A Novel Derivative of the Natural Agent Deguelin for Cancer Chemoprevention and Therapy

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Abstract The natural compound deguelin has promising preventive and therapeutic activity against diverse cancers by directly binding to heat shock protein-90 and thus suppressing its function. Potential side effects of deguelin over a certain dose, however, could be a substantial obstacle to its clinical use. To develop a derivative(s) of deguelin with reduced potential side effects, we synthesized five deguelin analogues (SH-02, SH-03, SH-09, SH-14, and SH-15) and compared them with the parent compound and each other for structural and biochemical features; solubility; and antiproliferative effects on normal, premalignant, and malignant human bronchial epithelial (HBE) and non–small-cell lung cancer (NSCLC) cell lines. Four derivatives destabilized hypoxia-inducible factor-1α as potently as did deguelin. Reverse-phase protein array (RPPA) analysis in H460 NSCLC cells revealed that deguelin and the derivatives suppressed expression of a number of proteins including heat shock protein-90 clients and proteins involved in the phosphoinositide 3-kinase/Akt pathway. One derivative, SH-14, showed several features of potential superiority for clinical use: the highest apoptotic activity; no detectable influence on Src/signal transducer and activator of transcription signaling, which can promote cancer progression and is closely related to pathogenesis of Parkinson's disease (deguelin, SH-02 and SH-03 strongly activated this signaling); better aqueous solubility; and less cytotoxicity to immortalized HBE cells (versus deguelin) at a dose (1 μmol/L) that induced apoptotic activity in most premalignant and malignant HBE and NSCLC cell lines. These collective results suggest that the novel derivative SH-14 has strong potential for cancer chemoprevention and therapy, with equivalent efficacy and lesser toxicity (versus deguelin).

Cancer remains one of the leading causes of death worldwide despite several decades of intensive efforts to prevent and treat it. Most effective cancer therapy agents are toxic in normal tissue, indicating the need for novel preventive and therapeutic drugs with no or low toxicity. Deguelin, a rotenoid isolated from the African plant *Mundulea sericea* (Leguminosae) and other plants (1), is a heat shock protein-90 (Hsp90) inhibitor with potent apoptotic and antiangiogenic effects on transformed cells and a variety of cancer cells but without cytotoxicity in normal cells (2, 3), making it a promising cancer prevention and therapy agent. We recently showed that deguelin has promising activity against a number of human cancers, at least in part, because it binds to the ATP pocket of Hsp90α, which leads to decreased expression of a number of Hsp90 client proteins, including mutated p53, cyclin-dependent kinase 4, mitogen-activated protein kinase kinase-1/2, Akt, and hypoxia-inducible factor (HIF)-1α, in addition to decreased expression of previously known deguelin targets such as cyclooxygenase-2, necrosis factor κB, and nucleoporin 98 kDa (Nup98).

Hsp90 facilitates the adaptation of cancer cells to many environmental stresses owing to its function as a molecular chaperone required for the stability and proper functioning of many cellular proteins. Therefore, cancer cells conceivably could rely on Hsp90 for their growth and survival. Indeed, small-molecule inhibitors of the chaperone function of Hsp90, such as 17-allylamino-17-demethoxygeldanamycin,

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have had promising effects on cancer cell proliferation, survival, and angiogenesis in preclinical models by accelerating degradation of numerous oncogenic Hsp90 client proteins. Phase II clinical trials of 17-allylamino-17-demethoxygeldanamycin have begun, and several second-generation analogues of 17-allylamino-17-demethoxygeldanamycin are now in late preclinical development. Several clinical trials of Hsp90 inhibitors have revealed various side effects with constitutional clinical symptoms (4–6). Deguelin has antitumor activity *in vitro* or *in vivo* at doses producing no toxic effects to normal cells or tissues and thus may be a promising cancer preventive and therapeutic agent (7). Researchers originally identified deguelin as a potent mitochondria complex I, NADH dehydrogenase inhibitor and implicated mitochondrial dysfunction and diminished complex I activity as factors in the pathophysiology of Parkinson's disease (8, 9), indicating a potential for noncytologic toxicity with this drug. Indeed, long-term or high-dose deguelin treatment results in a Parkinson's disease–like syndrome (associated with reduced tyrosine hydroxylase–positive neurons) in rats (10).

Despite the promising anticancer activity of deguelin *in vivo* and *in vitro*, the potential toxicity of this drug observed in rats has raised the need for new derivatives with stronger efficacy or less toxicity than that of deguelin. Therefore, we conducted the present study to synthesize deguelin derivatives and to compare them with deguelin and each other for molecular effects associated with anticancer effects and toxicity. We used Western blotting, reverse-phase protein array (RPPA) and structural analyses, and a proliferation assay for these assessments.

Materials and Methods

Cells and reagents

The BEAS2B human bronchial epithelial (HBE) cell line and its derivatives, including premalignant (1799 and 1198) and malignant (1170-I) cell lines; derivatives of immortalized HBE cells (HBEC3), including p53 short interfering (Si) RNA-expressing (HBEC3/p53i) and RAS^{v12}-expressing (HBEC3/RAS^{v12}) HBEC3 cells; and H1299 and H460 non–small cell lung cancer (NSCLC) cell lines were previously described (11–13). Antibodies were obtained from Cell Signaling Technology, Epitomics, Inc., and Santa Cruz Biotechnology, Inc. The antibodies used in the assay are listed in Supplementary Table S1.

Synthesis of deguelin and its derivatives

Deguelin was synthesized as described previously(10) and the detail procedures are described in Supplemental Methods. The synthesis of the five derivatives is described in Supplemental Methods as well.

Effects of deguelin and derivatives on proliferation of normal, premalignant, and malignant HBE cells and NSCLC cells

BEAS2B, 1799, 1198, 1170-I, HBEC3/p53i, HBEC3/RAS^{v12}, H460, and H1299 cells were seeded into 96-well culture plates (5×10^3 cells/well) and allowed to adhere overnight. The next day, the cells were treated with various concentrations of deguelin or SH-14 or with 0.1% DMSO as a control for both drug treatments. Cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide assay after 3 d. Six replicate wells were used for each analysis, and data for replicate wells are presented as the mean with ± SD.

Western blot analysis for deguelin and its derivatives on HIF-1 expression

Western blot analysis of HIF-1α was done to asses the anti-HSP90 function of the drugs after similar treatment. H460 cells were untreated or treated with the derivatives or deguelin at 100 nmol/L concentration for 16 h and then incubated under hypoxic condition (1% O_2) for 12 h. The resulting cell lysates were submitted to Western blot analysis with monoclonal antibody against HIF-1α (BD Pharmingen)

Lysate preparation and array spotting for RPPA

H460 cells were cultured in optimal medium with 10% fetal bovine serum in six-well plates. The cells were treated with 50 nmol/L deguelin, the derivatives, or vehicle (DMSO) for 24 or 48 h. All the samples were replicated in two to four independent experiments. Cells were then washed twice with PBS and lysed in ice-cold lysis buffer [1% Triton X-100, 50 mm HEPES (pH 7.4), 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L egtazic acid, 100 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 1 mmol/L Na_3VO_4, 10% glycerol] supplemented with proteinase inhibitors (Roche Applied Science). The cellular protein concentration in the lysates was determined using the Bradford reaction (Bio-Rad). The lysates (three parts) were boiled with a solution (one part) of 4× SDS (90%)/β-mercaptoethanol (10%). Serial dilutions of the cell lysates were done in lysis buffer containing 1% SDS (dilution buffer). The diluted lysates were spotted on nitrocellulose-coated glass slides (FAST slides; Whatman, Inc.) using an Aushon Biosystems 2470 microarrayer.

Antibody probing and signal detection of RPPA

The DAKO-catalyzed signal amplification system (DakoCytomation) was used for antibody blotting. Briefly, the nonspecific binding sites on nitrocellulose were blocked using ReBlot (mild; Chemicon International) and I-Block (Applied Biosystems). Endogenous peroxidase was blocked using hydrogen peroxide, and avidin and biotin were blocked using a biotin blocking system (DakoCytomation). Each slide was then incubated with a primary antibody at the appropriate dilution. The signal was captured by a biotin-conjugated secondary antibody and amplified using tyramide deposition. The analyte was detected using avidin-conjugated peroxidase reactive with the substrate chromogen diaminobenzidine. Subsequently, the slides were individually scanned, analyzed, and quantitated using the MicroVigene software program (VigeneTech, Inc.). This program enables automated spot identification, background correction, individual spot intensity determination (expressed in logarithmic units), and curve fitting using a logistic fit model such as $\ln(y) = a+(b - a)/1 + \exp\{c^*[d - \ln(x)]\}$. Serial dilution curves were then constructed for all of the samples, and each sample on each slide was assigned a relative quantification value as described previously (14). Each protein and phosphoprotein measurement was subsequently corrected for loading using the average expression of all 85 measured proteins in each sample.

Docking modeling

Modeling of the docking of deguelin and the derivatives with the ATP pocket of Hsp90 was done as previously described (7). Flexible docking of deguelin and the derivatives to a target receptor Hsp90 has been done with the Sybyl 7.0 software package (Tripos, Inc.) based on Red-Hat Linux 7.0. The chemical structures of deguelin and the derivatives were prepared in Mol2 format using the sketcher module in SYBYL, and partial atomic charges were calculated using the Gasteiger-Huckel method and assigned to the ligand atoms. To optimize the ligand structures, the conjugate gradient energy minimization of the ligand was run until the value converged to a maximum derivative of 0.001 kcal/mol Å; the final coordinates were stored in a database. The X-ray crystal structure (15) of human Hsp90 complexed with geldanamycin (Protein Data Bank entry 1YET) was used as a target for the docking. All crystallographic water molecules were removed from the 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 the FlexX run were set in 1,000 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 using a consensus scoring method (CScore) based on the following five scoring functions: FlexX score, G_score, D_score, ChemScore, and PMF (Potential of Mean Force) score (16). After visual inspection, final docking models were created by selecting one of the conformers with the highest consensus scores (CScore = 5) and then docking into the active site of Hsp90.

Generation of molecular surface

Surface hydrophobicity (lipophilicity) potential maps of the ligand binding site for Hsp90 were generated on the solvent assessable (connolly) surface using the MOLCAD module in SYBYL.

Laser nephelometry

To determine the kinetic solubility of deguelin and its derivative, stock solutions were prepared at 10 mmol/L in 5% DMSO:95% PBS buffer manually. For a single batch of compounds, the stock solutions were diluted to decreasing molarity across the well plates with 5% DMSO:95% PBS buffer. Each plate was read vertically, with a gain of 30 and a laser intensity of 90% to produce raw data of counts per well. All raw data were processed using the BMG LabTech NEPHELOstar Galaxy evaluation software.

Results

Synthesis of five derivatives of deguelin that inhibit Hsp90 function and lung cancer cell proliferation

We synthesized SH-02, SH-03, SH-09, SH-14, and SH-15 to develop derivatives of deguelin with improved efficacy. All five derivatives have intact backbone rings and saturation of terminal 1,2-unsaturated benzopyran ring; hydroxylation of 7a carbon or changes of 7 carbon were allowed. The structures of synthesized deguelin derivatives are shown in Fig. 1A.

To validate the anticancer activity of the derivatives, we analyzed them for two known effects of deguelin: inhibition of Hsp90 function and NSCLC cell proliferation. Because HIF-1α was one of the most sensitive Hsp90 client proteins in response to deguelin treatment (7), we assessed the effects of deguelin and the derivatives on HIF-1α protein level in H460 cells (Fig. 1B). H460 cells were untreated or treated with

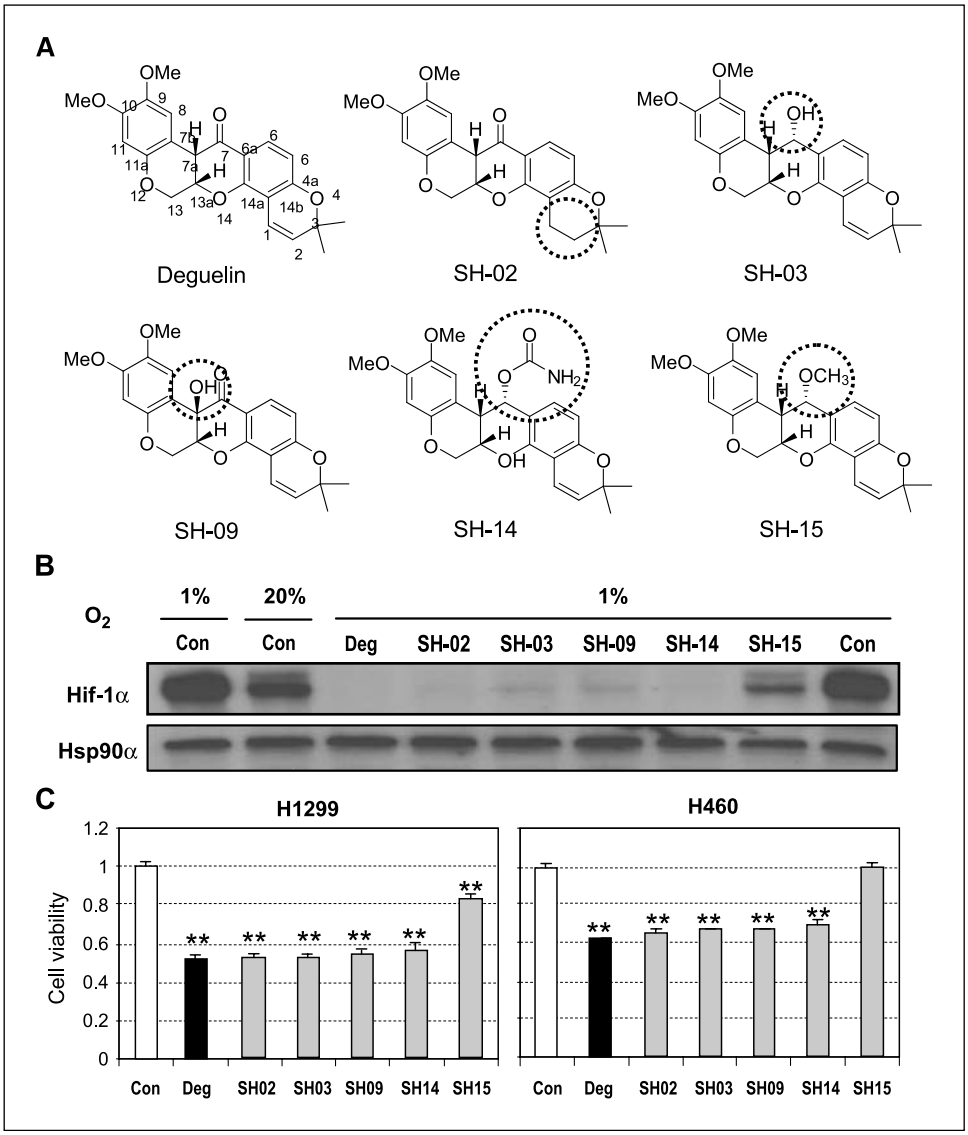

Fig. 1. Assessment of activity of novel deguelin derivatives. *A*, chemical structure of deguelin and the derivatives used in the study. The regions that changed from the mother compound, deguelin, are marked with dotted circles. *B*, Western blotting of HIF-1α pretreated with the 32 deguelin (*Deg*) derivatives. Of note, SH-01, SH-03, SH-09, SH-14, and SH-15 were as effective as deguelin in suppressing HIF-1α expression under hypoxic conditions. *C*, effects of deguelin and the derivatives on the proliferation of the NSCLC cell lines H460 and H1299. Results are expressed as relative cell proliferation compared with the DMSO-treated control cells (*Con*). *Columns*, mean of six identical wells from a representative single experiment ($n = 6$); *bars*, SE. Drug concentration, 10^{-7} mol/L. **, $P < 0.01$.

deguelin or the derivatives, incubated under hypoxic conditions, and then analyzed for HIF-1α expression. Western blot analysis revealed that HIF-1α protein almost disappeared in the cells treated with any of the compounds except SH-15, which showed a moderate effect on the protein expression. We further examined the cytotoxic effects of the five derivatives on H1299 and H460 NSCLC cell lines. SH-02, SH-03, SH-09, and SH-14, but not SH-15, significantly decreased proliferation of both cell lines in a manner similar to that of deguelin (Fig. 1C). Therefore, we concluded that SH-02, SH-03, SH-09, and SH-14 may have similar effects on HIF-1α expression and cytotoxic activity in NSCLC cells compared with deguelin.

Use of RPPA to scan molecular-level changes induced by deguelin derivatives systematically

We confirmed that two known effects of deguelin on NSCLC cells, inhibition of cell proliferation and HIF-1α expression, are conserved in the derivatives as we identified previously with deguelin. However, it is not clear yet whether other many molecular changes of the cancer cells that were treated with these derivatives may be distinctive or similar among them. In addition, the results on proliferation inhibition, mainly from the cumulated effects of the drugs, cannot lead us to identify molecular-level changes in the cells that were treated by the derivative drugs. To find the derivatives with better efficacy or less off-target activity than the parent compound, we used the RPPA, a new technology that allows more systematic scanning of the molecular changes in the cells rather than several known activities of the parent compound. We were able to get the expression profile of more than 80 cellular proteins of the H460 cells that had been treated with deguelin or the derivatives for 1 or 2 days at the dose (50 nmol/L) the cells do not die yet. Because SH-09, a major metabolite of deguelin (10), was expected to have similar results to that of deguelin, we excluded the derivative from the analysis. The clustered heat map of RPPA shown in Fig. 2 revealed five major clusters of molecules according to the patterns of expression level compared with control cells. Interestingly, we observed a distinguishable pattern of protein expression among the cells treated with different derivatives. In addition, deguelin, SH-02, SH-03, and SH-14 regulated the expression of many proteins in similar patterns. We observed no significant differences in protein expression between the SH-15–treated and control cells. The proteins in the cluster I included increased proteins of an apoptosis marker–cleaved form of caspase-7, activated Src, and activated signal transducer and activator of transcription (STAT). Expression of the molecules in cluster II increased on the first day of treatment but decreased or were similar to that induced by control treatment on the second day. This cluster included phosphorylated ribosomal protein 6 (S6), cadherin, and the early apoptosis marker cleaved poly(ADP-ribose) polymerase (17). Cluster III contained proteins whose expression did not change on the first day of treatment but decreased significantly on the second day. Interestingly, this cluster included mammalian target of rapamycin (mTOR), glycogen synthase kinase 3, AMP-activated protein kinase, tuberous sclerosis complex 2, mitogen-activated protein kinase, phosphoinositide-dependent protein kinase 1, S6, Src, P21, and phosphorylated 4E-binding protein 1. Expression of most of the proteins in the phosphoinositide 3-kinase (PI3K)/mTOR signaling pathway was down-regulated by deguelin and the derivatives in cluster III. Of note is that we observed major changes in the expression of unphosphorylated proteins in this cluster. Cluster IV contained proteins whose expression levels did change only mildly after the treatment. Finally, cluster V contained proteins whose expression was down-regulated on the first day of treatment but recovered pretty much to the original level on the second day. As we observed in the cell proliferation assay and HIF-1α expression Western blotting in Fig. 2, the levels of protein expression induced by SH-15 were not different significantly from those in control treatment, whereas the levels induced by deguelin, SH-01, SH-03, and SH-14 were similar in each cluster. Collectively, the RPPA showed that the SH-15 is the least effective deguelin derivative in the changes of protein expression whereas others are similarly potent as we have seen using Western blotting of HIF-1 and cell proliferation assay.

Similar but distinctive molecular signatures of the deguelin derivatives

We classified the proteins whose expression differed by treatment as determined using RPPA according to the known function of the proteins. We and others have previously found that treatment with deguelin induced apoptosis of cancer cells. In the present study, we examined several proteins known to be associated with programmed cell death in the H460 NSCLC cells. We found that the expression of cleaved caspase-7 (2nd day) and cleaved poly(ADP-ribose) polymerase (1st day) was increased by treatment with SH-02, SH-03, and SH-14, as well as deguelin (Fig. 3A). Of the four derivatives we studied, SH-14 was most effective on regulating the expression of proteins related to apoptosis, whereas SH-02 and SH-03 had marginal or variable effects on those, and SH-15 did not induce any effects on these proteins. We also sought to determine whether the derivatives shared the Hsp90-inhibitory effect of deguelin that we previously observed (7). We found that expression of epidermal growth factor receptor, cyclin-dependent kinase 4, mitogen-activated protein kinase kinase 1, vascular endothelial growth factor receptor 2, insulin-like growth factor type I receptor, and AKT, the known HSP90 client proteins, were down-regulated by treatment with deguelin, SH-02, SH-03, and SH-14, but not with SH-15 (Fig. 3B). Interestingly, SH-02, SH-03, and SH-14 similarly decreased the expression of Hsp90 client proteins. In addition to the regulation of the expression of proteins involved in apoptosis and Hsp90 function, we observed that treatment with deguelin and its derivatives significantly and consistently changed the expression of a series of proteins in the PI3K/mTOR pathway (Fig. 3C). Treatment with deguelin, SH-02, SH-03, or SH-14 down-regulated the expression of almost every proteins in the PI3K/mTOR signaling axis. However, SH-03 was much less effective than SH-02, SH-14, and deguelin. Interestingly, the two hallmarks of the translation signaling downstream from mTOR, the phosphorylated form of 4E-binding protein and S6, had distinctive patterns of expression induced by the drugs, deguelin, SH-02, SH-03, or SH-14.

We have observed that the activity of SH-02, SH-03, and SH-14 mimics that of deguelin. In addition to the expression of apoptosis markers, Hsp90 client proteins, and PI3K/mTOR signaling–related proteins, treatment with deguelin, SH-02,

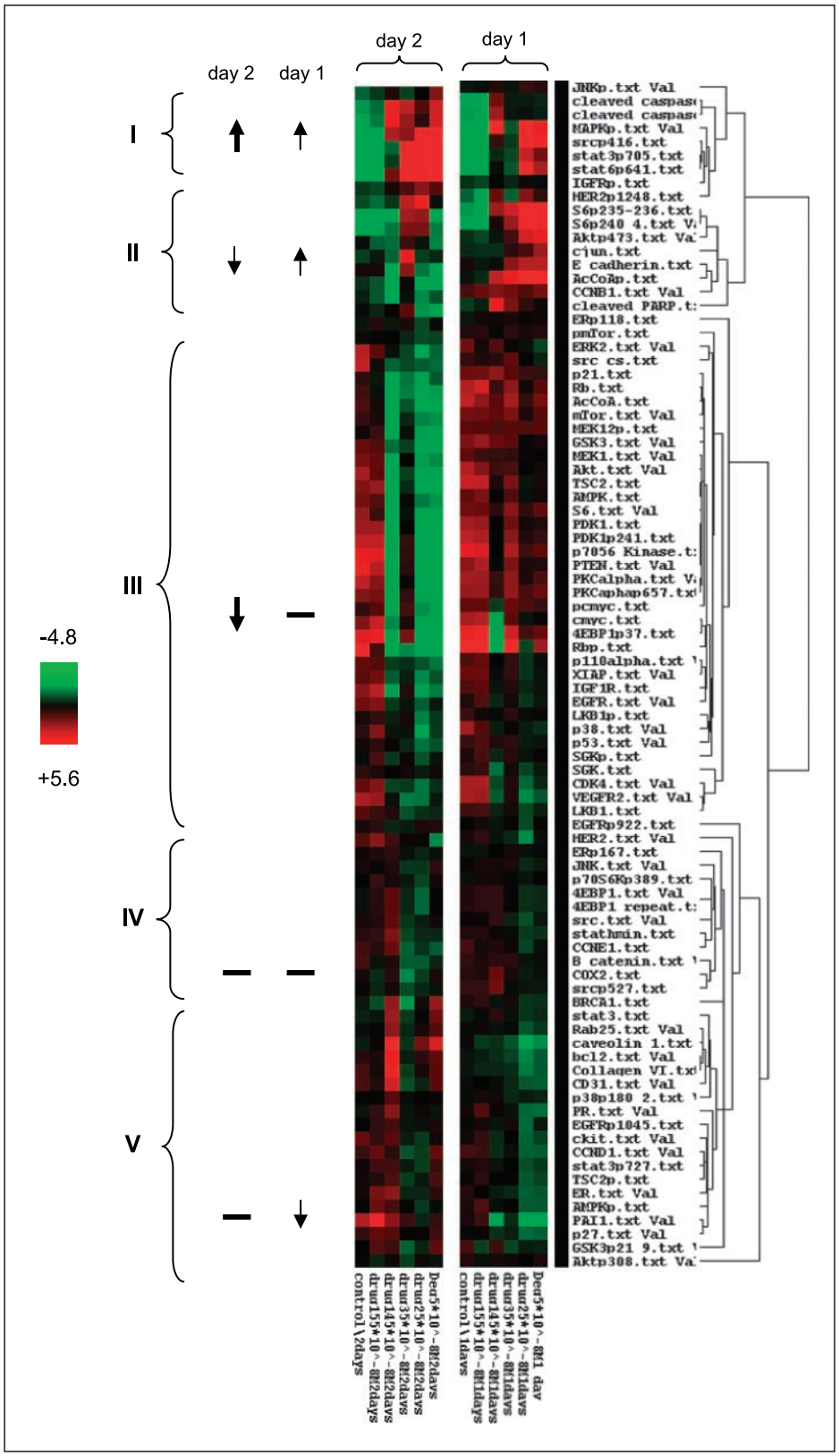

Fig. 2. Clustered heat map for the RPPA analysis. The average binding values in two to four independent samples on days 1 and 2 of treatment were analyzed and are illustrated. The proteins were categorized into five clusters based on changes in their expression pattern.

SH-03, and SH-14 down-regulated the expression of a series of proteins mainly related to cell signaling (Supplementary Fig. S1). These proteins, including phosphorylated Rb and myc, may be related to suppression of Hsp90 function and/or arrest of the proapoptotic/cell cycle by these drugs. In addition, we observed that expression of caveolin, which is related to the endocytosis and caveolar structure and also associated with increased apoptosis in macrophages (18),

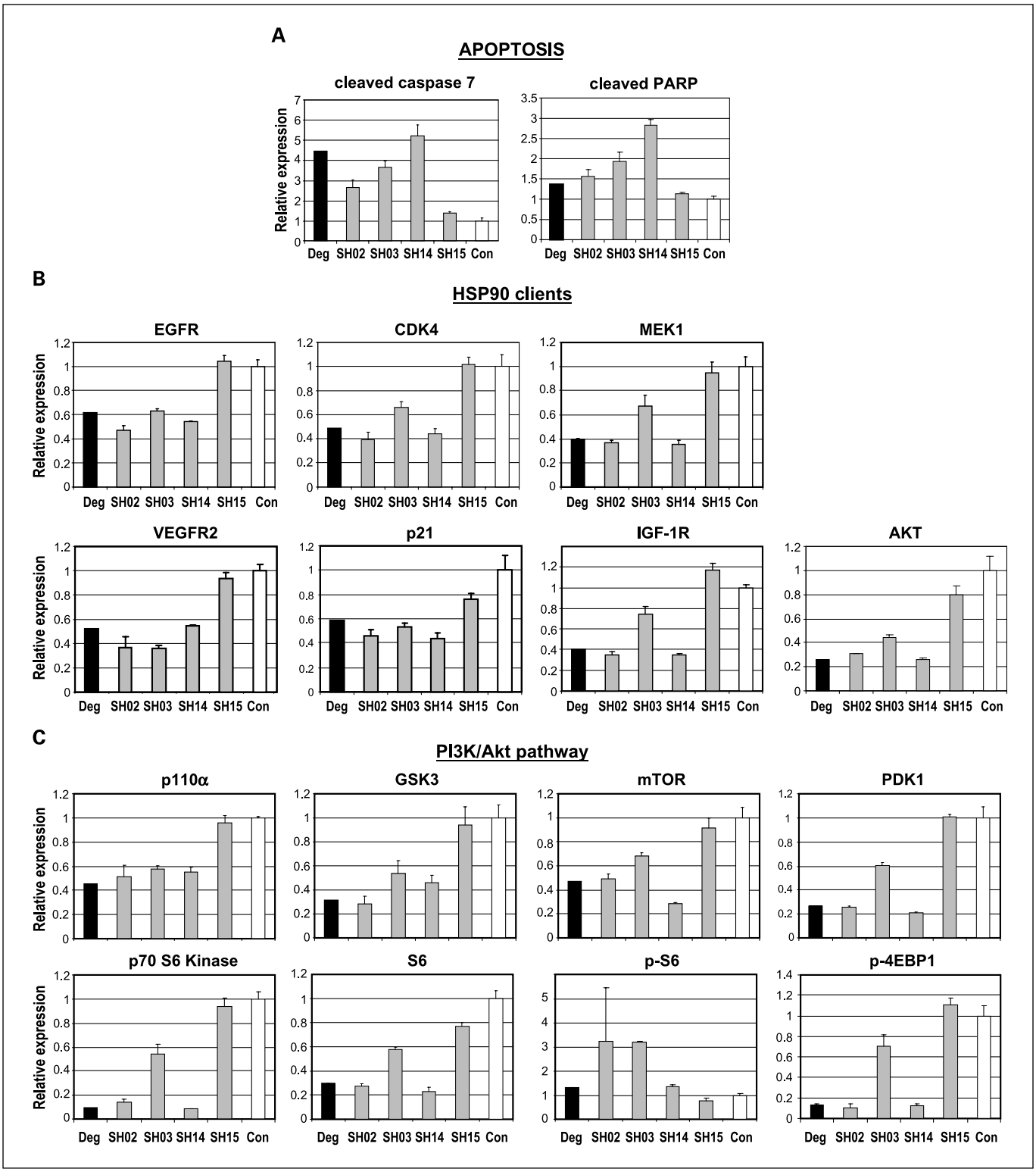

Fig. 3. Protein expression changes induced by deguelin and its derivatives. The relative levels of expression of the proteins analyzed using the RPPA were plotted. The expression level in the control sample was set to 1, and the relative level of expression is shown. *Columns,* mean of the repeated experiments and the SEs. *A,* apoptosis markers. *B,* Hsp90 client proteins. *C,* PI3K/mTOR signaling molecules.

was increased by treatment with the compounds. In most of the results, SH-02 was as potent as deguelin in down-regulating the expression of the proteins. In comparison, SH-14 occasionally showed stronger activity than deguelin did, and the activity of SH-03 was similar to that of deguelin in induction of apoptosis markers and inhibition of cell proliferation. However, SH-03 was less active than deguelin, SH-02, and SH-14 in down-regulating the expression of Hsp90 client proteins and

PI3K/mTOR axis proteins and the other protein expression. Overall, deguelin, SH-02, SH-03, and SH-14 regulate cellular protein expression in similar patterns at different levels.

Unlike most of the other proteins we analyzed, deguelin and the derivatives differentially regulated expression of a group of proteins related to *Src*, a proto-oncogene (19), and the activity of one of its downstream proteins, STAT (Fig. 4). We found that deguelin, SH-02, or SH-03 increased expression of the active form of Src (Src-p416) and STAT3 (STAT3-p705) 8- to 20-fold with no significant changes in the total form of the proteins. Surprisingly, treatment with SH-14 showed very mild effects on the expression of Src-p416 and STAT3-p705. In addition, unlike with the active form of phosphorylation of Src, treatment with deguelin, SH-02, or SH-03 did not change the negative regulatory phosphorylation of Src-p527 or STAT3-p727. Additionally, treatment with deguelin, SH-02, or SH-03 increased STAT6 phosphorylation 40 to 100 times, but treatment with SH-14 only very mildly increased this phosphorylation. Therefore, it was clear that SH-14 did not induce Src and/or STAT signaling as strongly as deguelin or the other active derivatives did.

We then investigated whether the derivatives can bind to the ATP-binding pocket of Hsp90 and inhibit the association between HIF-1α and Hsp90 as we described previously (7). We conducted docking analysis of deguelin and its derivatives (SH-02, SH-03, SH-09, and SH-14) by docking into the X-ray crystal structure of human Hsp90 complexed with geldanamycin using the FlexX program to determine whether these compounds bind to this pocket. The docked conformer of deguelin and each SH-compound (white) with highest consensus score (CScore = 5) was retrieved from FlexX results, and shown with geldanamycin (yellow) in the active site of

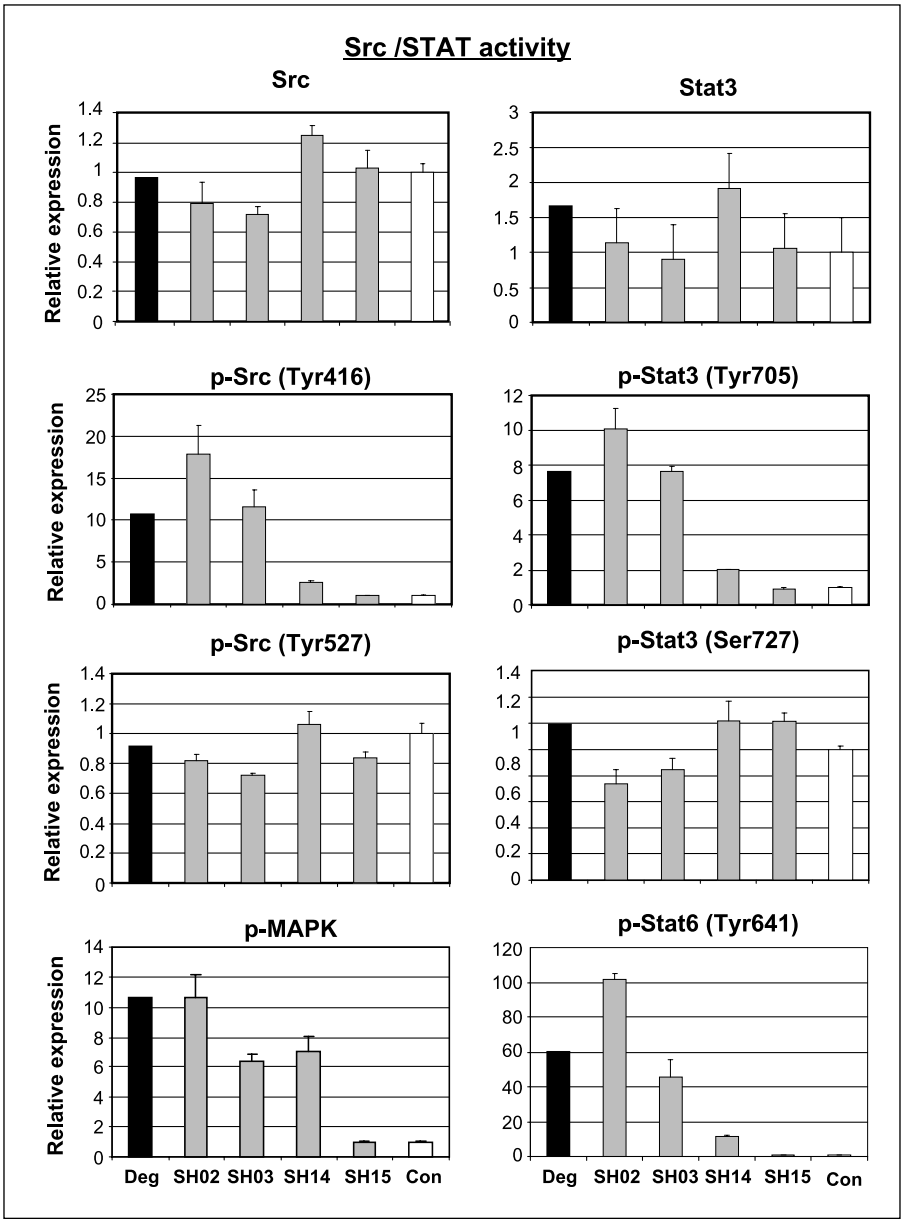

Fig. 4. Src and STAT activation induced by deguelin and its derivatives. The expression level in the control sample was set to 1, and the relative level of expression is shown. *Columns*, mean of repeated experiments; *bars*, SD.

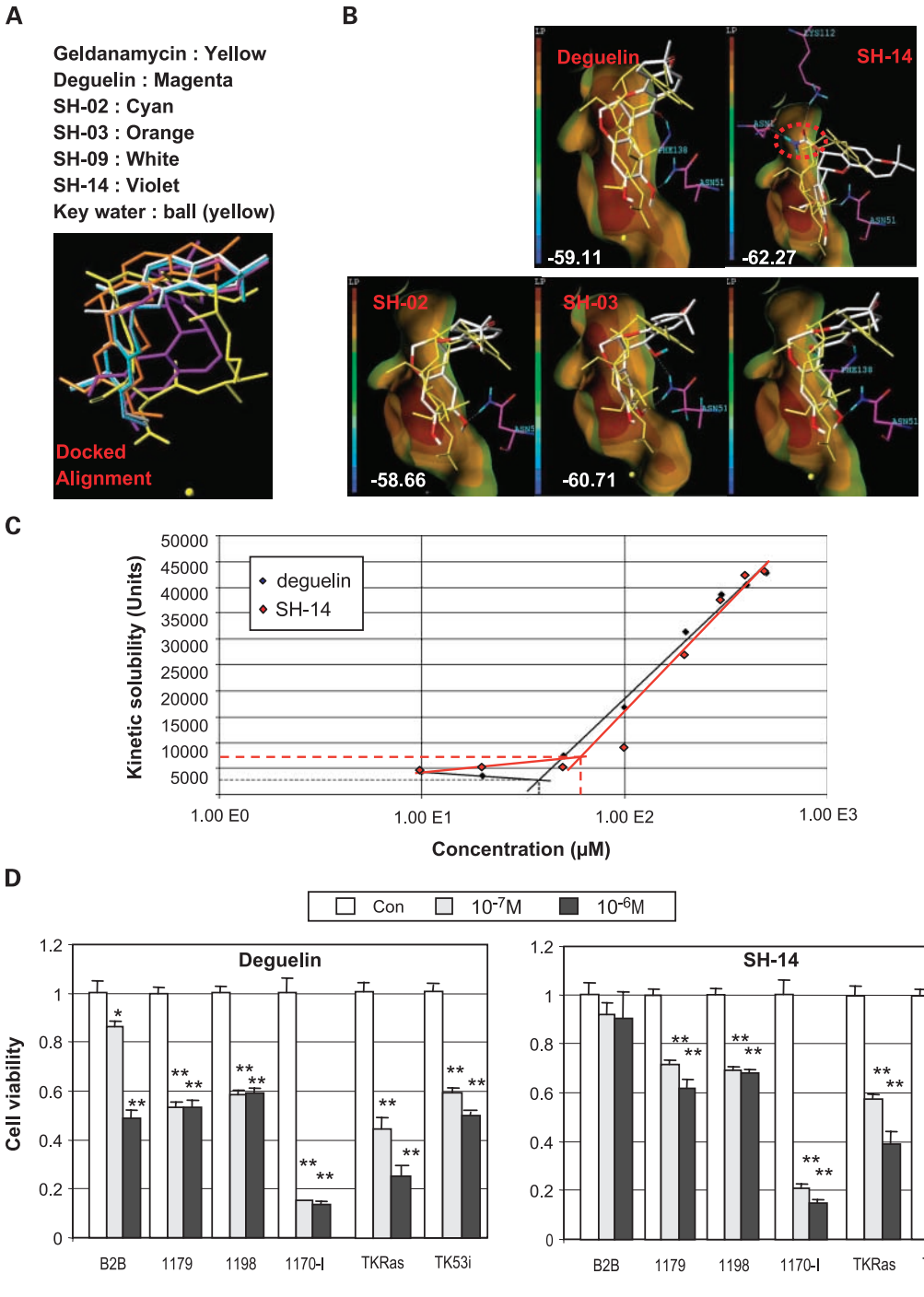

Fig. 5. Physical characteristics and chemopreventive cytotoxicity of the derivatives (SH compounds) compared with deguelin. *A,* structural analysis of deguelin and the derivatives, which bind to the ATP-binding pocket of Hsp90. Docked poses of deguelin (*magenta*) and SH compounds superimposed over the X-ray pose of geldanamycin (*yellow*) within the ATP-binding pocket of Hsp90. The hydrogen atoms are not displayed for clarity. *B,* binding pose of deguelin and SH compounds (white carbon) in comparison with the X-ray pose of geldanamycin (yellow) in the active site of Hsp90. The binding energy score value is the average of binding energy calculated by five scoring functions. Ligands are surrounded by the molecular surface (lipophilicity map) of the amino acid residues in the active site pocket. The color-code for molecular surface is shown on the left: lipophilicity increases from blue (hydrophilic) to brown (lipophilic). *C,* kinetic solubility of deguelin and SH-14. Relative kinetic solubility was determined by nephelometric analysis. Two linear lines are fitted to the data and the point at which they across is taken as the kinetic solubility. Graph of the kinetic solubility for deguelin (*dark blue diamond*). Graph of kinetic solubility for SH-14 (*red diamond*). *D,* effects of deguelin and SH-14 on the proliferation of virally immortalized (B2B), immortalized premalignant (1799 and 1198), malignant (1170-I), and genetically immortalized premalignant (TKRas and TK53i) HBE cell lines. Results are expressed as relative cell proliferation compared with the proliferation of DMSO-treated control cells (*Con*). In the same cell line, the same control was used for both drug treatments. *Columns,* mean of six identical wells from a representative single experiment ($n = 6$); *bars,* SDs. *Open columns,* DMSO treated control (*Con*); *light gray columns,* 10^{-7} mol/L; *dark gray columns,* 10^{-6} mol/L; *, $P < 0.05$; **, $P < 0.01$, for all cells treated with drug relative to control cells.

Hsp90. The binding mode of deguelin and its derivatives is almost identical to the bound conformation of geldanamycin in the X-ray structure (Fig. 5A). The D and E rings of ligands were matched well with the benzoquinone moiety, and the *cis*-fused ring system of ligands confers a C-shaped conformation, similar to the overall shape of the ansamycin ring of Hsp90-bound geldanamycin. As shown in Fig. 5B, all ligands formed H-bonding with Phe^{138} and/or Asn^{152} inside the binding pocket. Noticeably, the carbamate moiety of SH-14 formed additional H-bondings with the Lys^{112} and Asn^{1} residues located at the surface of the binding pocket, and the SH-14 had lower binding energy score than deguelin and other derivatives. Tight H-bonding network in the ATP-binding pocket of Hsp90 formed by SH-14 may be related to the improved binding affinity and biological activity of this compound.

SH-14 has better aqueous solubility than deguelin

Aqueous solubility is one of the most important physical properties in drug discovery (20). Developing low-soluble and lipophilic compounds to drug candidates having proper pharmacokinetic profiles is not only difficult but also more time-consuming and costly. Nephelometry is a reliable technique for the measurement of kinetic solubility of low-soluble compounds (21). Laser nephelometry measures the intensity of the scattered light when a laser beam is passed through a solution. The more insoluble particles there are in the solution, the greater the amount of scattered light measured as counts. We conducted the measurement of kinetic solubility for deguelin and SH-14 through the nephelometric analysis using NEPHELOstar Galaxy (BGM LabTechnologies). Through this nephelometric analysis, we obtained the kinetic solubility for deguelin (37.69 μmol/L, 14.94 μg/mL) and SH-14 (62.08 μmol/L, 27.28 μg/mL) in PBS buffer solution containing 5% DMSO (Fig. 5C). This Nephelometric analysis showed that SH-14 is more soluble than deguelin in aqueous media. In addition, we performed a calculation of LogP (cLogP) using CemBioDraw Ultra 11.0 program (CambridgeSoft Corporation) to determine lipophilicity for deguelin and SH-14. Compared with deguelin (cLogP: 4.38), SH-14 showed somewhat low lipophilicity (cLogP: 3.87). Consequently, compared with deguelin, SH-14 shows better physical properties as a drug candidate, as well as different molecular responses and similar efficacy for cancer therapeutics.

SH-14 and deguelin cytotoxicity in premalignant lung epithelial cells

We compared the cytotoxic effects of SH-14 and deguelin on the growth of virally immortalized BEAS-2B (B2B) and its premalignant (1799 and 1198) and malignant (1170-I) derivative HBE cells; these different cells constitute an *in vitro* progressive lung carcinogenesis model. We also tested the effects of deguelin and SH-14 on derivatives of genetically immortalized premalignant HBE cells (HBEC3), expressing RAS^{v12} (HBEC3/RAS^{v12}, TKRas) or p53-Si RNA (HBEC3/p53i, TK53i). The growth of most premalignant and malignant HBE cell lines was inhibited by both deguelin and SH-14 in a dose-dependent manner (Fig. 5D). Of note, BEAS-2B cells, which do not have premalignant characteristics, were sensitive to deguelin but not to SH-14, suggesting that SH-14 may be less cytotoxic to normal lung epithelial cells than deguelin. All of the cells that mimicked bronchial epithelial cells in premalignant and malignant lung carcinogenesis were sensitive to deguelin and SH-14, suggesting the chemopreventive and therapeutic potential of SH-14.

Discussion

In this study, we synthesized five derivatives (SH-02, SH-03, SH-09, SH-14, and SH-15) of deguelin, a well-studied chemopreventive and therapeutic agent (3, 13, 22–26). Although we found similar overall activity among the derivatives in two independent assays (HIF-1α Western blotting and inhibition of premalignant and malignant HBE and NSCLC cell proliferation), systematic screening with RPPA and study of physical characteristics revealed significant differences between SH-14 and the other derivatives in effects at the molecular level (summarized in Table 1). Our results present preclinical evidence that the deguelin derivative SH-14 has potential as a cancer preventive and therapeutic drug with similar efficacy but fewer side effects compared with deguelin or other analogues we tested.

Deguelin has shown cancer chemopreventive and therapeutic activities in a variety of cancer types (3, 13, 27, 28). However, its potential side effects could limit its use in clinical cancer chemoprevention and treatment. Therefore, we attempted to develop derivatives of deguelin with better efficacy and/or fewer side effects than those of deguelin. Most

Table 1. Summary of activity of the derivatives compared with deguelin

Formula	Deguelin	SH-02	SH-03	SH-09	SH-14
	$C_{23}H_{22}O_6$	$C_{23}H_{24}O_6$	$C_{23}H_{24}O_6$	$C_{23}H_{22}O_7$	$C_{23}H_{25}O_7$
Docking in ATP pocket	++	++	+++	++	++
Hif-1	++	++	++	++	++
Cytotoxicity	++	++	++	++	++
Apoptosis/RPPA	++	++	++	ND	+++
Hsp90/RPPA	++	++	++	ND	++
mTOR/RPPA	++	++	+	ND	++
Src/STAT Activation/RPPA	+++	+++	+++	ND	+
Aqueous solubility (μmol/L)	37.69	ND	ND	ND	62.08

of the activity induced by deguelin and the derivatives we examined consisted of inhibition of proliferation, suppression of survival, promotion of apoptosis, and inhibition of angiogenesis. Our study showed that the derivatives SH-02, SH-03, and SH-14 were similar to deguelin in inhibiting HIF-1α expression and premalignant and malignant HBE and NSCLC cell proliferation. Overall, these derivatives and deguelin showed similar effects on Hsp90 clients and proteins involved in PI3K/mTOR signaling, and we found no strong evidence that the derivatives would be superior to deguelin in producing these effects. However, we found evidence that SH-14 could be more potent in inducing apoptosis. Furthermore, deguelin, SH-02, and SH-03 induced activation of Src and its substrate STAT, whereas SH-14 produced no significant increase in Src/STAT activity.

The viral form of *Src* (*v-Src*) was the first oncogene ever discovered (19). Src is a nonreceptor tyrosine kinase and is downstream from the EGF and a number of other growth factor receptors (29, 30). The activated signal from Src is transferred to PI3K, STAT3, mitogen-activated protein kinase kinase, and Jun and results in cancer cell survival, proliferation, angiogenesis, migration, and invasion (31). The transcription factor STAT also is closely related to oncogenesis. Therefore, targeting this kinase could be a strategy for cancer therapy and prevention as well (32–37). Our result showing activation of Src/STAT3 after treatment with deguelin suggests that the drug evokes a pathway that could induce survival, angiogenesis, or proliferation of cancer cells. Src and STAT also are associated with the pathogenesis of Parkinson's disease; α-synuclein, a presynaptic protein involved in the pathogenesis of Parkinson's disease, is phosphorylated by Src (38–41). Furthermore, pharmacologic inhibition of Src-family tyrosine kinase has been shown to enhance ionomycin-induced dopamine release (42), suggesting that activated Src suppresses dopamine release. Reactive oxygen species have also been suggested to activate STAT1 and STAT3 in neurons (43), resulting in the pathogenesis of many neurologic diseases (44) including Parkinson's disease (45). These findings suggest a potential involvement of Src/STAT3 in Parkinson's disease, and our present results indicating that SH-14 did not activate the Src/STAT pathway are encouraging. Although further research is required to confirm a relationship between Src/STAT activation and loss of the dopaminergic neuron (in rodents treated with deguelin over a certain dose), the lack of Src/STAT activation with SH-14 could allow this analogue to circumvent neuronal toxicity, distinguishing it from the other derivatives and deguelin.

Another beneficial feature of SH-14 for clinical use is its relatively improved stability. The instability of rotenoids including deguelin results from exposure to air and light, which causes significant decomposition in chemical structure and a decrease in biological activity (46). Deguelin decomposes to tephrosin (C7a-hydroxy deguelin) in various vehicles exposed to air and light (47) and is destabilized by the auto-oxidation process of light-catalyzed radical reaction of the oxygen molecule to deguelin. Deguelin contains a proton at a tertiary carbon in the C7a position adjacent to a ketone as well as a benzylic. This acidic proton is susceptible to attack by a base to form anion and hydrogen abstraction by a radical derived from light to generate a stabilized radical at the C7a position. As a result, deguelin can be oxidized/decomposed to tephrosin by a combination of light and air and also racemized to an optically inactive structure, (±)-deguelin, under a basic or acidic condition. SH-14 differs from deguelin and other analogues not only in molecular effects but in chemical structure as well. We derived SH-14 by converting a ketone to a carbamate group at the C7 position; SH-14 has no more acidic proton at the 7a tertiary carbon, which makes SH-14 more chemically stable than deguelin under basic and acidic conditions and when exposed to light and air.

Small-molecule inhibitors have been changing the paradigm of cancer treatment. Achieving the least off-target effects without losing the efficacy against the target is a major goal in developing better small-molecule inhibitors. Many drug development approaches test for known biological activity, such as HIF-1 down-regulation or cell proliferation inhibition, as examined in our present study. More systematic and molecular approaches for comparing the activity of new drugs with that of preexisting drugs are needed, however, because small-molecule drugs may have unknown molecular effects linked to side effects or resistance. The new proteomic assay RPPA has the potential to discover new drugs for targeted therapy through comprehensive analyses of protein expression levels and activation statuses (48). RPPA allowed us to compare deguelin and its derivatives for desired activity and potential side effects in multiple independent samples both systematically and simultaneously. Overall, we found that RPPA is an effective, informative high-throughput system for drug discovery that allows for rapid, large-scale proteomic analysis of protein expression and cell signaling.

In conclusion, we found evidences suggesting that the novel derivative SH-14 may be less toxic than, and similar in efficacy to, its parent, deguelin, for clinical cancer prevention and therapy. SH-14 also seemed to have better solubility in aqueous solution and to be more chemically stable under various conditions (versus deguelin). Our present study warrants further systemic evaluation of the *in vivo* activity and neuronal toxicity of SH-14.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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