Deguelin Analogue SH-1242 Inhibits Hsp90 Activity and Exerts Potent Anticancer Efficacy with Limited Neurotoxicity

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Abstract

The Hsp90 facilitates proper folding of signaling proteins associated with cancer progression, gaining attention as a target for therapeutic intervention. The natural rotenoid deguelin was identified as an Hsp90 inhibitor, but concerns about neurotoxicity have limited prospects for clinical development. In this study, we report progress on deguelin analogues that address this limitation, focusing on the novel analogue SH-1242 as a candidate to broadly target human lung cancer cells, including those that are chemoresistant or harboring KRAS mutations. In a KRAS-driven mouse model of lung cancer, SH-1242 administration reduced tumor multiplicity, volume, and load. Similarly, in human cell line-based or patient-derived tumor xenograft models, SH-1242 induced apoptosis and reduced tumor vasculature in the absence of detectable toxicity. In contrast to deguelin, SH-1242 toxicity was greatly reduced in normal cells and when administered to rats did not produce obvious histopathologic features in the brain. Mechanistic studies revealed that SH-1242 bound to the C-terminal ATP-binding pocket of Hsp90, disrupting the ability to interact with its co-chaperones and clients and triggering a degradation of client proteins without affecting Hsp70 expression. Taken together, our findings illustrate the superior properties of SH-1242 as an Hsp90 inhibitor and as an effective antitumor and minimally toxic agent, providing a foundation for advancing further preclinical and clinical studies.

Introduction

Numerous proteins involved in tumor signaling networks depend on the 90-kDa Hsp90 to mediate cell proliferation and survival (1, 2). Thus, Hsp90 inhibition has been proposed to target client proteins associated with hallmarks of cancer (3) and to overcome resistance to conventional or targeted anticancer agents (4–6). Several natural, synthetic, and semisynthetic Hsp90 inhibitors with various structural backbones, such as radicicol (7), novobiocin (2, 11) and cisplatin (12), have demonstrated antiproliferative effect and, in some cases, apoptotic activities showing a propensity for inducing a heat shock response (HSR) that ultimately leads to increases in Hsp90 and antiapoptotic proteins, expression. Taken together, our findings illustrate the superior properties of SH-1242 as an Hsp90 inhibitor and as an effective antitumor and minimally toxic agent, providing a foundation for advancing further preclinical and clinical studies.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Published Online First December 8, 2015; DOI: 10.1158/0008-5472.CAN-15-1492

Cancer Res; 76(3); 686-99. © 2015 AACR.
Parkinsonism-like neurotoxicity may be an obstacle to further clinical development. Hence, we have synthesized a variety of deguelin analogues; of these, SH-1242, a B- and C-ring truncated analogue (25), displayed a potent antiproliferative effect against human cancer cells (25) and suppressed hypoxia-mediated retinal neovascularization and vascular leakage in diabetic retinas by suppressing hypoxia-mediated upregulation of HIF1α and VEGF expression without any obvious toxicity (26). These promising effects imply the potential of SH-1242 as a lead candidate for developing Hsp90 inhibitors.

Here, we demonstrate the preclinical evidence of potent antitumor effects of SH-1242 with significantly reduced toxicity. SH-1242 has broad antitumor and antiangiogenic activities in various therapy-naïve and drug-resistant non–small cell lung cancer (NSCLC) cell lines and in clinically relevant experimental models, including Kras transgenic mice and patient-derived xenograft tumors (PDX). In addition, SH-1242 displayed significantly reduced toxic effects on various normal cells and showed no obvious Parkinsonism-like toxicity in the rat brain compared with deguelin. Furthermore, SH-1242 effectively disrupted Hsp90 function by interacting with its C-terminal domain, thereby leading to degradation of its client proteins without Hsp70 induction. Collectively, these results suggest that SH-1242 is a novel C-terminal Hsp90 inhibitor and has broad application against various cancers in the first- and second-line treatment.

Materials and Methods

Additional or detailed methods are described in the Supplementary Materials and Methods.

Synthesis of SH-1242

Detailed procedure for synthesis of SH-1242 is described in our previous report (25).

Cell culture

H1299, H460, H292, and BEAS-2B cells were purchased from the ATCC. The other NSCLC cells were kindly provided by Dr. John V. Heymach (MD Anderson Cancer Center, Houston, TX); HUVECs were purchased from Invitrogen. Human bronchial epithelial (HBE) cells were kindly provided by Dr. John Minna (The University of Texas Southwestern Medical Center, Dallas, TX). Human retinal pigment epithelial (RPE) cells were kindly provided by Dr. Jeong Hun Kim (College of Medicine, Seoul National University, Seoul, Republic of Korea). HT-22 cells were provided by Dr. Dong Gyu Jo (College of Pharmacy, Sungkyunkwan University, Suwon, Republic of Korea). Cell lines were authenticated and validated at the Korean Cell Line Bank using AmpliFLSTR identifier PCR Amplification Kit (Applied Biosystems; cat. No. 4322288) in 2013. Cells passed for fewer than 6 months after receipt or resuscitation of validated cells were used in this study.

Immunoprecipitation and pull-down assay

The following assays were performed following procedures described in our previous report (24): immunoprecipitation, purification of Hsp90 proteins, and a pull-down assay to identify the competitive ATP-binding pocket using ATP-agarose (Innova Biosciences) and the binding of biotinylated SH-1242 to each domain of Hsp90 in the presence or absence of a known Hsp90 inhibitor.

Animal studies

All animal procedures were performed according to a protocol approved by the Seoul National University Institutional Animal Care and Use Committee (approval numbers SNU-121207-2 and SNU-130820-6). For xenograft and PDX experiments, the tumor-bearing mice were treated with treated with vehicle (10% DMSO in corn oil), SH-1242 (4 or 20 mg/kg), deguelin (4 mg/kg), or geldanamycin (4 mg/kg) by oral gavage (for H292 xenografts) or intraperitoneal injection (for H1299 xenografts and PDX) six times per week for 3 weeks. In the experiment using Kras transgenic mice, vehicle or SH-1242 was administered by intraperitoneal injection (20 mg/kg) once a day for 8 weeks. To examine the parkinsonism-like neurotoxic effects of SH-1242 and deguelin, 6- to 10-week-old Sprague–Dawley rats were treated with SH-1242 or deguelin (4 mg/kg, dissolved in corn oil) by oral gavage every day for 20 days.

IHC and immunofluorescence

IHC and immunofluorescence analyses to detect CD31 and cleaved caspase-3 expression in the tumors and to evaluate tyrosine hydroxylase immunoactivity in the rat brain were performed as previously described (24).

Statistical analysis

Data are presented as the mean ± SD. Data were calculated with Microsoft Excel (Microsoft Corp.) or GraphPad Prism 6 (GraphPad Software Inc.). The statistical significance of in vitro or in vivo data was determined using either two-sided Student t test or ANOVA followed by Dunnett post hoc test. Unless otherwise indicated, statistical significance was determined by comparison with vehicle-treated control. A P value less than 0.05 was considered to be statistically significant.

Results

SH-1242 displays potent antiproliferative and antiangiogenic activities in several types of human cancer cells

On the basis of the established structure–activity relationship (SAR) of deguelin, we synthesized a series of potent deguelin analogues to identify a novel anticancer drug targeting Hsp90 (25). Among these compounds, we chose SH-1242, a B- and C-ring truncated analogue (Supplementary Fig. S1). SH-1242 displayed potent inhibitory effects on HIF1α expression and viability of H1299 human NSCLC cells and on hypoxia-mediated retinal neovascularization and vascular leakage in a diabetic retina without developmental defects in zebrafish embryos (25, 26). We first assessed the potency of SH-1242 against various human NSCLC cell lines. Considering the clinical utility of targeted anticancer drugs as a second-line therapy, we included several cancer sublines (designated "R") with acquired resistance to anticancer therapeutics, including the chemotherapeutic drug paclitaxel (H226B/R and H460/R), the EGFR TKI gefitinib (PC9/GR), and the IGF1R TKI linsitinib (H292/R; Supplementary Table S1). SH-1242 significantly inhibited the viability of the drug-naïve as well as the drug-resistant sublines (Supplementary Fig. S2). SH-1242 also showed significant inhibitory effects on anchorage-dependent (Fig. 1A) and anchorage-independent (Fig. 1B) colony-forming...
Figure 1.

Effects of SH-1242 on the anchorage-dependent or independent colony forming ability of various cancer cells and on the tube formation of vascular endothelial cells. A, the effect of SH-1242 on the anchorage-dependent colony formation of lung cancer cells with or without anticancer drug resistance. Bottom, representative images of colony formation. B, the anchorage-independent growth of cells treated with increasing concentrations of SH-1242 was determined using a soft agar colony formation assay. Bottom, representative images of colony formation. C, the expression levels of cleaved PARP (cl-PARP), cleaved caspase-3 (cl-Cas3), and Hsp70 in NSCLC cells treated with SH-1242 were evaluated by Western blot analysis. D, the cell-cycle distribution of NSCLC cells treated with SH-1242 (10 μmol/L) for 2 days was analyzed by flow cytometry. E, the effect of SH-1242 on the anchorage-independent colony formation of H226B cells overexpressing mutant KRAS was determined using a soft agar colony formation assay. F, HUVECs were treated with the CM from H1299 cells treated with SH-1242. Tube formation was analyzed as described in Materials and Methods. Con, control; SH, SH-1242. *, P < 0.05; **, P < 0.01; ***, P < 0.001, compared with vehicle-treated control.
abilities of both naive and their drug-resistant sublines. These cell lines underwent apoptosis after the treatment with SH-1242, as evidenced by increased cleavage of caspase-3 and its substrate PARP (Fig. 1C) and by an increased distribution of the cell population in the sub-G0–G1 phase (Fig. 1D). Because Hsp90 inhibitors that block the N-terminal ATP-binding pocket induce cytoprotective Hsp70 expression (9, 10), we also examined the effects of SH-1242 on Hsp70 induction. However, Hsp70 expression remained unchanged or slightly decreased by treatment with SH-1242 (Fig. 1C).

It is known that alterations in cellular redox status can affect cell fates, including cell proliferation, growth, and death (27). Interestingly, a recent study suggested that deguelin induces apoptosis of lung cancer cells via ROS-induced Akt dephosphorylation (28). To confirm whether this mechanism could be associated with the proapoptotic effect of SH-1242, NSCLC cells were treated with SH-1242, alone or in combination, with an antioxidant N-acetyl-L-cysteine (NAC). Consistent with previous findings, treatment with SH-1242 markedly suppressed the phosphorylation of Akt and GSK3β and induced PARP cleavage. However, treatment with NAC in combination only partially reversed these SH-1242’s effects in H1299 cells and displayed no obvious effects in H226B and H226R cells (Supplementary Fig. S3). These results suggest that the effect of SH-1242 on the Akt dephosphorylation and apoptosis induction appears to be unrelated to ROS generation, which is consistent with our previous findings on the ROS-independent HIF regulation by deguelin (24).

Because Ras mediates the signal transduction from growth factor receptors to downstream mediators, many (e.g., Raf and MEK) of which are clients of Hsp90 (29, 30), and Ras mutations are involved in resistance to various anticancer drugs (31, 32), we next tested the effects of SH-1242 on cancer cells harboring mutant KRAS. As shown in Fig. 1E, SH-1242 exhibited significant and comparable inhibitory effect on the anchorage-independent colony formation of mutant KRAS–overexpressing H226B (H226B/KRAS) cells; this inhibitory effect of SH-1242 was comparable with its effect on parental H226B cells carrying wild-type KRAS.

On the basis of the potent antiangiogenic effects of deguelin and its derivatives (23, 26), we further assessed the effect of SH-1242 on angiogenic activities of NSCLC cells. Because tumor angiogenesis is mediated at least in part by tumor-secreted angiogenic growth factors that interact with their surface receptors expressed on endothelial cells (33), we analyzed the effects of conditioned medium (CM) derived from H1299 cells treated with increasing concentrations of SH-1242 on the tube formation of HUVECs. HUVECs incubated with the conditioned medium (CM) derived from H1299 cells, which had been treated with SH-1242 under normoxic or hypoxic conditions, formed significantly fewer tubes than did those treated with CM from untreated H1299 cells (Fig. 1F). Collectively, these results demonstrate the broad anticancer activities of SH-1242.

Anticancer effect of SH-1242 on the growth of KRAS–driven spontaneous lung tumors and NSCLC cell line- and patient-derived xenograft tumors

Because conventional cancer cell lines can adapt to artificial in vitro culture conditions and the interaction of tumor cells with stromal components supports cancer cell survival (34), we explored whether SH-1242 suppresses lung tumor growth in vivo using Kras transgenic mice that develop spontaneous lung tumors with a 100% incidence (35). We first tested effective doses of SH-1242 by performing dose determining experiments using a NSCLC xenograft tumor model. We found that intraperitoneal treatment with SH-1242 at the dose of 20 mg/kg had significant antitumor activities without detectable toxicities. Hence, we chose this treatment schedule. Difference in the tumor growth was monitored by microscopic tomography (Fig. 2A, a) and fluorescence-based image analyses (Fig. 2A, b; refs. 21, 36). Two representative mice showed markedly decreased lung tumor growth by SH-1242 treatment. Postmortem examination of the mice revealed that SH-1242–treated mice had fewer lung tumor nodules than vehicle-treated mice (Fig. 2A, c and B, left). Microscopic analysis of hematoxylin and eosin (H&E)-stained lung tissues (Fig. 2A, d) showed significantly decreased tumor multiplicity (Fig. 2B, right), especially tumor nodules bigger than 1 mm3 (Fig. 2C) in SH-1242–treated mice compared with the control mice. Tumor volume (Fig. 2D) and load (Fig. 2E) in SH-1242–treated mice were also significantly less compared with those in the control mice. These data indicate the significant inhibitory effect of SH-1242 on mutant KRAS–driven lung tumor growth.

We next evaluated the effects of SH-1242 on the growth of human NSCLC cell–derived xenograft tumors. Compared with vehicle-treated control tumors, SH-1242–treated H1299 (Fig. 3A) xenograft tumors showed significantly reduced growth. We assured the clinical applicability of SH-1242 by confirming its significant inhibitory effects on the growth of PDX tumors (Fig. 3B). Because 4 mg/kg deguelin via oral gavage administration was the maximum tolerated dose, we additionally chose the treatment schedule (4 mg/kg by oral gavage) and compared the antitumor effects of SH-1242, geldanamycin, and deguelin. As illustrated in Fig. 3C, we observed significant antitumor effect of SH-1242, which was comparable with deguelin and geldanamycin. We further confirmed the effects of SH-1242 on apoptosis and angiogenesis in vivo by measuring the expression of cleaved caspase-3 (a marker of apoptosis) and CD31 (a marker of microvessel formation) in xenograft tumors of H1299 cells and patient-derived tissues. We observed that SH-1242 significantly increased cleaved caspase-3 and decreased CD31 expression in the H1299 and PDX tumors (Fig. 3D and E). During the treatment period for these three animal experiments, body weight was not significantly different between the control and SH-1242–treated groups. Moreover, tissue samples obtained from several organs (liver, lung, heart, kidneys, spleen, urinary bladder, ovary, stomach, pancreas, colon, and rectum) of the SH-1242–treated mice revealed no remarkable histopathological changes, which is consistent with the lack of major toxic effects observed in A/J mice after 19 weeks of oral deguelin administration (twice daily) during a previous study (21). These overall results suggest that SH-1242 has effective and clinically applicable antitumor activities with minimal toxicities in NSCLC.

Reduced toxicity profile of SH-1242 compared with deguelin

A concern on the use of deguelin as an anticancer drug is potential toxicities. In a previous study, deguelin caused...
parkinsonism-like syndrome, which was manifested by decreased tyrosine hydroxylase immunoreactivity in the rat brain (37). In addition, it was reported that Hsp90 inhibitors may induce ocular and liver toxicity (38). Although it is not clear whether therapeutic doses of deguelin would induce the side effects and whether the side effects can be relieved after drug withdrawal, the potential toxicities of deguelin can be a considerable obstacle to its clinical use. On the basis of this notion, we evaluated whether SH-1242 harbors less or no potential toxicity compared with deguelin. We first examined toxicity of SH-1242 at the cellular levels by testing the effects of SH-1242 on the viability of several normal cells, including human normal lung epithelial cells (HBE and BEAS-2B; Fig. 4A), hippocampal cells (HT-22; Fig. 4B), retinal pigment epithelial cells (Fig. 4C), and vascular endothelial cells (HUVEC; Fig. 4D). Compared with deguelin, SH-1242 showed significantly reduced cytotoxicity in the tested normal cells (Fig. 4A–D). We next compared the effects of SH-1242 and deguelin on tyrosine hydroxylase immunoreactivity in the substantia nigra in rats. The tyrosine hydroxylase immunoreactivity was significantly decreased by deguelin treatment while it was minimally affected by SH-1242 treatment,
suggesting markedly reduced potential neurotoxicity of SH-1242 compared with deguelin (Fig. 4E). SH-1242 treatment also showed minimal influence on the body weight of rats (Fig. 4F). In contrast, the body weight in the deguelin-treated rats was significantly reduced compared with control. Together, these results indicate the markedly improved safety profile of SH-1242.

Effect of SH-1242 on Hsp90 function and associated client proteins

We investigated whether Hsp90 is involved in the molecular mechanism of action of SH-1242. During our first assessment of the effects of SH-1242 on Hsp90 function, we performed an in vitro pull-down experiment using bacterial proteins expressing the HLH/PAS/ODD domain of the HIF1α, which
is essential for complex formation with Hsp90 and sensitivity to Hsp90 inhibitors (39–41). Consistent with previous findings (39, 42), the HLH-PAS-ODD domain of HIF1α interacted strongly with Hsp90, and this interaction was inhibited by SH-1242 in a dose-dependent manner (Fig. 5A). Inhibition of the Hsp90–HIF1α interaction by SH-1242 was more potent than that of the same concentration of deguelin, suggesting that SH-1242 more potently inhibits Hsp90 function than did deguelin (Fig. 5B). We further performed immunoprecipitation assay to analyze the interaction between Hsp90 and client proteins. Hsp90 coprecipitated with its client proteins, including Akt, MEK, and HIF1α. The interactions were markedly

**Figure 4.** Effects of SH-1242 on the viability of normal cells and on the neuropathologic lesions in rat brains. A–D, the effect of SH-1242 on the viability of normal cells originated from lung epithelium (HBE and BEAS-2B; A), mouse hippocampus (HT-22; B), human retinal pigment epithelium (RPE; C), and human vascular endothelium (HUVEC; D) was determined by the MTT assay. A, the concentration of SH-1242 (SH) and deguelin (Deg) was 5 μmol/L. E, the reduced neuropathologic lesions in rat brains treated with SH-1242, determined by immunofluorescence analysis. Left, a representative image of tyrosine hydroxylase (TH) immunoreactivity. Right, quantitative analysis of tyrosine hydroxylase immunoreactivity. F, body weight changes of vehicle- or drug-treated rats. Con, control; SH, SH-1242; Deg, deguelin. *, P < 0.05; **, P < 0.01; ***, P < 0.001, compared with vehicle-treated control. Scale bar, 10 μm.
diminished in H1299 cells treated with SH-1242 without obvious changes in the total protein expression of each client protein (Fig. 5C). Furthermore, SH-1242 dose dependently inhibited the expression of Hsp90 client proteins, including HIF1α, ErbB2, Akt, and MEK (Fig. 5D and E), and the target genes of HIF1α, including VEGF and IGF2 (Fig. 5F). These

Figure 5. Inhibition of Hsp90 function by treatment with SH-1242. A, evaluation of the effect of SH-1242 on the interaction between Hsp90 and the HLH-PAS-ODD domain of HIF1α. B and C, H1299 cells were treated with SH-1242 for 1 hour and then incubated under hypoxic conditions for 4 hours. B, comparison of the effect of SH-1242 (1 μmol/L) and deguelin (1 μmol/L) on the interaction between Hsp90 and HIF1α under identical experimental conditions. C, the interaction between Hsp90 and its client proteins, Akt and MEK, was analyzed by immunoprecipitation analysis. D and E, the effects of SH-1242 on the expression of several Hsp90 client proteins, including HIF1α (D), ErbB2, Akt, and MEK (E), were determined by Western blot analysis. F, the effect of SH-1242 on the expression of VEGF and IGF2 was determined by RT-PCR analysis. Con, control; SH, SH-1242; Deg, deguelin.
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Figure 6.
Binding of SH-1242 to the ATP-binding pocket in the C-terminal domain of Hsp90. A, an illustration of the Hsp90 domains (top). Bottom, the binding of each Hsp90 domain to ATP agarose with or without coincubation with ATP or ATPγS was determined by the pull-down of recombinant proteins bound to agarose beads and subsequent Western blot analysis. B, the competition between FL and each Hsp90 domain was determined. C, fluorescence-based analysis of SH-1242 and Hsp90 binding. D, the binding of SH-1242 to each domain of Hsp90. E, competition between SH-1242 and cisplatin (Cis; left) or geldanamycin (GAA; right) binding to the FL, N, and C domains of Hsp90. F, the inhibitory effect of SH-1242 on the interaction between Hsp90 and a C-terminal-specific co-chaperone, Hop. G, the effect of SH-1242, deguelin, and 17-AAG on Hsp70 expression was evaluated by Western blot analysis. H, top, Western blot analysis evaluating Hsp90 binding to SH-1242 in H1299 cells. Bottom, a silver-stained gel showing Hsp90 protein that has come down as the result of binding to SH-1242 and the high and low molecular weight of proteins that have also come down as the result of binding to Hsp90 is included. Con, control; SH, SH-1242; Deg, deguelin.
findings suggest that SH-1242 has the capacity to disrupt Hsp90 function and to decrease expression of Hsp90 client proteins.

Competitive inhibition of ATP binding to the C-terminal potential nucleotide-binding pocket of Hsp90 by SH-1242

Hsp90 possesses the ATP-binding pockets in the N-terminal domain (2, 29). The middle domain plays a role in the regulation of Hsp90 function such as client recognition (29) and interacts with γ-phosphate of ATP bound to the N-terminal ATP pocket (29, 43). The C-terminal domain of Hsp90 is crucial for dimerization of Hsp90 (2, 29). Previous reports suggest the interaction between ATP and the Hsp90 C-terminal domain (43–45). However, the potential ATP-interacting region in the Hsp90 C-terminal domain could be a hydrophobic pocket, and it is largely unknown whether Hsp90 possess structurally defined C-terminal ATP binding sites with a function. Hence, additional investigation to prove the structural, functional, and biochemical aspects of the C-terminal ATP-binding sites are required. To obtain direct evidence for SH-1242 binding to Hsp90, recombinant Hsp90 proteins containing the full length (FL), truncated N-terminal (N), middle (M), and C-terminal (C) domains were prepared (Fig. 6A, top). We first confirmed the capacity of these Hsp90 domains to ATP by using ATP-agarose and ATPγS as a competitive agent. The FL, N, M, and C domains of Hsp90 bound to ATP-agarose, and these interactions were decreased by coincubation with ATPγS (Fig. 6A, down). Subsequent competition analysis demonstrated that the increasing amount of the N and, to a lesser extent, C domain gradually decreased ATP binding to the FL domain, but this effect was not observed for the M domain of Hsp90 (Fig. 6B). ATP binding to the N domain was unaffected by increasing the molar ratio of the C domain, whereas ATP binding to the C domain was markedly decreased by increasing levels of the N domain. These results were consistent with previous findings that the ATP-binding region exists in both the N and C domains of Hsp90 and that the affinity of ATP binding is higher to the Hsp90 N-terminal than to the Hsp90 C-terminal (45).

We next performed fluorescence-based equilibrium binding experiments to assess whether SH-1242–binding sites are localized within the N and/or C domains of Hsp90. The calculated KD value of SH-1242 binding to FL, N, and C domains of Hsp90 was obtained by exciting the proteins at 285 nm and monitoring the emission change at 340 nm. The fluorescence emission spectrum produced by Hsp90 excited at 258 nm is shown in Fig. 6C. When Hsp90 proteins were titrated with SH-1242, the emission at 340 nm decreased after ligand binding. SH-1242 binding to Hsp90 proteins was analyzed by monitoring the change in fluorescence intensity. After saturation of the protein with SH-1242, the protein fluorescence quenching levels observed at 340 nm were 57.5% (full length), 83.4% (N-terminal domain), and 61.9% (C-terminal domain). The Kd values for the fragmented N and C domains were 27.3 μmol/L and 16.4 μmol/L, respectively. Titration of FL Hsp90 with SH-1242 displayed a two-phase binding curve with Kd values of 13.95 μmol/L and 197.1 μmol/L. We then assessed the Kd values after blockade of the N-terminal ATP binding domain of Hsp90 by preincubation with geldanamycin (GAA). We observed one-phase binding curve with Kd values of 15.7 μmol/L. We next assessed the capacity of SH-1242 to bind the ATP-interacting region in the N and C domains of Hsp90. The FL, N, M, and C domains of Hsp90 were preincubated with SH-1242, and then ATP-agarose resin was added. The ATP binding to the Hsp90 FL domain was clearly diminished by treatment with SH-1242 (Fig. 6D). SH-1242 disrupted the interaction of ATP with the C domain, and, to a much lesser extent, with the N domain of Hsp90. These results suggest that SH-1242 binds to both the N and C domains, presumably with a higher affinity to the C-terminal ATP-interacting region than to the N-terminal ATP-binding pocket of Hsp90. The difference between the two Kd values obtained from FL and those obtained from the fragmented N and C domains also suggests that the second binding site in FL Hsp90 may become accessible when the first site is occupied.

To ensure that SH-1242 is binding to the C-terminal ATP-binding region, the FL and C domains were preincubated with C-terminal (cisplatin) or N-terminal (geldanamycin) Hsp90 inhibitors, then biotinylated SH-1242 was added. We observed that preincubation with cisplatin effectively blocked the binding of SH-1242 to the FL and C domains of Hsp90, whereas SH-1242 binding to the FL and N domains was moderately suppressed by incubation with relatively higher concentrations of geldanamycin (Fig. 6E). Coimmunoprecipitation assays revealed that SH-1242 blocked the interaction between Hsp90 and Hop, a co-chaperone known to bind to the C domain of Hsp90 (Fig. 6F; ref. 46). We also compared the effects of SH-1242, deguelin, and 17-AAG on Hsp70 protein levels in H1299 cells. Hsp70 expression was increased after 17-AAG treatment, whereas it remained unchanged by treatment with SH-1242 or deguelin (Fig. 6G). We further ensured the Hsp90-binding capacity of SH-1242 in vivo. To this end, biotinylated SH-1242 was incubated with H1299 cell lysates and immunoblotted with an anti-Hsp90 antibody. H1299 cell lysates revealed an obvious Hsp90 binding to biotinylated SH-1242 (Fig. 6H, top). The Hsp90 protein as the result of binding to SH-1242 and the high and low molecular weight of proteins that have also come down non-specifically or as the result of binding to Hsp90 was also detected by a silver stained gel (Fig. 6H, bottom). Collectively, these results indicate that the inhibitory effect of SH-1242 on Hsp90 function is mediated mainly by its direct association with the ATP-interacting region in the Hsp90 C-terminal domain.

Docking modeling of SH-1242 bound to human Hsp90

We evaluated the binding capability of SH-1242 for the site known as Hsp90 C-terminal nucleotide-binding region by molecular docking analysis using the Surflex-Dock program. To corroborate the reliability of our homology model of hHsp90, docking study of Hsp90 C- (EGCG, paclitaxel, novobiocin, and KU130) and N-terminal inhibitors (geldanamycin and 17-AAG; ref. 47) was conducted. As shown in Supplementary Table S2, the ranking of Surflex-Dock binding scores of SH-1242, deguelin, and the well-known Hsp90 inhibitors are consistent with the γ-phosphate–linked ATP-agarose binding assay results. All the compounds were docked into the active site of Hsp90 homodimer, which is positioned in close proximity to the dimerization interface. The cavity involving the residues known as C-terminal ATP-binding region, was assigned as the active site for docking (48). As shown in Fig. 7A, all the C-terminal inhibitors and ATP fit well in the active site of chain A, occupying the neighboring pocket in the dimerization interface. To examine the binding pose of SH-1242 (Fig. 7B), the drug interacts with key amino acid residues
for ATP binding in the C-terminal. The oxygen atom of the methoxy group forms a hydrogen bond with the side chain hydroxy group at Ser677 in chain A, and the oxygen in the benzopyran ring forms an additional hydrogen bond with the side-chain amine at Lys615, one of key residues of the ATP-binding region in chain B. The binding pose of SH-1242 is quite similar to that of an analogous C-terminal inhibitor reported recently (49). Considering the overall shape of the Hsp90-SH1242 complex (Fig. 7C), SH-1242 was likely to bind the central region of dimerization in the C-terminal and may stabilize an open conformation of the Hsp90 homodimer, which inhibits N-terminal dimerization and suppresses ATP binding to the active site in the N-terminal of Hsp90. To validate whether the predicted amino acid residues within the C domain of Hsp90 play a role in ATP binding, we assessed the ATP-agarose binding assay by using the C domain of Hsp90, in which Glu611, Lys615, and Ser677 were mutated to valine, glutamine, and alanine, respectively. Mutation at K615 or S677 almost completely abolished the Hsp90 binding to ATP-agarose (Fig. 7D), indicating the importance of K615 and S677 within the Hsp90 C-terminal domain to ATP binding.

Discussion

Extensive efforts have been directed to develop potent anticancer therapies, and molecularly targeted therapies blocking Hsp90 have raised the hope of developing effective therapeutic strategies. To date, several clinical trials evaluating the effectiveness of Hsp90 inhibitors have been performed (16), and development of several derivatives of known Hsp90 inhibitors to improve efficacy and safety has been extensively investigated (15, 16). Consistent with this notion, the studies reported herein demonstrate that SH-1242, an analogue of a naturally occurring deguelin, has promising anticancer activities with minimal toxicities at least in part by blocking Hsp90’s C-terminal function. Our results provide preclinical support for the use of Hsp90 C-terminal inhibitors in the treatment of lung cancer.

Hsp90 plays major roles in cancer progression, tumor angiogenesis, and therapy resistance by functioning as a molecular chaperone whose association is necessitated for the stability and function of numerous client oncogenic proteins (1, 2). Several clinical trials are underway to evaluate the effectiveness of Hsp90 inhibitors, mainly those that block the N-terminal ATP pocket (16). Previous reports have indicated that: (i) Hsp90 presumably has a second C-terminal nucleotide-binding site; (ii) the nucleotide-binding region in the C-terminus mediates Hsp90 dimerization and is necessary for the close association between the two N-terminals in the ATP-bound state (50); (iii) Hsp90 dimerization is critical for its chaperone function and interaction with co-chaperones; (iv) the Hsp90 C-terminal inhibitors minimally cause cytoprotective Hsp70 induction (1, 16). Following these findings, we hypothesized...
that Hsp90 C-terminal inhibitors may offer a novel opportunity for cancer therapy.

We have demonstrated that deguelin, a rotenoid derived from natural products, has potent antitumor and antiangiogenic effects mediated through its disruption of Hsp90 function, thereby affecting the stability of numerous oncogenic cellular client proteins, including HIF1α (24). Despite the promising anticancer efficacy of deguelin in various in vitro and in vivo models, its widespread use could be hindered by concerns regarding its potential neurotoxicity (37). We have therefore attempted to develop potent, but safe, deguelin analogues, and SH-1242 was found to be one such lead candidate.

Our results in the current study demonstrate that a novel deguelin analogue, designated SH-1242, offers reasonable efficacy and safety profile by showing the following results, including: (i) SH-1242 inhibits viability, colony forming abilities, and angiogenic activity of a panel of NSCLC cell lines and their sublines that had acquired resistance to chemo- or molecular-targeted therapies. SH-1242 effectively induced apoptosis in these drug-naïve and drug-resistant sublines; and (ii) SH-1242 effectively suppressed the growth of lung tumors in in three different experimental models of lung cancer, including of mutant KRAS-driven lung tumors and human NSCLC cell line- and PDX tumors. The in vitro antitumor activities of SH-1242 were comparable with those of 17-AAG and deguelin; (iii) SH-1242 exhibited considerably lower cytotoxicity than deguelin in viability of several normal cells originated from various organs; (iv) SH-1242 was well tolerated in mice without any detectable inflammation and injury in major organs, including lung, liver, kidney, spleen, stomach, and ovary, after several weeks of treatment at therapeutic doses; and (v) compared with deguelin, SH-1242 exhibited markedly reduced effects on tyrosine hydroxylase immunoreactivity in rat brains. These collective findings indicated that SH-1242 could be an effective cancer therapeutic agent through its orchestrated inhibitory actions on tumor growth and angiogenesis with minimal histologic toxicities and neuropathologic disorders.

We investigated the mechanism of SH-1242–mediated antitumor activities. We first confirmed decreased interaction between Hsp90 and its client proteins and reduced expression of various Hsp90 client proteins in NSCLC cancer cells after treatment with SH-1242. We then identified the following features of SH-1242 as a novel C-terminal Hsp90 inhibitor: (i) direct binding of SH-1242 to the potential C-terminal nucleotide-binding region of Hsp90; (ii) the disruption of the interaction between Hsp90 and a C-terminal specific co-chaperone Hop by SH-1242 treatment; (iii) lack of the cytoprotective Hsp70 induction by SH-1242, which is the known feature of C-terminal Hsp90 inhibitors (13); and (iv) lower Kd value toward Hsp90 C-terminal domain compared with N-terminal domain. Our computational modeling using structure–function analysis further supports the mechanistic insights. Our docking model suggests that SH-1242 competes with ATP for a C-terminal ATP-binding site. In addition, SH-1242 forms hydrogen bonds with the residues both in chain A and chain B, which may stabilize the open state of the Hsp90 homodimer. We also compared the Surflex-Dock docking scores of known Hsp90 C- or N-terminal inhibitors, including SH-1242, after docking into the C-terminal region. As shown in Supplementary Table S2, most of the known Hsp90 C-terminal inhibitors were high-ranked, except the N-terminal inhibitor ganetespib. SH-1242 also ranked higher than the well-defined C-terminal inhibitor novobiocin. In addition, the calculated molecular volume of SH-1242 is approximately 70% of that of cisplatin. Cisplatin binding to Hsp90 requires a large conformational change that carries a high entropic penalty. The molecular size and structural rigidity of SH-1242 could be more entropically favorable when binding to the Hsp90 C-terminal compared with those of cisplatin. Collectively, these findings suggest that SH-1242 is a potential lead candidate for the development of Hsp90 C-terminal inhibitors.

Taken together, our findings provide evidence that SH-1242 effectively disrupts Hsp90 function by directly binding to the C-terminal ATP-binding site of Hsp90 and inhibiting its chaperone activity. A concomitant degradation of several Hsp90 client proteins induced by SH-1242 could have resulted in the potent antitumor activities of the drug against various NSCLC cell lines in vitro and the growth of KRAS-driven spontaneous lung tumor and human NSCLC cell line- and PDX tumors in mice. Moreover, when tested in vitro in various normal cells and in vivo in mice and rats, SH-1242 revealed an excellent toxicity profiles. These findings suggest the potential of SH-1242 as an effective anticancer agent targeting the C-terminal domain of Hsp90. The antitumor activities of SH-1242 in naïve and drug-resistant NSCLC cells also suggest a broad application of the drug in the first- and second-line treatment. Further studies are warranted to evaluate the efficacy of SH-1242 in additional preclinical and clinical settings.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Grant Support
This work was supported by grants from the National Research Foundation of Korea (NRF), the Ministry of Science, ICT and Future Planning (MSIP), Republic of Korea (Nos. NRF-2011-0017639 and NRF-2011-0019400).

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Received June 1, 2015; revised October 6, 2015; accepted October 23, 2015; published OnlineFirst December 8, 2015.
References


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