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# A study on the mechanism of DAA induced apoptosis and cell cycle arrest in gastric cancer

조선대학교 대학원

생명과학과

김 원 진

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# 김원진의 석사학위논문을 인준함

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## 위암세포에서 세포사멸과 세포주기 정지를 유도하는 DAA의 기작에 대한 연구

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위암은 전 세계적으로 높은 발병률을 나타내는 심각한 질병 중 하나이다. 화학요법과 방사선치료가 위암 치료에 많이 사용되고 있지만, 현재 사용되고 있는 항암제로는 효과가 제한되어 있기 때문에 새로운 항암제의 개발이 중요하게 여겨진다. Dehydroabietic acid (DAA) 는 많은 종류의 나무 송진에서 발견되는 단일 화합물 중 다이테르펜 형태의 한 종류이다. 이러한 다이테르펜은 항균, 항염증 그리고 항암 효과가 있다고 알려졌지만, 위암에서는 그 효과가 아직 보고되지 않았다. 이번 연구를 통해서 우리는 DAA가 인체 위암 세포주에서 성장을 억제하는 효과를 확인하였다. 그 결과는 DAA가 인체 위암 세포주 (AGS, MKN-28, YCC-2, SNU-216, SNU-601, 그리고 SNU-668) 의 세포 증식을 농도 의존적으로 감소시켰다. 또한 AGS 세포에서는 PI 염색을 이용한 유세포분석을 통해 DAA가 Sub-G1을 증가시키고, G1기의 세포 주기 정지를 유도한다는 것을 확인하였고, Annexin V-FITC/PI 염색을 이용하여, DAA에 의해 세포사멸이 유도된다는 것을 확인하였다. AGS 세포에서 DAA를 처리하였을 때, 세포사멸을 유도할 수 있는 단백질인 Bax의 발현을 상향 조절하였고, 세포사멸을 억제하는 것으로 알려진 Bcl-2 단백질을 하향 조절하였다. 게다가 Caspase-9, Caspase-3, Poly (ADP-ribose) polymerase (PARP) 의 활성화를 유도하였다. 이러한 결과는 위암세포에서 DAA가 세포 주기를 정지함으로, 미토콘

드리아 매개 신호전달 경로를 통하여 세포사멸을 유도한다는 것을 나타낸다. 추가로 RNA sequencing을 수행하여, DAA를 처리하였을 때 변화하는 다양한 유전자의 발현을 확인하였다. 결과적으로 DAA는 잘 알려진 세포주기 정지와 세포사멸 경로를 통하여 암세포의 세포성장을 억제하는 것뿐만 아니라, 세포성장을 억제하는 다양한 유전자의 발현을 조절한다는 것을 확인하였다. 이러한 발견들은 DAA가 intrinsic 경로를 매개로 한 apoptosis를 통하여 잠재적인 항암효과를 가질 수 있다는 점을 제안하고, 위암 치료에 강력한 치료약이 될 수 있는 가능성을 제시해준다.

# ABSTRACT

## A study on the mechanism of DAA induced apoptosis and cell cycle arrest in gastric cancer

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Gastric cancer is one of the serious diseases with a high incidence worldwide. Chemotherapy and radiation therapy are commonly used to treat gastric cancer. Conventional anti-cancer drugs have limited effectiveness. Therefore, the development of new anti-cancer drugs is important. Dehydroabietic acid (DAA) is a type of diterpene and is a single compound found in many tree rosin. These diterpenes are known to have anti-bacterial, anti-inflammatory and anti-cancer effects. However, the effects on human gastric cancer cell lines have not been reported much. In this study, i examined the effects of DAA on cell growth inhibition in human gastric cancer cell lines. The results showed that DAA could inhibit the proliferation of gastric cancer cells (AGS, MKN-28, YCC-2, SNU-216, SNU-601, and SNU-668) in a dose-dependent manner. Also followed DAA induced accumulation of Sub-G1 DNA content and G1 arrest, apoptotic cell population in AGS cells. In addition, the expression of Bax known as pro-apoptotic protein was up-regulated, Bcl-2 known as anti-apoptotic protein

was down-regulated in DAA treated AGS cells. Moreover, induce cleavage of Caspase-9 and –3, Poly (ADP-ribose) polymerase (PARP). These results suggest that DAA induced cell death though the mitochondrial mediated apoptosis & G1 cell cycle arrest. Furthermore, i want to which gene was changed through the DAA treatment. So i performed RNA sequencing was DAA and DMSO treatment in AGS cells. Not only does DAA suppress cancer cell growth through the well-known cell cycle arrest and apoptosis pathways, but it also regulates the expression of various genes to suppress cancer cell growth. Take the results suggest that DAA has a potential anti-cancer effect via intrinsic pathway-mediated apoptosis, possibly making it a strong therapeutic agent for human gastric cancer.

# I. INTRODUCTION

Cancer is a group of diseases caused by the abnormal growth regulation and proliferation of cells. Especially, gastric cancer is an important cancer worldwide, with more than 1,000,000 reported in 2018, with 783,000 deaths, the fifth highest incidence and the third highest [1]. However, the mortality rates have decreased considerably in recent years [2]. In Korea, despite a decline in the incidence of gastric cancer, it remains the second most common cancer [3]. Therefore, the development of new anti-cancer drugs and effective therapeutic strategies for patients with gastric cancer is needed to increase the efficacy of treatment. Almost the whole anti-cancer drug is accompanied by certain side effects [4]. Phytochemicals are selective in their functions and acts specifically on tumor cells without cytotoxicity normal cells [5].

Apoptosis by cell cycle arrest induction are important in cancer therapeutic strategies [6]. The ability to modulate survival or death of a cell is known for therapeutic potential in treating cancer cells [7]. Thus, i focus on research has been directed toward the cell cycle and programmed cell death mechanisms. Cell cycle regulatory factor are frequently modified in human cancer cells [8]. The cyclin-dependent kinases (CDKs) are key regulator that binding with cyclin control the initiation, progression and completion of the cell cycle [9]. Inhibiting CDK activity is expected to obstruct cell cycle progression and lead to cell cycle arrest [10]. Many compounds operate as anti-cancer agents at multiple steps in the cell cycle [11].

Apoptosis is another critical process, and abnormal induction leads to many diseases, including cancer [12]. The induction of apoptosis can be done in two pathway; intrinsic and extrinsic pathway, these ways active a caspase [13]. The Bcl-2 family proteins are activators of these two ways and are called pro-apoptotic or Bax-like death factors. Bax and Bcl-2 are each of representatively members that act as pro-apoptotic and anti-apoptotic proteins, respectively [14]. Studying both pro- and anti-apoptotic proteins and pathways can

provide an opportunity for the treatment of diseases, especially cancer therapy [15].

Terpenes are a variety of organic compounds widely distributed in the plant. Among them, Dehydroabietic acid (DAA) is a single compound that is one of the diterpenes found in tree rosin. Various terpenes are known to effectively inhibit the growth of cancer cells [16]. However, the effect of DAA on cancer cells is unknown.

To determine the anti-cancer effect of the DAA, i performed cell proliferation assay. The cell growth inhibition of DAA was then examined in six different gastric cancer cells. I found that DAA can significantly inhibit the growth of all gastric cancer cells in a time- and dose-dependent manner. Among these, AGS and YCC-2 cells were selected, and their efficacy was verified by crystal violet and microscopic observation. Finally, AGS cells were selected. Cell cycle analysis was performed through flow cytometry. The result was cell cycle arrest. Annexin V/FITC and PI staining were performed to confirm apoptosis following cell cycle arrest. As a result, apoptosis was induced by cell cycle arrest. Western blot was performed to confirm the expression of proteins that regulate cell cycle progression and apoptosis. Both cell cycle arrest and apoptosis increased or decreased expression of proteins. In addition, RNA sequencing was performed. The result was increased expression of various genes that regulate cell cycle and apoptosis. I explain the potential of new anti-cancer drugs by DAA inducing apoptosis by cell cycle arrest.

# II. MATERIALS AND METHODS

## II-1. Chemicals

The compounds Dehydroabietic acid (DAA) were purchased from Chengdu Biopurify phytochemicals Ltd (Chengdu, China).

## II-2. Cell culture

Six human gastric cancer cell lines (AGS, YCC-2, MKN-28, SNU-216, SNU-601, and SNU-668) obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea). The cell lines cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS; CORNING, NY State, USA) and 1% penicillin-streptomycin (Gibco, Waltham, USA). All cells incubated at 37°C and $CO_2$ atmosphere of 5%.

## II-3. Cell proliferation assay

AGS, YCC-2, MKN-28, SNU-216, SNU-601, and SNU-668 cells were plated in 96-well culture plates ($1\times10^4$ per well). After incubation for 24 h, AGS, YCC-2, MKN28, SNU-216, SNU-601, and SNU-668 cells were treated DAA. After treated for 24, 48 h, WST-8 solution (Daeil, Seoul, Korea) was subsequently added to each well. After 30 min of additional incubation, the plate was shaken gently. The absorbance was measured wavelength of 450 nm.

## II-4. Crystal violet staining assay

AGS, YCC-2 cells were plated in 6-well culture plates and treated with DAA ($IC_{50}$) for 48 h. Washing the cells with 1X PBS and fixed by 10 min exposure

to 1% glutaraldehyde (Sigma, St.Louis, USA). After fixation, washing with 1X PBS. Stain cells with 0.5% crystal violet (Sigma, St.Louis, USA) for 10 min at room temperature.

## II-5. Cell cycle analysis

AGS cells were plated in 100 mm cell culture dish and treated with DAA 85 μM for time course 48 h. Cells were harvested and washed twice with cold DPBS, and then resuspended cells in 5 ml 70% EtOH 5 h to overnight at -20°C. After fixation, the cells were washed twice with cold DPBS and resuspended in propidium iodide staining solution (PI solution; RNaseA 50 μg/ml, PI 50 μg/ml in PBS) and transferred to FACS filter tubes. Cell cycle distribution after DAA treatment was measured by PI staining using fluorescence activated cell sorting (Guava® easyCyte™, Luminex, Austin, USA).

## II-6. Annexin V-FITC/PI apoptotic analysis

AGS cells were plated in 100 mm cell culture dish and treated with DAA 85 μM for time course 48 h. Cells were collected by trypsinization and washed with cold DPBS. Then collected cells were resuspended in 100 μl of binding buffer and stained with 2.5 μl of Annexin V-FITC (BD pharmingen, CA, USA) and 2.5 μl (2 mg/ml) of PI (Sigma, USA) for 15 min at room temperature in the dark. Analysis was performed by FACSCalibur flow cytometer (BECKMAN COULTER, CA, USA) with 10,000 events each time. The data were analyzed by the FLOWJO software (BD, CA, US).

## II-7. RNA isolation & cDNA synthesis

AGS cells were collected by centrifugation and total RNA was isolated from DAA-treated cells using RNAiso Plus (Takara, Shiga, Japan) according to

protocol. Prepare the incubated cell culture dish, remove the media. After cold DPBS washing, 1 ml of RNAiso Plus reagent added to the dish, use a cell scraper. Collect the cells with pipette and transfer them to the new 1.5 ml tube, adding to chloroform 0.2 ml, after inverting 5-10 times, room temperature incubate for 5 min. centrifuge at 12,000 ×*g* for 15 min at 4°C. Transfer the top liquid layer to new 1.5 ml tube without touching middle layer. Add up to 0.5 ml of isopropanol of the top layer. Mix together well. Room temperature incubate for 10 min. Centrifuge at 12,000 ×*g* for 10 min at 4°C. Carefully remove the supernatant, add an amount of 75% cold ethanol that was equivalent to the supernatant. Centrifuge the solution at 12,000 ×*g* for 5 min at 4°C and discard supernatant. Dry the precipitate by leaving the tube open for several min. After the precipitate is dry, dissolved it with appropriate amount of DEPC water. AGS cells cDNA synthesis using cDNA synthesis kits (TOYOBO, Osaka, Japan) according to standard protocol.

## II-8. Reverse transcription polymerase chain reaction (RT-PCR)

The PCR protocol for the Ex-Taq (Takara, Shiga, Japan) was carried out in 20 µl total volume, containing 0.2 µl of Ex-Taq (5 units/µl), 2 µl of 10X Ex Taq buffer, 2 µl dNTP Mixture (2.5 mM each), 1 µl of each primer (10 pmol/µl), an appropriate amount of cDNA 1500 ng/µl, and sterilized distilled water up to 20 µl. PCR thermal cycler conditions were set to one cycle for an initial denaturation at 95°C for 3 min, followed by 28-31 cycles at 95°C for 30 sec, annealing at 58-60°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 5 min. DNA bands (PCR products) were examined using 1% agarose gel electrophoresis at 100V for 20 min.

**Table 1. Sequence of apoptosis regulatory genes primer design**

| | | |
|---|---|---|
| *BAX* | Forward | 5'- AAGAAGCTGAGCGAGTGTCT -3' |
| | Reverse | 5'- GTTCTGATCAGTTCCGGCAC -3' |
| *CASP3* | Forward | 5'- TTTTTCAGAGGGGATCGTTG -3' |
| | Reverse | 5'- CGGCCTCCACTGGTATTTTA -3' |
| *CASP9* | Forward | 5'- GAGGGAGTCAGGCTCTTCCT -3' |
| | Reverse | 5'- TCACCAAATCCTCCAGAACC -3' |
| *TP53* | Forward | 5'- CCTCACCATCATCACACTGG -3' |
| | Reverse | 5'- CCTCATTCAGCTCTCGGAAC -3' |
| *GAPDH* | Forward | 5'- GGCTGCTTTTAACTCTGGTA -3' |
| | Reverse | 5'- ACTTGATTTTGGAGGGATCT -3' |

**Table 2. Sequence of cell cycle regulatory genes primer design**

| | | |
|---|---|---|
| *CCNB1* | Forward | 5'- TGAGGAAGAGCAAGCAGTCA -3' |
| | Reverse | 5'- AAACATGGCAGTGACACCAA -3' |
| *CCND1* | Forward | 5'- TGAGGAAGAGCAAGCAGTCA -3' |
| | Reverse | 5'- AAACATGGCAGTGACACCAA -3' |
| *CDK1* | Forward | 5'- TGAGGAAGAGCAAGCAGTCA -3' |
| | Reverse | 5'- AAACATGGCAGTGACACCAA -3' |
| *CDKN1A* | Forward | 5'- CACCACTGGAGGGTGACTTC -3' |
| | Reverse | 5'- ATCTGTCATGCTGGTCTGCC -3' |
| *CDKN1B* | Forward | 5'- AGATGTCAAACGTGCGAGTG -3' |
| | Reverse | 5'- TCTCTGCAGTGCTTCTCCAA -3' |
| *GAPDH* | Forward | 5'- GGCTGCTTTTAACTCTGGTA -3' |
| | Reverse | 5'- ACTTGATTTTGGAGGGATCT -3' |

## II-9. Western blotting

Cell lysate extractions were prepared with RIPA buffer (1% NP-40; 0.1% sodium dodecyl sulfate; 0.5% desoxycholate; 150 mM NaCl; 50 mM Tris, pH 7.5) and a protease inhibitor cocktail. 50 μg of total protein was resolved in SDS PAGE gels and electro transferred to PVDF membranes, and then blocked in 5% skim milk in 1X PBS with 0.05% Tween-20 (PBST). Primary antibodies were incubated with the blots at a 1:1000 dilution in minimal volumes of 5% Bovine serum albumin (BSA; Bovogen, Keilor East, Australia) in PBST buffer for 1 h at room temperature or over-night at 4°C. Anti-mouse or anti-rabbit HRP-conjugated secondary antibodies were incubated at a 1:5000 dilution in PBST buffer for 1.5 h at room temperature. Antibodies used in this study were anti-Bcl-2, anti-Bcl-$X_L$, anti-Bax, anti-Caspase-9, anti-p53, anti-Cyclin B1, anti-Cyclin D1, anti-Cdk1, anti-Cdk2, anti-Cdk4, anti-p21, anti-Rb, anti-p-Rb and anti-beta-actin that were purchased from Santa Cruz Biotechnology (Texas, USA). Anti-caspase-3, anti-PARP were obtained from GeneTex (CA, USA). Anti-mouse and anti-rabbit polyclonal immunoglobulins were purchased from Bethyl Laboratories (AL, USA). Membranes that were probed with primary antibodies and secondary antibodies were detected by ECL solution (BIO-RAD, CA, USA) using a Supernova-Q1800 ChemiDoc (Lugen™, Bucheon, Korea) detector, according to the manufacturer's directions.

**Table 3. Information of primary antibody used in western blot**

| Primary antibody name | Manufacturer | Host | Dilution rate |
|---|---|---|---|
| Bcl-2 | Santa Cruz | Mouse | 1 : 1000 |
| Bcl-$X_L$ | Santa Cruz | Rabbit | 1 : 1000 |
| Bax | Santa Cruz | Rabbit | 1 : 1000 |
| Caspase-9 | Santa Cruz | Mouse | 1 : 1000 |
| Caspase-3 | GeneTex | Rabbit | 1 : 1000 |
| PARP | GeneTex | Rabbit | 1 : 1000 |
| p53 (DO-1) | Santa Cruz | Mouse | 1 : 1000 |
| Cyclin B1 | Santa Cruz | Rabbit | 1 : 1000 |
| Cyclin D1 | Santa Cruz | Rabbit | 1 : 1000 |
| Cdk1 | Santa Cruz | Rabbit | 1 : 1000 |
| Cdk2 | Santa Cruz | Mouse | 1 : 1000 |
| Cdk4 | Santa Cruz | Rabbit | 1 : 1000 |
| p21 (H164) | Santa Cruz | Rabbit | 1 : 1000 |
| Rb (C-15) | Santa Cruz | Mouse | 1 : 1000 |
| p-Rb (Ser 807/811) | Santa Cruz | Mouse | 1 : 1000 |
| β-actin | Santa Cruz | Mouse | 1 : 5000 |

## II-10. Library preparation and RNA sequencing

Library preparation and RNA sequencing a library was constructed using SENSE mRNA-Seq Library Prep Kit (Lexogen, Vienna Austria) as per manufacturers recommendations. High-throughput sequencing was then performed as paired-end 100 sequencing using a Agilent's 2100 Bioanalyzer system (Illumina, San Diego, CA). RNA-Seq reads were mapped using Exdega software to obtain the alignment file. The alignment file was used for assembling transcripts, estimating abundances, and detecting differential expression of genes/isoforms using cufflinks. Gene classification was based on searches done by DAVID (david.abcc.ncifcrf.gov) and Medline databases (ncbi.nlm.nih.gov). The library preparation and RNA sequencing were performed as the NGS services provided by Ebiogen Inc. (Seoul, Korea).

## II-11. Statistical analysis

All data are the mean ± SEM of five experiments. Fisher's exact test was adopted for statistical evaluation of the results. A t-test was used for the analysis of protein level measurements. All *P*-values are two-sided. Precise *p*-values are given. All statistical analyses were performed in GraphPad prism version 5 (GraphPad Software, San Diego, CA).

# III. RESULTS

## III-1. Dehydroabietic acid (DAA) inhibits cell proliferation in gastric cancer cells

To determine the function of Dehydroabietic acid (DAA) (Fig. 1) in the proliferation of gastric cancer cells, i performed WST-8 assay on human gastric cancer cell lines (AGS, YCC-2, MKN-28, SNU-216, SNU-601 and SNU-668). After DAA treatment by concentration (0, 20, 40, 60, 80, 100, 120, 140, and 160 μM), after 24, 48 h confirmed the cell viability, as the treatment time of DAA increased, the growth curve of cell viability decreased significantly in a concentration-dependent manner (Fig. 2). The six of gastric cancer cell lines $IC_{50}$ values are summarized in Table 4. These findings support that DAA has anti-cancer effects on human gastric cancer cells in *in vitro*.

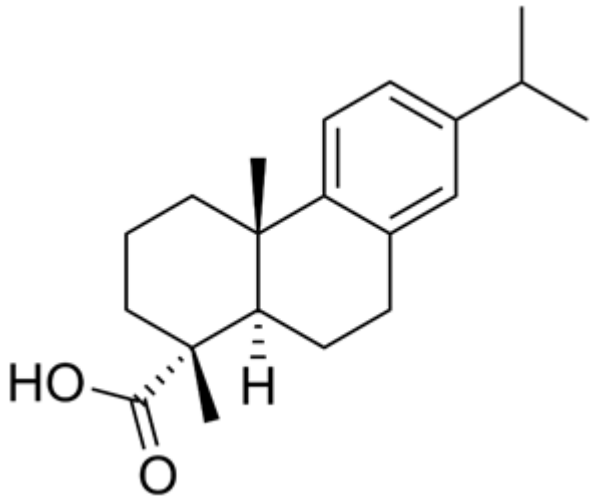



**Fig. 1. Chemical structure of Dehydroabietic acid (DAA).** Dehydroabietic acid, a derivative of abietic acid. $C_{20}H_{28}O_2$, molecular formula. 300.44, average molecular mass.

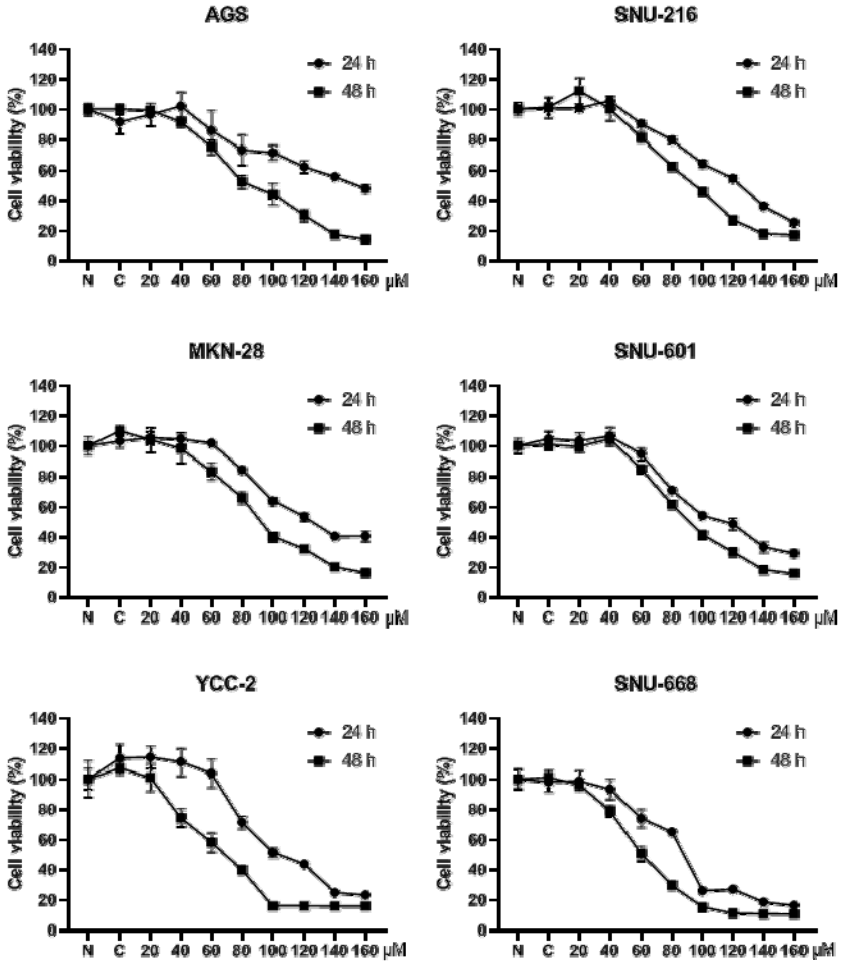



**Fig. 2. Effects on cell growth by treatment of DAA in six gastric cancer cell lines.** Cell growth inhibition of gastric cancer cells by DAA. Cells were treated with 20-160 μM DAA for 24, 48 h. Cell viability was measured by the WST-8 assay. Data are shown as the mean of five independent experiments.

**Table 4. $IC_{50}$ values for DAA in viability with gastric cancer cell lines**

| Cell line | $IC_{50}$ (95% CI) | $R^2$ |
|---|---|---|
| AGS | 148.2 (142.8 - 153.8) μM | 0.9211 |
| MKN-28 | 126.1 (122.3 - 130.1) μM | 0.9598 |
| YCC-2 | 99.2 (96.1 - 102.4) μM | 0.9691 |
| SNU-216 | 120 (117.7 - 122.4) μM | 0.9828 |
| SNU-601 | 109.7 (106.4 - 113) μM | 0.9688 |
| SNU-668 | 87.35 (83.84 - 91.02) μM | 0.9582 |

- $IC_{50}$ values of human gastric cancer cell lines (DAA 24 h treatment)
- 95% CI = 95% Confidence Intervals

| Cell line | $IC_{50}$ (95% CI) | $R^2$ |
|---|---|---|
| AGS | 87.23 (85.05 - 89.46) μM | 0.9846 |
| MKN-28 | 87.45 (84.96 - 90.01) μM | 0.9784 |
| YCC-2 | 60.46 (57.56 - 63.51) μM | 0.9639 |
| SNU-216 | 92.83 (89.75 - 96.02) μM | 0.9715 |
| SNU-601 | 92.97 (90.95 - 95.03) μM | 0.9865 |
| SNU-668 | 59.95 (58.44 - 61.50) μM | 0.9895 |

- $IC_{50}$ values of human gastric cancer cell lines (DAA 48 h treatment)
- 95% CI = 95% Confidence Intervals

### III-2. DAA inhibits cell proliferation in time-dependent manner in AGS and YCC-2 cells

AGS and YCC-2 cells were selected from gastric cancer cell lines. AGS and YCC-2 cells were treated with 48 h $IC_{50}$ (AGS-85 μM, YCC-2-60 μM) value of DAA. 24 h and 48 h after DAA treatment, cell viability was confirmed by WST-8 assay. Both AGS and YCC-2 cells showed reduced cell viability in a time-dependent manner, with a 50% survival rate at 48 h (Fig. 3). Crystal violet staining was performed 48 h after DAA $IC_{50}$ treatment in AGS and YCC-2 cells (Fig. 4). It was confirmed that the survival rate is significantly reduced in the DAA treatment group. Morphological changes of AGS and YCC-2 cells by DAA $IC_{50}$ treatment were observed under a microscope (Fig. 5). The results showed that DAA inhibited gastric cancer cell proliferation and induces morphological modification.

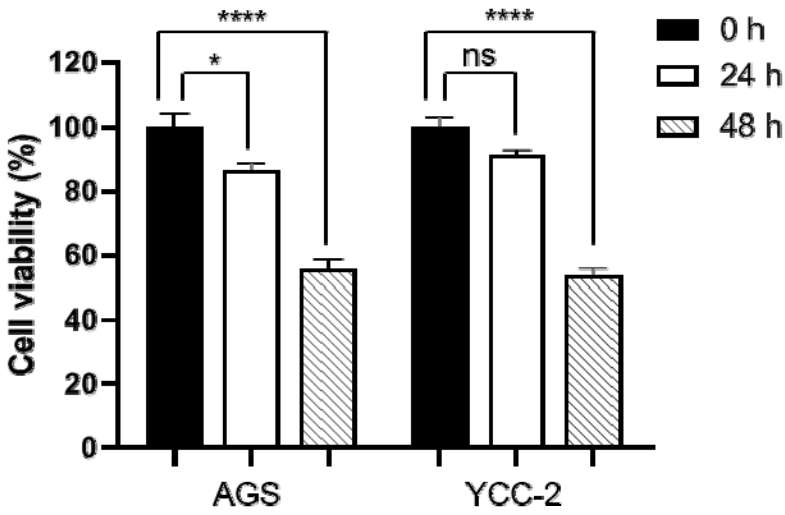



**Fig. 3. Effects on growth by treatment of DAA of AGS and YCC-2 cells.** Cell growth inhibition of gastric cancer cells by DAA. Cells were treated with DAA $IC_{50}$ (AGS-85 μM, YCC-2-60 μM) for 24, 48 h. Cell viability was measured by the WST-8 assay. The error bars indicate 95% confidence interval; ****$P$<0.0001, *$P$<0.0334 using the two-sided t-test.

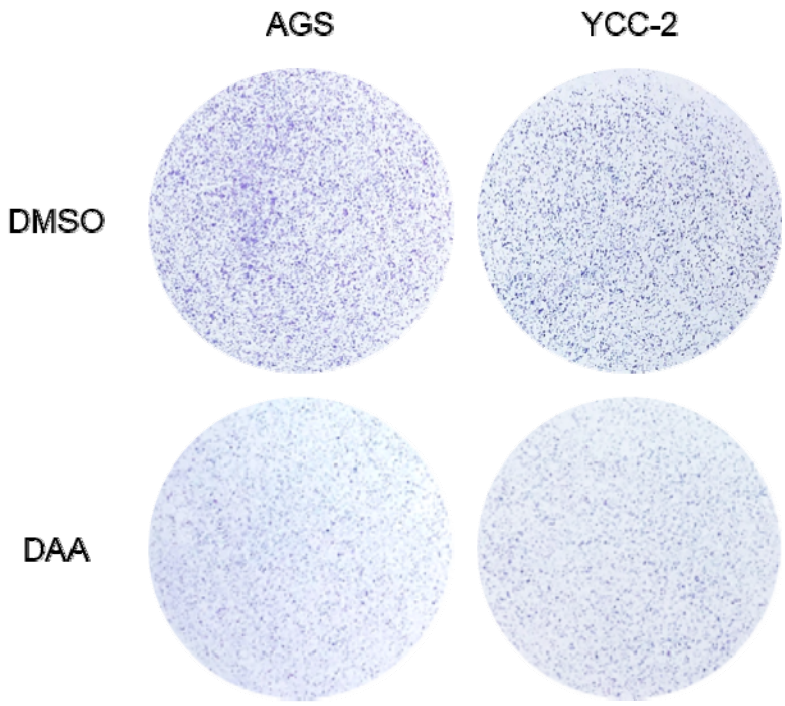



**Fig. 4. Decreased cell proliferation in AGS and YCC-2 cells.** AGS and YCC-2 cells were seeded and treated with DAA after 24 h. After DAA treatment (AGS-85 μM, YCC-2-60 μM) for 48 h, density of AGS and YCC-2 cells were observed by digital camera system and crystal violet staining.

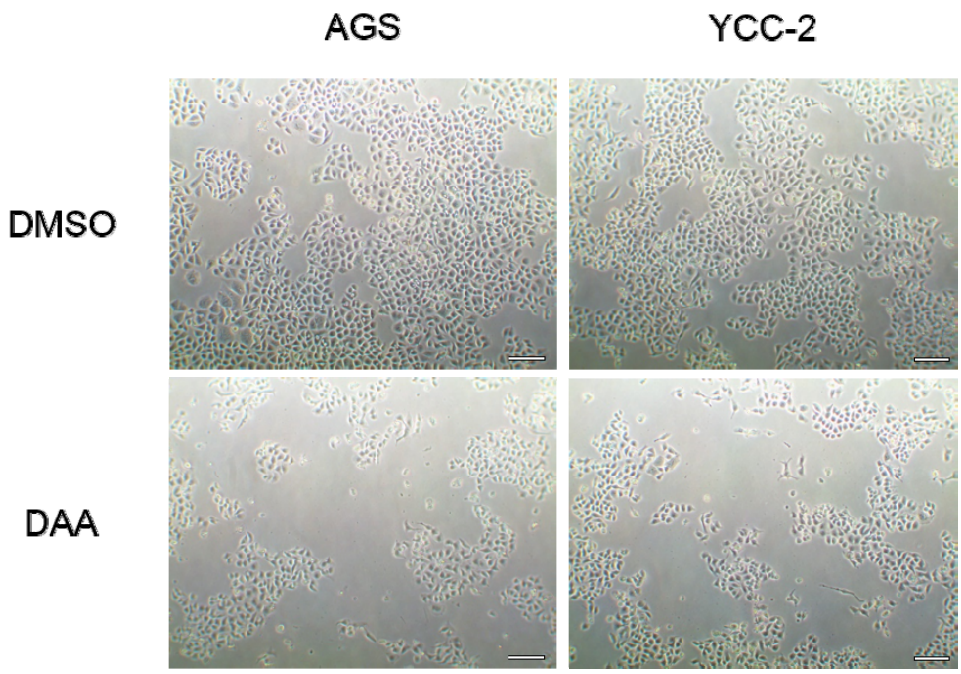



**Fig. 5. DAA affected the morphology of viable AGS and YCC-2 cells.** The cells were treated DAA (AGS-85 μM, YCC-2-60 μM) for 48 h. The cells were examined and photographed under phase-contrast microscope (Scale bar; 100 μM).

### III-3. DAA induces G1 phase cell cycle arrest

To evaluate whether growth inhibition effect of DAA on gastric cancer cell proliferation was due to cell cycle arrest, the cell cycle was analyzed by PI staining in AGS cells. After 48 h of DAA 85 μM treatment in AGS cells, flow cytometry was performed. As a result, it was confirmed that the Sub-G1, G1 population increased (Fig. 6). RT-PCR analysis confirmed the expression of the *CCNB1*, *CCND1*, *CDK1*, *CDKN1A, CDKN1B* and *GAPDH* gene. The expression of *CCNB1*, *CCND1*, and *CDK1* genes that induce cell cycle progression was decreased, expression of the *CDKN1A* and *CDKN1B* genes, which induce cell cycle arrest, was increased (Fig. 7). I performed western blot analysis to identify expression in cell cycle-related proteins. The results indicated that the expression of G2/M phase related proteins Cdk1 and Cyclin B1 were significantly reduced, and that of G1/S phase related proteins Cdk2, Cdk4, Cyclin D1 and phospho-Rb were also decreased. Also, not only the expression of cell cycle regulatory related proteins p53 was significantly increased, but also CDK inhibitor related p21 was increased (Fig. 8). These results suggest that DAA induces cell cycle arrest and inhibits cell proliferation.

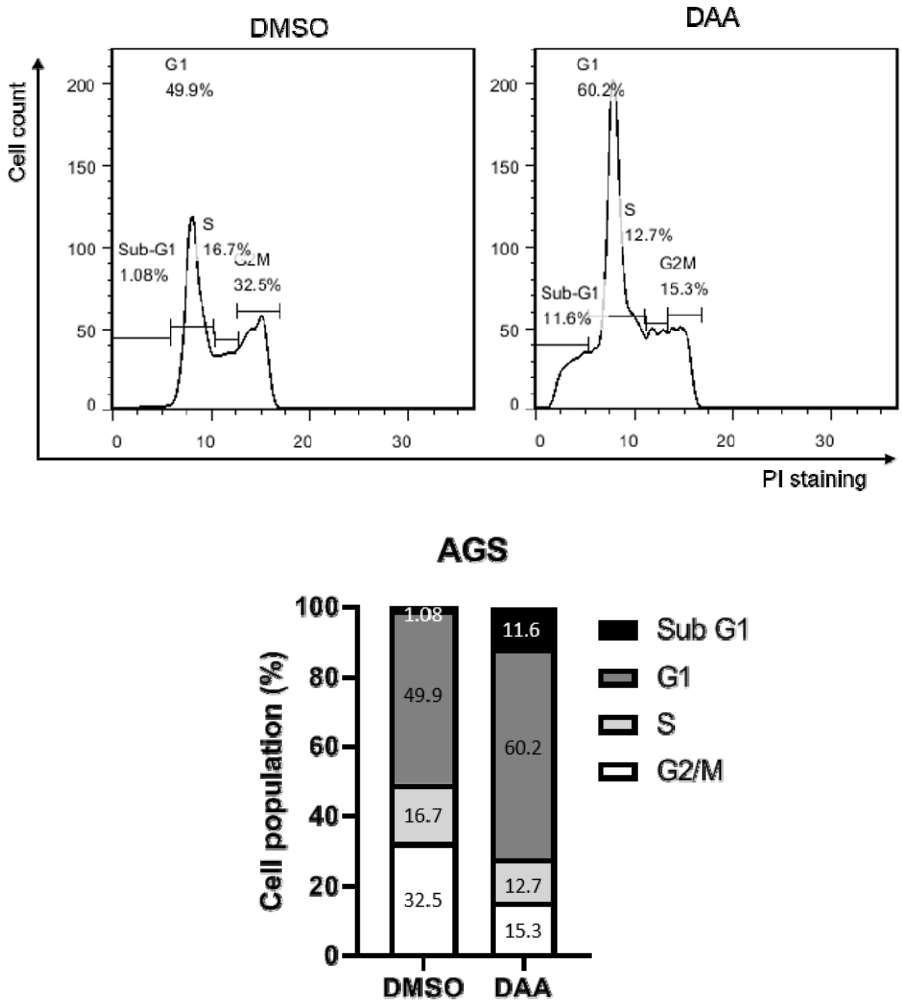



**Fig. 6. Cell cycle arrest by treatment of DAA in AGS cells.** Sub-G1, G1 population is induced after DAA (85 μM) 48 h treatment. Cell cycle distribution was analyzed using a flow cytometer, percentages of cell cycle phase cells which were determined based on DNA content histogram. Bar graphs indicated each percentage.

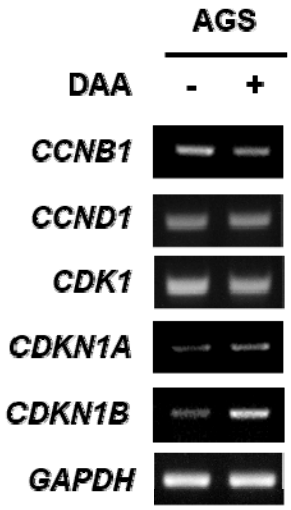



**Fig. 7. mRNA expression levels of cell cycle related genes in AGS cells treated with DAA.** Effects of treatment with DAA on *CCNB1, CCND1, CDK1, CDKN1A, CDKN1B* and *GAPDH* in AGS cells. Cells were treated with DAA (85 μM) for 48 h and then RNA were prepared and analyzed by RT-PCR.

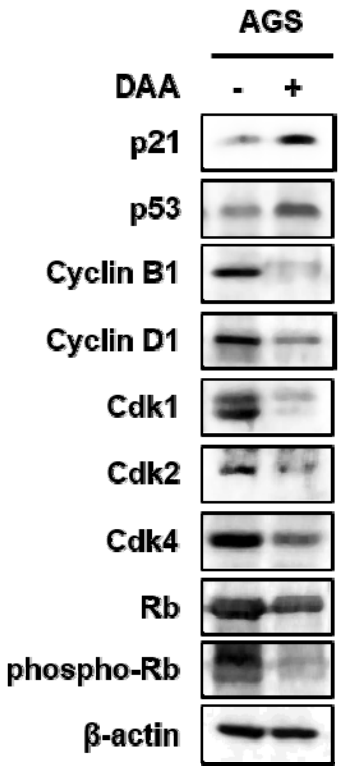



**Fig. 8. Protein expression levels of cell cycle related genes in AGS cells with DAA treatment.** Effects of treatment with DAA on p21, p53, Cyclin D1, Cyclin B1, Cdk4, Cdk2, Cdk1, Rb, phospho-Rb and β-actin in AGS cells. Cells were treated with DAA (85 μM) for 48 h and then cell lysates were prepared and analyzed by western blot.

## III-4. DAA induces apoptosis by regulating caspase, Bcl-2 family

To investigate the mechanism of DAA's growth inhibition, i performed flow cytometry. 48 h after DAA 85 μM treatment in AGS cells, it was measured by Annexin V/PI staining. In the DMSO treatment group, the early apoptosis ratio was 2.92% and the late apoptosis ratio was 1.14%, while in the DAA treatment group, the early apoptosis ratio was 7.52% and the late apoptosis ratio was 5.87% (Fig. 9). RT-PCR analysis showed that the expression of pro-apoptotic *BAX* gene was increased (There are no difference in the gene expression of CASP3 and CASP9) (Fig. 10). As a result of western blot analysis, The expression of anti-apoptotic Bcl-2 and Bcl-$X_L$ proteins was decreased. It also increases pro-apoptotic Bax. DAA treatment increases the cleaved form of Caspase-9 and Caspase-3 and induces apoptosis by increasing the cleaved form of PARP (Fig. 11). These results indicate that DAA alters the expression of Caspase and Bcl-2 family proteins by apoptosis stimulation in AGS cells.

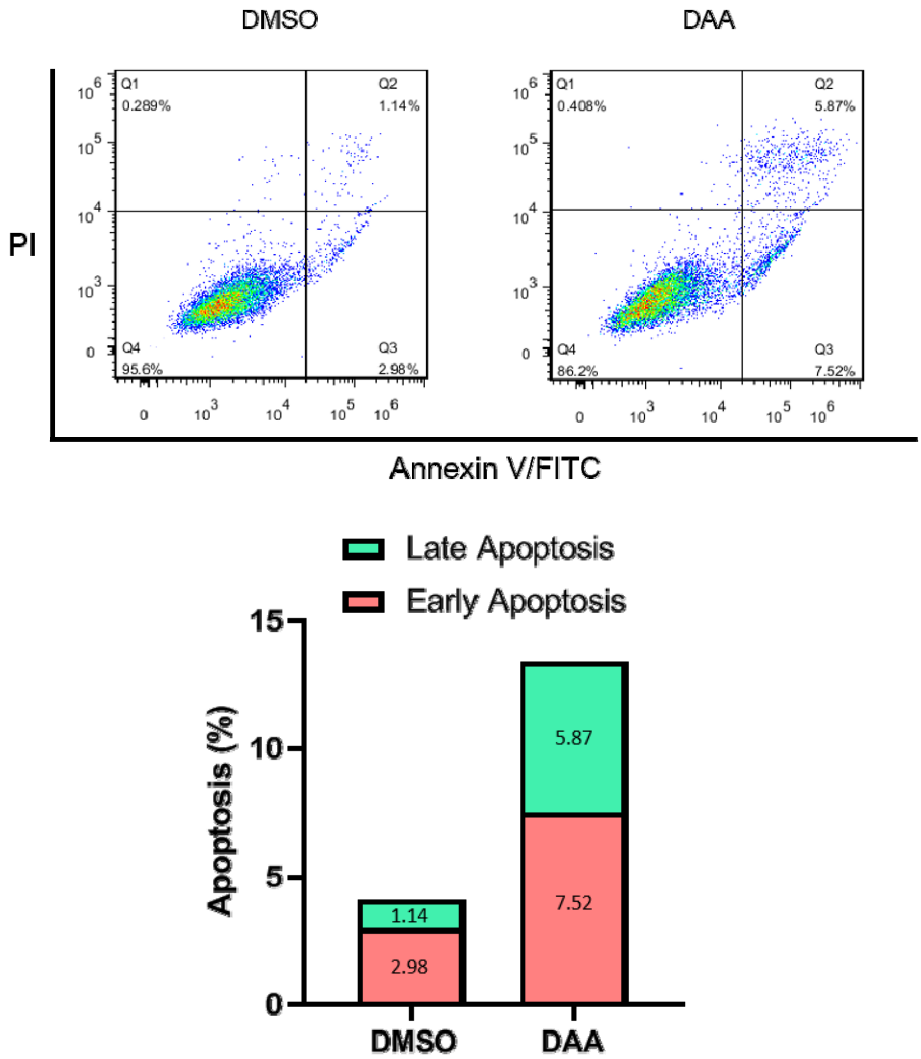



**Fig. 9. DAA induced apoptosis in AGS cells, measured by flow cytometry.** Percentage of vitality, apoptosis, and dead cell was detected by flow cytometer. The total ratio of apoptosis (Q2 and Q3 quadrants) was higher in treated DAA (85 μM) groups. The bar graph shows the ratio of early apoptosis and late apoptosis.

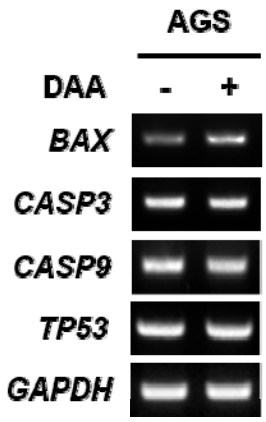



**Fig. 10. mRNA expression levels of apoptosis related genes in AGS cells treated with DAA.** Effects of treatment with DAA on *BAX, CASP3, CASP9, TP53* and *GAPDH* in gastric cancer cells. Cells were treated with DAA (85 μM) for 48 h and then RNA were prepared and analyzed by RT-PCR.

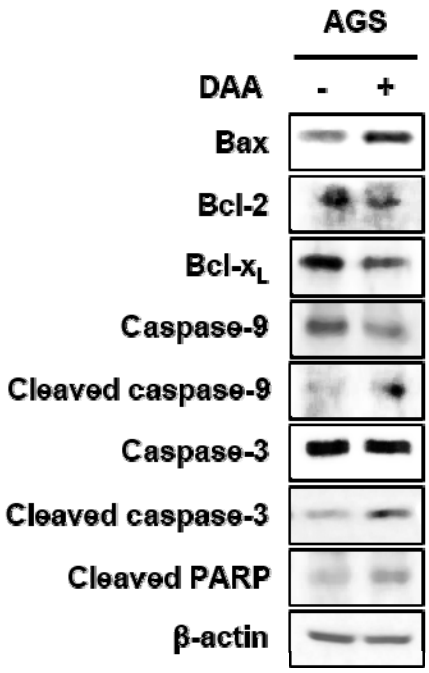



**Fig. 11. Protein expression levels of apoptosis related genes in AGS cells treated with DAA.** Effects of treatment with DAA on Bax, Bcl-2, Bcl-$X_L$, Caspase-9, Cleaved caspase-9, Caspase-3, Cleaved caspase-3, Cleaved PARP and β-actin in AGS cells. Cells were treated with DAA (85 μM) for 48 h, and then cell lysates were prepared and analyzed by western blot.

### III-5. DAA and 5-FU combination treatment showed synergistic effect of growth inhibition in AGS cells

I confirmed that DAA can be applied to treatment as an anti-cancer drug. 5-Fluorouracil (5-FU) and DAA, which are currently used as anti-cancer drug, were treated in AGS cells. DAA (85 μM), 5-FU (0.5 μg/ml) was single treated and combined treatment in AGS cells. 48 h after treatment with the drug, the results were confirmed by the WST-8 assay. Treatment with DAA alone resulted in higher growth inhibition than 5-FU treatment. The combination of DAA and 5-FU showed the highest cell proliferation inhibition. DAA has a higher cell proliferation inhibition rate than 5-FU, which is used for cancer treatment and can show higher efficiency in combination treatment (Fig. 12).

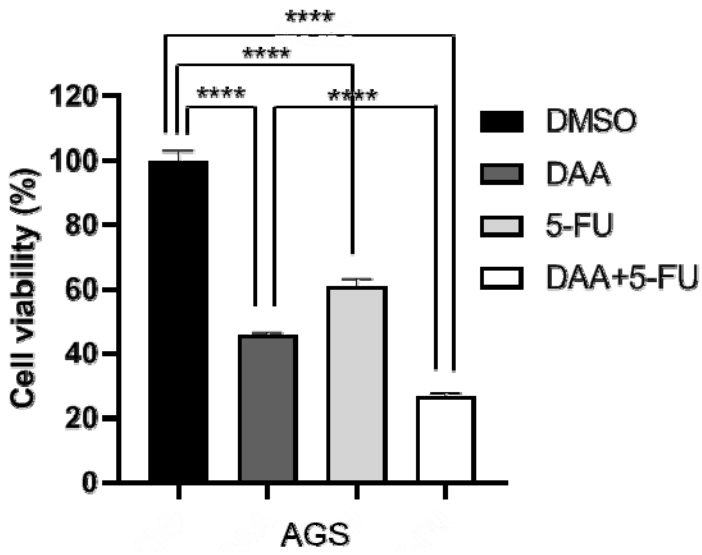
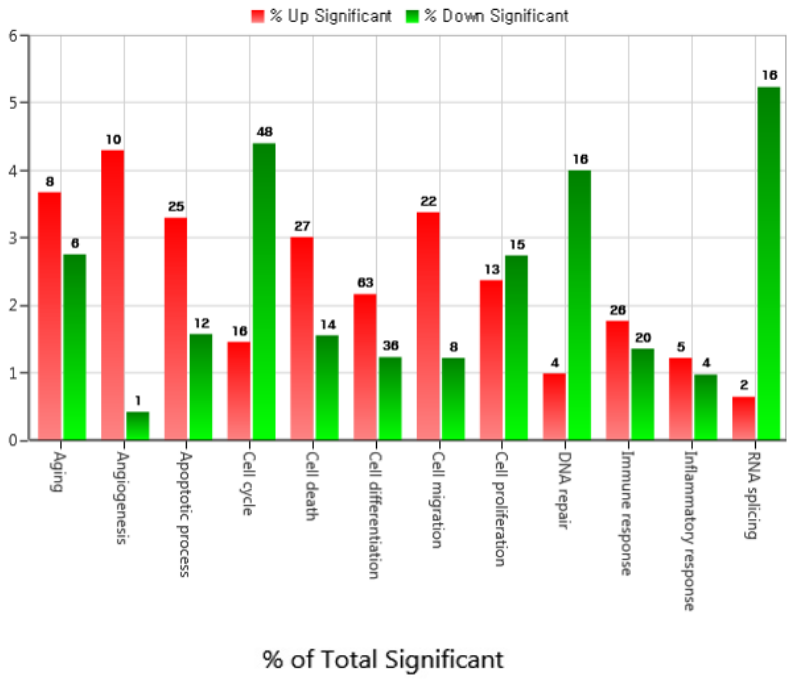



**Fig. 12. Combined treatment with DAA and anti-cancer drug in AGS cells.** Combined treatment with DAA (85 μM) and anti-cancer agents 5-FU (0.5 μg/ml) enhanced the therapeutic effects on AGS cells. The error bars indicate 95% confidence intervals; ****$P$<0.0001 using the two-sided t-test.

### III-6. DAA regulates various genes related to apoptosis and cell cycle

It was investigated that DAA induces apoptosis through cell cycle arrest in AGS cells. I performed RNA sequencing to identify various gene expressions regulated by DAA. First, specific changes were selected by filtering RNA sequencing results (Fold change: 1.5, $Log_2$: 9). Gene ontology was performed using Exdega's DEG analysis tool. In the apoptotic process, a total of 37 genes were regulated, in the cell cycle, a total of 64 genes were regulated (Fig. 13). Through these results, i selected genes related to cell cycle and apoptosis. 33 up-regulated genes and 33 down-regulated genes were selected and classified (Table 5 and 6). The expression level of the 66 genes selected was shown using heatmap (Fig. 14). RT-PCR was performed on the classified genes to identify up-regulated and down-regulated genes. Among them, 24 of 66 genes were up-regulated and 22 were down-regulated (Fig. 15 and 16). These results suggest that DAA regulates the expression of various genes that regulate apoptosis and the cell cycle.



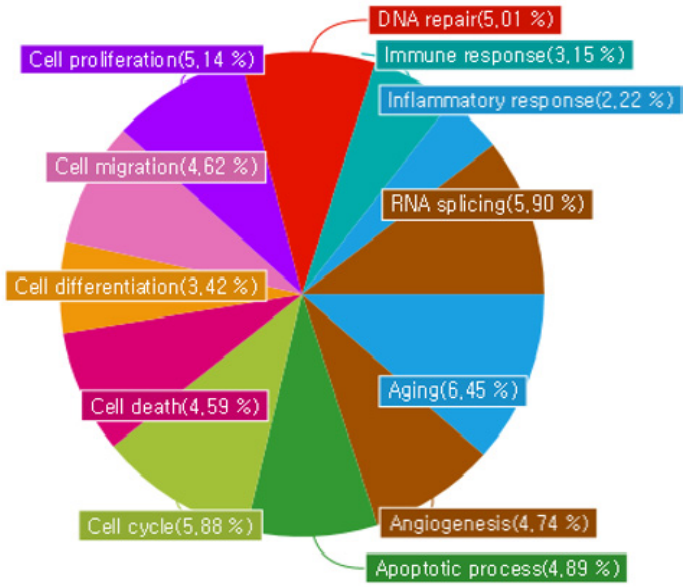




**Fig. 13. Gene category chart of performed RNA sequencing with DAA treatment in AGS cells.** Gene ontology was performed using Exdega's DEG analysis tool (Fold change: 1.5, $Log_2$; 9). A total of 613 genes were classified by category.

**Table 5. Up-regulated genes by DAA treatment in AGS cells**

| Gene symbol | Accession number | Gene name | Fold change |
|---|---|---|---|
| **Cell cycle** | | | |
| *CDKN2B* | NM_004936 | cyclin-dependent kinase inhibitor 2B | 2.484 |
| *CDKN1A* | NM_001220777 | cyclin-dependent kinase inhibitor 1A | 2.095 |
| *GADD45A* | NM_001924 | growth arrest and DNA damage inducible alpha | 1.701 |
| *PARD6B* | NM_032521 | par-6 family cell polarity regulator beta | 1.621 |
| **Cell proliferation** | | | |
| *HMOX1* | NM_002133 | heme oxygenase 1 | 2.661 |
| *ARHGEF2* | NM_004723 | Rho/Rac guanine nucleotide exchange factor 2 | 2.493 |
| *EGFR* | NM_005228 | epidermal growth factor receptor | 2.278 |
| *VEGFA* | NM_001171625 | vascular endothelial growth factor A | 2.175 |
| *JAG1* | NM_000214 | jagged 1 | 2.094 |
| *ITGA2* | NM_002203 | integrin subunit alpha 2 | 1.966 |
| *BTG1* | NM_001731 | B-cell translocation gene 1, anti-proliferative | 1.781 |
| **Apoptosis** | | | |
| *HSPA5* | NM_005347 | heat shock protein family A (Hsp70) member 5 | 2.806 |
| *AMIGO2* | NM_181847 | adhesion molecule with Ig-like domain 2 | 2.116 |
| *IER3* | NM_003897_4 | immediate early response 3 | 1.838 |
| *IGF2R* | NM_000876 | insulin-like growth factor 2 receptor | 1.665 |
| **DNA replication** | | | |
| *CTGF* | NM_001901 | connective tissue growth factor | 1.936 |

**Table 6. Down-regulated genes by DAA treatment in AGS cells**

| Gene symbol | Accession number | Gene name | Fold change |
|---|---|---|---|
| **Cell cycle** | | | |
| *CDK1* | NM_001170406 | cyclin-dependent kinase 1 | 0.657 |
| *CCNE1* | NM_001238 | cyclin E1 | 0.559 |
| *CHAF1A* | NM_005483 | chromatin assembly factor 1 subunit A | 0.538 |
| *AURKA* | NM_198434 | aurora kinase A | 0.537 |
| *CDKN3* | NM_001130851 | cyclin-dependent kinase inhibitor 3 | 0.527 |
| *GTSE1* | NM_016426 | G2 and S-phase expressed 1 | 0.524 |
| *CENPE* | NM_001813 | centromere protein E | 0.524 |
| *MKI67* | NM_001145966 | marker of proliferation Ki-67 | 0.512 |
| *KIFC1* | NM_002263 | kinesin family member C1 | 0.486 |
| *CCNB1* | NM_031966 | cyclin B1 | 0.451 |
| **Cell proliferation** | | | |
| *STAT1* | NM_007315 | signal transducer and activator of transcription 1 | 0.657 |
| *NME1* | NM_198175 | NME/NM23 nucleoside diphosphate kinase 1 | 0.559 |
| *LIFR* | NM_001127671 | leukemia inhibitory factor receptor alpha | 0.547 |
| *FOXM1* | NM_001243088 | forkhead box M1 | 0.521 |
| *DHCR24* | NM_014762 | 24-dehydrocholesterol reductase | 0.496 |
| *CDX2* | NM_001265 | caudal type homeobox 2 | 0.444 |
| *DHRS2* | NM_005794 | dehydrogenase/reductase (SDR family) member 2 | 0.356 |
| *SOX2* | NM_003106 | SRY-box 2 | 0.264 |
| **Apoptosis** | | | |
| *HMGB1* | NM_002128 | high mobility group box 1 | 0.621 |
| *BIRC5* | NM_001168 | baculoviral IAP repeat containing 5 | 0.449 |
| **DNA replication** | | | |
| *PCNA* | NM_182649 | proliferating cell nuclear antigen | 0.55 |
| *CHAF1A* | NM_005483 | chromatin assembly factor 1 subunit A | 0.538 |
| *TOP2A* | NM_001067 | topoisomerase (DNA) II alpha 170kDa | 0.511 |
| *RRM2* | NM_001165931 | ribonucleotide reductase regulatory subunit M2 | 0.507 |


**Fig. 14. List of genes that up-regulation and down-regulation after DAA treatment in AGS cells.** AGS cells were treated with DAA and RNA was extracted. Then, mRNA expression was analyzed by RNA-sequencing. Visualization was done using the JAVA Treeview software.

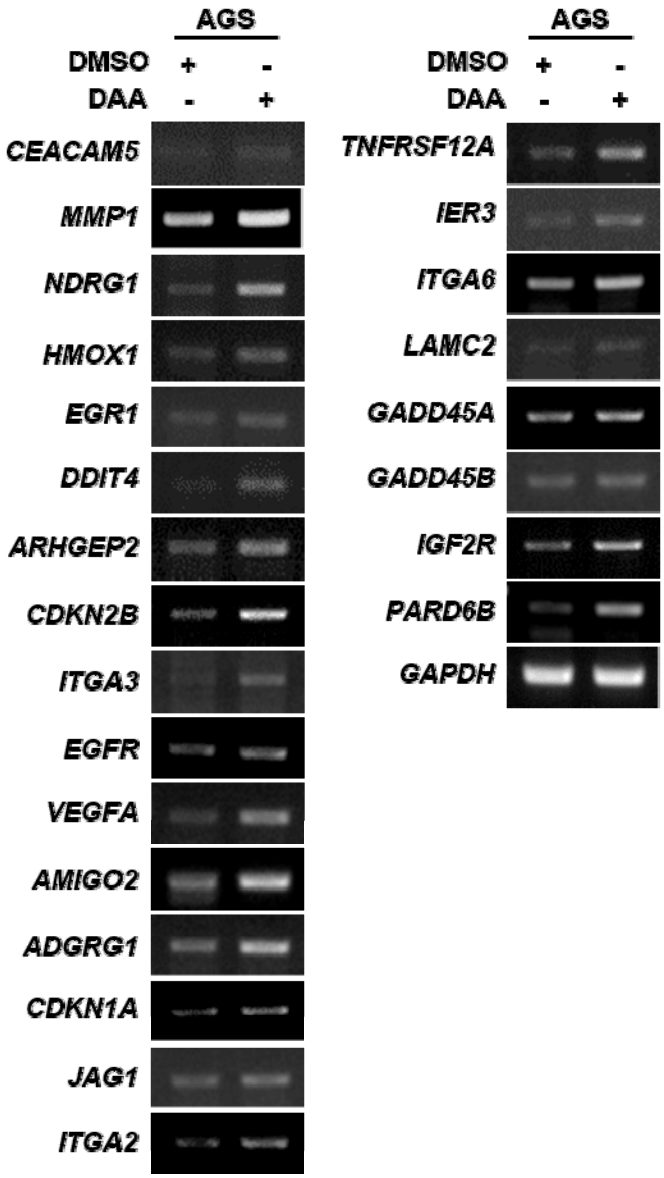



**Fig. 15. Up-regulated genes by DAA treatment in AGS cells.** Up-regulated genes were selected from the RNA-sequencing and analyzed by RT-PCR. *GAPDH* was used as a loading control.

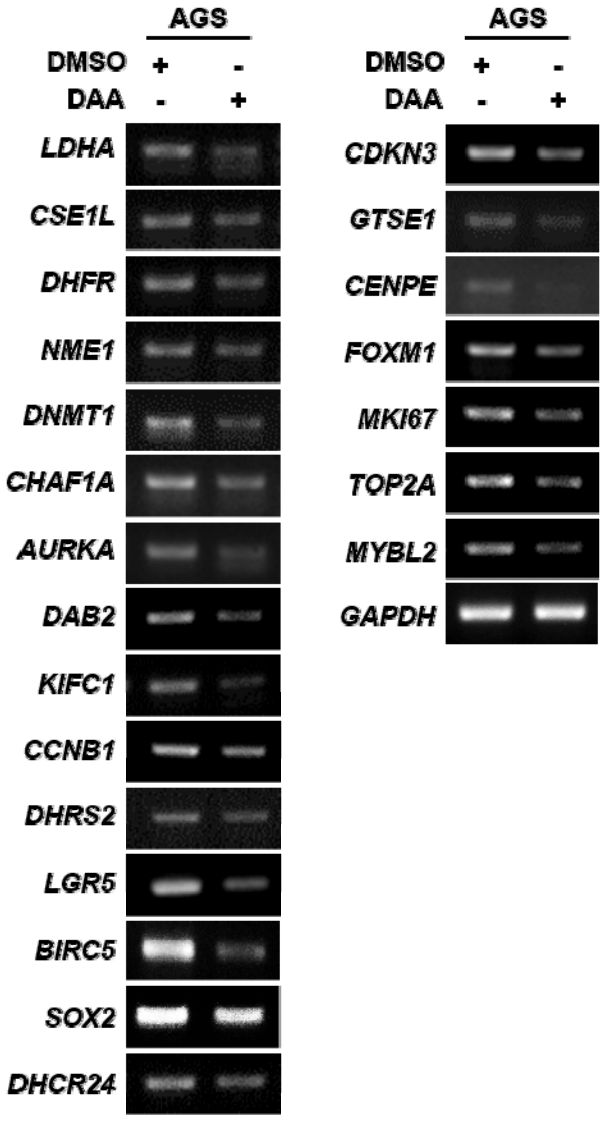



**Fig. 16. Down-regulated genes by DAA treatment in AGS cells.** Down-regulated genes were selected from the RNA-sequencing and analyzed by RT-PCR. *GAPDH* was used as a loading control.

# IV. DISCUSSION

Gastric cancer is characterized as one of the most malignant and fatal cancers [17]. Recently, there was no report on the effects of Dehydroabietic acid (DAA). In this study, i investigated the effects of DAA on cell proliferation in gastric cancer cells. I confirmed the growth inhibition effects of DAA in gastric cancer cells using WST-8 and crystal violet assays. The results show that DAA treatment considerably inhibited the viability of gastric cancer cell lines in a dose- and time-dependent manner.

Apoptosis by cell cycle arrest is an important target in cancer cell therapy [6]. Induction of apoptosis results in morphological changes [18]. Annexin V/PI staining was performed to identify the morphological changes caused by apoptosis. Through the cell cycle analysis demonstrated that the Sub-G1, G1 phase increases in DAA treatment. Next, the percentages of cells in apoptotic stages (early, late) significantly increased following DAA treatment. There are two signaling pathways for apoptosis. The signaling pathways are the intrinsic and extrinsic pathways. Both pathways occur through Caspase-3 [19]. The Bcl-2 family are important role in the intrinsic pathway [20]. In response to chemotherapy-induced apoptosis stimulation, cytochrome c is released into the cytoplasm inside the mitochondria [21]. The released cytochrome c interacts with Apaf-1 and activates caspase-9 [22]. Then caspase-3 is activated by caspase-9. Caspase-3 is a protein that can cleave PARP [23]. Apoptosis is induced by cleaved PARP. In this study, i found that DAA regulates proteins involved in the intrinsic pathway. In this study, i found that DAA increased pro-apoptotic Bax and decreased anti-apoptotic Bcl-2. I compared 5-FU and DAA, which are used as anti-cancer drugs. The combination of 5-FU and DAA was found to be more effective than single treatment.

Diterpene has been recently studied because of its inhibitory effect on tumor cells, such as induction of tumor cell apoptosis [24]. However, no study of DAA has been reported. Previous studies have confirmed that QC4, a derivative of

DAA, induces apoptosis in gastric cancer cells [25]. Although QC4 is a derivative made by DAA, no studies on the anti-cancer effect on DAA itself have been conducted. In this study, DAA was block the cell cycle progression in G1 phase, and increased Sub-G1 phase. Apoptosis was induced through regulation of the Bcl-2 family (Bax, Bcl-2, Bcl-$X_L$). In addition, various genes that regulated cell cycle and apoptosis were changed through RNA sequencing. Among them, the expression of the *BIRC5* gene was reduced. *BIRC5* is known to inhibit apoptosis [26]. DAA regulates various genes that induce cell cycle arrest and apoptosis, and *BIRC5* known as anti-apoptotic gene was inhibited in AGS cells. As a result, DAA demonstrated the potential for new anti-cancer drug.

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# 감사의 글

먼저, 짧은 시간이었지만 초조해하는 저를 가르치고 이끌어 주신 김석준 교수님께 감사의 말씀을 전합니다. 대학원 생활에 있어서 힘든 일과 고민에 빠져있을 때 마다, 올바른 방향으로 지도해주시고, 초조해 하지 않도록 잘 이끌어주셔서 여기까지 올 수 있었습니다. 평소 이 마음을 잘 표현할 수 는 없었지만 이러한 기회를 통하여 감사의 마음을 전합니다.

부끄러움 많고 이러지도 저러지도 못하는 저를 연구자로서의 기회를 주시고, 응원해주시고, 항상 믿어주신 정현숙 교수님께도 감사합니다. 교수님께서 학문의 길을 열어주시고, 잘 이끌어 주셨기에 이렇게 학위과정을 잘 마무리 할 수 있었습니다. 정말 감사합니다.

부족한 논문임에도 불구하고, 꼼꼼히 살펴봐주시고, 아낌없는 조언으로 부족함을 채워주신 박윤경 교수님, 졸업으로 모든게 끝나버리면 안된다는 말씀 마음에 잘 새기겠습니다. 전공 분야가 아닌 부분에 있어서 보완할 부분과 더 좋은 방향을 제시해주신 김정수 교수님 감사합니다. 앞으로 더 발전된 모습으로 보답하겠습니다.

학과 교수님이신 이정섭 교수님, 전홍성 교수님, 이건호 교수님 과학자로서 가야할 길을 제시해주시고, 아낌없이 조언해주셔서 감사합니다.

늦게 연구실에 들어온 저에게 항상 관심 가져주시고, 실험에 대해 많은 조언 해주신 이석철 박사님 감사합니다. 든든한 실험실의 버팀목이 되어주고, 항상 현실적인 조언으로 실험에 집중할 수 있게 해준 현구, 덕분에 정신차리고 실험 잘 할 수 있어서 고맙고, 항상 부지런히 실험실 내 부족한 것들 채워주고, 막내로서 헌신해준 하나 덕분에 실험 할 때마다 마음 편하게 할 수 있었고, 앞으로도 그렇게 열심히 하면서 마무리 잘 할 수 있기를 바란다.

항상 저를 걱정했던 PMB 식구들에게도 감사합니다. 실험실 운영에 있어서 항상 관심가지시고, 최선을 다 해주셨던 박재영 박사님 덕분에 2년 남짓한 시간을 걱정 없이 연구에 매진 할 수 있었습니다. 감사합니다. 실험 할 때마다, 세세하게 설명해주시고 다양한 실험방법들을 알려주시고 숙련시켜주신 김영순 박사님께도 감사의 말을 전합니다. 언제나 지쳐있을 때, 힘이 되어준

웅이형, 궁금한 건 함께 찾아보고, 실험에 대한 많은 고민을 함께 나누었던 범기형, 항상 에너지 넘치는 모습으로 활력을 돋게 해준 철우형, 좋은 형들이 있었기에 중간에 포기하지 않고 이렇게 마무리 잘 할 수 있었습니다. 감사합니다.

대학원 진학에 있어서 걱정이 많으셨던 부모님, 그래도 이렇게 잘 마무리하고 졸업합니다. 어머니 아버지께서 물심양면으로 지원해주신 덕분에 여기까지 올 수 있었습니다. 졸업이 끝이 아닌 새로운 마음으로 더 열심히 해서 자랑스러운 아들 되겠습니다. 감사하고 사랑합니다.

이 외에도 저의 졸업을 위해 마음써주시고, 관심을 가져주신 많은 분들에게 이 자리를 빌어 감사하다는 말을 전하고 싶습니다. 감사합니다.