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Ring truncated deguelin analogue induces apoptosis and anti-angiogenesis in non-small cell lung carcinoma cells

비소세포성 폐암세포에서 고리구조가 절단된 deguelin 유도체의 세포자살과 항 혈관신생 유도활성에 관한 연구

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분자의학 및 바이오제약전공
최훈
ABSTRACT

Ring-truncated deguelin analogue induces apoptosis and anti-angiogenesis in non-small cell lung carcinoma cells

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The natural compound deguelin, a rotenoid isolated from several plant species including Mundeulea sericea, structurally have 5 rings (A, B, C, D, E rings). It has reported anti-proliferation against diverse cancer cells by directly binding to heat
shock protein-90 and thus suppressing its function. Hsp90 is highly expressed in cancer cells, and it plays a significant role in various oncogenic proteins stability.

Despite this anti-tumor effect, there have several problems to clinical use because of its high cytotoxicity in normal cells. We are trying to solve this problem using ring-truncated deguelin analogue (SH-1242) compound synthesized by joint research. SH-1242 is structurally B- and C- ring truncated compound (Figure 4B). I therefore, assessed in non-small cell lung cells the anti-proliferation activity, anti-angiogenesis inhibition and mechanism of SH-1242. The effect of SH-1242 on cytotoxicity of NSCLCs and normal bronchial epithelial cells were assessed by MTT assay. SH-1242 has cytotoxicity against NSCLCs in a dose-dependent manner, but less than deguelin does in normal cells. Also, the effect of SH-1242 on colony formation was assessed by anchorage dependent and independent colony formation assay.
Both colony formation was inhibited by SH–1242 in a dose–dependent manner. The inhibitory effect of SH–1242 on proliferation of NSCLCs related to apoptosis was assessed by flow cytometric analysis and western blot. The proportion of sub–G1 apoptotic cells increased and caspase–3 signaling pathway inhibited.

Also, the effect of SH–1242 on anti–angiogenic activity was assessed by western blot and tube formation assay. Treatment of H1299 cells with SH–1242 for 4h under hypoxia conditions reduced the expression of the hypoxia–inducible factor 1ɑ protein. In a tube formation assay, SH–1242 remarkably reduced the capillary network formation of human umbilical vein endothelial cells (HUVECs) on Matrigel beds.

These effects of apoptosis and anti–angiogenesis were investigated whether SH–1242 affects on Hsp90 malfunction. As a result, SH–1242 dose–dependently inhibited the expression of Hsp90 client proteins, including ErbB2, Akt, and MEK,
Finally, I confirmed that SH–1242 has apoptotic and anti-angiogenic activity in H1299 or patient-derived xenograft (PDX) tumor tissues. SH–1242 treatment significantly increased cleaved caspase–3 and decreased CD31 expression in both tumor tissues.

Taken together, SH–1242 showed little cytotoxicity in normal lung epithelial cells, but may represent apoptosis and anti-angiogenesis in NSCLCs through Hsp90 inhibition.

**Key words:** deguelin, SH–1242, NSCLC, HSP90, HIF–1α

**Student Number:** 2012–22856
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I. INTRODUCTION

1. Heat Shock Protein 90 (Hsp90)

Molecular chaperones are proteins concerned with maintaining normal folding, function and stability of client proteins. Some molecular chaperones are particularly significant under hazardous conditions such as proteotoxic heat shock and other cellular stresses [1], however, molecular chaperones are also critical for maintaining normal cellular homeostasis [2]. Particularly, Heat Shock Protein 90 (Hsp90) has emerged over the last decade as one of the attractive targets for the development of novel molecular cancer therapeutics [2–5]. Several detailed reviews are available that demonstrate diverse functions of Hsp90 in cancer [6–9].

1–1 Hsp90 in normal cellular homeostasis

Despite the name 'heat-shock' protein, Hsp90 is highly abundant, comprising as much as 1–2% of total cellular protein component even under non-stressed conditions [2]. Hsp90 is
required for essential housekeeping functions, such as appropriate protein folding, translocation of proteins across the membranous structures, maintaining proteins in a metastable state, and normal protein turnover (Figure 1) [8,10]. To conclude, Hsp90 is able to have a strong influence on the signaling pathways that regulate cellular outcome, including cell growth, division, differentiation, movement and death [2].

Wearying studies in various model organism, Hsp90 seems to play a key role in development and evolution by acting as a buffer of phenotypic variation. Retaining function of the mutant protein by Hsp90 makes complex developmental process into discrete outcomes despite underlying genotypic variation. [11] Under stressful conditions, however, some of the unstable client proteins of Hsp90 may become more unstable. In this situation, demand for Hsp90 to promote the refolding of its usual client proteins, as well as stress-destabilized client proteins are increasing. In certain individuals the cache of genetic variation exceeds the capacity of Hsp90 buffer, previously hidden genetic variations become available for natural selection to enhance the survival.

1–2 Hsp90 structure and function
Hsp90 protein has three principal domains (Figure 2) [8,12]. The N-terminal domain attracted the majority of attention because it was the domain whose X-ray crystal structure was determined first [13,14]. The N-terminal domain possesses an ATP-binding site, which is unique and distinct from the ATP-binding site of Hsp70 or other protein kinases. The backbone of the N-terminal domain has an eight-stranded β sheet and the ATP-binding site is formed by four α helices, referred to as a Bergerat fold.

The ATP hydrolysis capacity of N-terminal domain is negligible as it lacks a key catalytic residue for orienting and polarizing the γ-phosphate of ATP. This function is provided by Arg380 in the middle domain. The X-ray crystal structure of the middle domain was also determined for the yeast Hsp90. A large hydrophobic surface in the middle domain is known as binding site for client proteins. The C-terminal domain is responsible for Hsp90 dimerization [15–17]. It also contains binding sites for various co-chaperones as part of a much larger super-chaperone machine. Recently, Blagg and coworkers reported the elucidation of Hsp90 C-terminal inhibitor binding site by protease fingerprinting and photoaffinity labeling utilizing LC-MS/MS [18].

The hydrolysis of ATP is required to induce a 'chaperone
cycle' in which ATP/ADP binding status dictates the nature of the client proteins or co-chaperones that bind to and are released from Hsp90. ATP-dependent dimerization of the N-terminal domain is critical point of the chaperone cycle. Inhibition of ATP binding and hydrolysis gives rise to the recruitment of a ubiquitin ligase entailing the proteasomal degradation of client proteins.

1–3 Hsp90 in cancer

Hsp90 plays a significant role in the development, maintenance and progression of cancer [1–3]. Hsp90 expression level is higher in oncogene-transformed and malignant cells than normal cells [19]. Upregulation of Hsp90 may be engendered by the stress induced by the microenvironment of the solid tumor and the alternative cellular stress associated with the activation of oncogenes. The most convincing evidence of the involvement of Hsp90 in cancer is that oncogene products or proteins known to be concerned in oncogenic pathways are client proteins of Hsp90. Hsp90 client proteins contribute functionally to each of the distinctive hallmark traits of malignancy (Table 1) [2,20].
Figure 1. Normal chaperone biology.

Newly synthesized, conformationally labile client proteins associate with multi-protein complexes that contain various chaperones, co-chaperones and accessory molecules. The particular components of a complex vary according to the client and also help specify the function of a particular complex. Dynamic association of a client with chaperone complexes can prevent its aggregation.
Figure 2. Structure of Hsp90 dimer

The numbering 1–732 indicates the approximate positions in the amino acid sequence of the human protein that define its functional domains. ‘CR’ refers to a charged region which serves as a flexible linker between the N–terminal and middle domains. The locations where various small molecules bind HSP90 (heat–shock protein of 90 kDa) and modulate its function are indicated. The biochemical functions of each domain are also shown. 17AAG, 17–allylaminogeldanamycin; GA, geldanamycin.
### Table 1. Hsp90 and the six hallmark traits of cancer

Six acquired capabilities of cancer cells are illustrated. These hallmarks of cancer cells are established by malfunctions of many Hsp90-dependent signal transduction molecules.

<table>
<thead>
<tr>
<th>Hallmark trait</th>
<th>Examples of relevant Hsp90 client proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Self-sufficiency in growth signals</td>
<td>ErbB2, Raf-1, Akt</td>
</tr>
<tr>
<td>Insensitivity to growth suppression signals</td>
<td>PItk, Wee1, Myt1</td>
</tr>
<tr>
<td>Evasion of apoptosis</td>
<td>PIP, Akt</td>
</tr>
<tr>
<td>Acquisition of limitless replicative potential</td>
<td>hTERT</td>
</tr>
<tr>
<td>Sustained angiogenesis</td>
<td>HIF-1α, FAK, Akt</td>
</tr>
<tr>
<td>Invasion and metastasis</td>
<td>Met</td>
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</tbody>
</table>
2. Hypoxia-Inducible Factor-1 (HIF-1)

Hypoxia-inducible factor (HIF) is one of the most potent pro-angiogenic proteins regulated by Hsp90. Most solid tumor develop hypoxic regions as they grow and surpass their blood supply. To survive in the hypoxic condition, tumor cells have developed a set of responses which organizes the adaptation to hypoxia. A critical mediator of the hypoxic response is HIF-1, the transcription factor that regulates the expression of proteins promote angiogenesis, anaerobic metabolism, and other survival pathways.

2-1 Tumor hypoxia

In tumor, proliferating cells often surpass the vascular supply because of their rapid expansion rate, resulting in impaired oxygen delivery. In addition, blood vessels formed by tumor neovascularization are disorganized and have poor blood flow, contributing to hypoxic regions in the tumor (Figure 3) [21]. Although severe hypoxia will lead to apoptosis many tumor cells undergo genetic alterations to survive in limited conditions. The tumor response to hypoxia includes the induction of
angiogenesis, a switch from aerobic metabolism to anaerobic glycolysis, and the regulation of stress proteins concerning cell death or survival [22,23]. Hypoxia–mediated responses are activated by a variety of hypoxia–inducible transcription factors, including nuclear factor (NF–kB), activator protein 1 (AP–1) and p53. However, HIF is the master regulator of hypoxic responses.

2–2 HIF–1 and Cancer

Angiogenesis is the physiological process involving the growth of new blood vessels from pre–existing vessels. It is a normal and vital process in growth and development, as well as in wound healing and in granulation tissue. However, in tumor, excessive angiogenesis is occurred. An oxygen tension is the most important factor to sustain oxygen homeostasis, which plays an essential role in angiogenesis, the formation of new vessels. Interestingly, oxygen tension is a crucial element for cancer proliferation. The major stimulus for a tumor's recruitment of additional blood vessels in cellular hypoxia, a condition which is especially pronounced in neoplasm in cancer. Hypoxia induces transcriptional activation of genes that alter
cellular metabolism and promote neo-angiogenesis. A highly-angiogenic response in this neoplasm correlates with increased tumor growth, increased metastasis, and decreased survival [24].

The Hypoxia Inducible Factor-1 (HIF-1) is an important factor involved in the cellular response to hypoxia, and it has been studied for the last decade [25-27]. HIF-1 is composed of α and β subunits. The α subunit is over-expressed in hypoxia condition and is maintained at low levels in most cells under normoxic condition. Unlike the α subunit, β subunit is constitutively expressed and its activity is controlled in an oxygen-independent manner [28].

HIF-1α is a transcription factor, and its stability is modulated by oxygen levels. In hypoxia condition, it binds to hypoxia-response element (HRE), thereby activating the several genes, such as proangiogenic growth factors, like a vascular endothelial growth factor (VEGF) [29]. These genes, stimulated by HIF-1α, are related to angiogenesis, cell proliferation, and survival. Moreover, it was reported that the HIF-1α is related to many types of tumor [26]. However, in normoxia condition, von Hippel-Lindau (VHL) binds to HIF-1α and induces the proteasomal degradation (Figure 4) [30-32]. Furthermore, HIF-1α is related to other disease. Retina ischemia, one of the
ischemic cardiovascular disorders, which is caused by diabetes, pregnancy disorders, pulmonary hypertension and cancers are deeply related to the HIF-1α [33]. Among them, cancer comes into the attention. Hypoxia condition is an important force in the clonal evolution of tumor [34] and HIF-1α is over-expressed in common human cancers [35,36]. Thus, overexpressed HIF-1 α is a marker of aggressive disease behavior in several types of tumors [27]. Therefore, to reduce the HIF-1α expression can be a good target for cancer therapy.
Figure 3. Tumor hypoxia

When a small, localized tumor outgrows its vascular supply (distances >100 µm) tumor hypoxia arises in regions with impaired oxygen delivery. Consequently, hypoxic cells switch on target genes involved in angiogenesis [vascular endothelial growth factor (VEGF)], glucose transport [glucose transporter 1 (GLUT-1)] and cell migration [urokinase-type plasminogen activator receptor (u-PAR) and plasminogen activator inhibitor 1 (PAI-1)]. Increased vascular supply to the tumor via the induction of new blood vessel formation (angiogenesis) encourages tumor growth and facilitates metastasis to distant sites.
Figure 4. HIF–1 regulation

(a) In normoxia, hypoxia-inducible factor (HIF)–1α is hydroxylated by proline hydroxylases (PHD1, 2 and 3) in the presence of O₂, Fe²⁺, 2-oxoglutarate (2-OG) and ascorbate. Hydroxylated HIF–1α (OH) is recognised by pVHL (the product of the von Hippel–Lindau tumour suppressor gene), which, together with a multisubunit ubiquitin ligase complex, tags HIF–1α with polyubiquitin: this allows recognition by the proteasome and subsequent degradation. Acetylation of HIF–1α (OAc) also promotes pVHL binding.

(b) In response to hypoxia, proline hydroxylation is inhibited. VHL is
no longer able to bind and target HIF–1α for proteasomal degradation, which leads to HIF–1α accumulation and translocation to the nucleus. There, HIF–1α dimerises with HIF–1β, binds to hypoxia–response elements (HREs) within the promoters of target genes and recruits transcriptional co–activators such as p300/CBP for full transcriptional activity. A range of cell functions are regulated by the target genes, as indicated. Abbreviation: CBP, CREB binding protein; Ub, ubiquitin.
3. Deguelin and SH-1242

A deguelin is a plant derived rotenoids which is a natural antibiotics [37]. It is isolated from a constituent of the bark of the African plant *Mundulea seicea*. *Mundulea seicea* has been used as an insecticide and an aphrodisiac for decades of years. However, deguelin was found to inhibit ornithine decarboxylase (ODC) activity induced by 12-0-tetradecanoylphorbol-13-acetate (TPA) in cultured mouse epidermal 308 cancer cells [38]. It was highly effective in the mouse two-stage DMBA/TPA skin carcinogenesis model, and in rats, mammary tumors induced by *N*-methylnitrosourea were inhibited in a dose-dependent manner [39]. Given the demonstrated effectiveness of deguelin as a cancer chemopreventive agent in two animal models, the previous study was undertaken to evaluate the pharmacokinetic parameters in Sprague-Dawley rats. While this agent was initially developed as a chemopreventive agent, previous studies appear to show that deguelin has potential as a chemotherapeutic agent in melanoma [39]. A deguelin blocks proliferation of premalignant and malignant human bronchial epithelia (HBE) cells and it induces apoptosis in malignant and premalignant HBE cells. A deguelin also displays anti-cancer
activity by inhibiting the growth and angiogenesis of pre-cancerous and cancerous cells especially for lung cancer. A deguelin inhibits HSP90 activity which can fold a lot of protein related to tumor growth, survival and angiogenesis. When deuglein inhibits the HSP90 activation, AKT signal pathway and the expression of HIF-1α is blocked [40]. Targeting AKT and HIF-1α with deguelin may increase the apoptotic potential of Non-small-cell lung carcinoma (NSCLC) cells [41]. Moreover, a deguelin strongly inhibited COX-2 expression in HBE cells, without affecting the COX-1 protein level [42].

But, deguelin has strong side effect. It has cytotoxicity in normal cells. For that reason, Our joint research team professor Young-Ger Suh's group made deguelin analogues. SH-1242 is a structurally modified derivative of deguelin, which is B- and C- ring truncated compound (Figure 5).
Figure 4. Structure of deguelin and SH–1242

(A) Deguelin has 5 (A, B, C, D, E) rings. (B) SH–1242 is a structurally modified derivative of deguelin, which is B– and C– ring truncated compound.
II. MATERIALS AND METHODS

1. Cell culture and hypoxia condition

Human umbilical vein endothelial cells (HUVECs) were maintained in gelatin coated dish at 37°C in a humidified atmosphere of 5% CO₂ / 95% air in endothelial cell growth medium (EGM-2, Lonza) with full supplements (EGM-2 bullet kits: 2% FBS, 0.4% hFGF-2, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% hEGF, 0.04% hydrocortisone, 0.1% ascorbic acid, 0.1% heparin, and 0.1%-GA-100). HUVECs used in this study were taken from passages 6 to 7. BEAS-2B cells were maintained in Keratinocyte serum free medium (KSFM) (Invitrogen), supplemented with 5 µg/L of human recombinant EGF and 50 mg/L of bovine pituitary extract in plates coated with fibronectin. Non-small-cell lung carcinoma (NSCLC). H1299 cells were maintained in RPMI 1640 medium contained 10% fetal bovine serum (Life Technologies Inc., Gaithersburg, MD, USA) and 1% antibiotics. For hypoxic conditions, cells were incubated at 5% CO₂ level with 1% O₂ balanced with N2 in hypoxic chamber. (Forma, Marietta, OH, USA).
2. Antibodies

Each mouse monoclonal antibody against HIF−1α/ CD31, α−tubulin was purchased from BD Bioscience(San Diego, CA), BioGenex (Fremont, CA). Rabbit monoclonal antibody against AKT, Cleaved PARP, Caspase3, Cleaved caspase−3, MEK1/2, ERK1/2 were purchased from Cell signaling(Beverly, MA). ErbB2 antibody were purchased from Santa Cruz Biotechnology(Santa Cruz, CA).

3. Cell Treatments

To assess the effect of SH−1242 on HIF−1α protein expression, H1299 cells were seeded in 60−mm diameter dishes, at 5×10^5 cells per dishes, 1 day before the start of treatment. When the culture was 80−90% confluent, cells were treated with SH−1242 in complete medium for 1 day. After 1 day, cells were additionally treated for 4 hours under normoxic (20% O_2) or hypoxic(1% O_2) conditions.

To assess the effects of SH−1242 on apoptosis and Hsp90 client protein expression, H1299 cells were seeded in 60−mm diameter dishes, at 3×10^5 cells per dishes, 1 day before the start of treatment. Cells were treated with SH−1242 in complete medium for 3 day.
4. MTT assay

To measure the effects of SH-1242 on cell proliferation, H1299, BEAS-2B cells were plated at a concentration of $6 \times 10^3$ to $1 \times 10^4$ cells/well in 96-well plates. After incubation for one day, cells were treated with either DMSO as a control or various concentrations of SH-1242. The final concentration of DMSO in the medium was $\leq 0.5\%$ (v/v); at this concentration, DMSO had no effect on cell growth. After the cells were incubated for 72h, 20ul 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (4mg/ml) was added to each well for another 4h at 37°C. The formed formazan crystals were dissolved in DMSO (100ul/well) for 2h. The plate was then read on a microplate reader at 570nm. Three replicate wells were used for each analysis.

5. Western blot

Whole cellular protein was extracted by incubation in lysis buffer (Cell signaling Technology) for 30 minutes on ice, and then centrifuged to remove cellular debris the protein in the resulting supernatant was quantified by the bicichonic acid method (Pierce Chemical Co., Rockford, IL, USA). A total of 50ug protein was loaded onto 8-15% gel and transferred to
protein nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was incubated in blocking buffer (3% skim milk in TBS-T) at room temperature. Then filter was incubated with specific primary antibody at 4°C for overnight and washed with TBS-T three times every 10min, followed by incubation with anti-mouse or rabbit polyclonal antibody at RT for 1h. Immunoreactive bands were visualized using chemiluminescent reagent as recommended by Americham Pharmacia Biotechnology. Equal amount of protein loaded on the gel was quantitatively verified using tubulin as control.

6. Preparation of conditioned medium (CM)

To obtain conditioned medium from SH–1242–treated H1299 NSCLC cells, we plated 10^6 H1299 cells in a 10cm diameter plate containing RPMI 1640 medium with 10% fetal bovine serum. After 24 hours, the medium on these cells was replaced with fresh growth medium containing SH–1242 (0, 0.1, 1, 10uM). The plates were then incubated under hypoxic conditions for 1day, and cells were washed with phosphate-buffered saline, and then serum–free medium containing the same concentration of SH–1242 was added. After 2 days of incubation, conditioned medium was removed and
centrifuged at 4000g for 20 minutes at 4°C through an Amicon Ultra-4 centrifugal filter (Millipore) to remove any trace of SH-1242.

7. Tube Formation Assay

HUVECs (1×10^5 cells) were inoculated on 40ul Matrigel (BD Biosciences, San Jose, CA) surfaces. Cell were incubated in 50ul of EBM containing 50ul of conditioned medium from H1299 cells cultured under normoxic conditions. After incubation at 37°C for 4 hours, capillary tube formation was assessed. Morphologic changes in the cells were assessed under a microscope and photographed at x40 magnification. Tube formation was scored: A complete ring was scored as one tube. The experiment was repeated three times, each with similar results.

8. Flow Cytometry Analysis

H1299 cells were plated in 60 mm-diameter culture dishes. The next day, cells were treated with various concentrations of SH-1242 or vehicle (0.1% DMSO) for 3 days. Floating and adherent cells were collected and fixed in cold 70% ethanol at
4°C overnight. After washing, the cells were subsequently stained with 20μg/ml PI and 100μg/ml RNase A for 1h in the dark and subjected to FACS analysis to determine the percentage of cells at subG1 phase. Flow cytometric analysis was performed using a BD FACSVerse. Event (~10,000) were evaluated for each sample. The results are presented as the number of cells versus the amount of DNA as indicated by the intensity of the fluorescence signal.

9. Clonogenic growth assay

The anchorage-dependent clonogenic growth assay was performed by seeding H1299 cells into 6 well plates at a low density (200–500cells/well). Cells were treated with different SH-1242 concentrations (1, 2, and 5μM) in RPMI 1640 medium supplemented with 10% FBS for 10–15 days in a humidified atmosphere with 5% CO₂ at 37°C. The colonies were stained with 0.1% crystal violet in dH₂O for 20 minutes. We then counted the number of colonies.

10. Soft agar colony formation assay

To test the effect of SH-1242 on anchorage-independent colony formation, H1299 cells were suspended in 0.4% top agar
dissolved in growth medium at $2 \times 10^3$ cells/ml and then placed on top of solidified 1% base agar in 12-well plates. After the top cell layer solidified, growth medium containing DMSO or SH-1242 was overlaid. Two weeks later, colonies greater than 125μm in diameter were counted using an inverted microscope at 40× magnification after staining with an MTT solution.

11. Generation of Xenograft Tumors and Immunofluorescence

Xenograft tumors were generated by subcutaneous injecting nude mice with H1299 cells at a single dorsal flank site ($5 \times 10^7$ cells/mouse in 100μl of PBS). For patient-derived tumors, primary human lung tumor specimens were collected from an untreated patient. Tumors were minced into 2-mm$^3$ pieces and implanted under the skin of nude mice (one piece per mouse). When tumors reached a volume of 80mm$^3$ to 100mm$^3$ (termed day 0 for our experiments), mice were treated with SH-1242 (20mg/kg, i.p., twice a day). 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. Tumors were separated into several parts for analyses immediately after they were removed. Each part was frozen. Cryostat sections of 10μm were
attached on the gelation coated slide. The samples were stained with primary antibodies against CD31 (BD), cleaved-caspase 3 (Cell Signaling) overnight at 4°C followed by Alexa 488, 546 (Invitrogen) secondary antibodies at RT for 1h. Nuclear-staining were obtained with an ApoTome microscope (Carl Zeiss, Axiovert M200) or confocal microscopy (Carl Zeiss, LSM700).

12. Statistical analysis
The Student's t-test was used for analyzing statistical significance between the two groups. A p value of less than .05 was considered to be statistically significant at least.
III. RESULTS

1. SH–1242 has cytotoxicity against NSCLCs, but less than deguelin does in normal bronchial epithelial cells.

We first assessed the effect of SH–1242 on cytotoxicity by treating NSCLCs (Non–small–cell lung carcinoma cells) such as H1299 and normal bronchial epithelial cell line (BEAS–2B) with a range of various SH–1242 concentrations. All the concentrations of SH–1242 tested inhibited NSCLCs growth, with statistically significant differences after 72h exposure. NSCLC growth was inhibited in a dose–dependent manner (Figure 6A). In contrast, SH–1242 exhibited markedly lower cytotoxicity in normal bronchial epithelial cell lines (BEAS–2B) than deguelin did (Figure 6B).
Figure 6. Effects of SH-1242 on cytotoxicity of NSCLCs and normal bronchial epithelial cells

H1299 and BEAS-2B cells were seeded in 96-well culture plates. After incubation of 24 h, the cells were treated with various concentrations of SH-1242 or 0.1% DMSO as a control. After incubation for 72 h, they were subjected to MTT assay. Each value represents the mean (±SD) of a representative of 3 independent experiments.
2. **SH-1242 exhibits inhibitory effects on colony formation in a dose dependent manner**

Consistent with the MTT assay results (Figure 6A), SH-1242 treatment also affected the anchorage-dependent colony-forming ability of these cells in a dose-dependent manner (Figure 7A). Because the behavior, growth, and internal and external signal response of cells grown in vivo in three-dimensional conditions are largely different from those of cells grown in vitro in a monolayer on tissue culture plates, we tested the effects of SH-1242 on H1299 cells grown in soft agar, a 3D culture system. SH-1242 significantly reduced the colony forming ability in a dose-dependent manner (Figure 7B). SH-1242 treatment in soft agar at concentrations higher than 5uM caused a more than 50% inhibition in the colony forming ability of H1299 cells. These in vitro findings suggest that SH-1242 is capable of suppressing the tumorigenicity of non-small-lung cancer carcinoma cells.
Figure 7. Effect of SH–1242 on colony formation.

(A) An anchorage-dependent clonogenic growth assay was performed using H1299 cells. Cells were treated with different SH–1242 concentrations (1, 2, and 5 μM) in RPMI1640 supplemented with 10% FBS for 7–10 days, and then colonies were fixed with 0.1% crystal violet. Each value represents the mean (±SD) of a representative of 3 independent experiments.

(B) Cells were plated in RPMI 1640 medium containing 0.4% agar overlaid on a base of 1.0% agar in culture medium. After the upper cell agar solidified, deguelin-containing medium was overlaid. After 2 weeks, colonies were counted. Each value represents the mean (±SD) of a representative of 3 independent experiments.
3. **SH–1242 induces apoptosis**

Deguelin inhibits the growth of tumor cells by inducing apoptosis and cell cycle arrest in the G2/M phase in various cancer cells. To investigate whether the anti-proliferative effects of SH–1242 were accompanied by activation of caspase, processing of caspase–3 was examined by Western blot analysis. Treatment of H1299 cells with SH–1242 for 72h increased caspase–3 cleavage (Figure 8A). To confirm the activation of caspase–3, we examined cleavage of the DNA repair enzyme poly(ADP–ribose) polymerase (PARP), one of the major substrates of activated caspase–3, which is cleaved into an N-terminal DNA-binding domain and C-terminal catalytic domain. SH–1242–induced cleavage of PARP was also observed in 10μM concentration (Figure 8A).

Also, the relative DNA content of H1299 cells was measured using flow cytometry. SH–1242 treatment resulted in an evident increase in subG1 phase population. As shown Figure. 8B, there was little change in the subG1 portion until the SH–1242 concentration was 1μM. However, H1299 treated with 10μM SH–1242 for 72h had 14.08% cells in subG1 phase, as compared with only 2.27% in the control.
Figure 8. Effects of SH−1242 on apoptosis of H1299 cells.

(A) The protein expression of apoptosis−related protein such as PARP, caspase−3 cleavage fragments and caspase3 were evaluated in H1299 cells after 72 h of SH−1242 treatment.

(B) H1299 cells were incubated with the different concentration of SH−1242 for 72 h and analyzed by flow cytometry. The percentage of cells in subG1 phase by means of PI staining were determined.
4. **SH−1242 inhibits HIF−1α stability and angiogenic activities**

Because HIF−1 plays a crucial role in tumor progression by regulating the expression of key apoptotic and angiogenic factors, including VEGF, I investigated the effects of SH−1242 on HIF−1α expression under normoxic condition (20% O₂) or by exposure to hypoxic condition (1% O₂). Treatment with SH−1242 reduced HIF−1α protein expression in H1299 cells in a dose−dependent manner (Figure 9A).

The in vitro capillary tube formation assay uses HUVECs to assess endothelial cell morphogenesis into capillaries on matrigel−coated plates. I used conditioned medium from untreated or SH−1242−treated H1299 cells incubated under hypoxic (1% O₂) or normoxic (20% O₂) conditions. Conditioned medium from cells treated with various conditions stimulated endothelial cell morphogenesis statistically significantly less than conditioned medium from untreated control cells (Figure 9B). These findings indicate that SH−1242 inhibited the angiogenic activities of H1299 cells.
Figure 9. Effect of SH−1242 on HIF−1α protein stability and tube formation.

(A) H1299 cells were untreated or treated with different SH−1242 concentrations under normoxic conditions for 24 h. After 24 h, cells incubated under normoxic or hypoxic conditions for additional 4 hours.

(B) Hypoxic conditioned medium was prepared using H1299 cells. Cells were treated with different SH−1242 concentrations (1, 2, and 5 μM) in RPMI1640 supplemented with 10% FBS. After 24 h, the medium on these cells was replaced with fresh growth medium
containing SH-1242. The plates were then incubated under hypoxic conditions for 1 day, and cells were washed with phosphate-buffered saline, and then serum-free medium containing the same concentration of SH-1242 was added. After 2 days of incubation, conditioned medium was harvested.

(C) Angiogenic activity in conditioned medium from H1299 cells tested by plating human umbilical vein endothelial cells (HUVECs) onto Matrigel-coated 96well-plates. After 4 h, images of capillary tube formation were captured. Each value represents the mean (±SD) of a representative of 3 independent experiments.
5. SH-1242 induce degradation of Hsp90 client proteins

Heat shock protein 90 (Hsp90) is an evolutionarily conserved molecular chaperone that participates in stabilizing and activating more than 200 proteins—referred to as HSP90 'clients'—many of which are essential for cancer progression, survival. Deguelin has shown effective cancer chemopreventive and therapeutic activities by disrupting ATP binding to Hsp90, resulting in destabilization of its client proteins, such as Akt and HIF-1α [40]. In previous study, our group found that SH-1242 interrupted the interaction between HIF-1α and Hsp90 in vitro, which was reversed by addition of excess ATP. These results strongly support that SH-1242 may inhibit the activity of HSP90 by competing with ATP at the ATP-binding site [43]. When we tested SH-1242 as an inhibitor of Hsp90, we observed decreased expression of several Hsp90 client proteins (including ErbB2, Akt, MEK1/2, ERK1/2) in H1299 cells (Figure 10).
Figure 10. Effect of SH-1242 on Hsp90 client protein expression.

(A) Inhibition of heat shock protein 90 (Hsp90) chaperone function by SH-1242 through Hsp90 client proteins destabilization. Western blot analysis of the indicated Hsp90 client protein expression in H1299 cells untreated or treated with 0.1, 1 and 10μM SH-1242 for 3 days.

(B) Band intensities in the 3 independent experiments including (A) result were measured with NIH software Image J. Each value represents the mean (±SD) of a representative of 3 independent experiments.
6. **SH-1242 has apoptotic and antiangiogenic activities in vivo**

I further investigated the in vivo effect of SH-1242 treatment on apoptotic and anti-angiogenic proteins such as cleaved caspase3 (a marker of apoptosis) and CD31 (a marker of microvessel formation) in H1299 or patient-derived xenograft tumors. In immunofluorescence studies, I found that the expression of CD31 in the tumors from SH-1242 treated mice was lower than that in a control tumors from both control mice (Figure 11A, B). Conversely, tumors from SH-1242-treated mice showed higher cleaved caspase3 staining than tumors from both untreated mice (Figure 11A, B).
Figure 11. Effect of SH–1242 on H1299 and patient-derived xenograft tumors.

(A) Xenograft tumors were generated by subcutaneous injection of H1299 cells. Briefly, nude mice were injected at a single dorsal flank site with $5 \times 10^7$ H1299 cells in 100 µL of phosphate-buffered saline (PBS). Injection of these cells into nude mice induced exponentially growing tumors. After 10 days, mice were treated orally with vehicle (50 µL of cotton seed oil and 50 µL of dimethyl
sulfoxide) or SH–1242 at 20 mg/kg for 20 days.

(B) Immunofluorescence analysis. CD31 and cleaved caspase–3 expression in H1299 xenograft tumors obtained from vehicle control (Con) or SH–1242–treated nude mice.

(C) Images were represented by quantification. Each value represents the mean (±SD) of a representative of 3 different mice.
IV. DISCUSSION

Many cancer chemopreventive agents, including naturally occurring and synthetic compounds, have been studied for their in vivo and in vitro antitumor efficacy [44]. Most drugs currently available for the treatment of cancer are based on the inhibition of cell proliferation and induction of apoptosis [45]. Deguelin has known for a chemopreventive agent against various cancer. However, it has high cytotoxicity in normal cells.

In my studies, I examined effects of SH-1242 on cytotoxicity of NSCLCs and normal bronchial epithelial cells. The growth of H1299 cells were inhibited in a dose-dependent manner less than deguelin, but SH-1242 has a less cytotoxicity in normal lung epithelial cell line such as BEAS-2B. Also, SH-1242 exhibited dose-dependent inhibitory effect of colony forming ability. This indicates that SH-1242 may potential tumor growth and metastasis inhibition.

Chemotherapeutic agents causing apoptosis have been increasingly appreciated as ideal compounds for the management of cancer [46]. Previous studies have reported that deguelin can trigger apoptosis in various tumor cells in vitro [47]. Therefore, in order to investigate the possible pathway that was involved
with the anti-tumor effect of SH-1242, the apoptosis of NSCLC was analyzed in vitro. Treatment of H1299 cells with SH-1242 increased caspase-3 and PARP cleavage, and confirmed by flow cytometry analysis.

Another possible pathway that was involved with the anticancer effect of SH-1242 in vitro. Angiogenesis, the formation of new blood vessels from pre-existing vessel, is integral to tumor growth and metastases [48]. Deguelin has been reported to be antiangiogenic targeting HIF-1α in NSCLC [49]. Therefore, I investigated antiangiogenic activities of SH-1242 in NSCLC. As a result, SH-1242 inhibited HIF-1α in a dose-dependent manner. Furthermore, our study reveals that SH-1242 inhibits angiogenic features of HUVEC in vitro as revealed by a capillary-like tube formation.

I finally assured the clinical applicability of SH-1242 by confirming its inhibitory effects on the growth of patient-derived xenograft tumors. SH-1242 significantly increased cleaved caspase-3 and decreased CD31 expression in the H1299 and patient-derived xenograft tumors.

Taken together, I demonstrated that SH-1242 induced apoptosis as well as inhibited angiogenesis in NSCLC. Therefore, I suggest that SH-1242 is potentially useful as a chemotherapeutic agent in NSCLC.
V. REFERENCES


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VI. 국문 초록

Deguelin은 Mundeulea sericea를 포함하는 식물에서 추출한 rotenoid 화합물이며 구조적으로 5개의 고리(A, B, C, D, E)를 가지고 있다. 이들은 열 충격 단백질 90 (Hsp90)에 결합하여 이들의 기능의 억제를 통해 여러 암세포에서 증식억제를 유도한다고 알려져 있다. 열 충격 단백질 90은 암세포에서 과발현되어있으며 여러 가지의 종양단백질의 안정성에 기여한다고 알려져 있다.

그러나, deguelin은 이러한 항암효과에도 불구하고 정상세포에서 높은 독성으로 인해 임상적으로 사용되기 어려운 문제점이 가지고 있다. 그리하여 공동연구를 통해 합성된 deguelin 유도체인 SH-1242 화합물을 이용하여 이러한 부작용을 극복하고자 하였다. SH-1242는 deguelin 구조에서 B와 C 고리를 제거한 화합물이다.

비소세포성 폐암세포에서 SH-1242가 암세포 증식억제 활성 및 항 혈관신생 억제 그리고 그 기전에 대해서 연구하였다. 비소세포성 폐암세포와 정상 기관지 상피세포에서 SH-1242를 처리하였을 때, 세포 독성을 확인하기 위하여 MTT 분석을 수행하였다. 그 결과, 비소세포성 폐암세포에서는 높고 의존적으로 세포 독성을 나타내었으며, 정상 폐 상피세포에서는 deguelin과 비교하여 독성이 거의 없는 것으로 나타났다. 또한 SH-1242가 비소세포성 폐암세포 클로로니 형성에 어떠한 영향을 주는지 보기 위해 부착 의존적인 또는 비 의존적인 클로로니 형성 분석 실험을 진행하였다. 두 실험에서 SH-1242의 높고 의존적으로 클로로니 형성이 저해되었다. 이러한 암
세포 증식억제는 세포자살과 관련되어지는지를 확인하기 위하여 유세포 분류기 분석과 western blot을 수행하였다. sub-G1기의 사멸세포가 증가하였고 caspase-3 신호전달경로가 억제되었다.

또한 SH-1242가 항혈관신생억제 활성에 관여하는지 보기 위해서 western blot과 관 형성 분석을 수행하였다. 저산소상태에서 H1299 세포에 SH-1242를 처리하였을 때 저산소 유도 인자-1ɑ의 발현이 감소되었다. 관 형성 분석에서는 SH-1242를 처리하였을 때 matrigel 위에 HUVEC 세포의 모세관 형성이 현저하게 줄어들었다.

이러한 암세포 사멸과 항혈관신생억제가 열 충격 단백질 90의 기능 저하에 관여되는지를 조사하였다. 그 결과, SH-1242 농도 의존적으로 ErbB2, Akt, MEK, ERK 등 열 충격 단백질 90 클라이언트단백질들의 발현이 억제되었다.

마지막으로 SH-1242가 H1299 또는 환자 유래 이식암 동물에서 세포 자살 또는 항혈관신생 활성이 있는지 조사하였다. 그 결과, SH-1242를 처리한 암 조직에서 cleaved caspase3의 발현은 증가되었고 CD31의 발현은 감소되었다.

종합적으로, SH-1242가 정상 기관지 상피세포에는 독성이 없지만, 비소세포성 폐암세포에서 Hsp90의 억제를 통해 암세포의 증식과 혈관신생 활성을 억제하는 것으로 보여진다.

주요어 : deguelin, SH-1242, 비소세포성 폐암세포, 열 충격 단백질 90, 저산소 유도인자 1-ɑ
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