA-MB-231 mRNA expression levels following deguelin treatment. MDA-MB-231 mRNA was harvested after 24 hours of deguelin treatment at 1 μmol/L. Using the 1.5-fold change as a cutoff, 2,355 genes were found to be differentially expressed and were subsequently imported into Ingenuity Pathway Analysis 4.0. To enable the identification of biological mechanisms, pathways, and functions most relevant to the genes of interest altered by deguelin treatment in MDA-MB-231 cells, we identified an array of main canonical pathways regulated by deguelin in human breast cancer cells (Fig. 2C). The G_1-S cell cycle checkpoint pathway was one of the canonical pathways identified by Ingenuity to be altered by deguelin action (e.g., cyclin D1). This correlated with our previous findings described above (Fig. 2A; Table 1). The apoptosis cell death cascade (Fig. 2D) was another prominent pathway identified to be altered by deguelin treatment in MDA-MB-231 cells, again confirming our above results (Fig. 2B). Other notable pathways identified to be altered by deguelin were those of the NF-κB pathway (Fig. 2D) and the p38 mitogen-activated protein kinase/c-*jun*-NH_2-kinase signaling pathway. The expression of CK2 was downregulated by >13-fold, which is essential for the nuclear interactions of NF-κB.

The identification of gene alterations in the Wnt/β-catenin pathway provides a novel route by which deguelin can mediate the chemotherapeutic effects in breast cancer cells. Two of

the major inhibitors of the Wnt/β-catenin pathways, WIF-1 (5.3) and DKK4(8.0), were found to be upregulated by deguelin, whereas several of the Wnt/β-catenin pathway activators (Wnt14, Wnt2B, and Wnt3) were shown to be downregulated at the mRNA level (Table 2). The microarray data show a fold change of 25.4, 5.0, and 2.4 for WNT14, WNT2B, and WNT3A, respectively (Table 2). Moreover, Snail 1, the zinc finger transcription factor reported to be a predictor of breast cancer metastatic potential and recurrence, was found to be downregulated in deguelin-treated cells [–8.1] (19). In addition, several members of the cadherin family (CDH3, CDH7, and CDH9) were all found to be upregulated with treatment. Whereas the cadherin/β-catenin adhesion complex and the Wnt signaling pathway share β-catenin as a common member, the role of the cadherins remains to be established. P-cadherin has been reported to have negative growth control of the mammary gland and the Wnt signaling pathway is involved (20). Results were confirmed by real-time PCR for several of the Wnt signaling genes and several of the apoptosis-related genes (Table 2; Fig. 3). Real-time PCR show a dose-dependent

Table 2. Wnt signaling–related genes regulated by deguelin

Genbank accession no. (UniGene symbol)	Fold change	Involvement in the Wnt signaling pathway
NM_003395 (WNT14)	–25.4	WNT14 was significantly upregulated in one of nine cases of primary breast cancer
AF125377 (SNAIL1)	–8.1	Snail1 is a potent EMT inducer in normal and neoplastic cells, as well as an important predictor of breast cancer metastatic potential and recurrence
NM_014421 (DKK2)	–6.2	Can activate or inhibit the Wnt signaling pathway
NM_004185 (WNT2B)	–5.0	Positive regulator of the WNT–β-catenin–TCF signaling pathway
NM_003013 (SFRP2)	–3.8	Secreted Frizzled-related protein 2 (SFRP2) has been reported to be abundantly expressed in canine mammary tumor cells but not in normal canine mammary gland cells
NM_033131 (Wnt3A)	–2.4	Wnt-3a initiates Dvl-3, Akt/PKB, and GSK-3β hyperphosphorylation and β-catenin activation
NR_024047 (WNT2)	30.5	WNT2 is present in normal human breast tissue
NM_007017 (SOX30)	25.5	Expression of SOX30 in the breast could be a marker of differentiation and part of the signature induced by pregnancy in the breast epithelial cells
NM_016279 (CDH9)	21.9	Cadherin/catenin adhesion complex
AB059569 (WNT10A)	21.6	Although upregulation of WNT10A mRNA might play key roles in some cases of esophageal, gastric, and colorectal cancer, its role in breast cancer is not well defined
NM_001083962 (TCF4)	19.2	Unbound to nuclear β-catenin, it functions to repress genes involved in cell proliferation; however, when bound to β-catenin, it coactivates downstream target genes in diverse systems including the breast
NM_001130861 (CLDN5)	18.9	β-Catenin nuclear localization is inversely related to claudin-5 expression
NM_020384 (CLDN2)	17.4	These results suggest that CLDN2 is implicated in the progression as well as the development of breast carcinoma, indicating that CLDN2 is a possible tumor suppressor gene product; gene expression mediated by the promoter of the human tight junction protein claudin-2 is regulated by factors involved in Wnt signaling
NM_031866 (FZD8)	16.9	Wnt activators in several types of cancers; function in breast cancer unknown
NM_007197 (FZD10)	11.1	No data are available for MDA_MB-231 cells. Furthermore, FZD10 mRNA was undetectable in MCF-7 cells but significantly upregulated by β-estradiol in MCF-7 cells. Further research is necessary to better elucidate its role in MDA-MB-231 cells
NM_004189 (SOX14)	8.2	Transcription factors; function not well defined for breast cancer
NM_014420 (DKK4)	8.0	Wnt/β-catenin inhibitors
AJ007611 (CDH7)	7.5	Cadherin/catenin adhesion complex; cadherin was found to be expressed in human mammary epithelial cells
Y12692 (WNT11)	7.2	Involved in the transformation of mammary epithelial cells
NM_007191 (WIF1)	5.3	Binds to WNT proteins and inhibits their activities. WIF1 interacts with structurally diverse extracellular inhibitors, presumably to fine-tune the spatial and temporal patterns of WNT activity
NM_012193 (FZD4)	4.4	Wnt activators in several types of cancer; function in breast cancer unknown
NM_002181 (IHH)	4.4	Expression of Ihh has been statistically correlated with increased proliferating index of Ki-67 in invasive ductal carcinoma. Hedgehog signaling molecules play an important role in the progression of invasive ductal carcinoma of breast
CR456713 (SOX5)	4.1	Transcription factors; function not well defined for breast cancer
NM_001463 (FRZB)	3.5	Acts as a soluble WNT-binding protein that antagonizes WNT signaling
NM_001793 (CDH3)	2.8	Activation of β-catenin signaling has been found to correlate with upregulation of P-cadherin expression

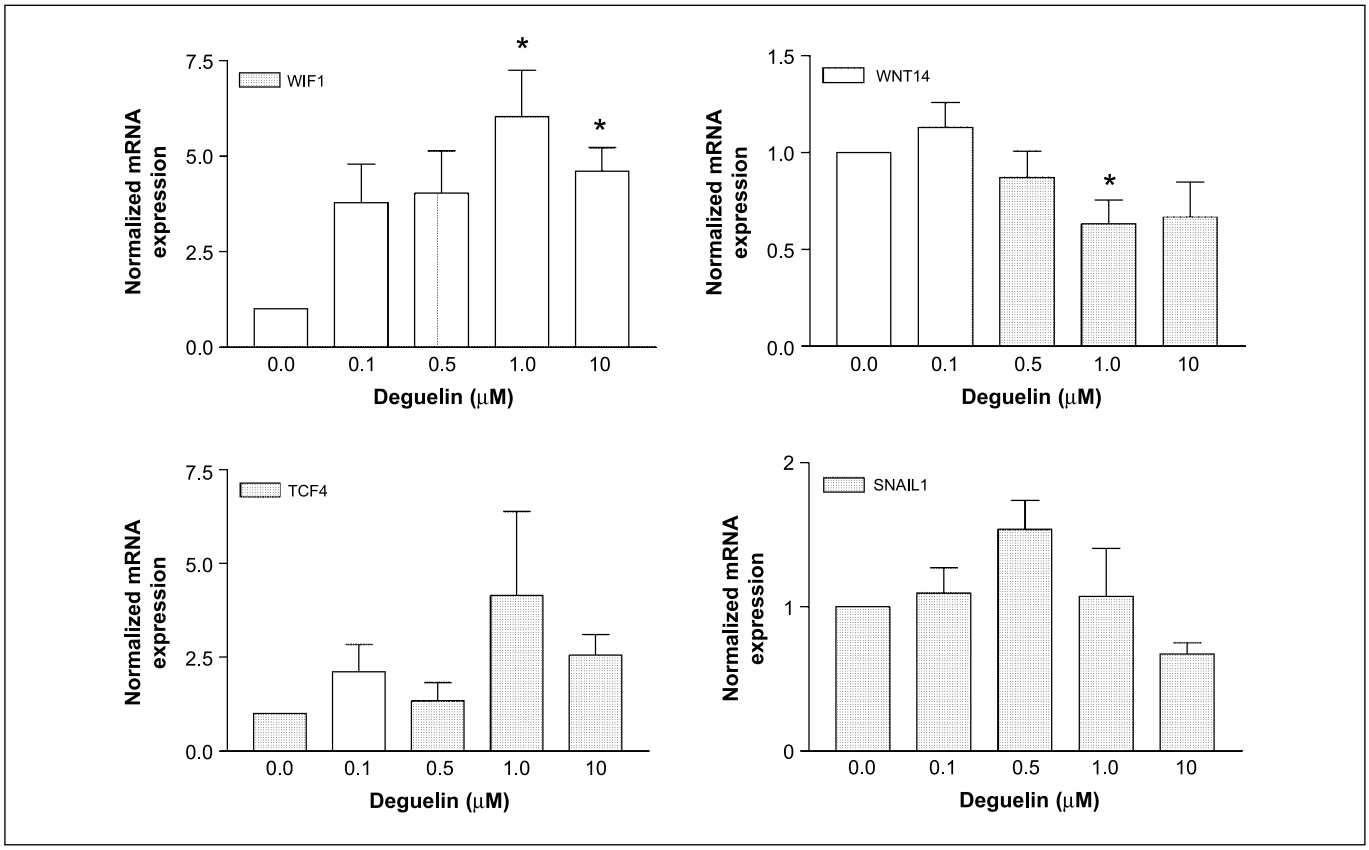

Fig. 3. Effects of deguelin on Wnt signaling genes. Dose-dependent effect of deguelin on WIF1 and WNT14 mRNA expression. MDA-MB231 cells were cultured to 50% confluence and treated with different concentrations of deguelin for 24 h; total RNA isolated from deguelin-treated cells were then subjected to quantitative RT-PCR analysis. *Columns*, mean fold of control (DMSO)–treated cells ($n = 3$) after normalization to β-actin expression; *bars*, SEM. *, $P < 0.05$, compared with control (Student's *t* test: paired two samples for means).

effect by deguelin (24-hour treatment) for WIF1 and WNT14 (Fig. 3). Although not statistically significant, a trend in dose-dependent effects by deguelin were also noted for TCF4, and Snail1 mRNA expression levels. In summary, the microarray results clearly show that deguelin regulates key components of the Wnt signaling family; however, many interactions between these members remain to be studied.

To further investigate the role of deguelin in the Wnt/β-catenin signaling, we sought to examine the protein expression levels of several key members of this pathway. First, we examined the level of total GSK3β, a negative regulator of β-catenin, as well as its phosphorylated form (Ser^9), which represents the inactivated form of GSK3β. Using Western blot analysis, we examined the expression levels of GSK3β with increasing concentrations of deguelin (0-5.0 μmol/L; 24 hours). No significant change in total GSK3β levels was noted with deguelin treatment, but a significant decrease in its inactive GSK3β (Ser^9) form was detected for 1 and 5 μmol/L doses of deguelin (Fig. 4A). Next, we examined the protein expression levels of total β-catenin. These studies show that treatment with deguelin at a concentration of 0.5 or 1 μmol/L for 24 hours significantly decreases the levels of expression of β-catenin. Taken together, these results suggest that in the presence of deguelin, β-catenin protein may be targeted for destruction through GSK3β-mediated phosphorylation. Studies have shown that GSK-3β plays a critical role by regulating the degradation of cyclin D1 (21). Thus, activation of GSK-3β is expected to lead to a reduction in the level of cyclin D1. Hence, Western blot analysis was used to examine the expression levels of cyclin D1. As shown in Fig. 4B, cyclin D1 was found to be significantly downregulated with deguelin treatment. Cumulatively, the data suggest that deguelin acts as an inhibitor of the Wnt/β-catenin signaling pathway, in part through activation of GSK-3β.

Discussion

Breast cancer is a common disease and its incidence is increasing worldwide. In the United States, breast cancer affects one in eight women over their lifetime. In the last three decades, the mortality rate has declined in part due to an increased emphasis on early detection and more effective treatments. Despite early detection, conventional and chemotherapeutic methods of treatment, about 7% of women still die every year. Moreover, current therapeutic modalities for breast cancer continue to be linked with toxicity and side effects. Hence, novel therapies continue to be warranted. In recent years, the discovery of active compounds from natural products has gained significant importance in the field of breast cancer therapy.

This study presents data showing that deguelin attenuated *in vitro* proliferation in breast cancer cells in part by inducing apoptosis. The ability of deguelin to induce programmed cell death provides an attractive and potentially promising approach for the treatment of breast cancer. The actions of deguelin in this apoptosis signaling pathway proceed through several key molecules, including the activation of caspase and recruitment of proteins in the Bcl-2 family. The action of deguelin in the caspase cascade is evident from genes such as *caspase-3*, *caspase-7*, *caspase-8*, and *caspase-9*, shown to be upregulated following a 24-hour treatment. Furthermore, we show that deguelin inhibits the mRNA expression of tumor necrosis factor-α, one of the most potent activators of the NF-κB pathway. Moreover, deguelin reduced the gene expression of phosphatidylinositol 3-kinase (PI3K), MAP3K8, PKAc, and CK2 as such treatment with deguelin blocked the PI3K–Akt–NF-κB signaling cascade. In addition, reduced expression of P65, the active form of the NF-κB family, was observed. Lastly, the expression of Bcl-X_L, a downstream target of NF-κB, decreased in deguelin-treated cells, cumulatively demonstrating the profound effect of deguelin on apoptosis-related genes.

The role of deguelin as a potential regulator of the Wnt/β-catenin signaling pathway provides a novel mode by which this agent can inhibit the growth of breast cancer cells. Accumulating evidence indicates that hyperactive Wnt signaling is associated with the development and progression of human breast cancer. In this signaling pathway, Wnt ligands bind to a coreceptor complex consisting of a seven-transmembrane domain, Frizzled receptor, and one of the low density lipoprotein (LDL) receptor–related proteins (1). A signaling cascade is then engaged that leads to formation of a complex between β-catenin and transcription factors of the TCF family. The end result is the activation of several target genes involved in proliferation and metastasis. In recent years, interest in developing regulators of the Wnt signaling pathway has intensified (22). Thus, deguelin actions on this pathway warrant further study. As shown in Fig. 3, MDA-MB-231 cells treated with deguelin exhibited increased mRNA expression levels for WIF1 and Wnt14 compared with control cells in a dose-dependent manner. Overexpression of WIF-1 has been linked to growth inhibition of various types of cancer cells (18, 23–25). In contrast, a number of lines of evidence show that some Wnt genes have growth-promoting properties. In particular increased expression of WNT14, WNT2B, and/or WNT3 are associated to cell proliferation and transformation of breast cancer cells (26–29). RT-PCR confirmed a decrease in expression of mRNA levels for WNT14 by deguelin treatment. Further studies are warranted to examine the effects of deguelin on the Wnt genes.

In summary, deguelin inhibits prosurvival signaling such as NF-κB, Bcl-X_L, PI3K, MAP3K8, PKAc, and CK2 with a simultaneous induction of proapoptotic proteins in breast cancer

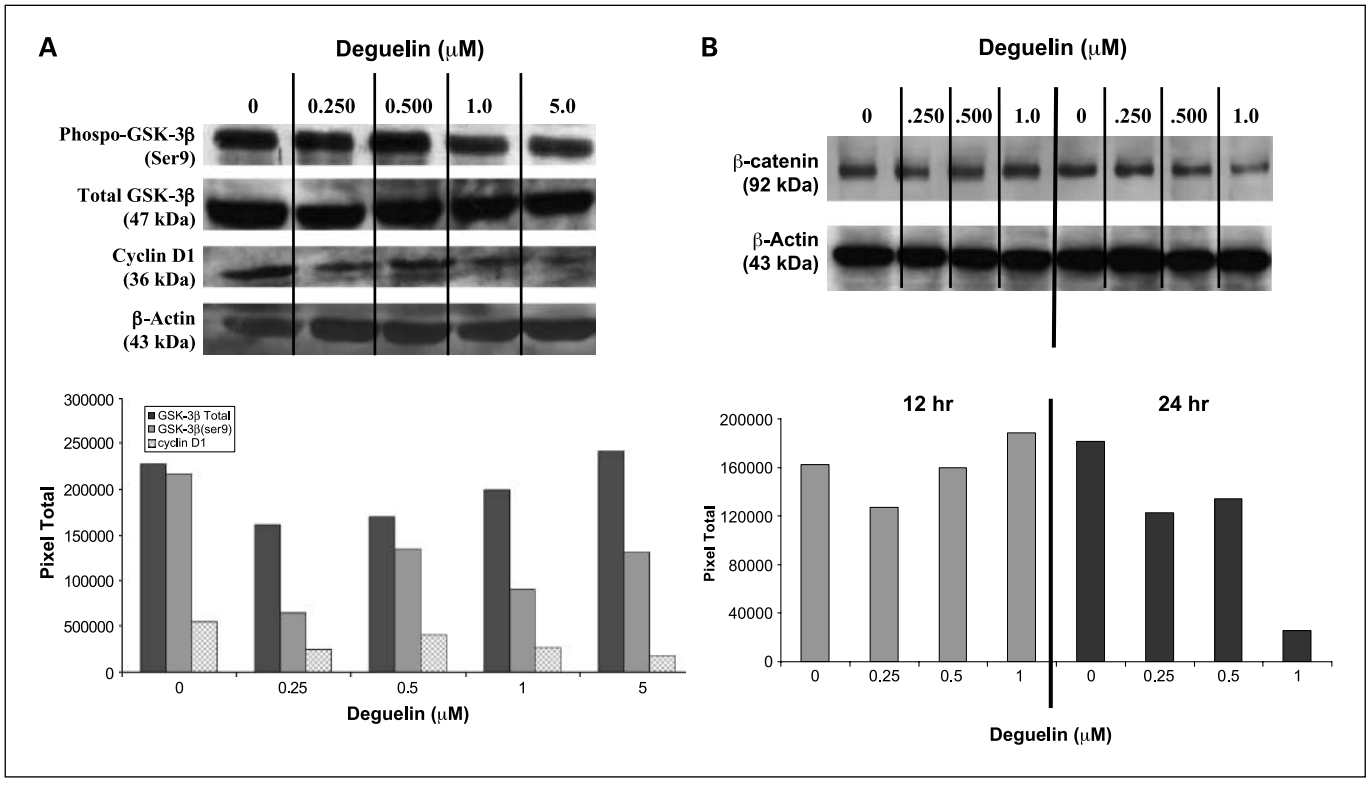

Fig. 4. Western blot analysis of Wnt signaling genes. Western blot analyses were completed to examine the effects of deguelin on proteins of interest. To account for the difference in protein loading during the experiment, the percentage of regulation was calculated after the intensity of each band was adjusted according to its respective actin band intensity using the UnScan-It gel program (Silk Scientific, Inc.). *A*, the expression levels of total GSK-3β and GSK-3β Ser^9, the phosphorylated form that represents the inactivated form of GSK3β. These studies show that deguelin (24-h treatment) increases expression levels of total GSK-3β protein while decreasing the levels of GSK-3β Ser^9. Results also show downregulation of cyclin D1 after 24 h of deguelin treatment at the concentrations studied (0.250-5.0 μmol/L). *B*, the effect of deguelin treatment on total β-catenin protein levels was examined following 12 and 24 h posttreatment. We found that 24 h of treatment significantly reduces the expression levels of β-catenin.

cells, resulting in inhibition of cell survival and proliferation. Additionally, deguelin causes cell cycle arrest in the MDA-MB231, MCF-7, and BT474 cells, which is also an important aspect for the treatment of cancer. Moreover, the novel actions by deguelin in the Wnt signaling pathway are presented in this report. Collectively, our results suggest that deguelin may have wide therapeutic application in the treatment of breast cancer exhibiting both ER- and PR-positive as well as ER- and PR-negative properties. Further investigation for deguelin efficacy *in vivo* models is warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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