Structural Basis for Depletion of Heat Shock Protein 90 Client Proteins by Deguelin

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Background
The molecular chaperone heat shock protein 90 (Hsp90) participates in preserving the expression and activity of various oncoproteins, including hypoxia-inducible factor 1α (HIF-1α) and Akt. Deguelin is a rotenoid with antitumor activities. We investigated whether the antitumor activities of deguelin involve the functional inhibition of Hsp90.

Method
Human xenograft tumors were generated in mice from H1299 (n = 6 per group) and A549 (n = 4 per group) non-small-cell lung cancer cells, UMSSC38 (n = 5 per group) head and neck cancer cells, MKN45 (n = 5 per group) stomach cancer cells, and PC-3 (n = 3 per group) prostate cancer cells. Tumor-bearing mice were treated with deguelin at 4 or 8 mg/kg or with vehicle (as a control) twice a day by oral gavage for 15–28 days. Protein expression was assessed by western blot analysis. Akt and Hsp90 were assessed by use of adenoviral vectors expressing constitutively active Akt or Hsp90. Binding of deguelin to Hsp90 was examined by docking analysis and by competition binding experiments with ATP-Sepharose beads. The proteasome inhibitor MG132 was used to investigate deguelin's effect on the induction of ubiquitin-mediated proteasomal degradation of HIF-1α. All statistical tests were two-sided.

Results
Deguelin bound to the ATP-binding pocket of Hsp90 and disrupted Hsp90 function, leading to ubiquitin-mediated degradation of HIF-1α. Administration of deguelin to xenograft-bearing mice statistically significantly decreased tumor growth by inducing apoptosis and decreasing the expression of Hsp90 client proteins, without detectable toxic effects. For example, at 15 days after the start of deguelin treatment, the volume of untreated control H1299 xenograft tumors was 798 mm³ and that of xenograft tumors treated with deguelin at 4 mg/kg was 115.9 mm³ (difference = 682.1 mm³, 95% confidence interval = 480.4 to 883.9 mm³; \( P < .001 \)).

Conclusions
The antitumor activities of deguelin appear to involve its binding to the ATP-binding pocket of Hsp90, which suppresses Hsp90 function.

Context and Caveats

Prior Knowledge
Heat shock protein 90 (Hsp90) is a molecular chaperone that is required for the stability and function of various normal proteins and oncogenes (i.e., their client proteins), including hypoxia-inducible factor 1α (HIF-1α). Deguelin is a rotenoid with antitumor activity in a variety of tumor types.

Study Design
The molecular mechanism of action of deguelin was investigated in human xenograft mouse model systems for five different tumors and by computer modeling the binding of deguelin to the ATP-binding pocket of Hsp90.

Contribution
The antitumor activities of deguelin appear to involve its binding to the ATP-binding pocket of Hsp90, which suppresses Hsp90 function.

Implications
Deguelin appears to be an effective antitumor agent that targets Hsp90. Complete and extensive screening for toxic effects in clinical trials is required before further development because of the wide expression of Hsp90 in normal tissues.

Limitations
Deguelin’s mechanism of action on various Hsp90 client proteins has not been fully elucidated.

degradation of numerous oncogenic client proteins that participate in the regulation of cell proliferation, survival, and angiogenesis (6). Chemically distinct inhibitors of Hsp90 have been used as probes to define the biologic functions of Hsp90 at the molecular level and/or to validate Hsp90 as a novel target for anticancer drugs in clinical trials (7,8). Phase II clinical trials evaluating one of these agents, 17-AAG, have begun, and several second-generation compounds are now in late preclinical development (7,9).

We have previously shown (10,11) that the rotenoid deguelin effectively prevents tobacco carcinogen–induced lung carcinogenesis by blocking Akt activation. Deguelin has also shown potential as a chemopreventive agent against breast, colon, and skin cancers (12–14), and it has shown antiproliferative and apoptotic activities in some non–small-cell lung cancer (NSCLC) cell lines in vitro (12). However, antitumor activities of deguelin and its mechanism of action have not been defined. In this study, we investigated the therapeutic efficacy of deguelin in several human xenograft tumor models and potential mechanisms of its antitumor activity.

Materials and Methods

Cells, Animals, Adenoviral Vectors, Antibodies, and Materials
The human NSCLC cell lines H1299, A549, and H322; stomach cancer cell line MKN45; and prostate cancer cell line PC-3 were obtained from the American Type Culture Collection (Manassas, VA). The human head and neck squamous cell carcinoma cell line UM-SCC38, which was established originally by Dr Thaddeus E. Carey (University of Michigan, Ann Arbor), was obtained from Dr Reuben Lotan (15). These cancer cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (GIBCO-BRL Life Technologies, Gaithersburg, MD).

Adenoviral vectors expressing constitutively active, hemagglutinin (HA)-tagged Akt (Ad-HA-MyrAkt, in which Myr is the Src myristylation signal) (10), HA-tagged Hsp90 (Ad-HA-Hsp90) (16), and an empty adenoviral vector (Ad-EV) were amplified as described previously (10). Briefly, an adenoviral vector expressing a full-length human Akt1 sequence with the Src myristylation signal fused in-frame to the c-Akt–coding sequence or expressing a full-length human Hsp90 sequence, each fused with HA, under the control of the cytomegalovirus promoter was constructed by use of the corresponding pAd-shuttle vector system. The viruses were amplified in human embryonic kidney 293 cells that were maintained in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum and purified by use of centrifugation through a CsCl gradient (17). Viral titers were determined by plaque assays. The expression vector for HIF-1α mutated at two proline hydroxylation sites (Pro-402 and Pro-564) was a kind gift from Dr Len Neckers (National Institutes of Health, Rockville, MD) (18).

Female nude mice, 6 weeks old, were purchased from Harlan-Sprague Dawley (Indianapolis, IN). Deguelin was purchased from Gaia Chemical Corp (Gayslorsville, CT). The Hsp90 inhibitor 17-AAG was from Invivogen (San Diego, CA). Bovine serum albumin, gelatin, the proteasome inhibitor MG132, CoCl2, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were from Sigma-Aldrich (St Louis, MO). Recombinant Hsp90 protein was from Stressgen (San Diego, CA).

The following antibodies were used for western blot or immunohistochemical analyses: mouse monoclonal antibodies against HIF-1α (BD Transduction Laboratories, Lexington, KY), HIF-2α (Novus, Littleton, CO), p53 (BD Pharmingen, San Diego, CA), or HIF-1β (BD Transduction Laboratories); rabbit polyclonal antibodies against phosphorylated Akt (Ser-473) (Cell Signaling, Beverly, MA), Akt (Cell Signaling), Hsp90α (Stressgen), and endothelial nitric oxide synthase (eNOS) (BD Pharmingen); goat polyclonal antibodies against CDK4, HA, MEK1/2, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA); and a rabbit anti-mouse immunoglobulin G (IgG)—horseradish peroxidase conjugate, a goat anti-rabbit IgG—horseradish peroxidase conjugate, and a rabbit anti-goat IgG—horseradish peroxidase conjugate (Santa Cruz Biotechnology).

Generation of Xenograft Tumors and Immunohistochemical Staining
All animal procedures were performed in accordance with a protocol approved by the M. D. Anderson Institutional Animal Care and Usage Committee. Xenograft tumors were generated by subcutaneous injection of H1299, A549 UM-SCC38, MKN45, or PC-3 cells, as described elsewhere (19). Briefly, nude mice were injected at a single dorsal flank site with 5 × 106 H1299 (n = 12 mice), A549 (n = 8 mice), UM-SCC38 (n = 10 mice), MKN45 (n = 10 mice), or PC-3 (n = 6 mice) cells in 100 µL of phosphate-buffered saline (PBS). Injection of these cells into nude mice induced exponentially growing tumors. When tumors reached a volume of 40–80 mm³ (termed day 0 for our experiments), mice were treated orally with vehicle (50 µL of cotton seed oil and 50 µL of dimethyl sulfoxide)
or deguelin at 4 mg/kg—which is the maximum tolerable dose of intragastrically administered deguelin for rats (14)—twice a day for 15–28 days. We also included a deguelin dose of 8 mg/kg for H1299 xenografts to obtain the maximum antitumor activity against this tumor. Tumor size was measured every 2–4 days, and tumor growth was quantified by measuring the tumors in two dimensions. Volumes were calculated by the formula \(0.5 \times a \times b^2\), where \(a\) and \(b\) are the longer and shorter diameters, respectively. Tumor volumes were expressed as the mean and 95% confidence interval (CI). Mice with necrotic tumors or with tumors that had a diameter of more than 1.5 cm were killed by use of CO\(_2\), and tumors were removed. Histopathologic evidence of pulmonary toxicity (i.e., edema or inflammation of the bronchial epithelium and alveoli), inflammation, and injury in other organs, such as liver, kidney, spleen, stomach, and ovary, were evaluated by a veterinary pathologist. Tumors were separated into three parts for analyses immediately after they were removed. One part was fixed in 10% buffered formalin, embedded in paraffin, and sectioned for CDK4 staining. Another part was frozen and sectioned for terminal deoxynucleotidyltransferase-mediated 3′-terminal dideoxynucleotide labeling (TUNEL) staining, a measure of apoptosis. Immunohistochemical staining for CDK4 and TUNEL was as previously described (15). For apoptosis analysis, we counted all nuclei in the field, as well as all TUNEL-positive cells in three to five randomly selected fields of view, at a magnification of ×100. The third part of the tumor was frozen for western blot analysis.

**Flow Cytometry Analysis of Reactive Oxygen Species Production**

H1299 cells (3 × 10\(^5\) cells) were preincubated under normoxic (20% O\(_2\)) or hypoxic (1% O\(_2\)) conditions for 6 hours and then incubated with 5 mM N-acetylcyesteine (a reactive oxygen species [ROS] scavenger; Sigma-Aldrich), 4 mM succinate (a substrate of mitochondrial complex II; Sigma-Aldrich), or 100 nM deguelin for an additional 6 hours. The relative cellular ROS concentration was obtained by measuring the hydrogen peroxide concentration. Briefly, cells were treated with 20 \(\mu\)M dichlorofluorescin diacetate (Molecular Probe; Invitrogen Corporation, Carlsbad, CA) for 30 minutes at 37 °C, washed with PBS, trypsinized, and collected in 500 \(\mu\)L of PBS. The hydrogen peroxide content in 2000 cells per sample was analyzed by flow cytometry (FACS Calibur; Becton Dickinson, San Jose, CA).

**Cell Treatments**

To assess the effects of deguelin on protein and mRNA expression, H1299 cells were seeded in six-well plates, at 4 × 10\(^5\) to 5 × 10\(^5\) cells per well, 1 day before the start of treatment. When the culture was 80%–90% confluent, cells were treated with deguelin (0.1–100.0 nM) or 17-AAG (10 \(\mu\)M) in complete medium for 16 hours under hypoxic or normoxic conditions, and then prolyl hydroxylase (PHD)– or von Hippel–Lindau protein (pVHL)–independent HIF-1α regulation by deguelin was investigated. H1299 cells (2 × 10\(^5\) cells per well in six-well plates) that were transfected with control or VHL small-interfering RNA (siRNA; 60 nmol) or with an expression vector for HIF-1α mutated at two proline hydroxylation sites (Pro-402 and Pro-564; 1 \(\mu\)g), which exhibits diminished hydroxylation by PHD, were treated with insulin-like growth factor I (IGF-I; 100 ng/mL) or CoCl\(_2\) (100 \(\mu\)M) under normoxic conditions (20% O\(_2\)) or exposed to hypoxia for 6 hours and then incubated with deguelin. We treated cells with IGF-I or CoCl\(_2\), under normoxic conditions or exposed them to hypoxia for 6 hours, which showed the great induction of HIF-1α in most of the cell lines used in our study. We did not test what happened after 6 hours. To assess the role of ROS in deguelin-mediated decreased expression of HIF-1α, H1299 cells were preincubated under hypoxic conditions for 6 hours and then incubated with 4 mM succinate in the presence or absence of 100 nM deguelin for an additional 6 hours.

To assess the effects of deguelin on the expression of HIF-1α and to investigate the roles of Akt, mitogen-activated protein kinase, and Hsp90 in deguelin action on HIF-1α stability, H1299 cells were infected or infected with Ad-HA-MyrAkt, Ad-MEK, Ad-HA-Hsp90, or Ad-EV (each at 25–50 plaque-forming units per cell) as described previously (15); treated with IGF-I or CoCl\(_2\), under normoxic conditions or exposed to hypoxia (1% O\(_2\)) for 6 hours; and then incubated with complete medium containing deguelin for 1–6 hours. To test the effect of deguelin on proteasome-mediated HIF-1α degradation, H1299 cells were infected with Ad-EV or Ad-HA-Hsp90 for 2 days and then treated with 100 nM deguelin in the absence or presence of 10 \(\mu\)M MG132 (Z-Leu-Leu-Leu-al; a proteasome inhibitor) for 6 hours.

The effects of deguelin on the degradation of Hsp90 client proteins were tested by western blot analysis in H1299 cells treated with 100 nM deguelin for 1 or 2 days in the absence or presence of 10 \(\mu\)M MG132 in complete medium under hypoxic conditions.

**Western Blot and Coimmunoprecipitation Analyses**

Western blot analysis was performed as described previously (15). Briefly, whole-cell lysates were prepared in a lysis buffer containing 20 mM Tris–HCl (pH 7.6), 1 mM EDTA, 140 mM NaCl, 1% Nonidet P-40, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium vanadate as described elsewhere (16). Equivalent amounts of protein (20–80 \(\mu\)g) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in 7.5% to 12% gels and transferred by electroblotting to a polyvinylidene fluoride membrane. Nonspecific binding to the blot was blocked by incubation in Tris-buffered saline containing 0.1% Tween-20 (TBST) supplemented with 5% nonfat powdered milk. The blot was incubated with primary antibody against HIF-1α, HIF-1β, phosphorylated Akt (Ser-473), Akt, CDK4, HA, MEK1/2, β-actin, or Hsp90α (each at an appropriate dilution) in TBST containing 5% bovine serum albumin at 4 °C for 16 hours. The membrane was then washed three times with TBST and incubated with the appropriate horseradish peroxidase–conjugated secondary antibody for 2 hours at room temperature. The protein–antibody complexes were detected by using enhanced chemiluminescence (Amersham, Arlington Heights, IL), according to the manufacturer’s recommended protocol.

For coimmunoprecipitation assays, H1299 cells were uninfected or infected with the indicated adenoviral vectors, treated with IGF-I or CoCl\(_2\) (100 \(\mu\)M) under normoxic conditions to induce HIF-1α expression or incubated under hypoxic conditions, and then untreated or treated with deguelin (100 nM) as described above. Cells were lysed in a buffer containing 50 mM Tris–HCl...
(pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture by pulse sonication of 2 watts for 5–10 seconds (Vibra Sonic sonicator, Sonics & Materials, Danbury, CT). The level of HIF-1α ubiquitination in deguelin-treated H1299 cells was determined by immunoprecipitation with an anti–HIF-1α antibody and western blot analysis with an anti-ubiquitin antibody. To investigate the interaction between HIF-1α and Hsp90, 200 µg of H1299 cellular protein was immunoprecipitated with anti–HIF-1α antibodies overnight at 4 °C in a binding buffer containing 25 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 5 mM EDTA. Thereafter, protein-G agrose beads were added and incubated for 1 hour. The beads were collected and washed three times with the same buffer, 30 µL of 2× SDS–PAGE sample buffer was added, and the mixture was boiled at 95 °C for 10 minutes to release bound proteins. Bound proteins were resolved by SDS–PAGE gel and then subjected to western blot analysis with an anti-Hsp90 antibody.

Reverse Transcription–Polymerase Chain Reaction
Total RNA was prepared from H1299 cells, and cDNA was synthesized from the total RNA, as described previously (15). Primer sequences used for reverse transcription–polymerase chain reaction assays were as follows: for HIF-1α, 5′-CTCAAGTGCGGACAG CCTCA-3′ (sense) and 5′-CCCTGCGTAGTTCTGCT-3′ (antisense); for VHL, 5′-GAGATGCAGGGACACGAT-3′ (sense) and 5′-TCACACTGTAGATGTCAACC-3′ (antisense); and for β-actin, 5′-ACTACCTCATGGAATC-3′ (sense) and 5′-GATCCACATCTGCTGGA-3′ (antisense). The thermocycler conditions used for amplification were 94 °C for 5 minutes and then 28–32 cycles of 94 °C for 45 seconds, 54–60 °C for 45 seconds, and 72 °C for 1 minute.

Expression and Purification of Epitope-Tagged Proteins
Glutathione S-transferase–tagged JNK1 proteins and hexahistidine (His)-tagged HIF-1α proteins were expressed in Escherichia coli strain BL21 (DE3) pLysS (Novagen, Madison, WI) and cultured in Luria-Bertani (LB) medium as previously described (19). Cells were lysed with a lysis buffer containing 20 mM sodium phosphate (pH 7.8), 500 mM NaCl, lysozyme (1 mg/mL), 0.1 M phenylmethylsulfonyl fluoride, leupeptin (0.5 mg/mL), pepstatin (1 mg/mL), and aprotinin (1 mg/mL) by sonification. After centrifugation (15 000g for 5 minutes at 4 °C), the lysate was loaded on columns of glutathione–Sephase beads (Pharmacia-LKB Biotechnology, Uppsala, Sweden) or of nickel-nitrirotiocetic acid (Ni-NTA)–agarose beads (Qiagen, Valencia, CA). Glutathione S-transferase–tagged JNK1 and His-tagged HIF-1α proteins were eluted from the matrix by the addition of 20 mM glutathione or 500 mM imidazole, respectively. Eluted proteins were dialyzed against 1× PBS overnight at 4 °C.

Binding of Deguelin to Heat Shock Protein 90
We investigated whether deguelin could disrupt the interaction between HIF-1α and Hsp90 by comparing the amounts of HIF-1α–mutant recombinant protein (1 = amino acid residues 1–603; 2 = amino acid residues 401–827; 3 = amino acid residues 604–827) bound to Hsp90 in the presence or absence of deguelin. Briefly, H1299 cells were scraped off, lysed in 150 µL of buffer A, and sonicated with a standard probe-type sonicator (Vibra Sonic sonicator, Sonics & Materials) twice for 15 seconds, followed by centrifugation (15 000g, 15 minutes, at 4 °C). To investigate the interaction between Hsp90 and HIF-1α, 200 µg of H1299 cellular protein was added to recombinant His-HIF-1α bound with Ni-NTA–agarose beads (Qiagen) (5 mg/mL of resin in a total volume of 0.5 mL) and deguelin (100 nM) in a binding buffer containing 25 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 0.2% Triton X-100; and the mixture was incubated for 1 hour at 4 °C. Beads were then collected and washed three times with the same buffer, 30 µL of 5× SDS–PAGE sample buffer was added, and the mixture was boiled at 95 °C for 10 minutes to release bound proteins. These proteins were resolved by SDS–PAGE and subjected to western blotting for Hsp90α as described above.

Competition between deguelin and Hsp90 recombinant protein or between deguelin and JNK1 recombinant protein (as a negative control) for binding to ATP–Sepharose was examined as described previously (20). Briefly, 100 ng of each recombinant protein was incubated with vehicle (0.1% dimethyl sulfoxide), 10 mM ATP, deguelin (at 1, 5, and 10 µM), or 17-AAG (at 1, 5, and 10 µM) in 200 µL of TNE buffer (20 mM Tris–HCl at pH 7.4, 150 mM NaCl, 1 mM EDTA, 10% glycerol, and 1 mM dithiothreitol) for 2 hours, and then 50 µL of γ-phosphate–linked ATP–agarose (Fluka, Milwaukee, WI) was added. Samples were incubated for another 2 hours at 4 °C and washed three times with ice-cold TNE buffer. Material bound to the agarose beads was eluted in 5× SDS–PAGE sample buffer and the concentrations of JNK1 and Hsp90 proteins bound to ATP–Sepharose were determined by western blot analysis with antibodies against JNK1 or Hsp90, respectively. Hsp90 protein bound to the beads was quantified by densitometry analysis.

Docking Modeling
Flexible docking, a method to dock the conformational ensemble (i.e., collection) of a ligand into a target receptor, of deguelin onto Hsp90 was performed by use of the SYBYL version 7.0 software program (SYBYL molecular modeling software, Tripos, Inc, version 7.0, St Louis, MO) and the Red Hat Linux operating system, version 7.0. The chemical structures of deguelin, 17-AAG, and geldanamycin were prepared in MOL2 format by use of the sketcher module in SYBYL, and partial atomic charges were calculated by the Gasteiger–Huckel method (21) and assigned to the ligand atoms. To optimize the ligand structures, the conjugate gradient energy minimization (22) of the ligand was run until the value converged to a maximum derivative of 0.001 kcal/mol·Å, and the final coordinates were stored in a database. The x-ray crystal structure (23) of complex between human Hsp90 and geldanamycin (Protein Data Bank entry = 1YET) was used as a target for the docking of flexible ligand by FlexX algorithm in SYBYL. All crystallographic water molecules were removed from target structure except for one that was involved in the hydrogen bond network inside the binding pocket. The active site was defined as all the amino acid residues enclosed within 6.5-Å radius sphere centered by the bound geldanamycin. The main settings for FlexX run were set in 1000 solutions to generate the maximum number of conformers for each compound during FlexX docking. For database ranking, the scores for all FlexX solutions were calculated by use of a consensus scoring method (Cscore) that was based on the following five scoring functions: FlexX score, G_score, D_score, ChemScore, and potential of mean force score.
After visual inspection, final docking models were created by selecting the conformers with the highest consensus scores (Cscore = 5) and then docking these conformers into the active site of Hsp90.

Generation of the Molecular Surface Map
To visualize the hydrophobic interactions between the ligand (17-AAG and deguelin) and Hsp90, surface hydrophobicity (lipophilicity) potential physicochemical property maps of the ligand-binding sites of Hsp90 were generated on the solvent-accessible surface of Hsp90 (25) by use of the MOLCAD module in SYBYL. Surface colors ranged from dark blue (hydrophilic) to dark brown (lipophilic).

Statistical Analysis
Data are expressed as the mean and 95% confidence interval from at least triplicate samples. Data were calculated with the Microsoft Excel software program (version 5.0; Microsoft Corporation, Redmond, WA). The statistical significance of differences between groups was analyzed with Student’s t test after we determined the normal distribution of the data. Shapiro-Wilk’s test was applied for testing the normality of the data in each experimental group. The test of normality was not rejected at 5% level. All statistical tests were two-sided. A P value of less than .05 was considered to be statistically significant.

Results
Deguelin and Tumor Growth
We evaluated the effect of deguelin treatment on the growth of H1299 NSCLC (Fig. 1, A), UMCC38 head and neck (Fig. 1, B), MKN45 stomach (Fig. 1, C), and PC-3 prostate (Fig. 1, D) xenograft tumors in athymic nude mice (six mice with H1299 tumors, five with UMCC38 tumors, five with MKN45 tumors, and three with PC-3 tumors). Mice bearing xenograft tumors were treated with deguelin at 4 or 8 mg/kg or with vehicle (as a control) twice a day by oral gavage. Tumor growth was statistically significantly suppressed by deguelin treatment throughout the 15- or 28-day treatment regimen, indicating the potent therapeutic efficacy of deguelin in various human cancers (Fig. 1 and Table 1). For example, at 15 days after the start of treatment, the volume of control H1299 xenografts was 798 mm³ and that of xenografts treated with deguelin at 4 mg/kg was 115.9 mm³ (difference = 682.1 mm³, 95% CI = 480.4 to 883.9 mm³; P < .001).

Table 1. Xenograft tumor volume at 15 days after the start of treatment in mice treated with vehicle (control) or deguelin

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>Mean tumor volume, mm³ (95% confidence interval)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1299</td>
<td>Control</td>
<td>798 (807.8 to 988.3)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td></td>
<td>Deguelin (4 mg/kg)</td>
<td>115.9 (48.6 to 183.1)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td></td>
<td>Deguelin (8 mg/kg)</td>
<td>190.9 (139.7 to 242.2)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>PC-3</td>
<td>Control</td>
<td>854.3 (781.7 to 926.9)</td>
<td>.044</td>
</tr>
<tr>
<td></td>
<td>Deguelin (4 mg/kg)</td>
<td>533.0 (369.5 to 696.5)</td>
<td>.037</td>
</tr>
<tr>
<td>UMCC38</td>
<td>Control</td>
<td>746.9 (533.9 to 966.0)</td>
<td>.010</td>
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<tr>
<td></td>
<td>Deguelin (4 mg/kg)</td>
<td>379.0 (296.1 to 462.8)</td>
<td>.010</td>
</tr>
<tr>
<td>MKN45</td>
<td>Control</td>
<td>993.3 (783.3 to 1203.2)</td>
<td>.010</td>
</tr>
<tr>
<td></td>
<td>Deguelin (4 mg/kg)</td>
<td>492.4 (326.0 to 658.9)</td>
<td>.010</td>
</tr>
</tbody>
</table>

* The statistical significance of differences between groups was analyzed with Student’s t test (two-sided).
Deguelin and Expression of Proteins Downstream of Hypoxia-Inducible Factor 1α

To explore the antitumor mechanisms of deguelin, we first investigated its effect on proteins whose transcription is stimulated by HIF-1α, such as vascular endothelial growth factor (VEGF) and aldolase, which are used by cancer cells to adapt to environmental stress (26). Under normoxic conditions, HIF-1α protein was rapidly degraded, and so its expression was very weak (27). To stabilize HIF-1α protein in cells, we treated cells with IGF-I or CoCl2 under normoxic conditions (20% O2) or incubated cells under hypoxic conditions (1% O2). Treatment of H1299 cells with 0.1–100 nM deguelin for 16 hours under all conditions reduced the expression of HIF-1α protein (Fig. 2, A) and VEGF mRNA (Fig. 2, B) in a dose-dependent manner without affecting the expression of HIF-1β protein, which is expressed constitutively (Fig. 2, A). Deguelin also inhibited the expression of aldolase A, another HIF-1α-regulated gene (Fig. 2, C).

We then investigated whether deguelin regulates HIF-1α expression transcriptionally or posttranscriptionally in H1299 cells. After treatment with 100 nM deguelin for 6 hours, HIF-1α mRNA expression changed only marginally (data not shown), whereas the expression of HIF-1α protein was effectively suppressed (Fig. 2, A). Thus, deguelin appears to regulate the expression of HIF-1α protein posttranscriptionally, and so we focused our attention on whether deguelin treatment changed the stability of HIF-1α protein.

Hypoxia-induced ROS may be involved in the stability of HIF-1α, and because complex I inhibitors, such as rotenone and diphenylene iodonium, block the electron flow of the mitochondrial respiratory chain and thus ablate the production of hypoxia-induced ROS (26,28–32). We, therefore, investigated whether ROS are involved in deguelin modulation of HIF-1α expression by comparing the effect of deguelin on HIF-1α expression with its effect on the generation of hydrogen peroxide. Hydrogen peroxide
transmits an oxygen-sensitive signal that regulates HIF-1α (29,32,33). H1299 cells that were grown under hypoxic conditions produced substantially more hydrogen peroxide than H1299 cells that were grown under normoxic conditions. Hydrogen peroxide production was further increased by treatment with 4 mM succin- nate (a mitochondrial complex II substrate for an indirect route of electron flow to complex III) and was decreased by treatment with 5 mM N-acetylcysteine (an ROS scavenger) (Fig. 2, D). Treatment with 100 nM deguelin for 6 hours resulted in a slight increase in hydrogen peroxide production under normoxic conditions but not under hypoxic conditions. Moreover, treatment with succinate failed to restore HIF-1α protein expression under hypoxic conditions in the presence of 100 nM deguelin (Fig. 2, E). Thus, a deguelin-mediated ROS-independent mechanism appears to regulate HIF-1α expression.

HIF-1α protein expression is regulated by many oxygen-dependent and -independent mechanisms (27,34). Under normoxic conditions, two proline sites (Pro-402 and Pro-564) within the oxygen-dependent degradation (ODD) domain of HIF-1α are subjected to PHD-mediated hydroxylation, which triggers binding of the pVHL and ubiquitin-mediated protein degradation (34–36). For these reasons, we investigated whether PHDs and pVHL are involved in the effect of deguelin on HIF-1α regulation. For these experiments, H1299 cells were transiently transfected with an expression vector containing HIF-1α mutated at two proline hydroxylation sites (Pro-402 and Pro-564). We found that the mutated HIF-1α protein expression was also decreased by the deguelin treatment (Fig. 2, F). Moreover, treatment with deguelin decreased normoxic IGF-induced or hypoxia-induced HIF-1α protein expression in H1299 cells transfected with a pVHL-specific siRNA to inhibit pVHL expression (Fig. 2, G). Thus, PHDs and pVHL are not required for the deguelin-mediated decrease in HIF-1α expression. We then explored whether the phosphatidylinositol 3-kinase (PI3K)–Akt signaling pathway, which induces cap-dependent mRNA translation and stabilization of HIF-1α protein (26,37–39), was involved in deguelin-mediated HIF-1α decrease. Our findings were consistent with previous findings (10); i.e., treatment with 100 nM deguelin for 16 hours decreased the expression of phosphorylated Akt in normoxic IGF-I–stimulated or normoxic CoCl2–stimulated or hypoxic H1299 cells (Fig. 2, H, left). However, after a 6-hour treatment with 100 nM deguelin, when HIF-1α protein was almost completely depleted, the level of phosphorylated Akt was similar to that in untreated cells (Fig. 2, H, right). Moreover, when constitutively active Akt (expressed from Ad-HA-MyrAkt) was overexpressed (10), deguelin had no effect on the level of HIF-1α (Fig. 2, H, lower). Thus, the PHD, pVHL, and PI3K–Akt pathways do not appear to be involved in the effect of deguelin on HIF-1α expression.

Heat Shock Protein 90 and Deguelin-Mediated Regulation of Hypoxia-Inducible Factor 1α Stability

Hsp90 physically interacts with HIF-1α and protects it from oxygen-independent (i.e., pVHL independent) degradation by an unidentified proteasome pathway (15,18,38,40). We next investigated the role of Hsp90 in deguelin-mediated destabilization of HIF-1α. As shown in Fig. 3, A, the levels of HIF-1α were restored in normoxic IGF–CoCl2–stimulated and hypoxic H1299 cells cultured with 100 nM deguelin and infected with Hsp90-expressing adenovirus (Ad-HA-Hsp90) (41). The total Hsp90 protein levels (endogenous intracellular and HA-tagged virus–induced), as determined by western blot analysis of Hsp90 and HA, showed no change after deguelin treatment. Moreover, overexpression of Hsp90 resulted in a rapid decrease in HIF-1α ubiquitination after a 1 hour of treatment with 100 nM deguelin and 10 µM MG132 (a proteasome inhibitor) (Fig. 3, B), indicating that Hsp90 is involved in the deguelin-mediated HIF-1α ubiquitination and destabilization.

We next investigate the involvement of Hsp90 in deguelin-mediated regulation of HIF-1α expression. Because treatment with deguelin for up to 16 hours did not change the total level of Hsp90 protein in H1299 cells, unlike the deguelin-mediated destabilization of HIF-1α (Fig. 3, C), we investigated whether HIF-1α interacted with Hsp90 in H1299 cells by use of coimmunoprecipitation assays and whether deguelin treatment modified the interaction. We found that Hsp90 was co-precipitated with anti-HIF-1α antibodies. The interaction between Hsp90 and HIF-1α was abolished in cells that had been treated with 100 nM deguelin for 1 hour, but HIF-1α protein expression was not affected (Fig. 3, D).

We further confirmed the effects of deguelin on the interaction between HIF-1α and Hsp90 by use of various HIF-1α mutant proteins. Because the helix–loop–helix and Per–Amt–Sim (HLH–PAS) domain of HIF-1α is essential for complex formation with Hsp90 and sensitivity to Hsp90 inhibitors (38,40,42), we examined the interaction between Hsp90 and various recombinant proteins containing the HLH–PAS–ODD–N-transactivation domain (N-TAD) domain, the ODD–N-TAD–C-terminal transactivation (C-TAD) domain, or C-TAD domains of HIF-1α, instead of intact HIF-1α. Consistent with previous findings (40,43), the HLH–PAS–ODD–N-TAD domain of HIF-1α interacted strongly with Hsp90, and this interaction was inhibited by the addition of 100 nM deguelin to the H1299 cells (Fig. 3, E). In contrast, recombinant ODD–N-TAD–C-TAD and C-TAD domains bound weakly to Hsp90, and this binding was not affected by deguelin. Thus, deguelin appears to interfere with the function of Hsp90 by inhibiting the interaction between Hsp90 and HIF-1α.

Molecular Modeling of Interaction Between Deguelin and Heat Shock Protein 90

We investigated whether deguelin could bind to the ATP-binding pocket of Hsp90 and inhibit the association between HIF-1α and Hsp90. By using the FlexX computer program, docking analysis of deguelin was conducted to determine whether deguelin could bind to the ATP-binding pocket of Hsp90. We used the structures of geldanamycin and 17-AAG, Hsp90 inhibitors that bind to the ATP-binding pocket of Hsp90 (44), as model structures. As shown in Fig. 4, A, the conformation of deguelin with the highest binding score among the FlexX docking results is a good match for the benzoquinone moiety and polyketide backbone of the geldanamycin and 17-AAG structures (23). The cis-fused ring system of deguelin conferred a C-shaped conformation that is similar to the overall shape of the Hsp90-bound geldanamycin or 17-AAG. The docked conformations of deguelin and 17-AAG with the highest

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Deguelin and the interaction between heat shock protein 90 (Hsp90) and hypoxia-inducible factor 1α (HIF-1α) under expression in normoxic insulin-like growth factor I (IGF)- or CoCl₂-stimulated or hypoxic conditions. A) Deguelin and Hsp90-mediated HIF-1α protein stability. Normoxic IGF- or CoCl₂-treated or hypoxic H1299 cells were infected with adenoviruses Ad-EV (control) or Ad-HA-Hsp90 expressing HA-tagged Hsp90 and then untreated or treated with 100 nM deguelin for 6 hours. Western blot analysis of HIF-1α, Hsp90, HA, and β-actin was conducted. B) Deguelin and ubiquitination of HIF-1α. H1299 cells were infected with an empty adenoviral vector (Ad-EV) or Ad-HA-Hsp90 and then treated with 100 nM deguelin and/or 10 μM MG132 for 1 hour, as indicated. The level of HIF-1α ubiquitination in deguelin-treated H1299 cells was determined by immunoprecipitation (IP) with an anti-HIF-1α antibody and western blot analysis (WB) with antibodies against ubiquitin or HIF-1α. Ub = ubiquitin. C) Deguelin and Hsp90 protein expression. Normoxic IGF- and CoCl₂-stimulated or hypoxic H1299 cells were untreated or treated with deguelin, as indicated, for 16 hours. The expression of Hsp90 was determined by western blot analysis. Overall, consensus binding scores showed that 17-AAG bound to the ATP-binding pocket of Hsp90 through various hydrophobic interactions (Fig. 4, B). In addition, we predicted that the carbonyl oxygen atom of deguelin formed hydrogen bonds both with the backbone amide of Phe-138 and side-chain amide of Asn-51 (Fig. 4, C) and that the oxygen atom of the methoxy group also formed a hydrogen bond with the side-chain amide of Asn-51 (Fig. 4, C). The carbonyl oxygen atom of deguelin forms hydrogen bonds both with the backbone amide and side-chain amide of Asn-51.

![Deguelin Binding to Heat Shock Protein 90 and Inhibition of the Chaperone Function of Heat Shock Protein 90](https://example.com/deguelin-binding.png)

**Deguelin Binding to Heat Shock Protein 90 and Inhibition of the Chaperone Function of Heat Shock Protein 90**

To further investigate the mechanism through which deguelin influences Hsp90 function, we examined the effects of deguelin on ATP binding to Hsp90 in vitro by use of ATP–Sepharose. The binding of Hsp90 to ATP–Sepharose was more strongly inhibited by deguelin (difference = 95.65%, 95% CI = 93.58% to 97.71%; *P* < 0.001) than by 17-AAG (difference = 80.67%, 95% CI = 67.5% to 93.8%; *P* < 0.001) (Fig. 5, A and B). To confirm that the effects of deguelin on Hsp90 were specific to ATP binding, we used the same assay to test the effects of deguelin on ATP binding to JNK1, which has an ATP-binding pocket that is structurally different from that of Hsp90, as a negative control. Deguelin did not alter the binding of JNK1 to ATP–Sepharose, whereas soluble ATP completely inhibited the binding of JNK1 to ATP–Sepharose (Fig. 5, B). In addition, consistent with the finding that ATP binding to Hsp90 was inhibited better by deguelin than by 17-AAG, treatment of H1299 cells with 100 nM deguelin for 16 hours significantly inhibited the binding of JNK1 to ATP–Sepharose. This suggests that deguelin is able to bind to the Hsp90 pocket and inhibit its chaperone function.
Fig. 4. Structural analysis of deguelin and heat shock protein 90 (Hsp90). Geldanamycin and 17-allylamino,17-demethoxygeldanamycin (17-AAG), which bind to the ATP-binding pocket of Hsp90, were used as model structures. A) Docked conformation of deguelin (yellow carbon) with the highest binding score in comparison with that of 17-AAG (magenta carbon) and geldanamycin (white carbon). The colors of the other atoms are red for oxygen and blue for nitrogen. The hydrogen atoms are not displayed for the sake of clarity. B and C) Structures of 17-AAG and deguelin bound to the ATP-binding pocket of Hsp90. Amino acid residues that form hydrogen bonds within the binding site are represented in line form, and the bound 17-AAG (B) or deguelin (C) are shown by capped stick models. The yellow dotted lines are hydrogen bond interactions (<2.5 Å). D and E) Space-filling model of 17-AAG (D) and deguelin (E) in the ATP-binding pocket of Hsp90. The binding pocket is a molecular surface (hydrophobicity map) of the surrounding amino acid residues. A trapped water molecule in the x-ray structure of the geldanamycin complex is shown as a magenta sphere. F) Orthogonal view of the complex between deguelin and Hsp90, as obtained by docking simulations.

Discussion

We have shown in this study that deguelin binds directly to Hsp90 and inhibits its chaperone activity, resulting in ubiquitin-mediated degradation of Hsp90 client proteins. We have also shown that deguelin has antitumor activity against xenografts of many human cancers, including lung, prostate, head and neck, and stomach cancers. These results are important because intense research efforts have been directed at developing more effective targeted anticancer therapies. Hsp90 is a molecular chaperone that has many client proteins that play critical roles in cancer progression, tumor angiogenesis, and therapy resistance. An interaction between Hsp90 and its client proteins is required for the stability and function of these client proteins. Consequently, Hsp90 is a potentially promising target for the development of new cancer treatments, and several small-molecule inhibitors of Hsp90 have shown promising antitumor activity in preclinical and/or clinical systems (8,46).

In this article, we also present evidence that 1) deguelin reduces the expression of a subset of Hsp90 client proteins, including the HIF-1α protein, under normoxic IGF-stimulated and hypoxic conditions; 2) deguelin-induced degradation of HIF-1α protein is independent of ROS, PHD, pVHL, and PI3K–Akt pathways under normoxic and hypoxic conditions; and 3) deguelin binds directly to the ATP-binding pocket of Hsp90 and thus interferes with the chaperone function of Hsp90. Thus, deguelin appears to be a novel Hsp90 inhibitor with potential chemotherapeutic activity in many cancers.

HIF-1α plays an important role in tumor growth and angiogenesis by regulating the transcription of several genes in response to hypoxic stress and changes in growth factor expression (26). Because we found that deguelin treatment affected the expression of HIF-1α, we investigated whether HIF-1α is involved in antitumor activities of deguelin. Our observations showed that deguelin treatment decreases the half-life of HIF-1α. Because ROS production may be involved in the HIF-1α stability and because rotenone (a rotenoid) inhibits the complex I mitochondrial respiratory chain and thus blocks hypoxia-induced ROS production (26,28–32),

decreased HIF-1α expression more than treatment with 5 µM 17-AAG (Fig. 5, C).

We then investigated whether blocking Hsp90 function would lead to the proteasome-mediated degradation of Hsp90 client proteins. As shown in Fig. 6, A, after treatment of A439, H1299, and H322 cells with 0, 10, or 100 nM deguelin for 0–2 days, the expression of many Hsp90 client proteins was reduced—including that of CDK4, Akt, eNOS, MEK1/2, and mutant p53 proteins (in H322 cells)—in a time- and dose-dependent manner, with no detectable effect on the expression of Hsp90 and β-actin. We found that prolonged treatment with deguelin also decreased the expression of HIF-2α, another HIF-α subunit and a Hsp90 client protein (45). However, HIF-2α was less sensitive than HIF-1α to the deguelin treatment because treatment with more than 100 nM deguelin for longer than 48 hours was required to suppress HIF-2α expression in H1299 and H322 cells (Fig. 6, A). Treatment with the proteasome inhibitor MG132 prevented the deguelin-mediated decreased expression of CDK4 and Akt in H1299 cells (Fig. 6, B). Thus, the ability of deguelin to disrupt Hsp90 function appears to have altered the expression of many signaling components that have roles in cancer cell proliferation, survival, and angiogenic activities.

deguelin has antitumor activity against xenografts of many human cancers.
ROS could have contributed to deguelin’s activity on HIF-1α protein expression. However, we found that the decreased HIF-1α expression mediated by deguelin treatment was not associated with ROS production and was not affected by the treatment with succinate, indicating that deguelin acts through a ROS-independent mechanism to destabilize HIF-1α.

Because the PHD, pVHL, and PI3K–Akt pathways have roles in the expression or stabilization of HIF-1α (27,35,36), we next investigated whether PHD, pVHL, and Akt are involved in deguelin-mediated regulation of HIF-1α expression by examining HIF-1α proteins mutated at two proline hydroxylation sites (Pro-402 and Pro-564) or HIF-1α proteins expressed in pVHL siRNA–transfected H1299 cells. In both cases, the expression of HIF-1α protein was decreased by deguelin treatment through a PHD- and pVHL-independent mechanism. Moreover, the effect of deguelin on HIF-1α expression was not abrogated by overexpression of constitutively active Akt, indicating that PI3K-Akt–independent mechanisms may mediate the effect of deguelin on HIF-1α expression. One such mechanism could involve Hsp90, which increases the stability of HIF-1α protein (15,40,42,43). Indeed, we found that the deguelin-mediated decreased expression of HIF-1α protein was attenuated by overexpression of Hsp90. Furthermore, the results of our structure–function analyses (computer modeling and ATP-binding assay) support a mechanism through which deguelin inhibits Hsp90 function by 1) inhibiting the binding of Hsp90 to the PAS domain of HIF-1α, which mediates the interaction between HIF-1α and Hsp90 and thus the sensitivity of HIF-1α to Hsp90 inhibitors (40,43); 2) binding to the ATP-binding pocket of Hsp90; 3) competing with ATP for binding with Hsp90; and 4) destabilizing HIF-1α and many Hsp90 client proteins.

A limitation of this study is that the mechanisms through which deguelin mediates its effects on various Hsp90 client proteins have
not been fully elucidated. When we tested deguelin as an inhibitor of Hsp90, we observed decreased expression of several Hsp90 client proteins (including CDK4, Akt, eNOS, MEK1/2, mutant p53, and HIF-2α) in a subset of deguelin-treated cancer cell lines. Each client protein showed different levels of sensitivity to the deguelin treatment, depending on the cellular context. We previously showed that treating premalignant human bronchial epithelial cells with deguelin for less than 1 day reduced the level of phosphorylated Akt (Ser-473) but did not affect that of total Akt protein (10). However, in this study prolonged treatment with deguelin decreased Akt expression in NSCLC cell lines, consistent with the observation that the Hsp90 inhibitor 17-AAG has a greater effect on phosphorylated Akt than on Akt (47,48). Of the client proteins that we tested for deguelin sensitivity, HIF-1α and phosphorylated Akt were the most sensitive. The differential levels of deguelin sensitivity may depend on various factors, including the half-life and expression of each client protein, the intracellular level and activity of the various E3 ubiquitin ligases and/or deubiquitylases [which are involved in polyubiquitination, which marks proteins for degradation by the proteasome (49)] required by the client proteins, and the metabolism of deguelin. The conformation of Hsp90 in multichaperone complexes could be another factor that affects the sensitivity of various cells to deguelin, as has been found for other Hsp90 inhibitors (50).

Our findings provide evidence that deguelin binds directly to the ATP-binding pocket of Hsp90, interferes with Hsp90 chaperone function, decreases the expression of many Hsp90 client proteins, and induces apoptosis in cancer cells, which reduces tumor growth. A concomitant reduction in the expression of Hsp90 client proteins induced by deguelin could have resulted in the broad antitumor activities of deguelin in vitro and in vivo in various types of cancer cells.

Fig. 7. Deguelin and heat shock protein 90 function in vivo. A) Immunohistochemical analysis. Cyclin-dependent kinase 4 (CDK4) expression and terminal deoxynucleotidyltransferase-mediated UTP end-labeling (TUNEL), both detected immunohistochemically, and 4′,6-diamidino-2-phenylindole (DAPI) staining for nucleus identification in H1299, A549, and MKN45 xenograft tumors obtained from vehicle control (Con) or deguelin-treated nude mice after 15–28 days are shown. B) Apoptosis. Percentage of apoptotic (TUNEL-positive) cells compared with DAPI-positive cells in H1299, A549, and MKN45 xenograft tumors obtained from vehicle (Con) or deguelin (Deg)-treated nude mice after 15–28 days is shown. Data are expressed as the mean; error bars are 95% confidence intervals.
Deguelin has several features that make it an attractive targeted antineoplastic drug in clinical trials. The calculated molecular volume of deguelin is approximately 70% of that of 17-AAG, an Hsp90 antagonist that is currently being evaluated in clinical trials but is proving unsatisfactory because of problems related to its metabolism and poor solubility (51). The binding of the ansamycin ring of 17-AAG to Hsp90 requires a large conformational change with a high entropic penalty (52). The molecular size and structural rigidity of deguelin could make its binding to Hsp90 more entropically favorable than the binding of 17-AAG. Indeed, deguelin is more potent than 17-AAG in vivo. For example, at 100 nM—a dose that is considerably below that achievable in rats (13,14,53)—deguelin inhibited expression of multiple Hsp90 client proteins and induced apoptosis in NSCLC cells, whereas 1 µM 17-AAG had antiproliferative and apoptotic effects in cancer cells (10,12). Moreover, when toxic effects, including inflammation and injury, in organs (including lung, liver, kidney, spleen, stomach, and ovary) were evaluated by a veterinary pathologist in xenograft models, deguelin was well tolerated, with no detectable organ or systemic toxic effects after a 4-week treatment to achieve therapeutic efficacy or after a 19-week treatment to achieve effective chemopreventive activities (11). Several clinical trials of Hsp90 inhibitors that have been completed or are in progress have observed various side effects, including liver, gastrointestinal, and constitutional symptoms, although it is not clear whether these side effects are related to the Hsp90 mechanism per se, to the specific chemical nature of the benzoquinone ansamycin structure, or to the formulation of the drug used (39,54,55).

Thus, deguelin appears to be an effective antineoplastic drug targeting Hsp90 client proteins. However, extensive and complete chronic toxicity testing in clinical trials is warranted before the further development of deguelin as an anticancer therapeutic agent.

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Notes
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