이학석사 학위논문

Biological Screening of Natural Compounds for Developing Anticancer Agents in Non-Small Cell Lung Cancer

비소세포성 폐암에서 항암제 개발을 위한 천연물 스크리닝

2016 년 2 월

서울대학교 대학원

협동과정 유전공학전공

정 유 진

ABSTRACT

Biological Screening of Natural Compounds for Developing Anticancer Agents in Non-Small Cell Lung Cancer

Yujin Jung

Interdisciplinary Graduate Program in Genetic Engineering

The Graduate School

Seoul National University

After surgery or chemoradiation therapy, recurrence of non-small cell lung cancer is considered as a barrier to effective treatment. Several studies have reported that cancer metastasis and cancer metabolism augment NSCLC recurrence. Integrin α5β1 is overexpressed in metastatic NSCLC, and it leads to decrease survival rates. In terms of cancer metabolism, mitochondria play a role not only in ATP provision but also in aggressive behaviors of cancer cells. Therefore, novel compounds targeting cancer metastasis or cancer metabolism are in demand to prevent failure of primary treatment. In this study, we discovered compound A and compound B as targeting integrin α5β1 and mitochondria, respectively, through cell-based screens of a 160 natural compound library. Compound A suppresses cell adhesion on fibronectin which is an integrin α5β1 specific ligand, and shows acceptable values with integrin α5β1 on SwissDock docking. We identify that

compound A reduces phosphorylation of FAK-Y397, an integrin dependent activation site, and its intracellular signaling molecules such as Src and AKT. Moreover, compound A disrupts the interaction between integrin α5β1 and Src. When it comes to cancer metabolism, compound B decreases intracellular ATP levels mediated by mitochondrial damage. Mitotracker, Mitosox, and TMRM data support that compound B negatively modulates mitochondrial function. Both compound A and compound B have anticancer activities confirmed by colony formation assays and propidium Iodide(PI) staining. In summary, our data suggest that compound A and compound B can be further developed to combat cancer metastasis and cancer metabolism as to cancer recurrence.

Keywords: Integrin α5β1, Mitochondria, NSCLC, Recurrence, cancer metastasis, cancer metabolism

Student Number: 2014-20336

CONTENTS

LISF OF FIGURES

INTRODUCTION

Lung cancer is one of the leading causes of cancer death worldwide regardless of gender. According to american cancer society, lung cancer is ranked at the first and second places in estimated cancer deaths and estimated new cancer cases respectively in 2015 (American cancer society, 2015). In Korea, lung cancer was ranked first as a cause of cancer death in 2014 (National cancer information center, 2015). Cigarette smoking is the main cause of lung caner, but other risk factors also exist in our daily life such as air pollution, radiation, family history and aging. Lung cancer is divided into two main groups, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for 83% of all lung cancer cases, 13% for SCLC (American cancer society, 2015). Surgery, radiation therapy or chemotherapy would be introduced depending on the types and stages of lung cancer. In spite of the efforts to cure lung cancer patients, the 1- and 5-year survival rates for lung cancer are 44% and 17%, respectively (American cancer society, 2015).

When lung cancer undergoes metastasis to distant organs, 5-year survival rate of lung cancer is only 4% (American cancer society, 2015). Many studies have reported the positive correlation between metastasis and integrins (AK Mitra et al., 2011; Yang GY et al., 2008; Casey Trimmer et al., 2010; Kim SA et al., 2011). Integrins are transmembrane receptors which interact with extracellular metrix (ECM), and function as cell adhesion, migration, and signal transduction. In NSCLC, integrin α5β1 is commonly expressed in the case of lymph node metastasis (Han JY et al., 2003). Dingemans and colleagues reported that integrin β1 showed the highest expression in NSCLC patient tissues, and NSCLC patients expressing integrin α3, α5, β1, or β3 above median levels had a shorter overall

survival compared to patients whose expressions of the integrin were below the median (Dingemans et al., 2010).

Activation of oncogenes and mutation of tumor suppressor genes force cancer cells to undergo uncontrolled proliferation. To support this uncontrolled proliferation, cancer cells modify their energy metabolism to make lots of ATP. Therefore, targeting a key mechanism in cancer metabolism is a one of the approaches to develop a novel drug. Cancer cells have 2 main ways to get energy, glycolysis and oxidative phosphorylation in mitochondria. 2-deoxy-d-glucose (2-DG), a glycolysis inhibitor, enhances therapeutic effectiveness of antimycin and paclitaxel in NSCLC (Gregory et el., 2004). Mitochondria complex I inhibitor, metformin, sensitizes NSCLC cells to radiation response through ATM and AMPK (Y Storozhuk et al., 2013). To accelerate activity and to reduce toxicity and non-specificity, setting up a database for anti-metabolic drugs is considered importantly. This is because there is a strategic significance that we can optimize the structure of chemical compounds on the basis of this database.

In this study, we screened 160 natural compounds provided by Prof Won Keun Oh. In chapter 1, we showed that compound A inhibits integrin α5β1 and integrin-Src/FAK-AKT signaling by binding its extracellular domain in NSCLC cell lines, thereby suppressing NSCLC migration and colony formation. In chapter 2, we found that compound B caused mitochondria malfunction and ATP depletion, leading cancer cell death. Our findings may assist the future development of optimized anticancer drugs for cancer metastasis and cancer metabolism in NSCLC

MATERIALS AND METHODS

Cell lines and Cultures

H1299, 226B, H460 and A549 (human Non-small cell lung cancer, NSCLC) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI-1640(Welgene Inc., Gyeongsan-si, Republic of Korea) supplemented with 10% FBS and 1% antibiotics at 37℃ in a humidified 5% CO2 incubator.

MTT assay

1000 of cells were placed into 96-well plates(NUNC) for 24 hours incubation with RPMI-1640 medium supplemented with 10% FBS and 1% antibiotics. Cells were incubated with increasing concentrations of compounds for 3 days. MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (4mg/ml) was treated for 2 hours at 37℃ to measure cell proliferation rate at 570nm. DMSO was added into each well to desolve the formazan products.

Adhesion assay

70ul of fibronectin or laminin in PBS was added into each well of 96-well plates to coat the plates at 4℃ overnight (10ug/ml for fibronectin, 20ug/ml for laminin). The plates were washed 2 times with washing buffer (0.1% of BSA in RPMI-1640 medium, serum free). For blocking the plate, the washing buffer was replaced with blocking buffer(0.5% of BSA in RPMI-1640 medium, serum free), and then the plates were incubated for 1 hour at 37℃. 20000 of cells were incubated in each well of the plates for 30 minutes in a 37℃ CO2 incubator. After

that, each well of the plates was washed 3 times with the washing buffer. Attached cells were further stained by MTT solution.

Luminescence ATP detection assay

ATPlite™(PerkinElmer) kit was used for this assay. 1000 of cells were seeded into each well of 96-well plates (costar black clear bottom plates), and incubated for 24 hours. Compounds were treated for 6 hours, and drug treated media were removed completely. ATP standard solution was diluted with PBS by a generalized ten-fold serial dilution. Cell lysis buffer and substrates were added. Luminescence for each well was measured by Gemini XS (Molecular Devices).

Western blot and Coimmunoprecipitation analysis

Cells were lysed with modified RIPA lysis buffer [50mM Tris-HCl (pH7.5), 150mM NaCl, 1mM EDTA, 0.25% Sodium deoxycholate, 1% Triton X-100, 1mM Na_3VO_4, 100mM NaF, 0.5mM DTT, 1mM PMSF, 1μg/ml Leupeptin, 20mM β−glycerophosphate, 1μg/ml Aprotinin]. The lysates were centrifuged at 13000 rpm for 30 minutes at 4℃. For coimmunoprecipitation analysis, cells were lysed with EBC1 lysis buffer [40mM Tris-HCl (pH7.5), 120mM NaCl, 0.5% NP-40, 2mM EDTA, 1mM $MgCl_2$, 1mM Na_3VO_4, 100mM NaF, 0.5mM DTT, 1mM PMSF, 1μg/ml Leupeptin, 20mM β−glycerophosphate, 1μg/ml Aprotinin]. 250~500ug of cell lysates were incubated with antibody (250~500ng) at 4℃ overnight. Protein concentrations of supernatants were measured by BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amount of protein was loaded into a 8~10% SDS-PAGE gel, and transferred from gel to PVDF membrane.

The membrane was blocked with 3% bovine serum albumin in Tris-buffered saline-0.1% Tween-20 (TBST) containing 0.02% sodium azaide for 1 hour at room temperature, and then incubated with a primary antibody(1:1000 dilution in blocking BSA) overnight. The membrane was washed with TBST for 1 hour (four times per 1 hour) and incubated with a secondary antibody (1:5000 in 3% skim milk). The membrane was washed again with TBST for 1 hour(four times per 1 hour), and the protein band was detected by using ECL solution (Thermoscientific INC, Bremen, Germany).

Anchorage-dependent and -independent assays

For anchorage dependent assay, 300 of cells were seeded per well in 6-well plates with RPMI-1640 medium supplemented with 10% FBS and 1% antibiotics. Next day, cells were treated with increasing concentrations of compounds. Media were exchanged every 3 days. After 10~14 days, cells were fixed by 100% methanol for 15 minutes, and stained with 0.01% crystal violet.

For anchorage independent assay, 500 of cells in 0.4% top agar (0.5ml) were seeded onto 1% solid bottom agar in 24-well plates. Different concentrations of compounds (0, 1, 10uM) were treated, and the media were changed every 3 days. After colonies were grown for 10~14 days, they were stained by MTT solution.

FACS analysis

Adherent cells were trypsinized and pelleted by centrifugation with floating cells (1500rpm, 1.5 minutes). Cells were washed with PBS 2 times and fixed by ice-cold 100% methanol at -20℃ overnight. Cells were stained with 50μl

of propidium iodide (50μg/ml) in the presence of 50μg/ml RNase for 30 minutes at room temperature. Apoptotic cells were measured using a FACS Calibur flow cytometer (BD Biosciences).

Wound healing assay

$2X10^5$ cells were seeded in six-well plates to be grown to full confluence. After 24 hours incubation, we incubated the cells for 12 hours in starvation medium. Cell monolayer surface was scratched with a sterile 200μl tip and washed with starvation medium to remove detached cells from the plates. Attached cells were treated or untreated with 5μM of compound A for indicated times.

SwissDock program

SwissDock Docking calculations were undertaken using SwissDock (http://www.swissdock.ch) web site. SwissDock is a web-based docking service which is easy to assess, because protein and ligand structures can be inputted directly. PDB files of integrin α5β1 were downloaded from protein data bank, and SDF file of compound A was obtained from PubChem and converted to Mol2 file by Chimera 1.10.1. The image of binding structure was viewed in UCSF Chimera package.

Mitotracker, TMRM, and Mitosox staining

Cells were seeded on round coverslips in 12-well plates, and allowed to attach for overnight. Compounds were treated for indicated time periods. Cells were treated with an indicated fluorescence dye [100nM Mitotracker (M-7514,

ThermoFisher) and 100nM TMRM (T-668, ThermoFisher) for 30 minutes, 5uM Mitosox (M36008, Invitrogen) for 15 minutes]. For mitosox, cells were counterstained with DAPI ($1\mu g/ml^{-1}$). Cells were washed with DPBS for 2 times, and coverslips were mounted using Vectamount (H-5000, Vector laboratories). All samples were photomicrographed using Nuance fluorescence microscope (Perkin Elmer).

RESULTS

CHAPTER 1.

1.1. A cell-based compound screen identifies an effective inhibitor of integrin α5β1

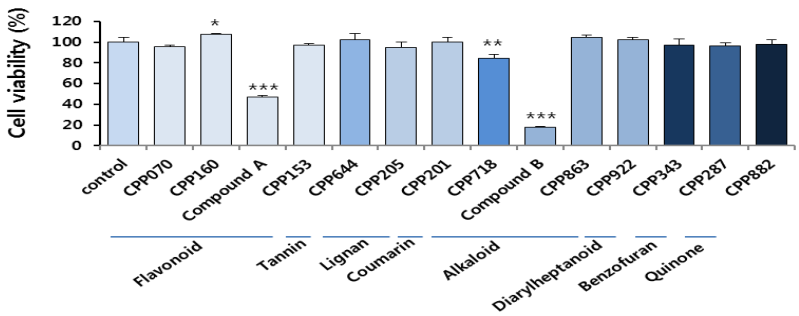
To screen a potential natural compound inhibiting integrin α5β1 activity, we first performed adhesion assay using fibronectin with H1299 NSCLC cell line. Fibronectin is reported as a ligand specific for integrin α5β1. H1299 cell line was selected for this cell-based screening, because it had a p53 null phenotype which demonstrated a resistant response to chemotherapy. We provided a 160 natural compound library from Prof Won Keun Oh. 14 compounds from 8 different classes were randomly selected depending on their natural toxic activities reported by other studies. Among 14 compounds, we could find a compound, compound A, from flavonoid class shown a most prominent adhesion inhibition (figure 1B). Moreover, compound A showed anticancer activity in MTT assay (figure 1A). Thus, we decided to screen the 12 numbers of flavonoids with adhesion assay (figure 1D) and MTT assay (figure 1C). In the 12 numbers of flavonoids, compound A had profound cell adhesion and viability suppression. Hence, we chose compound A to proceed following experiments.

To make sure that the inhibition of cell adhesion by compound A was due to its effect on interfering cell adhesion and not for a reduction in cell viability, we checked a cytotoxicity effect of compound A with NSCLC cell lines by MTT assay (figure 2A). As shown in figure 2A, treatment with various concentration of compound A on NSCLC cell lines for 1 or 2 days has no effect on NSCLC viability. And then, we performed the adhesion assay with those NSCLC cell lines, H460,

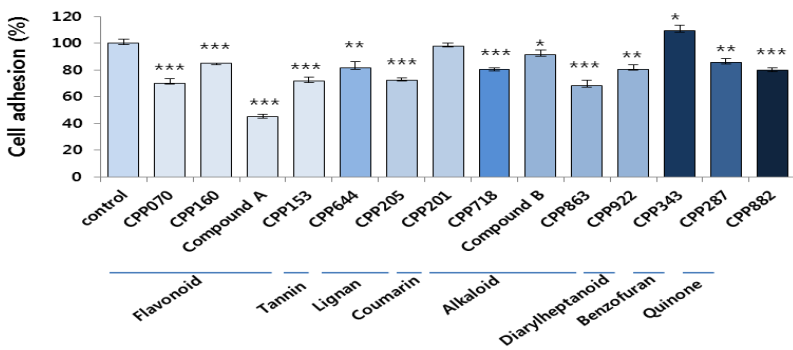
226B, and H1299 (figure 2B). Compound A also interfered cell adhesion to fibronectin in above mentioned NSCLC cell lines (Fig 2B). Taken together, these results suggest that compound A could be an integrin α5β1 targeting compound along with an anti-cancer effect.

.

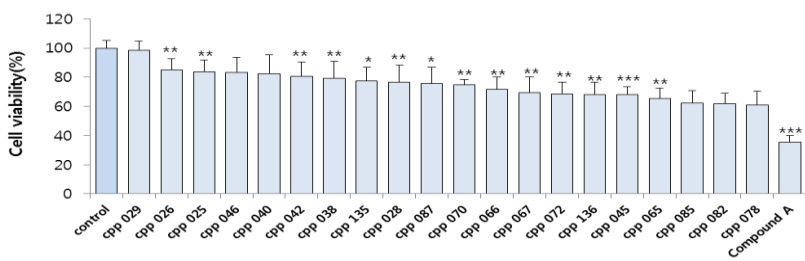
A


Cell viability (%)
120
100
80
60
40
20
0
control
CPP070
CPP160
Compound A
CPP153
CPP644
CPP205
CPP201
CPP718
Compound B
CPP863
CPP922
CPP343
CPP287
CPP882
Flavonoid
Tannin
Lignan
Coumarin
Alkaloid
Diarylheptanoid
Benzofuran
Quinone

B


Cell adhesion (%)
120
100
80
60
40
20
0
control
CPP070
CPP160
Compound A
CPP153
CPP644
CPP205
CPP201
CPP718
Compound B
CPP863
CPP922
CPP343
CPP287
CPP882
Flavonoid
Tannin
Lignan
Coumarin
Alkaloid
Diarylheptanoid
Benzofuran
Quinone

C


Cell viability(%)
120
100
80
60
40
20
0
control
cpp 029
cpp 026
cpp 025
cpp 046
cpp 040
cpp 042
cpp 038
cpp 135
cpp 028
cpp 087
cpp 070
cpp 066
cpp 067
cpp 072
cpp 136
cpp 045
cpp 065
cpp 085
cpp 082
cpp 078
Compound A

D

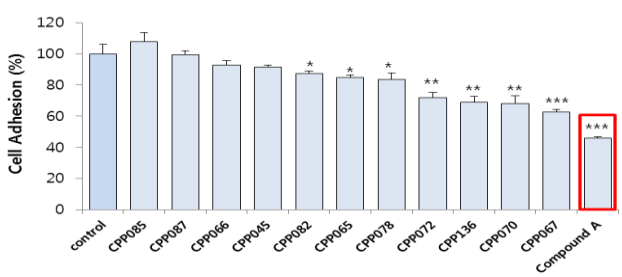

Cell Adhesion (%)
120
100
80
60
40
20
0
control
CPP085
CPP087
CPP066
CPP045
CPP082
CPP065
CPP078
CPP072
CPP136
CPP070
CPP067
Compound A

Figure 1.1. Screening of natural compounds targeting integrin α5β1

A, C. H1299 cells were seeded in 96-well plates and treated with 10uM of various natural compounds for 3 days. Cell viability was measured by MTT assay

B, D. 20000 of H1299 cells underwent adhesion assay with 10uM of various natural compounds for 30 minutes. The amount of adherent cells were determined by MTT assay

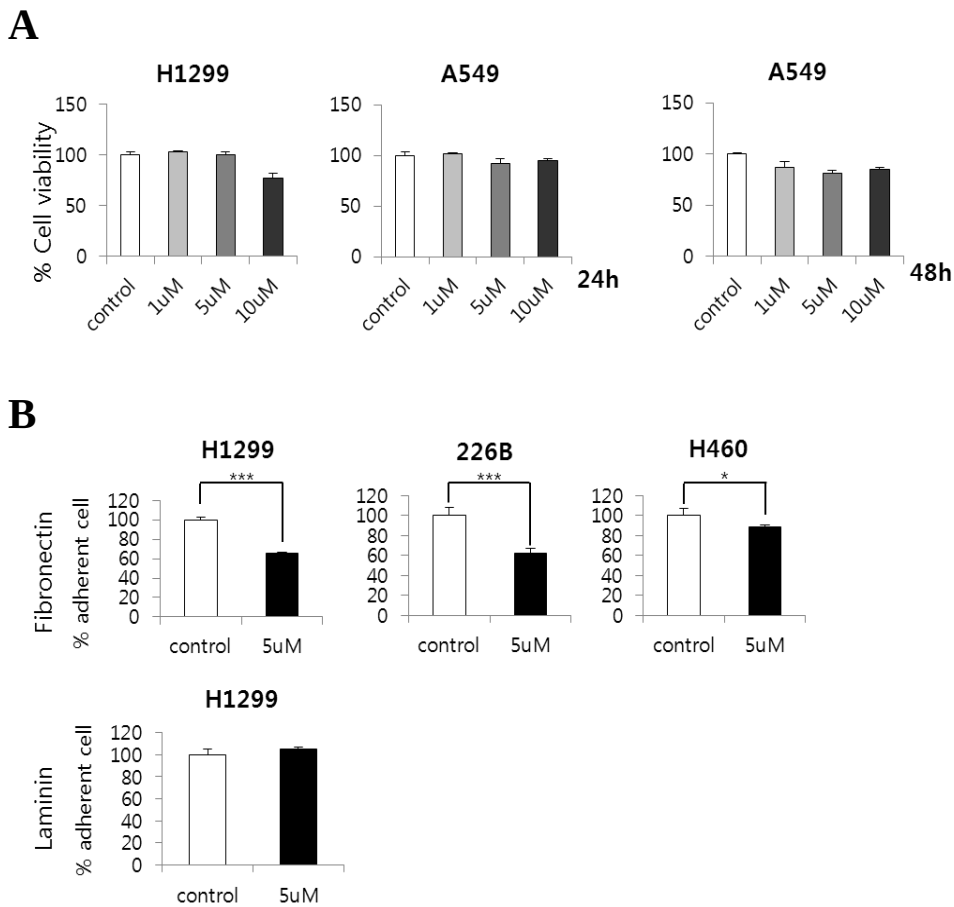


Figure 1.2. Effect of compound A on cell adhesion

A. MTT data show that compound A doesn't have an affect on cell viability in 1 or 2 days.

B. Compound A inhibits NSCLC adhesion specific to integrin-α5β1 ligand fibronectin.

1.2. Compound A suppresses NSCLC migration

Integrins are well known as regulators of cell migration, differentiation, and proliferation. To examine the effect of compound A on NSCLC migration, we conducted wound healing assay with H1299 and A549 as described in materials and methods. As shown in figure 3A, 5uM of compound A reduced the motility of H1299 and A549 cells in wound healing assay, compared to control cells. The wound healing spaces were completely closed by migration of H1299 cells and A549 cells, which were not treated with compound A, after 24h and 48h respectively. However, the gap spaces were not occupied by migration in compound A 5uM treatment groups for 24h or 48h. These observations indicate that compound A restricts NSCLC migration.

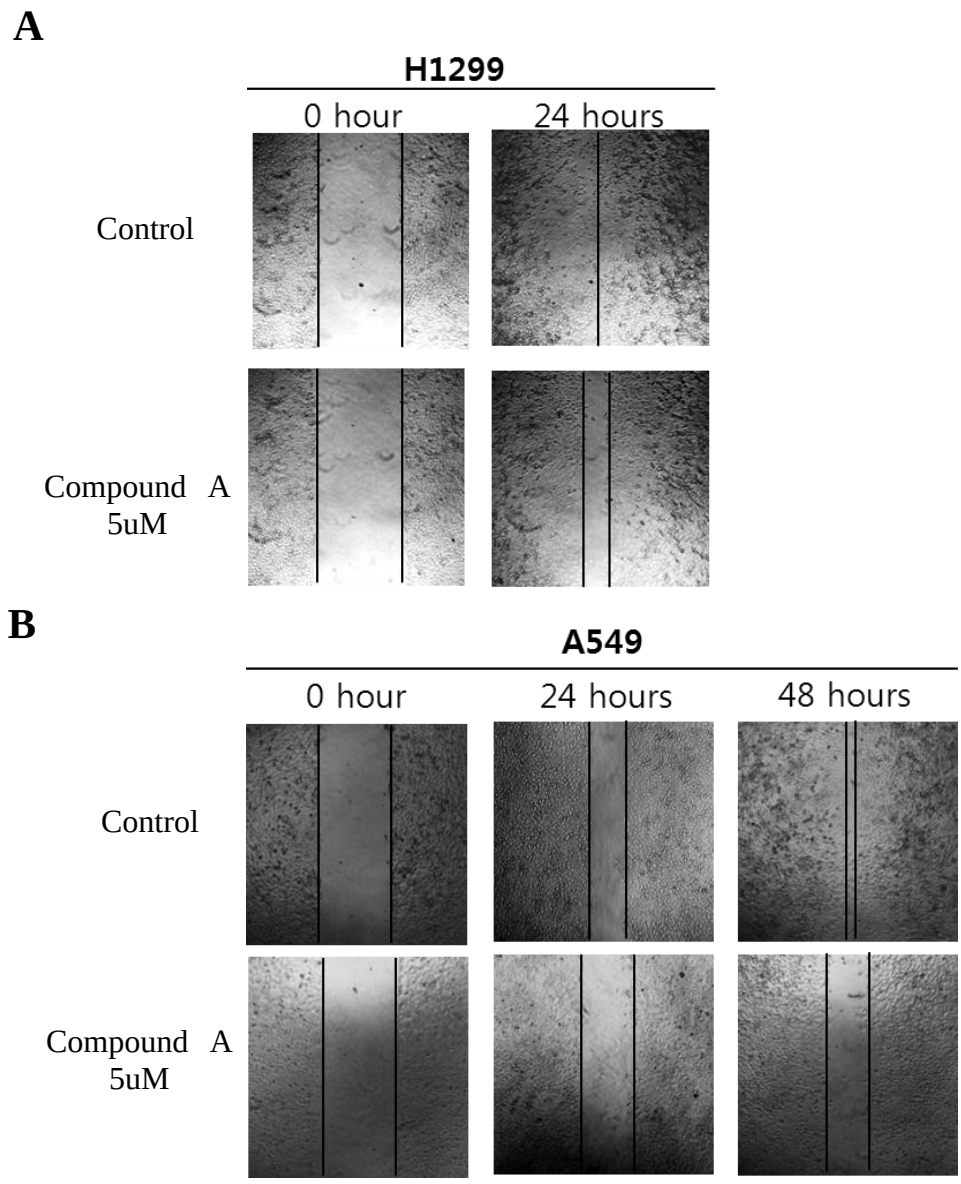


Figure 1.3. Down-regulation of NSCLC migration

A. Compound A delays NSCLC migration. 0 hour parallels show the original space after making a wound. The space between parallels indicates unoccupied area by NSCLC.

1.3. Compound A represses anchorage-dependent and –independent colony formation.

To investigate NSCLC cells lose its capacity to make a colony from a single cell by compound A, we carried out anchorage-dependent colony formation assay with NSCLC cell lines, H1299, 226B, and H460. As shown in figure 4A, the number of colonies formed decreased in a dose-dependent manner (0, 1, 10uM) by compound A. 1uM of compound A treatment led to more than 30% of reduction in anchorage-dependent colony formation compared to control groups in all tested NSCLC cell lines. Compound A inhibited colony formation completely at 10uM in H1299, 226B and H460 cell lines.

To further characterize compound A effect on NSCLC growth inhibition, we performed anchorage-independent colony formation assay (soft agar assay) to examine NSCLC ability to grow in a unattached condition under compound A treatment. In common with anchorage-dependent colony formation assay, the number of colonies over 80 pixel^2 was less than the control group in a dose-dependent manner (0, 1 10uM) in all NSCLC cell lines (figure 4B). These data support that compound A is capable of disrupting colony formation in NSCLC cell lines.

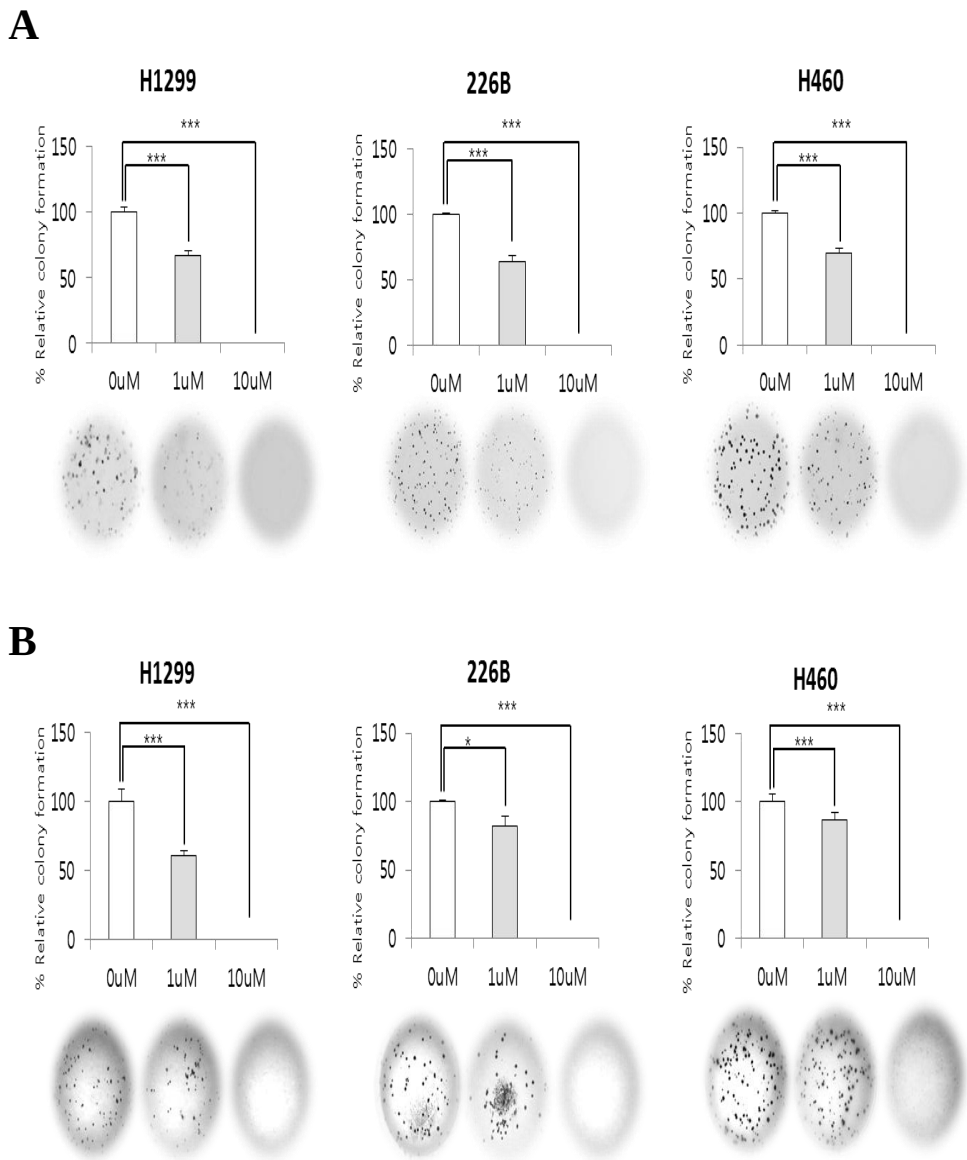


Figure 1.4. Decrease of colony formation

A, B. The inhibitory effects of compound A on anchorage-dependent (A) and – independent (B) colony formation.

1.4. Compound A induces apoptosis in NSCLC

Integrin α5β1 activation stimulates auto-phosphorylation of FAK(Tyr397), leading to FAK activation. This activation leads to PI3K-AKT survival pathway activation. AKT signaling cascade is described as one of the best cell survival pathways. For example, NF-kB activated by AKT is a transcription factor up-regulating several anti-apoptotic proteins such as Bcl-xl, and XIAP. As the apoptotic function of blocking integrin α5β1, we then explored whether compound A treatment could progress cell death via apoptosis. FACS analysis data showed the marked increment of sub-G0/G1 apoptotic phase from 5uM of compound A treatment in H1299 cell line (figure 5A, 5B). Next, we used western blot assay to detect the expression level of cleaved parp which is a critical marker for apoptosis. As shown in figure 5C, 5uM and 10uM treatments of compound A elevated cleaved parp level markedly. These findings suggest that compound A triggers apoptosis in NSCLC.

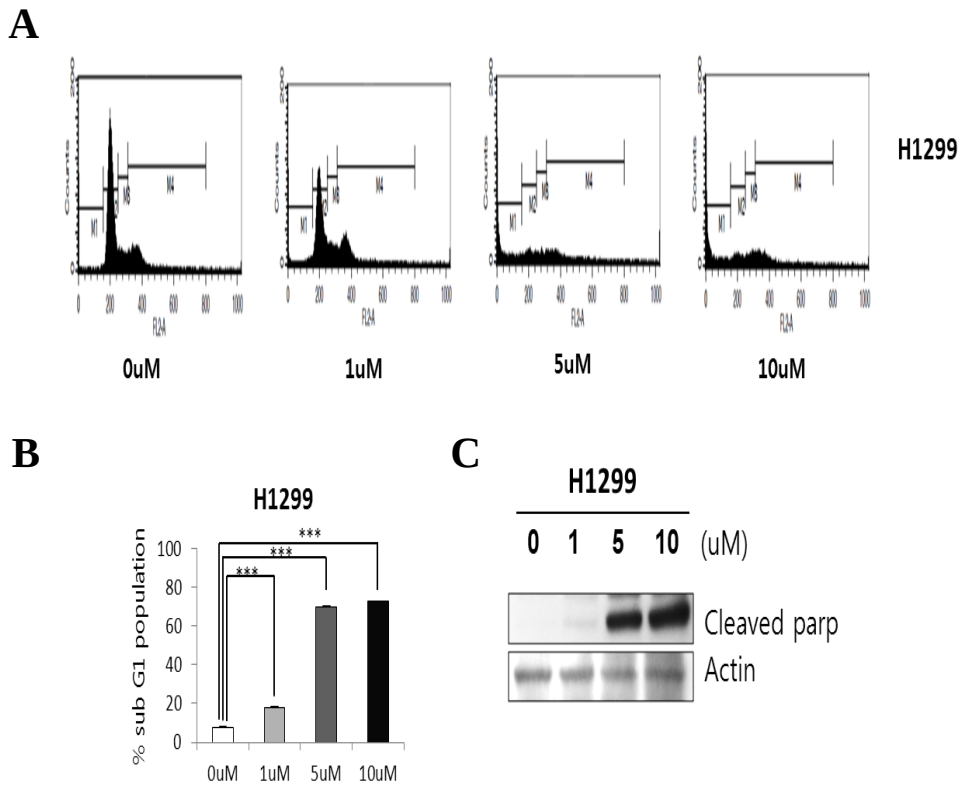


Figure 1.5. Inhibition of NSCLC viability

A, B. The decrease in cell viability by compound A was measured by PI staining with FACS analysis.

C. Compound A increases PARP cleavage, an indicator of apoptosis, in a dose-dependent manner.

1.5. Compound A suppresses integrin-mediated FAK phosphorylation

Upon integrin α5β1 binding to fibronectin, initial auto-phosphorylation of FAK Tyr-397 is followed. To further explore the role of compound A on suppressing integrin α5β1, we conducted western blot to compare the changes in phosphorylation FAK Tyr-397 between control and compound A time-dependent treated groups. Our analysis showed that 5uM of compound A treatment attenuated the phosphorylation of FAK Tyr-397 in H1299, A549, and 226B in a time-dependent manner, while expression of total FAK remained unchanged (figure 6A, 6B, 6C). These results suggest that compound A disturbs integrin α5β1 activation which results in decrease of FAK Tyr-397 phosphorylation.

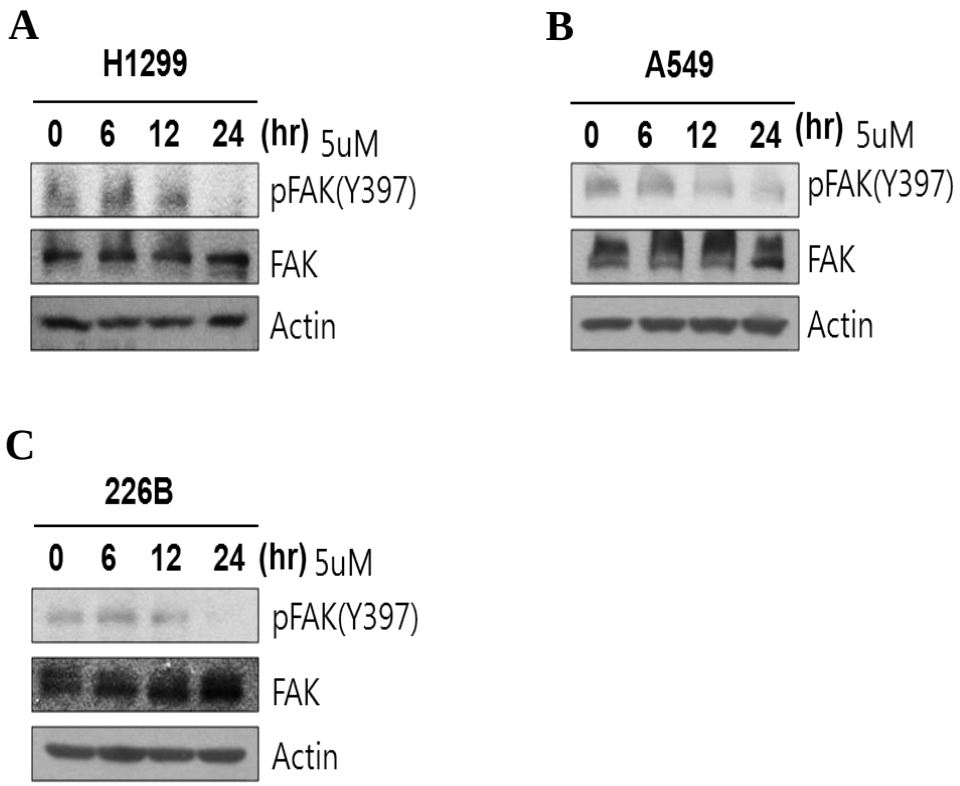


Figure 1.6. Suppression of FAK-Y397 phosphorylation

A, B, and C. Expression of FAK-Y397 was suppressed in a time-dependent manner by compound A in H1299, A549 and 226B cell lines.

1.6. Compound A interferes with integrin-driving signaling pathways

Activation of integrin α5β1 provokes not only FAK Tyr-397 phosphorylation but also Src Tyr-416 activation. The activation of Src leads FAK Tyr-576 phosphorylation. Those activations (FAK Tyr-397, Src Tyr-416, FAK Tyr-576) lead to AKT signaling pathway. To examine the regulation of intracellular signaling molecules mentioned above by compound A, we performed western blot assay to detect the phosphorylation levels of above mentioned signaling molecules in NSCLC cell lines, H1299 and A549. As shown in figure 7A and 7B, 5uM of compound A diminished the phosphorylation levels of Src, FAK, and AKT noticeably in a time-dependent manner, whereas total protein levels of the intracellular signaling molecules were not altered. These results indicate that repression of integrin α5β1 by compound A decreases activation of Src, FAK, and AKT.

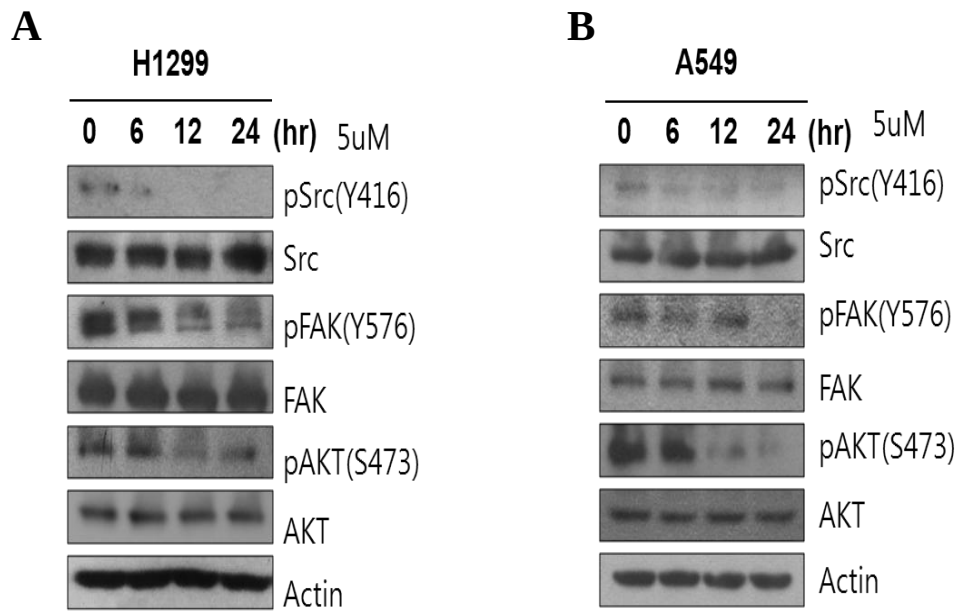


Figure 1.7. Inhibition of integrin β1 downstream signaling: Src, FAK, and AKT

A, B. 5uM of compound A decreased phosphorylation of integrin β1 downstream signaling in a time-dependent manner.

1.7. Compound A targets integrin β1 extracellular domain, and inhibits interaction with Src

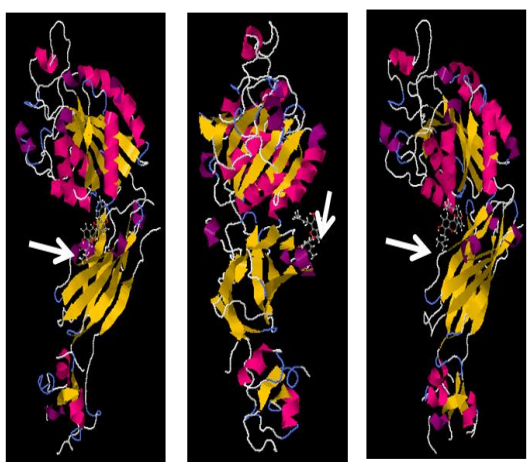
In order to predict the putative binding modes of compound A to integrin α5β1, SwissDock service was carried out. SwissDock is a web-based service which estimates the affinity of docking between a small molecule and a protein. The values were provided in terms of Fullfitness(kcal/mol). The cristal structures of an integrin α5β1 extracellular domain (PDB ID: 4wk0, 4wk2, 4wjk) were obtained from protein data bank (http://www.rcsb.- org/pdb/home/home.do). 3D structure of compound A was obtained from PubChem, and converted into Mol2 File by Chimera 1.10.1. Fullfitness docking scores (kcal/mol) of compound A to integrin α5β1 were shown in figure 7A. These Fullfitness docking scores support the hypothesis that compound A may bind to an integrin α5β1 extracellular domain. Figure 7B shows the images of docking between compound A and integrin α5β1.

I next investigated whether the binding of compound A to integrin extracellular domain would decrease the interaction between integrin and intracellular signaling molecules. Src protein kinase is activated by integrin β1 in the presence of ECM binding. Therefore, we examined whether binding of compound A to integrin could interfere the association of integrin with Src. Co-immunoprecipitation assay demonstrates that the association of Src to integrin becomes weaken under 12 hours of compound A 5uM treatment. These results imply that Compound A appears to inactivate Src by disrupting the integrin-mediated adhesion

A

Integrin PDB code	Full fitness (kcal/mol)
	CPP041
4wk0	-2192.46
4wk2	-2346.74
4wjk	-2495.74

B



C

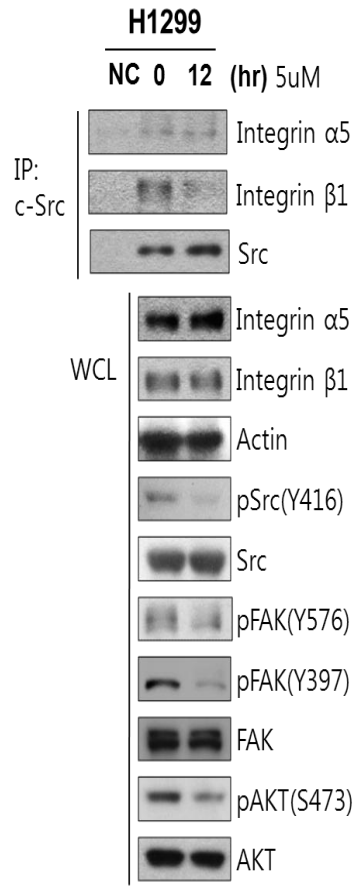


Figure 1.8. Inhibition of interaction between integrin α5β1 and Src

A, B. SwissDock results show that compound A has a high affinity for the integrin β1 extracellular domain.

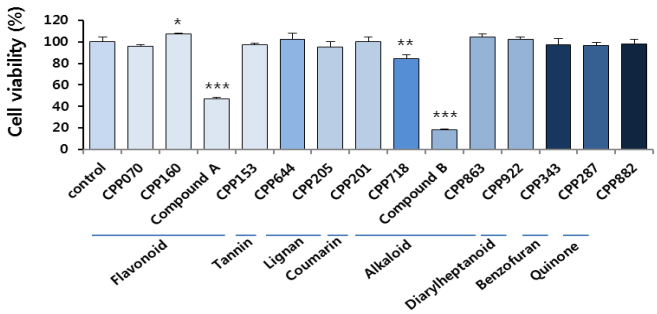
C. Co-immunoprecipitation assay indicates that compound A interferes the interaction between integrin and Src, leading to suppression of Src activation.

CHAPTER 2.

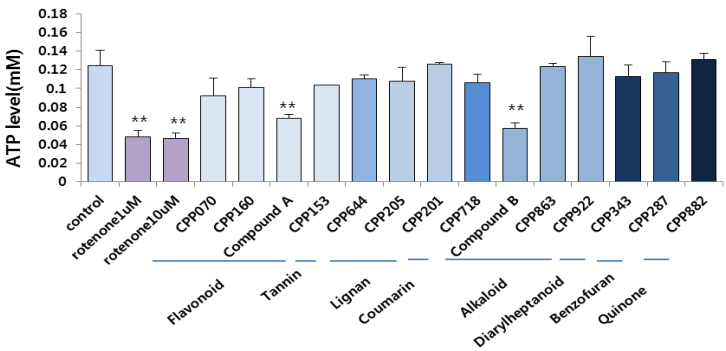
2.1. Screening natural products for cancer metabolism

We first conducted luminescence ATP detection assay (ATPlite) to identify a novel natural compound repressing cancer metabolism. This luminescence assay is to evaluate intracellular ATP levels based on the emission of light from the reaction between intracellular ATP with added D-luciferin and luciferase. We started the screen with H1299 cell line because null phenotype of p53 would weaken the effect of chemotherapy. A 160 natural compound library was obtained from Prof Won Keun Oh. According to their natural-toxicity reports by other papers, 14 compounds were chosen from 8 different classes in the library. Interestingly, compound B from alkaloid class was found to suppress intracellular ATP level conspicuously (figure 1B). Also, compound B was most effective to repress H1299 cell viability in MTT assay (figure 1A). Therefore, we included 14 alkaloid compounds in subsequent luminescence and MTT assays (figure 1C, 1D). From the subsequent assays, compound B was confirmed to be most active in the case of decreasing intracellular ATP level and cell viability. In addition, we confirmed the activity of compound B in a dose and time dependent luminescence assay in H1299 cell line (figure 1E). For these reasons, we selected compound B to proceed following experiments.

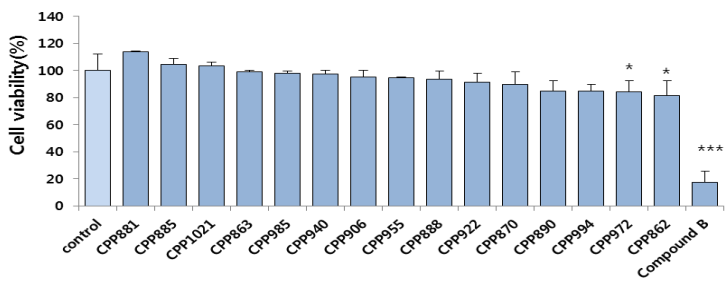
A


Cell viability (%)
120
100
80
60
40
20
0
control
CPP070
CPP160
Compound A
CPP153
CPP644
CPP205
CPP201
CPP718
Compound B
CPP863
CPP922
CPP343
CPP287
CPP882
Flavonoid
Tannin
Lignan
Coumarin
Alkaloid
Diarylheptanoid
Benzofuran
Quinone

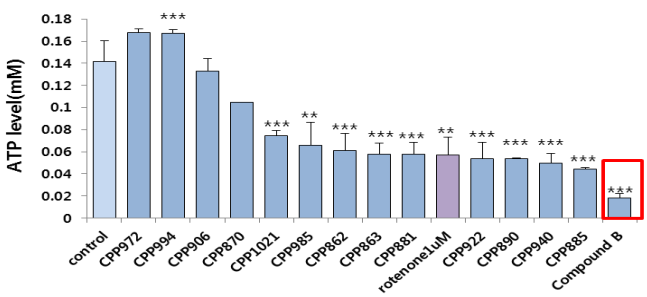
B


ATP level(mM)
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
control
rotenone1uM
rotenone10uM
CPP070
CPP160
Compound A
CPP153
CPP644
CPP205
CPP201
CPP718
Compound B
CPP863
CPP922
CPP343
CPP287
CPP882
Flavonoid
Tannin
Lignan
Coumarin
Alkaloid
Diarylheptanoid
Benzofuran
Quinone

C


Cell viability(%)
140
120
100
80
60
40
20
0
control
CPP881
CPP885
CPP1021
CPP863
CPP985
CPP940
CPP906
CPP955
CPP888
CPP922
CPP870
CPP890
CPP994
CPP972
CPP862
Compound B

D


ATP level(mM)
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
control
CPP972
CPP994
CPP906
CPP870
CPP1021
CPP985
CPP862
CPP863
CPP881
rotenone1uM
CPP922
CPP890
CPP940
CPP885
Compound B

E

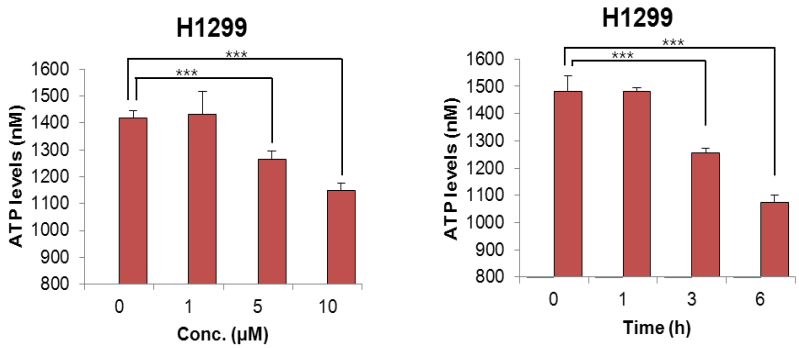


Figure 2.1. Screening of natural compounds for cancer metabolism

A, C. MTT assay was conducted at 10uM of various natural compounds with H1299 cells for 3 days.

B, D, E. Intracellular ATP levels were measured by luminescence ATP detection assay (ATPlite). Rotenone was used as a positive control.

2.2. Compound B activates AMPK and regulates downstream signaling

AMPK acts as a sensor of intracellular ATP:ADP ratio and plays an important role in energy homeostasis. AMPK exists as a heterotrimer which is composed of 3 obligate subunits, catalytic α subunit, regulatory β and γ subunits. Under energy starvation, AMP or ADP can favorably bind, and cause conformation change on the regulatory γ subunit. This conformation change leads to phosphorylation of threonine 172, resulting in activation of AMPK. Based on this knowledge, we tested whether compound B would affect phosphorylation level of AMPK (Thr 172) by western blot in H1299 and A549 cell lines. As shown in figure 2A and 2B, compound B provokes AMPK Thr-172 phosphorylation in a dose and time-dependent manner, while expression of total AMPK remains unchanged. Activation of AMPK negatively regulates mTOR complex activation by serine-2448 dephosphorylation. To examine the regulation of mTOR complex by compound B, we performed western blot to check the variation of mTOR phosphorylation level and its downstream signaling molecule, p70 S6 kinase. Our data show that 5uM of compound B attenuates the phosphorylation of above mentioned molecules in time- and dose-dependent manners, whereas total protein levels of the molecules were not altered (figure 2A, 2B). These observations suggest that ATP depression by compound B induces AMPK activation, mTOR/p70 S6 kinase pathway suppression.

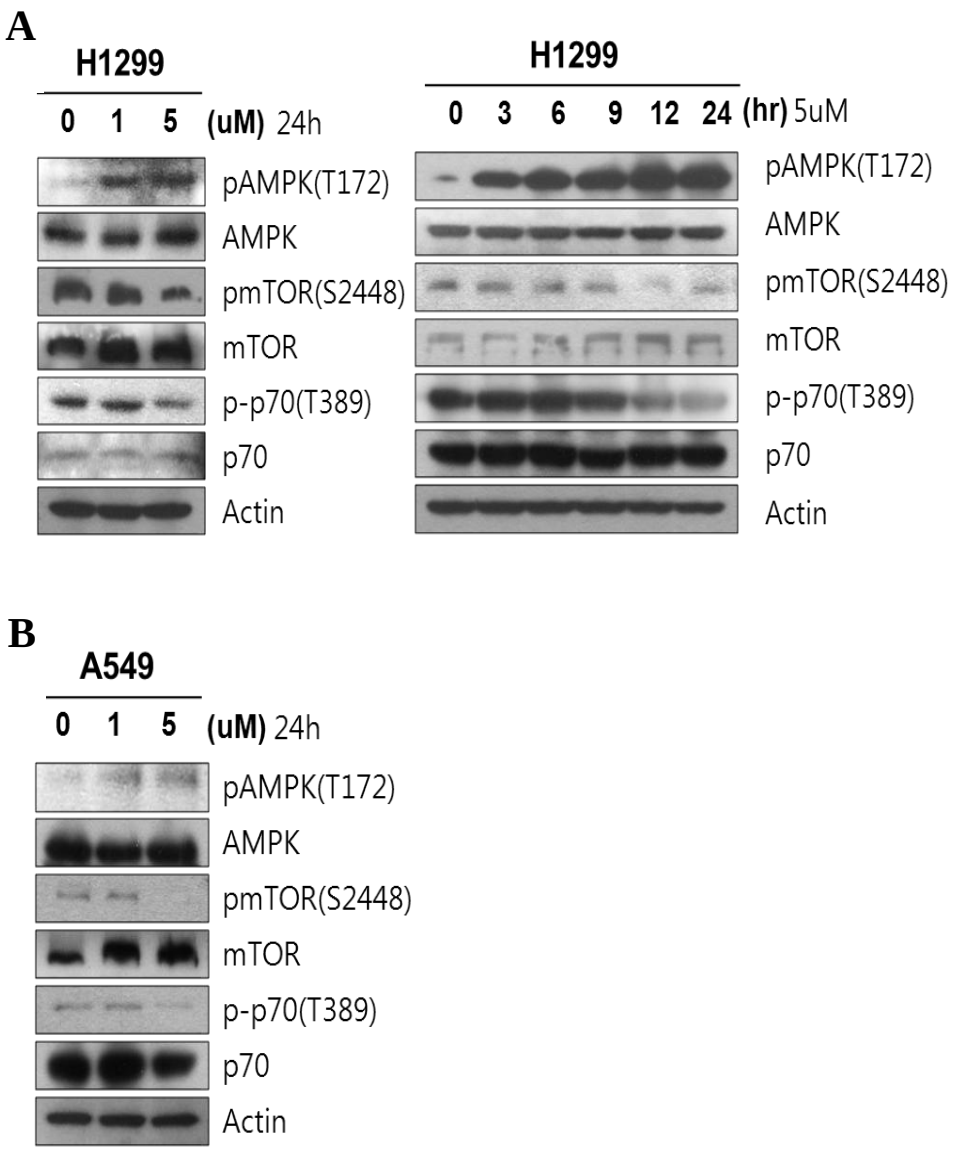


Figure 2.2. Stimulation of AMPK signaling cascades

A, B. 5uM of compound B altered AMPK, mTOR and p70 phosphorylation in H1299 and A549 cell lines.

2.3. Compound B causes mitochondria damage

Mitochondria is a cellular ATP synthesis factory, produces majority of intracellular ATP by oxidative phosphorylation. Because compound B diminished intracellular ATP and activated AMPK, we determined to check activity of mitochondria by mitochondria-selective dyes, Mitotracker and TMRM. First, we examined mitochondrial network structure by Mitotracker. We observed net-like well organized mitochondrial networks in control (figure 3A). However, 5uM of compound B led shrinkage and disrupted networks in a time-dependent manner (figure 3A). Second, we used TMRM which accumulates within polarized mitochondria to check mitochondria membrane depolarization by compound B. As shown in figure 3B, fluorescent red color become faded in compound B treated cells, indicating the mitochondria membrane depolarization by compound B. Third, we introduced Mitosox to identify mitochondrial reactive oxygen species (ROS) production by compound B. With the treatment of compound B, we detected purple color resulted from mitochondria ROS reaction with nucleic acid (DAPI) (firue 3C). Therefore, Mitosox result demonstrates that compound B generates mitochondria ROS in a time-dependent manner (figure 3C). All together, these data suggest that compound B damages mitochondria, and causes mitochondria ROS generation.

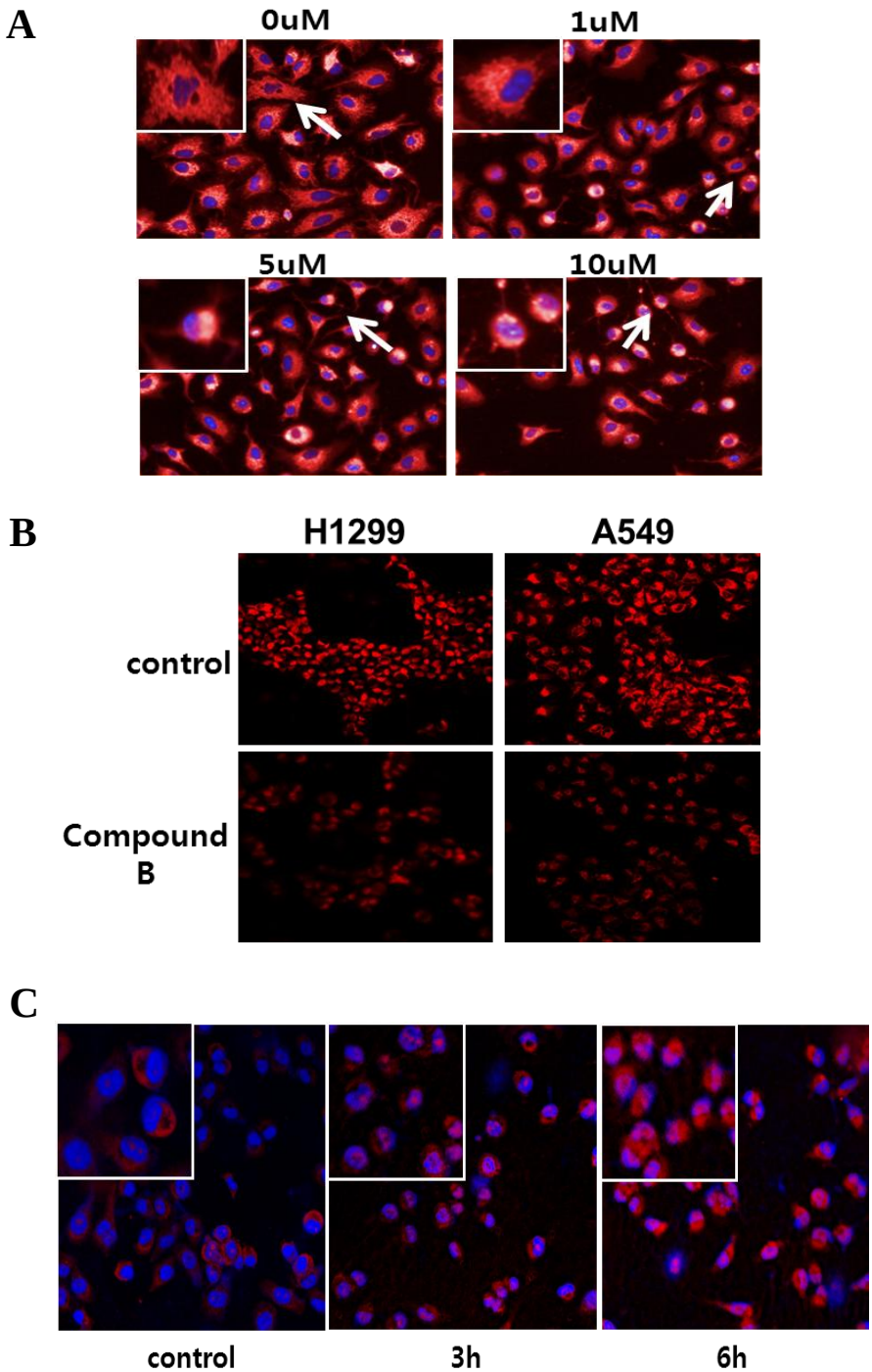

A
0uM
1uM
5uM
10uM
B
H1299
A549
control
Compound B
C
control
3h
6h

Figure 2.3. Effect of compound B on mitochondria dysfunction

A. A549 cells were treated with compound B in a dose-dependent manner and stained with mitotracker (red color) and DAPI (blue color).

B. 5uM of compound B was treated for 3 hours. Mitochondria depolarization was detected by TMRM staining..

C. H1299 cells were treated with 5uM of compound B in a time-dependent manner, and stained by Mitosox and DAPI to measure the generation of mitochondria ROS.

2.4. Compound B inhibits NSCLC cell viability

Mitochondria damage is one of the main causes of cell death. To check the mitochondria damage triggered by compound B regulates cell viability, we performed MTT assay with NSCLC cell lines, H1299, 226B, and A549. MTT is a yellow tetrazolium dye which is reduced by mitochondrial reductase in living cells. The reduction turns MTT dye into insoluble purple formazan product. Therefore, the more cells alive, the stronger purple color we get. Compound B treatment for 3 days leads dose-dependent viability inhibition in above mentioned cell lines (figure 4A). 5uM of compound B exposure decreased cell viability to less than 40% in all-tested NSCLC cell lines (figure 4A). These results represent that compound B suppresses NSCLC cell viability

A

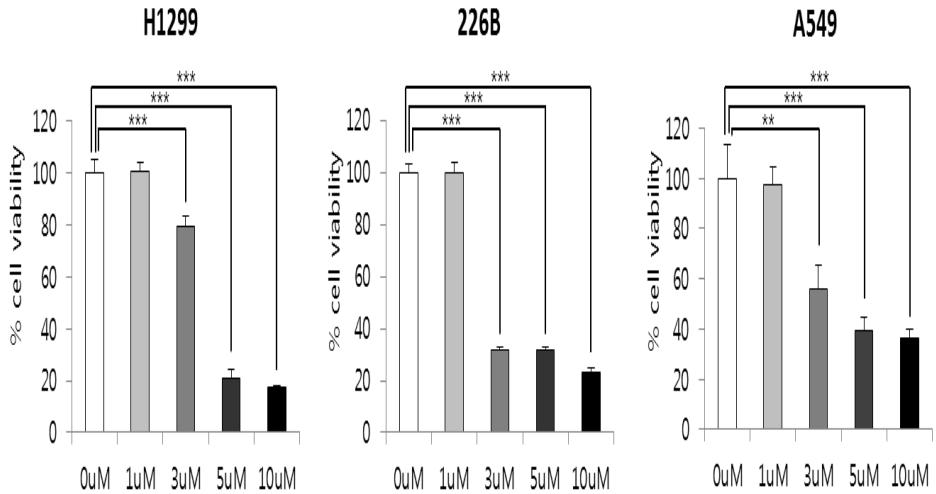


Figure 2.4. Inhibition of NSCLC viability

A. 3 days of compound B treatment decreased NSCLC viability in a dose-dependent manner.

2.5. Compound B suppresses anchorage-dependent and -independent colony formation

To examine NSCLC cells lose its ability to form a colony from a single cell, we conducted anchorage-dependent colony formation assay with NSCLC cell lines, H1299, 226B and A549. Figure 5A shows that compound B reduces anchorage-dependent colony formation at 1uM. As shown in figure 5A, the number of colonies formed declined in a dose-dependent manner (0, 1, 10uM) by compound B. 1uM of compound B treatment inhibited more than 50% of anchorage dependent colony formation compared to a untreated control group in H1299 (figure 5A). At a concentration of 10uM, compound B suppressed colony formation completely in all-tested NSCLC cell lines (figure 5A).

To further examine the impact of compound B on anchorage-independent growth, we assessed soft agar assay with NSCLC cell lines, H1299 and 226B. Similar to anchorage-dependent colony formation assay, the number of colonies over 80 pixel^2 was reduced by compound B in a dose-dependent manner in all tested cell lines (figure 5B). The inhibition effect of compound B in softagar assay at 1uM was less than in anchorage-dependent assay. All together, these observations indicate that compound B prevents anchorage-dependent and -independent colony formation.

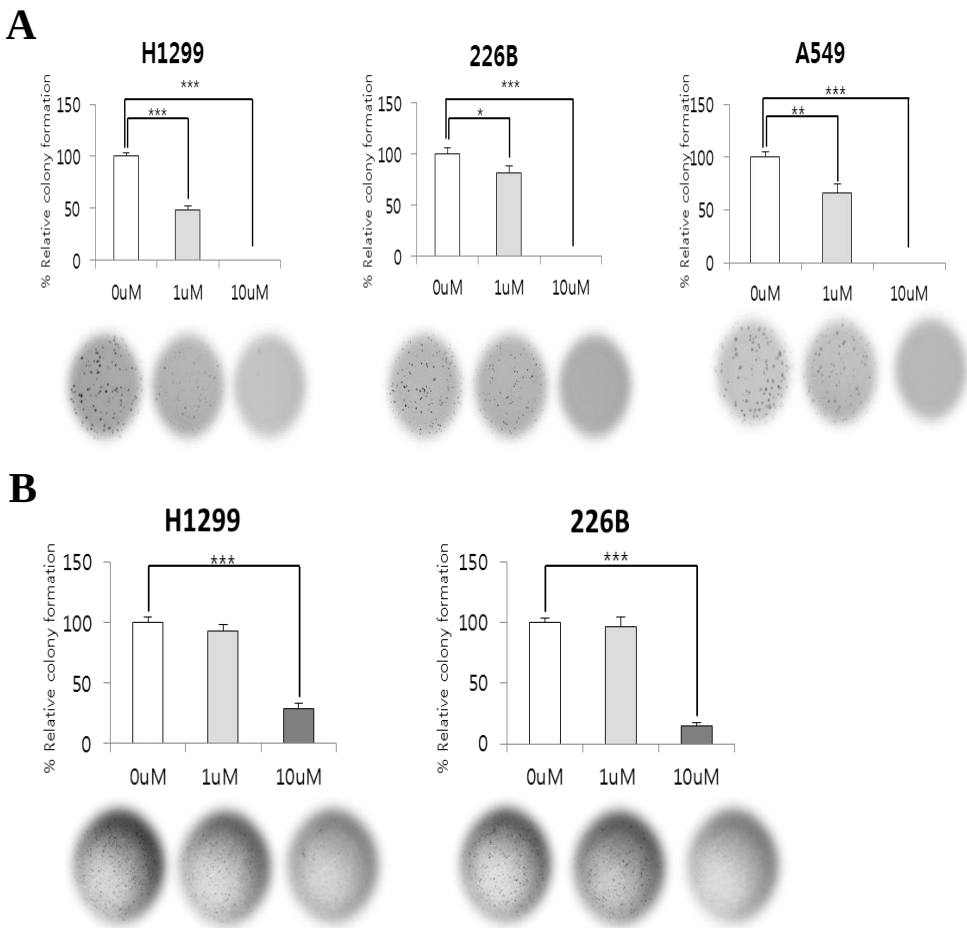


Figure 2.5. Decrease of colony formation

A, B. Compound B reduced anchorage-dependent (A) and –independent (B) colony formation.

2.6. Compound B induces apoptosis

Mitochondrion is the start of intrinsic apoptosis pathway signaling, and one of the reasons causing intrinsic apoptosis is mitochondria dysfunction. In intrinsic apoptosis, mitochondrial outer membrane permeabilization (MOMP) occurs. MOMP leads the release of pro-apoptotic proteins from mitochondrial inter-membrane space into cytosol. The pro-apoptotic proteins include cytochrome C and Smac. Cytochrome C binds APAF1(apoptotic protease-activating factor 1), and undergoes its conformation change and oligomerization to form apoptosome. This apoptosome is required to activate caspase 9, leading activation of caspase 3. Activation caspase 3 upregulates Caspase-Activated DNase(CAD) activity which causes DNA fragmentation. Propidium Iodide(PI) is a fluorescent DNA intercalating agent which shows red color. PI staining is a popular apoptosis detecting method, because PI cannot intercalate fragmented DNA, showed as sub G0/G1, by apoptosis. As shown in figure 6A and 6B, compound B increased sub G0/G1 phase in 5uM treatment. Moreover, compound B augmented cleaved caspase 3 level in a dose-dependent manner (figure 6C). Collectively, these results support that compound B causes intrinsic apoptosis.

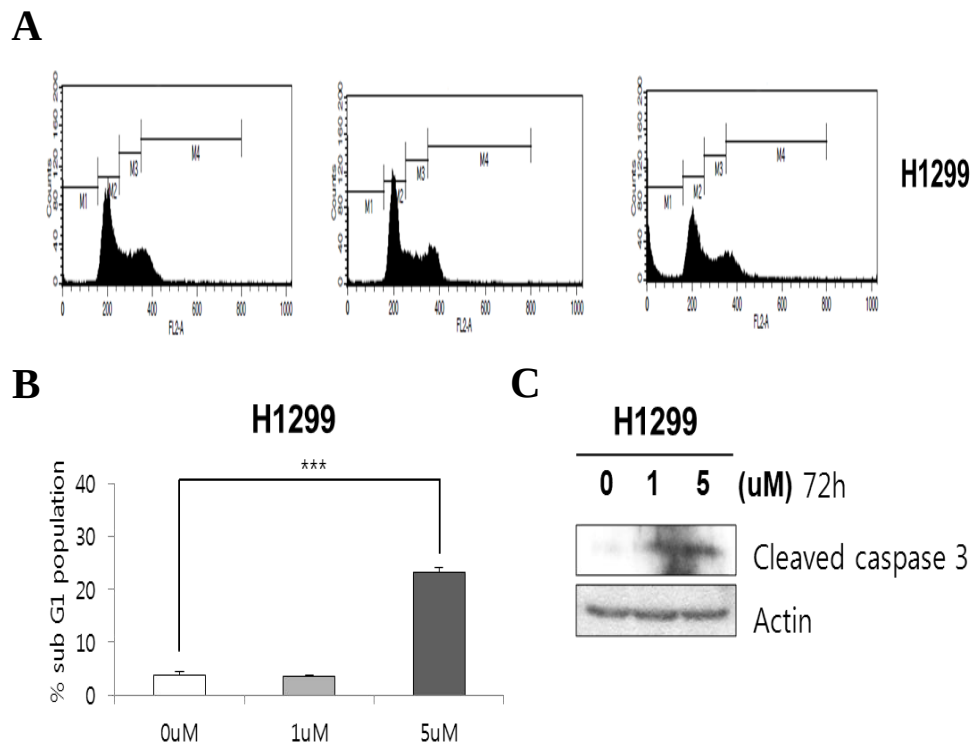


Figure 2.6. Stimulation of NSCLC apoptosis

A, B. Compound B reduced anchorage-dependent (A) and –independent (B) colony formation.

C. Compound B up-regulates cleaved caspase 3 level.

DISCUSSION

Surgery and chemoradiation therapy not only improve survival but also alleviate symptoms in NSCLC patients. In spite of substantial advances in surgery and chemoradiation therapy, patients face with cancer recurrence, meaning a return of cancer after treatment and after a period of remission. According to Lou and colleagues, recurrence developed in 257 NSCLC patients (20%), and second primary lung cancer was detected in 91 patients (9%) during the study period (Low et al., 2013). There are lots of causes which make recurrence happen, despite the efforts to get rid of cancer. In that respect, cancer metastasis and cancer metabolism have been gaining more and more attentions. In this study, we suggest compound A and B as chemotherapeutic agents targeting cancer metastasis and cancer metabolism, respectively.

We found compound A and compound B from a 160 natural compound library. Nature is considered that it harbors the largest diversity resource of therapeutic agents. From 1940s to December 2010, natural compounds or their derivates occupy 48.6% (85 compounds) of all anticancer drugs (175 compounds) (David et al., 2012). For example, paclitaxel isolated from yew tree has been widely used for NSCLC (Sakkaraiappan et al., 2004). Based on these precedents, we decided to find novel compounds for targeting cancer metastasis and cancer metabolism in NSCLC from natural compounds.

Integrin is a trans-membrane molecule which mediates cell to extracellular matrix (ECM) interaction and transmits signals to activate various cellular responses such as cell adhesion, cell motility, and cell migration. Integrins have two different subunits, α and β, and 18 α and 8 β subunits are found in humans. Combination of α and β subunits form heterodimers (24 heterodimers in

humans), and each heterodimer binds to specific type of ECM. In the case of integrin α5β1 expressed in metastatic NSCLC, it binds to fibronectin (Han JY et al., 2003; Anne et al., 2010). For these reasons, we focused on finding a novel compound targeting integrin α5β1 in NSCLC.

According to Lee et al., mAb 33B6, an anti-β1 integrin antibody, suppressed PC3-mm2 cell adhesion on fibronectin (Lee et al., 2013). Therefore we conducted adhesion assay with fibronectin for screening from the natural compound library. In the adhesion assay, 5uM of compound A in flavonoid class showed 35% adhesion inhibition in H1299 cell line (figure 1.2B). Compound A also suppressed adhesion of other NSCLC cell lines, 226B for 38% and H460 for 12% (figure 1.2B). But, there was no detachment by compound A in laminin coated plate, a negative control (figure 1.2B). Since integrins are involved in cell migration (Hood et al., 2002), we launched wound healing assay. As shown in figure 1.3A, wound closing was delayed by compound A in H1299 and A549 cell lines. Next, to evaluate integrin α5β1 silencing effects by compound A on cancer cell proliferation, anchorage-dependent and anchorage-independent assays were performed (figure 1.4). Compared with control groups, compound A reduced colony formations in H1299, H460 and 226B cell lines (figure 1.4A, 1.4B). Many studies have demonstrated that integrin binding to ECM ligands activates pro-survival signalings to inhibit apoptosis (Hood et al., 2002; Desgrosellier et al., 2009). Therefore, we tested the effect of compound A on apoptosis using PI staining in NSCLC cell lines (figure 1.5). FACS analysis confirmed that compound A increased sub G0/G1 fractions (figure 1.5A, 1.5B), indicating apoptotic cell deaths. Consistent with PI staining, PARP cleavage was increased by compound A in a dose-dependent manner (figure 1.5C). FAK-Y397 is reported as an integrin

mediated auto-phosphorylation site (Hood et al., 2002). Therefore, we checked whether compound A could disrupt auto-phosphorylation of FAK-Y397 in NSCLC (figure 1.6). As expected, the phosphorylation level of FAK-Y397 was notably decreased by compound A in a time-dependent manner (figure 1.6). The cytoplasmic domain of integrin interacts with lots of intracellular signaling molecules, such as FAK, talin, and Src (Calderwood et al., 2003). With this in mind, we examined phosphorylation levels of Src-Y416 in NSCLC cell lines, H1299 and A549, under compound A treatment (figure 1.7). Interestingly, compound A suppressed phosphorylation levels not only Src but also its downstream signaling molecule, AKT, in tested cell lines (figure 1.7). Based on adhesion assay results, we expected that compound A may interact with the extracellular domain of integrin α5β1. Thus, we used SwissDock service to check the interaction between compound A and extracellular domain of integrin, and got valuable results (figure 1.8A, 1.8B). Moreover, my findings in co-immunoprecipitation assay describe that compound A disturbs the interaction of integrin α5β1 and its intracellular signaling molecule,Src, and represses activation of the signaling molecules (figure 1.8C).

Mitochondria occupy the major contribution to cellular metabolism and intrinsic apoptosis (Galluzzi et al., 2012). Mitochondria produce lots of ATP from various carbon fuels most efficiently through oxidative phosphorylation; they generate 36 ATP molecules from 1 glucose molecule (Dias et al., 2005). Recent studies have reported that mitochondria metabolism is essential for cancer development, and aggressive behaviors of cancer cells (Weinberg, F. et al., 2010; Fogal, V. et al, 2010; Guo, J.Y. et al., 2011; Wang et al., 2011). In addition to ATP synthesis, mitochondria participate in intrinsic apoptosis. DNA damage, oxidative stress, cytosolic Ca^{2+} overload and endoplasmic reticulum stress can trigger

mitochondria-mediated intrinsic apoptosis (Dias et al., 2005). For these reasons, mitochondria become attractive targets for anti-cancer therapies. In this paper, we discovered compound B which could induce mitochondrial dysfunction.

Mitotracker data show damaged mitochondrial networks in a dose-dependent manner by compound B (figure 2.3A). TMRM staining results also support that compound B is associated with mitochondria damage (figure 2.3B), and depolarization of mitochondrial membrane potential would be one of the mechanisms. Mitochondrial ROS over-generation is not only a stimulus but also a response of mitochondrial stress (Galluzzi et al., 2012; Hu et al., 2011). Mitosox data indicate that compound B leads mitochondrial ROS over-generation which is the indicator of mitochondrial impairment (figure 2.3C). Previous report shows that mitochondria dysfunction reduced ATP production rate (Hu et al., 2011). Consistent with this reference, compound B showed a dose- and time-dependent decrease in intracellular ATP levels in H1299 cell line (figure 2.1E), and these decreases confirmed by activation of AMPK (T172) (figure 2.2). Moreover, mTOR and p70, downstream signaling molecules of AMPK, were also affected by the AMPK activation (figure 2.2). It is well known that cells including cancer cells use ATP as an energy currency to synthesis macromolecules to grow and divide (Cairns et al., 2011). Based on this evidence, we checked the effect of compound B on anchorage-dependent and anchorage-independent growths (figure 2.5). As shown in figure 2.5, compound B inhibits colony formation in dose- and time-dependent manners. Numerous studies reported that mitochondria play a key role in triggering intrinsic apoptosis (Dias et al., 2005; Olszewska et al., 2013). Thus, we examined cell viability, cleaved caspase-3, and sub G0/G1 levels with a dose-dependent

treatment of compound B (figure 2.4, 2.6). These examined data demonstrate that compound B causes intrinsic apoptosis, leading cell death.

Targeted cancer therapy refers to a chemotherapy designed to disrupt a specific target protein which is involved in cancer progression (Wu et al., 2006). There are three largest approaches for the targeted therapy, antibody-targeted therapy, ligand-targeted therapy, and small molecule inhibitor therapy (Wu et al., 2006). These approaches have their own advantages and disadvantages. When it comes to antibody-targeted therapy and ligand-targeted therapy, high-specificity and activity are strong advantages. However, they are unstable, and oral bioavailability is very low (injection is required) (Rohlena et al., 2012; Wu et al., 2006). Moreover, they cost a lot which can be a big burden to patients (Rohlena et al., 2012; Gerber et al., 2008). These disadvantages can be overcome by small molecule inhibitor therapy (Imai et al., 2006). In this study, we found compound A and B for targeting integrin α5β1 and mitochondria, respectively. Small molecule inhibitor therapy also has limitations, such as toxicity and non-specificity (Rohlena et al., 2012). More research is needed to optimize compound A and B to their targets and to reduce their toxicity.

In conclusion, cell-based screens yield 2 novel compounds targeting integrin α5β1 or mitochondria, respectively. Both of these novel compounds suppressed NSCLC proliferation, and induced apoptosis. Therefore, compound A and B may new chemotherapeutic agents against NSCLC.

REFERENCES

AK Mitra, K Sawada, P Tiwari, K Mui, K Gwin, E Lengyel. Ligand independent activation of c-Met by fibronectin and α5β1-integrin regulates ovarian cancer invasion and metastasis. Oncogene. 2011; 30(13):1566-1576.

American Cancer Society. Cancer Facts & Figures 2015. American Cancer Society 2015;1-52

Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. Nat Rev Cancer. 2011;11(2):85-95.

Calderwood DA, Ginsberg MH. Talin forges the links between integrins and actin. Nat Cell Biol. 2003;5(8):694-7.

David J. Newman, Gordon M. Cragg. Natural products as sources of new drugs over the 30 years from 1981 to 2010. J Nat Prod. 2012;75(3):311-35.

Desgrosellier JS, Barnes LA, Shields DJ, Huang M, Lau SK, Prévost N, Tarin D, Shattil SJ, Cheresh DA. An integrin alpha(v)beta(3)-c-Src oncogenic unit promotes anchorage-independence and tumor progression. Nat Med. 2009 Oct;15(10):1163-9.

Dias N, Bailly C. Drugs targeting mitochondrial functions to control tumor cell growth. Biochem Pharmacol. 2005;70(1):1-12.

Dingemans AM, van den Boogaart V, Vosse BA, van Suylen RJ, Griffioen AW, Thijssen VL. Integrin expression profiling identifies integrin alpha5 and beta1 as prognostic factors in early stage non-small cell lung cancer. Mol Cancer. 2010;9:152.

Fogal V, Richardson AD, Karmali PP, Scheffler IE, Smith JW, Ruoslahti E. Mitochondrial p32 protein is a critical regulator of tumor metabolism via maintenance of oxidative phosphorylation. Mol Cell Biol. 2010;30(6):1303-18.

Galluzzi L, Kepp O, Trojel-Hansen C, Kroemer G. Mitochondrial control of cellular life, stress, and death. Circ Res. 2012;111(9):1198-207.

Gerber DE. Targeted therapies: a new generation of cancer treatments. Am Fam Physician. 2008;77(3):311-9.

Gregory Maschek, Niramol Savaraj, Waldemar Priebe, Paul Braunschweiger, Kara Hamilton , George F. Tidmarsh , Linda R. De Young, Theodore J. Lampidis. 2-Deoxy-D-glucose Increases the Efficacy of Adriamycin and Paclitaxel in Human Osteosarcoma and Non-Small Cell Lung Cancers In Vivo. Cancer Res. 2004;64(1):31-4.

Guo JY, Chen HY, Mathew R, Fan J, Strohecker AM, Karsli-Uzunbas G, Kamphorst JJ, Chen G, Lemons JM, Karantza V, Coller HA, Dipaola RS, Gelinas C, Rabinowitz JD, White E. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. Genes Dev. 2011 Mar 1;25(5):460-70.

Hood JD, Cheresh DA. Role of integrins in cell invasion and migration. Nat Rev Cancer. 2002;2(2):91-100.

Hu F, Liu F. Mitochondrial stress: a bridge between mitochondrial dysfunction and metabolic diseases? Cell Signal. 2011;23(10):1528-33.

Imai K, Takaoka A. Comparing antibody and small-molecule therapies for cancer. Nat Rev Cancer. 2006;6(9):714-27.

Kim SA, Kwon SM, Kim JA, Kang KW, Yoon JH, Ahn SG. 5'-Nitro-indirubinoxime, an indirubin derivative, suppresses metastatic ability of human head and neck cancer cells through the inhibition of Integrin β1/FAK/Akt signaling. Cancer Lett. 2011;306(2):197-204.

Lee YC, Jin JK, Cheng CJ, Huang CF, Song JH, Huang M, Brown WS, Zhang S, Yu-Lee LY, Yeh ET, McIntyre BW, Logothetis CJ, Gallick GE, Lin SH. Targeting constitutively activated β1 integrins inhibits prostate cancer metastasis. Mol Cancer Res. 2013;11(4):405-17.

Lou F, Huang J, Sima CS, Dycoco J, Rusch V, Bach PB. Patterns of recurrence and second primary lung cancer in early-stage lung cancer survivors followed with routine computed tomography surveillance. J Thorac Cardiovasc Surg. 2013;145(1):75-81.

National cancer information center, Statistics in Cancer 2014, National cancer information center Korea, 2015

Olszewska A, Szewczyk A. Mitochondria as a pharmacological target: magnum overview. IUBMB Life. 2013;65(3):273-81.

Rohlena J, Dong LF, Neuzil J. Targeting the mitochondrial electron transport chain complexes for the induction of apoptosis and cancer treatment. Curr Pharm Biotechnol. 2013;14(3):377-89.

Sakkaraiappan Ramalingama, Chandra P Belanib. Paclitaxel for non-small cell lung cancer. Expert Opin Pharmacother. 2004;5(8):1771-80.

Simon L. Goodman, Martin Picard. Integrins as therapeutic targets. Trends Pharmacol Sci. 2012;33(7):405-12.

Trimmer C, Whitaker-Menezes D, Bonuccelli G, Milliman JN, Daumer KM, Aplin AE, Pestell RG, Sotgia F, Lisanti MP, Capozza F. CAV1 inhibits metastatic potential in melanomas through suppression of the integrin/Src/FAK signaling pathway. Cancer Res. 2010;70(19):7489-99.

Wang Y, Dong L, Cui H, Shen DH, Wang Y, Chang XH, Fu TY, Ye X, Yao YY. Up-regulation of mitochondrial antioxidation signals in ovarian cancer cells with aggressive biologic behavior. J Zhejiang Univ Sci B. 2011;12(5):346-56.

Weinberg F, Hamanaka R, Wheaton WW, Weinberg S, Joseph J, Lopez M, Kalyanaraman B, Mutlu GM, Budinger GR, Chandel NS. Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. Proc Natl Acad Sci U S A. 2010;107(19):8788-93.

Wu HC, Chang DK, Huang CT. Targeted Therapy for Cancer. . J. Cancer Mol. 2006;2(2): 57-66.

Y Storozhuk, S N Hopmans, T Sanli, C Barron, E Tsiani, J-C Cutz, G Pond, J Wright, G Singh, T Tsakiridis. Metformin inhibits growth and enhances radiation response of non-small cell lung cancer (NSCLC) through ATM and AMPK. Br J Cancer. 2013;108(10):2021–2032.

Yang GY, Xu KS, Pan ZQ, Zhang ZY, Mi YT, Wang JS, Chen R, Niu J. Integrin alphavbeta6 mediates the potential for colon cancer cells to colonize in and metastasize to the liver. Cancer Science. 2008;99(5):879-887.

국문초록

수술요법이나 화학·방사선 요법에도 불구하고 비소세포 폐암의 재발은 빈번하게 일어나는 실정이다. 그 중에서 암세포의 전이와 대사의 증가가 비소세포 폐암의 재발의 원인으로 알려져 있다. 따라서 본 연구에서는 비소세포 폐암의 전이 또는 대사의 증가를 억제하는 약물을 천연물 library를 스크리닝 함으로써 찾고자 하였다.

Integrin $\alpha 5\beta 1$은 전이된 비소세포 폐암세포에서 발현이 높게 나타날 뿐만 아니라 환자의 생존율도 크게 줄인다고 보고되었다. 따라서 비소세포 폐암의 전이를 억제하기 위한 신약의 target으로써 Integrin $\alpha 5\beta 1$을 선정 하였다. Integrin $\alpha 5\beta 1$을 표적으로 하는 천연물을 library로부터 찾아내기 위해, Integrin $\alpha 5\beta 1$의 기질인 fibronectin을 이용한 adhesion assay를 수행하였고 compound A를 찾아내었다. Compound A는 비소세포 폐암세포가 fibronectin 위에 부착하는 것을 억제하였을 뿐만 아니라 비소세포 폐암의 전이도 억제하는 것을 wound healing assay를 통해 확인할 수 있었다. 또한, compound A는 integrin $\alpha 5\beta 1$의 하위신호전달 단백질인 Focal Adhesion Kinase(FAK)과 Src 그리고 AKT 인산화를 억제하는 것이 본 연구를 통해 확인 되었다. 더 나아가, immunoprecipitation assay를 통해 compound A가 integrin $\alpha 5\beta 1$과 Src간의 결합을 억제함을 알 수 있었다. Swissdock 결과는 compound A와 integrin $\alpha 5\beta 1$의 결합가능성을 유의성 있게 보여주었다.

비소세포 폐암을 비롯한 암세포는 암유전자의 활성화 또는 종양억제유전자의 돌연변이로 인해 비정상적인 세포분열을 한다고 알려져 있다. 그

결과 암세포는 많은 양의 에너지를 필요로 하고, 세포 대사는 매우 활발하게 이루어지게 된다. 따라서, 암세포의 대사를 억제하는 것이 신약개발의 한 전략이 될 수 있다. 본 연구에서는 암 세포의 대사를 억제할 수 있는 천연물을 찾기 위해 luminescence ATP detection assay를 수행하였고 compound B를 찾아내었다. Compound B는 비소세포 폐암의 대사를 효과적으로 억제하여 세포 내의 ATP 양을 줄였을 뿐만 아니라 에너지 항상성 센서 단백질인 AMPK의 인산화를 일으키는 것을 확인하였다. Compound B가 미토콘드리아를 손상시킴으로써 세포 내 에너지 대사를 억제하는지 확인해 본 결과, 미토콘드리아의 shrinkage를 일으키며 탈분극을 일으키고 ROS생성을 증가시키는 것을 확인할 수 있었다.

Compound A와 compound B 모두 비소세포 폐암의 proliferation 그리고 colony formation을 억제하였으며 세포 사멸을 유도하는 것을 확인할 수 있었다. 연구결과를 종합하여 볼 때, compound A와 compound B는 천연물 유래의 잠재적 신약후보 물질로 제시될 수 있을 것으로 기대된다.

주요어: 비소세포폐암, Integrin $\alpha 5\beta 1$, 미토콘드리아, 암 전이, 암 대사
학번: 2014-20336