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Antiproliferative Mechanism of a Cannabinoid Agonist by Cell Cycle Arrest in Human Gastric Cancer Cells

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ABSTRACT

For gastric cancers, the antineoplastic activity of cannabinoids has been investigated in only a few reports and knowledge regarding the mechanisms involved is limited. We have reported previously that treatment of gastric cancer cells with a cannabinoid agonist significantly decreased cell proliferation and induced apoptosis. Here, we evaluated the effects of cannabinoids on various cellular mediators involved in cell cycle arrest in gastric cancer cells. AGS and MKN-1 cell lines were used as human gastric cancer cells and WIN 55,212-2 as a cannabinoid agonist. Cell cycles were analyzed by flow cytometry and western blotting. Treatment with WIN 55,212-2 arrested the cell cycle in the G0/G1 phase. WIN 55,212-2 also upregulated phospho-ERK1/2, induced Kip1/p27 and Cip1/WAF1/p21 expression, decreased cyclin D1 and cyclin E expression, decreased Cdk 2, Cdk 4, and Cdk 6 expression levels, and decreased phospho-Rb and E2F-1 expression. ERK inhibitor decreased the proportion of G0/G1 phase which was induced by WIN 55,212-2. Inhibition of pAKT led to cell cycle arrest in gastric cancer cells. Cell cycle arrest preceded apoptotic response. Thus, this cannabinoid agonist can reduce gastric cancer cell proliferation via G1 phase cell cycle arrest, which is mediated via activation of the MAPK pathway and inhibition of pAKT. J. Cell. Biochem. 112: 1192–1205, 2011.

KEY WORDS: CANNABINOIDS; GASTRIC CANCER; CELL PROLIFERATION; CELL CYCLE; WIN 55212-2

Gastric cancer is one of the most common malignant tumors and the second cause of cancer-related death in the world [Parkin et al., 2005]. For patients with unresectable advanced or recurrent gastric cancers, the main therapeutic option is systemic chemotherapy. Although a series of trials have produced evidence that chemotherapy can prolong the survival of patients with advanced gastric cancers, a globally accepted standard chemotherapy and the optimal regimen have not yet been determined [Cervantes et al., 2008; Nishiyama and Eguchi, 2009]. Furthermore, systemic chemotherapy usually results in resistance arising in gastric cancer cells, which is associated with poor prognosis. Therefore, novel targets are necessary for the treatment of gastric cancers.

Cannabinoids exert diverse effects not only on the central nervous system but also on peripheral targets. Two receptors are biologically important and have been studied widely: type 1 receptor (CB_1) and type 2 receptor (CB_2) [Matsuda et al., 1990; Munro et al., 1993]. They are currently used to prevent nausea, vomiting, pain, and to stimulate the appetite in patients with cancers [Guzmán, 2003]. Cannabinoids have been known to exert antineoplastic effects in oncology since the early 1970s, when it was discovered that cannabinoids inhibit Lewis lung carcinoma growth [Munson et al., 1975]. In subsequent studies, several plant-derived cannabinoids, such as Δ^9-tetrahydrocannabinol and cannabidiol, synthetic cannabinoids such as WIN 55,212-2 and HU-210, and endogenous cannabinoids such as anandamide and 2-arachidonoylglycerol inhibit tumor cell growth and induce apoptosis by modulating different cell signaling pathways in gliomas [Sánchez et al., 2001; Carracedo et al., 2006b], lymphomas [McKallip et al., 2002; Gustafsson et al., 2006], and in cancers of the prostate [Mimeault et al., 2003; Sarfaraz et al., 2006], breast [De Petrocellis et al., 1998; Ligresti et al., 2006], skin [Casanova et al., 2003;

Jae Myung Park and Xiang-Shu Xian contributed equally to this study.

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Blázquez et al., 2006], pancreas [Carracedo et al., 2006a], and colorectal region [Ligresti et al., 2003; Greenhough et al., 2007]. Some studies showed that cannabinoids inhibit tumor cell proliferation by cell cycle arrest [Blázquez et al., 2006; Sarfaraz et al., 2006; Galanti et al., 2008].

For gastric cancers, the antineoplastic activity of cannabinoids has been investigated in only a few reports. Although recent studies including ours have reported that cannabinoid agonists such as Δ^9-tetrahydrocannabinol, anandamide, and WIN 55,212-2 inhibit gastric cancer cell proliferation and induce cancer cell apoptosis [Ligresti et al., 2006; Miyato et al., 2009; Xian et al., 2010], the exact mechanisms involved are poorly understood. In particular, there is no information available regarding their effects on the cell cycle. In this study, we investigated the effects of WIN 55,212-2 on various cellular mediators related with the cell cycle in gastric cancer cells.

MATERIALS AND METHODS

DRUGS

The mixed CB_1/CB_2 agonist (R)-(+)-WIN 55,212-2 ((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmet-hyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate, $C_{27}H_{26}N_2O_3$-CH_3SO_3H) and ERK1/2 inhibitor U0126 were purchased from Tocris Bioscience (Bristol, UK). Dimethyl sulfoxide (DMSO) was purchased from Sigma–Aldrich (St. Louis, MO). We selected WIN 55,212-2 as an agonist to extend our previous experiment [Xian et al., 2010].

CELL CULTURE

The human gastric cancer cell lines, AGS and MKN-1, were obtained from the Korean Cell Line Bank (Seoul, S. Korea). We selected these cells to extend our previous experiment. RPMI 1640 medium and fetal bovine serum (FBS) were purchased from WelGENE (Daegu, S. Korea) and penicillin–streptomycin was purchased from Sigma–Aldrich. AGS and MKN-1 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. The cells were maintained under standard cell culture conditions at 37 °C and 5% CO_2 in a humid environment.

PLASMID TRANSFECTION

Active (myristoylated) AKT plasmid pcDNA3 Myr HA Akt1 was purchased from Addgene (Cambridge, MA), and was used according to the manufacturer's instructions. Briefly, Aliquots of 2×10^5 cells were plated in 24-well plates (the cell culture medium without serum and antibiotics). After an overnight incubation for the cells to adhere, pcDNA3 Myr HA Akt1 was transfected into AGS cells by Lipofectamine 2000 (Invitrogen, Carlsbad, MA). Stable cell line was confirmed by Western blot analysis.

CELL CYCLE ANALYSIS BY FLOW CYTOMETRY

Aliquots of 1×10^6 cells were plated in 60 mm culture dishes. After an overnight incubation for the cells to adhere, cells were treated with different concentrations of the agents (1, 5, or 10 μM of WIN 55,212-2), and control cells were treated with an equivalent amount of vehicle (DMSO). After incubation for the specified time, the final concentration of DMSO was 0.1% (v/v) for each treatment. The cells were then washed twice with cold phosphate-buffered saline (PBS), detached with 0.25% trypsin–EDTA, and pelleted. The pellet was suspended in cold PBS and the cells were fixed in a final concentration of 70% ethanol for 1 h at 4°C. The cells were washed with cold PBS and incubated with 100 μg/ml RNase A for 15 min. at 37°C. Nuclei were stained with 50 μg/ml propidium iodide (PI; Sigma–Aldrich) for 30 min. at 37°C in the dark. Samples were analyzed by flow cytometry using a FACSCalibur apparatus (Becton Dickinson, San Jose, CA). For flow cytometric evaluation of cell cycle, 10,000 events corrected for debris and aggregate were analyzed for each sample. The assay was carried out in three replicates.

APOPTOSIS ANALYSIS BY FLOW CYTOMETRY

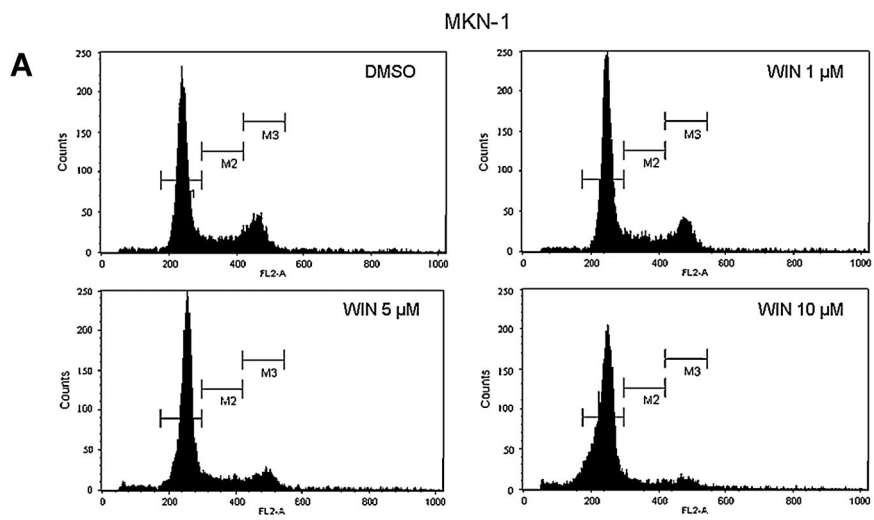
The Phycoerythrin (PE)-annexin V apoptosis detection kit from BD Biosciences (Bedford, MA) was used according to the manufacturer's instructions. Briefly, the cells were treated with WIN 55,212-2 (1, 5, or 10 μM) or an equivalent amount of DMSO for the specified time. The cells were then washed twice with cold PBS and resuspended in 1× binding buffer (0.01 M HEPES (pH 7.4), 0.14 M NaCl, 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. One hundred microliters of the cell suspension was transferred to a 5 ml culture tube, and 5 μl of PE-annexin V and 5 μl of 7-amino-actinomycin D (7-AAD) were added. The cells were incubated at room temperature for 15 min. in the dark, and 400 μl of 1× binding buffer was added. Apoptosis was analyzed by flow cytometry using a FACSCalibur apparatus (Becton Dickinson).

PREPARATION OF CELL EXTRACTS

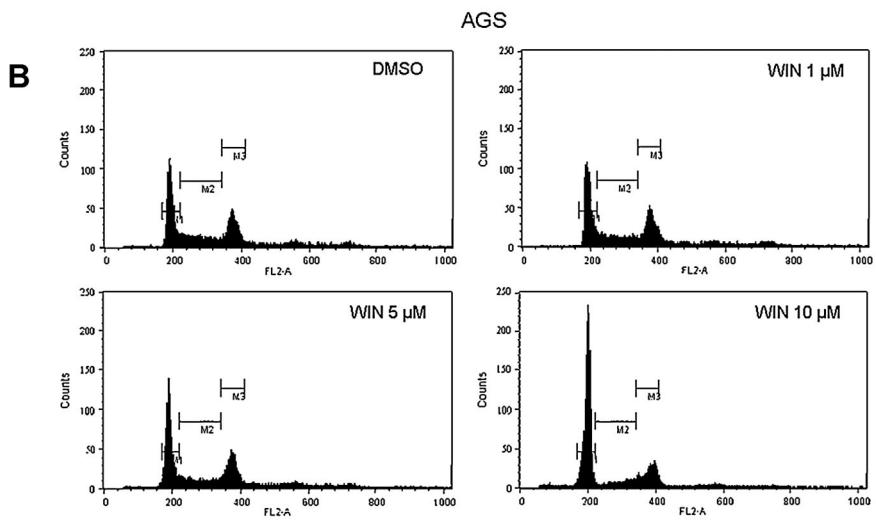
The cells were plated in 100 mm culture dishes. After an overnight incubation for the cells to adhere, cells were treated with WIN 55,212-2 (1, 2.5, 5, 7.5 or 10 μM) or an equivalent amount of DMSO for 24 h. The cells were then washed with cold PBS, harvested, disrupted on ice for 5 min. using lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin; Cell Signaling Technology, Beverly, MA) supplemented with protease inhibitors (Sigma–Aldrich) and centrifuged at $14{,}000 \times g$ for 15 min. at 4°C. The supernatants were removed, flash frozen in clean 1.5 ml tubes, and stored at −70°C for subsequent western blot analysis.

WESTERN BLOT ANALYSIS

The cell protein concentration was determined using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). For western blotting, equal amounts of protein samples were loaded onto 10% sodium dodecyl sulfate polyacrylamide electrophoresis gels and subjected to electrophoresis. Then, proteins were transferred onto nitrocellulose membranes at 350 mA for 1 h at room temperature. The membranes were blocked with 5% non-fat dry milk/Tris-buffered saline containing 0.1% Triton X-100 (TBST) for 1 h at room temperature and washed thrice for 5 min in TBST. The membranes were incubated overnight at 4°C with specific primary antibodies then



WIN 55,212-2 (μM)	0	1	5	10
G0/G1 (M1)%	66.3±0.2	67.1±1.7	72.6±0.5*	85.1±0.5***
S (M2)%	12.7±0.2	12.6±1.1	11.5±0.6	6.4±0.3**
G2/M (M3)%	16.5±0.2	16.1±0.7	11.4±0.2***	3.4±0.1***



WIN 55,212-2 (μM)	0	1	5	10
G0/G1 (M1)%	32.0±1.1	33.0±0.9	36.8±1.1*	61.4±0.7***
S (M2)%	18.5±1.3	16.5±2.3	16.5±0.5	10.1±0.1*
G2/M (M3)%	25.9±0.8	26.6±0.9	24.6±0.3	18.2±0.5***

Fig. 1. Effects of the cannabinoid agonist WIN 55,212-2 on the cell cycle in human gastric cancer cells. Gastric cancer cell lines MKN-1 (A) and AGS (B) cells were treated with WIN 55,212-2 (1, 5, or 10 μM) or equivalent concentrations of the vehicle DMSO for 24 h. Cell cycle analysis was conducted using flow cytometry as described in the Materials and Methods Section. Results are from three independent experiments and the data are expressed as the mean ± SEM. Statistical analyses used comparisons with vehicle values ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

washed thrice for 5 min. in TBST and hybridized with the secondary antibodies conjugated with horseradish peroxidase (HRP) for 1 h at room temperature. Then, the membranes were washed thrice for 5 min. in TBST. Reactions were detected using West-Q Chemiluminescent Substrate Plus Kits (GenDEPOT, Barker, TX). Antibody binding detection and intensities of the different proteins were quantified using a western blotting analysis system (Amersham Pharmacia Biotech, Piscataway, NJ). All experiments were repeated at least three times and yielded similar results. Antibodies to phospho-ERK1/2, ERK1/2, AKT, and E2F-1 were from Cell Signaling Technology. Antibodies to Kip1/p27, cyclin D1, cyclin E, Cdk2, Cdk4, Cdk6, phospho-Rb, and β-actin (loading control) were from Santa Cruz Biotechnology (Santa Cruz, CA). Cip1/WAF1/p21 antibody was from Millipore (Billerica, MA).

STATISTICAL ANALYSIS

Data are expressed as the mean ± standard error of the mean (SEM). Data were analyzed using analysis of variance (ANOVA) and the two-sample independent *t*-test. A $P < 0.05$ was considered significant. Statistical analysis was performed with SAS software (SAS Institute, Cary, NC).

RESULTS

EFFECTS OF WIN 55,212-2 ON CELL CYCLE PROGRESSION

We have shown previously that treatment of MKN-1 and AGS cells with WIN 55,212-2 significantly decreased cell survival and induced apoptosis [Xian et al., 2010]. Therefore, in this study we analyzed the cell cycle to characterize the inhibition of gastric cancer cell growth induced by WIN 55,212-2 and to relate this to cell cycle progression. As shown in Figure 1, compared with vehicle treatment, WIN 55,212-2 treatment resulted in a dose-dependent accumulation of MKN-1 and AGS cells in the G0/G1 phase of the cell cycle. When the concentration of WIN 55,212-2 was increased to 5 μM, the levels of accumulation of cells in the G0/G1 phase relative to control (vehicle only) values were 72.6 ± 0.5% in MKN-1 cells and 36.8 ± 1.1% in AGS cells (both $P < 0.05$). For treatment at 10 μM, the levels of accumulation of cells in the G0/G1 phase relative to vehicle values were 85.1 ± 0.5% in MKN-1 cells and 61.4 ± 0.7% in AGS cells (both $P < 0.001$).

UPREGULATION OF PHOSPHO-ERK1/2 BY WIN 55,212-2 TREATMENT IN HUMAN GASTRIC CANCER CELLS

To investigate the molecular mechanism by which WIN 55,212-2 leads to gastric cancer cell cycle arrest, we studied its effects on signal transduction through the ERK/mitogen-activated protein kinase (MAPK) pathway. As shown in Figure 2, treatment of MKN-1 and AGS cells with different concentrations of WIN 55,212-2 for 24 h led to a dose-dependent increase in the phospho-ERK1/2 protein expression.

ERK INHIBITOR DECREASES THE PROPORTION OF G0/G1 PHASE INDUCED BY WIN 55,212-2

To assess whether cell cycle arrest is mediated via activation of ERK1/2, we performed blocking of ERK1/2 activation by its inhibitor U0126 and quantification of cell cycle by flow cytometry. As shown in Figure 3, simultaneous treatment of WIN 55,212-2 and ERK1/2 inhibitor U0126 decreased the number of cells in the G0/G1 phase of cell cycle in MKN-1 cells and AGS cells, compared with the number of those treated with WIN 55,212-2 alone in MKN-1 cells and AGS cells, respectively.

INHIBITION OF PAKT LEADS TO CELL CYCLE ARREST IN GASTRIC CANCER CELLS

To assess whether cell cycle arrest is mediated via inhibition of pAKT, we performed cell cycle analysis in active (myristoylated)

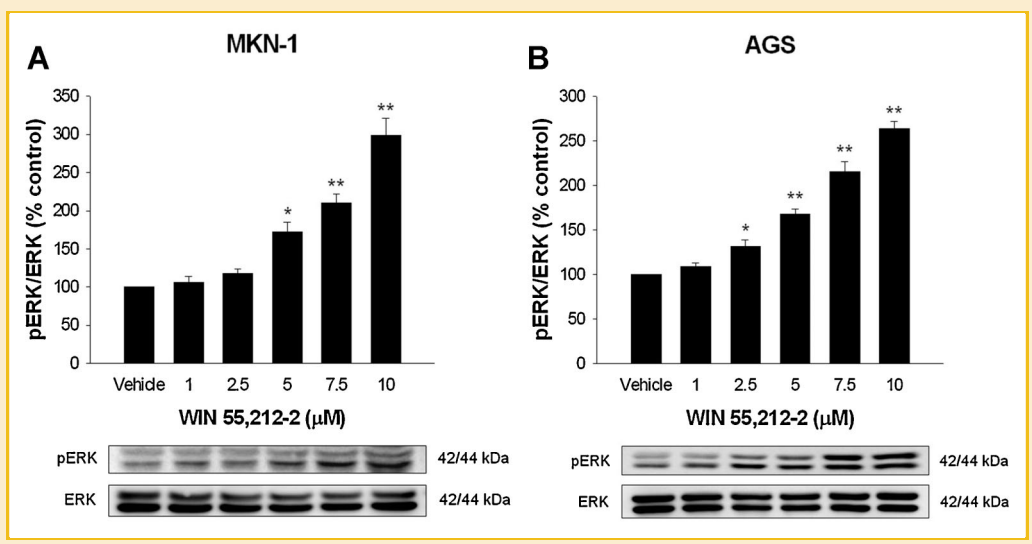


Fig. 2. Effects of WIN 55,212-2 on the expression of ERK1/2 in human gastric cancer cells. Western blot analysis of total ERK1/2 and phospho-ERK1/2 expressed in MKN-1 (A) and AGS (B) cells. MKN-1 and AGS cells were treated with WIN 55,212-2 (1, 2.5, 5, 7.5, or 10 μM) or an equivalent concentration of DMSO vehicle for 24 h. Densitometric analyses were obtained from three independent experiments and the data are shown as the mean ± SEM. Statistical analyses used comparisons with vehicle values ($^{*}P < 0.01$ and $^{**}P < 0.001$).

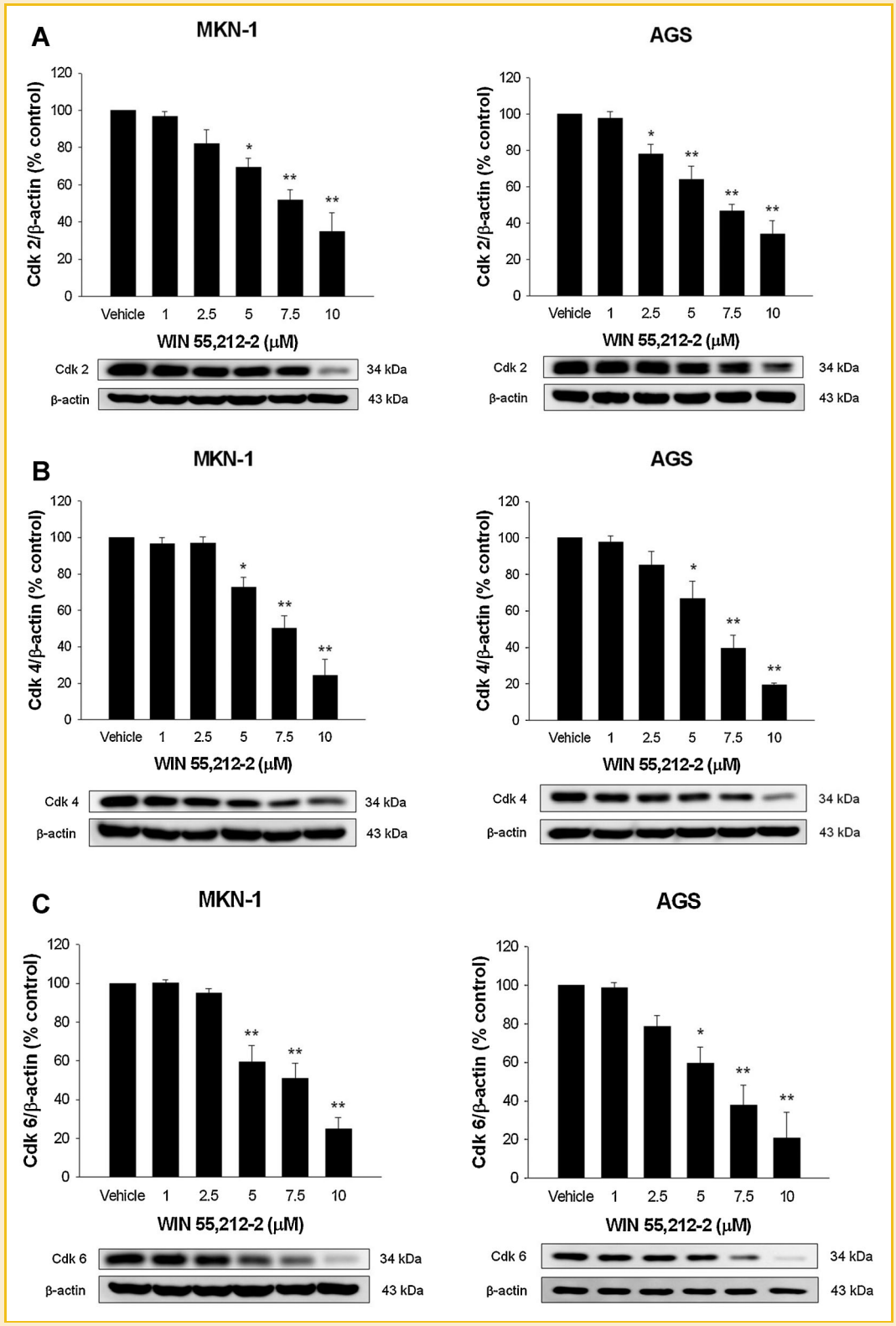


Fig. 3. Effect of simultaneous treatment of WIN 55,212-2 and ERK1/2 inhibitor on cell cycle in human gastric cancer cells. MKN-1 (A) and AGS (B) cells were pretreated for 1 h with 10 μM of U0126 or equivalent DMSO, followed by incubation with 10 μM WIN 55,212-2 or equivalent DMSO for 24 h. Cell cycle analysis by flow cytometry was conducted as described in the Materials and Methods Section. Data from representative experiments repeated thrice with similar results.

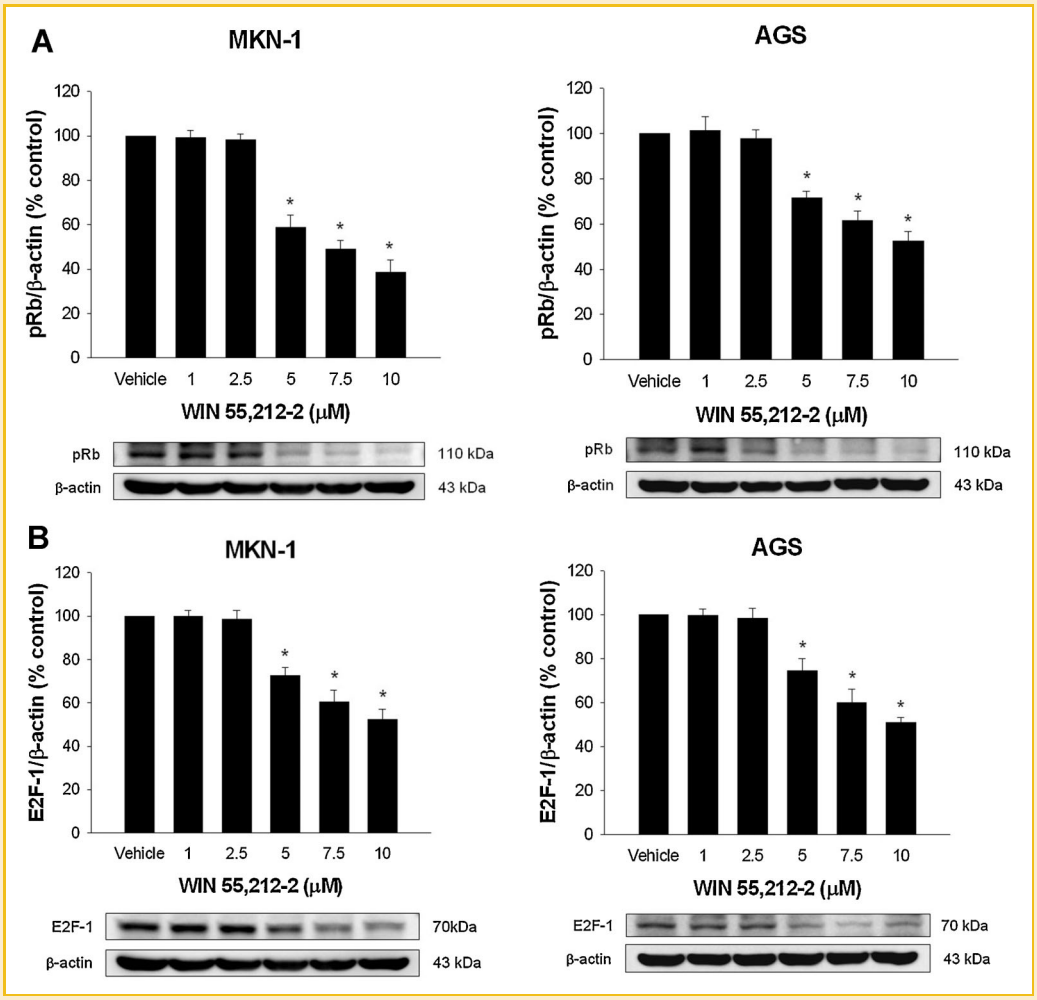


Fig. 4. Effect of WIN 55,212-2 in AGS/MyrAKT cells. (A) Western blot analysis of AKT expressed in AGS/MyrAKT cell samples. WT: wild-type AGS. Lane 1-5: AGS/MyrAKT cell samples. (B) Quantification of cell cycle by flow cytometry. AGS/MyrAKT cells were treated with WIN 55,212-2 (1, 5, and 10 μM) or equivalent DMSO for 24 h. Cell cycle analysis by flow cytometry was conducted as described in the Materials and Methods Section. Results are obtained from three independent experiments. Data reflect the mean ± SEM. Statistical analyses were conducted by comparisons with vehicle values ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

AKT of AGS (AGS/MyrAKT) cells. First, stable cell lines were selected (AGS/MyrAKT cell sample 2, Fig. 4A). As shown in Figure 4B, treatment of AGS/MyrAKT cells with 10 μM of WIN 55,212-2 for 24 h increased the number of cells in the G0/G1 phase compared with vehicle treatment (49.6 ± 0.9% and 40.4 ± 0.8%, respectively; $P < 0.01$). The number of cells in G0/G1 phase of cell cycle was significantly decreased when compared with treatment of wild-type AGS as shown in Figure 1.

WIN 55,212-2 TREATMENT INDUCES KIP1/P27 AND CIP1/WAF1/P21 EXPRESSION IN HUMAN GASTRIC CANCER CELLS

Our previous study showed that WIN 55,212-2 treatment significantly downregulated phosphorylated (phospho)-AKT (Ser473) in human gastric cancer cells [Xian et al., 2010]. Therefore, we examined the effect of WIN-55,212-2 on cell cycle regulatory molecules in the G1 phase of the cell cycle. First, we examined the levels of protein expression of Kip1/p27 and Cip1/WAF1/p21. As shown in Figure 5, treatment of MKN-1 and AGS cells with different concentrations of WIN 55,212-2 for 24 h led to a dose-dependent increase in Kip1/p27 and Cip1/WAF1/p21 levels. The Kip1/p27 protein level was significantly increased after treatment with WIN 55,212-2 at 5–10 μM in MKN-1 and AGS cells (Fig. 5A). For the protein expression of Kip/p27 at 5.0, 7.5, and 10 μM of WIN-55,212-2, relative density data showed 1.5, 1.8, and 2.5 fold increases in MKN-1 and 2.8, 7.3, and 11.1 fold increases in AGS, respectively. The Cip1/WAF1/p21 protein level was also significantly increased after treatment with WIN 55,212-2 at 5–10 μM in MKN-1 and AGS cells (Fig. 5B).

WIN 55,212-2 TREATMENT DECREASES CYCLIN D1 AND CYCLIN E EXPRESSION IN HUMAN GASTRIC CANCER CELLS

The cyclins and cyclin-dependent kinase (Cdks) are negatively regulated by Kip1/p27 and Cip1/WAF1/p21. Therefore, we examined the effect of WIN 55,212-2 on the expressed levels of cyclin D1 and cyclin E. Treatment of MKN-1 and AGS cells

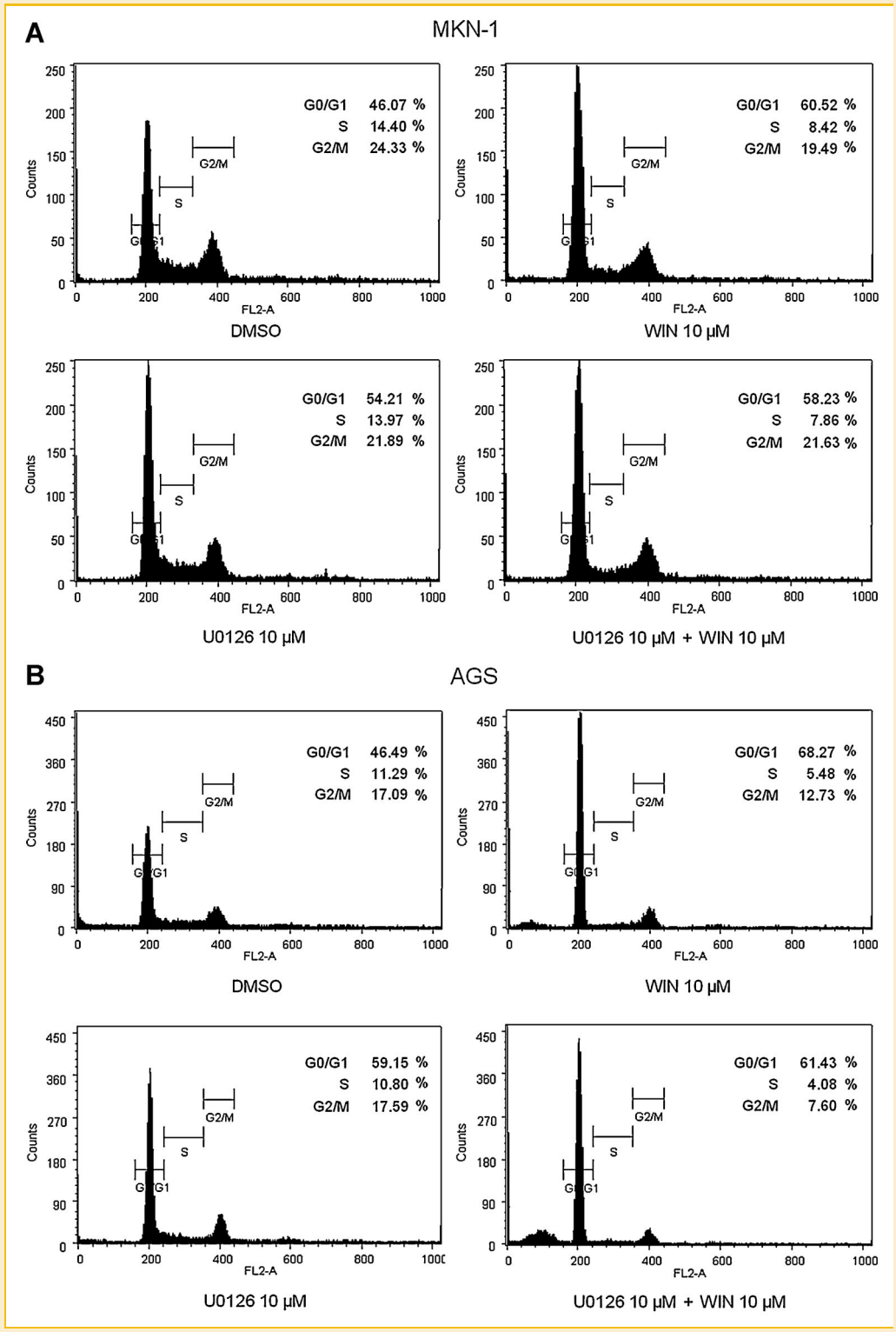
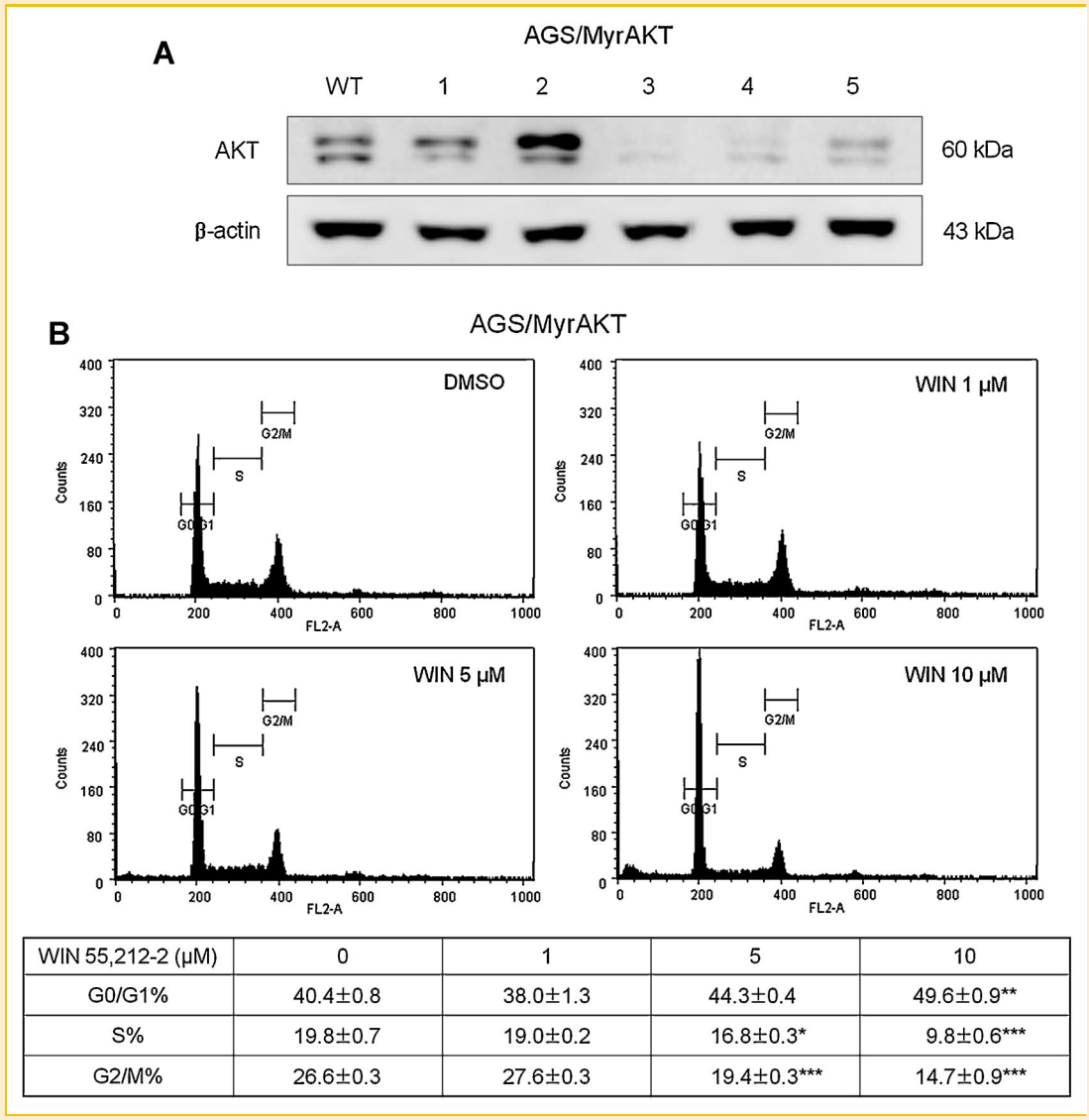


Fig. 5. Effects of WIN 55,212-2 on the expression of Kip1/p27 and Cip1/WAF1/p21 in human gastric cancer cells. (A) Western blot analysis of Kip1/p27 expressed in MKN-1 and AGS cells. (B) Western blot analysis of Cip1/WAF1/p21 expressed in MKN-1 and AGS cells. MKN-1 and AGS cells were treated with WIN 55,212-2 (1, 2.5, 5, 7.5, or 10 μM) or equivalent concentrations of DMSO for 24 h; β-actin was used as a loading control. Densitometric analyses were obtained from three independent experiments. Data are shown as the mean ± SEM. Statistical analyses used comparisons with vehicle values ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).



WIN 55,212-2 (μM)	0	1	5	10
G0/G1%	40.4±0.8	38.0±1.3	44.3±0.4	49.6±0.9**
S%	19.8±0.7	19.0±0.2	16.8±0.3*	9.8±0.6***
G2/M%	26.6±0.3	27.6±0.3	19.4±0.3***	14.7±0.9***

Fig. 6. Effects of WIN 55,212-2 on the expression of cyclin D1 and cyclin E in human gastric cancer cells. (A) Western blot analysis of cyclin D1 expressed in MKN-1 and AGS cells. (B) Western blot analysis of cyclin E expressed in MKN-1 and AGS cells. MKN-1 and AGS cells were treated with WIN 55,212-2 (1, 2.5, 5, 7.5, or 10 μM) or equivalent concentrations of DMSO for 24 h; β-actin was used as a loading control. Densitometric analyses were obtained from three independent experiments. Data are shown as the mean ± SEM. Statistical analyses used comparisons with vehicle values ($^{*}P < 0.01$ and $^{**}P < 0.001$).

with different concentrations of WIN 55,212-2 for 24 h led to a dose-dependent decrease in the cyclin D1 and cyclin E protein levels. As shown in Figure 6A, the cyclin D1 protein level was significantly decreased after treatment with WIN 55,212-2 at 5–10 μM in MKN-1 and AGS cells. Densitometric analysis data revealed a significant decrease in the expression of cyclin D1 in MKN-1 cells to 42.8 ± 6.1%, 29.7 ± 4.8%, and 21.3 ± 5.3% of control values at 5, 7.5, and 10.0 μM of WIN-55,212-2, respectively. In AGS cells, expression of cyclin D1 was 59.7 ± 3.1%, 25.8 ± 2.8%, and 15.3 ± 2.7% at 5, 7.5, and 10.0 μM WIN-55,212-2, respectively. As shown in Figure 6B, the cyclin E protein level was significantly decreased after treatment with WIN 55,212-2 at concentrations of 2.5–10 μM in MKN-1 and AGS cells.

WIN 55,212-2 TREATMENT DECREASES Cdk 2, Cdk 4, AND Cdk 6 EXPRESSION IN HUMAN GASTRIC CANCER CELLS

Next, we examined the effect of WIN 55,212-2 on the protein expression levels of Cdk 2, Cdk 4, and Cdk 6. Treatment of MKN-1 and AGS cells with different concentrations of WIN 55,212-2 for 24 h led to a dose-dependent decrease in the Cdk 2, Cdk 4, and Cdk 6 protein expression levels (Fig. 7). Cdk 2 protein level was significantly decreased after treatment with WIN 55,212-2 at >5 μM in MKN-1 and AGS cells (Fig. 7A). At 5 μM of WIN 55,212-2, densitometry analysis showed that the expression levels of Cdk 2 were 69.2% ± 5.1% and 64.2% ± 7.1% relative to the vehicle control in MKN-1 and AGS cells ($P < 0.01$ and $P < 0.001$, respectively). As shown in Figure 7B and C, Cdk 4 and Cdk 6 protein levels were also significantly decreased after treatment with WIN 55,212-2 at >5 μM

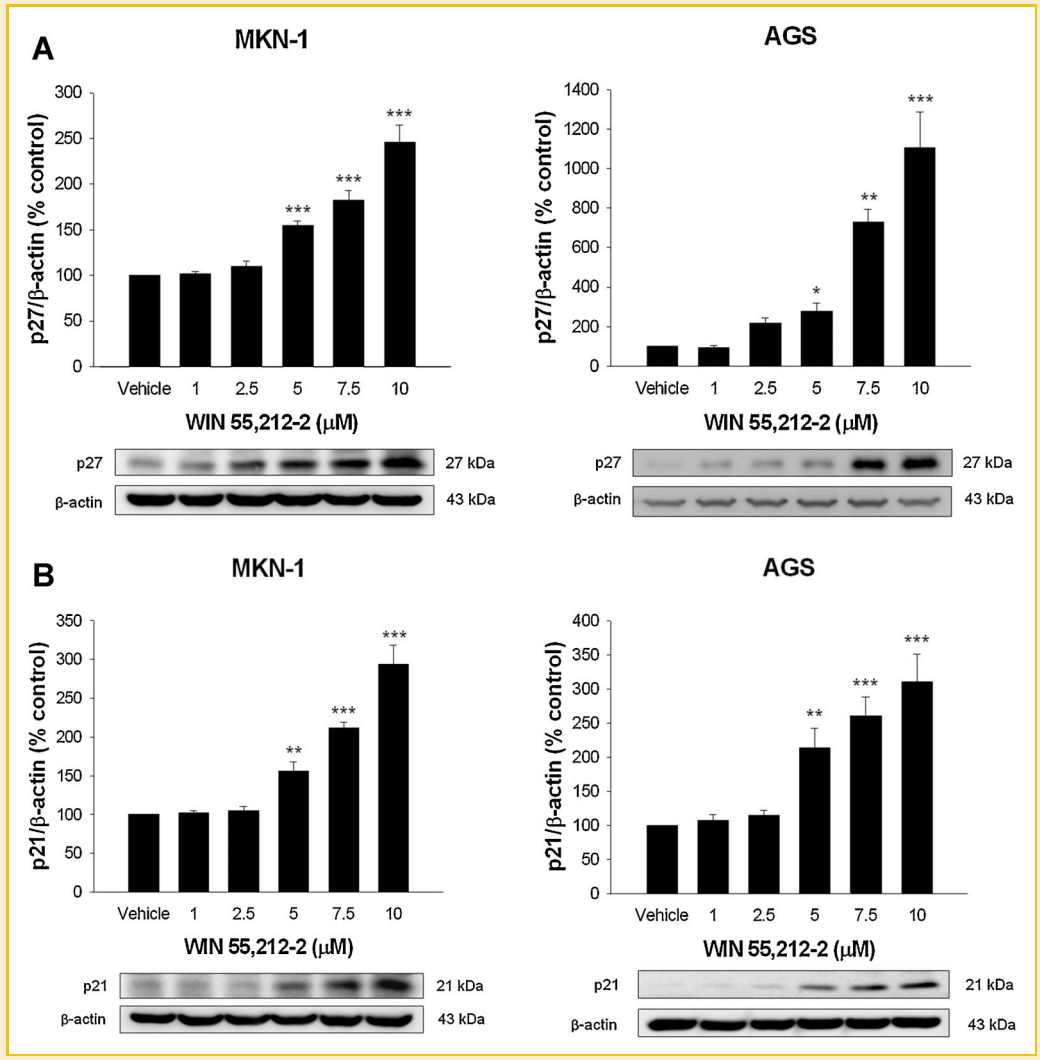


Fig. 7. Effects of WIN 55,212-2 on the expression of Cdk 2, Cdk 4, and Cdk 6 in human gastric cancer cells. (A) Western blot analysis of Cdk 2 expressed in MKN-1 and AGS cells. (B) Western blot analysis of Cdk 4 expressed in MKN-1 and AGS cells. (C) Western blot analysis of Cdk 6 expressed in MKN-1 and AGS cells. MKN-1 and AGS cells were treated with WIN 55,212-2 (1, 2.5, 5, 7.5, or 10 μM) or equivalent concentrations of DMSO for 24 h; β-actin was used as a loading control. Densitometric analyses were obtained from three independent experiments. Data are shown as the mean ± SEM. Statistical analyses used comparisons with vehicle values ($^{*}P < 0.01$ and $^{**}P < 0.001$).

in MKN-1 and AGS cells. At 5 μM WIN 55,212-2, densitometric analysis revealed that the mean expression levels of Cdk 4 and Cdk 6 were 72.8% ± 5.4% and 59.6% ± 8.3%, respectively, in MKN-1 cells, and 66.9% ± 9.5% and 59.7% ± 8.1%, respectively, in AGS cells.

WIN 55,212-2 TREATMENT DECREASES PHOSPHO-Rb AND E2F-1 EXPRESSION IN HUMAN GASTRIC CANCER CELLS

The retinoblastoma (Rb) protein is a key regulator of the G1/S transition in the cell cycle and it is associated with Cdks such as Cdk 2, Cdk 4 and Cdk 6. Treatment of MKN-1 and AGS cells with different concentrations of WIN 55,212-2 for 24 h led to a dose-dependent decrease in the phospho-Rb protein expression (Fig. 8A). This was significantly decreased after treatment with >5 μM of WIN 55,212-2 in MKN-1 and AGS cells. At 5 μM, mean expression levels relative to vehicle were 58.7% ± 5.7% and 71.5% ± 3.2% in MKN-1 and AGS, respectively (both $P < 0.001$).

Phosphorylated (phospho-) Rb controls cell cycle by binding to and inhibiting the E2F transcription factor. Treatment of MKN-1 and AGS cells with different concentrations of WIN 55,212-2 for 24 h led to a dose-dependent decrease in the E2F-1 protein expression level (Fig. 8B), which was significantly decreased after treatment with >5 μM of WIN 55,212-2 in MKN-1 and AGS cells. At this concentration, the mean expression levels relative to vehicle were 72.7% ± 3.5% and 74.4% ± 5.5% in MKN-1 and AGS cells, respectively (both $P < 0.001$).

CELL CYCLE ARREST BY WIN 55,212-2 PRECEDES APOPTOTIC RESPONSE IN GASTRIC CANCER CELLS

To assess distributions of gastric cancer cells with respect to cell cycle arrest and apoptosis during their exposure to WIN 55, 212-2, we treated gastric cancer cells with WIN 55,212-2 for 6 and 12 h, and quantified apoptosis and cell cycles. As shown

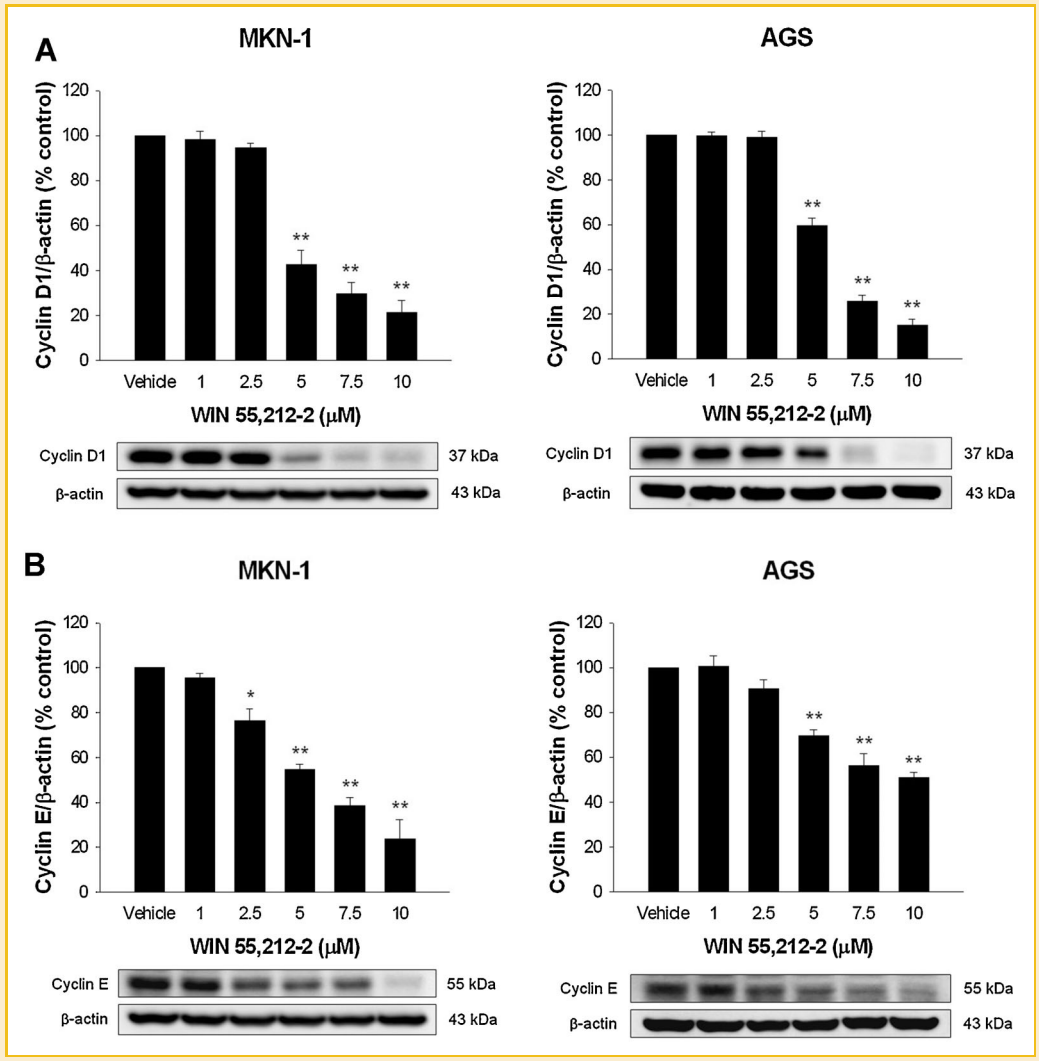


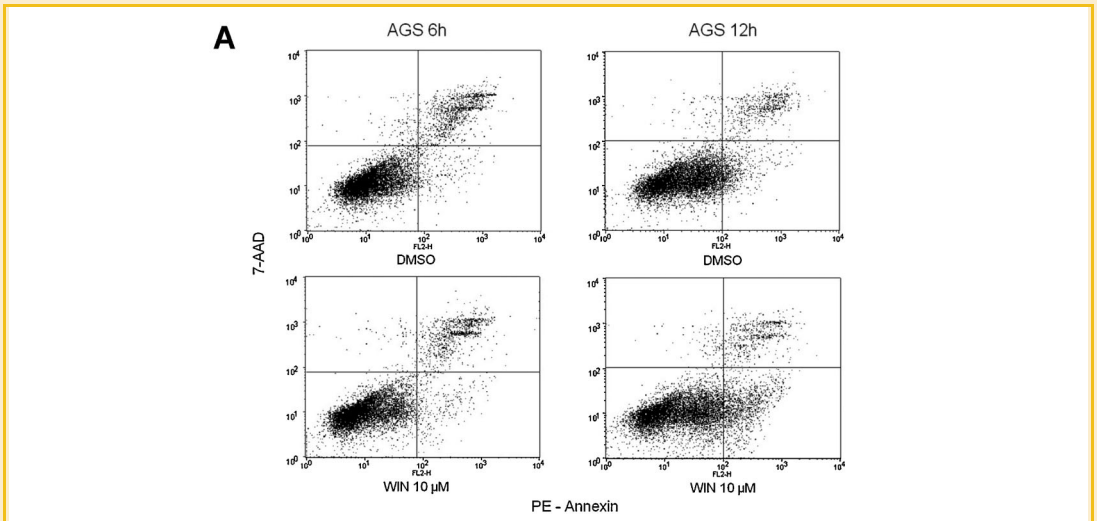
Fig. 8. Effects of WIN 55,212-2 on the expression of phospho-Rb and E2F-1 in human gastric cancer cells. (A) Western blot analysis of phospho-Rb expressed in MKN-1 and AGS cells. (B) Western blot analysis of E2F-1 expressed in MKN-1 and AGS cells. MKN-1 and AGS cells were treated with WIN 55,212-2 (1, 2.5, 5, 7.5, or 10 μM) or equivalent concentrations of DMSO for 24 h; β-Actin was used as a loading control. Densitometric analyses were obtained from three independent experiments. Data are shown as the mean ± SEM. Statistical analyses used comparisons with vehicle values ($^*P < 0.001$).

in Figure 9A, significant proportion of AGS cells were early apoptotic cells after 12 h (12.1 ± 0.1%, $P < 0.001$; compared with control 4.8 ± 0.1%). MKN-1 cells showed no significant evidence of apoptosis during 6 and 12 h incubation with 10 μM of WIN 55,212-2. As shown in Figure 9B, significant proportion of AGS cells was in G0/G1 phase of cell cycle after 6 h (46.5 ± 0.3%, $P < 0.001$; compared with control 41.5 ± 0.2%). In MKN-1 cells, significant proportion was in G0/G1 phase after 6 h incubation with 10 μM of WIN 55,212-2 (38.2 ± 0.5%, $P < 0.05$; compared with control 36.5 ± 0.2%). Furthermore, significant proportion of MKN-1 cells was in G0/G1 phase after 12 h incubation with 10 μM of WIN 55,212-2 (62.5 ± 1.1%, $P < 0.001$; compared with control 41.7 ± 0.4%). Considering Figure 9A and B, the timing of G0/G1 phase of cell cycle arrest preceded the timing of apoptosis.

DISCUSSION

We have reported previously that cannabinoid agonists inhibit the proliferation of gastric cancer cells. Here we showed that the antiproliferative effect of WIN 55,212-2 of human gastric cancer cells could be explained by cell cycle arrest in the G0/G1 phase. The treatment upregulated phospho-ERK1/2, induced Kip1/p27 and Cip1/WAF1/p21 expression, decreased cyclin D1 and cyclin E expression, decreased Cdk 2, Cdk 4, and Cdk 6 expression, and decreased phospho-Rb and E2F-1 expression.

Inhibition of the cell cycle has been a target for the management of cancer [McDonald and El-Deiry, 2000; Owa et al., 2001]. Several studies have shown that the induction of apoptosis may be cell cycle-dependent [Hartwell and Kastan, 1994; King and Cidlowski, 1998]. Exact knowledge regarding the antiproliferative mechanisms

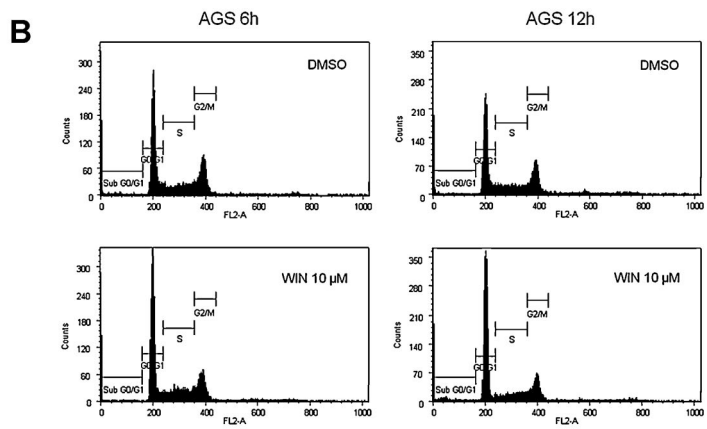


WIN 55,212-2 (μM)	0	1	5	10
AGS 6h				
Early apoptosis%	2.4±0.2	2.5±0.1	2.7±0.1	3.2±0.2
Late apoptosis%	11.0±0.4	11.2±0.2	10.5±0.2	9.6±0.4
AGS 12h				
Early apoptosis%	4.8±0.1	6.5±0.2	6.3±0.1	12.1±0.1%*
Late apoptosis%	11.7±0.1	9.4±0.2	9.1±0.3	9.9±0.3
MKN-1 6h				
Early apoptosis%	3.2±0.1	2.9±0.1	4.7±0.3	4.9±0.2
Late apoptosis%	11.6±0.2	11.0±0.2	9.0±0.2	10.5±0.2
MKN-1 12h				
Early apoptosis%	3.9±0.1	3.8±0.1	3.8±0.1	3.2±0.1
Late apoptosis%	13.3±0.3	12.2±0.1	14.3±0.4	12.7±0.3

Fig. 9. Distributions of gastric cancer cells with respect to cell cycle arrest and apoptosis during their exposure to WIN 55,212-2. (A) Quantification of apoptosis by flow cytometry, with PE-annexin V staining. The dual parametric dot plots combining PE Annexin V and 7-AAD fluorescence show the viable cell population in the lower left quadrant (PE Annexin V and 7-AAD negative), the early apoptotic cells in the lower right quadrant (PE Annexin V positive and 7-AAD negative), and the late apoptotic cells in the upper right quadrant (PE Annexin V and 7-AAD positive). AGS and MKN-1 cells were treated with WIN 55,212-2 (1, 5, and 10 μM) or equivalent DMSO for 6 or 12 h. Results are obtained from three independent experiments. Data reflect the mean ± SEM. Statistical analyses were conducted by comparisons with vehicle values ($^{*}P < 0.001$). (B) Quantification of cell cycle by flow cytometry. MKN-1 and AGS cells were treated with WIN 55,212-2 (1, 5, and 10 μM) or equivalent DMSO for 6 or 12 h. Cell cycle analysis by flow cytometry was conducted as described in the Materials and Methods Section. Results are obtained from three independent experiments. Data reflect the mean ± SEM. Statistical analyses were conducted by comparisons with vehicle values ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

of cannabinoids for cancer cells is limited. In melanoma cells, WIN 55,212-2 and Δ^9-tetrahydrocannabinol treatment inhibit cell cycle progression [Blázquez et al., 2006]. In prostate cancer cells and glioblastoma cells, cannabinoid agonist treatment resulted in an arrest in the G0/G1 phase of the cell cycle [Sarfaraz et al., 2006; Galanti et al., 2008]. For gastric cancers, there is little information about the action mechanism of cannabinoid agonists. Here we found that WIN 55,212-2 treatment resulted in a dose-dependent accumulation of cells in the G1 phase of the cell cycle in MKN-1 and AGS cells. A dose of >5 μM was statistically significant in producing cell cycle arrest. This dose was consistent with the result of a proliferation assay in our previous study [Xian et al., 2010].

We analyzed the potential mechanisms that could induce these effects. We studied the effects of WIN 55,212-2 treatment on signal transduction through the ERK/MAPK pathway. ERK1/2 activation leads to cell proliferation [Yuan and Yankner, 2000; Anjum and Blenis, 2008]. However, the link between activation of the ERK cascade and cell proliferation can depend on the duration of the stimulus [Marshall, 1998]. Prolonged ERK1/2 activation can mediate cell cycle arrest and cell death [Pumiglia and Decker, 1997; Guzmán, 2003]. Recent studies have been reported that cannabinoids leads to the activation of ERK1/2 signaling and AKT inhibition in gliomas and prostate cancers [Galve-Roperh et al., 2000; Sarfaraz et al., 2006]. Those results



WIN 55,212-2 (μM)	0	1	5	10
AGS 6h				
Sub G0/G1 %	1.2±0.6	0.5±0.1	0.6±0.1	1.1±0.1
G0/G1 %	41.5±0.2	43.0±0.2	43.2±0.1	46.5±0.3***
S %	21.8±0.2	23.0±0.1	23.2±0.6	22.4±0.4
G2/M %	26.0±0.5	25.3±0.1	23.7±0.3*	20.4±0.3***
AGS 12h				
Sub G0/G1 %	1.7±0.3	1.0±0.1	1.4±0.1	3.9±0.1*
G0/G1 %	38.4±0.2	40.4±0.2	44.8±0.1**	48.6±0.2***
S %	21.4±0.3	22.6±0.4	18.7±0.1**	16.5±0.3***
G2/M %	24.4±0.5	24.1±0.5	23.4±0.1	19.4±0.4***
MKN-1 6h				
Sub G0/G1 %	2.7±0.2	2.6±0.1	4.3±0.3	4.2±0.1
G0/G1 %	36.5±0.2	37.0±0.1	37.2±0.3	38.2±0.5*
S %	11.7±0.2	11.8±0.1	13.7±0.4	14.0±0.3*
G2/M %	33.7±0.3	34.0±0.2	27.1±0.5	28.4±0.3**
MKN-1 12h				
Sub G0/G1 %	2.9±0.1	3.4±0.1	2.9±0.1	3.4±0.1
G0/G1 %	41.7±0.4	42.9±0.6	56.4±0.1***	62.5±1.1***
S %	19.7±0.2	19.7±0.3	19.7±0.2	18.5±0.4
G2/M %	23.6±0.3	22.2±0.6	12.1±0.1***	8.5±0.3***

Fig. 9. (*Continued*).

are consistent with our findings that phospho-ERK1/2 protein level was dose-dependently increased after WIN 55,212-2 treatment, and that ERK1/2 inhibitor decreased the number of cells in the G0/G1 phase of cell cycle in gastric cancer cells. Our results suggest that activation of ERK1/2 signaling by cannabinoid agonists mediates the accumulation of gastric cancer cells in the G0/G1 phase.

Our previous and present data showed that phospho-AKT (Ser473) protein level was dose-dependently decreased and phospho-ERK1/2 protein level was dose-dependently increased after treatment with WIN 55,212-2 in gastric cancer cells. Treatment of human gastric cancer cells with WIN 55,212-2 upregulated phospho-ERK1/2, induced Kip1/p27 and Cip1/WAF1/p21 expression, decreased cyclin D1 and cyclin E expression, decreased Cdk 2, Cdk 4, and Cdk 6 expression, and decreased phospho-Rb and E2F-1 expression. These results were similar to that of a previous study on human prostate cancer cells [Sarfaraz et al., 2006]. Δ^9-tetrahydrocannabinol induced cell cycle arrest by downregulation of E2F-1 in human glioblastoma multiforme cells [Galanti et al., 2008]. However, the effects of such drugs could be different in other types of cancer. WIN 55,212-2 acts on melanoma cells by arresting the cell cycle at the G1–S transition by inhibiting phospho-AKT and phospho-Rb, but no significant effect of WIN 55,212-2 challenge was observed on the levels of other classical members of the G1–S transition machinery, such as Cdk2/3, Cdk4, Kip1/p27, Cip1/WAF1/p21, or cyclin D1 [Blázquez et al., 2006]. Our results showed that inhibition of pAKT led to cell cycle arrest in gastric cancer cells, which was consistent with the previous studies [Blázquez et al., 2006; Sarfaraz et al., 2006]. We suspect that these different effects produced by cannabinoid agonists might be related to the different cell types used.

Based on these results, we cannot rule out the possibility that cannabinoids also act on additional cell cycle regulators in gastric cancer cells, and that other mechanisms might inhibit cell proliferation, apart from cell cycle arrest. Although we did not study whether WIN 55,212-2 arrested via CB_1 or CB_2 receptors, our previous study showed that the antiproliferative action was produced via CB_1. The concentrations needed for WIN55,212-2 to inhibit proliferation were too high considering that the Ki values for CB_1 and CB_2 receptors for this compound are in the nanomolar range. Experimental data suggest that WIN 55,212-2 at high mM concentrations possibly interacts with CB_2 receptors and transient receptor potential vanilloid 1 receptors [Contassot et al., 2004; Akopian et al., 2009]. Finally, clinical relevance will be increased and should be followed by in vivo experiments.

Drugs controlling the cell cycle in malignant tumors have been major pharmaceutical targets. We do not yet know the exact antiproliferative mechanism of WIN 55,212-2. However, it was clear from this study that this cannabinoid receptor agonist can inhibit gastric cancer cells by cell cycle arrest. This study supports the renewed interest in cannabinoids as potential therapeutic agents for treating gastric cancers.

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