약학석사 학위논문

A Novel Synthetic Derivative of Deguelin, SH-174, Exhibits Anti-Cancer Activities Against Non-small-cell Lung Cancer Cells

새로운 deguelin 합성 유도체인 SH-174의 비소세포성 폐암세포에서의 항암 활성 연구

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Abstract

The identification of a novel anti-cancer agent against non-small-cell lung cancer cells

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The naturally occurring rotenoid, deguelin, has been studied to have a strong anti-cancer efficacy in various types of cancer for more than a decade. However, its intolerable toxicity at therapeutic dose and light-labile physical property deterred its development to be a clinical drug of use. Here, I report that SH-174, a novel synthetic derivative of deguelin, has a strong anti-cancer activity against several non-small-cell lung cancer (NSCLC) cells while having no or a less toxicity to human non-cancerous normal cells (e.g. BEAS-2B, ARPE-19, and HUVEC) than deguelin has. Several NSCLC cell lines were treated with or without SH-174 and subject to MTT assay, colony formation assay, and apoptosis assay; all of which treated with SH-174 exhibited significant inhibited results on the proliferation

and survival of those cancer cells. Moreover, SH-174 reduced the ability of cancer cells to adapt to and survive under hypoxic cancer microenvironment by downregulating phosphorylation of Akt and MEK1/2, as well as HIF-1α protein level. To elucidate the target of oncogenic signaling in NSCLC cells by SH-174, I examined the AMPK-mTORC1 signaling axis which is harnessed by cancers for their rapid proliferation and survival. As a result, SH-174 induced the phosphorylation of AMPKα at Thr172, resulting in the inhibition of mTORC1 activity on cellular proliferation. This suggests SH-174 blocks the unchecked proliferation of cancer cells by modulating AMPK-mTORC1 signaling pathway. Thus, SH-174 is a new promising candidate for lung cancer therapy and further *in vivo* studies in animals are warranted.

Keyword: non-small-cell lung cancer (NSCLC), deguelin, anti-cancer drug, apoptosis, mTORC1, AMPK, hypoxia

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Table of Contents

Chapter 1. Introduction

1-1. Study Background

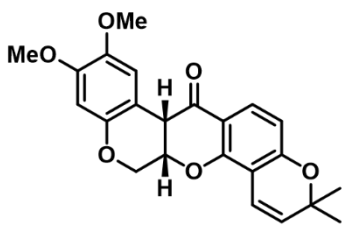
Among all human cancers, lung cancer is the most prevalent one in the world and the primary cause of cancer-related deaths worldwide[1]. It has the highest mortality among all types of human cancers, almost 1.8 million people are diagnosed and 1.6 million die every year owing to lung cancer[1]. Based on the histopathological characteristics, lung cancers are divided into two categories; small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC), the latter comprising more than 80% of total lung cancer with only 15.9% of 5-year survival rate[2]. NSCLC is divided into 3 major categories by histology: adenocarcinoma (ADC), squamous cell carcinoma (SCC), and large cell carcinoma. Among these, ADC and SCC comprises up to 90% of total NSCLC[3-4]. ADC arises relatively in distal airways, while SCC does in proximal ones lining trachea and upper airways[3-4]. When it comes to large cell carcinoma, which is diagnosed when the carcinoma is not thought to be ADC or SCC, it is not known whether the genetic backgrounds of large cell carcinoma is different from those of ADC or SCC[4]. In spite of acknowledging the fatality of lung cancer to people for more than a half century and vigorous efforts to develop effective medications, people are still suffering from lung cancer owing to its high rate of recurrence[5-6]. In addition, the extreme heterogeneity of genetic backgrounds of NSCLC makes it more intractable disease[7]. Therefore, the pursuit of diversity of anti-cancer agents is in urgent demand to deal with the extreme heterogeneity of NSCLC and its intransigence.

The natural products from various plants have been promising

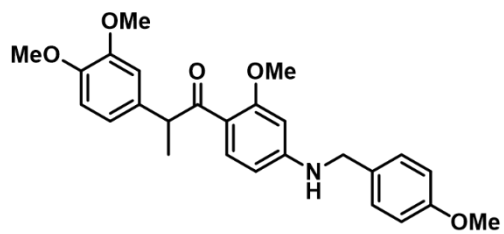
sources of drugs for human diseases, including cancer[8]. Deguelin, the naturally occurring rotenoid, has been shown to have a potent anti-cancer activity against several cancers arising from lung, liver, skin, prostate, breast, pancreas, and head and neck, etc., by us and other groups[9-20]. It has been reported that deguelin inhibits a variety of carcinogenic activity—angiogenesis, proliferation, survival, migration and EMT—while inducing apoptosis and autophagy of cancer cells[9-20]. However, its toxicity to normal cell lines *in vitro* and to animals' neurons *in vivo* kept it from being developed into a clinical drug for anti-cancer therapy[21, 23]. To address this issue, I had screened derivatives of deguelin synthesized by Prof. Suh Young-Ger's team (Seoul National University) to find one with a reduced toxicity while retaining its potent anti-cancer activity. Of those, SH-174 was chosen based on its inhibitory activity on the viability of H1299 NSCLC cells.

Here, I demonstrate that SH-174, a novel synthetic derivative of deguelin, has a strong anti-cancer activity against NSCLC cell lines including three major subtypes of NSCLC: adenocarcinoma (H1299), squamous cell carcinoma (H292) and large cell carcinoma (H460). It is found that apoptosis is involved in its mode of anti-cancer action. Moreover, SH-174 showed no or a less toxicity than deguelin to several human cell lines such as BEAS-2B, ARPE-19, and HUVEC. To elucidate the mechanism of anti-proliferative effect by SH-174, I investigated AMPK-mTORC1 signaling axis which is critical players for regulating ribosome biogenesis and protein synthesis for cancer cells to proliferate. As a result, SH-174 induced the phosphorylation of AMPKα^{T172}, causing downregulation of mTORC1 activity by directly phosphorylating Raptor^{S792}, which in turn making its two major downstream target dysfunctional: p70S6K and 4E-BP1.

Collectively, these findings thereby warranty the further *in vivo* animal study of SH-174 as a chemotherapeutic agent for anti-lung cancer therapy.



(-)-Deguelin



SH-174

The structure of (−)−Deguelin and its synthetic derivative, SH−174

1-2. Purpose of Study

Keeping abreast with the exigent demand of new drugs for chemotherapy of lung cancer, we and other groups have been searching for new chemotherapeutic agents that could aid to manage the NSCLC better. The natural compound deguelin has shown its promising efficacy as an agent for NSCLC treatment. This study aims to excavate a new agent succeeding to the efficacy of deguelin, while reducing the cytotoxicity of deguelin to normal cells.

Chapter 2. Materials and Methods

Cell Lines. ARPE-19 and the NSCLC cell lines—H1299, H292, and H460—were purchased from ATCC (Manassas, VA, USA) and H226B, H226B/R, HUVEC, ARPE-19, and BEAS-2B were kindly provided by Prof. Lee, Ho-Young (Seoul National University). All NSCLC cell lines were maintained in RPMI1640 medium (cat. CM059-050) from GenDEPOT (Katy, TX, USA). ARPE-19 cells were maintained in DMEM/F-12 (1:1) from Invitrogen (Carlsbad, CA, USA) and used in the experiment below the passage 30. BEAS-2B cells were maintained in Keratinocyte-SFM (cat. 17005-042) from Invitrogen supplemented with bovine pituitary extracts (50 μg/ml) and epidermal growth factor (5 ng/ml). The human breast cancer cell MCF-7 was kindly provided by Prof. Surh, Young-Joon (Seoul National University) and maintained in DMEM. The media for all cancer cells and ARPE-19 contain 10% heat-inactivated FBS (cat. F0600-050) and 1% antibiotics (cat. CA002-010) from GenDEPOT to their final volume. HUVEC was cultured on 0.15% gelatin-coated plastic dish and maintained in EGM-2 medium (cat. cc-3162) from Lonza (Walkersville, MD, USA). In the experiment, HUVEC with passage below 10 was used. All cells were incubated in 37℃, 5% CO_2 humidified chamber.

Reagents. Antibodies against caspase 9 (#9508), caspase 7 (#9494), XIAP (#2042), survivin (#2808), AMPKα (#5831), phospho-AMPKα (Thr172) (#2535) were from Cell Signaling Technology, Inc. (Danvers, MA, USA) Antibody against caspase 3 was from Cell Signaling Technology, Inc. (#9664) and Santa Cruz Biotechnology, Inc. (sc-56053, Dallas, Texas, USA) GAPDH antibody (sc-365062)

was from Santa Cruz Biotechnology, Inc. Tubulin α antibody (cat. MU121-UC) was from BioGenex (Fremont, CA, USA) and those against actin (cat. A2066) and vinculin (cat. V9131) were from Sigma-Aldrich (St. Louis, MO, USA). HIF-1α antibody was from BD Biosciences (#610959, San Jose, CA, USA). For MTT assay, MTS solution (cat. G3581) was purchased from Promega (Madison, WI, USA). For soft agar assay, low melting temperature agarose (cat. 50100) was purchased from Lonza (Walkersville, MD, USA). SH-174 and deguelin was synthesized from Prof. Suh, Young-Ger's lab. The drugs were prepared in 10~100mM stock in DMSO and treated not exceeding 0.1% (v/v) to its final volume diluted in the suitable medium for each cell line. Thus, 0.1% DMSO was used as a positive control for all experiments. Unless indicated, when treated to cells, the compounds were diluted in whole medium containing 10% FBS and 1% antibiotics.

Cell Viability Assay. To measure the cell viability, optimized cell number empirically 3×10^3 or 10^4 cells were seeded on 96-well plate. After 24 hours, drugs were treated to each well as indicated concentration in the fresh medium suitable for each cell line. After indicated amount of time, MTS solution was treated and the absorbance at 492nm was measured to assess the viability according to the manufacturer's protocol.

Anchorage-dependent Colony Formation Assay. 2×10^3 cancer cells as singlets were seeded on 10-cm culture dish, incubated for 24 hours, and then treated with indicated concentration of SH-174 in 12ml of fresh medium. The medium was not changed until the detection of colony. 7-8 days later, the previously published method[22] was slightly modified to detect the colonies. Briefly, the dish was washed with warm 1x PBS, fixed with methanol for 10 mins, and then

0.5% ethidium bromide in 50% ethanol was treated for 20 seconds. The dishes were drained and then subject to UV light by LAS-4000 (Fujifilm, Tokyo, Japan).

Soft Agar Colony Formation Assay. For the base layer, 0.8% agarose gel was layered on 12-well plate. After 3 hours for agarose gel to be fully solidified, 2×10^3 cancer cells as singlets in the whole medium containing 0.4% agarose were seeded. After another 3 hours for the solidification of upper agarose layer, the drug with indicated concentration diluted in the whole medium was treated. The medium containing drug was replaced with fresh one every 4 days to protect cells from being dried. 8-21 days later, the colonies were fixed with 4% PFA for 10 mins, stained with 0.05% crystal violet for 30 mins and washed out with current tap water. Then the colonies were counted under the light microscope. Colonies with the diameter exceeding 450 μm were counted using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Western blot assay. Cells were washed with ice-cold 1x PBS for once and the dish was drained and an adequate amount of lysis buffer (#9803, Cell Signaling Technology, Inc.) supplemented with phosphatase inhibitor cocktail B (cat. Sc-45045, Santa Cruz Biotechnology, Inc.) as well as protease inhibitor cocktail was treated. In case of apoptosis assay, floating cells were also collected and treated with lysis buffer. For HIF-1α, cells were lysed by high-salt buffer (10 mM HEPES pH 7.9, 1 mM EDTA, 400 mM NaCl, 5% Glycerol). Cells scraped off from the dish were centrifuged by 18,000 g for 30 mins at 4℃. Supernatant were collected and subject to BCA assay to quantify the amount of total proteins in cell lysates. Equal amount of proteins were subject to SDS-PAGE and transferred to a nitrocellulose membrane (cat. 10600004, GE Healthcare). The

membranes were blocked by 5% skim milk for 20 mins and incubated more than 16 hours with the appropriate primary antibodies at 4℃. The membranes were washed with TBS-T (0.1% Tween20), and incubated with secondary antibodies for 1 hour at room temperature. Unbound antibodies were washed off by TBS-T. To detect the bands, membranes were treated with enhanced chemiluminescence solution and subject to LAS-4000.

Hypoxia and Obtaining Conditioned Medium. Cells were seeded on plastic culture dish and incubated for 24 hours. When the confluency reached nearly 80%, SH-174 or DMSO 0.1% was treated for the next 24 hours, followed by 4 hour incubation under hypoxic (1.5% O_2, 5% CO_2, 93.5% N_2, 37℃) conditions. Cells were harvested at the end of hypoxic incubation and subject to western blot assay.

To examine the inhibitory effect of growth factors secretion, SH-174 or DMSO 0.1% diluted in serum-free medium was treated to cells with 80% confluency and incubated simultaneously under hypoxic conditions as above for 24 hours. Then, the medium in dish was collected and filtered by centrifugal filter units (Amicon Ultra, Cat. UFC800324, Merck Millipore, Darmstadt, Germany) under 3,000 g, 1 h, at 4℃. By the centrifugation, approximately 40-fold (v/v) concentrated conditioned medium (CM) was obtained with being filtered out with molecules larger than 3 kDa.

Statistical Analysis. Values were presented as the mean ± SD or SEM as indicated. Statistical significance was determined by Student's two-tailed paired t test. Values were regarded biologically significant when p value was less than 0.05 compared to control set; $*p<0.05$, $**p<0.01$, $***p<0.001$. To calculate IC_{50} value, Prism 6.0 (GraphPad Software, Inc, La Jolla, CA, USA) was used. All other statistical values were calculated by Excel (Microsoft,

Redmond, WA, USA).

Chapter 3. Results

3-1. SH-174 shows a potent anti-proliferative activity against NSCLC cells

The initial screening of 18 synthetic derivatives of deguelin by measuring the inhibitory effect on the viability of H1299 cells identified SH-174 as a promising candidate for anti-NSCLC agent (Fig. 1). H1299 cells were treated with 10 μM of each compound for 72 hours and the viability was measured by MTT assay (Fig. 1). Because the major problem of deguelin as an anti-cancer agent was the safety issue to normal cell, I also examined the effect of SH-174 on the viability of non-cancerous human cells such as ARPE-19 (retinal pigment epithelial cell), BEAS-2B (lung bronchial epithelial cell), and HUVEC (human umbilical vein endothelial cell). As a result, SH-174 exhibited no or if existed, a little toxicity to such cells with nearly 0 or 20% inhibition of the cell viability (Fig.2). Thus, I chose SH-174 as a strong candidate for the further anti-cancer study.

To investigate if SH-174 has such an efficacy on overall NSCLC cell lines, some of them including H1299 (adenocarcinoma cell), H460 (large cell carcinoma cell), and H292 (squamous cell carcinoma cell) were treated with various concentrations of SH-174 (0.1 μM, 0.5 μM, 1 μM, 5 μM and 10 μM) or DMSO 0.1% and the viability was measured by MTT assay every 24 hour up to 3 days. As a result, it showed a potent inhibitory effect on the viability of all subject cell lines with its IC_{50} values at 72 hour as follows: 0.44 μM for H1299, 0.40 μM for H460 and 0.37 μM for H292 (Fig. 3i)-iii)). The viability of H226B (EGFR-propagated H226) and its paclitaxel resistance-

acquiring subline, H226B/R[23] were also reduced by SH-174 (10 μM, 72 h, Fig. 4i)-ii)). To see whether this result could be applied to other type of cancer, breast cancer cell line MCF-7 was also subject to MTT assay. However, its viability was reduced marginally even at relatively high concentration (50 μM), indicating that SH-174 has a degree of selectivity on NSCLC cells (Fig. 4iii)).

The decrease of viability of NSCLC cells was in accordance with the result of colony formation assays on two different types of bottom: plastic dish and soft agar. H1299, H460, and H292 cells were seeded on plastic culture dish for cell culture, followed by the treatment of SH-174 (1 μM, 5 μM, 10 μM) for 8-9 days and the colonies with their diameter exceeding 450 μm were counted. As shown in the figure 5i), the number of colony was significantly decreased with the treatment of 5 μM and 10 μM of SH-174 for all 3 NSCLC cell lines. Those 3 types of NSCLC cells were also seeded on double-layered soft agar as singlets (see Materials and Methods for further experimental details), followed by the treatment of SH-174 as above. The number of colony was reduced as well, indicating that SH-174 has such an anti-cancer activity for both types of cancer growth: anchorage-independently as well as anchorage-dependently (Fig. 5ii)). Collectively, the data indicate that SH-174 inhibits the growth and survival of NSCLC cells with no or a less toxicity than deguelin to normal cells.

3-2. SH-174 induces apoptosis in H460 cells

Apoptosis has been known to be one of the major mechanisms of the anti-cancer activity[24]. Since deguelin was shown to induce apoptosis in several cancers, including lung cancer[9, 11, 16-18, 20], I examined whether apoptosis is involved in the anti-cancer activity of SH-174 as likely in that of deguelin. H460 cells were treated with SH-174 (1 μM, 2 μM, 5 μM, 10 μM) for various time longer than 48 hours of treatment, since the viability of H460 significantly plummeted only after 48 hours of treatment (Fig.3i)). Of those, at 54 hour after treatment, several apoptotic markers were observed by western blot assay (Fig. 6); when H460 cells were treated with SH-174 (2 μM, 54 h), cleaved forms of the effector caspase 3 and 7 and the initiator caspase 9 were detected while cleaved forms of caspase 8 were not detected (data not shown), indicating that SH-174 induces apoptosis through the cellular intrinsic pathway. In addition, XIAP and survivin, two of the Inhibitor Apoptosis Protein (IAP) family, were also downregulated in this conditions. Various timepoints between 48-72 hours, treated with 5 or 10 μM of SH-174 were also examined to detect apoptotic markers. However, any of cytoskeleton proteins generally used for loading controls as housekeeping genes (i.e. tubulin, actin) as well as any cleaved form of caspases was not detected by western blot assay, so that I excluded these data as a proof of inducing apoptosis by SH-174. Consequently, the data in figure 6 show that apoptosis is involved at least in part in the anti-cancer activity of SH-174 against H460 cells.

3-3. SH-174 reduces the ability of H1299 cells to adapt to and survive hypoxic conditions

Cancer microenvironment is nutrients and oxygen-restrictive[25, 32, 33] due to the competition between highly proliferative cancer cells and other stromal cells (e.g., immune cells[28-30]). This forces cancer cells to adapt to and survive under its hypoxic microenvironment by exploiting several molecules such as HIF-1α[25], Akt[31], and MEK1/2[34], etc. Thus, targeting these molecules of cancer to interfere with its adaptability could be a valid approach as a cancer therapy. For example, deguelin was reported as a prominent anti-angiogenic agent by reducing the protein level of HIF-1α[15, 17, 20, 27]. Moreover, deguelin was renowned for its negative effect on the phosphorylation of Akt[16, 26]. Thus, to investigate whether SH-174 has a negative effect on the stability of HIF-1α and phosphorylation level of Akt and MEK1/2 as likely as deguelin does. H1299 cells were treated with SH-174 or DMSO 0.1% for 24 hours, followed by the incubation under 1.5% O_2 for 4 hours and then subject to western blot assay. As a result, HIF-1α protein level was downregulated and the phosphorylation level of Akt^{S473} and $MEK1/2^{S217/221}$ was also diminished by SH-174 (Fig. 7i)).

Cancer cells can make their microenvironment advantageous to themselves. For example, they can secrete several factors to stimulate the proliferation of endothelial cells to give rise to angiogenesis to supply adequate amount of nutrients and oxygen[35, 36]. Since SH-174 downregulated HIF-1α, p-Akt^{S473}, and p-$MEK1/2^{S217/221}$ (Fig. 7i)), I hypothesized that SH-174 would decrease the ability of cancer cells to make their niche favorable to themselves, especially the ability to stimulate the proliferation of

endothelial cells to which HIF-1α is deeply related. To test my hypothesis, SH-174 was treated in serum-free medium to H1299 cells and simultaneously, incubated under 1.5% O_2 for 24 hours. Then, the medium was collected and filtered by centrifugation (3,000 g, 1 h, 4℃). This conditioned medium (CM) obtained from H1299 cells was treated to HUVEC (for details, see Materials and Methods). After 24-hour incubation, the viability of HUVEC was assessed by MTT assay. As shown in figure 7ii), the growth rate of HUVEC for 24 hours was decreased by the CM from SH-174-treated H1299 cells dose-dependently. This indicates that SH-174 decreased the ability of H1299 cells to secrete factors that could stimulate the proliferation of endothelial cells. Together, figure 7 shows that SH-174 reduces the ability of H1299 cells to survive and be accustomed to hypoxic conditions.

3-4. SH-174 modulates the AMPK-mTORC1 signaling pathway in H1299 cells

To elucidate the target attributed to the mode of anti-cancer activity by SH-174, I had checked if SH-174 inhibits the phosphorylation of Akt^{S473}, previously reported as one of the anti-cancer mechanism by deguelin[16, 26]. However, unlike under hypoxic conditions (Fig. 7i)), SH-174 neither reduced the phosphorylation level of Akt at Ser473 site nor altered the protein level of unphosphorylated Akt (pan) even at the lethal concentration (5 μM) within 24 hours (data not shown), unlikely does deguelin[26, 27]. Moreover, none of the HSP90 client protein levels, including PRMT5, ERK1/2, and EGFR was downregulated by SH-174 (5 μM, 24 h, data not shown), the proposed mode of action of deguelin[27]. Thus, I hypothesized different modes of action would be involved in its anti-cancer efficacy. This was plausible because the structure of SH-174 is quite different from its mother compound, deguelin (see Introduction).

The mammalian target of rapamycin (mTOR) has been revealed to regulate cell proliferation by controlling protein translation, ribosome biogenesis, autophagy, etc. for the past few decades[37]. Since the mTOR kinase is at the central position of cellular signal transduction network, it is one of the key molecules in cancer for its survival and proliferation, which makes it an attractive therapeutic target for cancer therapy[38-40]. There are two types of complex that mTOR kinase comprises: mTORC1 and mTORC2. Of those, mTORC1 is less dependent upon Akt than mTORC2[38]. Thus, being independent of Akt, I presumed that the inhibition of mTORC1 signal might be involved in the anti-cancer activity of SH-174.

Besides Akt, AMP-activated protein kinase (AMPK), which

regulates numerous cellular signaling related to energy metabolism, lipogenesis, autophagy, exercise etc.[41-46] has been revealed to control mTORC1 activity by direct phosphorylation of scaffold protein Raptor—a component of mTORC1—at serine 792[46]. Therefore, I examined whether SH-174 could modulate the activity of mTORC1 via the activation of AMPK.

To test my hypothesis above, H1299 cells were treated with SH-174 (5 μM) by time-dependent manner: 0, 1, 3, 9, 24 hours (Fig. 8). To exclude the effects that could be induced by growth factors in serum, cells were serum-starved for 24 hours and SH-174 or DMSO 0.1% was treated in serum-free medium. As a result shown in figure 8, 24-hour treatment of SH-174 induced the phosphorylation of AMPKα^{T172}, in turn directly phosphorylating Raptor^{S792} and causing dephosphorylation of p-mTOR^{S2448}, which made mTORC1 dysfunctional. Consequently, the phosphorylation level of 4E-BP1 and p70S6K—two downstream targets of mTORC1—was decreased, which would cause halt of ribosome biogenesis, protein translation and in the end, blockage of cell proliferation. This indicates that the modulation of AMPK-mTORC1 signaling axis is involved in the anti-cancer effect by SH-174.

3-5. Phosphorylation level of 4E-BP1 might dictate the inhibitory response of cells by SH-174

It has been known that cancer relies on a very few driver genes, or oncogenes, for its carcinogenesis, called oncogene addiction[65-67]. One of such oncogenes in lung cancer is eIF4E[68-70]. eIF4E serves as the rate-limiting factor in eIF4F complex formation, which regulates the cap-dependent mRNA translation in eukaryotic cell, so as to control numerous protein expression level[68-70]. Moreover, eIF4E dose is critical especially in carcinogenesis, unlike in normal tissue development[71]. Thus, restraining the eIF4E availability is a reasonable strategy in cancer treatment[72-74]. 4E-BP1, one of the mTORC1 downstream targets, regulates eIF4E availability by, when phosphorylated, releasing eIF4E from its complex[75-77]. This makes one to speculate that inhibition of 4E-BP1 phosphorylation could be a therapeutic target for cancer treatment. As a matter of fact, it has been reported that the phosphorylation level of 4E-BP1 is correlated with the prognosis of several cancer[78, 79], so as to be an oncotarget[80] in that it is a key effector of the oncogenic activation[81], as well as it predicts the sensitivity to anti-cancer agent of cancer cells[82, 83].

Figure 9 shows the phosphorylation level of 4E-BP1 is higher in cancer cells than normal cells. In line with this, the viability of cells with high 4E-BP1 phosphorylation level is more vulnerable to SH-174 (Fig. 2, 3, 9). Given that SH-174 abrogates the phosphorylation of 4E-BP1 via mTORC1 inhibition, it could be reasoned that cells with higher p-4E-BP1 level would have more damage on cellular proliferation. In this vein, 4E-BP1 phosphorylation level could explain the strong toxicity selectively on cancer cells by SH-174.

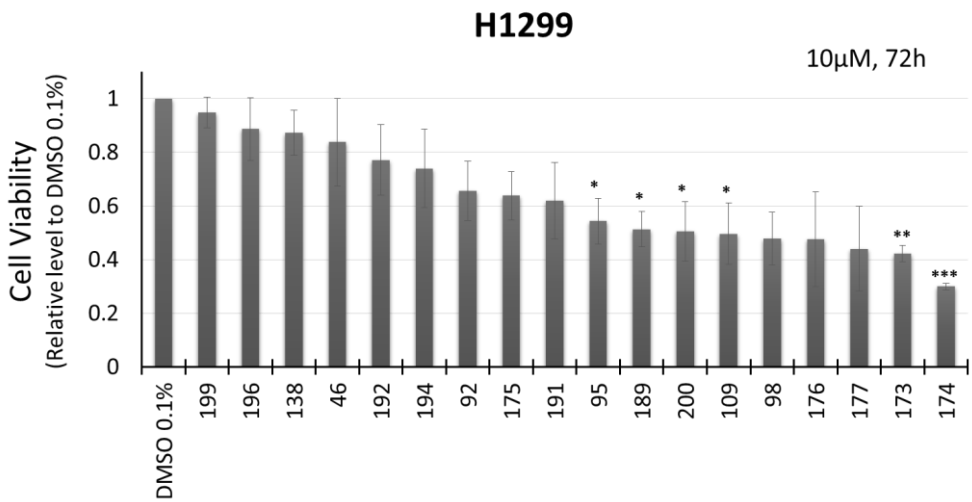


Figure 1. The identification of SH-174 as a promising candidate for anti-cancer agent against NSCLC cell

Inhibitory activities on the viability of H1299 cells by a series of synthetic derivatives of deguelin. Compounds were treated for 72 hours with 10μM concentration. The viability was assessed by MTS assay with 3 independent experiments as triplicates for one experiment. Mean ± SEM; n=3; paired Student's t test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

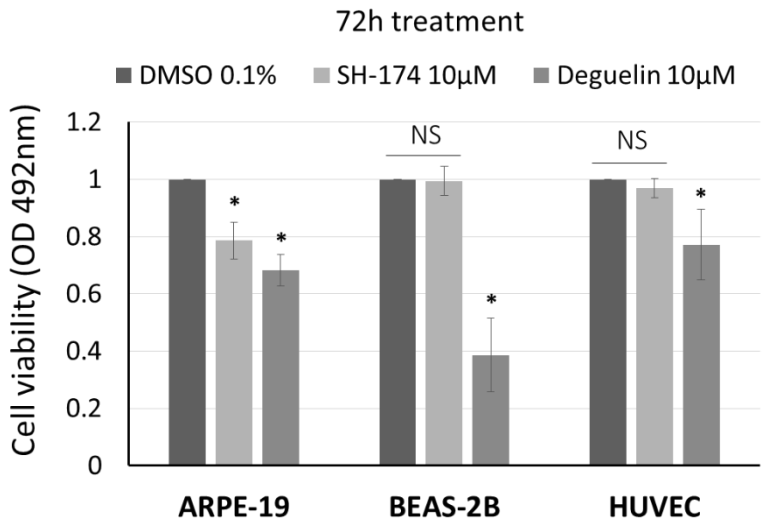


Figure 2. SH-174 has no or a less toxicity than deguelin to several non-cancerous human cell lines

The result of MTT assay on several human cell lines. Cells were treated with DMSO 0.1% or SH-174 or deguelin for 72 hours. Mean ± SD; n=3, 4; paired Student's t test; $*p < 0.05$

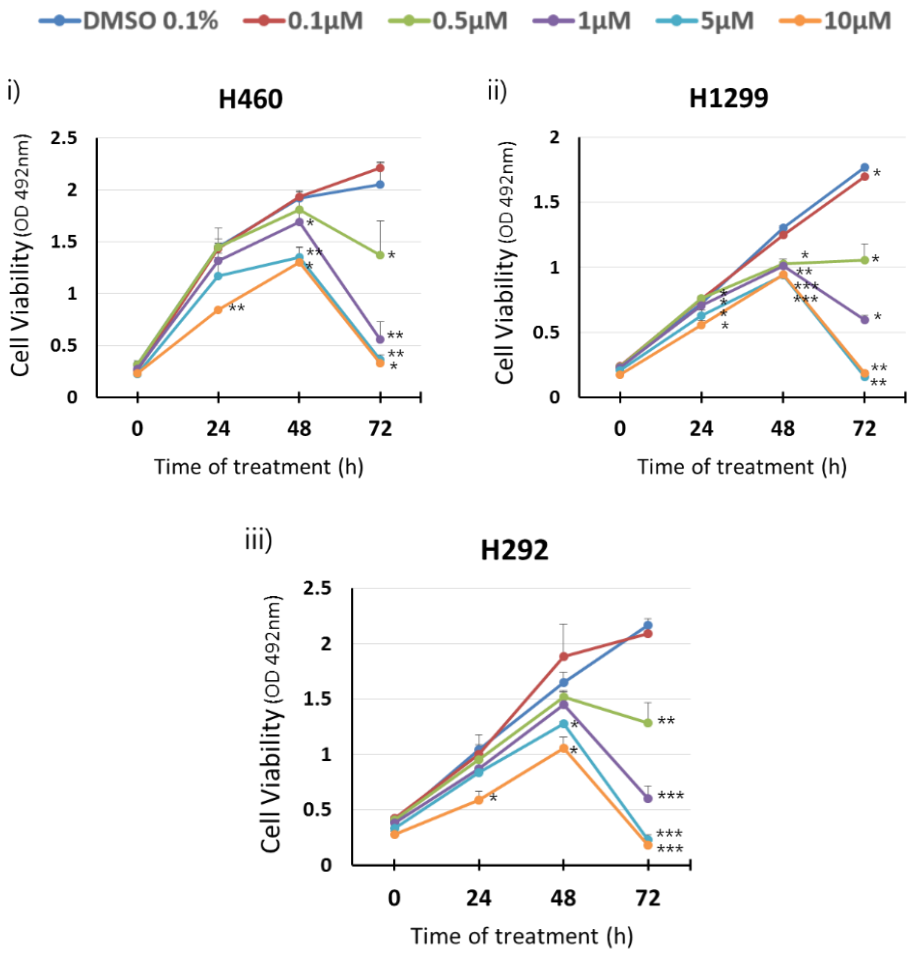


Figure 3. SH−174 exhibits a potent inhibitory activity on the viability of NSCLC cell lines

3 NSCLC cell lines were subject to MTT assay, treated with various concentrations indicated of SH−174 every 24 hour. Each cell line represents large cell carcinoma cell (H460), adenocarcinoma (H1299), and squamous cell carcinoma cell (H292). Mean ± SD; n=3, 4; paired Student's t test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

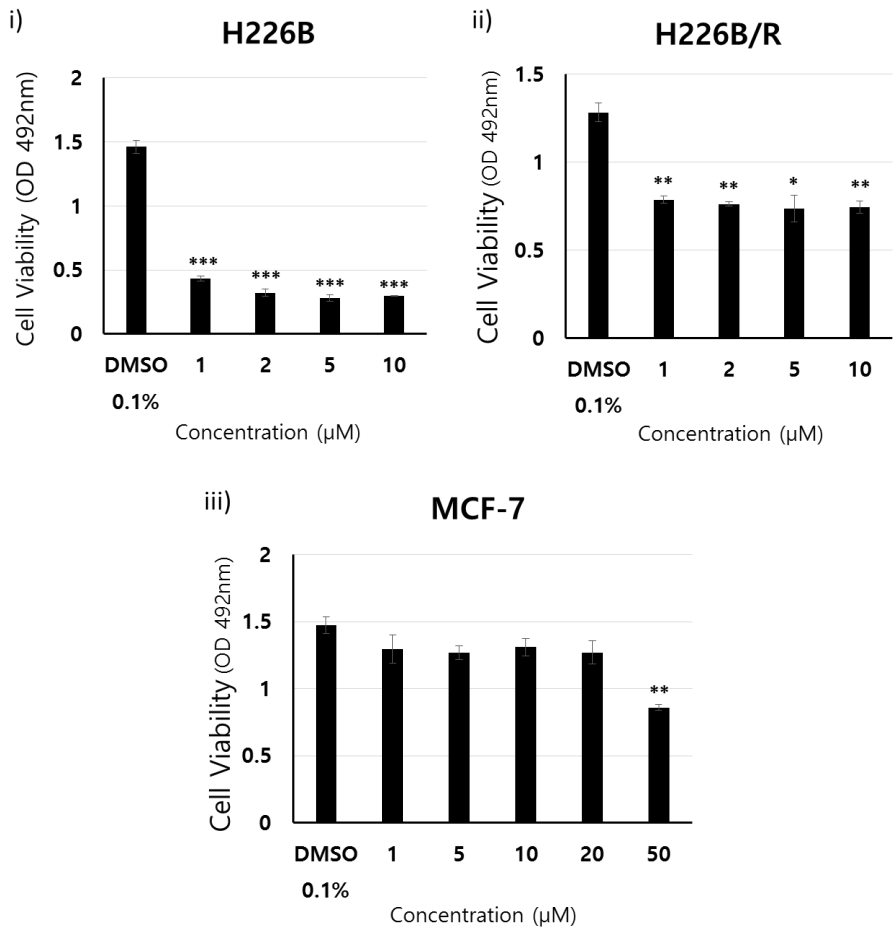


Figure 4. Evaluation of anti-proliferative activity by SH-174 against several other cancer cell lines

SH-174 with various concentrations indicated was treated to each cell line for 72 hours and then the viability of each cell lines was assessed by MTT assay. Each cell line represents EGFR-propagated H226 (H226B), paclitaxel-resistant H226B (H226B/R), malignant breast cancer cell (MCF-7). Mean ± SD; n=3, 4; paired Student's t test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

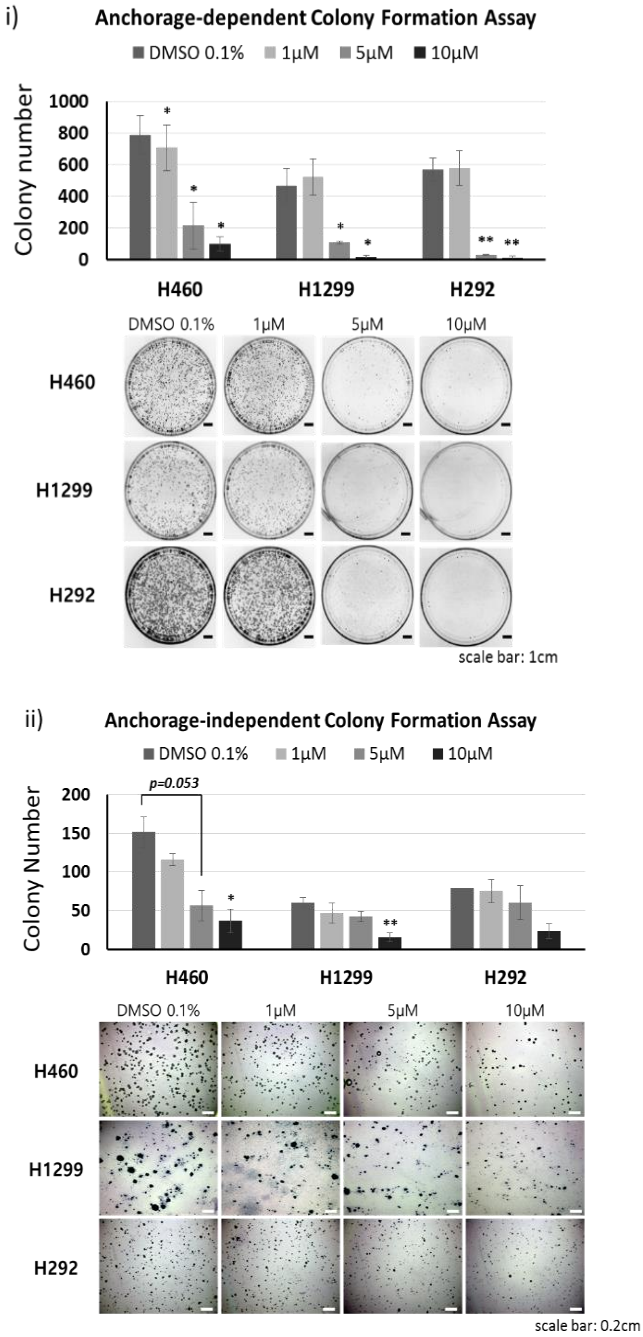


Figure 5. SH-174 reduces the colony-forming ability of NSCLC cells

i) Cells cultured on normal plastic dishes were treated with SH-174 or DMSO 0.1%. 8-9 days later, the number of colony was assessed by ImageJ software.

ii) Cells cultured on soft agar were treated with SH-174 or DMSO 0.1%. 8-21 days later, the number of colony was assessed by ImageJ software. In case of H292, two of DMSO 0.1%-treated dishes were lost during preparation so that they were excluded from the statistical analysis.

The images are representatives of 3 independent wells. Mean ± SD; n=3; paired Student's t test; $*p < 0.05$, $**p < 0.01$

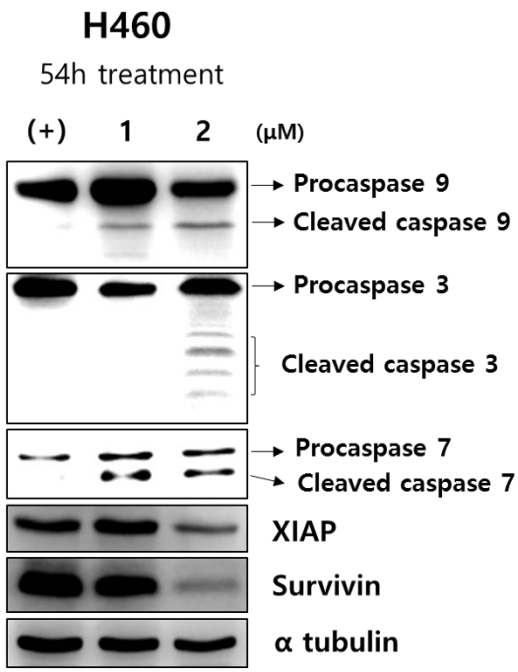


Figure 6. SH-174 induces apoptosis in H460 cells

H460 cells were treated with various indicated concentrations of SH-174 for 54 hours and several apoptosis molecules were assessed by western blot assay. The image is a representative of 2 independent experiments; (+) is DMSO 0.1%.

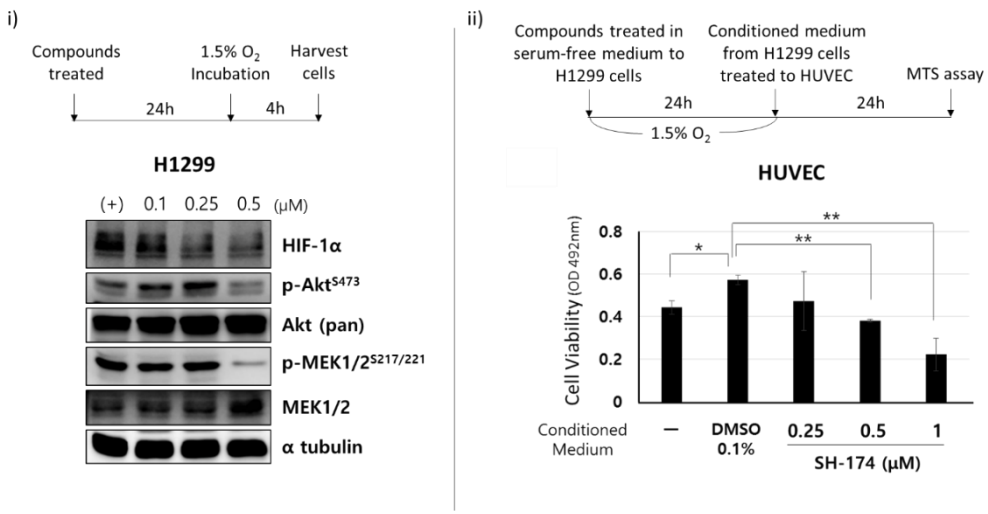


Figure 7. SH-174 reduces survival signaling of H1299 cells under hypoxic conditions

i) Several molecules of SH-174-treated H1299 cells were assessed by western blot assay under hypoxic conditions. The image is a representative of 2 independent experiments; (+) is DMSO 0.1%.

ii) Conditioned medium from SH-174-treated H1299 cells were treated to HUVEC and the viability of HUVEC was assessed by MTT assay. Mean ± SD; n=3; paired Student's t test; $*p < 0.05$, $**p < 0.01$

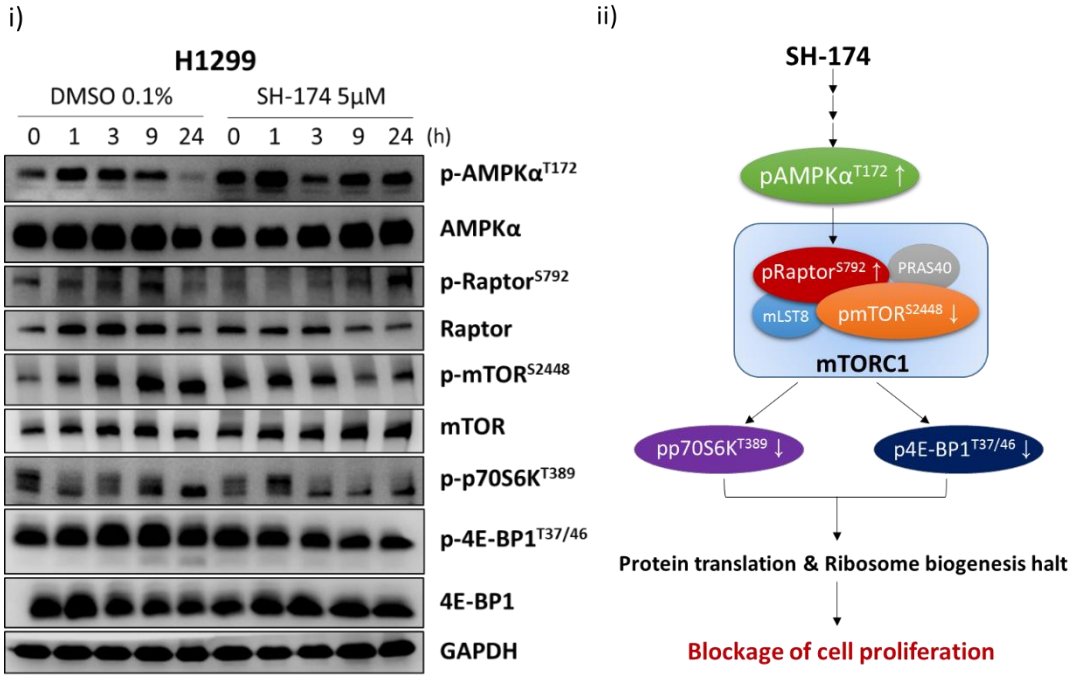


Figure 8. SH-174 modulates AMPK-mTORC1 signaling pathway in H1299 cells

i) H1299 cells were treated with SH-174 5 μM or DMSO 0.1% and the whole-cell lysates were subject to western blot assay at various timepoints. Harvesting cells right after the treatment of compounds, which took less than 10 seconds, was regarded as 0 hour. The image is a representative of 3 independent experiments.

ii) Summary of figure 8i).

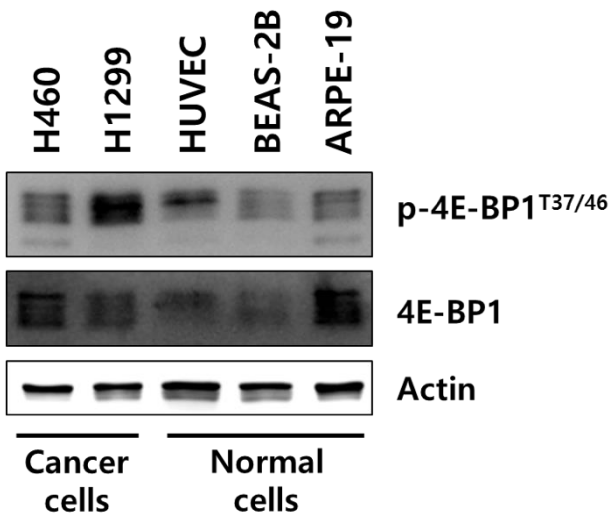


Figure 9. Correlation between the phosphorylation level of 4E-BP1 and the inhibitory effects of SH-174 on cellular viability

Each type of cells was cultured to reach 80% of its confluency, harvested, and subject to western blot analysis.

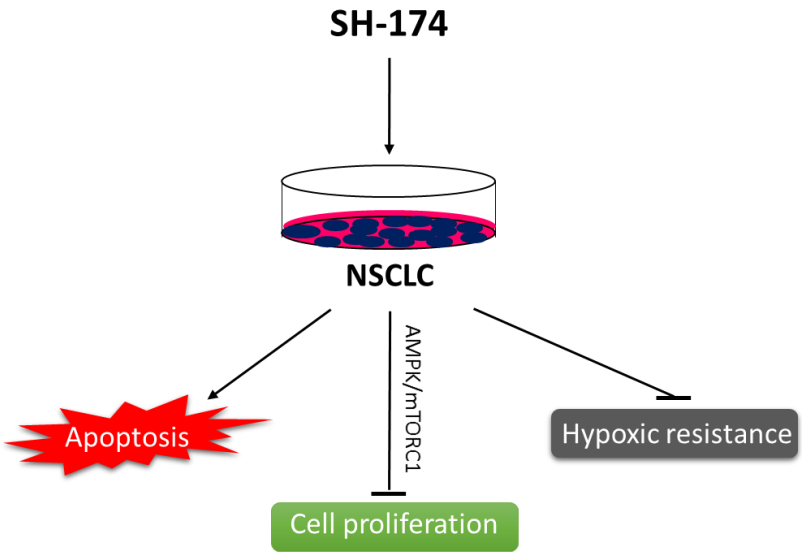


Figure 10. Summary of anti-cancer activities by SH-174 against NSCLC cells

Chapter 4. Discussion

Lung cancer was merely a reportable disease a hundred years ago, however, now being the number one cause of cancer-related deaths all over the world[1]. The naturally occuring rotenoid, deguelin, has been studied to have diverse anti-cancer activity[9-20, 26, 27] (e.g. inducing apoptosis, inhibiting tumor angiogenesis, perturbing the quality control of oncogenic protein by inhibiting HSP90 chaperone activity, etc.), however, its neurotoxicity which gives rise to Parkinson-like symptoms in rat and cytotoxicity to normal cells were obstacles to be developed as a clinically available drug[21, 23]. In this study, I examined the possible candidate derivatives of deguelin that could be developed as a new agent for anti-NSCLC therapy. Of those, SH-174 was identified as a promising anti-cancer agent against NSCLC cells in this study. Several NSCLC cell lines were treated with SH-174 and subject to MTT assay, immunoblot assay and colony formation assay; not only did it show a strong inhibitory effect on the viability of the cancer cells with a little or no cytotoxicity to normal cells (i.e. BEAS-2B, ARPE-19, and HUVEC), but also it inhibited the growth and survival of cancer cells and induced the apoptosis.

Owing to significant anti-cancer activity by rapamycin, mTOR, the mammalian target of rapamycin, has been an attractive target for anti-cancer therapy[37-40]. Among several pathways to control the activity of mTOR complexes, AMPK has been examined as a regulator of mTORC1 activity in this study.

AMPK has been thought to be one of the tumor suppressors because diabetic patients who had been under the long-term

prescription of metformin, known to be an AMPK agonist, had rarely cancers in their late years of lifetime[47]. However, there are several rebuttals by some of the recent researches that it is an oncogenic protein indeed[48-50], while others suggest that it is a tumor suppressor in diverse circumstances[51-57], which triggered the controversy on the role of AMPK in cancer[58-63].

In this study, SH-174 induced the phosphorylation of AMPKα at T172 site by unknown mechanism. One explanation could be SH-174 reduces the cellular ATP level so that AMPKα^{T172} is phosphorylated by increased AMP:ATP ratio. It is noteworthy that the onset of action of SH-174 is after 48 hours from its initial treatment to cause cell death, in contrast to other typical anti-cancer agents (e.g. geldanamycin) with their immediate action on causing cell death. In this vein, there could be several possible explanations for its delayed onset on cell death. One is that SH-174 needs to be metabolized to be 'activated' to exhibit its anti-cancer activity. Another is that, if my presumption that SH-174 decreases the cellular ATP level is true, it causes crisis on management of energy metabolism in cancer cells, leading to cell deaths, which would take relatively longer time until the nutrients in medium is depleted. Lastly, there is a chance that the MTT assay does not truly reflect the onset of cell deaths since it only shows the summation of cell viability with mixed status of dead, alive but cell cycle arrested, or normal proliferative ones in a single well. Given that SH-174 induced cell cycle arrest at the stage of G1/S progression in H1299 cells when treated for 24 hours (data not shown), the first explanation can be dismissed. In addition, provided that SH-174 activated AMPK between 9 and 24 hours from its treatment (Fig. 8), it could be speculated that its onset on challenging the management of cellular signaling modulation is

thought to be at least after 9 hours from being treated.

Another possible mechanism of inducing AMPKα^{T172} phosphorylation by SH-174 is modulating its upstream kinases: LKB1, TAK1, and CaMKKβ. Among them, LKB1 is best known for its tumor suppressor function as well as one to phosphorylate AMPKα^{T172} directly. Thus, I checked indirectly if LKB1 is a bona fide target of SH-174 by comparing two different cell lines: H1299 (LKB1 wildtype) and H460 (LKB1 null) (data not shown). As a result, SH-174 induced marginal phosphorylation of AMPK in H460 cells while clearly dephosphorylated 4E-BP1$^{T37/46}$, one of the downstream target of mTORC1. Therefore, it can't be excluded that the direct target of SH-174 is neither AMPK nor LKB1.

SH-174 has a relatively high IC_{50} value on H1299 cells compared to that of deguelin, as reported[64]: 0.44 μM (SH-174) versus 0.11 μM (deguelin) with 72-hour treatment. However, it had a significantly lower cytotoxicity on several human normal cells than deguelin. This could be due to the different phosphorylation level of 4E-BP1 in each cell line as mentioned in Results 3-5.

Another interesting point is the type of cell death induced by SH-174. In this study, apoptosis is shown to be involved in it, but other types of cell death, at least caspase-independent apoptosis, must be involved, regarding that the viability of NSCLC cells was little to be rescued by pan-caspase inhibitor, z-VAD-fmk (20 μM, 72 h, data not shown). Normally, when cells are under deadly circumstances, it is unlikely that the death process is performed by a single type of cell death. Rather, it would be mixed one with a variety of mechanisms[84]. In this vein, I also investigated that if autophagy is involved in it, however I could not detect any difference on LC3B-I/II ratio between control and subject groups. Therefore, other than

autophagy would be involved in the type of cell death induced by SH-174.

In addition to the cell death type induced by SH-174, the last point to be considered is the fact that only H460 cell line is used as a proof of apoptosis induced by SH-174 (Fig. 6), despite the efficacy of SH-174 on more than 3 different NSCLC cell lines (Fig. 3 and Fig. 4). As a matter of fact, H1299 cells were also subject to western blot assay to detect apoptotic markers in various conditions. Nonetheless, it failed to detect the cleaved forms of effector caspase, neither 3 nor 7, the definitive evidence of apoptosis (data not shown). Given that H1299 is the only cell line with $p53^{-/-}$ among the NSCLC cell lines used in this study, it could be said that H1299 is more resistant to apoptosis induction than other cell lines. However, SH-174 had relatively similar IC_{50} values on each 3 NSCLC cell lines as discussed above. This fact also corroborates my presumption that different types of cell deaths other than caspase-dependent apoptosis must be involved in the mode of anti-cancer action by SH-174.

All in all, SH-174 is a promising candidate as an anti-NSCLC agent and encouraged to be studied further *in vivo* using animal models.

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국 문 초 록

새로운 Deguelin 합성 유도체인 SH-174의 비소세포성 폐암세포에대한 항암 활성 연구

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천연에서 얻어지는 rotenoid 계열의 화합물인 deguelin은 과거 십여년이 넘는 기간 동안 여러가지 암에 대해 강력한 항암 효과가 있음이 입증되어왔다. 그러나 치료용량에서 관찰되는 쥐에서의 신경독성, 정상조직에의 세포독성, 그리고 가시광선에 의해 쉽게 분해되는 광분해성 때문에 실제 임상에서 쓰이는 약으로의 개발이 어려웠다. 본 연구에서는 이러한 deguelin의 약점을 극복하고자, 항암 효과는 유지하면서 정상세포에 대한 독성이 줄어든 합성 유도체를 발굴하는 실험을 하였다. 본 연구를 통해 동정한 SH-174는 deguelin의 합성 유도체로서, 비소세포성 폐암세포 (H1299, H460, H292, H226B)에 다소 특이적으로 강력한 항암 효과를 갖고 있음이 관찰되었다. 또한, SH-174는 사람의 정상 폐의 상피세포 (BEAS-2B), 탯줄정맥 내피세포 (HUVEC)에서는 세포 독성을 일으키지 않고 망막색소 상피세포에서는 세포 활성에 대해 20% 미만의 독성을 일으켰다. 이는 암세포에 일으키는 독성의 1/8 수준으로 치료용

량에서의 암세포 선택성이 뛰어나며, 합성 모체인 deguelin과 비교했을 때도 정상 세포에의 그 독성이 현저히 낮다. 이와 같은 항암 활성은 SH-174가 처리된 비소세포성 폐암세포의 세포 활성, 군락형성능력, 세포자살사 여부의 관찰을 통해 발견되었다. 또한, 본 연구에서는 SH-174가 저산소 상태인 종양미세환경에서 암세포의 생존과 적응을 돕는 신호전달을 차단함으로써 항암 효과를 나타낸다는 것을 기전으로 제시하였다. 이는 암세포의 Akt, AMPK-mTORC1, MEK1/2의 신호전달경로를 억제함으로써 나타나는 것임을 밝혔다. 따라서, SH-174는 비소세포성 폐암치료에 촉망되는 신규 화합물로서 추후 개체 수준에서의 연구가 장려된다.

주요어: 비소세포성 폐암세포, deguelin, 항암제, 세포자살사, AMPK-mTORC1

학 번: 2012-22840

약학석사 학위논문

A Novel Synthetic Derivative of Deguelin, SH-174, Exhibits Anti-Cancer Activities Against Non-small-cell Lung Cancer Cells

새로운 deguelin 합성 유도체인 SH-174의 비소세포성 폐암세포에서의 항암 활성 연구

2015 년 12 월

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김 준 용

Abstract

The identification of a novel anti-cancer agent against non-small-cell lung cancer cells

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The naturally occurring rotenoid, deguelin, has been studied to have a strong anti-cancer efficacy in various types of cancer for more than a decade. However, its intolerable toxicity at therapeutic dose and light-labile physical property deterred its development to be a clinical drug of use. Here, I report that SH-174, a novel synthetic derivative of deguelin, has a strong anti-cancer activity against several non-small-cell lung cancer (NSCLC) cells while having no or a less toxicity to human non-cancerous normal cells (e.g. BEAS-2B, ARPE-19, and HUVEC) than deguelin has. Several NSCLC cell lines were treated with or without SH-174 and subject to MTT assay, colony formation assay, and apoptosis assay; all of which treated with SH-174 exhibited significant inhibited results on the proliferation

and survival of those cancer cells. Moreover, SH-174 reduced the ability of cancer cells to adapt to and survive under hypoxic cancer microenvironment by downregulating phosphorylation of Akt and MEK1/2, as well as HIF-1α protein level. To elucidate the target of oncogenic signaling in NSCLC cells by SH-174, I examined the AMPK-mTORC1 signaling axis which is harnessed by cancers for their rapid proliferation and survival. As a result, SH-174 induced the phosphorylation of AMPKα at Thr172, resulting in the inhibition of mTORC1 activity on cellular proliferation. This suggests SH-174 blocks the unchecked proliferation of cancer cells by modulating AMPK-mTORC1 signaling pathway. Thus, SH-174 is a new promising candidate for lung cancer therapy and further *in vivo* studies in animals are warranted.

Keyword: non-small-cell lung cancer (NSCLC), deguelin, anti-cancer drug, apoptosis, mTORC1, AMPK, hypoxia

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Table of Contents

Chapter 1. Introduction

1-1. Study Background

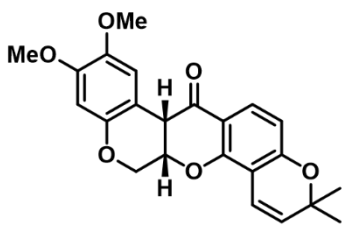
Among all human cancers, lung cancer is the most prevalent one in the world and the primary cause of cancer-related deaths worldwide[1]. It has the highest mortality among all types of human cancers, almost 1.8 million people are diagnosed and 1.6 million die every year owing to lung cancer[1]. Based on the histopathological characteristics, lung cancers are divided into two categories; small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC), the latter comprising more than 80% of total lung cancer with only 15.9% of 5-year survival rate[2]. NSCLC is divided into 3 major categories by histology: adenocarcinoma (ADC), squamous cell carcinoma (SCC), and large cell carcinoma. Among these, ADC and SCC comprises up to 90% of total NSCLC[3-4]. ADC arises relatively in distal airways, while SCC does in proximal ones lining trachea and upper airways[3-4]. When it comes to large cell carcinoma, which is diagnosed when the carcinoma is not thought to be ADC or SCC, it is not known whether the genetic backgrounds of large cell carcinoma is different from those of ADC or SCC[4]. In spite of acknowledging the fatality of lung cancer to people for more than a half century and vigorous efforts to develop effective medications, people are still suffering from lung cancer owing to its high rate of recurrence[5-6]. In addition, the extreme heterogeneity of genetic backgrounds of NSCLC makes it more intractable disease[7]. Therefore, the pursuit of diversity of anti-cancer agents is in urgent demand to deal with the extreme heterogeneity of NSCLC and its intransigence.

The natural products from various plants have been promising

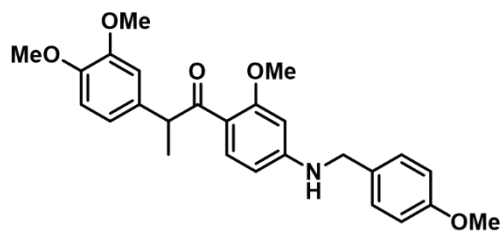
sources of drugs for human diseases, including cancer[8]. Deguelin, the naturally occurring rotenoid, has been shown to have a potent anti-cancer activity against several cancers arising from lung, liver, skin, prostate, breast, pancreas, and head and neck, etc., by us and other groups[9-20]. It has been reported that deguelin inhibits a variety of carcinogenic activity—angiogenesis, proliferation, survival, migration and EMT—while inducing apoptosis and autophagy of cancer cells[9-20]. However, its toxicity to normal cell lines *in vitro* and to animals' neurons *in vivo* kept it from being developed into a clinical drug for anti-cancer therapy[21, 23]. To address this issue, I had screened derivatives of deguelin synthesized by Prof. Suh Young-Ger's team (Seoul National University) to find one with a reduced toxicity while retaining its potent anti-cancer activity. Of those, SH-174 was chosen based on its inhibitory activity on the viability of H1299 NSCLC cells.

Here, I demonstrate that SH-174, a novel synthetic derivative of deguelin, has a strong anti-cancer activity against NSCLC cell lines including three major subtypes of NSCLC: adenocarcinoma (H1299), squamous cell carcinoma (H292) and large cell carcinoma (H460). It is found that apoptosis is involved in its mode of anti-cancer action. Moreover, SH-174 showed no or a less toxicity than deguelin to several human cell lines such as BEAS-2B, ARPE-19, and HUVEC. To elucidate the mechanism of anti-proliferative effect by SH-174, I investigated AMPK-mTORC1 signaling axis which is critical players for regulating ribosome biogenesis and protein synthesis for cancer cells to proliferate. As a result, SH-174 induced the phosphorylation of AMPKα^{T172}, causing downregulation of mTORC1 activity by directly phosphorylating Raptor^{S792}, which in turn making its two major downstream target dysfunctional: p70S6K and 4E-BP1.

Collectively, these findings thereby warranty the further *in vivo* animal study of SH-174 as a chemotherapeutic agent for anti-lung cancer therapy.



(-)-Deguelin



SH-174

The structure of (−)−Deguelin and its synthetic derivative, SH−174

1-2. Purpose of Study

Keeping abreast with the exigent demand of new drugs for chemotherapy of lung cancer, we and other groups have been searching for new chemotherapeutic agents that could aid to manage the NSCLC better. The natural compound deguelin has shown its promising efficacy as an agent for NSCLC treatment. This study aims to excavate a new agent succeeding to the efficacy of deguelin, while reducing the cytotoxicity of deguelin to normal cells.

Chapter 2. Materials and Methods

Cell Lines. ARPE-19 and the NSCLC cell lines—H1299, H292, and H460—were purchased from ATCC (Manassas, VA, USA) and H226B, H226B/R, HUVEC, ARPE-19, and BEAS-2B were kindly provided by Prof. Lee, Ho-Young (Seoul National University). All NSCLC cell lines were maintained in RPMI1640 medium (cat. CM059-050) from GenDEPOT (Katy, TX, USA). ARPE-19 cells were maintained in DMEM/F-12 (1:1) from Invitrogen (Carlsbad, CA, USA) and used in the experiment below the passage 30. BEAS-2B cells were maintained in Keratinocyte-SFM (cat. 17005-042) from Invitrogen supplemented with bovine pituitary extracts (50 μg/ml) and epidermal growth factor (5 ng/ml). The human breast cancer cell MCF-7 was kindly provided by Prof. Surh, Young-Joon (Seoul National University) and maintained in DMEM. The media for all cancer cells and ARPE-19 contain 10% heat-inactivated FBS (cat. F0600-050) and 1% antibiotics (cat. CA002-010) from GenDEPOT to their final volume. HUVEC was cultured on 0.15% gelatin-coated plastic dish and maintained in EGM-2 medium (cat. cc-3162) from Lonza (Walkersville, MD, USA). In the experiment, HUVEC with passage below 10 was used. All cells were incubated in 37℃, 5% CO_2 humidified chamber.

Reagents. Antibodies against caspase 9 (#9508), caspase 7 (#9494), XIAP (#2042), survivin (#2808), AMPKα (#5831), phospho-AMPKα (Thr172) (#2535) were from Cell Signaling Technology, Inc. (Danvers, MA, USA) Antibody against caspase 3 was from Cell Signaling Technology, Inc. (#9664) and Santa Cruz Biotechnology, Inc. (sc-56053, Dallas, Texas, USA) GAPDH antibody (sc-365062)

was from Santa Cruz Biotechnology, Inc. Tubulin α antibody (cat. MU121-UC) was from BioGenex (Fremont, CA, USA) and those against actin (cat. A2066) and vinculin (cat. V9131) were from Sigma-Aldrich (St. Louis, MO, USA). HIF-1α antibody was from BD Biosciences (#610959, San Jose, CA, USA). For MTT assay, MTS solution (cat. G3581) was purchased from Promega (Madison, WI, USA). For soft agar assay, low melting temperature agarose (cat. 50100) was purchased from Lonza (Walkersville, MD, USA). SH-174 and deguelin was synthesized from Prof. Suh, Young-Ger's lab. The drugs were prepared in 10~100mM stock in DMSO and treated not exceeding 0.1% (v/v) to its final volume diluted in the suitable medium for each cell line. Thus, 0.1% DMSO was used as a positive control for all experiments. Unless indicated, when treated to cells, the compounds were diluted in whole medium containing 10% FBS and 1% antibiotics.

Cell Viability Assay. To measure the cell viability, optimized cell number empirically 3×10^3 or 10^4 cells were seeded on 96-well plate. After 24 hours, drugs were treated to each well as indicated concentration in the fresh medium suitable for each cell line. After indicated amount of time, MTS solution was treated and the absorbance at 492nm was measured to assess the viability according to the manufacturer's protocol.

Anchorage-dependent Colony Formation Assay. 2×10^3 cancer cells as singlets were seeded on 10-cm culture dish, incubated for 24 hours, and then treated with indicated concentration of SH-174 in 12ml of fresh medium. The medium was not changed until the detection of colony. 7-8 days later, the previously published method[22] was slightly modified to detect the colonies. Briefly, the dish was washed with warm 1x PBS, fixed with methanol for 10 mins, and then

0.5% ethidium bromide in 50% ethanol was treated for 20 seconds. The dishes were drained and then subject to UV light by LAS-4000 (Fujifilm, Tokyo, Japan).

Soft Agar Colony Formation Assay. For the base layer, 0.8% agarose gel was layered on 12-well plate. After 3 hours for agarose gel to be fully solidified, 2×10^3 cancer cells as singlets in the whole medium containing 0.4% agarose were seeded. After another 3 hours for the solidification of upper agarose layer, the drug with indicated concentration diluted in the whole medium was treated. The medium containing drug was replaced with fresh one every 4 days to protect cells from being dried. 8-21 days later, the colonies were fixed with 4% PFA for 10 mins, stained with 0.05% crystal violet for 30 mins and washed out with current tap water. Then the colonies were counted under the light microscope. Colonies with the diameter exceeding 450 μm were counted using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Western blot assay. Cells were washed with ice-cold 1x PBS for once and the dish was drained and an adequate amount of lysis buffer (#9803, Cell Signaling Technology, Inc.) supplemented with phosphatase inhibitor cocktail B (cat. Sc-45045, Santa Cruz Biotechnology, Inc.) as well as protease inhibitor cocktail was treated. In case of apoptosis assay, floating cells were also collected and treated with lysis buffer. For HIF-1α, cells were lysed by high-salt buffer (10 mM HEPES pH 7.9, 1 mM EDTA, 400 mM NaCl, 5% Glycerol). Cells scraped off from the dish were centrifuged by 18,000 g for 30 mins at 4℃. Supernatant were collected and subject to BCA assay to quantify the amount of total proteins in cell lysates. Equal amount of proteins were subject to SDS-PAGE and transferred to a nitrocellulose membrane (cat. 10600004, GE Healthcare). The

membranes were blocked by 5% skim milk for 20 mins and incubated more than 16 hours with the appropriate primary antibodies at 4℃. The membranes were washed with TBS-T (0.1% Tween20), and incubated with secondary antibodies for 1 hour at room temperature. Unbound antibodies were washed off by TBS-T. To detect the bands, membranes were treated with enhanced chemiluminescence solution and subject to LAS-4000.

Hypoxia and Obtaining Conditioned Medium. Cells were seeded on plastic culture dish and incubated for 24 hours. When the confluency reached nearly 80%, SH-174 or DMSO 0.1% was treated for the next 24 hours, followed by 4 hour incubation under hypoxic (1.5% O_2, 5% CO_2, 93.5% N_2, 37℃) conditions. Cells were harvested at the end of hypoxic incubation and subject to western blot assay.

To examine the inhibitory effect of growth factors secretion, SH-174 or DMSO 0.1% diluted in serum-free medium was treated to cells with 80% confluency and incubated simultaneously under hypoxic conditions as above for 24 hours. Then, the medium in dish was collected and filtered by centrifugal filter units (Amicon Ultra, Cat. UFC800324, Merck Millipore, Darmstadt, Germany) under 3,000 g, 1 h, at 4℃. By the centrifugation, approximately 40-fold (v/v) concentrated conditioned medium (CM) was obtained with being filtered out with molecules larger than 3 kDa.

Statistical Analysis. Values were presented as the mean ± SD or SEM as indicated. Statistical significance was determined by Student's two-tailed paired t test. Values were regarded biologically significant when p value was less than 0.05 compared to control set; $*p<0.05$, $**p<0.01$, $***p<0.001$. To calculate IC_{50} value, Prism 6.0 (GraphPad Software, Inc, La Jolla, CA, USA) was used. All other statistical values were calculated by Excel (Microsoft,

Redmond, WA, USA).

Chapter 3. Results

3-1. SH-174 shows a potent anti-proliferative activity against NSCLC cells

The initial screening of 18 synthetic derivatives of deguelin by measuring the inhibitory effect on the viability of H1299 cells identified SH-174 as a promising candidate for anti-NSCLC agent (Fig. 1). H1299 cells were treated with 10 μM of each compound for 72 hours and the viability was measured by MTT assay (Fig. 1). Because the major problem of deguelin as an anti-cancer agent was the safety issue to normal cell, I also examined the effect of SH-174 on the viability of non-cancerous human cells such as ARPE-19 (retinal pigment epithelial cell), BEAS-2B (lung bronchial epithelial cell), and HUVEC (human umbilical vein endothelial cell). As a result, SH-174 exhibited no or if existed, a little toxicity to such cells with nearly 0 or 20% inhibition of the cell viability (Fig.2). Thus, I chose SH-174 as a strong candidate for the further anti-cancer study.

To investigate if SH-174 has such an efficacy on overall NSCLC cell lines, some of them including H1299 (adenocarcinoma cell), H460 (large cell carcinoma cell), and H292 (squamous cell carcinoma cell) were treated with various concentrations of SH-174 (0.1 μM, 0.5 μM, 1 μM, 5 μM and 10 μM) or DMSO 0.1% and the viability was measured by MTT assay every 24 hour up to 3 days. As a result, it showed a potent inhibitory effect on the viability of all subject cell lines with its IC_{50} values at 72 hour as follows: 0.44 μM for H1299, 0.40 μM for H460 and 0.37 μM for H292 (Fig. 3i)-iii)). The viability of H226B (EGFR-propagated H226) and its paclitaxel resistance-

acquiring subline, H226B/R[23] were also reduced by SH-174 (10 μM, 72 h, Fig. 4i)-ii)). To see whether this result could be applied to other type of cancer, breast cancer cell line MCF-7 was also subject to MTT assay. However, its viability was reduced marginally even at relatively high concentration (50 μM), indicating that SH-174 has a degree of selectivity on NSCLC cells (Fig. 4iii)).

The decrease of viability of NSCLC cells was in accordance with the result of colony formation assays on two different types of bottom: plastic dish and soft agar. H1299, H460, and H292 cells were seeded on plastic culture dish for cell culture, followed by the treatment of SH-174 (1 μM, 5 μM, 10 μM) for 8-9 days and the colonies with their diameter exceeding 450 μm were counted. As shown in the figure 5i), the number of colony was significantly decreased with the treatment of 5 μM and 10 μM of SH-174 for all 3 NSCLC cell lines. Those 3 types of NSCLC cells were also seeded on double-layered soft agar as singlets (see Materials and Methods for further experimental details), followed by the treatment of SH-174 as above. The number of colony was reduced as well, indicating that SH-174 has such an anti-cancer activity for both types of cancer growth: anchorage-independently as well as anchorage-dependently (Fig. 5ii)). Collectively, the data indicate that SH-174 inhibits the growth and survival of NSCLC cells with no or a less toxicity than deguelin to normal cells.

3-2. SH-174 induces apoptosis in H460 cells

Apoptosis has been known to be one of the major mechanisms of the anti-cancer activity[24]. Since deguelin was shown to induce apoptosis in several cancers, including lung cancer[9, 11, 16-18, 20], I examined whether apoptosis is involved in the anti-cancer activity of SH-174 as likely in that of deguelin. H460 cells were treated with SH-174 (1 μM, 2 μM, 5 μM, 10 μM) for various time longer than 48 hours of treatment, since the viability of H460 significantly plummeted only after 48 hours of treatment (Fig.3i)). Of those, at 54 hour after treatment, several apoptotic markers were observed by western blot assay (Fig. 6); when H460 cells were treated with SH-174 (2 μM, 54 h), cleaved forms of the effector caspase 3 and 7 and the initiator caspase 9 were detected while cleaved forms of caspase 8 were not detected (data not shown), indicating that SH-174 induces apoptosis through the cellular intrinsic pathway. In addition, XIAP and survivin, two of the Inhibitor Apoptosis Protein (IAP) family, were also downregulated in this conditions. Various timepoints between 48-72 hours, treated with 5 or 10 μM of SH-174 were also examined to detect apoptotic markers. However, any of cytoskeleton proteins generally used for loading controls as housekeeping genes (i.e. tubulin, actin) as well as any cleaved form of caspases was not detected by western blot assay, so that I excluded these data as a proof of inducing apoptosis by SH-174. Consequently, the data in figure 6 show that apoptosis is involved at least in part in the anti-cancer activity of SH-174 against H460 cells.

3-3. SH-174 reduces the ability of H1299 cells to adapt to and survive hypoxic conditions

Cancer microenvironment is nutrients and oxygen-restrictive[25, 32, 33] due to the competition between highly proliferative cancer cells and other stromal cells (e.g., immune cells[28-30]). This forces cancer cells to adapt to and survive under its hypoxic microenvironment by exploiting several molecules such as HIF-1α[25], Akt[31], and MEK1/2[34], etc. Thus, targeting these molecules of cancer to interfere with its adaptability could be a valid approach as a cancer therapy. For example, deguelin was reported as a prominent anti-angiogenic agent by reducing the protein level of HIF-1α[15, 17, 20, 27]. Moreover, deguelin was renowned for its negative effect on the phosphorylation of Akt[16, 26]. Thus, to investigate whether SH-174 has a negative effect on the stability of HIF-1α and phosphorylation level of Akt and MEK1/2 as likely as deguelin does. H1299 cells were treated with SH-174 or DMSO 0.1% for 24 hours, followed by the incubation under 1.5% O_2 for 4 hours and then subject to western blot assay. As a result, HIF-1α protein level was downregulated and the phosphorylation level of Akt^{S473} and $MEK1/2^{S217/221}$ was also diminished by SH-174 (Fig. 7i)).

Cancer cells can make their microenvironment advantageous to themselves. For example, they can secrete several factors to stimulate the proliferation of endothelial cells to give rise to angiogenesis to supply adequate amount of nutrients and oxygen[35, 36]. Since SH-174 downregulated HIF-1α, p-Akt^{S473}, and p-$MEK1/2^{S217/221}$ (Fig. 7i)), I hypothesized that SH-174 would decrease the ability of cancer cells to make their niche favorable to themselves, especially the ability to stimulate the proliferation of

endothelial cells to which HIF-1α is deeply related. To test my hypothesis, SH-174 was treated in serum-free medium to H1299 cells and simultaneously, incubated under 1.5% O_2 for 24 hours. Then, the medium was collected and filtered by centrifugation (3,000 g, 1 h, 4℃). This conditioned medium (CM) obtained from H1299 cells was treated to HUVEC (for details, see Materials and Methods). After 24-hour incubation, the viability of HUVEC was assessed by MTT assay. As shown in figure 7ii), the growth rate of HUVEC for 24 hours was decreased by the CM from SH-174-treated H1299 cells dose-dependently. This indicates that SH-174 decreased the ability of H1299 cells to secrete factors that could stimulate the proliferation of endothelial cells. Together, figure 7 shows that SH-174 reduces the ability of H1299 cells to survive and be accustomed to hypoxic conditions.

3-4. SH-174 modulates the AMPK-mTORC1 signaling pathway in H1299 cells

To elucidate the target attributed to the mode of anti-cancer activity by SH-174, I had checked if SH-174 inhibits the phosphorylation of Akt^{S473}, previously reported as one of the anti-cancer mechanism by deguelin[16, 26]. However, unlike under hypoxic conditions (Fig. 7i)), SH-174 neither reduced the phosphorylation level of Akt at Ser473 site nor altered the protein level of unphosphorylated Akt (pan) even at the lethal concentration (5 μM) within 24 hours (data not shown), unlikely does deguelin[26, 27]. Moreover, none of the HSP90 client protein levels, including PRMT5, ERK1/2, and EGFR was downregulated by SH-174 (5 μM, 24 h, data not shown), the proposed mode of action of deguelin[27]. Thus, I hypothesized different modes of action would be involved in its anti-cancer efficacy. This was plausible because the structure of SH-174 is quite different from its mother compound, deguelin (see Introduction).

The mammalian target of rapamycin (mTOR) has been revealed to regulate cell proliferation by controlling protein translation, ribosome biogenesis, autophagy, etc. for the past few decades[37]. Since the mTOR kinase is at the central position of cellular signal transduction network, it is one of the key molecules in cancer for its survival and proliferation, which makes it an attractive therapeutic target for cancer therapy[38-40]. There are two types of complex that mTOR kinase comprises: mTORC1 and mTORC2. Of those, mTORC1 is less dependent upon Akt than mTORC2[38]. Thus, being independent of Akt, I presumed that the inhibition of mTORC1 signal might be involved in the anti-cancer activity of SH-174.

Besides Akt, AMP-activated protein kinase (AMPK), which

regulates numerous cellular signaling related to energy metabolism, lipogenesis, autophagy, exercise etc.[41-46] has been revealed to control mTORC1 activity by direct phosphorylation of scaffold protein Raptor—a component of mTORC1—at serine 792[46]. Therefore, I examined whether SH-174 could modulate the activity of mTORC1 via the activation of AMPK.

To test my hypothesis above, H1299 cells were treated with SH-174 (5 μM) by time-dependent manner: 0, 1, 3, 9, 24 hours (Fig. 8). To exclude the effects that could be induced by growth factors in serum, cells were serum-starved for 24 hours and SH-174 or DMSO 0.1% was treated in serum-free medium. As a result shown in figure 8, 24-hour treatment of SH-174 induced the phosphorylation of AMPKα^{T172}, in turn directly phosphorylating Raptor^{S792} and causing dephosphorylation of p-mTOR^{S2448}, which made mTORC1 dysfunctional. Consequently, the phosphorylation level of 4E-BP1 and p70S6K—two downstream targets of mTORC1—was decreased, which would cause halt of ribosome biogenesis, protein translation and in the end, blockage of cell proliferation. This indicates that the modulation of AMPK-mTORC1 signaling axis is involved in the anti-cancer effect by SH-174.

3-5. Phosphorylation level of 4E-BP1 might dictate the inhibitory response of cells by SH-174

It has been known that cancer relies on a very few driver genes, or oncogenes, for its carcinogenesis, called oncogene addiction[65-67]. One of such oncogenes in lung cancer is eIF4E[68-70]. eIF4E serves as the rate-limiting factor in eIF4F complex formation, which regulates the cap-dependent mRNA translation in eukaryotic cell, so as to control numerous protein expression level[68-70]. Moreover, eIF4E dose is critical especially in carcinogenesis, unlike in normal tissue development[71]. Thus, restraining the eIF4E availability is a reasonable strategy in cancer treatment[72-74]. 4E-BP1, one of the mTORC1 downstream targets, regulates eIF4E availability by, when phosphorylated, releasing eIF4E from its complex[75-77]. This makes one to speculate that inhibition of 4E-BP1 phosphorylation could be a therapeutic target for cancer treatment. As a matter of fact, it has been reported that the phosphorylation level of 4E-BP1 is correlated with the prognosis of several cancer[78, 79], so as to be an oncotarget[80] in that it is a key effector of the oncogenic activation[81], as well as it predicts the sensitivity to anti-cancer agent of cancer cells[82, 83].

Figure 9 shows the phosphorylation level of 4E-BP1 is higher in cancer cells than normal cells. In line with this, the viability of cells with high 4E-BP1 phosphorylation level is more vulnerable to SH-174 (Fig. 2, 3, 9). Given that SH-174 abrogates the phosphorylation of 4E-BP1 via mTORC1 inhibition, it could be reasoned that cells with higher p-4E-BP1 level would have more damage on cellular proliferation. In this vein, 4E-BP1 phosphorylation level could explain the strong toxicity selectively on cancer cells by SH-174.

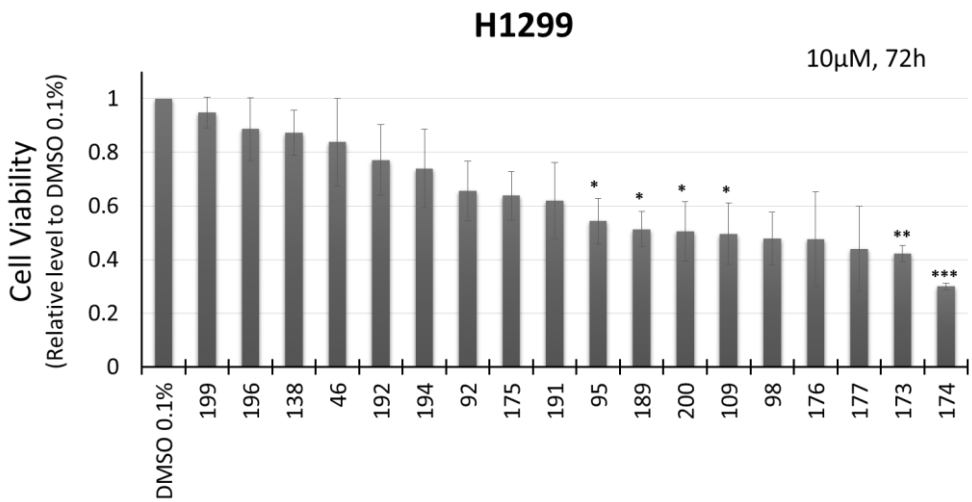


Figure 1. The identification of SH-174 as a promising candidate for anti-cancer agent against NSCLC cell

Inhibitory activities on the viability of H1299 cells by a series of synthetic derivatives of deguelin. Compounds were treated for 72 hours with 10μM concentration. The viability was assessed by MTS assay with 3 independent experiments as triplicates for one experiment. Mean ± SEM; n=3; paired Student's t test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

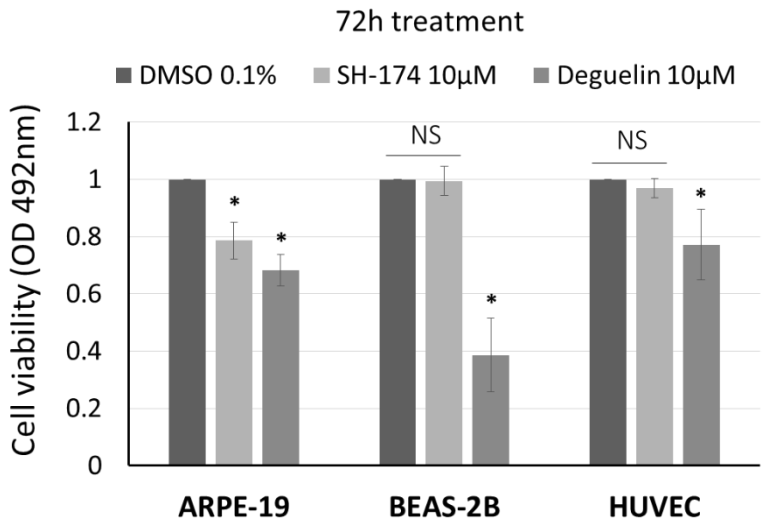


Figure 2. SH-174 has no or a less toxicity than deguelin to several non-cancerous human cell lines

The result of MTT assay on several human cell lines. Cells were treated with DMSO 0.1% or SH-174 or deguelin for 72 hours. Mean ± SD; n=3, 4; paired Student's t test; $*p < 0.05$

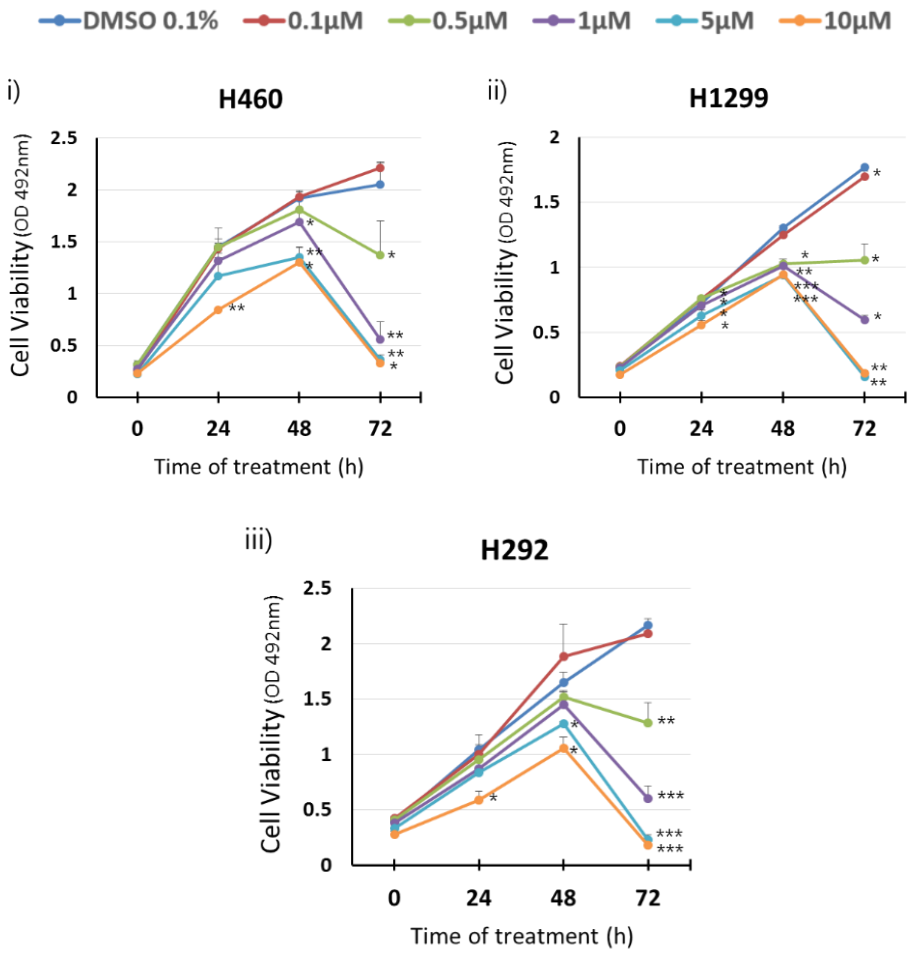


Figure 3. SH−174 exhibits a potent inhibitory activity on the viability of NSCLC cell lines

3 NSCLC cell lines were subject to MTT assay, treated with various concentrations indicated of SH−174 every 24 hour. Each cell line represents large cell carcinoma cell (H460), adenocarcinoma (H1299), and squamous cell carcinoma cell (H292). Mean ± SD; n=3, 4; paired Student's t test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

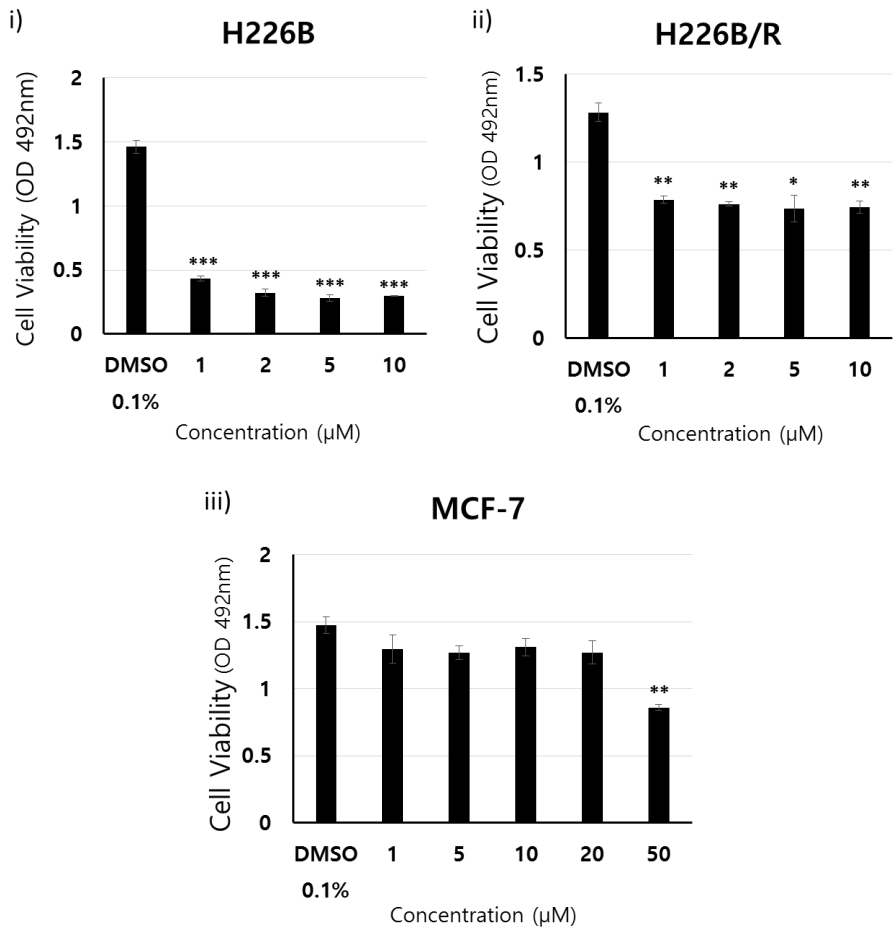


Figure 4. Evaluation of anti-proliferative activity by SH-174 against several other cancer cell lines

SH-174 with various concentrations indicated was treated to each cell line for 72 hours and then the viability of each cell lines was assessed by MTT assay. Each cell line represents EGFR-propagated H226 (H226B), paclitaxel-resistant H226B (H226B/R), malignant breast cancer cell (MCF-7). Mean ± SD; n=3, 4; paired Student's t test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

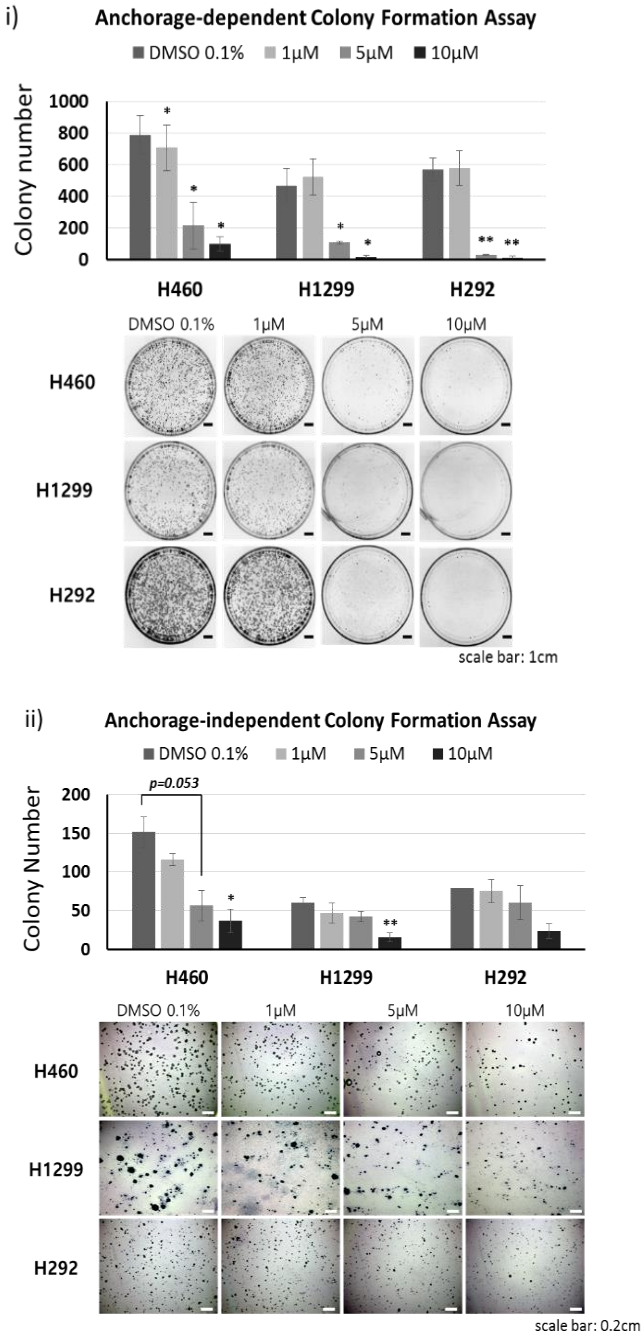


Figure 5. SH-174 reduces the colony-forming ability of NSCLC cells

i) Cells cultured on normal plastic dishes were treated with SH-174 or DMSO 0.1%. 8-9 days later, the number of colony was assessed by ImageJ software.

ii) Cells cultured on soft agar were treated with SH-174 or DMSO 0.1%. 8-21 days later, the number of colony was assessed by ImageJ software. In case of H292, two of DMSO 0.1%-treated dishes were lost during preparation so that they were excluded from the statistical analysis.

The images are representatives of 3 independent wells. Mean ± SD; n=3; paired Student's t test; $*p < 0.05$, $**p < 0.01$

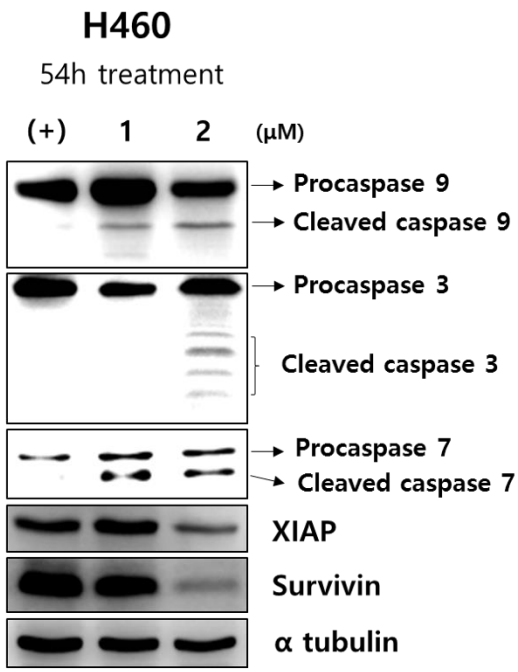


Figure 6. SH-174 induces apoptosis in H460 cells

H460 cells were treated with various indicated concentrations of SH-174 for 54 hours and several apoptosis molecules were assessed by western blot assay. The image is a representative of 2 independent experiments; (+) is DMSO 0.1%.

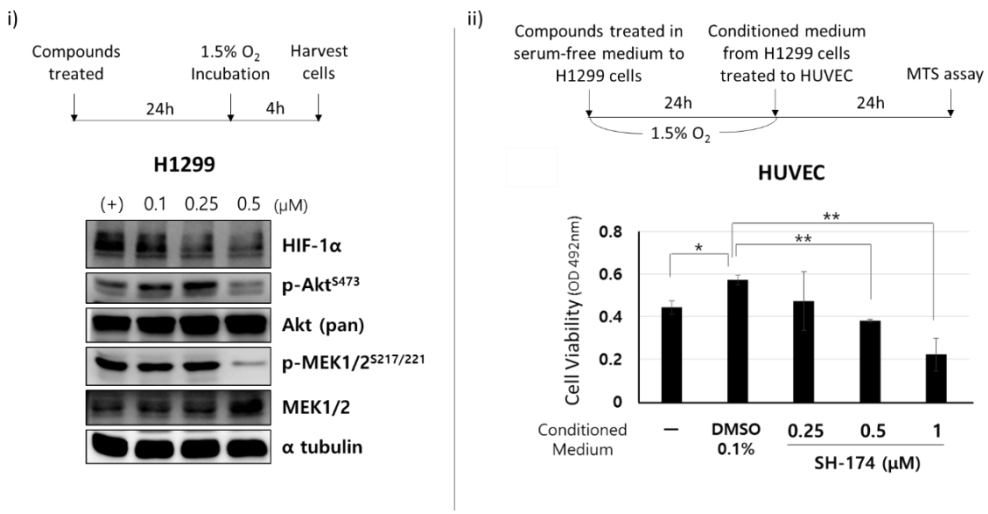


Figure 7. SH-174 reduces survival signaling of H1299 cells under hypoxic conditions

i) Several molecules of SH-174-treated H1299 cells were assessed by western blot assay under hypoxic conditions. The image is a representative of 2 independent experiments; (+) is DMSO 0.1%.

ii) Conditioned medium from SH-174-treated H1299 cells were treated to HUVEC and the viability of HUVEC was assessed by MTT assay. Mean ± SD; n=3; paired Student's t test; $*p < 0.05$, $**p < 0.01$

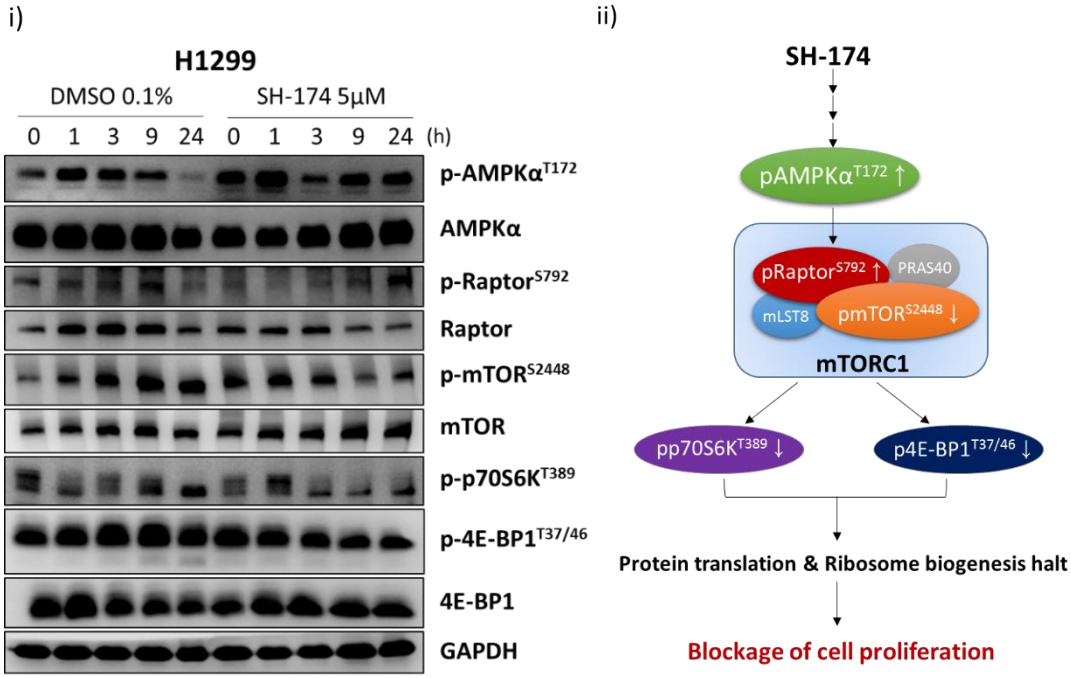


Figure 8. SH-174 modulates AMPK-mTORC1 signaling pathway in H1299 cells

i) H1299 cells were treated with SH-174 5 μM or DMSO 0.1% and the whole-cell lysates were subject to western blot assay at various timepoints. Harvesting cells right after the treatment of compounds, which took less than 10 seconds, was regarded as 0 hour. The image is a representative of 3 independent experiments.

ii) Summary of figure 8i).

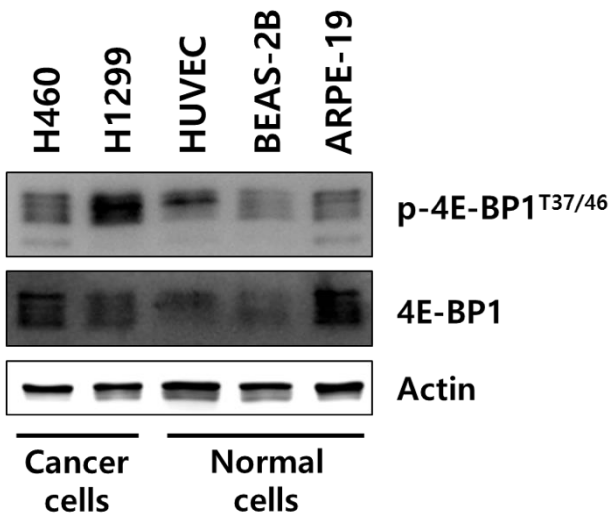


Figure 9. Correlation between the phosphorylation level of 4E-BP1 and the inhibitory effects of SH-174 on cellular viability

Each type of cells was cultured to reach 80% of its confluency, harvested, and subject to western blot analysis.

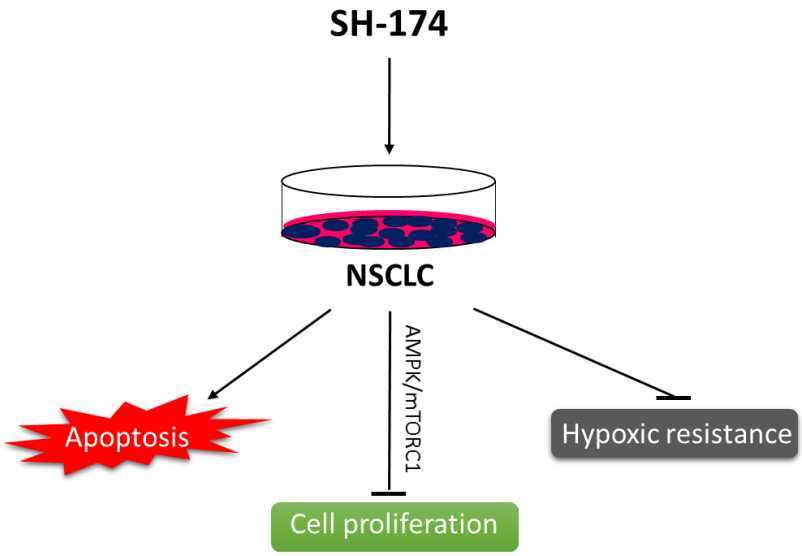


Figure 10. Summary of anti-cancer activities by SH-174 against NSCLC cells

Chapter 4. Discussion

Lung cancer was merely a reportable disease a hundred years ago, however, now being the number one cause of cancer-related deaths all over the world[1]. The naturally occuring rotenoid, deguelin, has been studied to have diverse anti-cancer activity[9-20, 26, 27] (e.g. inducing apoptosis, inhibiting tumor angiogenesis, perturbing the quality control of oncogenic protein by inhibiting HSP90 chaperone activity, etc.), however, its neurotoxicity which gives rise to Parkinson-like symptoms in rat and cytotoxicity to normal cells were obstacles to be developed as a clinically available drug[21, 23]. In this study, I examined the possible candidate derivatives of deguelin that could be developed as a new agent for anti-NSCLC therapy. Of those, SH-174 was identified as a promising anti-cancer agent against NSCLC cells in this study. Several NSCLC cell lines were treated with SH-174 and subject to MTT assay, immunoblot assay and colony formation assay; not only did it show a strong inhibitory effect on the viability of the cancer cells with a little or no cytotoxicity to normal cells (i.e. BEAS-2B, ARPE-19, and HUVEC), but also it inhibited the growth and survival of cancer cells and induced the apoptosis.

Owing to significant anti-cancer activity by rapamycin, mTOR, the mammalian target of rapamycin, has been an attractive target for anti-cancer therapy[37-40]. Among several pathways to control the activity of mTOR complexes, AMPK has been examined as a regulator of mTORC1 activity in this study.

AMPK has been thought to be one of the tumor suppressors because diabetic patients who had been under the long-term

prescription of metformin, known to be an AMPK agonist, had rarely cancers in their late years of lifetime[47]. However, there are several rebuttals by some of the recent researches that it is an oncogenic protein indeed[48-50], while others suggest that it is a tumor suppressor in diverse circumstances[51-57], which triggered the controversy on the role of AMPK in cancer[58-63].

In this study, SH-174 induced the phosphorylation of AMPKα at T172 site by unknown mechanism. One explanation could be SH-174 reduces the cellular ATP level so that AMPKα^{T172} is phosphorylated by increased AMP:ATP ratio. It is noteworthy that the onset of action of SH-174 is after 48 hours from its initial treatment to cause cell death, in contrast to other typical anti-cancer agents (e.g. geldanamycin) with their immediate action on causing cell death. In this vein, there could be several possible explanations for its delayed onset on cell death. One is that SH-174 needs to be metabolized to be 'activated' to exhibit its anti-cancer activity. Another is that, if my presumption that SH-174 decreases the cellular ATP level is true, it causes crisis on management of energy metabolism in cancer cells, leading to cell deaths, which would take relatively longer time until the nutrients in medium is depleted. Lastly, there is a chance that the MTT assay does not truly reflect the onset of cell deaths since it only shows the summation of cell viability with mixed status of dead, alive but cell cycle arrested, or normal proliferative ones in a single well. Given that SH-174 induced cell cycle arrest at the stage of G1/S progression in H1299 cells when treated for 24 hours (data not shown), the first explanation can be dismissed. In addition, provided that SH-174 activated AMPK between 9 and 24 hours from its treatment (Fig. 8), it could be speculated that its onset on challenging the management of cellular signaling modulation is

thought to be at least after 9 hours from being treated.

Another possible mechanism of inducing AMPKα^{T172} phosphorylation by SH-174 is modulating its upstream kinases: LKB1, TAK1, and CaMKKβ. Among them, LKB1 is best known for its tumor suppressor function as well as one to phosphorylate AMPKα^{T172} directly. Thus, I checked indirectly if LKB1 is a bona fide target of SH-174 by comparing two different cell lines: H1299 (LKB1 wildtype) and H460 (LKB1 null) (data not shown). As a result, SH-174 induced marginal phosphorylation of AMPK in H460 cells while clearly dephosphorylated 4E-BP1$^{T37/46}$, one of the downstream target of mTORC1. Therefore, it can't be excluded that the direct target of SH-174 is neither AMPK nor LKB1.

SH-174 has a relatively high IC_{50} value on H1299 cells compared to that of deguelin, as reported[64]: 0.44 μM (SH-174) versus 0.11 μM (deguelin) with 72-hour treatment. However, it had a significantly lower cytotoxicity on several human normal cells than deguelin. This could be due to the different phosphorylation level of 4E-BP1 in each cell line as mentioned in Results 3-5.

Another interesting point is the type of cell death induced by SH-174. In this study, apoptosis is shown to be involved in it, but other types of cell death, at least caspase-independent apoptosis, must be involved, regarding that the viability of NSCLC cells was little to be rescued by pan-caspase inhibitor, z-VAD-fmk (20 μM, 72 h, data not shown). Normally, when cells are under deadly circumstances, it is unlikely that the death process is performed by a single type of cell death. Rather, it would be mixed one with a variety of mechanisms[84]. In this vein, I also investigated that if autophagy is involved in it, however I could not detect any difference on LC3B-I/II ratio between control and subject groups. Therefore, other than

autophagy would be involved in the type of cell death induced by SH-174.

In addition to the cell death type induced by SH-174, the last point to be considered is the fact that only H460 cell line is used as a proof of apoptosis induced by SH-174 (Fig. 6), despite the efficacy of SH-174 on more than 3 different NSCLC cell lines (Fig. 3 and Fig. 4). As a matter of fact, H1299 cells were also subject to western blot assay to detect apoptotic markers in various conditions. Nonetheless, it failed to detect the cleaved forms of effector caspase, neither 3 nor 7, the definitive evidence of apoptosis (data not shown). Given that H1299 is the only cell line with $p53^{-/-}$ among the NSCLC cell lines used in this study, it could be said that H1299 is more resistant to apoptosis induction than other cell lines. However, SH-174 had relatively similar IC_{50} values on each 3 NSCLC cell lines as discussed above. This fact also corroborates my presumption that different types of cell deaths other than caspase-dependent apoptosis must be involved in the mode of anti-cancer action by SH-174.

All in all, SH-174 is a promising candidate as an anti-NSCLC agent and encouraged to be studied further *in vivo* using animal models.

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국 문 초 록

새로운 Deguelin 합성 유도체인 SH-174의 비소세포성 폐암세포에대한 항암 활성 연구

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천연에서 얻어지는 rotenoid 계열의 화합물인 deguelin은 과거 십여년이 넘는 기간 동안 여러가지 암에 대해 강력한 항암 효과가 있음이 입증되어왔다. 그러나 치료용량에서 관찰되는 쥐에서의 신경독성, 정상조직에의 세포독성, 그리고 가시광선에 의해 쉽게 분해되는 광분해성 때문에 실제 임상에서 쓰이는 약으로의 개발이 어려웠다. 본 연구에서는 이러한 deguelin의 약점을 극복하고자, 항암 효과는 유지하면서 정상세포에 대한 독성이 줄어든 합성 유도체를 발굴하는 실험을 하였다. 본 연구를 통해 동정한 SH-174는 deguelin의 합성 유도체로서, 비소세포성 폐암세포 (H1299, H460, H292, H226B)에 다소 특이적으로 강력한 항암 효과를 갖고 있음이 관찰되었다. 또한, SH-174는 사람의 정상 폐의 상피세포 (BEAS-2B), 탯줄정맥 내피세포 (HUVEC)에서는 세포 독성을 일으키지 않고 망막색소 상피세포에서는 세포 활성에 대해 20% 미만의 독성을 일으켰다. 이는 암세포에 일으키는 독성의 1/8 수준으로 치료용

량에서의 암세포 선택성이 뛰어나며, 합성 모체인 deguelin과 비교했을 때도 정상 세포에의 그 독성이 현저히 낮다. 이와 같은 항암 활성은 SH-174가 처리된 비소세포성 폐암세포의 세포 활성, 군락형성능력, 세포자살사 여부의 관찰을 통해 발견되었다. 또한, 본 연구에서는 SH-174가 저산소 상태인 종양미세환경에서 암세포의 생존과 적응을 돕는 신호전달을 차단함으로써 항암 효과를 나타낸다는 것을 기전으로 제시하였다. 이는 암세포의 Akt, AMPK-mTORC1, MEK1/2의 신호전달경로를 억제함으로써 나타나는 것임을 밝혔다. 따라서, SH-174는 비소세포성 폐암치료에 촉망되는 신규 화합물로서 추후 개체 수준에서의 연구가 장려된다.

주요어: 비소세포성 폐암세포, deguelin, 항암제, 세포자살사, AMPK-mTORC1

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