A Master's Thesis

Quercetin inhibits Multidrug Resistance by down-regulation of CYR61 in human gastric adenocarcinoma AGS cells

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퀘르세틴의 인간 위암 세포주 AGS 에서 CYR61 억제를 통한 다약제내성 극복

지도교수 김 소 미

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Quercetin inhibits Multidrug Resistance by down-regulation of CYR61 in human gastric adenocarcinoma AGS cells

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The cysteine-rich angiogenic inducer 61 (CYR61), an extracellular matrix-associated protein, is involved in survival, tumorigenesis, angiogenesis, and drug resistance. There is an increasing demand for developing agents that target CYR61. We study here the effects of flavones against CYR61-overexpressing human gastric adenocarcinoma AGS (AGS-cyr61) cells. AGS-cyr61 cells exhibit resistance to 5-fluorouracil, adriamycin, tamoxifen, paclitaxel, and docetaxel. Overexpression of CYR61 in AGS cells leads to up-regulation of MRP1 (Multidrug resistance-associated protein 1, encoded by ABCC1, ATP-binding cassette sub-family C member 1), poly (ADP-ribose) polymerase (PARP), and nuclear factor-kappa B (p65). Treatment with 5-Fluorouracil at 25-50 μM or Adriamycin at 125-250 nM showed the dramatic cleavage of PARP in AGS cells but AGS-cyr61 cells. Among the various flavones, quercetin had lowest IC_{50} value of 27.3 ± 15.1 μM and it reduced the viability of AGS-cyr61 cells more than that of AGS cells. Quercetin down-regulates CYR61 and MRP1 in AGS-cyr61 cells and the multidrug resistance observed in AGS-cyr61 cells is revered by either the treatment of quercetin or CYR61 siRNA. In addition, quercetin synergizes actions of standard chemotherapeutics 5-Fluorouracil and adriamycin. Our results demonstrate for the first time that CYR61 can be potential regulator of ABC transporters and quercetin can be the novel agent that improves the efficacy of anticancer drugs by down-regulating CYR61 and ABC transporters.

요약문

CYR61은 extracellular matrix-associated protein으로써, 세포생존, 암유발, 신생혈관 형성, 약물내성을 발생시킨다. 이러한 CYR61을 타겟으로 하는 약물의 개발에 대한 수요가 증가하고 있다. 우리는 본 연구에서 CYR61을 과발현시킨 인간 위암세포주인 AGS세포에 효과가 있는 flavone들을 확인하였다. AGS-cyr61세포는 5-fluorouracil, adriamycin, tamoxifen, paclitaxel, docetaxel에 대해 내성을 나타냈다. CYR61의 과발현은 AGS세포에서 MRP1, PARP, p65의 증가를 유도하였다. 5-fluorouracil 25-50 μM 혹은 adriamycin 125-250 nM 처리에 의하여 AGS세포에서는 PARP의 절단이 일어났지만, AGS-cyr61세포에서는 일어나지 않았다. 다양한 flavone들 중에서는 quercetin이 27.3 ± 15.1 μM의 가장 낮은 IC50값을 보였고, AGS 세포보다 AGS-cyr61세포의 생존율을 더 감소 시켰다. 이러한 quercetin은 AGS-cyr61세포에서 CYR61과 MRP1을 억제시켰고, quercetin 혹은 CYR61 siRNA 처리에 의해 다약제내성이 극복되었다. 게다가 quercetin은 5-fluorouracil과 adriamycin과 시너지 효과를 보였다. 우리의 결과를 통하여 처음으로 CYR61이 ABC transporter를 조절할 수 있다는 것을 알 수 있었고, quercetin이 CYR61과 ABC transporter를 억제하여 항암제의 효과를 증가 시킬 수 있는 새로운 약물 될 수 있을 것임을 알 수 있었다.

1. INTRODUCTION

Gastric cancer (GC) is the second leading cause of death from malignant disease worldwide and is a major global public health problem (1). The 5-year survival rate for patients with gastric adenocarcinoma is only about 20%. Death from gastric cancer is mainly due to recurrent disease, and the most common form is loco-regional recurrence (2). Many evidences suggest that overexpression of the CCN family increased migration, peritoneal dissemination, and carcinogenic progresses in gastric cancer cells (3, 4). Cysteine-rich 61 (Cyr61) is a member of the CCN family (5), which comprises Cyr61/CCN1, connective tissue growth factor (CTGF/CCN2), nephroblastoma overexpressed (Nov/CCN3), Wisp-1/elm1 (CCN4), Wisp-2/rCop1 (CCN5), and Wisp-3 (CCN6). CYR61 controls p53 and NF (Nuclear factor) -κB expression in ovarian cancer cells, is involved in growth, migration, and metastasis of prostate cancer cells, and is critical for stemness of pancreatic cancer cells (6-8). A stromal CYR61 showed resistance to mitoxantrone drug in human acute myeloid leukaemia (9). Lin et al., found that Cyr61 was highly expressed in more advanced gastric adenocarcinoma specimens and overexpressed Cyr61 in human gastric cancer cell lines significantly promoted tumor cell motility/ invasion through activation of the integrin/NF-κB /Cox(cyclooxygenase)-2 signaling pathway (10). CYR61-overexpression conferred resistance via activation of NF-κB-XIAP pathway in breast cancer MCF-7 cells (11). However, there are very limited reports about the role of CYR61 in resistance of gastric cancer cells.

The molecular genetic basis of resistance to cancer therapeutics is complex, involving multiple processes, such as drug transport, drug metabolism, DNA repair and apoptosis (5). Due to the importance of chemotherapy in treating gastric cancer, the development of multidrug resistance (MDR) becomes a serious obstacle to effective chemotherapy. ATP-binding cassette

(ABC) transporters are a family of transporter proteins that contribute to drug resistance via ATP-dependent drug efflux pumps. The ABC transporter family is known to have at least 48 members in humans and 12 of these are recognized to be putative drug transporters, including the P-glycoprotein (P-gp, encoded by the ABCB1 gene) and MDR-associated protein 1 (MRP1, encoded by the ABCC1 gene) (12). Some ABC transporters confer chemo-resistance by causing the efflux of anti-cancer drugs (13). Previous studies have reported that the mechanisms of MDR were associated with the overexpression of P-glycoprotein (P-gp) encoded by MDR1 gene in tumor cells (14, 15). MRP1 protein was regulated by mutant p53 in prostate cancer cells (16). N-myc also regulated MRP1 mRNA and protein levels in human neuroblastoma (17). Activation of Notch1 was correlated with MRP1 expression in breast cancer cells (18). In lung cancer cells, MDR showed significantly higher Nrf2-ARE pathway activity and expression of MRP1 (19). In rat model, p38 MAPK may be involved drug resistance by regulated MRP1 (20).

Flavonoids are a group of naturally occurring low molecular weight polyphenolic compound that recently has been the subject of considerable scientific and therapeutic interest. They are found in a wide variety of plants and are present in fruits, roots, stems and flowers. Several beneficial effects of flavonoids on health are known and flavonoids are consumed in daily diets and in form of tea and wine (21). Flavone, 2-phenyl-4H-1-benzopyran-4-one, is the core structure of flavonoids. Flavones contain phenyl rings, hydroxyl or methoxy groups can be substituted. Importantly, a hydroxyl group can increase solubility, whereas a methoxy group increases cell permeability (22). Most of flavones are able to increase the sensitivity to chemopreventive drugs or to promote a synergistic action with other flavonoids and act as inhibitors of cell cycle, promotors of cancer cell apoptosis and inhibitors of cell migration (23).

Quercetin is the principal flavonoid compound (3,3',4',5,7-penta-hydroxy-flavone) commonly extracted from cranberries, blueberries, apples, and onions. Quercetin has bioactivities, such as reversed epithelial to mesenchymal transition and invasion, induced growth arrest, suppressed drug resistance via p38 MAPK apoptotic pathway, and inhibited MRP1 activity (24-27). Also, Quercetin sensitized to docetaxel, gemcitabine, and radiotherapy (28-30). However, the anticancer activity and mechanism of hydroxy or methoxy flavones are totally unknown. So, we investigated anticancer activity about several flavones in CYR61 overexpressed gastric adenocarcinoma cells.

As Cyr61 is, presumably, one of the major contributors to chemoresistance, the CCN targeting agents, including monoclonal antibodies, antisense oligonucleotides and RNA interference compounds, have been proposed (31). However, the safety and particularly the economics of these experimental strategies are yet to be clinically proven for their suitability. Considering the low cost and proven safety and pharmacological efficacy of phytochemicals like flavons, we aimed to find the phytochemicals that can target CYR61 and examined their anticancer activity against constitutively expressed Cyr61 in established gastric cancer cell line AGS. We demonstrated that quercetin is the effective agent which targets CYR61 in gastric cancer cells and it downregulates CYR61, NF-κB, and MRP1. We also investigated chemoadjuvant action of quercetin in combination with the standard chemotherapeutic agent, 5-Fluorouracil (5-FU) and adrianmycin (ADR). This study provides the evidence that quercetin is a novel agent to inhibit MDR and enhance drug sensitivity in CYR61 overexpressed gastric cancer patients.

2. MATERIALS AND METHODS

2.1 Reagents

RPMI 1640 medium, DMEM medium, trypsin/EDTA, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), was purchased from Sigma Chemical Co. (St. Louis, MO, USA). MRP1, p65, PARP and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). CYR61 was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

2.2 Cell culture

AGS was obtained from the Korean Cell Line Bank (Seoul, Korea). AGS and AGS-cyr61 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics at 37ºC in a humidified atmosphere containing 5% CO_2.

2.3 Gene Transfection

The expression vector CYR61 was constructed by placing human CYR61 cDNA in the pcDNA3.1 eukaryotic expression vector containing the neomycin gene under the control of the same promoter. The CYR61-sense expression constructs were transfected into AGS cells using lipofectamine 2000 (Invitrogen). At 24 h after transfection, the cells were serum-starved for 16 h and lysed for analysis for transient transfection. After G418 selection, we isolated one single clone, AGS-Cyr61. To silence the expression of CYR61, AGS cells were transfected using lipofectamine 2000 reagent (Invitrogen) with 50 pmol small interfering RNA (siRNA) against CYR61 (NM_001554) or control non-targeting siRNA (Santa cruz).

2.4 Measurement of cell viability

Cell viability was determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, which involves the conversion of MTT to formazan crystals by mitochondrial dehydrogenases. Briefly, cells were plated in 96-well plates at an initial density of 2×10^4 cells/mL per well. After 24 h incubation, the cells were treated various concentrations of samples were added to the cells. At the indicated time points, 20 μL of MTT solution (5 mg/mL) was added to each well and the cells were kept in a humidified environment for 3–4 h. The supernatant was removed and dissolved in 150 μL DMSO. Absorbance was detected in a microplate reader at 570 nm (Tecan, Salzburg, Austria).

2.5 Western blotting

After treatment, the cells were collected and washed twice with cold PBS. The cells were then lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM NaVO3, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 25 μg/mL aprotinin, and 25 μg/mL leupeptin) and kept on ice for 30 min. The lysates were then centrifuged at 13,000 × rpm at 4ºC for 30 min; the supernatants were stored at -70ºC until use. The protein concentration was determined by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Aliquots of the lysates were separated by 7.5–15% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Darmstadt, Germany) using a glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl, pH 8.8, and 20% methanol [v/v]). After blocking with 5% nonfat dried milk, the membrane was incubated for 2 h with primary antibodies followed by 30 min with secondary antibodies in

milk containing Tris-buffered saline (TBS) and 0.1% Tween 20. All primary antibodies were used at a dilution of 1 : 1,000; HRP-conjugated goat anti-rabbit IgG (H+L) and HRP-conjugated goat anti-mouse IgG were used as secondary antibodies at a dilution of 1 : 5,000. The PVDF membrane was then exposed to X-ray film (AGFA, Mortsel, Belgium), and the protein bands were detected using a BS ECL-Plus kit (Biosesang, Gyeonggi-do, Korea).

2.6 Microscopic Observation of Nuclear Morphology.

Cells, placed in 60 mm dish at 2×10^4 cells/mL, were treated with the samples. After 24 h, 10 μM of Hoechst 33342, a DNA specific fluorescent dye, was added to the solution in each well and the dishes were incubated for 20 min at 37ºC. The stained cells were then observed under an Olympus fluorescence microscope (Tokyo, Japan).

2.7 Wound-healing assays

Cells were seeded onto 12-well plate at 1×10^5 per well in growth medium. After Confluent monolayers, a single scratch wound was created using a micropipette tip. Cells were washed with PBS to remove cell debris, supplemented with growth medium with quercetin (0 ~ 10 μM), and monitored. Images were captured by phase microscopy at 0, 24, and 48 h post-wounding.

2.8 Clonogenic assay

Cells were seeded onto 12-well plated at 5×10^3 per well in growth medium. After 1day, cells were treated quercetin and anticancer drugs, and monitored. Colonies are fixed with 4%

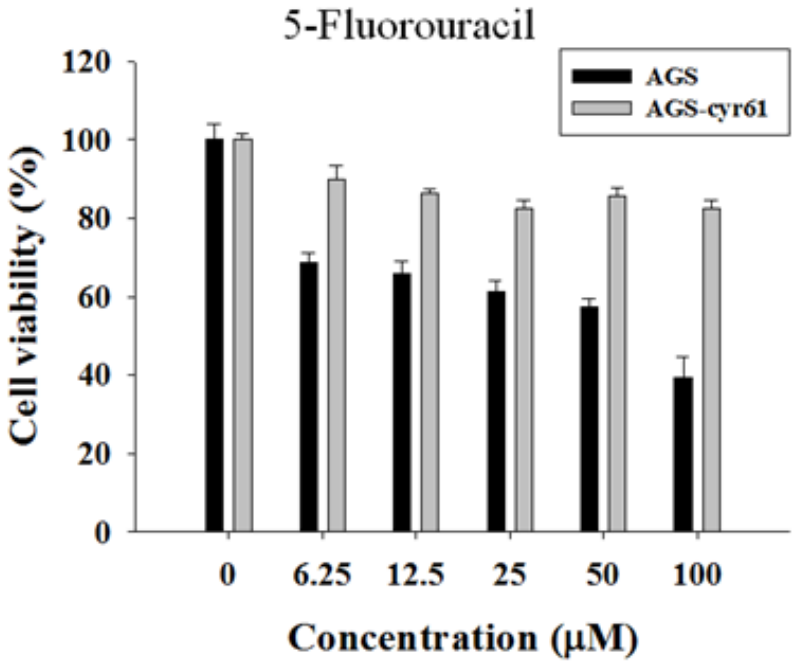
paraformaldehyde 500 μL per well at 30 min, stained with crystal violet (0.5% w/v) at 30 min and washed using PBS.

3. RESULTS

3.1 Overexpression of CYR61 confers Multi drug resistance in AGS-cyr61 cells.

Previous studies have shown that CYR61 associated paclitaxel, and adriamycin resistance in breast cancer cells (11). We first examined whether CYR61 could related to Multi drug resistance (MDR) in gastric cancer cells. We treated anti-cancer drugs in AGS and AGS-cyr61 cells, and observed cell viability. In our results showed AGS-cyr61 cells had resistance to 5-fluorouracil, adriamycin, tamoxifen, paclitaxel, and docetaxel, but not to cisplatin (Fig. 1A-F). Specially, 5-fluorouracil and Adriamycin showed strong resistance in AGS-cyr61 cells.

(A)



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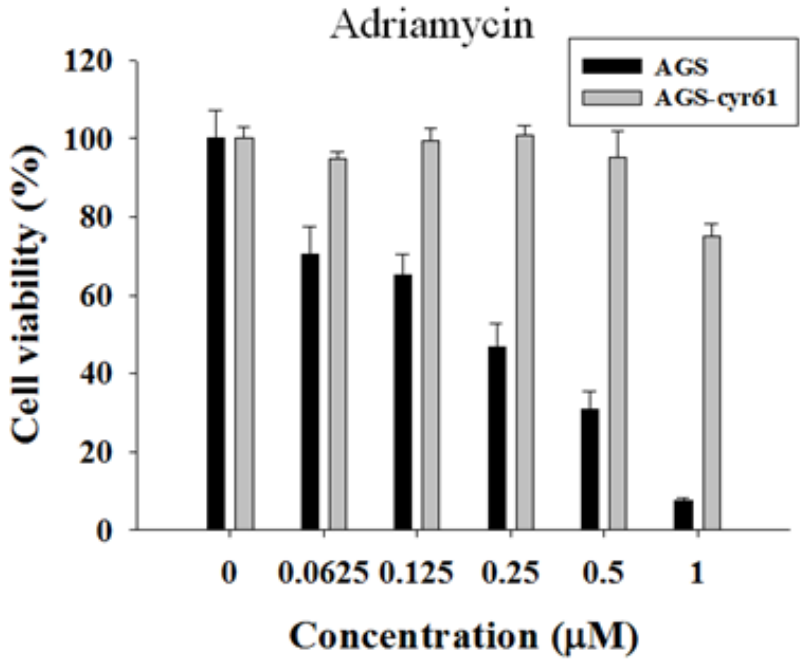

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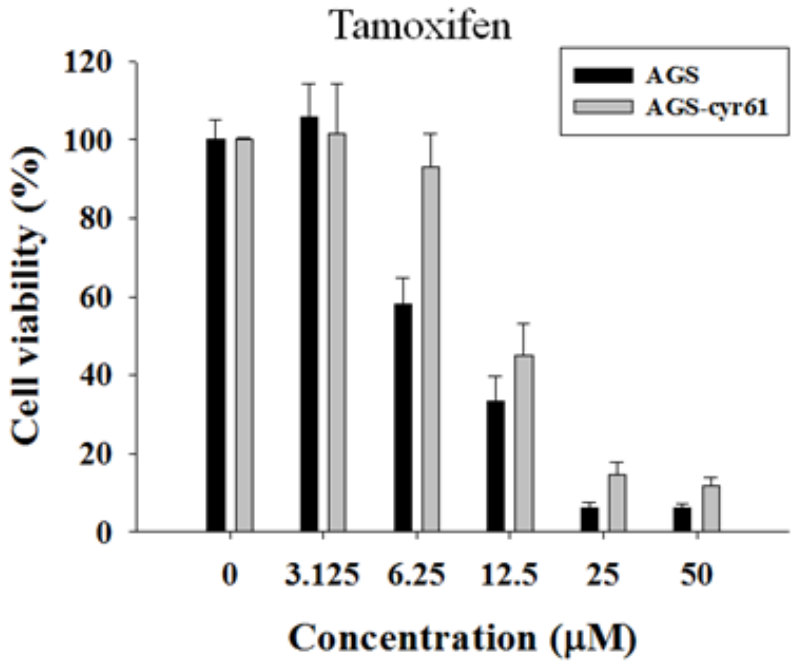

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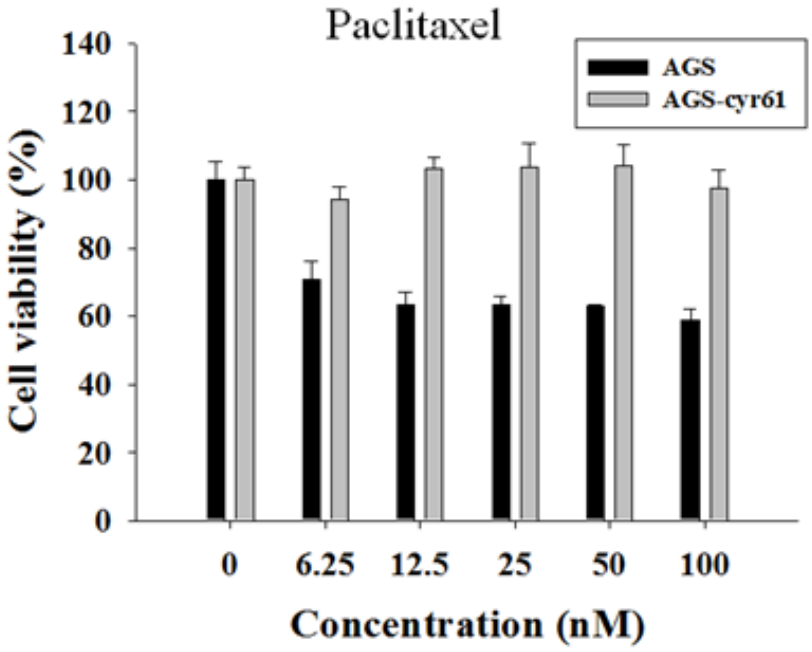

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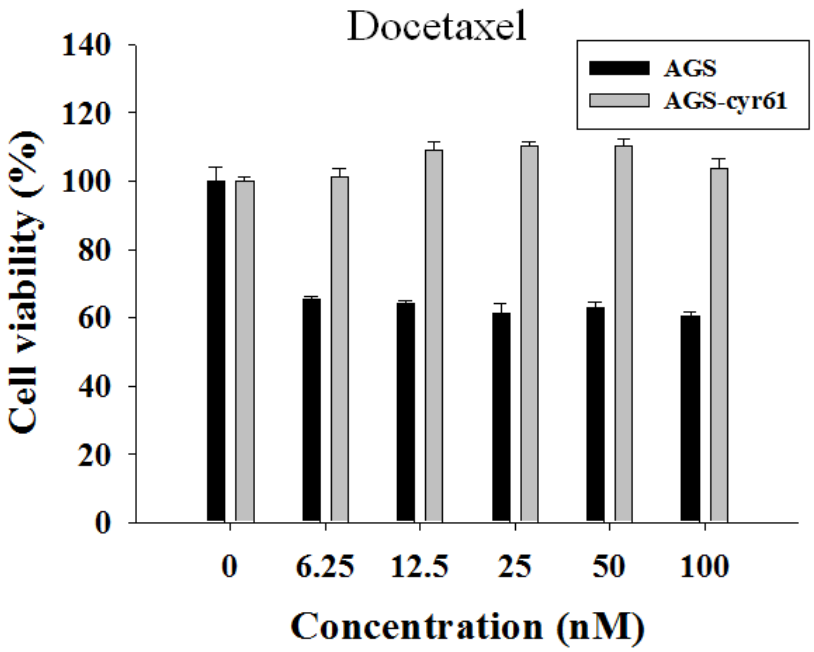

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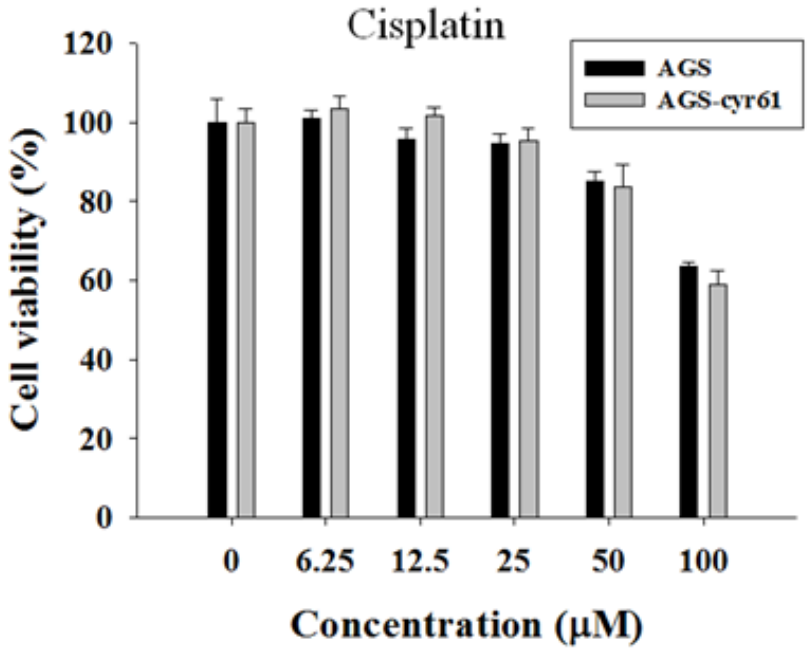


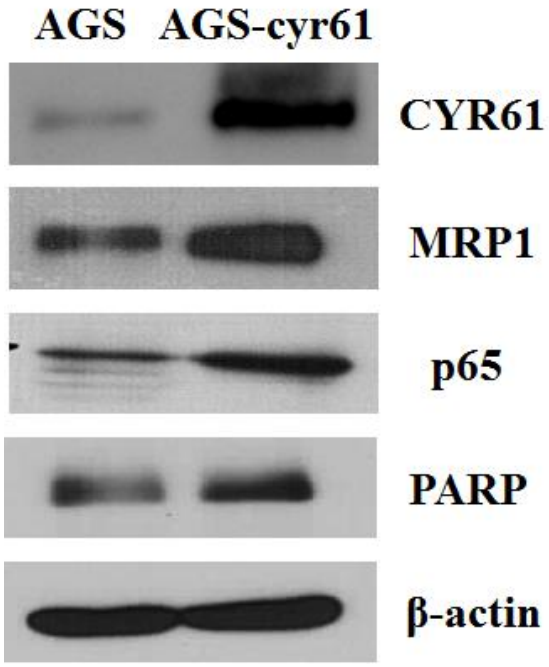
Figure 1. Cell viability of AGS-cyr61 cells treated standard chemotherapeutic drugs. (A-F) Cell growth inhibition by standard chemotherapeutic drugs. Cell viability was determined on the basis of MTT reduction assay against AGS and AGS-cyr61 cells for 48 h. The data shown represent the mean ± S.E. for one experiment performed in triplicate.

(A : 5-fluorouracil, B : Adriamycin, C : Tamoxifen, D : Paclitaxel, E : Docetaxel, F : Cisplatin)

3.2 Elevated drug resistance related protein involve MDR in AGS-cyr61 cells.

AGS-cyr61 cells showed highly expression of ABC transporters (MRP1), poly (ADP-ribose) polymerase (PARP), and nuclear factor-kappa B (p65). The role of PARP is to detect and signal single-strand DNA breaks (SSB) to the enzymatic machinery involved in the SSB repair. It was associated drug resistance in BRCA-mutant cancers (32). Also, p65 was linked to resistance in breast cancer cells (33) and CYR61 induced resistance by p65-dependent XIAP up-regulation (11) (Fig. 2A). To knockdown CYR61, we used siRNA transfection. After knockdown, MRP1, p65 and PARP expressions were also decreased in AGS-cyr61 cells (Fig. 2B). Next, we investigated the MDR mechanism after the treatment of AGS and AGS-cyr61 cells with 5-fluorouracil (5-FU) and Adriamycin (ADR) which concentrations were determined as judged from their around IC_{50} values in AGS cells (Fig. 2C, D). Treatment with 5-FU and ADR reduced MRP1 and increased cleaved PARP in AGS cells. However, CYR61, MRP1, and PARP levels were not changed in AGS-cyr61 cells. This result implicated that MDR comes from up-regulation of MRP1, p65, and PARP by CYR61 overexpression. Taken together, CYR61 could up-regulate MRP1, p65 and PARP and increase MDR in gastric adenocarcinoma cells.

(A)



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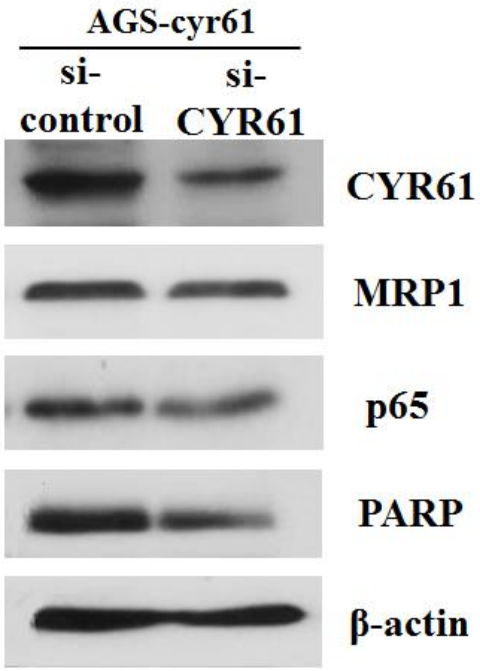

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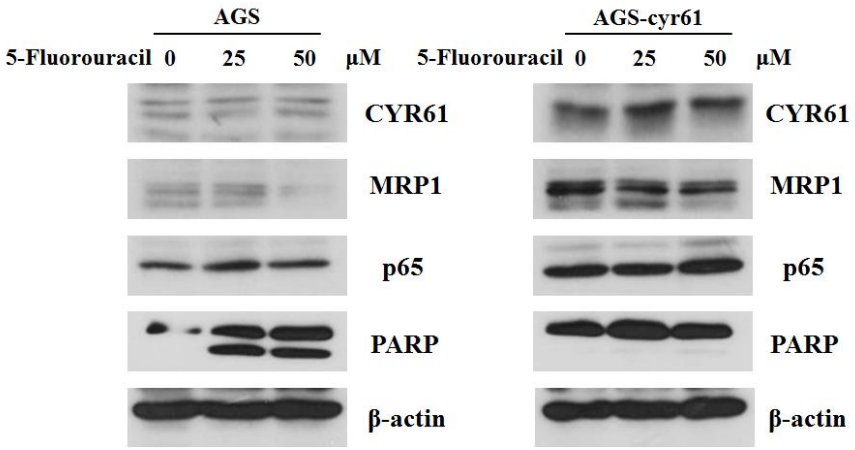

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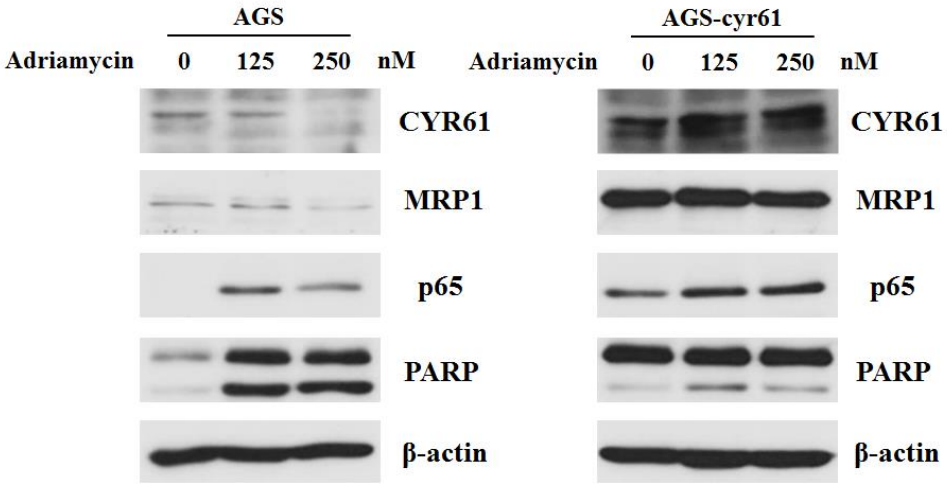
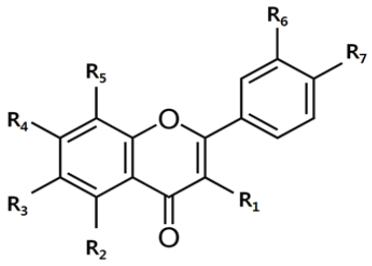


Figure 2. Effect of CYR61 overexpression and standard chemotherapeutics drugs on drug resistance related protein expression of AGS and AGS-cyr61 cells. (A) CYR61, MRP1, p65, and PARP proteins in AGS and AGS-cyr61 cells were analyzed by Western blot. (B) AGS-cyr61 cells transfected with control-siRNA (si-control) or CYR61-siRNA (si-CYR61) for 24 h and protein levels were then examined with Western blot. β-actin was used as an internal control. (C, D) AGS and AGS-cyr61 cells were treated 5-FU and ADR for 48 h, and protein levels were examined by Western blot.

3.3 Quercetin inhibits drug resistance related protein expression and cell migration.

To assess the biological activity of flavones (Table 1) in terms of cell proliferation, AGS and AGS-cyr61 cells were treated with flavones of dose-dependent for 48 h. Quercetin reduced cell viability of AGS-cyr61 cells more than AGS cells (2.6 fold change), but other flavones showed similar or less effects compared AGS and AGS-cyr61 cells. In addition, quercetin had lowest IC_{50} value (27.3 ± 15.1 μM) among the tested flavones (38.6 ~ >200 μM) (Table 2). So, we chose quercetin for next experiment, and determined the mechanism of quercetin in AGS-cyr61 cells. Treatment with quercetin significantly decreased CYR61, MRP1, and p65 levels and increased cleaved PARP levels in AGS-cyr61 cells (Fig. 3A). A number of studies have identified that CYR61 functioned as a ligand of integrin that play pivotal roles in cell migration (10, 34). Thus, we investigated the effects of quercetin on the mobility of AGS-cyr61 cells. Wound-healing results demonstrated that quercetin inhibited cell migration about 138% compared to control (100%) at 20 μM of quercetin (Fig. 3B). These data suggests that quercetin might be effective compound which can downregulate CYR61 levels and its downstream proteins and inhibit migration in gastric cancer cells.

Table 1. Molecular structures of flavones.



Flavone

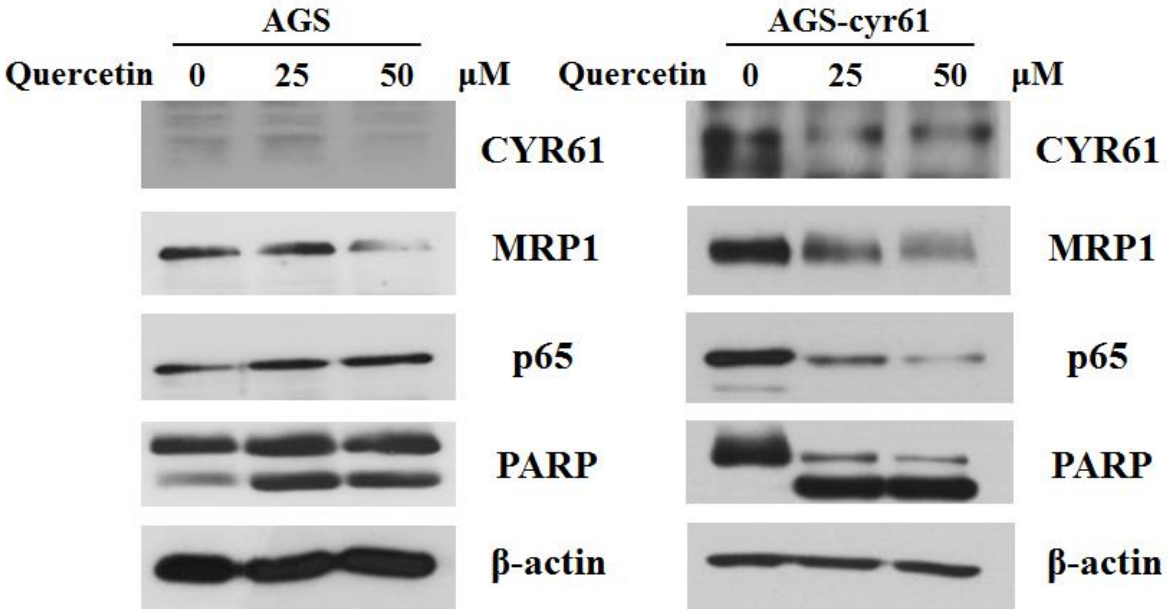
flavones	R_1	R_2	R_3	R_4	R_5	R_6	R_7	Number of methoxy /ethoxy group
3,5,7,3',4'-Pentahydroxyflavone (Quercetin)	OH	OH	H	OH	H	OH	OH	0
5,6,7,8,4'-Pentamethoxyflavone (Tangeritin)	H	OCH_3	OCH_3	OCH_3	OCH_3	H	OCH_3	5
3,5,7,3',4'-pentamethoxyflavone (PMF)	OCH_3	OCH_3	H	OCH_3	H	OCH_3	OCH_3	5
5,6,7,8,3',4'-Hexamethoxyflavone (Nobiletin)	H	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	6
5,3'-dihydroxy-3,7,4'-triethoxyflavone (TEF)	OC_2H_5	OH	H	OC_2H_5	H	OH	OC_2H_5	3
5-hydroxy-3,7,3',4'-tetraethoxyflavone (TeEF)	OC_2H_5	OH	H	OC_2H_5	H	OC_2H_5	OC_2H_5	4
3,5,7,3',4'-pentaethoxyflavone (PEF)	OC_2H_5	OC_2H_5	H	OC_2H_5	H	OC_2H_5	OC_2H_5	5

Table 2. IC_{50} value of flavones.

Flavone	IC_{50} (μM)	
	AGS	AGS-cyr61
3,5,7,3',4'-Pentahydroxyflavone (Quercetin)	71.2±9.1	27.3±15.1
5,6,7,8,4'-Pentamethoxyflavone (Tangeritin)	>100	>100
3,5,7,3',4'-pentamethoxyflavone (PMF)	49.8	>200
5,6,7,8,3',4'-hexamethoxyflavone (Nobiletin)	>100	>100
5,3'-dihydroxy-3,7,4'-triethoxyflavone (TEF)	90.5	71.1
5-hydroxy-3,7,3',4'-tetraethoxyflavone (TeEF)	>200	>200
3,5,7,3',4'-pentaethoxyflavone (PEF)	33.6	38.6

IC_{50} : Inhibitory concentration of 50%.

(A)



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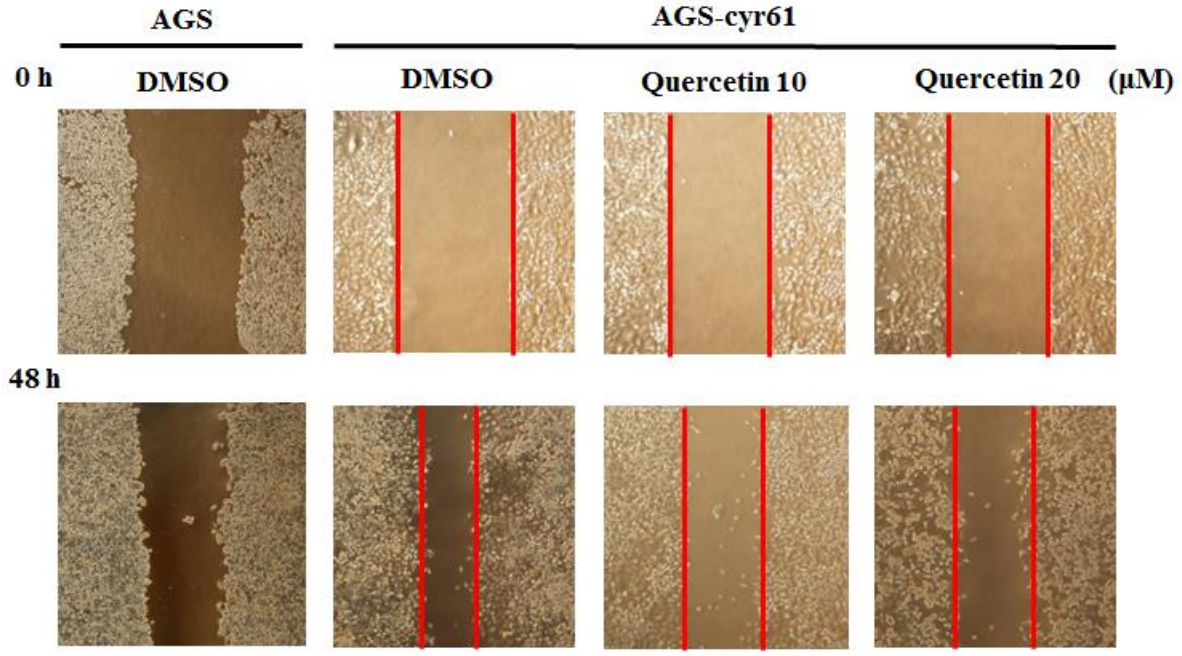

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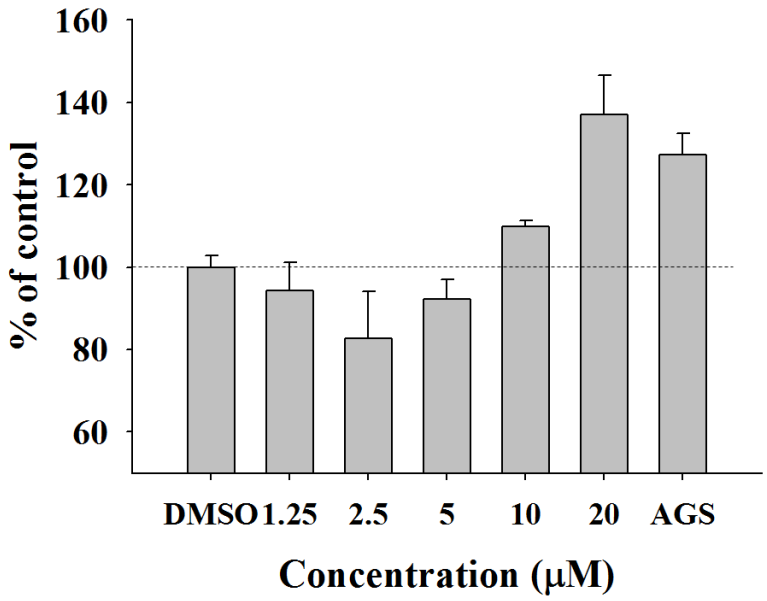


Figure 3. Effect of quercetin on drug resistance related protein and inhibits cell migration of AGS-cyr61 cells. (A) Protein levels were then examined with western blotting after treated quercetin for 48 h. β-actin was used as an internal control. (B) Wound-healing assay for AGS-cyr61 cells. AGS-cyr61 cells were treated with dose-dependent quercetin (0 ~ 20 μM) for 48 h. (C) Graph for migration % of quercetin compared control. Data are the mean ± standard error for one experiment performed in triplicate.

3.4 Quercetin inhibits colony formation in AGS-cyr61 cells.

The clonogenic assay is one of the most intensively studied *in vitro* methods for chemo-sensitivity testing. So, we tested effect of quercetin and anticancer drugs in AGS and AGS-cyr61 cells using clonogenic assay (Fig. 4A). 5-FU and ADR inhibited colony formation in AGS cells, while CYR61 overexpressed cells exhibited resistance to those drugs. However, quercetin significantly decreased colony formation ability in AGS-cyr61 cells.

(A)

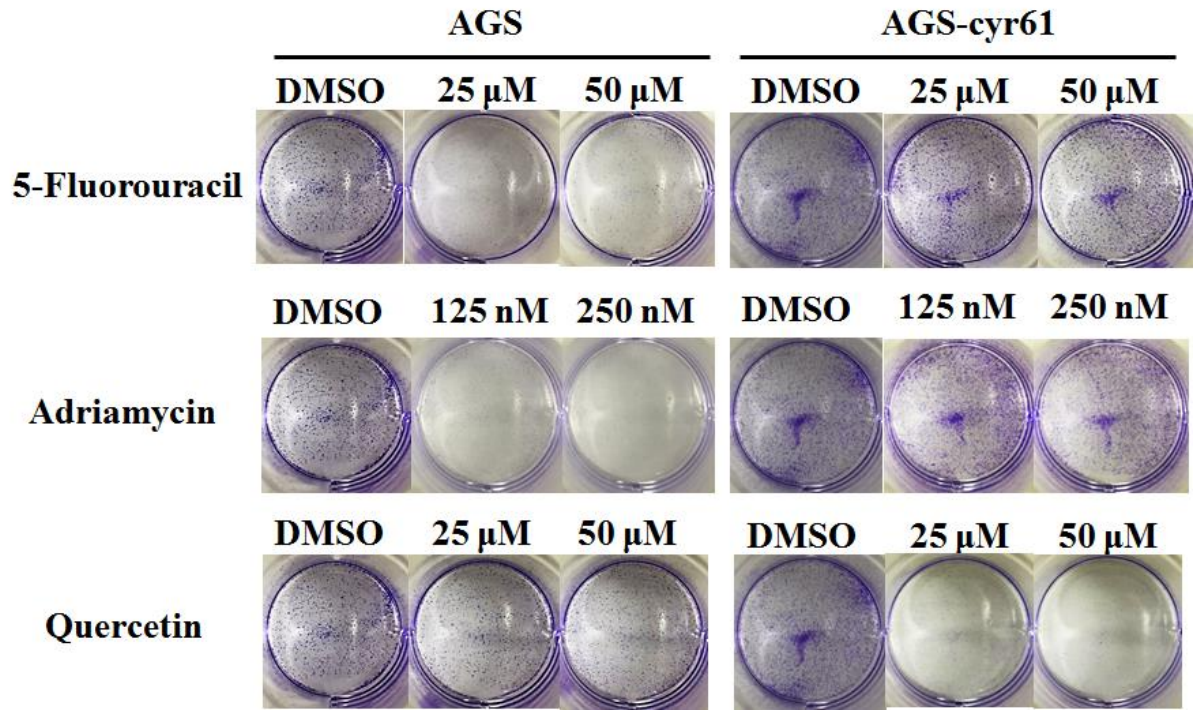
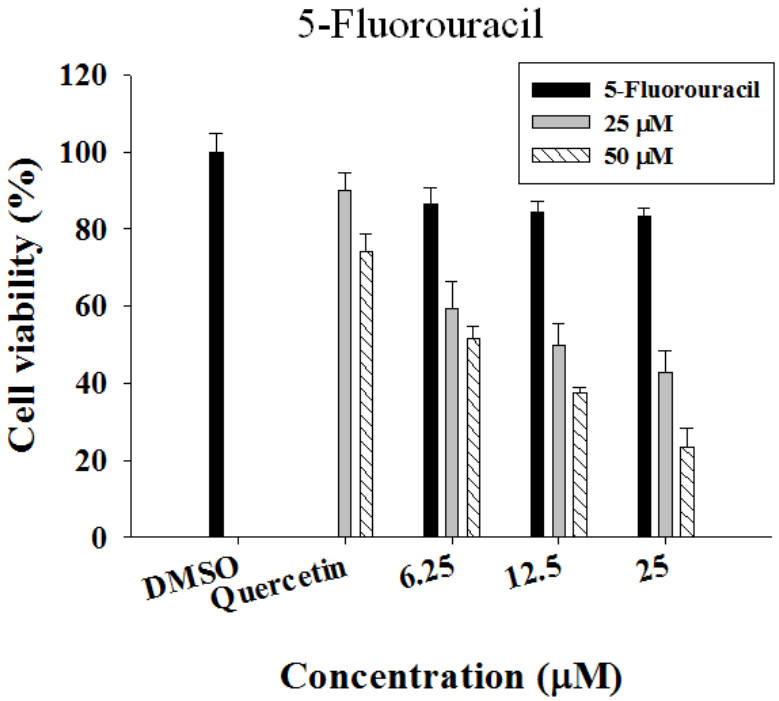


Figure 4. Effect of quercetin on colony formation of AGS and AGS-cyr61 cells. (A) AGS and AGS-cyr61 cells were seeded onto 12-well plate. Then treated with vehicle control (DMSO), quercetin, 5-Fluorouracil, Adriamycin for 5 days.

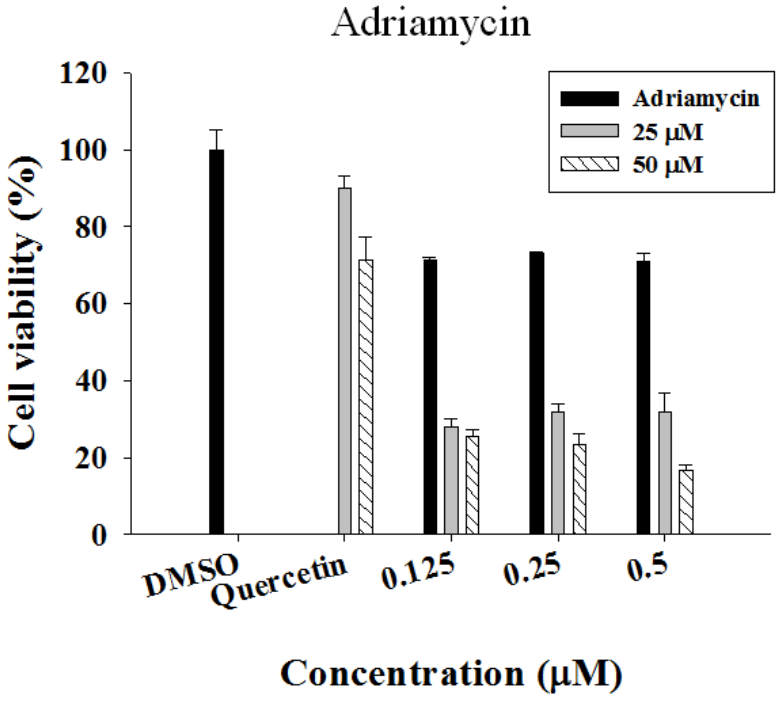
3.5 Quercetin synergizes actions of standard chemotherapeutics 5-fluorouracil and Adriamycin.

In previous studies, quercetin showed combination effect with some DNA damaging drugs in colorectal and prostate cancer cells (35, 36). But, there is no report about combination treatment with quercetin and the chemotherapy drugs on AGS gastric adenocarcinoma cells. Thus, we investigated whether quercetin acts synergistically with anti-cancer drugs in AGS-cyr61 cells. We found outstanding synergy with 5-FU and ADR in AGS-cyr61 cancer cells (Fig. 5A-F). We calculated combination index (CI) values using CalcuSyn program (Fig. 5G, Table. 3). CI values of 5-FU and ADR with quercetin showed 0.18 ~ 0.54.

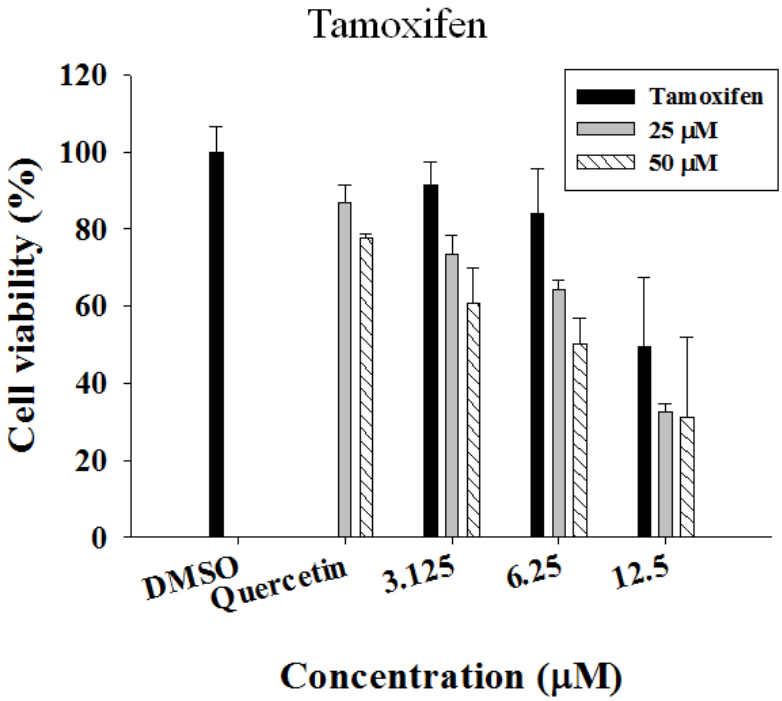
(A)



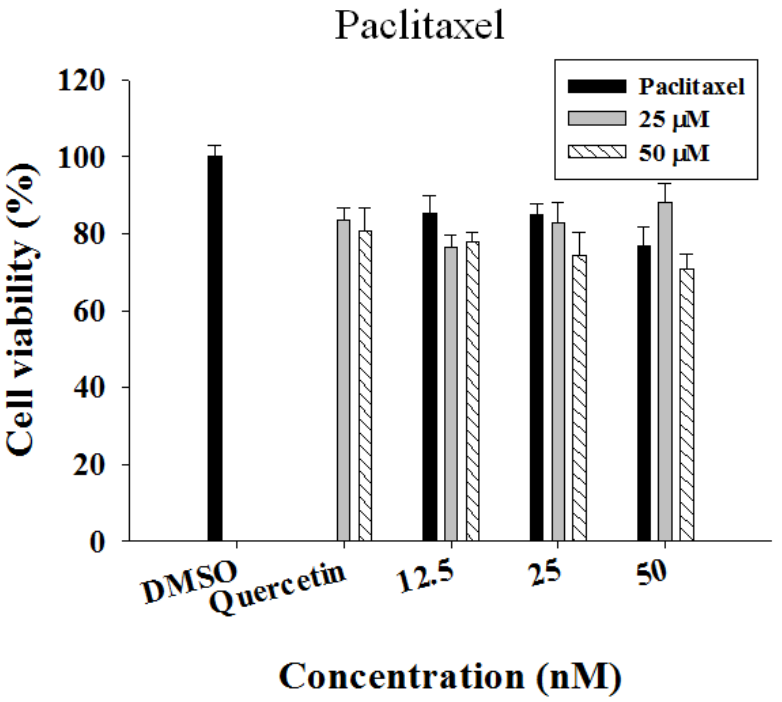
(B)



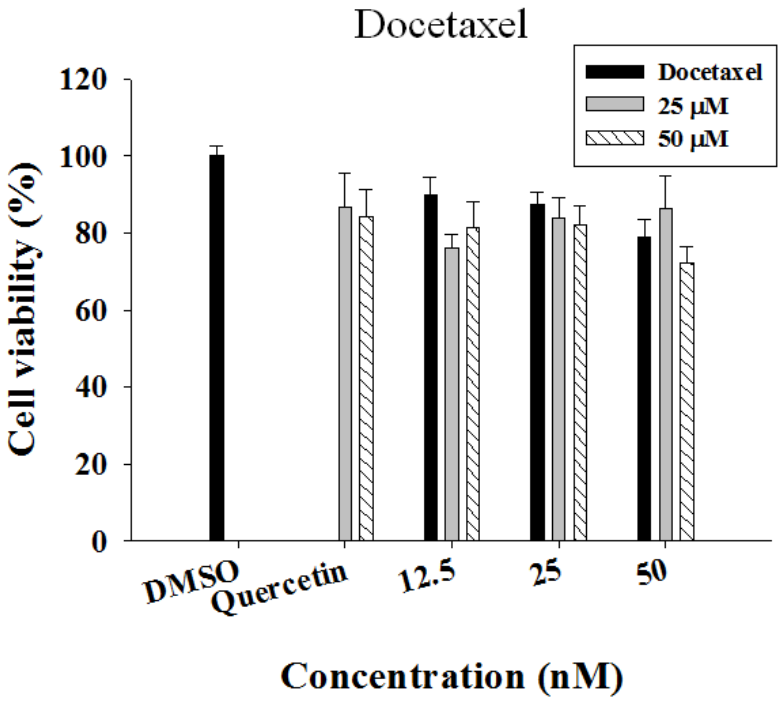
(C)



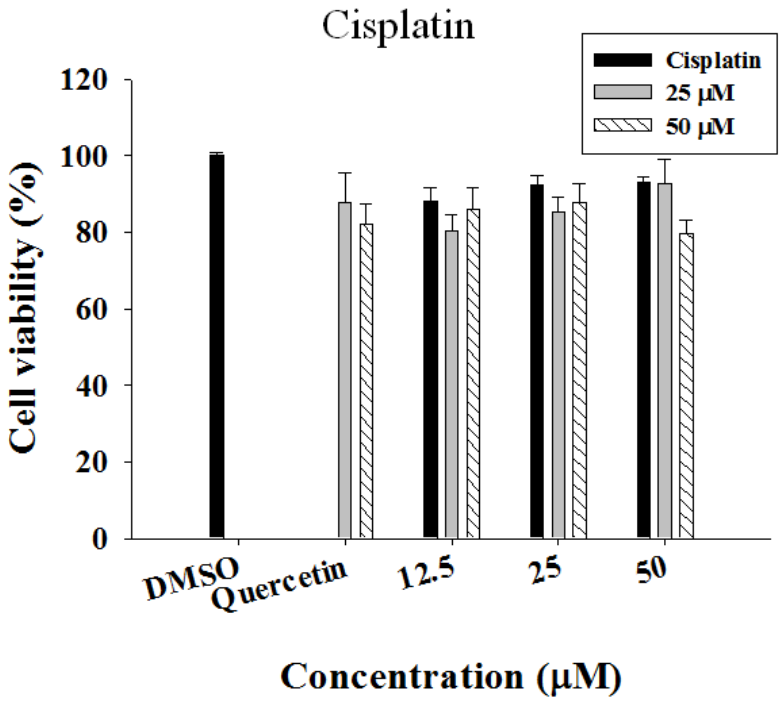
(D)



(E)



(F)



(G)

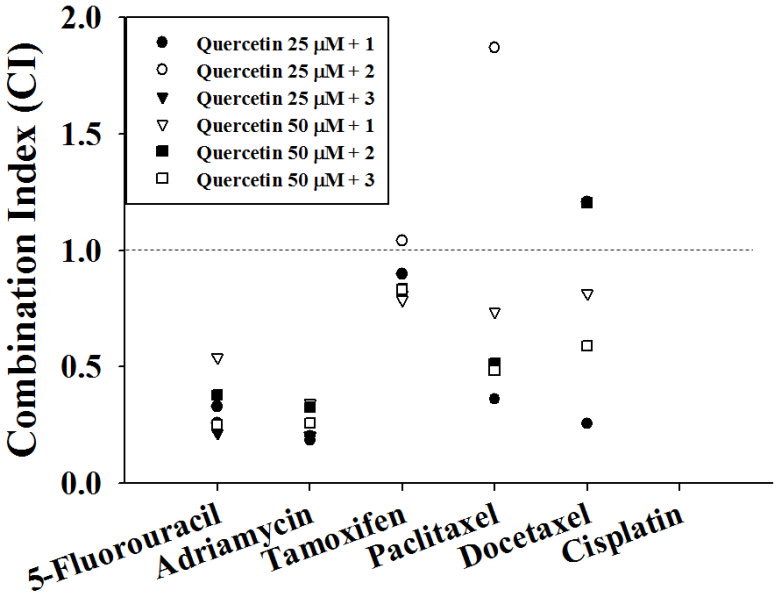


Figure 5. Quercetin synergizes actions of standard chemotherapeutics drugs in AGS-cyr61 cells. (A-F) Synergistic effects of quercetin and standard chemotherapeutics drugs in AGS-cyr61 cells for 48 h. MTT assay was used for measurement of cell viability. (G) Combination Index (CI) value was calculated by CalcuSyn program. (Addition : 1 = CI, Synergy: 1 > CI, Antagonist : 1 < CI)

Table 3. Combination index of quercetin with drugs.

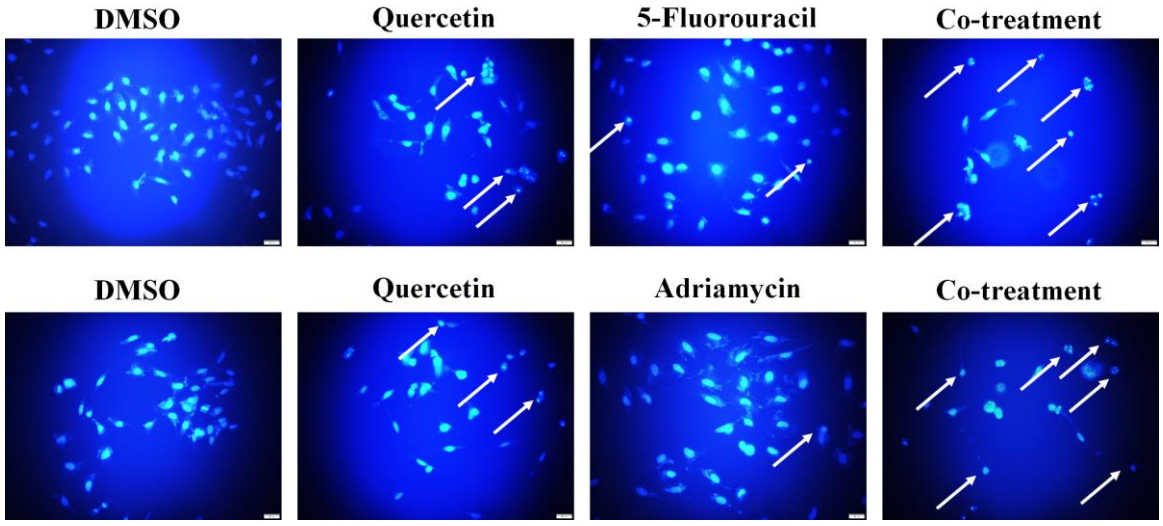
CI value	Quercetin 25 μM			Quercetin 50 μM		
	1	2	3	1	2	3
5-fluorourail	0.33	0.26	0.21	0.54	0.38	0.25
Adriamycin	0.18	0.20	0.20	0.34	0.32	0.25
Tamoxifen	0.89	1.04	0.81	0.78	0.83	0.83
Paclitaxel	0.36	1.86	6.87	0.73	0.51	0.48
Docetaxel	0.25	1.20	2.96	0.81	1.20	0.59
Cisplatin	5.37	2.83	3.68	3.42	4.55	21.76

* 1, 2, 3 : Different concentration of drugs, 5-flrououracil : 6.25, 12.5, 25 μM, Adriamycin : 0.125, 0.25, 0.5 μM, Tamoxifen : 3.125, 6.25, 12.5 μM, Paclitaxel and Docetaxel : 12.5, 25, 50 nM, Cisplatin : 12.5, 25, 50 μM

3.6 Effect of combining quercetin with 5-fluorouracil and adriamycin on induction of apoptotic body in AGS-cyr61 cells.

Moreover, we confirmed synergistic effects by Hoechst 33342 staining. The synergistic response was observed by remarkable increase of apoptotic body formation (Fig. 6A). Alone treatment of quercetin showed only 27.8 ± 6.7% and 5-FU increased 7.1 ± 3.2% of apoptotic bodies, but co-treatment showed about 60.5 ± 16.4% of apoptotic bodies. ADR also increased apoptotic bodies when we co-treated quercetin and ADR (25.4 ± 9.0% or 2.6 ± 0.4% to 55.1 ± 7.7%) (Fig. 6B). These results suggest that quercetin provides synergistic effect with selective anticancer drugs, such as 5-FU and ADR which causes DNA damage.

(A)



(B)

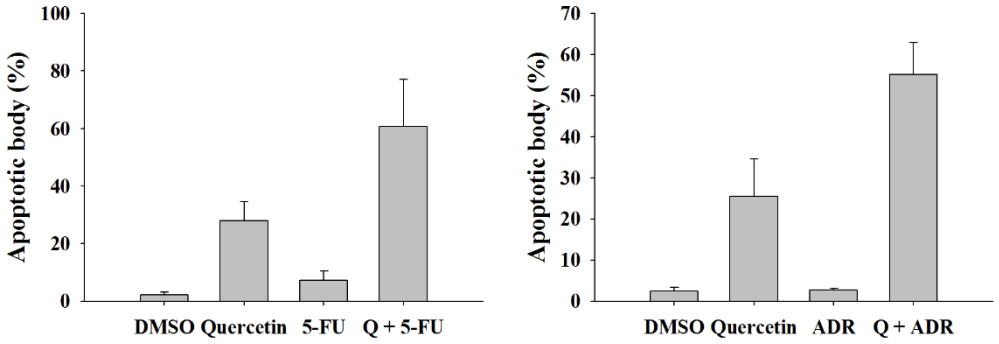


Figure 6. Quercetin increases apoptotic bodies with standard chemotherapeutics drugs in AGS-cyr61 cells. (A) Synergistic effects of quercetin and 5-fluorouracil and adriamycin on apoptosis in AGS-cyr61 cells for 24 h. We treated 25 μM quercetin, 25 μM 5-fluorouracil, and 125 nM adriamycin in AGS-cyr61 cells. Apoptotic body was determined using Hoechst 33342. Stained cells were visualized under a fluorescence microscope. (B) Graph for quantification of number of apoptotic bodies. Data are the mean ± standard error for one experiment performed in triplicate. Compared to the control. (Q : Quercetin, 5-FU : 5-Fluorouracil, ADR : Adriamycin)

4. DISCUSSION

CYR61 is well known for cell migration, growth, and metastasis (7, 34, 37). Previously, it was reported that CYR61 is associated with drug resistance (9, 11). But these mechanisms are not fully described. In this study, we transfected human gastric cancer AGS cells with CYR61 gene to elucidate the mechanism of CYR61-mediated drug resistance. CYR61-overexpressed AGS cells showed up-regulation of MRP1, p65, and PARP expression and these cells exhibited resistance to chemotherapeutic agents such as 5-FU, adriamycin, tamoxifen, paclitaxel, and docetaxel.

The development of MDR to cancer chemotherapy is a major obstacle to the effective treatment of gastric cancer. However, the mechanism of MDR remains obscure. Several laboratories have studied the mechanism(s) of MDR in the past decades and many possible mechanism(s) have been identified. These resistance mechanism(s) appears to fall into four major categories. The first category involves DNA damage/repair proteins. The second category involves drug retention (increased influx or decreased uptake). The third category involves increased drug inactivation or prevention of drug to reach the DNA target. The fourth category involves growth signaling via different pathways or increase in anti-apoptotic protein(s) (5).

Our results revealed that CYR61 is related to MDR via MRP1 (ABCC1) upregulation by CYR61 overexpression. MRP1 is a well characterized form of drug resistance which is drug efflux pump. This efflux pump belongs to ABC (ATP-binding-cassette) transporter superfamily, and is capable of efflux many different chemotherapeutic agents, hence it can induce the multidrug resistance. MRP1 can also efflux various endogenous and exogenous substances.

The overexpression of the MRP1 confers a various anticancer drug resistance, such as doxorubicin, topotecan, and vincristine (38). Therefore, ADR-resistance in AGS-cyr61 cells might be come from MRP1 efflux activity. However, other drugs, 5-FU, tamoxifen, paclitaxel, docetaxel, and cisplatin are not related to resistance by MRP1 expression. Paclitaxel and docetaxel are effluxed by ABCC10 and ABCB1 (38), while cisplatin is a substrate of ABCC2.

Poly (ADP-ribose) polymerase 1 (PARP-1) is a key eukaryotic stress sensor that responds in seconds to DNA single-strand breaks (SSBs), the most frequent genomic damage. SSBs are by far the most frequent form of DNA damage, resulting both directly from oxidative damage and as intermediates in other DNA repair pathways (39). Some cancer cells display low sensitivities to available chemotherapies and develop drug resistance partly via hyper-activation of some DNA repair functions (40). PARP inhibitors were developed to potentiate the cytotoxic effect of certain chemotherapeutic agents and are currently being investigated in combination with chemotherapy in diverse cancer types. In our results showed PARP expression was increased by CYR61 overexpression, thus we speculated that this is the one of mechanism of MDR in AGS-cyr61 cells.

Nuclear factor-kappa B (p65) was linked to resistance in breast cancer cells (33) and CYR61 induced resistance by p65-dependent XIAP up-regulation in MCF-7 breast cancer cells (11). In addition, p65 was regulated by CYR61 through PI3K/Akt/mTOR pathway in ovarian cancer cells (6). As CYR61 is involved also by NF-κB (41). We thought that in these cells, an autocrine loop between CYR61 and NF-κB maintains drug resistance.

There are many studies reported quercetin have bio-activities such as, growth arrest, cell cycle arrest, and suppress drug-resistance (25, 26, 42). Our results showed that quercetin

significantly decreased CYR61, MRP1, and p65 levels. In addition, quercetin remarkably increased cleaved PARP in AGS-cyr61 cells which were not respond to anticancer drugs. Based on these results, we tested whether quercetin can enhance the cytotoxicity of anticancer drugs. We measured combination effects of treatment with quercetin and drugs in AGS-cyr61 cells, and strong synergistic effects of quercetin with 5-FU and ADR were observed.

Taken together, our results provide a strong rationale for the ongoing study with quercetin in combination with drugs in patients, and suggest that combination treatment with quercetin and other anticancer drugs could allow dramatically improved therapy of a wide range of therapy-refractory human tumors carrying overexpression CYR61.

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