의학석사학위논문

Studies on the tumor invasion through JAK2-STATs pathway inhibition by ginsenoside 20(S)-Rh2 in human colorectal cancer cells

대장암세포에서 진세노사이드 20(S)-Rh2 의 JAK2-STATs 경로 억제에 따른 암 침윤에 관한 연구

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한 송 희

A thesis of the Master's Degree

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The Department of Biomedical Sciences

Seoul National University

College of Medicine

Songhee Han

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ABSTRACT

Ginsenoside Rh2 has been reported to inhibit cancer cell growth and induce apoptosis. The Rh2 has two stereoisomeric forms, 20(S) and 20(R) that have been reported different biological activities. 20(S)-Rh2 mainly has been focused of its anti-cancer and anti-inflammatory effects. However, it is poorly understood whether 20(S)-Rh2 and 20(R)-Rh2 can suppress tumor invasion in human colorectal cancer cells. Here, this study demonstrated that 20(S)-Rh2 strongly inhibited IL-6-induced signal transducer and activator of transcription 3 (STAT3) activation than that of 20(R)-Rh2. 20(S)-Rh2 inhibited STAT3 activation in HCT116 cells when cells were incubated with or without IL-6 stimulation. In addition, this compound effectively inhibited the levels of matrix metalloproteinases (*MMPs*), including *MMP-1, -2, -3* and *-9* that are known to be regulated by STAT3, resulting in suppression of the tumor cell invasion in colorectal cancer cells. In parallel, the expression levels of *MMPs* and tumor cell invasion were suppressed by siSTAT3. The pharmacological activities of 20(S)-Rh2 were identified to be associated with inhibition of the STAT3 upstream regulator JAK2 activation. Interestingly, these inhibitory activities of 20(S)-Rh2 were more potent than those of 20(R)-Rh2. This study further found that 20(S)-Rh2 synergistically enhanced cytotoxicity of HCT116 cells in combinational treatment with other anti-cancer agents, suggesting ginsenoside 20(S)-Rh2 has a promising therapeutic potential against colorectal tumor metastasis as well as to be a combinational partner with classic chemotherapeutic agents in treatment of colorectal cancer.

Keywords: 20(S)-Rh2, 20(R)-Rh2, STAT3, IL-6, invasion, colorectal cancer

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CONTENTS

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

CRC : Colorectal cancer

STAT3 : Signal transducer and activator of transcription 3

IL-6 : Interleukin 6

JAK : Janus kinase

MMP : Matrix metalloproteinase

INTRODUCTION

Ginseng, the root of *Panax ginseng C.A. Meyer*, is one of the most famous medicinal plants. It has been used for a long time in Oriental medicine for the treatment of various human diseases, especially cancer. Ginsenosides are the main effective components of ginseng extracts and have multiple biological activities without influencing normal cells. (1, 2) However, the bioavailability of ginsenosides in white ginseng is low because of incomplete compound absorption. Compared with white ginseng extracts, red ginseng extracts represented stronger anti-cancer activities, especially in colorectal cancer. The red ginseng has been shown the ability of cancer prevention and also treatment for colorectal cancer. (3, 4) The Rh2 is one of the major effective components of red ginseng extracts and classified into 20(S) and 20(R), according to the orientation of hydroxyl group of carbon at R3 position. Depending on the difference of stereochemistry, two stereoisomeric forms represent distinct biological effects. These Rh2 have been known for its anti-cancer effect by inhibiting cell growth and inducing apoptosis. (5, 6) The Rh2 reduces cell proliferation through modulation of mitogen-activated protein kinases (MAPKs) in prostate cancer cells and arresting G1 to S phase cell cycle in breast and intestinal cancer cells. These compounds can also increase apoptosis by activation of caspase-3 and Fas signaling pathway in hepatoma and colon cancer cells. (5) Recent studies mainly have been focused on the anti-cancer effects of 20(S)-Rh2. However, these biological effects of 20(S)-

Rh2 and 20(R)-Rh2 are poorly identified in colorectal cancer cell invasion and signaling cascades.

Colorectal cancer (CRC) has a high incidence rate worldwide and mortality with malignancy as lethal cancer. Occurrence rate of CRC increases annually and almost 50% of CRC patients eventually die from this cancer. (7) The main reason of cancer-related death is not the primary tumor, but the distant metastasis. It has been demonstrated that tumor invasion plays a critical role in CRC malignancy by contributing to tumor metastasis. Colorectal cancer is prone to metastasis, which is the main cause for CRC patients who were related with death within 5 years after diagnosis. (8, 9) However, there is no clear developed therapy for CRC treatment even surgical treatment after the cancer metastasis. Many metastatic CRC patients rely on substitute treatment for conventional chemotherapy using natural products such as ginseng. Since conventional therapy agents for CRC have side effect and limited efficacy, study for the development of new treatment is necessary in order to increase the survival of CRC patients. Ginsenoside has been studied as an alternative treatment in CRC reducing cancer cell proliferation and using as chemoprevention. (3, 4) Recent evidence suggests that signal transducer and activator of transcription 3 (STAT3) can be a therapeutic target for CRC treatment. STAT3 activation has been known as a major factor in colon carcinogenesis and is also involved in the process of metastasis. (10)

The transcription factor STAT3 is crucial for cellular responses to cytokines and growth factors in normal conditions which, contributes to a numbers of biological functions such as cell differentiation, proliferation,

development, hematopoiesis, and immune responses. (11) However, consistently activated STAT3 signaling is observed in human cancer patients and tumor-derived cell lines which implies the importance of STAT3 activation in tumorigenesis and cancer progression. These evidences indicate that inappropriate activation of STAT3 signaling occurs with surprisingly high frequency in human cancers, including CRC. (7, 12) Therefore, regulation of abnormally activated STAT3 signaling is valuable therapeutic targets for the treatment of human cancers. Binding of ligands to their cognate receptors phosphorylates tyrosine-705 residue in STAT3 protein, forms stable homo- or hetero-dimers with other STAT proteins, translocates to the nucleus, and binds to specific promoter region of DNA. (13, 14) A series of signaling cascades regulate the expression of a wide range of genes, including oncogenic genes, which are associated with cell proliferation, survival, migration, invasion, and tumor metastasis. Interleukin-6 (IL-6) is a well-known and most studied cytokine in tumor-associated STAT3 signaling. (15) A major signature cytokine in tumor development and metastasis, IL-6 is a pro-inflammatory cytokine associated in invasive colorectal cancer through crosstalk between tumor and immune cells in tumor microenvironment. (16) In addition, IL-6 activates STAT3 signaling that is associated with tumorigenesis, especially intestinal and metastasis. (17, 18) Actually, 72.6% of primary colon carcinoma tissues showed immunopositivity for phosphorylated STAT3 and also correlated with the expression of IL-6 in invasive cancer phenotype. (19) These findings suggest that IL-6-induced STAT3 signaling is activated and correlates with tumor invasion in CRC. (20)

In this study, I demonstrated the inhibitory effects of 20(S)-Rh2 and 20(R)-Rh2 on STAT3 activation and tumor invasion in CRC. 20(S)-Rh2 effectively inhibited STAT3 activation in CRC cells independent of IL-6 stimulation. This result was associated with the attenuation of STAT3 upstream regulator JAK2 activation. As a results, 20(S)-Rh2 inhibited the expression of STAT3 target genes matrix metalloproteinases (*MMPs*), including *MMP-1, -2, -3* and *-9*, resulting in suppression of cancer cell invasion. These inhibitory effects of 20(S)-Rh2 were more potent than those of 20(R)-Rh2. I further observed that 20(S)-Rh2 sensitized synergistic cytotoxicity of other anti-cancer agents, such as cisplatin and doxorubicin in colorectal cancer cells. These results suggest that ginsenoside 20(S)-Rh2 is a promising therapeutic candidate with combinational treatment in colorectal tumor metastasis and may be relevant for the treatment in patients with CRC.

MATERIALS AND METHODS

Cell lines and culture conditions

Human colorectal cancer cell line HCT116 was purchased from the Korean Cell Line Bank (KCLB, Korea) and maintained in RPMI media (Life Technologies, Inc., Gaithersbrug, MD, USA) containing 10% fetal bovine serum (FBS, Life Techologies Inc.) and 1% penicillin/streptomycin solution (Life Technologies Inc.). Another human colorectal cancer cell line SW620 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), and maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS and 1% penicillin/streptomycin solution. These cells were cultured at 37°C with 5% CO_2 atmosphere in a humidified incubator.

Western blot analysis

After treatment, cells were washed twice with ice-cold PBS, and lysed in a lysis buffer (50mM Tris-HCl, pH 7.4, 350 mM NaCl, 0.5% NonidetP-40, 10% glycerol, 0.1% sodium dodecyl sulfate (SDS), and 1% Triton X-100). The lysates were collected by centrifuge for 20 min at 13,000 rpm at 4°C and boiled with SDS loading buffer. The amounts of protein were quantified using a bio-rad protein assay (Bio-rad, Hercules, CA, USA). Proteins were separated by SDS-poly acrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose (NC) membranes (Whatman, Atlanta, GA, USA). The membranes were blocked in blocking buffer (5% skim milk in 150 mM NaCl, 25 mM Tris, pH 7.4, and 0.1% Tween20) and subsequently

incubated with specific primary antibodies for the target molecules. The membranes were then washed with Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and further incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. After washing with TBS-T, the signals were visualized using the Super Signal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

Cell viability assay

The cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Sigma-aldrich, Saint Louis, MO, USA). To examine the effects of reagents on cell viability, cells were seeded at a density of 10,000 cells per well in 96-well plates. After overnight, the cells were incubated with either dimethyl sulfoxide (DMSO) as a vehicle or various concentrations of 20(S)-Rh2 or 20(R)-Rh2 in the presence or absence of anti-cancer agent for 24h. After add 100 μl of MTT (5 mg/ml), the cells were further incubated for 4 h at 37ºC incubator and removed the MTT solution. The MTT formazan was dissolved in DMSO and absorbance was measured at 540nm with an ELISA plate reader (Molecular Devices, CA, USA).

RNA isolation and quantitative real-time PCR

Total RNA was isolated using a Trizol reagent (Favorgen biotech corp., Taiwan) and cDNA was synthesized using ReverTra Ace qPCR RT Master Mix kit (TOYOBO, Osaka, JAPAN). Quantitative real time-PCR was

performed using the SYBR Green PCR mix (Abm, Richmond, Canada) with an Applied Biosystems 7300 Real-time PCR system (Life Technologies, Inc.). Primers were purchased from Qiagen (Qiagen, Valencia, CA, USA) and the raw data were analyzed using comparative Ct quantification.

Cell invasion assay

Cell invasion assay was performed in a Boyden chamber system (Neuro Probe, Gaithersburg, MD, USA). Cells (4×10^4) were placed on top of matrigel (BD Biosciences, San Jose, CA, USA) layer inside the chamber with 100 μl of RPMI containing 1% FBS in the presence or absence of 20(S)-Rh2 or 20(R)-Rh2. The lower well was filled with 500 μl of 10% RPMI containing epidermal growth factor (EGF, 5 ng/ml). Chambers then incubated at 37°C for 48 h. After migration of cells, matrigel in upper chamber was wiped off. The upper chamber was dipped into fixer and transferred into Diff-Quik solution (Sysmex Corp. Kobe, Japan). The membranes detached from upper chamber were rinsed in distilled water to remove excess stain and gently moved onto slideglass. The migrated cells were captured using an inverted microscope (Olympus, Germany).

Statistical analysis

Data are represented as the mean ±standard error of the mean (SEM). Statistical significance was determined by a two-tailed Student's *t*-test and analyzed Graph Pad Prism 6 (Graph Pad Software, Inc., San Diego, CA, USA). The differences were considered significant for $p<0.05$.

RESULTS

Differential cytotoxic effects of ginsenosides, 20(S)-Rh2 and 20(R)-Rh2

Ginsenosides are the main components that are extracted from ginseng. Among the ginsenosides, Rh2 is a major effective component because of its high bioavailability and contains two stereomeric forms as alpha and beta by different arrangement of hydroxyl group at C-20 position. 20(S)-Rh2 contains beta form, while 20(R)-Rh2 contains alpha form, respectively (Figure 1A). Although numerous biological activities of Rh2 were identified, the differential biological activities are not well understood. To compare their differential activities, I first performed MTT assay to determine the effects of 20(S)- and 20(R)-Rh2 on cell viability. The 20(S)-Rh2 exhibited cytotoxic activity *in vitro* at concentrations of 15 to 30 μM in colorectal cancer cell line HCT116 when cells were incubated for 24 h. However, 20(R)-Rh2 did not affect cell viability at concentrations of up to 30 μM (Figure 1B). Cell morphology was photographed when cell were incubated with either 20(S)-Rh2 or 20(R)-Rh2 at 10 μM for 24 h. Consistent with MTT assay, the cell viability and morphology were not influenced, compared with DMSO-treated group (Figure 1C). These results suggest that the biological activities of Rh2 may depend on the position of hydroxyl group at C-20. To exclude cytotoxic effects of 20(S)-Rh2, the following *in vitro* experiments were performed up to 10 μM concentration of 20(S)-Rh2.

20(S)-Rh2 inhibited STAT3 activation in CRC cells

To verify the ability of 20(S)- and 20(R)-Rh2 in CRC cells, I first examined the effects of both Rh2 compounds on STAT3 phosphorylation, which contributes to CRC malignancy. (21) 20(S)-Rh2 effectively inhibited tyrosine phosphorylation of STAT3 in a dose- and time-dependent manner without affecting total STAT3 expression in CRC cell line HCT116 (Figures 2A and B). STAT3 phosphorylation was dramatically inhibited at 5 and 10 μM of 20(S)-Rh2 for 3 h of incubation. IL-6 plays an important role in activating STAT3 signaling and contributes tumor progression and metastasis in CRC. Indeed, elevated IL-6 expression was observed in patients with colorectal cancer tissues, suggesting the importance of IL-6 in CRC initiation and development. (18, 22) Therefore, I investigated whether 20(S)- and 20(R)-Rh2 could inhibit IL-6-induced STAT3 activation in HCT116 cells. Western blot analysis showed that 20(S)-Rh2 effectively inhibited IL-6-induced tyrosine phosphorylation of STAT3 in a dose- and time-dependent manner and these results were similar with those of endogenous STAT3 activation (Figures 2C and D). Although this compound effectively inhibited tyrosine phosphorylation of STAT3 in both CRC cell lines HCT116 and SW620, 20(R)-Rh2 exhibited no inhibitory activity in those cells. Interestingly, 20(S)-Rh2 did not affect phosphorylation of serine residue in STAT3. The inhibitory effects of both Rh2 compounds were compared with AG-490, a pan-JAK inhibitor. AG-490 effectively inhibited STAT3 phosphorylation in both cells. (Figures 2E and F). These results indicate that 20(S)-Rh2 is a small molecule

STAT3 inhibitor, specifically by inhibition of tyrosine residue, but not serine residue.

20(S)-Rh2 inhibited JAK-2-mediated STATs signaling cascades

Janus kinases (JAKs) are upstream signaling of cytokine-induced STAT activation. Specifically, JAK2 is an important upstream signaling of IL-6-mediated STAT3 activation. I therefore examined whether inhibition of STAT3 activation by 20(S)-Rh2 is mediated by its upstream JAK2 signaling. JAK2 phosphorylation was markedly increased by IL-6 stimulation in HCT116 cells, whereas 20(S)-Rh2, similarly to AG-490, inhibited this phosphorylation of JAK2, but not that of 20(R)-Rh2. (Figure 3A). In parallel with this result, 20(S)-Rh2 effectively inhibited tyrosine phosphorylation of IL-6-induced STAT1 and STAT3. However, 20(R)-Rh2 did not inhibit phosphorylation of these proteins (Figure 3B). These results indicated that the inhibition of STAT1 and STAT3 activation by 20(S)-Rh2 was involved in suppression of JAK2 phosphorylation.

Targeted-STAT3 silencing suppressed *MMPs* expression and tumor cell invasion

Activation of STAT3 signaling cascades increases migration and invasion of tumor cells by induction of various genes such as *MMPs*, resulting in tumor metastasis. However, STAT1 signaling has been known for no significant influence on *MMPs* expression and tumor metastasis. (23, 24) To examine the functional role of STAT3 in CRC invasion, STAT3 levels were silenced by transfection with STAT3-specific siRNA in HCT116 cells. STAT3 expression and activation were effectively silenced by STAT3-specific siRNA, which was identified by RT-PCR and Western blot analysis (Figures 4A and B). To investigate the functional role of STAT3 in CRC, quantitative real-time PCR was performed to measure the mRNA levels of *MMPs*, which are STAT3 target genes and essential for tumor cell invasion in CRC. (21) The mRNA levels of *MMPs*, including *MMP-1, -2, -3* and *-9* were effectively suppressed by STAT3 knockdown (Figure 4C). In addition, CRC cell invasion was also decreased by STAT3 knockdown in the cells, compared with control siRNA (Figure 4D). These results clearly indicate that activation of STAT3 and downstream signaling play important role in CRC invasion and targeting this signaling is a promising therapeutic potential in patients with CRC.

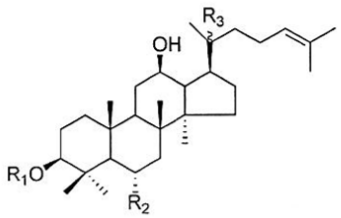
20(S)-Rh2 inhibited *MMPs* expression and tumor cell invasion

The aforementioned results suggested that STAT3 activation is essential for CRC invasion and suggested to determine whether Rh2 compounds could inhibit *MMPs* expression and tumor cell invasion. Stimulation of IL-6 increases the mRNA levels of *MMPs*, including *MMP-1, -2, -3* and *-9* in HCT116 cells. I observed that treatment with 20(S)-Rh2 effectively inhibited the levels of these genes and the inhibitory activities were much stronger than those of 20(R)-Rh2 (Figures 5A-D). In the case of *MMP3*, 20(R)-Rh2 revealed a significant decrease (Figure 5C). To identify the functional effects of Rh2 compounds, I performed a matrigel invasion assay. Results showed that CRC cell invasion was increased by IL-6 stimulation, compared with DMSO-treated control group. Treatment with 20(S)-Rh2 dramatically suppressed tumor cell invasion, while 20(R)-Rh2 exhibited marginally to no inhibitory effect (Figure 5D). These results indicate that the inhibition of STAT3 signaling by 20(S)-Rh2 plays an important role in the suppression of CRC invasion and tumor metastasis.

Combination with 20(S)-Rh2 and anti-cancer reagents enhanced anti-cancer effect

To investigate whether the combination therapy of 20(S)-Rh2 with other anti-cancer agents can increase the cytotoxic effects on human colorectal cancer cells, MTT assay was performed on HCT116 cells. HCT116 cells were treated with 5 or 10 μM of 20(S)-Rh2 in combination with either cisplatin or doxorubicin with various concentrations. The combination with 20(S)-Rh2 exhibited stronger cytotoxic activities than single treatment of each anti-cancer reagent when cells were incubated for 24 h (Figure 6). As shown the results, combination treatment with 5 μM of 20(S)-Rh2 and 2.5 μM of cisplatin or doxorubicin decreased HCT116 cell viability similarly when treated with 5 μM of each agent by itself. Furthermore, 1.25 μM of cisplatin or doxorubicin with 10 μM of 20(S)-Rh2 have decreased cell viability similarly to 5 μM of each agent. 20(S)-Rh2 can reduce the treatment concentration of anti-cancer agents, acting synergistically with conventional chemotherapeutic agents on HCT116 cells. These results suggested that 20(S)-Rh2 might have potential therapeutic benefits in combinational treatment with other anti-cancer agents to increase the therapeutic effects in CRC.

(A)



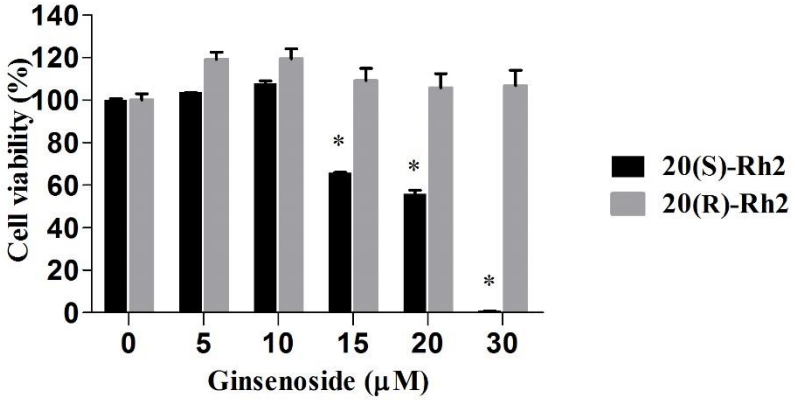
20(S)-Rh2

R1: Glc
R2: H
R3: b-OH

20(R)-Rh2

R1: Glc
R2: H
R3: a-OH

(B)



(C)

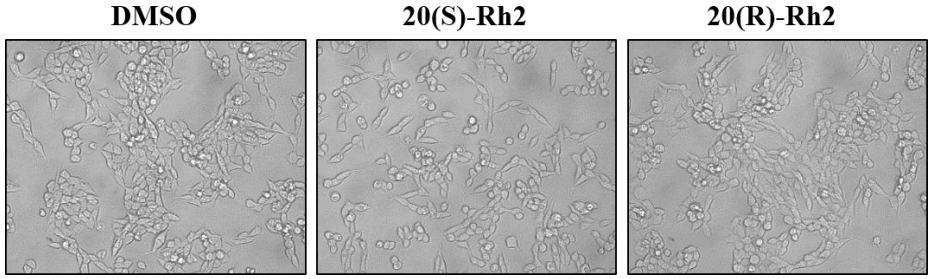


Figure 1. Differential cytotoxic effects of ginsenosides, 20(S)-Rh2 and 20(R)-Rh2. (A) Chemical structures of two stereomeric forms of ginsenoside Rh2. (B) HCT116 cells were treated with various concentrations of 20(S)-Rh2 or 20(R)-Rh2 as Rh2 stereoisomers for 24 h, and cell viability was quantified by MTT assay. (±SEM indicated by error bar) (C) Morphologic changes were photographed when HCT116 cells were incubated with 20(S)-Rh2 or 20(R)-Rh2 at 10 μM for 24 h. *$p<0.05$ compared with DMSO-treated group. .

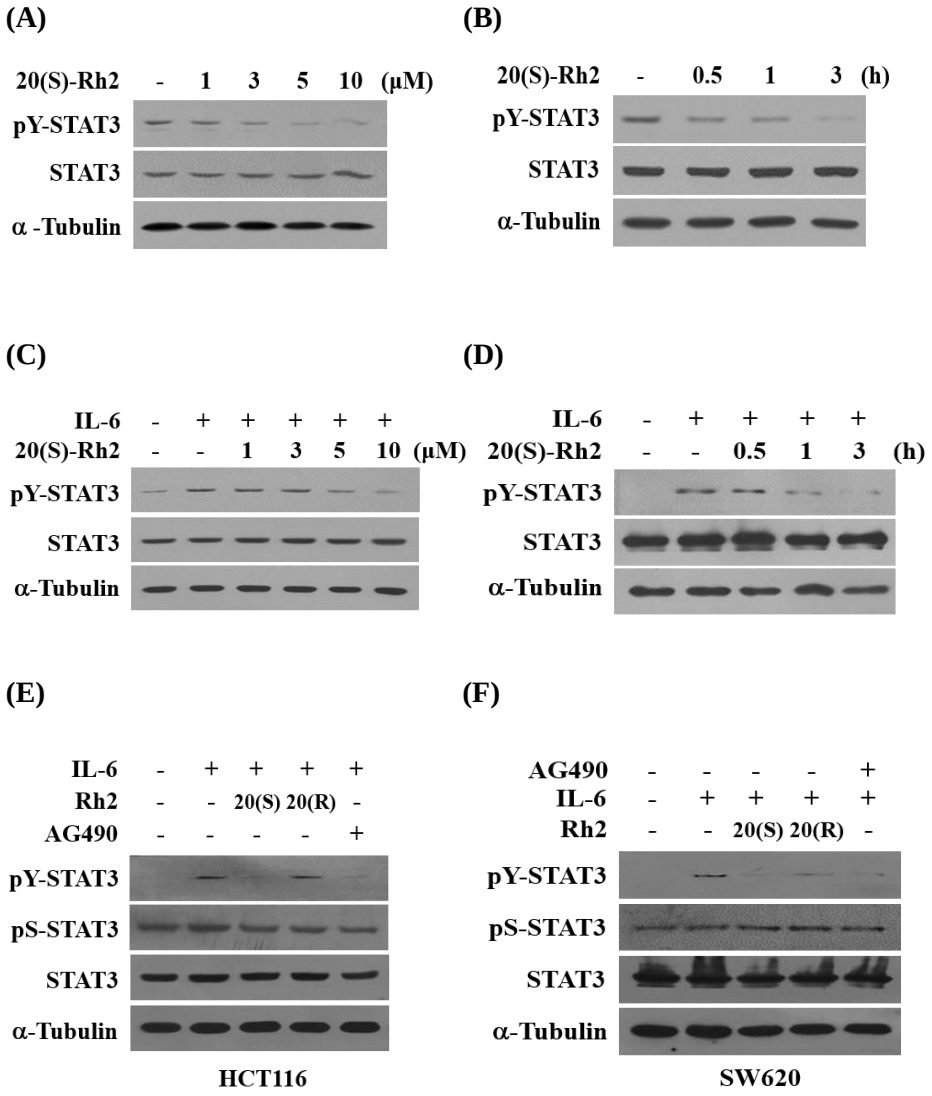

(A)
20(S)-Rh2 - 1 3 5 10 (μM)
pY-STAT3
STAT3
α -Tubulin
(B)
20(S)-Rh2 - 0.5 1 3 (h)
pY-STAT3
STAT3
α-Tubulin
(C)
IL-6 - + + + + +
20(S)-Rh2 - - 1 3 5 10 (μM)
pY-STAT3
STAT3
α-Tubulin
(D)
IL-6 - + + + +
20(S)-Rh2 - - 0.5 1 3 (h)
pY-STAT3
STAT3
α-Tubulin
(E)
IL-6 - + + + +
Rh2 - - 20(S) 20(R) -
AG490 - - - - +
pY-STAT3
pS-STAT3
STAT3
α-Tubulin
HCT116
(F)
AG490 - - - - +
IL-6 - + + + +
Rh2 - - 20(S) 20(R) -
pY-STAT3
pS-STAT3
STAT3
α-Tubulin
SW620

Figure 2. 20(S)-Rh2 inhibited STAT3 activity in dose- and time-dependent manner. HCT116 Cells were treated with 20(S)-Rh2 in dose-dependent manner for 3 h (A), and in time-dependent manner at 10 μM (B). IL-6 (20 ng/ml) was treated for 30 min, after HCT116 Cells were pre-treated with 20(S)-Rh2 in dose-dependent manner (C), and in time-dependent manner (D). (E and F) HCT116 cells and SW620 cells were pre-treated with 20(S)-Rh2 or 20(R)-Rh2 (10 μM) for 3 h and stimulated by IL-6. AG490 (200 μM), which is JAK2 inhibitor, was used as a negative control. Protein extracts were lysed and phosphorylation of STAT3 was estimated by western blotting.

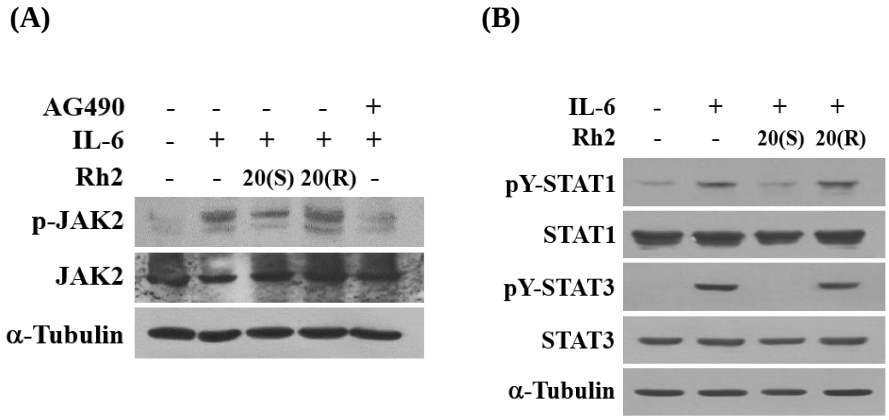
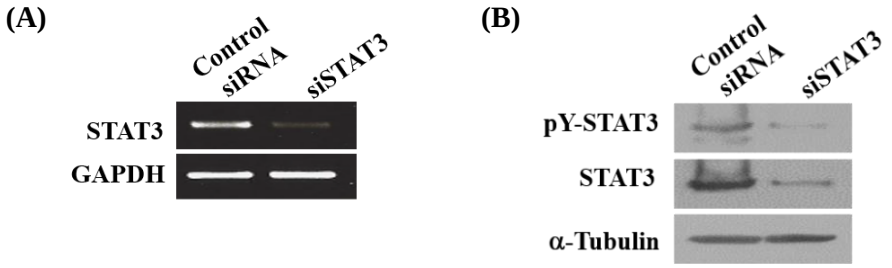
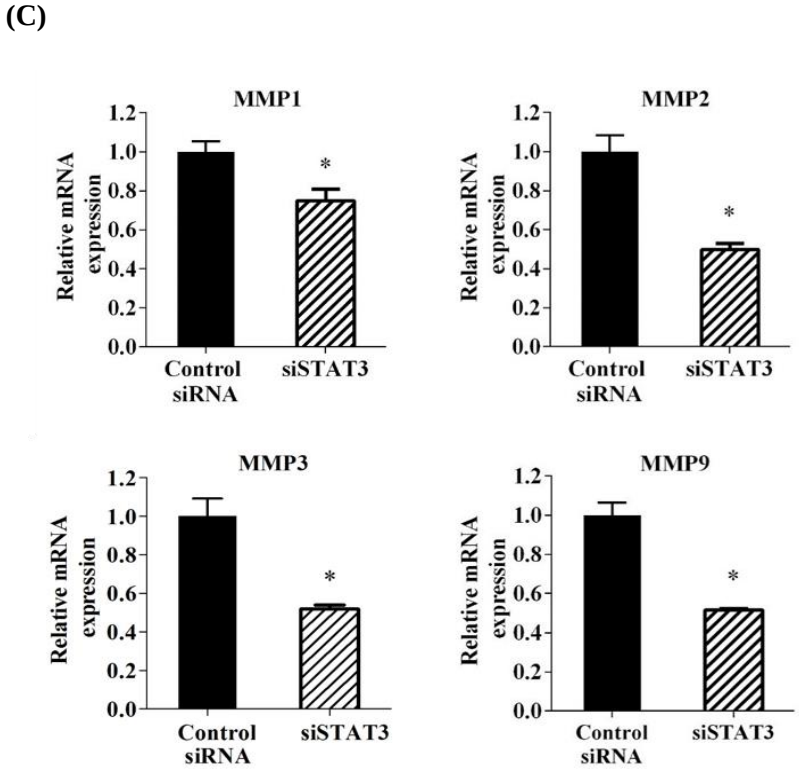


Figure 3. 20(S)-Rh2 inhibited JAK2 activation and JAK2-mediated STAT activation. (A and B) Pre-treated HCT116 cells with 20(S)-Rh2 or 20(R)-Rh2 (10 μM) for 3 h were stimulated with IL-6 (20 ng/ml) for 10 min (A), or for 30 min (B). Upstream regulator JAK2 activation and the phosphorylation of STAT1 and STAT3 were detected by western blotting.


(A)
Control siRNA
siSTAT3
STAT3
GAPDH
(B)
Control siRNA
siSTAT3
pY-STAT3
STAT3
α-Tubulin


(C)
MMP1
MMP2
MMP3
MMP9
Relative mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control siRNA
siSTAT3

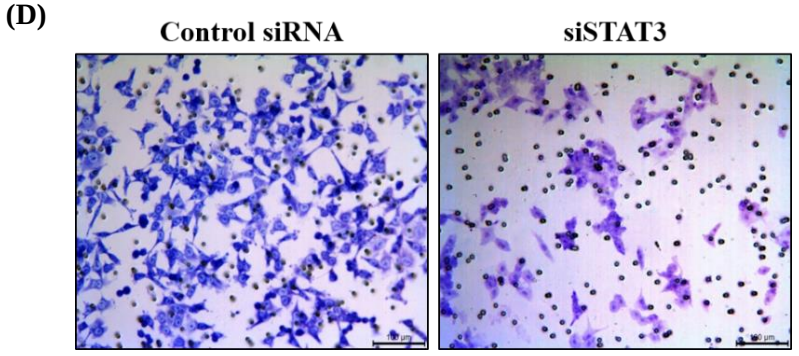

(D)
Control siRNA
siSTAT3

Figure 4. Targeted-STAT3 silencing suppressed *MMPs* expression and tumor cell invasion in HCT116 cells. HCT116 cells were transfected with siSTAT3 (50 nM) and control siRNA (50 nM, negative control). After transfection, total RNA was isolated and STAT3 levels were confirmed by RT-PCR (A) and western blot analysis (B). (C) Quantitative real-time PCR was performed to measure the levels of matrix metalloproteinases (*MMPs*) in siSTAT3 or control siRNA transfected cells. (D) siSTAT3 or control siRNA transfected cells were seeded into the matrigel-coated membrane. After 48 h, invading cells were fixed and stained using Diff-Quick solution (10x magnification). * $p<0.05$ compared with control siRNA group.

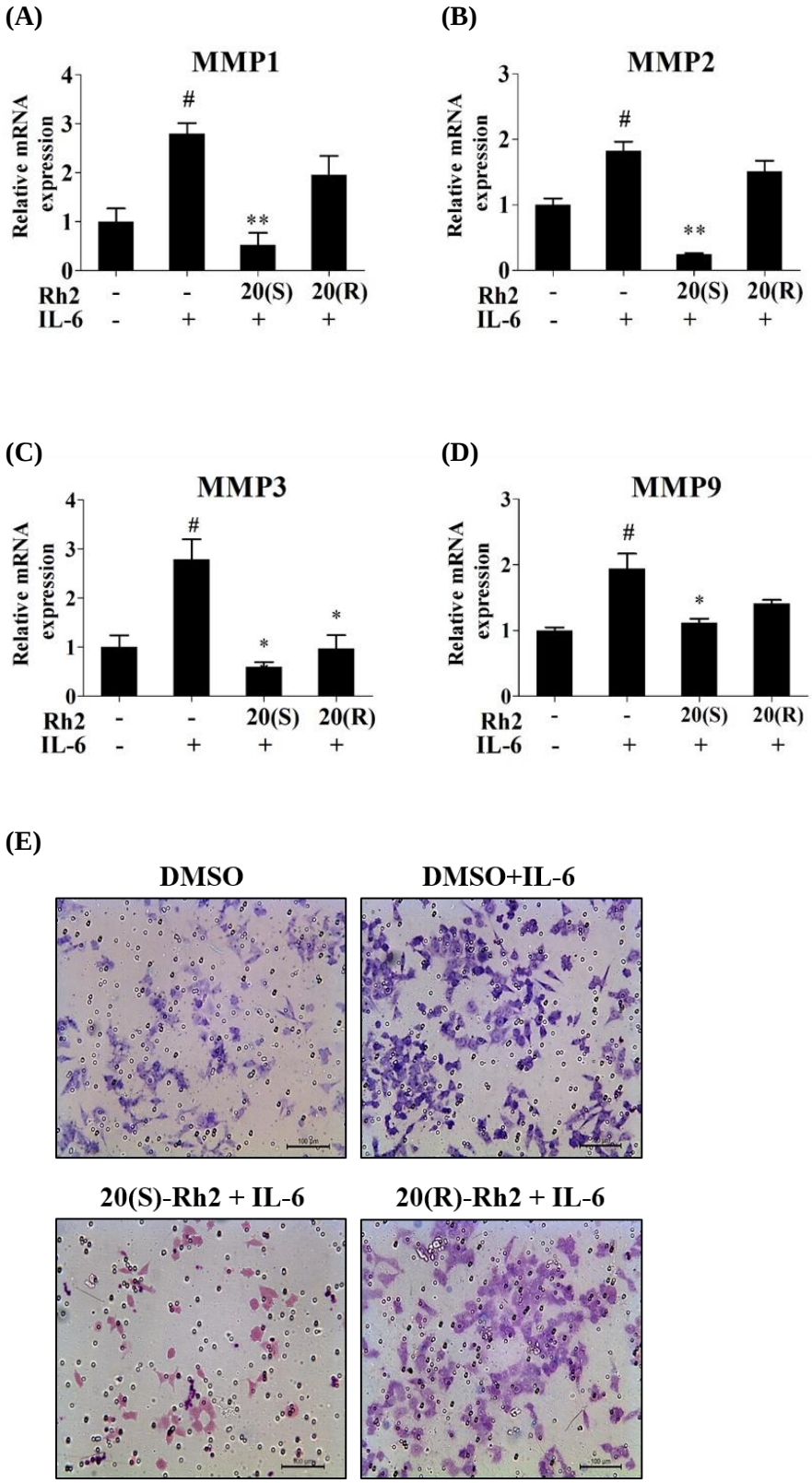
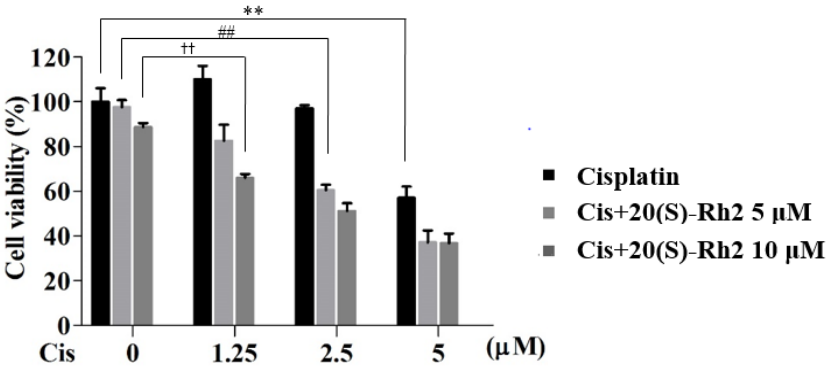

(A)
MMP1
Relative mRNA expression
Rh2 - - 20(S) 20(R)
IL-6 - + + +
(B)
MMP2
Relative mRNA expression
Rh2 - - 20(S) 20(R)
IL-6 - + + +
(C)
MMP3
Relative mRNA expression
Rh2 - - 20(S) 20(R)
IL-6 - + + +
(D)
MMP9
Relative mRNA expression
Rh2 - - 20(S) 20(R)
IL-6 - + + +
(E)
DMSO
DMSO+IL-6
20(S)-Rh2 + IL-6
20(R)-Rh2 + IL-6

Figure 5. 20(S)-Rh2 inhibited IL-6-induced *MMPs* expression and tumor cell invasion. (A-D) Pre-treated HCT116 cells with 20(S)-Rh2 or 20(R)-Rh2 (10 μM) were stimulated with IL-6 (20 ng/ml) for 6 h and total RNA were isolated. Quantitative real-time PCR was performed to measure the levels of *MMP-1, -2, -3* and *-9*. (E) Pre-treated HCT 116 Cells with 20(S)-Rh2 or 20(R)-Rh2 were seeded into the matrigel-coated membrane. After 48 h of IL-6 stimulation, invading cells were fixed and stained using Diff-Quick solution (10x magnification). # $p<0.05$ compared with control group, and * $p<0.05$ and ** $p<0.005$ compared with IL-6-stimulated group.

(A)



(B)

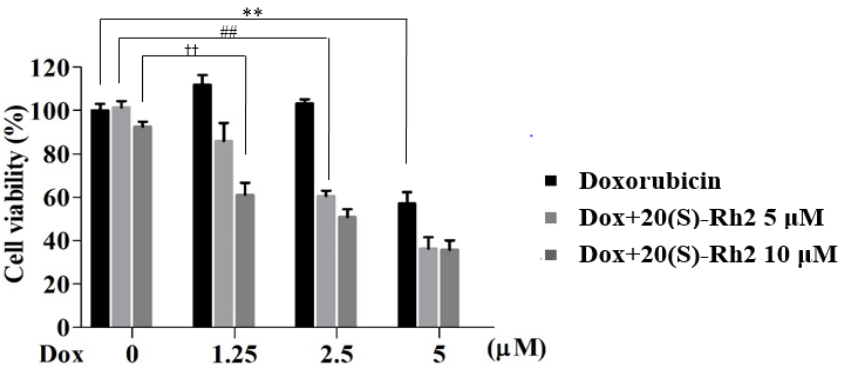


Figure 6. Combination with 20(S)-Rh2 and anti-cancer reagents enhanced synergistic cytotoxicity in HCT116. HCT116 cells were treated with 5 or 10 μM of 20(S)-Rh2 with or without different concentrations of other anti-cancer agents, such as cisplatin (A), doxorubicin (B) for 24 h. Cell viability was quantified by MTT assay (± SEM indicated by error bar). **p<0.005 compared with vehicle-treated group. ##p<0.005 compared with 20(S)-Rh2 5 μM treated group. ††p<0.005 compared with 20(S)-Rh2 10 μM treated group.

DISCUSSION

Most of ginsenosides have low absorption rate of 2%, this results in reduced bioavailability. However, Rh2, which is obtained from red ginseng, is characterized by its high bioavailability due to 98% of absorption rate. The Rh2 is a derivative of ginsenosides that is responsible for various biological effects of ginseng, including anti-cancer, anti-inflammatory and anti-photoaging activities. (2) Although two stereoisomers of Rh2 are identified, the differences of biological activities and anti-tumor activities between the isomers in CRC are not fully identified. In this study, I investigated the biological activities of both Rh2 compounds in human colorectal cancer cells. 20(S)-Rh2 inhibited JAK2-mediated STATs activation and also downstream signaling of STAT3, resulting in suppression of cancer cell invasion. These biological activities of 20(S)-Rh2 were much stronger than those of 20(R)-Rh2.

To the best of our knowledge, no previous investigations have indicated that 20(S)-Rh2 can inhibit STATs activation, despite activation of STATs and downstream signaling being pivotal roles in tumor invasion and metastasis. (25, 26) Activation of STATs signaling cascades is observed in many kinds of human cancers and tumor-derived primary cancer cells, especially indicating the importance of STAT3 signaling in cancers. (27) Abrogation of STAT3 signaling by small molecule inhibitor, such as STA-21, S31-201 induces growth arrest, apoptosis and tumor invasion in various types of tumors. This STAT3 is constitutively activated by IL-6 in CRC. A multifunctional cytokine

IL-6 plays a central role in the regulation of inflammatory and immune responses. (28) It also plays as a potent STAT3 activator and is highly released in response to various inflammatory stimuli such as infection and inflammation. Extensive surveys with clinical studies demonstrated that elevated levels of IL-6 and STAT3 activation are observed in various types of primary tumors, including sporadic and inflammation-associated CRC. (26, 29, 30) In addition, STAT1 can be one of the factors on carcinogenesis and survival of tumor cells under specific conditions, such as chronic inflammation in CRC. (24) In my study, 20(S)-Rh2 effectively inhibited STAT1 and STAT3 activation in colorectal cancer cell lines HCT116 and SW620 *via* inhibition of upstream regulator JAK2. The potential of 20(S)-Rh2 on STATs inhibition was similar when cells were activated constitutively or stimulated with IL-6 in Figures 2 and 3. These findings suggested that 20(S)-Rh2 had effects on CRC through inhibition of STATs.

CRC is characterized as an invasive tumor and spread to other organs, such as the lung and the liver, eventually resulting in poor prognosis. Metastatic CRC has been considered as one of the most lethal cancers. (3) Invasion of CRC cells is the first step to tumor metastasis and often associated with treatment failure despite improvement of therapeutic process. (8) These IL-6 and STAT3 are important factors for tumor progression and metastasis of CRC cells, and related to advanced stage of disease and decreased survival in CRC patients. (20, 29, 31) Accordingly, inhibition of STAT3 activation is a promising target than that of STAT1 activation to increase the survival of

CRC patients. Therefore, development of new treatment targeting IL-6/STAT3 signaling is needed to treat malignant human colorectal cancer.

To identify novel compounds that possess the potential of inhibiting abnormally activated IL-6/STAT3 signaling, ginsenosides, the most prominent constituent of ginseng saponin, was a potential candidate because of its well-known anti-cancer effect. Ginsenosides have been reported that these compounds reduce proliferation and sensitize cancer cells to chemotherapy in cancer cells, such as colorectal cancer cells. (32) These results suggested that ginsenosides have potential for substitute or supplement agents in colorectal cancer. The Rh2 is especially one of the main active components among the ginsenosides and wide ranges of biological functions have been reported. (5, 6) However, the roles of ginsenosides on STAT3 signaling were not fully understood and, specifically, no evidences of Rh2 in CRC. The Rh2 classified into alpha and beta forms as stereoisomers by different arrangement of hydroxyl group at C-20. Interestingly, two isoforms of Rh2 exhibited differential biological activities in CRC. In the present study, the beta form of Rh2, 20(S)-Rh2 effectively inhibited STAT3 activation and downstream signaling cascades through targeting upstream JAK2 activation, and exhibited strong cytotoxic activity at concentrations among 10~15 μM. However, alpha form of Rh2, 20(R)-Rh2 exhibited weak to inhibitory activities and no cytotoxic activity at concentrations of up to 30 μM. (Figures 1B and 3) It was also demonstrated that stereoisomers of Rh2 have different activities on cytotoxicity and STAT3 activation in CRC. These data provided that 20(S)-Rh2 had anti-cancer effect through inhibition of STAT3 activation.

The important features of colon cancer initiation and development are chronic inflammation and tumor-elicited inflammation, which results in colitis-associated colon cancers (CAC) and sporadic colon cancer (CRC). (33) Under this circumstances, many inflammatory mediators are produced and tumor microenvironment is being formed. These conditions sustain and strengthen chronic inflammation and mediate crosstalk between immune cells and cancer cells, thereby initiating tumor development. (33, 34) IL-6-mediated activation of STAT3 signaling cascades is a major factor related with these progresses and promotes tumor cell invasion and metastasis through induction of wide range of target genes, including *MMPs* in CRC. (35, 36) The present data provided striking evidence that *MMPs* expression were dependent on STAT3 using siSTAT3 in Figure 4B. From this perspective, regulation of *MMPs* expression is valuable therapeutic targets in CRC. *MMPs* are important for extracellular matrix (ECM) remodeling in normal conditions, whereas they increase tumor cell migration, invasion and metastasis in CRC. (37) As shown in Figure 4C, invasion was significantly inhibited when expression of *MMPs* was suppressed by siSTAT3. In fact, high levels of *MMPs*, including *MMP-1, -2, -3* and *-9* are observed in patients with tumor tissues of colon cancer, compared with normal tissues. (37) Results revealed that 20(S)-Rh2 effectively inhibited the expression of *MMPs*, including *MMP-1, -2, -3* and *-9* in IL-6-stimulated CRC cells, resulting in the suppression of tumor cell invasion. (Figure 5) However, 20(R)-Rh2 exhibited significant inhibition of *MMP3* expression but no biological activities in these levels and invasion. Thus, 20(S)-Rh2 has stronger anti-invasion effect than 20(R)-Rh2.

CRC is responsible for 12.2% of all cancer deaths. Approximately 50% of patients are known to have metastatic CRC, which is difficult to treat even with surgical treatment. Ultimately, most of CRC-related deaths are due to metastasis, increasing the mortality rates of CRC. (38, 39) Thus, it is necessary to develop therapeutic treatment for inhibiting the invasion in CRC. Furthermore, *in vivo* experiments are needed for clinical treatment of 20(S)-Rh2 as anti-invasion drug.

Only small populations of patients with metastasis are available for surgical resection, and the advances in regional and systemic chemotherapeutic combination treatment is necessary to treat metastatic CRC. Currently, there are approved drugs for patients with metastasis that are clinically used alone or combination with other reagents. In the case of CRC patients, alternative treatment has been developed using natural products, such as ginseng. (3, 4) Ginsenosides, including Rh2 are natural products, which have numerous biological activities, and side effects have not been reported in normal cells yet. On the contrary, most of the classic chemotherapeutic agents for cancer treatment exhibit toxicity and side effects, such as hair loss, vomiting and liver problems that caused damage in normal cells and tissues. (40) Combination therapy is potential for reducing side effects of conventional chemotherapeutic agents and enhancing cytotoxicity to tumor cells. The synergistic effects of 20(S)-Rh2 were observed by measuring cell viability in combination with cisplatin or doxorubicin in HCT116 cells as proposed in Figure 6. In addition, activated STAT3 signaling results in difficulties in treatment due to induced drug-resistance, chemo-resistance, and

radio-resistance. (41, 42) STAT3 activation is shown in 70% of solid and hematological tumors, associating with a poor survival in CRC. (19, 43) For these reasons, cancer treatment targeting STAT3 is almost a necessity. Therefore, further experiments are necessary to identify whether 20(S)-Rh2 synergistically inhibits STAT3 activation in combination treatment.

In conclusion, the results suggest that ginsenoside 20(S)-Rh2 inhibits STAT3 activation and downstream signaling cascades *via* upstream signal JAK2 inhibition in CRC cells. As a result, this compound subsequently inhibits the expression of *MMPs* and cell invasion. In addition, 20(S)-Rh2 increases tumor cell cytotoxicity in combination with chemotherapeutic agent, such as cisplatin or doxorubicin. These findings may provide that 20(S)-Rh2 possess strong anti-invasion properties by targeting STAT3 signaling, and can be used as a combinational partner, especially in combination therapy with classic chemotherapeutic agents in CRC.

REFERENCES

1. Li, B., et al., Ginsenoside Rh2 induces apoptosis and paraptosis-like cell death in colorectal cancer cells through activation of p53. *Cancer Lett*, 2011. 301(2): p. 185-92.
2. Park, E.K., et al., Induction of apoptosis by the ginsenoside Rh2 by internalization of lipid rafts and caveolae and inactivation of Akt. *Br J Pharmacol*, 2010. 160(5): p. 1212-23.
3. Wang CZ., et al., Letter Natural Products and Chemotherapeutic Agents on Cancer: Prevention vs. Treatment, *The journal of Chinese medicine*, 2014. 7: p. 1-4
4. Wang CZ., et al., Potential role of ginseng in the treatment of colorectal cancer: *The American journal of Chinese medicine*, 2008. 36(6): p. 1019-28
5. Nag, S.A., et al., Ginsenosides as Anticancer Agents: In vitro and in vivo Activities, Structure-Activity Relationships, and Molecular Mechanisms of Action. *Front Pharmacol*, 2012. 3: p. 25.
6. Zhang, C., H. Yu, and J. Hou, [Effects of 20 (S) -ginsenoside Rh2 and 20 (R) -ginsenoside Rh2 on proliferation and apoptosis of human lung adenocarcinoma A549 cells]. ZhongguoZhong Yao ZaZhi, 2011. 36(12): p. 1670-4.
7. Corvinus, F.M., et al., Persistent STAT3 activation in colon cancer is associated with enhanced cell proliferation and tumor growth. *Neoplasia*, 2005. 7(6): p. 545-55.

8. Helling, T.S. and M. Martin, Cause of death from liver metastases in colorectal cancer. *Ann SurgOncol*, 2014. 21(2): p. 501-6.
9. Seo, E.Y. and W.K. Kim, Red ginseng extract reduced metastasis of colon cancer cells in vitro and in vivo. *J Ginseng Res*, 2011. 35(3): p. 315-24.
10. Ma, X.T., et al., Constitutive activation of Stat3 signaling pathway in human colorectal carcinoma. *World J Gastroenterol*, 2004. 10(11): p. 1569-73.
11. Turkson, J. and R. Jove, STAT proteins: novel molecular targets for cancer drug discovery. *Oncogene*, 2000. 19(56): p. 6613-26.
12. Kim, B.H., et al., A small-molecule compound identified through a cell based screening inhibits JAK/STAT pathway signaling in human cancer cells. *Mol Cancer Ther*, 2008. 7(9): p. 2672-80.
13. Horvath, C.M., STAT proteins and transcriptional responses to extracellular signals. *Trends BiochemSci*, 2000. 25(10): p. 496-502.
14. Zhong, Z., Z. Wen, and J.E. Darnell, Jr., Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science*, 1994. 264(5155): p. 95-8.
15. Musteanu, M., et al., Stat3 is a negative regulator of intestinal tumor progression in Apc(Min) mice. *Gastroenterology*, 2010. 138(3): p. 1003-11 e1-5.
16. Reimers, M.S., et al., Biomarkers in precision therapy in colorectal cancer. *Gastroenterol Rep* (Oxf), 2013. 1(3): p. 166-83.

17. Grivennikov, S., et al., IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell*, 2009. 15(2): p. 103-13.

18. Waldner, M.J., S. Foersch, and M.F. Neurath, Interleukin-6--a key regulator of colorectal cancer development. *Int J BiolSci*, 2012. 8(9): p. 1248-53.

19. Kusaba, T., et al., Expression of p-STAT3 in human colorectal adenocarcinoma and adenoma; correlation with clinicopathological factors. *J ClinPathol*, 2005. 58(8): p. 833-8.

20. Gordziel, C., et al., Both STAT1 and STAT3 are favourable prognostic determinants in colorectal carcinoma. *Br J Cancer*, 2013. 109(1): p. 138-46.

21. Xiong, H., et al., Inhibition of JAK1, 2/STAT3 signaling induces apoptosis, cell cycle arrest, and reduces tumor cell invasion in colorectal cancer cells. *Neoplasia*, 2008. 10(3): p. 287-97.

22. Dimberg, L.Y., et al., Stat1 activation attenuates IL-6 induced Stat3 activity but does not alter apoptosis sensitivity in multiple myeloma. *BMC Cancer*, 2012. 12: p. 318.

23. Woelfle U., et al., Conditionally active STAT1 and its functional role in tumor progression and invasion. *Proceedings of the American Association for Cancer Research*, 2006. 2006(1): 430.

24. Pensa S., et al., STAT1 and STAT3 in Tumorigenesis. *JAK-STAT pathway in disease*, 2009, 2009: 100.

25. Devarajan, E. and S. Huang, STAT3 as a central regulator of tumor metastases. *CurrMol Med*, 2009. 9(5): p. 626-33.

26. Kamran, M.Z., P. Patil, and R.P. Gude, Role of STAT3 in cancer metastasis and translational advances. *Biomed Res Int*, 2013. 2013: p. 421821.

27. Leeman, R.J., V.W. Lui, and J.R. Grandis, STAT3 as a therapeutic target in head and neck cancer. *Expert OpinBiolTher*, 2006. 6(3): p. 231-41.

28. Romano, M., et al., Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity*, 1997. 6(3): p. 315-25.

29. Chang, Q., et al., The IL-6/JAK/Stat3 feed-forward loop drives tumorigenesis and metastasis. *Neoplasia*, 2013. 15(7): p. 848-62.

30. Teng, Y., J.L. Ross, and J.K. Cowell, The involvement of JAK-STAT3 in cell motility, invasion, and metastasis. *JAKSTAT*, 2014. 3(1): p. e28086.

31. Rokavec, M., et al., IL-6R/STAT3/miR-34a feedback loop promotes EMT-mediated colorectal cancer invasion and metastasis. *J Clin Invest*, 2014. 124(4): p. 1853-67.

32. Iishi H., et al., Inhibition by ginsenoside Rg3 of bombesin-enhanced peritoneal metastasis of intestinal adenocarcinomas induced by azoxymethane in Wistar rats. *Clinical & experimental metastasis*, 1997. 15(6): p. 603-11

33. Koltsova, E.K. and S.I. Grivennikov, IL-22 gets to the stem of colorectal cancer. *Immunity*, 2014. 40(5): p. 639-41.

34. Grivennikov, S.I., F.R. Greten, and M. Karin, Immunity, inflammation, and cancer. *Cell*, 2010. 140(6): p. 883-99.

35. Elander, N., P. Soderkvist, and K. Fransen, Matrix metalloproteinase (MMP) -1, -2, -3 and -9 promoter polymorphisms in colorectal cancer. *Anticancer Res*, 2006. 26(1B): p. 791-5.

36. Tsareva, S.A., et al., Signal transducer and activator of transcription 3 activation promotes invasive growth of colon carcinomas through matrix metalloproteinase induction. *Neoplasia*, 2007. 9(4): p. 279-91.

37. Baker, E.A., F.G. Bergin, and D.J. Leaper, Matrix metalloproteinases, their tissue inhibitors and colorectal cancer staging. *Br J Surg*, 2000. 87(9): p. 1215-21.

38. de Jong, P.R., et al., STAT3: An Anti-Invasive Factor in Colorectal Cancer? *Cancers* (Basel), 2014. 6(3): p. 1394-407.

39. Van Cutsem, E., et al., Advanced colorectal cancer: ESMO Clinical Practice Guidelines for treatment. *Ann Oncol*, 2010. 21 Suppl 5: p. v93-7.

40. Rao, B., et al., Evaluation of an Actinomycin D/VX-680 aurora kinase inhibitor combination in p53-based cyclotherapy.*Oncotarget*, 2010. 1(7): p. 639-50.

41. Yin, Z., et al., Prognostic significance of STAT3 expression and its correlation with chemoresistance of non-small cell lung cancer cells. *ActaHistochem*, 2012. 114(2): p. 151-8.

42. Aggarwal, B.B., et al., Signal transducer and activator of transcription-3, inflammation, and cancer: how intimate is the relationship? *Ann N Y AcadSci*, 2009. 1171: p. 59-76.

43. Morikawa, T., et al., STAT3 expression, molecular features, inflammation patterns, and prognosis in a database of 724 colorectal cancers. *Clin Cancer Res*, 2011. 17(6): p. 1452-62.

국문 초록

인삼 추출물 중 하나인 진세노사이드 Rh2 는 세포 주기를 막음으로써 세포 성장을 억제하고 더 나아가 세포 사멸을 증가시킨다. 이러한 Rh2 는 두 입체 이성체를 가지고 있으며, 수산기의 결합 방향에 따라 20(S)-Rh2 와 20(R)-Rh2 로 나뉘어진다. 두 입체 이성체는 구조 차이에 따른 생물학적 활성의 차이를 가지고 있다. 그 중 주로 20(S)-Rh2 의 항암, 항염증 효과에 대해 연구되어 왔다. 그러나 사람의 대장암 세포에서 높은 빈도로 나타나는 암 침윤에 대해 20(S)-Rh2 와 20(R)-Rh2 가 억제 효과를 가지는 지에 대해서는 거의 밝혀 진 바가 없다. 이에 대해, 본 연구에서 20(S)-Rh2 와 20(R)-Rh2 의 암 침윤 억제에 대한 효과를 알아보고자 하였다. IL-6 를 처리하였을 때, 20(S)-Rh2 가 20(R)-Rh2 에 비해 IL-6-induced STAT3 활성을 강력하게 억제하는 것을 확인하였다. 더 나아가 20(S)-Rh2 의 STAT3 활성을 억제 함으로써 암 침윤 또한 감소시키는 것을 발견 할 수 있었다. 20(S)-Rh2 는 HCT116 세포에서 endogenous STAT3 활성과 IL-6-induced STAT3 활성을 모두 억제하였다. 그 영향으로 STAT3 에 의해 조절되는 *MMP-1, -2, -3* 그리고 *-9* 의 발현을 억제하였으며, 결과적으로 대장암에서 암 침윤의 감소를 일으키게 된다. 동시에 HCT116 세포에서 siSTAT3 로 인해 *MMPs* 의 발현 수준과 암 침윤이 억제되는 것을 발견할 수 있었다. 이는 20(S)-Rh2 의 STAT3 활성 억제

효과가 암 침윤의 억제를 유도할 수 있음을 알 수 있었다. 이러한 20(S)-Rh2 의 약물학적 활성은 STAT3 의 상위 조절자인 JAK2 활성을 타깃하여 억제함으로써 나타났다. 흥미롭게도, 20(S)-Rh2 의 이러한 억제효과는 20(R)-Rh2 보다 강하게 나타났다. 또한, 20(S)-Rh2 는 HCT116 세포에서 다른 항암제들과의 병합 처리하였을 때, 세포의 독성에 대한 민감도를 증가시키는 것을 발견할 수 있었다. 이상의 연구 결과는 20(S)-Rh2 가 사람의 대장암 전이에 대한 치료 약물로써의 가능성과 다른 항암제와의 병합 처리를 통해 암세포의 항암제에 대한 세포 독성 민감도를 상승시키는 효과의 약물로써 가능성을 가지고 있다는 것을 의미하고 있다.

주요어 : 20(S)-Rh2, 20(R)-Rh2, STAT3, IL-6, 침윤, 대장암

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