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Rhizochalin from *Rhizochalina incrustata* induces apoptosis via activation of AMP-activated protein kinase in HT-29 cell

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약 학 과

조 해 국

HT-29 세포에서, Rhizochalin 에 의한 AMP-activated protein kinase 의 활성화 및 세포 사멸기전 연구

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이 논문을 약학 석사학위 신청논문으로 제출함.

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List of Abbreviations

AglRhz	Aglycon of rhizochalin
AMPK	AMP-activated protein kinase
ERK1/2	Extracellular signal-related kinase1/2
mTOR	Mammalian taget of rapamycin
IGF-1	Type 1 insulin-like growth facor
Raptor	Regulatory associated protein of mTOR
PARP	Poly (ADP-ribose) polymerase
DMSO	Dimethylsulfoxide
FBS	Fetal bovine serum
PBS	Phosphate-buffered saline
SDS-PAGE	Sodium dodecyl sulfate-polyacryamide gel electrophoresis

국문 초록

HT-29 세포에서, Rhizochalin에 의한 AMP-activated protein kinase의 활성화 및 세포 사멸기전 연구

조해국

지도교수: 최홍석

조선대학교 대학원 약학과

Rhizochalin은 sponge라 불리는 *Rhizochalina incrustata*라는 해양심층생물로부터 얻어진 물질이며 Rhizochalin과 이것의 유도체가 암 예방 또는 암 치료 물질로서의 가능성이 알려졌다. 하지만 이러한 Rhizochalin과 이것의 유도체의 암세포 사멸유도작용이 어떠한 메커니즘에 의한 것인지 알려지지 않았고 그것을 규명하고자 하였다.

Aglycon of rhizochalin (AglRhz)은 HT-29 세포에서 AMP-activated protein kinase (AMPK)의 활성화를 유도하였고 또한 이 화합물에 의한 AMPK의 활성화가 raptor의 인산화를 유도하여 mTOR/P70S6k/ERK signaling을 억제하였다. 또한 AglRhz가 poly(ADP-ribose) polymerase(PARP)과 caspase-3의 활성화를 통해 HT-29 세포의 DNA fragmentation과 apoptosis를 유도하였다. 이와 더불어

AglRhz의 처치가 soft agar에서 IGF-I로 유도된 JB6 Cl41 세포에서의 transformation과 HT-29 세포의 tumorigenicity를 억제함을 밝혔다.

이러한 결과들을 종합해볼 때, AglRhz은 AMPK 의 활성화를 가져와 mTOR/P70S6K/ERK pathway를 억제하고, 또 apoptosis 를 유도하여 암세포에서의 사멸을 일으키는 것으로 사료되며 AglRhz가 대장암 치료 물질로서 사용될 수도 있음을 확인하였다.

ABSTRACT

Rhizochalin from *Rhizochalina incrustata* induces apoptosis via activation of AMP-activated protein kinase in HT-29 cell

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Rhizochalin is a two-headed shingolipid like compound isolated from the sponge *Rhizochalina incrustata*. It was reported that rhizochalin and its derivates has chemopreventive and chemotherapeutic effect. However, its molecular mechanism on these effects is not understood. Here, we demonstrate that aglycon of rhizochaline (AglRhz) from *Rhizochalina incrustata* induced AMP-activated protein kinase (AMPK) phosphorylayion, and thereby inhibited mTOR/p70S6K/ERK signaling via phosphorylayion of raptor in HT-29 cells. In addition, AglRhz induced activation of caspase-3 and PARP, and DNA fragmentation in HT-29 cells, leading to induction of apoptosis as well as suppression of tumorigenicity of HT-29 cells. Notably, AglRhz inhibited cell transformation in JB6 Cl41 cells. Overall, our finding identify AMPK as

an important target protein for mediating the anti-tumor properties of AglRhz in HT-29 colon cancer cells and have important implication for sponges, the most important marine source, in colon cancer.

I. Introduction

Rhizochalin is a first two-headed shingolipid like compound isolated from the sponge *Rhizochalina incrustata*. It has been shown that rhizochalin and its derivatives have antibacterial, selective DNA-damaging, antifungal and cytotoxic activity [1]. In addition, accumulated evidences suggest that rhizochalin and its derivatives have potent antitumor activity in human cancer cell lines [2, 3]. However, its underline mechanism of anticarcinogenic activity has not yet been understood.

The AMP-activated protein kinase (AMPK), serine/theronine kinase present in all eukaryotes, is a sensor of cellular energy status [4]. Since the activation of AMPK leads to the apoptosis of many cancerous or non-cancerous cells, it is sometimes called as apoptotic molecule. Previous studies have reported that AMPK activators, AICAR and metformin, inhibited the cell growth in cultured tumor cells [5, 6] and mouse xenograft model [5, 7]. AMPK activation also induces apoptosis in cancer cell lines such as neuroblastoma cells, pancreatic cells, glioma cells, and endometrial cancer cells [8-11]. In addition, AMPK activation by phyto-chemicals, such as quercetin and 24-hydroxyursolic acid is involved in apoptosis in colon cancer cell line [12, 13]. Therefore, AMPK activation by naturally occurring compounds has attractive potentials for cancer therapy.

Here, we have investigated the role of aglycon of rhizochalin (AglRhz), two-headed sphingolipid like compound isolated from the sponge *Rhizochalina incrustata*, in the activation of AMPK and induction of apoptosis in HT-29 colon cancer cells. We

observed that AglRhz inhibited phosphoryation of p70S6K and its downstream ERK in a raptor-dependent manner via AMPK activation. We have also found that AglRhz induced the cleavage of PARP and caspase-3 and DNA fragmentation, resulted in the induction of apoptosis. In addition, AglRhz inhibited IGF-I induced cell neoplastic transformation in JB6 Cl41 cells and tumorigenicity in HT-29 cells. Taken together, our findings illustrate that AglRhz extracted from sponge *Rhizochalina incrustata*, has a potent antitumor activity against the HT-29 colon cancer cells and suggested a molecular mechanism that underlies the antitumor activity of AglRhz.

II. Materials & Methods

1. Reagents and Antibodies.

McCoy's 5A medium, L-glutamine, gentamicin, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Polyvinylidene difluoride (PVDF) membrane was obtained from Millipore (Bedford, MA, USA). Insulin-like growth factor-I (IGF-I), 3-[4, 5-Dimethylthiazol-2-thiazoyl]-2, 5-diphenyltetrazolium bromide (MTT) was from Sigma-Aldrich (St. Louis, MO, USA). Compound C were from EMD Chemicals Inc (Gibbstown, NJ, USA). Antibodies against phospho-p70S6K (Thr389), p70S6K, phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, phospho-raptor (Ser792), phospho-AMPK, cleaved caspase-3, cleaved PARP, and caspase-3 were from Cell Signaling Technology Inc. (Beverly, MA, USA); antibodies against PARP, AMPK, goat anti-mouse IgG HRP, goat anti-rabbit IgG HRP were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fixation/Permeabilization Solution (Cytofix/Cytoperm) was from BD Biosciences (San Jose, CA, USA).

2. Purification of Rhz and Preparation of Its aglycon

The chemical structure of aglycon of Rhizochalin is shown in Fig. 1A. The chemicals were extracted, purified, and prepared as described previously [2].

3. Cell culture

Human colon cancer HT-29 cells were obtained from the American Type Culture

Collection (ATCC, Manassas, VA, USA). The cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum, glutamine, and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). DMSO was used as a vehicle to dissolve the compound and a final concentration of 0.1% DMSO (v/v) was used for each treatment.

4. 3-[4, 5-Dimethylthiazol-2-thiazolyl]-2, 5-diphenyltetrazolium bromide (MTT) assay.

The MTT assay was performed to check cell viability. In brief, cells ($1x10^5$ cells/ml) were seeded in 96-well plates with 100 μl of cell suspension in each well. After culturing for 24 h, cells were treated with AglRhz. The cells were then treated with 5 μg/ml MTT solution (10 μl/well) and incubated for 3 h, after which the purple formazan formed by the live cells was dissolved in 0.04 N HCl in isopropanol (100 μl/well) and the absorbance was measured at 570 nm.

5. Immunoblot analysis.

The cells were disrupted in lysis buffer [50 mM Tris (pH 7.5), 5 mM ethylenediaminetetraacetic acid, 150 mM NaCl, 1 mM dithiothreitol, 0.01% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, and 1x protease inhibitor cocktail]. The proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were blocked and hybridized with the appropriate primary antibody overnight at 4°C. The protein bands were visualized using a chemiluminescence detection kit (Amersham

HRP Chemiluminescent Substrates, Amersham Biosciences, Piscataway, NJ) after hybridization with the HRP-conjugated secondary antibody from rabbits or mice. A LAS3000-mini (Fujifilm, Tokyo, Japan) was used for chemiluminescence detection.

6. Detection of apoptosis.

The induction of apoptosis was assessed by TUNEL staining and detected with an in situ Cell Death detection Kit (Roche Applied Science, Indianapolis, IN, USA) according to manufacturer's instructions. In short, $2x10^5$ cells were cultured for 24 h in 6-well plates. The cells were treated 1% FBS media for 24 h and treated with AglRhz for 6 h. The cells were then washed with PBS and fixed with Cytofix/Cytoperm at 4 ºC for 20 min. Then cells were stained with 50 µl TUNEL solution and incubated at 37 ºC for 1 h. The cells were washed with PBS twice and the cells were fixed. DNA fragmentation was detected using an Axiovert 200 M fluorescence microscope and quantified with Axio Vision software (Carl Zeiss Inc., Thornwood, NY).

7. Apoptosis assessed by flow cytometry.

The induction of early and late apoptosis was analyzed by flow cytometry using the Becton Dickinson FACS Calibur Flow Cytometer (BD Biosciences). HT-29 cells (5×10^5 per dish), were grown in 6-cm dishes for 24 h in 1% FBS media. Then, cells were cultured with 1% FBS media for 24 h and treated with AglRhz for 24h. The medium was collected and attached cells were harvested with 0.025% trypsin in 5 mM EDTA in PBS. Cells were washed by centrifugation at 1,000 rpm for 5 min and processed for

detection of early and late apoptosis using Annexin V-FITC and propidium iodide staining according to the manufacturer's protocol.

8. Anchorage-independent cell transformation assay (soft agar assay).

In brief, $8x10^3$ cells/ml were seeded in 1 ml of 0.3% basal medium Eagle (BME) agar containing 10% FBS, 2mM L-glutamine, and 25µg/ml gentamicin with AglRhz. The cultures were maintained at 37 ºC for 2 weeks, and cell colonies were scored using an Axiovert 200 M fluorescence microscope and Axio Vision software (Carl Zeiss Inc., Thornwood, NY).

9. Statistical Analysis.

Statistical calculations were carried out with Prism 4 for Macintosh software (GraphPad Software. Inc., La Jolla, CA). Results are expressed as the mean ± S.E.M. of triplicate measurements of two independent experiments. Student's t test was used for statistical analyses; *P* values < 0.05 were considered to be significant.

III. Results

1. AglRhz induces the cell death in HT-29 cells.

AglRhz is two-headed sphingolipid like compound and it has rare α,ω-bifuctional structure [3] (Fig. 1A). To determine the antitumor effect of AglRhz in HT-29 cell, we first examined cell viability after AglRhz treatment dose- and time-dependently by MTT assay. The treatment of AglRhz significantly inhibited viability of HT-29 cells (Fig. 1B).

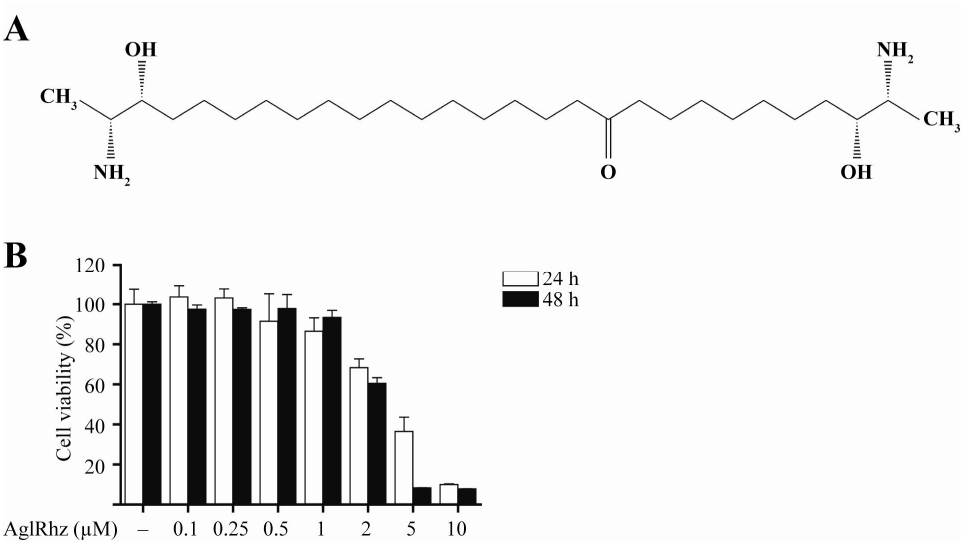

Fig. 1 Effects of AglRhz on cell viability in HT-29 cells.

(A) Structure of AglRhz isolated from the marine sponge *Rhizochalina incrustata*.

(B) Cells were seeded and cultured for 24 h in 10% FBS/McCoy's 5A at 37°C in 5% CO2 atmosphere. Then, the cells were treated with AglRhz dose- and time-dependently. Cell viability was measured by MTT assay, as described in Materials and Methods.

2. AglRhz induces phosphorylation of AMPK and inhibits mTOR/p70S6K/ERK signaling.

Next, we wish to investigate the mechanism of AglRhz induced cell death. AMPK plays a critical role in cell growth and it can be also target for cancer therapy [14]. Phyto-chemicals such as quercetin and 24-hydroxyursolic induce apoptosis by AMPK activation in HT-29 cells [12, 13]. First, we examined whether AglRhz induces AMPK phosphorylation. As shown in Fig 2A and 2B, AglRhz increased the phosphorylation of AMPK time-and dose-dependently. AMPK activation by AglRhz was partially blocked by a potent AMPK inhibitor, compound C (Fig. 2C).

AMPK inhibited mTORC1 through phosphorylation of TSC2 and raptor [15]. mTORC1 induces p70S6K phosphorylation and p70S6K is a mitogen activated Ser/Thr protein kinase that is required for regulation of cell growth, protein synthesis and G1/S transition [16, 17].

Our results show that AglRhz induced phosphorylation of raptor on S792 and inhibited phosphorylayion of p70S6K.

p70S6K interacts with ERK1/2 and enhances phosphorylation of ERK induced by insulin [18]. Thus, we investigated whether activation of AMPK by AglRhz regulates mTOR/p70S6K/ERK signaling. As a result in Fig 2E, phosphorylayion of ERK1/2 is downregulated by AglRhz dose-dependently. AglRhz also inhibited IGF-I induced phosphorylayion of p70S6K and ERK1/2 via activation of raptor (Fig 2F). These overall data suggest that AglRhz has inhibitory effects on p70S6K and ERK1/2 via phosphorylation of AMPK and raptor.

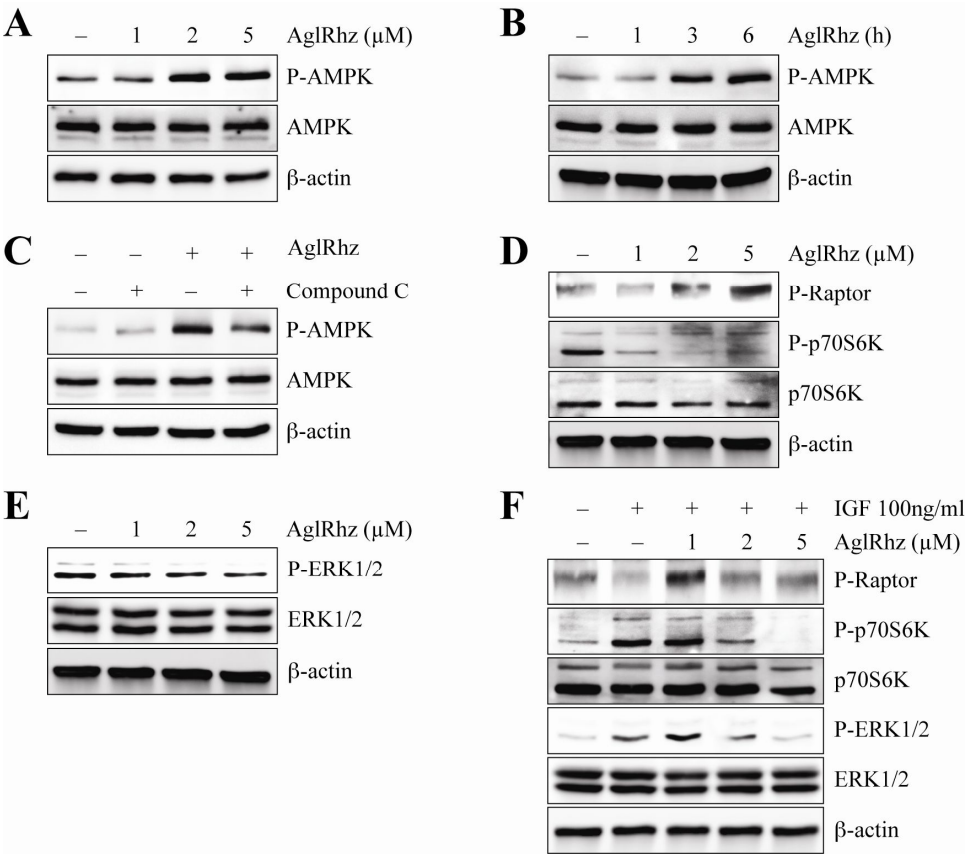
A
– 1 2 5 AglRhz (μM)
P-AMPK
AMPK
β-actin
B
– 1 3 6 AglRhz (h)
P-AMPK
AMPK
β-actin
C
– – + + AglRhz
– + – + Compound C
P-AMPK
AMPK
β-actin
D
– 1 2 5 AglRhz (μM)
P-Raptor
P-p70S6K
p70S6K
β-actin
E
– 1 2 5 AglRhz (μM)
P-ERK1/2
ERK1/2
β-actin
F
– + + + + IGF 100ng/ml
– – 1 2 5 AglRhz (μM)
P-Raptor
P-p70S6K
p70S6K
P-ERK1/2
ERK1/2
β-actin

Fig. 2 Effects of AglRhz on the AMPK, raptor, p70S6K, and ERK in HT-29 cells.

(A and B) Cells were seeded and cultured for 24 h, and then the cells were cultured in 1% FBS media for 24 h and treated with AglRhz dose-dependently for 6 h (*A*) or treated with 5μM of AglRhz time-dependently (*B*).

(C) Cells were seeded and cultured in 10% FBS media for 24 h. Then, the cells were pretreated or not pretreated with compound C (10uM) for 2h, then treated or not treated with AglRhz (5μM, 6 h).

(D and E) Cells were seeded and cultured in 10% FBS media for 24 h. Then, the cells were then treated with AglRhz dose-dependently for 6 h.

(F) Cells were seeded and cultured in 10% FBS media for 24 h. Then, the cells were pretreated or not pretreated with AglRhz dose-dependently. After 6 h, cells were treated or not treated with IGF-I (100ng/ml) for 30m. Phosphorylation and expression of AMPK, raptor, p70S6K, and ERK were measured by Western blot analysis.

3. AglRhz induces apoptosis signaling and DNA fragmentation in HT-29 cells.

We investigated whether AglRhz induced AMPK activation is associated with the apoptosis signaling pathway in HT-29 cells. AglRh induced cleavage of caspase-3 and PARP dose- and time- dependently (Fig. 3A and 3B). We also measured DNA fragmentation by TUNEL staining. The degree of TUNEL staining significantly increased after AglRhz treatment (Fig. 3C). AglRhz induced 2.82% apoptosis and 6.38% cell death at 5μM (Fig. 3D). These data indicate that AglRhz induced apoptosis via activation of caspase-3 and PARP, and DNA fragmentation in HT-29 cells.

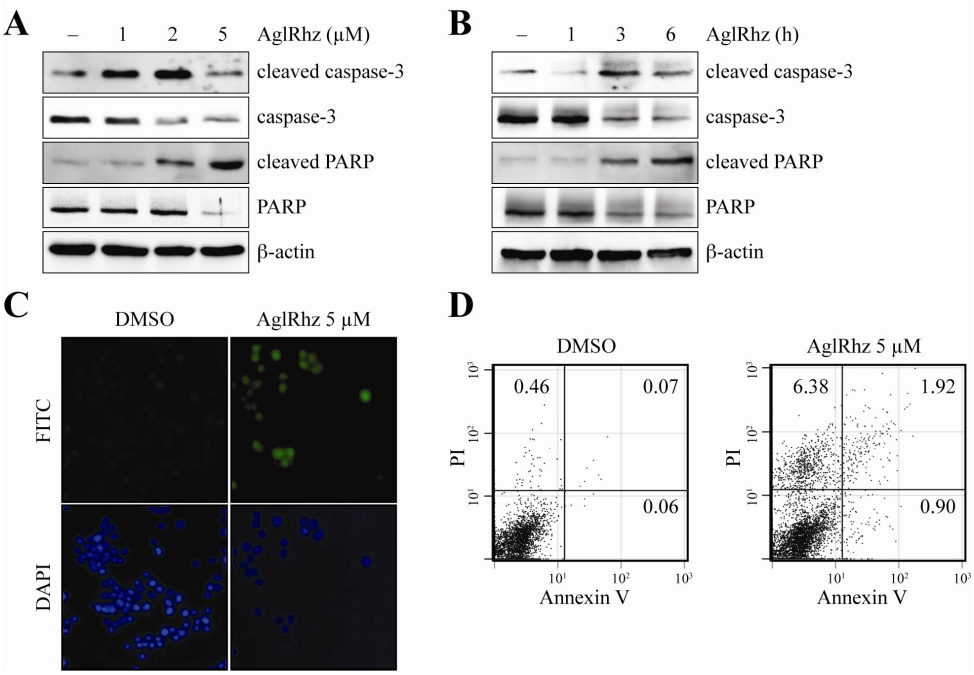

Fig. 3 Effects of AglRhz on the PARP, caspase-3, and DNA fragmentation in HT-29 cells.

(A and B) Cells were seeded and cultured for 24 h and then the cells were cultured with 1% FBS media for 24 h and were treated with AglRhz dose-dependently for 6 h (*A*) or treated with 5μM of AglRhz time-dependently (*B*). Cleaved PARP, cleaved caspase-3 were measured by Western blot analysis.

(C) Cells were cultured with 1% FBS media for 24 h and then the cells were treated or not treated with 5μM of AglRhz for 6 h. The cells were fixed and stained with TUNEL solution.

(D) Cells were cultured with 1% FBS media for 24 h and treated with 5μM of AglRhz for 24 h. The induction of early and late apoptosis rate induced by AglRhz was analyzed by flow cytometry. *Columns*, mean of triplicate samples; *bars*, SE. *, $P < 0.05$, compared with control cells.

4. AglRhz inhibits IGF-I induced neoplastic cell transformation of JB6 Cl41 cells and tumorigenicity of HT-29 cells via AMPK activation.

Next, we assessed the effect of AMPK activation by AglRhz on the cell transformation of JB6 Cl41 cells and tumorigenicity of HT-29 cells by soft agar colony formation.

The JB6 Cl41 cell system is a well developed model for studying tumor promotion induced by EGF under anchorage independent growth condition [19]. AglRhz inhibited IGF-I induced neoplastic cell transformation of JB6 Cl41 cells dose-dependently (Fig. 4A and 4B). To assess whether AglRhz inhibited tumorigenicity of HT-29 cells, the cells were treated with AglRhz (0.1, 0.25, and 0.5μM) in soft agar. AglRhz decreased colony numbers as well as colony sizes in HT-29 cells compared with control cells (4C and 4D).

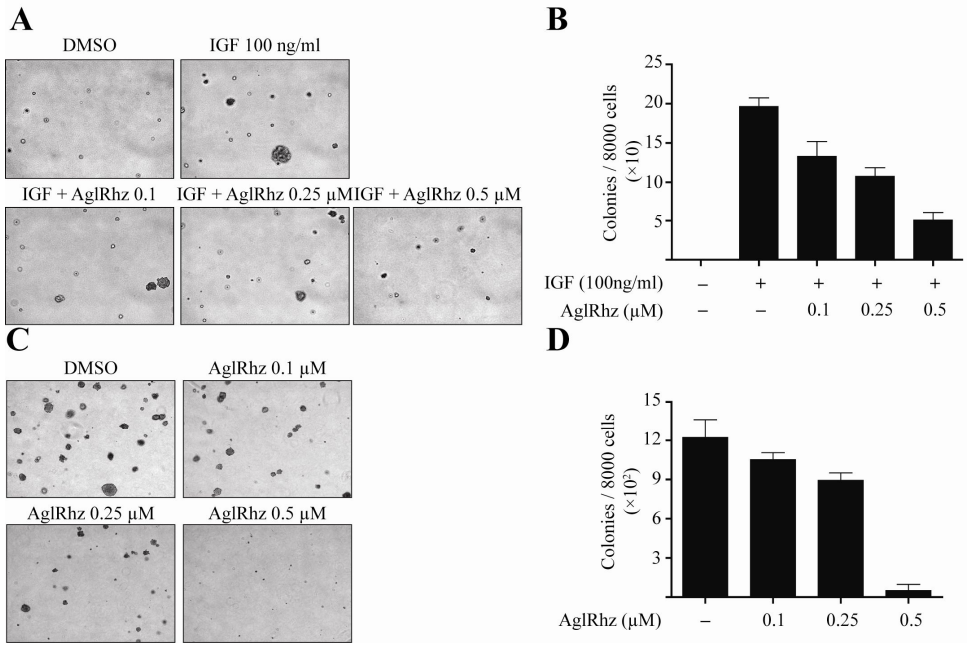

Fig. 4 Effects of AglRhz on IGF-I induced neoplastic cell transformation of JB6 Cl41 cells and effects on the tumorigenicity of HT-29 cells.

(A and B) JB6 Cl41 cells treated or not treated with AglRhz were exposed to IGF-I (100ng/ml) in soft agar matrix.

(C and D) HT-29 Cells were used in soft agar assays with AglRhz treatment. Columns, mean of triplicate measurements; bars, * $p < 0.05$, compared with only IGF-I treat cells (*A* and *B*) or control cells (*C* and *D*).

Ⅴ. Discussion

The chemical and biological diversity of the marine environment is immeasurable and therefore is an extraordinary resource for the discovery of new anticancer drugs [20]. The past decade has seen a dramatic increase in the number of preclinical anticancer lead compounds from diverse marine life enter human clinical trials [20]. Especially, marine sponges have been considered as a promising natural resource during the past 50 years, with respect to the diversity of their secondary metabolites. The biological effects of new metabolites from sponges have been reported, and sponges have the potential to provide future drugs against important diseases, such as cancer, a range of viral diseases, malaria, and inflammations [21].

Rhizochalin is two-headed sphingolipids from marine sponge *Rhizochalina incrustata*, and it was striking because of their rare structures and high biological activity [3]. After first isolation of rhizochalin, many other kind of two-headed sphingolipids are isolated form marine sponges, and some of them are studied as antitumor agent [2, 3]. AglRhz has been reported to inhibit the viability of cancer cells including leukemia [2, 3] , uterine cervical, and colon cancer [3]. However, anticancer mechanism remains poorly defined. Here, we show the first evidence that AMPK may play a role in mediating anticancer effect of AglRhz.

AMPK has a role in metabolism and cell growth regulation and emerges as a novel therapeutic target for the treatment of cancers or other metabolic disorders such as obesity and type 2 diabetes [14]. Quercetin, a major dietary flavonoid, induces

apoptosis via AMPK activation and p53-dependent apoptotic cell death in HT-29 cells [13]. In addition, resveratrol, a polyphenolic natural product [22], induces apoptosis in HT-29 cells shown chemoresistant to a cancer chemotherapeutic drug, etoposide via AMPK activation [23]. Furthermore, 24-hydroxyursolic acid from the leaves of the Diospyros kaki (persimmon) induces apoptosis by AMPK activation in HT-29 cells [12]. Our findings demonstrated that AglRhz is able to increase phosphorylayion of AMPK and decrease cell viability in HT-29 cells (Fig 1B and 2A and 2B). As stated above, AMPK is an important factor for cell apoptosis and growth, thus AglRhz may exert its antitumor effect in HT-29 cells via AMPK activation.

AMPK serves as a negative regulator for mTORC1, a functional complex of mTOR, via direct phosphorylation of both TSC2 tumor suppressor and the mTORC1 binding partner, Raptor [24, 25]. And blockade of mTORC1 by AMPK is vital in determining apoptotic or growth arrest in cells in response to glucose deprivation or hypoxia [26]. The p70S6K protein, which is a direct downstream of mTORC1, is a mitogen activated Ser/Thr protein kinase [27]. The mTOR/p70S6K signaling pathway has a major role in cell growth by intergarting growth factor and nutrient cascades [28]. This pathway is essential for cellular homeostasis and that aberrant modulation of this pathway can contribute to cancer and metabolic disorders such as obesity and type 2 diabetes [28]. In accordance with these previous reports, AglRhz induced phosphorylaytion of both AMPK and raptor. p70S6K activity is downregulated by AglRhz (Fig. 2D). These data indicate that AglRhz inhibited p70S6K phosphorylation through AMPK-raptor pathway. To

confirm AglRhz's effects on raptor/p70S6K pathway, we examined whether IGF-I induced p70S6K is affected by AglRhz. It is known that IGF-I is a potent activator of the AKT/mTOR/p70S6K signaling pathway and has a role in cell growth and proliferation, and anti apoptosis [29, 30]. As expected, IGF-I induced p70S6K phosphorylation and this was blocked by AglRhz treatment in HT-29 cells (Fig. 2F). Taken together, these data indicated that, upon both IGF-I stimulation and normal condition, AglRhz has effect on the mTOR/p70S6K signaling pathway via AMPK activation.

The ERK signaling pathway has been studied extensively and is a central MAPK pathway that plays a role in cell proliferation, differentiation and transformation [33]. Recently, it was reported that MAP kinase is regulated by mTOR/p70S6K signaling in hepatocacinoma cell [18]. More specifically, the insulin-induced interaction of p70S6K with ERK1/2 resulted in augmentation of it downstream target gene such as AP-1 and this interaction was blocked by rapamycin, suggesting that insulin-mediated crosstalk exists between p70S6K and ERK1/2 [18]. Our results demonstrates that AglRhz inhibited ERK phosphorylayion (Fig. 2E and 2F), suggesting that p70S6K/ERK signaling might be blocked through AglRhz's inhibitory effect on mTORC1 via AMPK-raptor activation.

Taken together, our study showed that AglRhz strongly activates AMPK, and thereby inhibits mTOR/p70S6K/ERK signaling via phosphorylation of raptor. In addition, AglRhz increases apoptosis via cleavage of caspase-3, PARP, and DNA fragmentation in HT-29 cells. Furthermore, AglRhz significantly inhibits IGF-I induced neoplastic cell transformation of JB6 Cl41 cells and the tumorigenicity of

HT-29 colon cancer cells in soft agar. The present study demonstrates that AMPK activation by AglRhz induces cellular apoptosis, suggesting that AMPK might be an important regulatory component of mTOR/p70S6K/ERK signaling in colon cancer cells.

V. References

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