Chemopreventive Effects of Deguelin, a Novel Akt Inhibitor, on Tobacco-Induced Lung Tumorigenesis

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Tobacco carcinogens induce Akt activation and lung carcinogenesis. We previously demonstrated that deguelin, a natural plant product, specifically inhibits the proliferation of premalignant and malignant human bronchial epithelial cells by blocking Akt activation. To evaluate the ability of deguelin to block tobacco carcinogen-induced lung tumorigenesis, we evaluated the in vivo effects of deguelin on Akt activation and lung tumorigenesis in transgenic mice in which Akt expression was induced by tamoxifen and in 4-(methylnitrosoamo)ino)-1-(3-pyridyl)-1-butane (NNK)/benzo(a)pyrene (BaP)-treated A/J mice. Deguelin suppressed Akt activation in vivo, as measured by immunohistochemistry and immunoblotting, and statistically significantly reduced NNK/BaP-induced lung tumor multiplicity, volume, and load in A/J mice, as monitored by microcomputed tomography image analysis, with no detectable toxicity. These results indicate that deguelin warrants consideration as a chemopreventive agent for early-stage lung carcinogenesis in a clinical lung cancer chemoprevention trial.

In the United States and western Europe, lung cancer leads all other cancers in both incidence and mortality rate (1), underscoring the need for effective lung cancer chemopreventive agents. Because tobacco smoking confers the greatest risk of developing lung cancer (2), molecules that target pathways involved in tobacco-mediated lung carcinogenesis could be effective lung cancer chemopreventive agents. The PI3K/Akt pathway could be such a target because Akt is activated in premalignant and malignant human bronchial epithelial cells, as well as non–small-cell lung cancer cells, through the activating mutation of ras, overexpression of the epidermal growth factor receptor and subunits of PI3K, inactivation of tumor suppressor genes such as PTEN, or exposure to tobacco carcinogens, all of which are frequent events in lung cancer (3–9). Akt is activated by phosphorylation at two key regulatory sites, Thr^308 and Ser^473 (10). Akt promotes cell survival by phosphorylating proapoptotic and antiapoptotic proteins, including the Bcl-2 family member BAD, caspase-9, cAMP response element-binding protein, inhibitor of kappaB kinase complex α, and forkhead transcription factor-1 (11–19).

We and others have shown that pharmacologic and genetic approaches targeting Akt suppress the proliferation of premalignant and malignant human bronchial epithelial cells and reverse characteristics of transformed human bronchial epithelial cells (20,21), indicating that inhibitors of Akt could be effective lung cancer chemopreventive agents. We have previously found that deguelin, isolated from several plant species, including *Mundula seireca* (*Leguminosae*), inhibits the PI3K/Akt pathway and decreases the expression of cyclooxygenase-2, which participates in xenobiotic metabolism, angiogenesis, and inhibition of immune surveillance and apoptosis during tumorigenesis (22).

Importantly, deguelin induces apoptosis in premalignant and malignant human bronchial epithelial cells, with minimal effects on normal human bronchial epithelial cells in vitro at dosages attainable in vivo (23). Deguelin has been shown to have cancer chemopreventive activities in the two-stage skin carcinogenesis model (24) and in the N-nitroso-N-methylurea-induced rat mammary carcinogenesis model (25). It also exhibits therapeutic activities in colon cancer, melanoma, and lung cancer (22,26,27). These findings led us to hypothesize that deguelin could be an effective lung cancer chemopreventive agent by blocking Akt activation. In the present study, we attempted to test our hypothesis in Akt-inducible transgenic mice, in which Akt is activated by tamoxifen (tmaAkt/Z;CAG::Cre) (28), and in A/J mice, in which lung tumors are induced by 4-(methylnitrosoamo)ino)-1-(3-pyridyl)-1-butane (NNK) and benzo(a)pyrene (BaP) (20,29).

To test the effects of deguelin on Akt activation in tmaAkt/Z;CAG::Cre mice, we performed immunohistochemical (Fig. 1, A) and immunoblot (Fig. 1, B) analyses with phosphorylated (p)Akt (at Ser^473) on lung tissues from tmaAkt/Z;CAG::Cre mice treated with 4 mg/kg of deguelin for 3 days. pAkt staining was homogeneous in the bronchial epithelium of the control and deguelin-treated mice; however, levels of pAkt in the lung tissues of control mice were higher than in that of deguelin-treated mice. Western blot analysis also showed decreased expression of pAkt in the lungs of deguelin-treated mice, indicating that deguelin affects Akt activation in vivo.

To evaluate the chemopreventive effects of deguelin in the A/J mice, we first evaluated the serum and tissue distribution of deguelin in A/J mice. Fig. 2, A, shows the concentration-time curve of deguelin in serum and various organs after oral gavage administration of 4 mg/kg deguelin, the maximum tolerated dose in rats (25). The total body clearance of deguelin was 0.33 L/kg/hour, the apparent volume of distribution was 1.86 L/kg, and the half-life was 3.98 hours. One hour after the treatment, concentrations of deguelin in...
commercial hematoxylin was used as a counterstain. pAkt (Ser473) (diluted 1:200) (Cell Signaling Technology, Beverly, MA) and were then processed using polyclonal anti-Akt (diluted 1:100 in 2.5% blocking serum) or rabbit polyclonal anti-phosphorylated Akt (Cell Signaling Technology, Beverly, MA) to reduce nonspecific endogenous peroxidase activity, and then incubated in blocking serum (Vector Laboratories, Burlingame, CA) to reduce nonspecific antibody binding. The sections were incubated overnight at 4 °C with rabbit polyclonal anti-Akt (diluted 1:100 in 2.5% blocking serum) or rabbit polyclonal anti-phosphorylated Akt (pAkt, Ser473) (diluted 1:200) (Cell Signaling Technology, Beverly, MA) and were then processed using standard avidin–biotin immunohistochemical techniques according to the manufacturer’s recommendations (Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as a chromagen, and commercial hematoxylin was used as a counterstain.

### Fig. 1. Effects of deguelin on Akt activation in the transgenic mice expressing tamoxifen-inducible Akt.

The mouse line expressing tamoxifen-inducible Akt (tmAkt/Z;CAG::Cre) was generated by crossing the “master” line (CAG::loxP::CAT::loxP::tmAkt::IRESLacZ) (provided by Thomas N. Sato, Weill Medical College of Cornell University, New York, NY) to CMV::Cre (distributed by the Mouse Resource Facility at M. D. Anderson Cancer Center, Houston, TX), which led to the expression of inactive (not phosphorylated) Akt by excising the floxed CAT. Six-week-old tmAkt/Z;CAG::Cre mice were orally treated with deguelin (4 mg/kg) during feeding twice a day for 3 days (five mice/group) and then were injected intraperitoneally with 5 mg of tamoxifen (Sigma, St. Louis, MO). The next day, the mice were killed by CO₂ asphyxiation. Lungs were surgically removed, and A) half of each lung was fixed with 10% formaldehyde, embedded in paraffin, sectioned (5-μm thick), and processed for immunohistochemical analysis. The sections were deparaffinized, immersed in methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase activity, and then incubated in blocking serum (Vector Laboratories, Burlingame, CA) to reduce nonspecific antibody binding. The sections were incubated overnight at 4 °C with rabbit polyclonal anti-Akt (diluted 1:100 in 2.5% blocking serum) or rabbit polyclonal anti-phosphorylated Akt (pAkt, Ser473) (diluted 1:200) (Cell Signaling Technology, Beverly, MA) and were then processed using standard avidin–biotin immunohistochemical techniques according to the manufacturer’s recommendations (Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as a chromagen, and commercial hematoxylin was used as a counterstain. B) The other half of each lung was lysed in 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 0.1 mM vanadate, and 1 μg/mL aprotinin by mechanical homogenization. Equivalent amounts of protein were resolved on sodium dodecyl sulfate–polyacrylamide gels (10%) and electrophoretically transferred to nitrocellulose membranes. After membranes were blocked in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) and 5% (w/v) nonfat powdered milk, they were incubated with primary antibodies against pAkt (Ser473) (1:1000) and unphosphorylated Akt (1:1000) (Cell Signaling Technology, Beverly, MA) or with a goat polyclonal anti-β-Actin (1:4000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in TBS–5% nonfat milk at 4 °C for 16 hours. The membranes were then washed three times with TBST and incubated with secondary antibody for 1 hour at room temperature. The goat anti-rabbit immunoglobulin G (IgG) or bovine anti-goat IgG horseradish peroxidase-conjugated complexes were detected using the enhanced chemiluminescence kit (Amersham, Arlington Heights, IL) according to the manufacturer’s recommended protocol.

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various organs ranged from less than 1 ng/mL in the brain tissue to 57.1 ng/mL in the kidneys. The peak concentration in these organs occurred between 1 hour (lung, heart, and kidney) and 6 hours (liver) after administration. These studies indicate that oral deguelin administration can achieve effective absorption and distribution in several organs, including the lung.

We next tested the chemopreventive effects of deguelin in the A/J mice, in which lung carcinogenesis was induced by NNK and BaP, as previously described (30). Cancer chemopreventive agents are classified as either blocking or suppressing agents (31). Blocking agents, which prevent the metabolic activation of carcinogens and reduce DNA damage, are tested by administering them before or simultaneously with the carcinogen. Suppressing agents, which inhibit the neoplastic progression of premalignant cells, are usually tested by administering them after the carcinogen (32). Hence, deguelin (4 mg/kg twice a day) was administered either 1 week after the first dose of NNK/BaP (entire period, group 3) or after completion of carcinogen administration (postcarcinogen, group 4) (Fig. 2, B). A/J mice untreated (group 1) or treated with NNK plus BaP (group 2) received only the vehicle (corn oil) during this period. Sixteen and/or 20 weeks after the first dose of NNK and BaP, representative A/J mice from groups 1, 2, and 3 were analyzed by microcomputed tomography to monitor changes in the number and size of lung tumor nodules. The lung structure in a control mouse (Fig. 2, C, 1) and tumor nodules (Fig. 2, C, 2) less than 1 mm in diameter in a NNK/BaP-treated mouse (10) were easily detected and were consistent with the block-faced image (Fig. 2, C, 3) at 16 weeks. A second NNK/BaP-treated mouse (33) had two tumor nodules (0.4 mm and 0.55 mm) at 16 weeks (Fig. 2, C, 4) that became larger (0.4 mm to 0.6 mm and 0.55 mm to 1 mm) at 20 weeks (Fig. 2, C, 5), when a new tumor nodule (0.8 mm) was observed. In contrast, a tumor nodule (1.1 mm) detected at 16 weeks (Fig. 2, C, 6) in the deguelin-treated mouse was not detectable at 20 weeks (Fig. 2, C, 7). All mice were killed at 20 weeks. Gross evaluation revealed no tumors in the lungs of control mice (group 1) and 100% lung tumor formation in NNK/BaP-treated mice (group 2) (Supplementary Fig. 1 and Supplementary Table 1 available at http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol97/issue22). Deguelin-treated mice had fewer lung tumors (Supplementary Fig. 1 and Supplementary Table 1 available at http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol97/issue22); mice in groups 3 and 4 had fewer lung tumor nodules than NNK/BaP-treated mice in group 2 (mean = 4.57 versus mean = 11.0, difference = 6.43, 95% confidence intervals
Six-week-old A/J mice were given 4 mg/kg deguelin orally. The serum and indicated organ samples were collected at 0 (baseline), 1 (purple), 2 (turquoise), 4 (blue), 6 (green), 12 (brown), or 24 (orange) hours after deguelin administration. Blood samples were immediately placed on ice and centrifuged at 2000 g at 4 °C for 15 minutes, and the serum was stored at −70 °C until analysis. The mice were killed prior to organ sample collection. Each organ sample was suspended in acetonitrile, sonicated, and then centrifuged at 1500 g for 3 minutes at 4 °C. The liquid-tissue extraction process was repeated three times, and each time, 100 μL of supernatant was collected. To the final 300-μL sample, 2700 μL of 0.1 M ammonium acetate, pH 5.5, was added. Deguelin was isolated from both serum and organ (liquid-extracted) samples by solid-phase chromatography (tC18 solid-phase extraction [SPE] cartridge, 100 mg, Waters Corp, Millford, MA). After the SPE cartridge was conditioned with 2 mL of methanol and 2 mL of high-performance liquid chromatography-grade water, either 100 μL of serum or 3 mL (a volume of 1 mL pulled through the column each time) of liquid-extracted sample was placed into the SPE cartridge. Deguelin was eluted with 1 mL of methanol, dried, and then reconstituted with a 100-μL mixture of methanol and 0.1% formic acid (50:50, v/v). Thirty microliters of the reconstituted sample was then injected onto a high-performance liquid chromatography mass spectrometer (LC/MS-ES+, MicroMass, Beverly, MA). Standard calibration curves for deguelin ranged from 5 to 1000 ng/mL. The lower limit of quantitation was 0.01 ng/mL. Pharmacokinetic modeling was completed using a noncompartment method (WinNonlin version 3.1, Pharsight Corporation, Mountain View, CA). The results are expressed as means and 95% confidence intervals (CIs), four mice/group.
In spite of its potential as a cancer chemopreventive/therapeutic agent, there is concern about possible side effects of deguelin treatment. Deguelin is derived from rotenone, which can inhibit NADH:ubiquinone oxidoreductase, an enzyme complex involved in mitochondrial oxidative phosphorylation (34), and induce cardiotoxicity, respiratory depression, and nerve conduction blockade at high doses (a dose that is lethal to 50% of those exposed = 10–100 g in humans). However, we did not observe major toxicity or substantial loss of body weight in the deguelin-treated A/J mice at the dose used in this study. Deguelin is also safer in terms of its mechanism of action, which differs from that of rotenone, which inhibits tubulin polymerization. Additionally, deguelin rapidly decomposes in light and air. All of these results suggest that deguelin would be harmless when orally administered. Moreover, in contrast to some natural products presently used in cancer chemoprevention and therapy, deguelin could be easily synthesized using commercially available rotenone as a starting material; therefore, its clinical use as a lung cancer chemopreventive agent is feasible. These collective findings provide a strong rationale for testing deguelin in a phase I clinical trial of lung cancer chemoprevention after its complete toxicity profile in humans is known.

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using a model RS-9 tablettop CT scanner (General Electric Medical Systems, London, Ontario) as previously described (35,36). The technique used was 80 kVp and 450 μA, with 720 views obtained at 0.5-degree increments at 400 msec per view. The radiation dose delivered during this scan was approximately 2.9 mGy. The data was transferred to a computer using a custom-designed software (National Instruments, Austin, TX) which provided an interface for selecting the mouse respiratory parameters. The Feldkamp reconstruction method was used to normalize the raw images and to correct for nonuniformities in the detector (35). Images were formed with isotropic 91-μm voxels. NNK/BaP-treated mice sections (10) were fast-frozen immediately after their micro-CT scan session to confirm their position in the images relative to block-faceted pathologic sections. A representative micro-CT (1, 2, 4–7) or block-
faced (3) image analysis of the lungs from a control mouse (4) or mice treated with NNK/BaP alone (2–5) or with NNK/BaP plus deguelin (group 3) at 16 (4, 6) and 20 (5, 7) weeks was 1 = lung; e = esophagus; v = thoracic vertebra; L = liver; s = spinal cord. D) Postmortem examinations were performed on the lungs of the A/J mice after mice were killed at 20 weeks. The lung tissues were fixed in Bouin’s solution, and the gross tumor nodules (lung tumors/slide) were counted. Microscopic evaluation of lung tissues was also performed to measure mean tumor number (N), volume (V), and total tumor load (N × V) in a blinded fashion. The tumor volume was calculated by the formula of V(mm³) = (long diameter × short diameter²)/2. The number and size of tumors in five sections distributed uniformly through each lung were calculated. Body weight of the mice was measured at 0 (baseline), 8, and 20 weeks (wks). Effects of deguelin on lung tumorigenesis were evaluated in 10% of the animals listed in Table 1, 2, 4–7) lesions in the A/J mice that were untreated (1, 2, 3) or treated with NNK/BaP alone (4, 5) or NNK/BaP plus deguelin (3, 5) were processed for immunohistochemical analysis with anti-pAkt (Ser473) as described in Fig. 1.


August 23, 2005; accepted September 7, 2005.


NOTES

Supported by National Institutes of Health grants R01 CA100816–01 and CA109520–01 (to H.-Y. Lee) and American Cancer Society grant RSG-04–082–01-TBE 01 (to H.-Y. Lee) and partly by Department of Defense grant W81XWH-04–1–0142–01–VITAL (to W. K. Hong) and National Institutes of Health Cancer core grant CA16672. We thank the staff of the Small Animal Cancer Research Imaging Facility for their diligence and devotion. WKH is an American Cancer Society clinical research professor.

Manuscript received March 29, 2005; revised August 23, 2005; accepted September 7, 2005.