# Deguelin-Induced Inhibition of Cyclooxygenase-2 Expression in Human Bronchial Epithelial Cells

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## ABSTRACT

**The increased expression of cyclooxygenase (COX)-2 significantly enhances carcinogenesis and inflammatory reactions, and its regulation may be a reasonable target for cancer chemoprevention. We demonstrated previously that deguelin inhibits proliferation of premalignant human bronchial epithelial (HBE) cells, such as 1799 cells and squamous HBE cells, by regulating phosphatidylinositol-3-kinase Akt activity, which is involved in COX-2 expression. We sought to determine the effect of deguelin on COX-2 expression in squamous HBE cells. Deguelin strongly inhibited COX-2 expression in squamous HBE cells, without affecting the COX-1 protein level. Deguelin inhibited proliferation of a variety of non-small cell lung carcinoma (NSCLC) cell lines through apoptosis and induced Bax expression in the H322 NSCLC and squamous HBE cells. Deguelin treatment did not affect Bcl-2 protein levels but increased expression levels of the proapoptotic protein p53 and the cyclin-dependent kinase inhibitors p21 and p27 in the squamous HBE cells. The sensitivity of the squamous HBE and NSCLC cells to deguelin and the inhibitory effects of deguelin on COX-2 expression in the squamous HBE cells indicate that regulation of COX-2 expression is involved in the chemopreventive action of deguelin in lung cancer.**

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## INTRODUCTION

In the United States, lung cancer leads all other cancers in both incidence and associated mortality rate (1). Despite recent advances in radiotherapy and chemotherapy, the severe morbidity associated with lung cancer and the 5-year survival rates have not improved (1). Hence, new approaches for the treatment of lung cancer are needed. Because early detection and effective chemoprevention together may constitute the most promising clinical approach, much effort has been focused on developing novel chemopreventive compounds, especially agents that inhibit enzyme activity associated with carcinogenesis.

An expanding body of evidence has demonstrated the chemopreventive effect of nonsteroidal anti-inflammatory drugs, such as indomethacin, aspirin, piroxicam, and sulindac, all of which inhibit cyclooxygenase (COX) and significantly reduce the risk of colorectal, esophageal, gastric, lung, and breast cancers (2–6).

Two isoforms of COX, each of which displays a distinct physiological profile, have been identified. COX-1, which is constitutively expressed in almost all tissues, is important for maintaining homeostatic function, whereas COX-2, an inducible isozyme, is up-regulated during certain pathological conditions (7).

The *cox-2* gene, an immediate early response gene, is rapidly induced in response to tumor promoters, cytokines, and growth factors. COX-2 is involved in the conversion of arachidonic acid to proinflammatory substances and activation of carcinogens to damage genetic material. Because increasing evidence has shown the critical role of COX-2 in carcinogenesis (7, 8), considerable interest has been focused on COX-2 inhibitors in the development of chemopreventive strategies. The importance of COX-2 in cancer chemoprevention is supported by data showing that COX-2 is dramatically up-regulated in transformed cells and in various forms of cancer, including colorectal adenocarcinoma (9–11), gastric carcinoma (12), prostate cancer (13), pancreatic adenocarcinoma, and pulmonary adenocarcinomas (14–17). The premise that COX-2 is involved in the pathological processes of cancer growth and progression is further supported by the results of animal studies showing that tumorigenesis is inhibited in COX-2 knockout mice (3, 18). These findings support the use of chemopreventive strategies that target COX-2. Although the pharmacological action of traditional nonsteroidal anti-inflammatory drugs has been widely accepted, the adverse side effects resulting from their inhibition of COX-1, a key enzyme in the production of physiologically important prostaglandins, are serious enough to restrict their use as chemopreventive agents (7, 19, 20). Thus, immense effort has been devoted to developing molecules that selectively and potently inhibit COX-2 expression while weakly affecting COX-1 expression.

We demonstrated previously that deguelin, a natural product isolated from *Mundulea sericea* Willd. (Leguminosae), inhibits the growth of premalignant human bronchial epithelial

(HBE) cell lines by inducing apoptosis but has minimal effects on normal HBE (NHBE) cells (21). We also showed that the ability of deguelin to inhibit phosphatidylinositol 3′-kinase (PI3K)/Akt-mediated signaling pathways contributes to its antiproliferative effects. Akt activity is largely responsible for the stabilization of COX-2 mRNA (22), and the protection from celecoxib-induced apoptosis by insulin-like growth factor type I correlates with an increase in the levels of activated Akt (23). These observations strongly suggest that deguelin has potential as a COX-2 inhibitor.

In the present study, we sought to elucidate the effects of deguelin on COX-2 expression in squamous HBE cells. In addition, we examined the growth-inhibitory effect of deguelin on non-small cell lung carcinoma (NSCLC) cells because cancer chemoprevention targets the multistep process of carcinogenesis with chemical agents that delay, reverse, or block cancer development (24), and inhibitors of cancer cell proliferation are known to be useful chemopreventive agents (25).

## MATERIALS AND METHODS

**Cells and Materials.** NSCLC cell lines were purchased from American Type Culture Collection and routinely maintained in RPMI 1640 supplemented with 10% FCS and 100 units/ml penicillin and streptomycin in a humidified environment with 5% $CO_2$. NHBE cells were prepared from bronchial epithelium harvested from fresh surgical specimens obtained from patients who had undergone pulmonary lobectomy procedures at The University of Texas M. D. Anderson Cancer Center, as described previously (26). For each experiment, NHBE cells from a single patient were used. Squamous differentiation was induced by growing HBE cells to confluence on 100-mm tissue culture plates coated with a matrix of fibronectin (Upstate Biotechnology, Lake Placid, NY) and collagen (Upstate Biotechnology) as described previously (27). Two days after the confluence was attained, deguelin was added. The stereospecific deguelin, which was synthesized in three steps from the natural product rotenone and conserves the cis-(7aS,13aS) configuration of the natural product, was used (28). The synthetic material conformed to the natural product as evidenced by identical $^{1}H$ and $^{13}C$ nuclear magnetic resonance, UV-visible, liquid chromatography-mass spectrophotometry, and optical rotation.

Western blot analysis was performed using murine polyclonal anti-COX-1 and anti-COX-2 antibodies (Cayman Chemical Co., Ann Arbor, MI), rabbit polyclonal anti-Bax, anti-Bcl-2, anti-p21, anti-p27, and anti-p53 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

**Immunoblotting.** Western blot analysis was performed using 30 μg of whole cell lysate as described previously (21). The immunoblots were visualized using the enhanced chemiluminescence kit (Amersham, Arlington Heights, IL), in accordance with the manufacturer's directions.

**Measurement of Cell Proliferation.** To measure the effects of deguelin on cell proliferation, NSCLC cell lines were seeded at $1 \times 10^3$ to $2 \times 10^3$ cells/well in 96-well plates. Squamous HBE cells were cultured on extracellular matrix-coated 96-well plates in confluence for 2 days, and then the cells were changed to fresh medium containing various concentrations of deguelin dissolved in DMSO (final concentration, 0.1%). Control cells received 0.1% DMSO. After the cells were incubated for 3 days, the growth of the treated cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, as described previously (26). Six replicate wells were used for each analysis. The drug concentration required to cause 50% cell growth inhibition ($IC_{50}$) was determined by interpolation from dose-response curves.

**Apoptosis Assay.** Apoptosis was measured using the APO-bromodeoxyuridine staining kit (Phoenix Flow Systems, San Diego, CA), as described previously (26). Briefly, $10^6$ H322 and squamous HBE cells were treated with deguelin at a concentration of $1 \times 10^{-6}$ M and $1 \times 10^{-7}$ M, respectively, and then allowed to grow for 0, 1, 2, and 3 days. Floating and adherent cells were analyzed using a fluorescence-activated cell sorter flow cytometer (Becton Dickinson, San Jose, CA) to determine the percentage of apoptotic cells. The percentage of dead cells was determined by fluorescence-activated cell-sorting analysis of propidium iodide-stained nuclei.

## RESULTS

**Deguelin Inhibits COX-2 Expression in Squamous HBE Cells.** COX-2 protein levels were determined in the NHBE and squamous HBE cells that were untreated or treated with $10^{-10}$ to $10^{-7}$ M deguelin for 1 day by Western blot analysis (Fig. 1). Squamous HBE cells mimic bronchial metaplasia, a potentially premalignant lesion induced in smokers (27). After induction of squamous differentiation, COX-1 expression was not changed, whereas COX-2 expression was induced (Fig. 1*A*). The nonspecific bands indicated equal protein loading. Deguelin inhibited COX-2 expression in the squamous HBE cells in a dose-dependent manner (Fig. 1*B*).

**Deguelin Inhibits the Growth of NSCLC Cell Lines.** In a previous study, we demonstrated that deguelin inhibits the growth of and induces apoptosis in premalignant and malignant HBE cell lines, with minimal effects on NHBE cells at *in vitro* dosages attainable *in vivo*, indicating the potential of deguelin as a chemopreventive agent and a therapeutic agent against lung cancer (21).

In this study, we further examined the growth-inhibitory effect of deguelin on NSCLC cells. Squamous HBE cells were

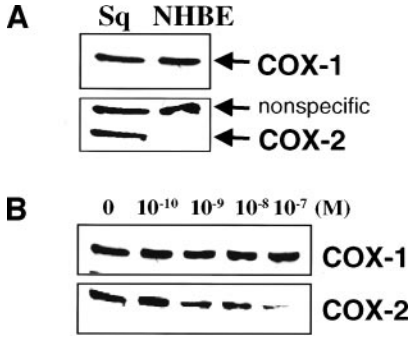


*Fig. 1* Regulation of cyclooxygenase-2 expression by deguelin in squamous human bronchial epithelial (HBE) cells. Western blot analysis was performed in (*A*) normal HBE cells (NHBE) and squamous HBE cells (Sq) and in (*B*) squamous HBE cells that were either untreated or treated with the indicated doses of deguelin for 1 day.

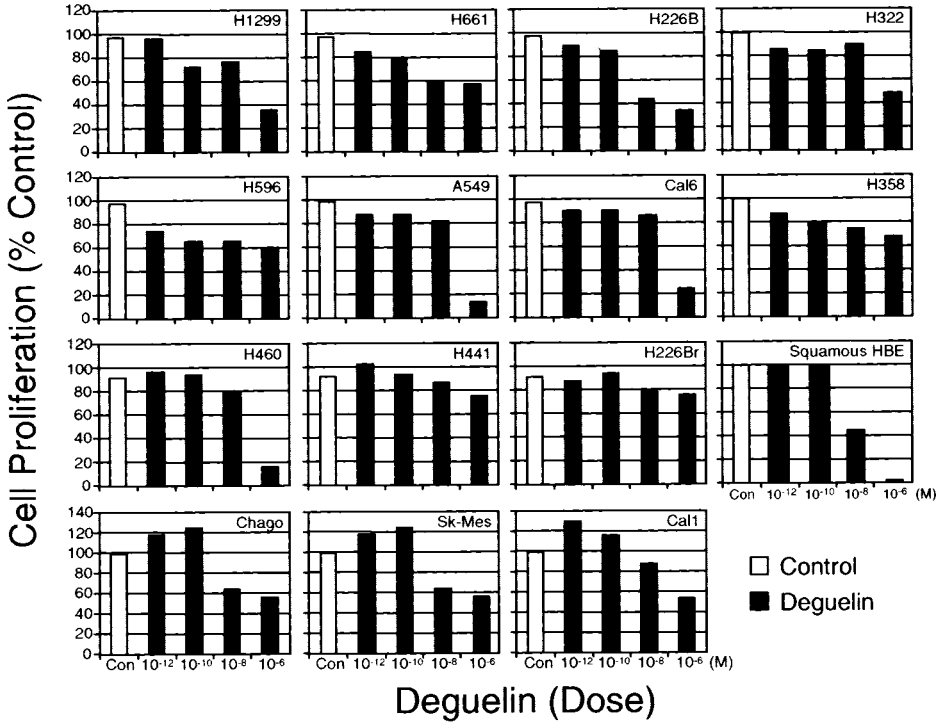


*Fig. 2* *A*, effects of deguelin treatment on non-small cell lung carcinoma (NSCLC) cells and squamous human bronchial epithelial cells. The indicated NSCLC cell lines and squamous human bronchial epithelial cells were treated with the indicated doses of deguelin for 3 days. The incubations were carried out in keratinocyte serum-free medium containing 2 ng/ml epidermal growth factor. After the treated cells were incubated for 3 days, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed. Results are expressed relative to the cell density of untreated cells. Each value is the mean of 5 identical wells.

also included. NSCLC cell lines and squamous HBE cells were treated with $10^{-12}$ to $10^{-6}$ M deguelin for 3 days, and then cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Fig. 2 depicts the dose-dependent growth-inhibitory effect of deguelin on the NSCLC cell lines after 3 days of treatment. The $IC_{50}$ of the deguelin in NSCLC cell lines ranged from $10^{-7}$ to $10^{-5}$ M, which is higher than that in the squamous HBE cells ($10^{-8}$ M). The growth of the NHBE cells was not affected by treatment with deguelin, consistent with previous reports (21).

**Induction of Apoptosis in NSCLC Cells by Deguelin.** We investigated the mechanism by which deguelin inhibited NSCLC cell proliferation. Apoptosis appears to be a major mechanism by which deguelin regulates proliferation of premalignant HBE cells (21). Therefore, the evidence of apoptosis was sought in the H322 NSCLC cell line. H322 cells were treated with $10^{-6}$ M deguelin for 1–3 days, and then the effect was compared with that in the squamous HBE cells. Flow cytometry after terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling showed that $10^{-6}$ M deguelin had a potent apoptotic activity in H322 cells in a time-dependent manner (Fig. 3). The ability of deguelin to induce apoptosis was stronger in squamous HBE cells than in H322 cells, confirming the effectiveness of deguelin as a chemopreventive agent in lung cancer.

In light of the importance of the expression of the Bcl protein family in cell survival and apoptosis (9–13), we evaluated the levels of Bcl-2 and Bax in H322 cells and squamous HBE cells treated with the indicated doses of deguelin for 3 days or with $10^{-8}$ M deguelin for the indicated times. Western blot analysis revealed that the Bcl-2 level in these cells remained virtually unchanged throughout the course of apoptotic death in response to deguelin (Fig. 4), whereas Bax expression was induced by deguelin treatment in a time- and dose-dependent manner, suggesting that deguelin alters the Bax:Bcl-2 ratio in NSCLC cells and squamous HBE cells.

To gain further insight into the mechanism by which deguelin induces apoptosis, we examined the effects of deguelin on p53, p21, and p27 protein levels in squamous HBE cells. Deguelin at a concentration of $10^{-8}$ M markedly increased p53

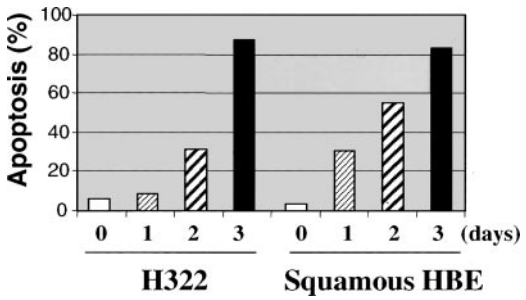


*Fig. 3* Apoptosis in H322 non-small cell lung carcinoma (NSCLC) cells and squamous human bronchial epithelial (HBE) cells. Flow cytometry was performed on H322 NSCLC cells and squamous HBE cells treated with $1 \times 10^{-6}$ M and $1 \times 10^{-7}$ M of deguelin, respectively, for the indicated time periods. Living gating of the forward and orthogonal scatter channels was used to exclude debris and selectively detect cellular events. All values presented are the percentage of cells as determined by light scatter. The percentage of dead cells was determined by fluorescence-activated cell sorter analysis of propidium iodide-stained nuclei.

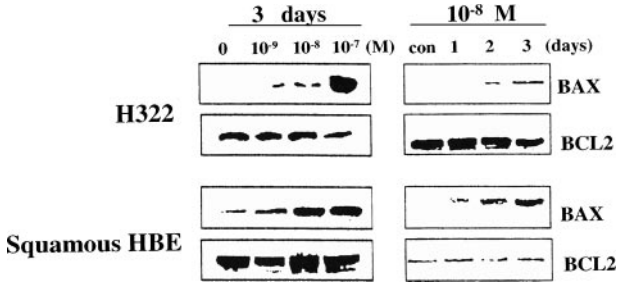


*Fig. 4* Expression of the Bcl protein family in deguelin-treated H322 non-small cell lung carcinoma cells and squamous human bronchial epithelial (HBE) cells. H322 cells and squamous HBE cells were treated with the indicated doses of deguelin for 3 days or with $10^{-8}$ M deguelin for the indicated times. Cells were lysed, and equal amounts of total protein were subjected to electrophoresis; Bax and Bcl-2 levels were determined by Western blot analysis. Bax expression levels were up-regulated in a dose- and time-dependent manner in the H322 cells and the squamous HBE cells, whereas Bcl-2 protein levels did not change appreciably throughout the 3-day period.

and p21 levels and slightly increased p27 levels in squamous HBE cells in a time-dependent manner (Fig. 5).

## DISCUSSION

Mounting evidence from several studies suggest that COX-2 is involved in the pathogenesis of lung cancer and that inhibition of COX-2 may help to prevent lung carcinogenesis. Because traditional nonsteroidal anti-inflammatory drugs inhibit both COX-1 and COX-2, side effects caused by the inhibition of COX-1, such as gastrointestinal and renal injuries, have resulted from nonsteroidal anti-inflammatory drug treatment (7). Thus, we sought to identify selective COX-2 inhibitors.

We showed previously that deguelin inhibits premalignant and malignant HBE cell proliferation by inducing apoptosis at *in vitro* dosages attainable *in vivo* (21) by inhibiting PI3K/Akt activity. Because it has been demonstrated that PI3K/Akt activity is largely responsible for COX-2 expression (22, 23), we investigated the effect of deguelin on COX-2 protein levels in squamous HBE cells. Our results showed that COX-2 expression is induced in squamous HBE cells that mimic bronchial metaplasia, a potentially premalignant lesion induced in smokers (27), and that treatment with deguelin inhibits the induced COX-2 expression, suggesting that the ability of deguelin to inhibit COX-2 expression may contribute to deguelin's chemopreventive action in lung cancer.

Because cancer chemoprevention targets the multistep process of carcinogenesis with chemical agents that delay, reverse, or block cancer development (24), and inhibitors of cancer cell proliferation are known to be useful chemopreventive agents (25), we also studied the effects of deguelin on the proliferation of a large number of NSCLC cells. The proliferation of a large number of NSCLC cell lines was suppressed by deguelin. The $IC_{50}$ of deguelin in NSCLC cell lines and squamous HBE cells was much lower than that of celecoxib, a COX-2 inhibitor, indicating the potential of deguelin as a therapeutic agent as well as a chemopreventive agent.

We further investigated the mechanism by which deguelin inhibits cell proliferation. Consistent with previous reports indicating apoptotic activities of selective COX-2 inhibitors in a variety of cancer cells, including those of the colon, stomach, prostate, and breast (9–13), we found clear evidence from flow cytometry and Western blot analysis that deguelin induced apoptosis in squamous HBE and NSCLC cells. Interestingly, compared with the strong induction of Bax levels by deguelin, Bcl-2 levels were marginally affected by deguelin treatment in these cells, indicating a role of deguelin in modulating the Bax:Bcl-2 ratio, which is a crucial determinant of cellular susceptibility to apoptosis (29). We showed previously that deguelin treatment decreases Bcl-2 levels in 1799 premalignant HBE cells (21), suggesting that the regulation of Bcl-2 levels by deguelin is dependent on the cellular context.

Deguelin also increased the expression of p53 and cyclin-dependent kinase inhibitors p21 and p27 in squamous HBE cells. *p21*$^{WAF1/CIP1}$ (29) and Bax (30) are downstream target genes of p53, and the importance of p53-mediated apoptosis through both transactivation-dependent and -independent mechanisms has been demonstrated (31). Moreover, it has been demonstrated that COX-2 expression is regulated by *p53* (32–34). These findings indicate that the p53 signaling pathway is partly responsible for the deguelin-induced apoptosis in squamous HBE cells.

In summary, we showed for the first time that nanomolar concentrations of deguelin reduced COX-2 expression in squamous HBE cells. We also demonstrated that deguelin effectively inhibited the proliferation of most of the NSCLC cell lines tested and of squamous HBE cells by inducing apoptosis, showing the potential of deguelin as a chemopreventive as well as chemotherapeutic reagent.

Deguelin has several characteristics as a potential cancer chemopreventive agent. First, deguelin preferentially affects the PI3K/Akt signaling pathway, which is important in regulating cell apoptosis and proliferation (21). Second, Akt activity is higher in premalignant and malignant HBE cell lines than in NHBE cells, and deguelin inhibits phosphatidylinositol 3′-kinase/Akt activity (21). Third, deguelin mediates the inhibition of 12-*O*-tetradecanoylphorbol-13-acetate-induced ornithine decarboxylase activity; ornithine decarboxylase is a key enzyme in the biosynthesis of polyamines and is highly inducible by growth-promoting stimuli, including growth factors, steroid hormones, cyclic adenosine 3′5′-monophosphate-elevating agents, and tumor promoters (35, 36). Both ornithine decarboxylase enzyme activity and the resulting polyamines are overexpressed

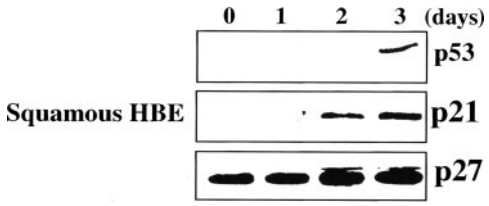


*Fig. 5* The regulation p53, p21, and p27 by deguelin in squamous human bronchial epithelial (HBE) cells. Squamous HBE cells were treated with $10^{-8}$ M deguelin for the indicated times, and the expression levels of p53, p21, and p27 were examined by Western blot analysis. p53, p21, and p27 expression levels were up-regulated in a dose-dependent manner in the squamous HBE cells.

in various cancer cells, and thus, agents that inhibit polyamine synthesis may be good candidates for use in cancer chemotherapy and chemoprevention. Fourth, deguelin reduces the formation of 7,12-dimethylbenz(*a*)anthracene-induced preneoplastic lesions in murine mammary glands in organ culture and suppresses skin and mammary carcinogenesis in 7,12-dimethylbenz(*a*)anthracene-induced, 12-*O*-tetradecanoylphorbol-13-acetate-promoted CD-1 mice and *N*-nitroso-*N*-methylurea-treated female Sprague Dawley rats, respectively (36, 37).

In addition, the insecticidal activity of deguelin and other rotenoids and the systemic toxicities, such as neurological toxicities including somnolence and ataxia (38) are probably due to the potent inhibition of NADH-dehydrogenase and concomitant depletion of intracellular ATP. Deguelin has shown an $IC_{50}$ as low as 33 nM for NADH-dehydrogenase. Although toxicity of deguelin in mouse skin was shown to be nonexistent at 660 μg, oral dosing shows toxicity at 5 mg/kg in mice. All of these problems might limit the use of deguelin in clinical application. Therefore, continued development of deguelin, such as aerosolized topical delivery, will be required. In addition, the mechanisms by which deguelin exerts its apoptotic effect in NSCLC cancer cells warrant further investigation.

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