Effects of Deguelin on the Phosphatidylinositol 3-Kinase/Akt Pathway and Apoptosis in Premalignant Human Bronchial Epithelial Cells

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Background: **Because lung cancer is the leading cause of cancer-related death, new approaches for preventing and controlling the disease are needed. Chemoprevention approaches are both feasible and effective. We evaluated the potential of deguelin, a natural plant product, as a lung cancer chemopreventive agent and investigated its mechanism of action.** ***Methods:*** **The effects of deguelin on proliferation and apoptosis of normal, premalignant, and malignant human bronchial epithelial (HBE) cells were assessed by using the MTT assay, a flow cytometry-based TUNEL assay, and western blot analyses. The effects of deguelin on the phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways were assessed by western blot analyses and with adenoviral vectors that expressed constitutively active Akt.** ***Results:*** **Deguelin treatment *in vitro* at doses attainable *in vivo* inhibited the growth of and induced apoptosis of premalignant and malignant HBE cells but had minimal effects on normal HBE cells. Levels of phosphorylated Akt (pAkt) were higher in premalignant HBE cells than in normal HBE cells. In premalignant HBE cells, deguelin inhibited PI3K activity and reduced pAkt levels and activity but had mimimal effects on the MAPK pathway. Although overexpression of a constitutively active Akt in premalignant and malignant HBE cells had no effect on growth inhibition mediated by *N*-(4-hydroxyphenyl)retinamide (4-HPR), a novel chemopreventive retinoid, it blocked deguelin-induced growth arrest and apoptosis.** ***Conclusions:*** **The ability of deguelin to inhibit PI3K/Akt-mediated signaling pathways may contribute to the potency and specificity of this pro-apoptotic drug. Because both premalignant and malignant HBE cells are more sensitive to deguelin than normal HBE cells, deguelin may have potential as both a chemopreventive agent for early stages of lung carcinogenesis and a therapeutic agent against lung cancer. [J Natl Cancer Inst 2003;95:291–302]**

In the United States, lung cancer is the leading cause of cancer-related mortality *(1).* Despite recent advances in radiotherapy and chemotherapy modalities, the severe morbidity from lung cancer and the low 5-year survival rates have not improved *(1).* Cancer chemoprevention is therefore a logical and obvious strategy to help alleviate the effects of this disease *(2,3).* Because clinical studies have shown that chemoprevention of aerodigestive tract cancers is both feasible and effective *(4–6)*, there has been a shift in interest toward developing strategies for early detection and effective chemoprevention of lung cancer. Much effort has been focused on the discovery and development of new chemopreventive agents, especially agents targeted at mechanisms known to be involved in the process of carcinogenesis. A large number of compounds with varying mechanisms, including retinoids, tyrosine kinase inhibitors, farnesyl transferase inhibitors, and the non-steroidal anti-inflammatory agents (NSAIDS), potent *in vivo* inhibitors of colon carcinogenesis, and agents such as difluoromethyl ornithine (DMFO), a polyamine synthase inhibitor, have a broad spectrum of preventive activity *in vitro* and *in vivo (2,3).* However, undesirable side effects and resistance of lung cancer cells to these agents have limited their long-term clinical use as chemopreventive agents *(7–11).* Therefore, we have sought to identify novel agents that can prevent lung carcinogenesis effectively but with minimal toxicity or resistance.

Several natural compounds, especially plant products and dietary constituents, have been found to exhibit chemopreventive activities both *in vitro* and *in vivo* in model systems *(12–14).* Their mechanisms of action vary widely, with many suppressing cell growth or modulating cell differentiation and a few also inducing apoptosis. Rotenoids, which constitute a class of compounds from the flavonoid family, have chemopreventive activity *(15)*, act by inhibiting NADH:ubiquinone oxidoreductase activity and by suppressing steady-state mRNA levels and enzymatic activity of 12-*O*-tetradecanoylphorbol 13-acetate-induced ODC *(15,16).* One rotenoid, deguelin, has been isolated from several plant species, including *Mundulea sericea* (*Leguminosae*) *(17).* Deguelin has been shown to have cancer-chemopreventive effects in models of both skin and mammary tumorigenesis *(18).*

We used an *in vitro* lung carcinogenesis model system that consists of premalignant and malignant human bronchial epithelial (HBE) cells to evaluate the potential of deguelin as a chemopreventive agent against lung cancer. Here, we report on the effects and molecular mechanism of action of deguelin on HBE cells representing different stages of lung carcinogenesis.

Materials and Methods

Preparation of Deguelin

Deguelin (Fig. 1) was synthesized in four steps from the natural product rotenone (Sigma-Aldrich, Milwaukee, WI), as

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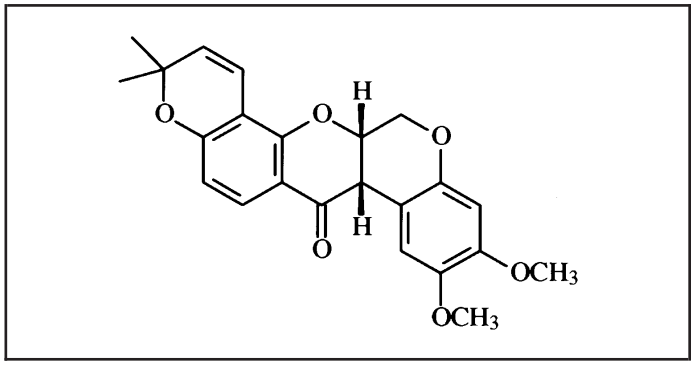

Fig. 1. Structure of deguelin.

previously described *(19).* The final product was more than 98% pure. Deguelin was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of $10^{-3}M$ and was stored in a nitrogen tank.

Cells and Cell Cultures

Normal HBE (NHBE) cells were purchased from Clontech (Palo Alto, CA) and maintained according to the manufacturer's recommended protocol. BEAS-2B cells, an HBE cell line immortalized with a hybrid adenovirus/simian virus 40 *(20)* (obtained from Dr. A. Klein-Szanto, Fox Chase Cancer Center, Philadelphia, PA), were previously used to derive both premalignant and malignant HBE cells *(21)* as follows. BEAS-2B cells were explanted, along with beeswax pellets or beeswax pellets containing cigarette smoke condensate (CSC), into rat tracheas that had been denuded of bronchial epithelium. The tracheas were then transplanted into the dorsal subcutaneous tissues of nude mice *(21).* Tumors developed after 6 months. From these tumors, a variety of cell lines were derived *in vitro* that exhibited different levels of tumorigenicity when transplanted into nude mice. For this study, we used three cell lines derived from these tumors, the characteristics of which have been described in detail *(21,22).* Two cell lines were premalignant: one (1799) was derived from BEAS-2B cells exposed to a beeswax control pellet, and one (1198) was derived from BEAS-2B cells exposed to a beeswax pellet containing CSC. The third cell line (1170-1) was malignant and was derived from BEAS-2B cells exposed to a beeswax pellet containing CSC. Although 1799 and 1198 cells are autonomous and grow indefinitely in culture, they are defined as premalignant because 1) neither was tumorigenic; 2) 1799 cell line was sensitive to serum in that it was growth inhibited and terminally differentiated into squamous cells similar to normal HBE and BEAS-2B cells; 3) *in vitro* invasiveness was detected after exposure of BEAS-2B cells (from which 1799 and 1198 cells were derived) to either phorbol myristate acetate or CSC; 4) *in vitro* exposure to the carcinogen *N*-nitrosamine-4-(methylnitrosamine)-1-(3 pyridyl)-1-butanone induced transformation of BEAS-2B cells and increased epidermal growth factor receptor (EGFR) expression in transformed BEAS-2B cells *(21,23).* Aberrant EGFR expression frequently occurs in bronchial preneoplasia, suggesting that squamous cell cancer development and EGFR expression are related *(24).* 1198 cells were not tumorigenic, although they were generated from CSC-treated BEAS-2B cells. The malignant 1170-1 cells were defined as a malignant cell line because they exhibit several features typical of invasive adenocarcinomas, including increased expression of EGFR and transforming growth factor-α (TGF-α) *(21).* In addition, 1170-1 cells had acquired resistance to serum-mediated growth inhibition *(21).*

NHBE cells were induced to differentiate into squamous cells by growing them to confluence on tissue culture plates coated with a thin matrix of fibronectin (10 μg/mL; Upstate Biotechnology, Inc., Lake Placid, NY) and collagen (30 μg/mL; Celtrix Laboratories, Inc., Palo Alto, CA) as described *(25).* HB56B cells, an immortalized HBE cell line induced by loss of a portion of chromosome 11p without p53 or K-ras gene mutations *(26)* obtained from Dr. R. Reddel (National Cancer Institute, Bethesda, MD), NHBE cells, 1799 cells, and squamous HBE cells were grown in keratinocyte serum-free medium (KSFM; Life Technologies Inc., Gaithersburg, MD) containing EGF (2 μg/mL) and bovine pituitary extract (BPE, 25 μg/mL) *(20).* 1198 and 1170-1 cells were maintained in KSFM supplemented with 3% fetal bovine serum.

For the analysis of growth inhibition by deguelin, NHBE cells, HBE cell lines, and squamous HBE cells were cultured in KSFM containing EGF and BPE. To induce activation of the phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways, H1299 non-small-cell lung cancer (NSCLC) cells, which were purchased from ATCC, and NHBE cells were cultured in the absence of serum and EGF for 1 day and then treated with 50 ng/mL insulin-like growth factor I (IGF-I) for 15 minutes.

Cell Treatment With Deguelin and Determination of Growth Inhibition

To measure the effects of deguelin on cell proliferation, NHBE, 1799, 1198, 1170-1, and HB56B cells were plated at concentrations of 2×10^3 to 4×10^3 cells/well in 96-well plates. The next day, cells were treated with either 0.1% DMSO as a diluent control or various concentrations of deguelin (final DMSO concentration = 0.1%). At the end of the assay time period, cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described *(27).* Six replicate wells were used for each analysis, and data from replicate wells are presented as means with 95% confidence intervals (CIs). For some experiments, the drug concentration required to inhibit cell growth by 50% (IC_{50}) was determined by interpolation from dose–response curves. At least three independent experiments were performed.

Cell Cycle Analysis

NHBE and 1799 cells were plated at a concentration of 2×10^5 cells/well in six-well plates. The next day, cells were treated with deguelin (various concentrations) or DMSO (0.1%) for 3 days to achieve maximal antiproliferative effects (determined from growth curves). All cells (nonadherent and adherent) were harvested, fixed with 1% paraformaldehyde and 70% ethanol, stained with 50 μg/mL propidium iodide, and subjected to flow cytometric analysis to determine the percentage of cells in specific phases of the cell cycle (G_1, S, and G_2/M) as described *(28).* Flow cytometric analysis was performed using a Coulter EPICS Profile II flow cytometer (Coulter Corp., Miami, FL) equipped with a 488-nm argon laser. Approximately 10 000 events (cells) were evaluated for each sample. Two independent experiments were performed, and one is presented.

Apoptosis Assays

NHBE, premalignant (1799 and 1198), and malignant (1170-1) HBE (2×10^6) cells were exposed to various doses of deguelin or to DMSO (0.1%) for 3 days. Apoptosis was assessed by morphology, by a flow cytometry-based, modified terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, and by the detection of fragmented DNA. For morphology, live cells were observed by light microscopy using a ×100 objective. For TUNEL analysis, both adherent and nonadherent cells were harvested and pooled, fixed with 1% paraformaldehyde and 70% ethanol, and processed using the APO-BrdU staining kit (Phoenix Flow Systems, San Diego, CA), a modified TUNEL assay, as described *(27)*. Cells treated with DMSO were used to gate the control nonapoptotic populations and as a reference for cells treated with deguelin. An internal control (HL-60 cells treated with camptothecin to induce apoptosis) provided in the apoptosis detection kit was also used to ensure that the TUNEL reaction was occurring during the staining procedure. For the detection of fragmented nucleosomal DNA, cells were processed using the TACS apoptotic DNA laddering kit (Trevigen Inc., Gaithersburg, MD), according to the manufacturer's recommended protocol.

Immunoblotting

Whole cell lysates from 1×10^6 cells were prepared in lysis buffer as described *(23)*. Equivalent amounts of protein were resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gels (7.5%–12%) and transferred to a nitrocellulose membrane. After the membrane was blocked in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST) and 5% (w/v) nonfat powdered milk, the membrane was incubated with primary antibody at the appropriate dilution in TBS–5% nonfat milk at 4 °C for 16 hours. The membrane was then washed multiple times with TBST and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. The protein–antibody complexes were detected by enhanced chemiluminescence (ECL kit; Amersham, Arlington Heights, IL), according to the manufacturer's recommended protocol.

The following antibodies and working dilutions were used for the western blots: rabbit polyclonal antibodies against human phosphorylated Akt (pAkt) (Ser473) (1 : 1000), Akt (1 : 1000), phosphorylated glycogen synthase kinase 3β (pGSK-3β) (Ser9) (1 : 1000), and mouse monoclonal antibody against human anti-phosphorylated MAPK (anti-pMAPK) (Thr202/Tyr204) (1 : 500) (Cell Signaling Technology, Beverly, MA); rabbit polyclonal anti-GSK-3α/β (1 : 1000) (BD Transduction Laboratories, Lexington, KY); rabbit polyclonal anti-Bax and anti-caspase-3 antibodies (1 : 2000) (Pharmingen, San Diego, CA); rabbit polyclonal anti-Bcl-2 (1 : 1000) and rabbit polyclonal anti-poly(ADP-ribose) polymerase (PARP) antibody (1 : 1000) (VIC 5; Roche Molecular Biochemicals, Indianapolis, IN); rabbit polyclonal anti-hemagglutinin (HA) antibody (1 : 1000), goat polyclonal antibodies against extracellular related kinase 1(Erk-1; 1 : 1000), Erk-2 (1 : 1000), and β-actin (1 : 4000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); rabbit anti-mouse immunoglobulin G (IgG)–horseradish peroxidase conjugate (1 : 2000) (DAKO, Carpinteria, CA); and donkey anti-rabbit IgG–horseradish peroxidase conjugate (1 : 2000) and rabbit anti-goat IgG–horseradish peroxidase conjugate (1 : 2000) (Amersham Pharmacia Biotech, Arlington Heights, IL).

Immune Complex Kinase Assay for MAPK

Approximately 5×10^6 1799 cells were treated with 10^{-7} *M* deguelin or 0.1% DMSO for various time periods in the absence of any additional stimulatory growth factors. The cells were then treated with 50 ng/mL IGF-I for 15 minutes to activate MAPK. Total cell extracts were prepared in lysis buffer *(23)*, and ERK-1/2 or c-Jun N-terminal kinase (JNK) were immunoprecipitated from 100 μg of each cell extract using antibodies (1 μg) that recognize ERK1/2 or JNK (Santa Cruz Biotechnology) and protein-A sepharose beads (20 μL) (Amersham Pharmacia Biotech). Kinase assays were performed by incubating the beads with 30 μL of kinase buffer to which 5 μCi of [γ^{32}P]ATP (2000 cpm/pmol) and 1 μg of substrate (myelin basic protein [MBP; Calbiochem, La Jolla, CA] or GST–cJun [1–79] [Santa Cruz Biotechnology]) were added, as previously described *(27)*. The samples were suspended in Laemmli buffer, boiled for 5 minutes, and then analyzed by SDS–polyacrylamide gel electrophoresis. The gel was dried and autoradiographed. Total cell lysates from Jurkat cells (Upstate Biotechnology, Inc.) and H1299 NSCLC treated with 50 ng/mL IGF-I for 15 minutes were used as positive controls. Changes in the level of phosphorylation in MBP or GST-cJun reflect changes in Erk1/2 and JNK activity, respectively.

PI3K Assay

Approximately 5×10^6 1799 cells were treated with 10^{-7} *M* deguelin or 0.1% DMSO for different time periods (0–24 hours) in the absence of any additional stimulatory growth factors. PI3K in these cells was then activated by treatment with 50 ng/mL IGF-I for 15 minutes. Cells were lysed, and PI3K was immunoprecipitated from 500 μg of total cell extracts with 5 μL of rabbit antibody against full-length rat p85 PI3K (Upstate Biotechnology, Inc.), which coprecipitates the p110 catalytic subunit of PI3K, and 20 μL of protein A–sepharose beads (Amersham Pharmacia Biotech). PI3K activity in the immunoprecipitates was analyzed using bovine brain extract (Type I; Sigma-Aldrich), which contains a mixture of phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate as a substrate, as described *(29)*. Jurkat cell lysates (Upstate Biotechnology, Inc.) and IGF-I-activated H1299 NSCLC cell lysates were used as positive controls.

Generation of Adenoviral Vectors and Cell Infection Protocol

The pCMV6.Myr.Akt.HA vector plasmid and the vectors for adenovirus construction were kindly provided by Dr. Gordon Mills and Dr. Jack A. Roth (The University of Texas M. D. Anderson Cancer Center, Houston), respectively. Ad5CMV (parental virus) was used as a viral control. An adenoviral vector expressing a full-length Akt (also known as human protein kinase B) with an Src myristoylation signal fused in-frame to the c-Akt coding sequence, and an HA epitope (MyrAkt-HA) *(29)* under the control of cytomegalovirus (CMV) promoter (Ad5CMV-MyrAkt-HA) was constructed using the pAd-shuttle vector system *(27)*. Viral titers were determined by standard plaque assays and spectrophotometric analysis of DNA content. The presence of MyrAkt-HA in viral DNA was confirmed by DNA sequencing of the vector.

Cells were untreated or infected with either Ad5CMV-MyrAkt-HA or Ad5CMV as a viral control. Infection was allowed to occur for 2 hours in the absence of serum, and then the infected cells were suspended in fresh medium. After 3 days of incubation, the induced expression of MyrAkt-HA in 1799 cells and squamous HBE cells by the adenoviral vector was examined by western blot analysis for Akt and HA. The function of Ad5CMV-MyrAkt-HA was examined by a western blot analysis of cell lysates for pGSK-3β (Ser9), which is a downstream target of Akt.

To determine whether deguelin-induced antiproliferative effects on premalignant HBE cells were mediated through the inhibition of the PI3K/Akt pathway, 2×10^5 1799 cells/well or 1×10^6 squamous HBE cells/well in six-well plates were infected with 5×10^3 particles/cell of a control virus (Ad5CMV) or with 1×10^3 or 5×10^3 particles/cell of Ad5CMV-MyrAkt-HA, an adenoviral vector that expresses a constitutively active Akt (MyrAkt) in KSFM. After 1 day of infection, cells were treated with 10^{-7} *M* or 10^{-6} *M* deguelin, 2×10^{-6} *M* or 4×10^{-6} *M N*-(4-hydroxyphenyl)retinamide (4-HPR), or 0.1 % DMSO as a control and then incubated for 1 or 2 days. Apoptosis was analyzed using the APO-BrdU staining kit for TUNEL, western blot analyses for caspase-3, and the cleavage of PARP.

Northern Analysis

Approximately 1×10^7 to 2×10^7 NHBE cells and squamous HBE cells were lysed in 4 *M* guanidinium isothiocyanate, and total cellular RNA was extracted as described *(22).* RNA (20 μg per sample) was electrophoresed through a 1% agarose gel containing 2% formaldehyde, transferred to a nylon membrane (Zeta-Probe; Bio-Rad Laboratories, Hercules, CA), and hybridized to a [γ-^{32}P] dCTP (2′-deoxycytidine 5′-triphosphate)-labeled transglutaminase (TG) *(30)* or involucrine (Inv) *(31)* complementary DNA (cDNA), as described *(31).* Loading and integrity of each RNA sample was examined by observing the intensity of 18S and 28S in ethidium bromide-stained gels.

Statistical Analysis

Cell survival among groups was compared using Student's *t* tests. All means and 95% CIs from triplicate samples were calculated using Microsoft Excel software (version 5.0; Microsoft Corporation, Seattle, WA). In all statistical analyses, two-sided *P* values of <.01 were considered statistically significant.

Results

Responses of Normal, Premalignant, and Malignant HBE Cells to Deguelin

To determine whether deguelin could be a potential lung cancer chemopreventive agent, we first examined its effects on the growth of normal, premalignant (1799 and 1198), and malignant (1170-1) HBE cells, which together constitute an *in vitro* progressive lung carcinogenesis model *(21,22).* We used a concentration range *in vitro* that was attainable *in vivo.* The growth of premalignant and malignant HBE cell lines was inhibited by deguelin in a dose- and time-dependent manner (Fig. 2, A). After testing a range of concentrations from 10^{-9} *M* to 10^{-7} *M,* we determined that the IC_{50} for deguelin was less than 10^{-8} *M.* Deguelin had minimal effect on the growth of NHBE cells. Of all the cell lines, premalignant 1799 cells, which represent the earliest stage in the lung cancer model, were the most sensitive to deguelin, with exposure to 10^{-7} *M* deguelin for 1 day decreasing cell growth by 67.1% (95% CI = 64.1% to 70.1%). Because BEAS-2B cells have only a few of the properties of premalignant HBE cells *in vivo,* we also tested the effects of deguelin on cells from another immortalized cell line, HB56B. Dose- and time-dependent growth-inhibitory effects of deguelin in these cells were also detected (Fig. 2, A). These results suggest that deguelin preferentially inhibits growth of premalignant HBE cells.

We also examined whether the antiproliferative effects of deguelin were reversible. 1799 cells were treated with 10^{-7} *M* deguelin for 1, 2, or 3 days and then cultured in medium without deguelin for an additional 5 days. The growth of cells preexposed to deguelin continued to decline during incubation in fresh medium, indicating that the effects of deguelin on cell growth were irreversible (data not shown).

We assessed how deguelin affected cell growth by determining the effects of deguelin on the cell cycle by flow cytometry. Cell lines 1799 (Fig. 2, B), 1198, and 1170-1 (data not shown) treated with deguelin (10^{-8} *M* or 10^{-7} *M*) for 3 days accumulated in the G_2/M phase of the cell cycle. No detectable cell cycle changes were noted in NHBE cells treated with deguelin (10^{-7} *M*) for 3 days.

Effects of Deguelin on Apoptosis *In Vitro*

Because cells that accumulate in the G_2/M phase of the cell cycle often enter apoptosis, we hypothesized that deguelin may have inhibited growth by inducing apoptosis. In fact, cells that were treated with deguelin at greater than 10^{-8} *M* for 1 day showed morphologic changes typical of apoptosis, including membrane blebbing, increased refractoriness, and chromatin condensation (data not shown). We confirmed that 1799 cells treated with deguelin were undergoing apoptosis by TUNEL staining and flow cytometry analysis (Fig. 3, A). Although less than 1% of 1799 cells treated with DMSO underwent apoptosis, approximately 3.3% of 1799 cells treated with 10^{-9} *M* deguelin, 68.5% of cells treated with 10^{-8} *M* deguelin, and 92.2% of cells treated with 10^{-7} *M* deguelin for 3 days underwent apoptosis (Fig. 3, A).

A second test for apoptosis, DNA fragmentation analysis, showed the generation of nucleosomal-sized DNA fragments in 1799 cells treated with deguelin, but not with DMSO (Fig. 3, B). Fragmented DNA was detectable in 1799 cells after treatment with deguelin for 1 day. 1198 and 1170-1 cells treated with deguelin (10^{-8} *M* or 10^{-7} *M*) for 3 days also showed patterns similar to those of 1799 cells in TUNEL and DNA fragmentation analyses; however, treatment with deguelin for 1 day did not induce detectable apoptotic events in these cells (data not shown). NHBE cells treated with deguelin showed neither a TUNEL-positive cell population nor fragmented DNA.

We next assessed the expression of apoptosis-related enzymes (caspase-3 and PARP) and Bcl family members (Bcl-2, Bax, Bcl-xL). There was a decrease in the 32-kd caspase-3 proenzyme and a concomitant increase in the cleavage of the 113-kd

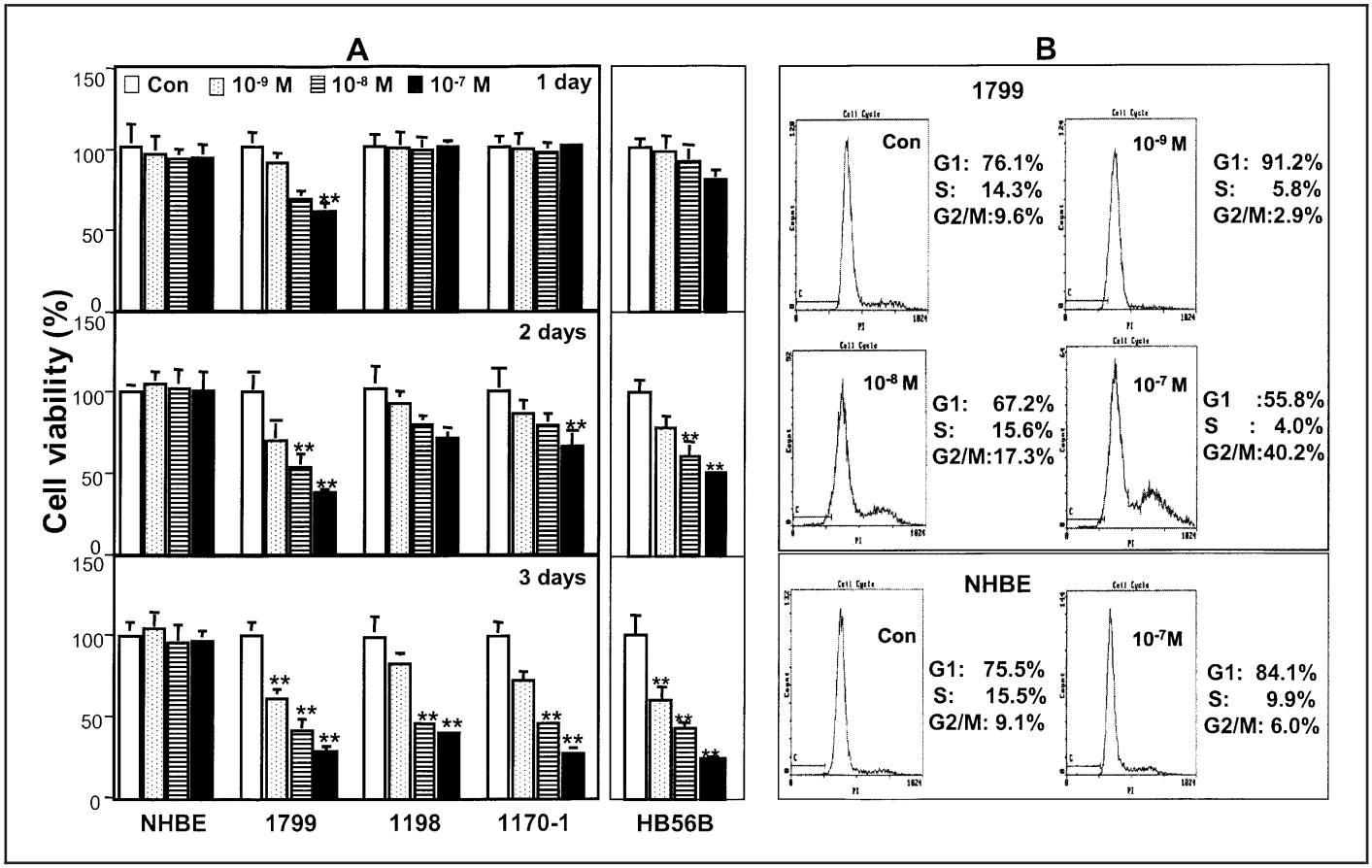

Fig 2. Effects of deguelin on the proliferation of normal human bronchial epithelial (NHBE), immortalized premalignant (1799 and 1198), malignant HBE (1170-1), and another immortalized HBE cell line, HB56B cells. **A**) Normal, 1799, 1198, 1170-1, and HB56B cells were seeded into 96-well culture plates (2×10^3 to 5×10^3 cells/well) and allowed to adhere overnight. The next day, the cells were treated with various concentrations of deguelin or with 0.1% dimethyl sulfoxide (DMSO) as a control. Cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay after 1, 2, or 3 days, as described *(27).* Results are expressed as percent cell proliferation relative to the proliferation of DMSO-treated cells (Con). Each bar represents the mean value of six identical wells from a representative single experiment (n = 3). **Error bars** show upper 95% confidence interval. **Open bars** = untreated control (Con); **dotted bars** = 10^{-9} *M* deguelin; **striped bars** = 10^{-8} *M* deguelin; **solid bars** = 10^{-7} *M* deguelin. ****, *P*<.001** for cells treated with deguelin relative to control cells for each series of experiments. **B**) Effects of deguelin on cell cycle distribution of 1799 cells and NHBE cells. 1799 cells and NHBE cells treated with 0.1% DMSO (Con) or the indicated concentrations of deguelin for 3 days were analyzed for DNA content (propidium iodide uptake) and for percentage of cells in specific phases of the cell cycle (G_1, S, and G_2/M) by flow cytometry as described *(28).* A representative experiment of two experiments is shown.

fragment of PARP to the 89-kd form in 1799 cells treated with deguelin (10^{-8} *M* or 10^{-7} *M*) for 3 days, indicating that deguelin activated caspase-3 (Fig. 3, C). Deguelin also induced a dose-dependent increase in the level of Bax and a slight decrease in Bcl-2 expression in 1799 cells (Fig. 3, C) but did not affect the level of Bcl-xL (Fig. 3, C). We observed changes in the levels of these proteins in 1198 and 1170-1 cells treated with deguelin that were similar to the changes observed in 1799 cells (data not shown).

Effect of Deguelin on Components of MAPK and PI3K/Akt Signaling Pathways in Premalignant HBE cells

We investigated whether MAPK and PI3K/Akt, which are important in regulating cell apoptosis and proliferation *(32–35)*, were involved in deguelin-mediated apoptosis in 1799 cells. Because the activities of MAPK and Akt are regulated by phosphorylation, we examined the levels of phosphorylated MAPK (pP44/42 MAPK) and pAkt in NHBE, premalignant (1799 and 1198), and malignant (1170-1) HBE cells treated with deguelin for different time periods. Basal levels of unphosphorylated or phosphorylated MAPK in NHBE, 1799, 1198, and 1170-1 cells were similar (Fig. 4, A). By contrast, basal levels of phosphorylated Akt were higher in premalignant (1799 and 1198) and malignant (1170-1) HBE cells than in NHBE cells (Fig. 4, A), although the levels of the unphosphorylated Akt and an unrelated protein (β-actin) were similar in these cells, suggesting that Akt was constitutively active in premalignant and malignant HBE cells. NHBE cells did not express a basal level of phosphorylated Akt; however, IGF-I could induce phosphorylation of Akt in NHBE cells (Fig. 4, B), indicating that the IGF-IR signaling pathway, which leads to Akt phosphorylation, is intact in NHBE cells.

To examine the effects of deguelin on MAPK and PI3K/Akt activities, 1799 cells were treated with 10^{-7} *M* deguelin for different time periods (0–24 hours), activated by treatment with 50 ng/mL IGF-I for 15 minutes, and then lysed. Erk1/2 was immunoprecipitated with anti-Erk1/2 antibody from the lysates, and kinase activity in the immunoprecipitates was analyzed by using MBP as a substrate. Deguelin had no discernible effect on

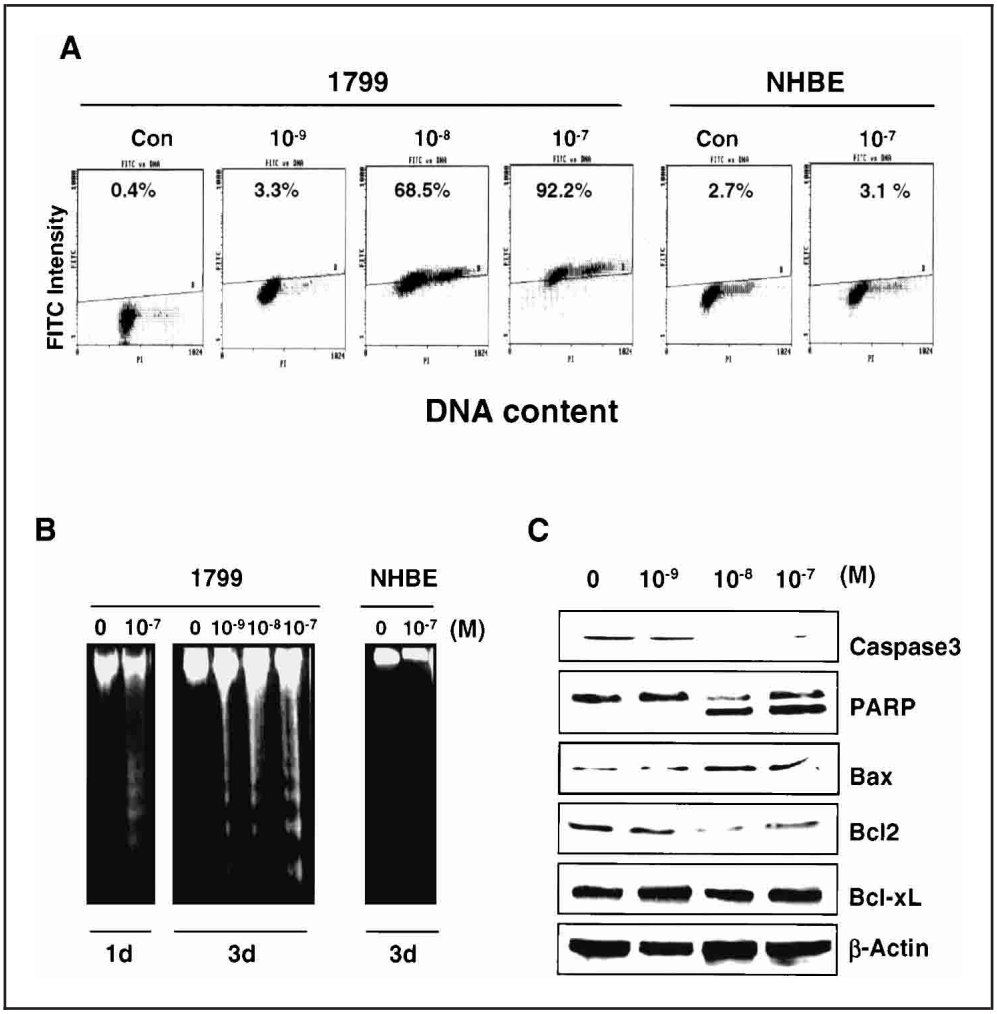

Fig. 3. Effect of deguelin on apoptosis in 1799 cells. **A**) 1799 and normal human bronchial epithelial (NHBE) cells were treated with deguelin (10^{-9} *M*, 10^{-8} *M*, or 10^{-7} *M*) or dimethyl sulfoxide (DMSO; 0.1%) for 3 days. Cells were processed for apoptosis with the APO-BrdU staining kit (Phoenix Flow Systems, San Diego, CA), a flow cytometry-based, modified terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay *(28)*. DNA content was determined by uptake of propidium iodide (*x*-axis). Apoptotic cells were determined by the intensity of fluorescein isothiocyanate (FITC) staining (*y*-axis). The number of apoptotic cells is represented by the number of FITC-positive cells of the total gated cells. Each value presented is the percentage of apoptotic cells. The percentage of dead cells was determined by flow cytometry analysis of propidium iodide-stained nuclei. Data shown are from a single representative experiment (n = 2). **B**) 1799 and NHBE cells were treated with deguelin or DMSO for 1 or 3 days. Nucleosomal DNA fragmentation was measured using the TACS apoptotic DNA laddering kit (Trevigen Inc., Gaithersburg, MD), according to the manufacturer's recommended protocol. Isolated DNA samples were electrophoresed through a 1.5% agarose gel and visualized by staining with ethidium bromide. **C**) 1799 cells were treated with DMSO (**lane 0**) or deguelin (10^{-9} *M*, 10^{-8} *M*, or 10^{-7} *M*) for 3 days. The expression of caspase-3 proenzyme (32K), cleavage of poly(ADP-ribose) polymerase (PARP), and expression of Bcl family members (Bax, Bcl-2, and Bcl-xL) were assessed by western blot analysis using rabbit polyclonal primary antibodies, horseradish peroxidase-conjugated anti-rabbit secondary antibodies, and enhanced chemiluminescence. Expression of β-actin was detected with a goat polyclonal antibody and was used as a loading comparison. Con = control.

ERK1/2 activity in 1799 cells (Fig. 4, C). In addition, the activity of JNK, a stress-induced MAPK that plays a role in regulating apoptosis *(36,37)*, was not affected by deguelin treatment (data not shown). Treatment of 1799 cells with 10^{-7} *M* deguelin resulted in a time-dependent decrease in the levels of pAkt and pGSK-3β, without affecting the levels of unphosphorylated proteins (Fig. 4, D).

We next measured the effect of deguelin on PI3K activity. PI3K was immunoprecipitated with anti-p85 antibody from total cell extracts derived from the 1799 cells treated with 10^{-7} *M* deguelin, activated with IGF-I, and tested in a kinase assay. Compared with PI3K activity from untreated cells, deguelin decreased PI3K activity approximately 55%; this decrease was not accompanied by decreased expression of the PI3K components (p85α and p110α) (Fig. 4, E). Total cell lysates from Jurkat cells and H1299 NSCLC cells treated with 50 ng/mL IGF-I for 15 minutes and from NHBE cells cultured in the absence of IGF-I activation were used as positive and negative controls, respectively. These findings indicated that deguelin appears to preferentially affect the PI3K/Akt signaling pathway in 1799 cells. Interestingly, the pAkt level was reduced relative to that in untreated cells after 7 hours of treatment (Fig. 4, D) and was virtually undetectable by 14 hours, although PI3K activity was still high during this time period (Fig. 4, E). This result suggests that deguelin may inhibit Akt activity through PI3K-independent pathways in addition to the PI3K-dependent pathway.

Effect of PI3K/Akt on Deguelin-Induced Death in Premalignant HBE Cells

To test the hypothesis that deguelin-induced apoptosis is mediated through the inhibition of the PI3K/Akt pathway, we constructed an adenovirus expressing a constitutively active form of Akt (Ad5CMV-MyrAkt-HA). We first tested its effects on endogenous Akt expression and activity in 1799 cells. Dose-dependent expression of the HA tag was detected in 1799 cells infected with Ad5CMV-MyrAkt-HA (Fig. 5, A). Compared with 1799 cells infected with a control adenovirus (Ad5CMV), expression of MyrAkt-HA, which had a slower mobility (i.e., larger molecular weight) than endogenous Akt, did not affect levels of endogenous Akt. However, the level of pGSK-3β, a downstream Akt target, was increased in 1799 cells infected with Ad5CMV-MyrAkt-HA, thus indicating an increase in Akt activity in these cells. Next, we infected 1799 cells with Ad5CMV-MyrAkt-HA and tested them for susceptibility to treatment with deguelin. 1799 cells infected with Ad5CMV-MyrAkt-HA showed an increase in cell survival, relative to cells

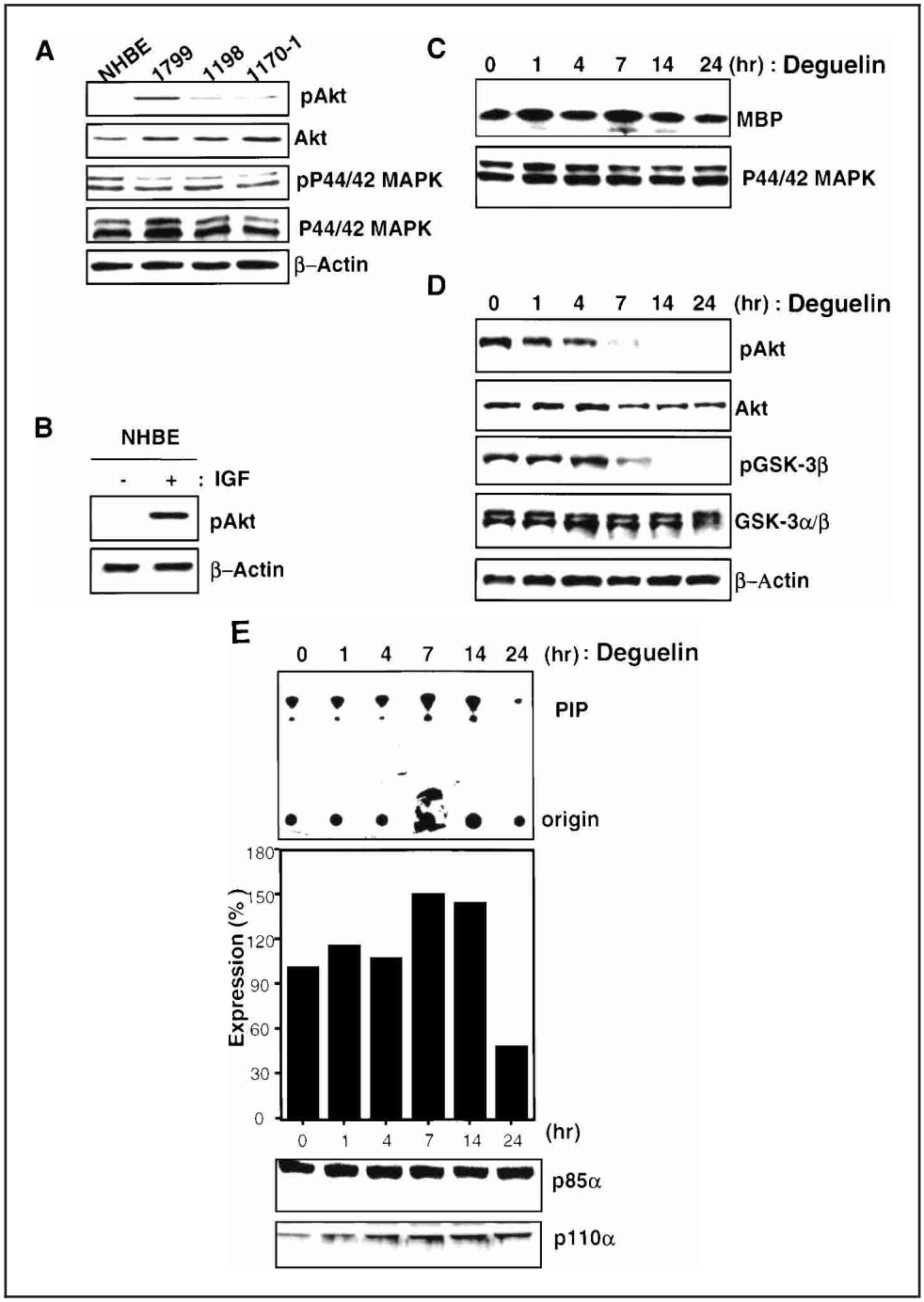

Fig. 4. Effect of deguelin on mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathway in human bronchial epithelial (HBE) cells. **A**) Basal levels of signaling proteins Akt and MAPK in normal, premalignant (1799 and 1198), and malignant (1170-1) HBE cells. Cells were grown for 24 hours in serum-free medium, and the expression of phosphorylated Akt (pAkt), Akt, phosphorylated MAPK (pP44/42 MAPK), and P44/42 MAPK proteins in 30 μg of whole cell extracts was assessed by western blot analysis. Western blots probed for β-actin were used to provide a loading comparison. **B**) Normal HBE (NHBE) cells that were cultured in serum-free medium for 24 hours and then treated with 50 ng/mL insulin-like growth factor I (IGF-I) for 15 minutes. Expression of pAkt in whole cell extracts from untreated (–) and IGF-I-treated (+) cells was examined by western blot analysis. **C–E**) 1799 cells were treated with 10^{-7} *M* deguelin for different time periods (0–24 hours) in serum-free medium and then with 50 ng/mL IGF-I for 15 minutes. The cells were lysed and centrifuged to remove cell debris. Aliquots of the supernatants were used for the analysis of MAPK activity with myelin basic protein (MBP) as a substrate and to determine levels of P44/42 MAPK (**C**). Western blot analyses were done to determine the levels of unphosphorylated Akt and pAkt and unphosphorylated glycogen synthase kinase 3α/β (GSK-3α/β) and phosphorylated GSK-3β (pGSK-3β), downstream mediators of PI3K (**D**), and analysis of PI3K activity (**E**). The PI3K activity of the immune complex was analyzed as described *(29)*. Because the substrate contained a mixture of phophatidylinositols, the PI3K products included mixtures of phosphatidylinositol phosphate (PIP) (**upper panel**). The data were quantified and expressed as a percentage of control (**middle panel**). The percentage of activity was calculated by using the following equation: % activity = A/C × 100, where A and C represent the density of the PIP on the phospholipase chromatography plate and the P85α protein band on the western blot for each time point and control, respectively. The expression levels of the PI3K components p85α and p110α for each time point were detected by western blot analysis (**lower panel**).

infected with Ad5CMV, in response to deguelin that was dependent on the viral load (Fig. 5, B). Compared with the growth of untreated control, growth of 1799 cells, which were uninfected or infected with 5×10^3 particles/cell of Ad5CMV and treated with 10^{-7} *M* deguelin for 2 days, was decreased by 54% (95% CI = 52.2% to 56.2%). However, growth of 1799 cells infected with 5×10^3 particles/cell of Ad5CMV-MyrAkt-HA and treated with 10^{-7} *M* deguelin was 85% (95% CI = 81.9% to 88.5%) that of control cell growth. Increasing the concentration of deguelin to 10^{-6} *M* did not decrease the growth of 1799 cells infected with Ad5CMV-MyrAkt-HA.

To test whether PI3K/Akt signaling is important in signal transduction pathways engaged by other pro-apoptotic agents that have effects similar to those of deguelin in HBE cells, we investigated the effects of constitutively active Akt on 4-HPR-induced apoptosis. In 1799 cells, 4-HPR (2–4 μ*M*)—concentrations that induce apoptosis—did not alter levels of pAkt and pGSK-3β (data not shown). Furthermore, among cells treated with 4-HPR, there was no difference in cell proliferation between control 1799 cells and 1799 cells overexpressing constitutively active Akt (Fig. 5, B). These data suggest that the Akt signaling pathway is not a generic response pathway for chemopreventive agents.

To determine whether expression of Ad5CMV-MyrAkt-HA in 1799 cells affects deguelin-induced apoptosis, we assessed apoptosis in 1799 cells infected with Ad5CMV-MyrAkt-HA and treated with deguelin. Treatment with deguelin (10^{-7} *M*) induced apoptosis in approximately 40% of 1799 cells or 1799 cells

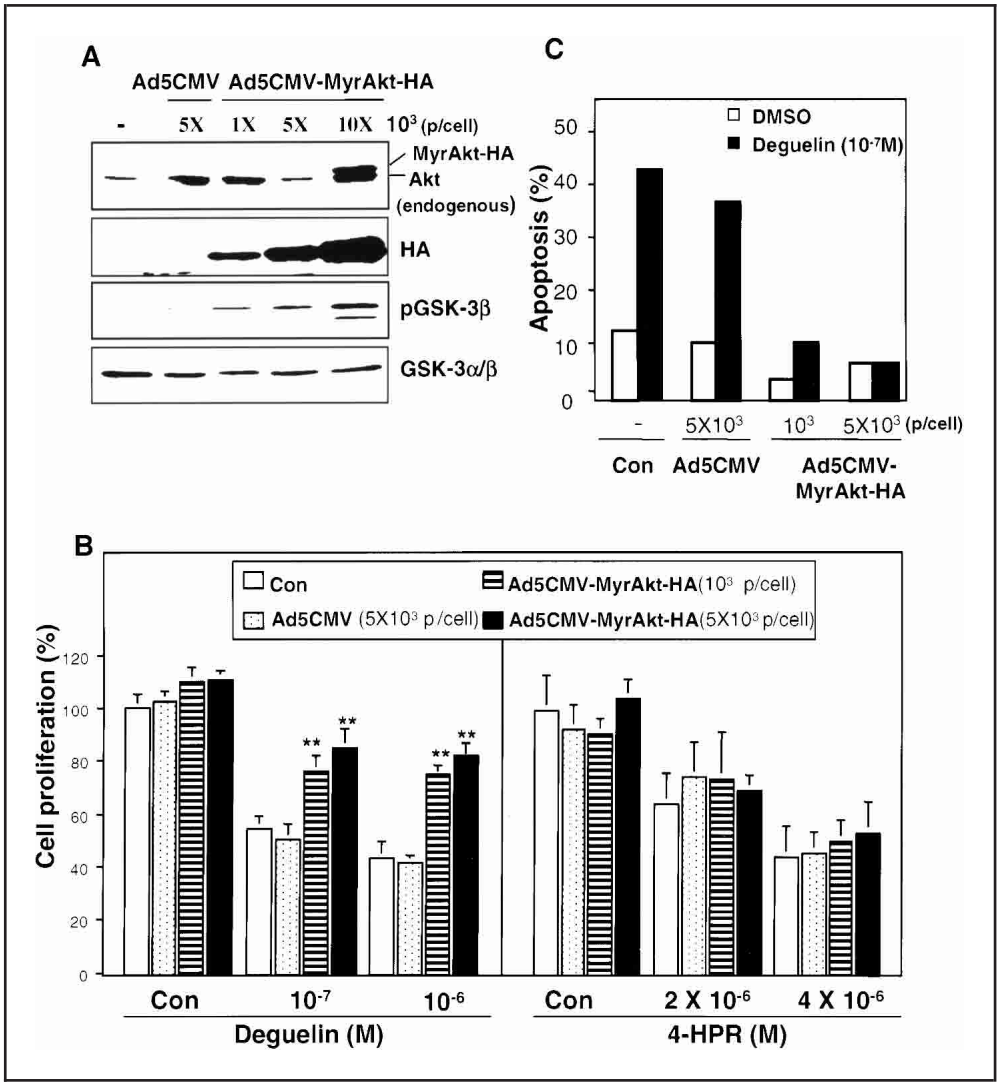

Fig. 5. Effect of deguelin on premalignant human bronchial epithelial (HBE, 1799) cells expressing a constitutively active Akt. **A**) 1799 cells were infected with Ad5CMV, a control adenovirus, or with Ad5CMV-MyrAkt-HA, an adenovirus that expresses a constitutively active Akt (MyrAkt) with a hemagglutinin (HA) tag. Cells were infected with different numbers of viral particles per cell (10^3 p/cell), and 3 days later, the expression levels of Akt, HA, phosphorylated glycogen synthase kinase (pGSK-3β), and GSK-3α/β were analyzed by western blot analysis using rabbit polyclonal (Akt, pGSK-3β, or GSK-3α/β) or mouse monoclonal (HA) antibodies, horseradish peroxidase-conjugated species-specific secondary antibodies, and enhanced chemiluminescence. Protein levels were compared with those from uninfected 1799 cells. **B**) Control (uninfected [Con]) 1799 cells or 1799 cells infected with Ad5CMV or different concentrations of Ad5CMV-MyrAkt-HA were treated with deguelin (10^{-7} *M* or 10^{-6} *M*) or *N*-(4-hydroxyphenyl)retinamide (4-HPR) (2×10^{-6} *M* or 4×10^{-6} *M*) for 2 days. Proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay *(27)*. Results are expressed as percent cell proliferation relative to the proliferation of dimethyl sulfoxide (DMSO)-treated uninfected cells. Each bar represents the mean value of six identical wells from a representative single experiment (n = 3). **Error bars** show upper 95% confidence intervals. ****, *P*<.001** for cells treated with deguelin relative to control cells for each series of experiments. **C**) Uninfected control 1799 cells or 1799 cells infected with Ad5CMV (5×10^3 viral p/cell) or Ad5CMV-MyrAkt-HA (1×10^3 or 5×10^3 viral p/cell) and treated with DMSO or deguelin (10^{-7} *M*) for 2 days. Cells were processed for apoptosis with the APO-BrdU staining kit (Phoenix Flow Systems, San Diego, CA), a flow cytometry-based, modified terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay. The number of apoptotic cells is represented by the number of fluorescein isothiocyanate (FITC)-positive cells of the total gated cells. Representative data are shown from a single experiment (n = 2).

infected with the control adenovirus but in less than 10% of 1799 cells infected with Ad5CMV-MyrAkt-HA (Fig. 5, C), which suggests that the induction of apoptosis by deguelin in 1799 cells results, at lease in part, from an inhibition of the PI3K/Akt-mediated anti-apoptotic pathway.

Effects of Deguelin on Squamous HBE Cells

The premalignant and malignant cell lines used in this study were derived from an HBE cell immortalized with a hybrid adenovirus/simian virus 40 *(21)*. Adenovirus interaction with α_v integrins, an event required for adenovirus internalization, also activates PI3K *(38)*. Thus, we sought to confirm that the increased level of pAkt in 1799 cells was related to the stage of disease and was not an artifact of the cell line's origin by assessing the level of pAkt in squamous HBE cells. These cells mimic bronchial metaplasia, a potentially premalignant lesion induced by tobacco smoke *(25)*.

Squamous HBE cells express higher levels of TG and Inv than NHBE cells *(25)*. To confirm that we had indeed induced squamous HBE cells by culturing them on fibronectin and collagen, we assessed expression of TG and Inv in NHBE and squamous HBE cells by northern blot analysis and found that both mRNAs were expressed (Fig. 6, A, left). We next examined expression of pAkt and pGSK-3β in squamous HBE cells to determine whether the PI3K/Akt pathway was constitutively active in these cells. Although the expression of unphosphorylated Akt and GSK-3α/β was similar in NHBE and squamous HBE cells, the level of pAkt and pGSK-3β was markedly higher in squamous HBE cells than in NHBE cells (Fig. 6, A, middle), suggesting that the PI3K/Akt pathway was activated in squamous HBE cells. We then examined whether deguelin would inhibit PI3K/Akt activity in squamous HBE cells. Treatment with deguelin decreased the levels of pAkt and pGSK-3β in a time-dependent manner (Fig. 6, A, right).

Next, we examined whether deguelin induced apoptosis in squamous HBE cells. Treatment of squamous HBE cells with deguelin (10^{-9} *M* to 10^{-7} *M*) for 1 day induced some of the morphologic changes typical of apoptosis (Fig. 6, B), decreased the inactive form of caspase-3, and concomitantly increased

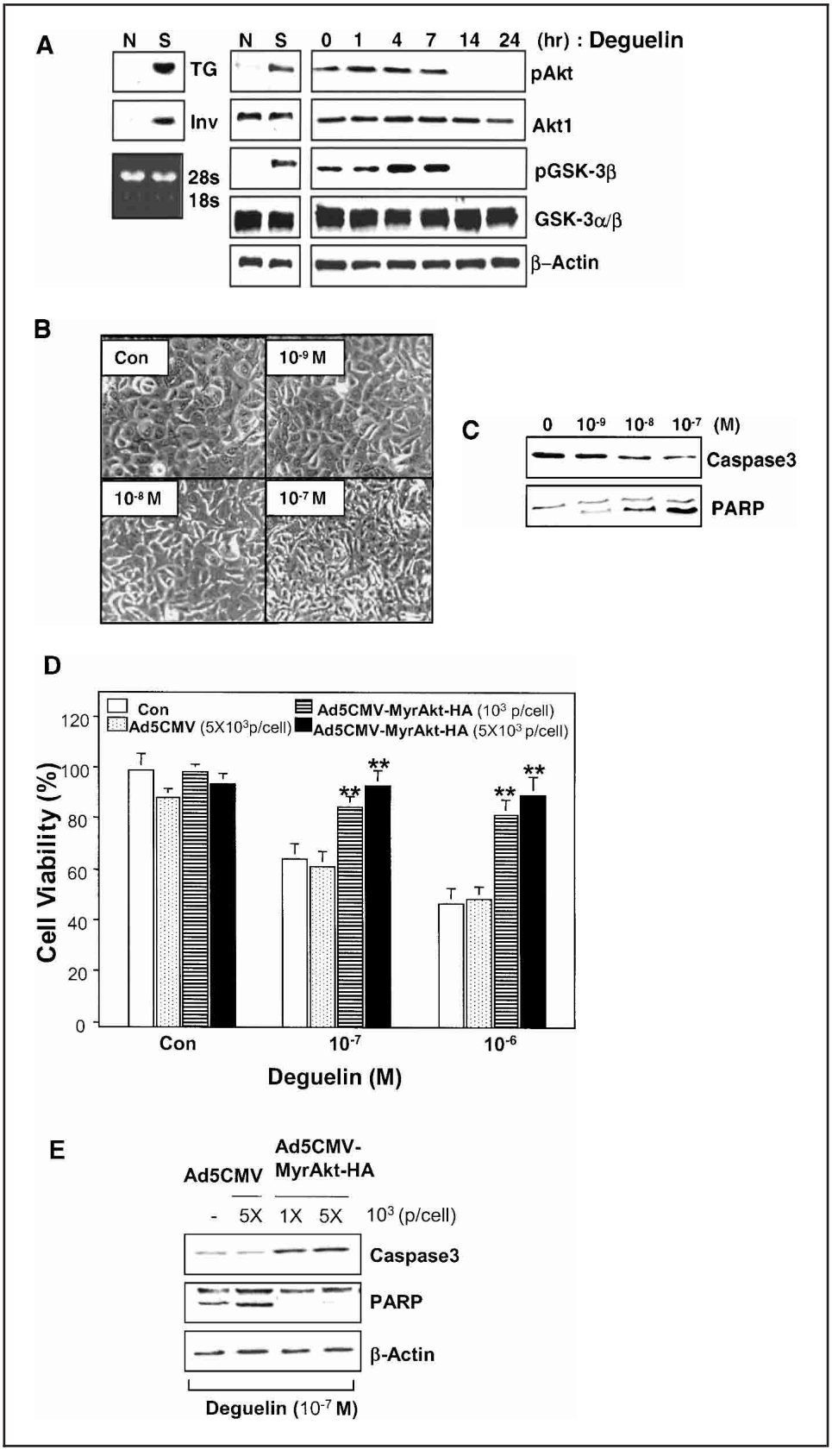

Fig. 6. Effects of deguelin on squamous human bronchial epithelial (HBE) cells. Squamous HBE cells were induced by culturing HBE cells on 96-well culture plates that were coated with fibronectin (10 μg/mL) and collagen (30 μg/mL) for 1 hour. **A**) Total RNA from normal (N) and squamous (S) HBE cells was subjected to northern blot analysis and probed for the mRNA expression of transglutaminase (TG) and involucrine (Inv) (**left panel**). Ethidium bromide staining of 28S and 18S ribosomal RNA is shown to provide a loading comparison. N and S HBE cells were untreated (**middle panel**) or incubated in serum-free medium in the absence or presence of deguelin (10^{-7} *M*) for 0–24 hours (**right panel**). Cells were lysed, and the lysates (30 μg) were used for western blot analysis to detect phosphorylated Akt (pAkt), Akt, phosphorylated glycogen synthase kinase (pGSK)-3β, and unphosphorylated GSK-3α/β using rabbit polyclonal (Akt, pGSK-3β, and GSK-3α/β) antibodies, horseradish peroxidase-conjugated anti-rabbit secondary antibodies, and enhanced chemiluminescence. β-Actin, detected using a goat polyclonal antibody, was included to provide a loading comparison. Squamous HBE cells that were untreated (Con) or treated with deguelin (10^{-9} *M*, 10^{-8} *M*, 10^{-7} *M*) for 1 day (**B**) were analyzed for the expression of the inactive form of caspase-3 and cleavage of poly(ADP-ribose) polymerase (PARP) by western blot analysis using rabbit polyclonal antibodies (**C**). **D**) Squamous HBE cells were left uninfected (Con), infected with a control adenovirus (Ad5CMV) at 5×10^3 particles per cell (p/cell), or infected with an adenovirus that expresses constitutively active Akt (Ad5CMV-MyrAkt-HA) at 1×10^3 or 5×10^3 p/cell. Cells were then treated with deguelin (10^{-7} *M* or 10^{-6} *M*) for 1 day. Proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay *(27)*. Results are expressed as percent cell proliferation relative to the proliferation of control uninfected cells. Each bar represents the mean value of six identical wells from a representative single experiment (n = 3). **Error bars** show upper 95% confidence intervals. ****,** *P*<.001 for cells treated with deguelin relative to control cells for each series of experiments. **E**) Uninfected squamous HBE cells or squamous HBE cells infected with Ad5CMV (5×10^3 p/cell) or Ad5CMV-MyrAkt-HA (1×10^3 or 5×10^3 p/cell) were treated with deguelin (10^{-7} *M*) for 1 day. Whole cell extracts were prepared and analyzed for expression of caspase-3 proenzyme (32K) and cleavage of PARP by western blot analysis. Expression of β-actin was included to provide a loading comparison.

PARP cleavage, all characteristics of cells undergoing apoptosis (Fig. 6, C).

To test whether deguelin induced apoptosis in squamous HBE cells by inhibiting the PI3K/Akt pathway, squamous HBE cells were infected with Ad5CMV or Ad5CMV-MyrAkt-HA and were treated with deguelin (10^{-7} *M* or 10^{-6} *M*) for 1 day. Deguelin decreased proliferation of control squamous HBE cells in a dose-dependent manner (Fig. 6, D). However, the growth of squamous HBE cells that overexpressed constitutively active Akt and were treated with deguelin (10^{-7} *M* to 10^{-6} *M*) for 1 day

was approximately 95% (95% CI = 92.8% to 96.6%) of the growth of untreated cells (Fig. 6, D). In addition, deguelin induced a loss of caspase-3 and a concomitant increase in PARP cleavage in squamous HBE cells (control or infected with Ad5CMV), but it induced an increase in caspase-3 and a decrease in PARP cleavage in squamous HBE cells infected with Ad5CMV-MyrAkt-HA (Fig. 6, E), indicating that deguelin induction of apoptosis in squamous HBE cells involved inhibition of the PI3K/Akt pathway.

Discussion

Chemoprevention targets the multistep process of carcinogenesis with chemical agents that delay, reverse, or block cancer development *(6).* The exposure of aerodigestive tract epithelium to carcinogenic and tumor-promoting agents often leads to histologic changes over large areas of the tissue, resulting in a field cancerization effect with potential multifocal, unsynchronized premalignant and primary malignant lesions *(39).* The cell lines used in this study, which included normal, premalignant (1799 and 1198), and malignant (1170-1) HBE cells, evolved from the same precursor cells and are therefore a useful *in vitro* lung carcinogenesis model for screening chemopreventive agents and investigating the mechanisms involved in lung carcinogenesis.

Our results demonstrate that deguelin inhibited the growth of premalignant and malignant HBE cells, with minimal effects on the growth of NHBE cells. The premalignant cell line 1799 was the most sensitive of the lines tested to deguelin-induced antiproliferative effects, indicating that deguelin may have potential as a chemopreventive agent against lung cancer. Premalignant and malignant HBE cells treated with deguelin accumulated in the G_2/M phase of the cell cycle and underwent apoptosis in a dose- and time-dependent manner. We found that deguelin increased the expression of Bax and decreased the expression of Bcl-2 in premalignant and malignant HBE cells, suggesting that changes in the ratio of Bax to Bcl-2 might contribute to the apoptosis-promoting activity of deguelin in these cells. However, changes in expression levels of Bcl family members were also observed in malignant HBE cells treated with deguelin, suggesting that other mechanisms may contribute to the sensitivity of premalignant HBE cells to deguelin.

In our ongoing efforts to determine the mechanism that mediates the effects of deguelin on premalignant HBE cells, we studied the involvement of the PI3K/Akt pathway. The PI3K/Akt pathway has been implicated in the development of multiple human cancers *(40–43).* The isolation of a retroviral oncogene, v-p3k, coding a homologue of the PI3K p110 catalytic subunit, established that PI3K has an active role in oncogenic transformation *(40).* PIK3CA, the alpha catalytic subunit of PI3K, encoding p110α, is amplified in human ovarian cancer cell lines *(41)*, and an oncogenic mutant of p85 can transform mammalian fibroblasts when introduced with the v-raf oncogene *(42).* In addition, mammalian fibroblasts transfected with a constitutively active form of p110α have a partially transformed phenotype *(43).* Akt, an important and probably essential downstream component of PI3K-mediated oncogenic signaling *(42–44)*, provides a critical cell survival signal for tumor progression by phosphorylating a number of proteins involved in cell cycle regulation and pro-apoptotic factors *(27,43–45).*

We found that Akt is constitutively active in premalignant and malignant HBE cells but not in NHBE cells. Because Akt activity is higher in 1799 cells (an immortalized HBE cell line) than in 1198 cells (a carcinogen-exposed immortalized HBE cell line) or in 1170-1 cells (a malignant HBE cell line), we suggest that Akt activation is an early event during lung carcinogenesis. Akt overexpression has been observed in early sporadic colon carcinogenesis *(46)*, in normal ovarian surface epithelium from women with germline BRCA mutations *(47)*, and in premalignant mammary hyperplasia, which has an increased risk of progressing to a tumor *(48).* These findings suggest that increased Akt activity resulting from Akt overexpression or constitutive activation from upstream molecules is a common feature during the early stages of carcinogenesis and that inhibition of Akt might be a potential target for a chemoprevention strategy.

Our data provide evidence that deguelin is a good potential chemopreventive agent, selectively blocking Akt activation and inducing apoptosis. The overexpression of constitutively active Akt blocked 1799 cells from undergoing deguelin-induced apoptosis, although it did not rescue these cells from 4-HPR-induced apoptosis, indicating that the inhibition of Akt activity by deguelin is an important mechanism of action in 1799 HBE cells. Exactly how deguelin inhibits Akt activity is unclear. Loss of Akt activity in response to deguelin was evident while substantial PI3K activity was still detectable, suggesting that deguelin inhibits Akt activity by a PI3K-independent mechanism. Indeed, Akt can be activated by a PI3K-independent mechanism that involves the Ca^{2+}/calmodulin-dependent protein kinase kinase, in which the increase in the intracellular Ca^{2+} concentration promotes survival of some cultured neurons *(49).* We also observed that treatment of deguelin inhibits PI3K/Akt activity in 1198 and 1170-1 cells and that constitutively active Akt reduced the apoptotic effects of deguelin (data not shown). We examined whether this unique mechanism applied to other PI3K inhibitors and found that LY294002, a representiative PI3K inhibitor that blocks ATP binding to the p110α PI3K catalytic domain *(50),* was less effective than deguelin at inhibiting the growth of premalignant HBE cells (Lee HY: unpublished data); LY294002 concentrations higher than 10 μ*M* were required to induce detectable growth inhibition in premalignant and malignant HBE cells, and those concentrations were cytotoxic to NHBE cells. The difference in efficacy between deguelin and LY294002 warrants investigation in view of the potential chemotherapeutic effect of deguelin.

Taken together, our results provide the first evidence, to our knowledge, that Akt is constitutively active in premalignant HBE cell lines and that deguelin can be a potential chemopreventive agent against lung cancer by targeting Akt activity. Deguelin blocks proliferation of premalignant and malignant HBE cells by inducing apoptosis. Deguelin is active at nanomolar levels, with no apparent cytotoxicity to normal HBE cells. Although rotenone is a pesticide, the U.S. Environmental Protection Agency, the National Center for Toxicological Research, and the National Toxics Rule have shown rotenone and other rotenoid-containing extracts to be noncarcinogenic and relatively nontoxic by oral administration (LD_{50} = 132 mg/kg in rats). A pharmacokinetic study in rats showed that a nontoxic dose of deguelin (1 μ*M*), delivered intravenously or by oral gavage, is achievable in a variety of tissues *in vivo,* including lung tissue *(51).* Therefore, dosages of deguelin that induce apoptosis *in vitro* may be attainable *in vivo* in other models without potential side effects. Deguelin selectively blocks Akt activity in a PI3K-dependent or -independent manner, thereby attenuating the activity of a major anti-apoptotic pathway. The role of

deguelin as an inhibitor of Akt activation also has clinical implications, especially in the treatment of NSCLC, in which constitutive activation of Akt occurs with high frequency *(52)*. It has been reported that the manipulation of Akt activity, addition of a PI3K inhibitor, or transfection of kinase-dead Akt into cells with high levels of Akt activity dramatically increased sensitivity of NSCLC cells to chemotherapy and irradiation *(52)*. Therefore, targeting Akt with deguelin may increase the efficacy of chemotherapy and radiation therapy and increase the apoptotic potential of NSCLC cells. How deguelin inhibits Akt activity in a PI3K-independent manner warrants further investigation.

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NOTES

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