Cannabinoid Receptor Agonist as an Alternative Drug in 5-Fluorouracil-resistant Gastric Cancer Cells

XIANG-SHU XIAN[1,2], HYEYEON PARK[1], MYUNG-GYU CHOI[1] and JAE MYUNG PARK[1]

[1]*Division of Gastroenterology, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea;*
[2]*Division of Gastroenterology, Department of Internal Medicine, Yantai Yuhuangding Hospital, Yantai, Shandong Province, China*

Abstract. *Fluorouracil is the main chemotherapeutic drug used for gastrointestinal cancers, which suffers the important problem of treatment resistance. There is little information whether cannabinoid agonists can be used as an alternative drug for fluorouracil-resistant gastric cancer cells. In this study, we investigated the effects of a cannabinoid agonist, WIN-55,212-2, on 5-fluorouracil (5-FU)-resistant human gastric cancer cells, to examine whether the cannabinoid agonist may be an alternative therapy. Survival of the 5-FU-resistant gastric cancer cell line, SNU-620-5FU/1000, was not significantly reduced even by a high dose of 5-FU treatment. However, WIN-55,212-2 inhibited the proliferation of SNU-620-5FU/1000 and enhanced their apoptosis, as indicated by an increase of apoptotic cell proportion, activated caspase-3 and Poly (ADP-ribose) polymerase cleavage. Furthermore, WIN-55,212-2 reduced phospho-extracellular-signal-regulated kinases (ERK) 1/2, phospho-Akt (protein kinase B), B-cell lymphoma-2 (BCL2) and BCL2-associated X (BAX) protein expression in 5-FU-resistant gastric cancer cells. These results indicate that a cannabinoid agonist may, indeed, be an alternative chemotherapeutic agent for 5-FU-resistant gastric cancer.*

Gastric cancer is one of the most common malignant tumors and the second cause of cancer-related death in the world (1). More than two thirds of patients diagnosed with advanced gastric cancer have unresectable stages and are treated with systemic chemotherapy (1). Even patients with an operable stage of gastric cancer have a high rate of both local and distant recurrence, which is associated with a median survival of two years and a 5-year survival rate lower than 30% (2). 5-Fluorouracil (5-FU) is the most extensively used chemotherapeutic agent in the treatment of gastric cancer (3). However, resistance is commonly found, which has resulted in an overall objective response rate of only 21% in patients with gastric cancer and which limits effective treatment in these patients (4, 5). Therefore, alternative drugs are necessary for patients with chemotherapy-resistant gastric cancer.

Cannabinoids are currently used to prevent nausea, vomiting, and pain, and to increase appetite in patients with cancer treated with chemotherapeutic agents (6). Cannabinoids have also been studied for their activity on the inhibition of tumor cell growth and induction of apoptosis by modulating diverse cell signaling pathways in many types of cancer cells (7-11). In gastrointestinal tumors, cannabinoids have also shown anti-neoplastic effects (12-16). We have reported that a cannabinoid agonist, WIN-55,212-2, reduces gastric cancer cell proliferation by induction of apoptosis and G_0/G_1 phase cell-cycle arrest (17, 18).

In this study, we aimed to investigate whether a cannabinoid agonist can be used as an alternative treatment modality for 5-FU-resistant gastric cancer. To show this, we assessed the reduction of proliferation and enhancement of apoptosis by WIN-55,212-2 in 5-FU-resistant gastric cancer cells.

Materials and Methods

Drugs. The mixed CB_1/CB_2 agonist R-(+)- WIN-55,212-2 (2,3 dihydro-5-methyl-3 [(morpholinyl)-methyl]pyrollo (1,2,3 de)-1,4-benzoxazinyl]-[1-naphthaleny]methanone, $C_{27}H_{26}N_2O_3{\cdot}CH_3SO_3H$) was purchased from Tocris Bioscience (Bristol, UK). 5-FU and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA).

Cell culture. The 5-FU-resistant cell line of the SNU-620 human gastric cancer cell line, SNU-620-5FU/1000, was obtained from the Korean Cell Line Bank (Seoul, Korea). RPMI-1640 medium and

Correspondence to: Jae Myung Park, MD, Division of Gastroenterology, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, 505, Banpo-Dong, Seocho-Gu, Seoul, 137-070, Korea. Tel: +82 222586023, Fax: +82 222582055; e-mail: parkjerry@catholic.ac.kr

Key Words: Gastric cancer, cannabinoids, fluorouracil, drug resistance, apoptosis.

fetal bovine serum (FBS) were purchased from WelGENE (Daegu, Korea), and penicillin–streptomycin was purchased from Sigma. SNU-620-5FU/1000 cells were cultured in RPMI-1640 medium, and 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.

CCK-8 assay. The Cell Counting Kit-8 (CCK-8) assay was performed to determine cell viability. CCK-8 assay detection kit from Dojindo Molecular Technologies (Rockville, MA, USA) was used according to the manufacturer's instructions. Briefly, cells were seeded in 96-multi-well plates and the cells were treated with WIN 55,212-2 (1, 2.5, 3, 3.5, 4, 4.5, 5, 10, 30 μM), 5-FU (0.1, 0.5, 1, 2.5, 5, 7.5, 10, 100 μM) or an equivalent amount of DMSO for 24 or 48 h. After incubation for the specified time, 10 μl of CCK-8 solution was added to each well in an assay plate. The plate was then placed in a CO_2 incubator for 4 h. After incubation, absorbance was recorded on a microplate reader (Spectra Max 250, Molecular Devices, Sunnyvale, CA, USA) at 450 nm. Six wells were used for each treatment condition.

Apoptosis analysis by flow cytometry. Phycoerythrin (PE)-annexin V apoptosis detection kit from BD Biosciences (Bedford, MA, USA) was used according to the manufacturer's instructions. Briefly, the cells were treated with WIN 55,212-2 (1, 3.4, or 5 μM) or an equivalent amount of DMSO for 48 h. The cells were then washed twice with cold phosphate-buffered saline (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 density 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 then added. Apoptosis was analyzed by flow cytometry using a FACSCalibur apparatus (Becton Dickinson, San Jose, CA, USA).

Cell-cycle analysis by flow cytometry. Aliquots of 1×10^6 cells were plated in 60-mm culture dishes. The cells were treated with WIN 55,212-2 (1, 3.4, or 5 μM) or an equivalent amount of DMSO for 48 h. After incubation for the specified time, the cells were then washed twice with cold PBS 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 then analyzed by flow cytometry using a FACSCalibur apparatus (Becton Dickinson). For flow cytometric evaluation of the cell cycle, 10,000 events corrected for debris and aggregate were analyzed for each sample. The assay was carried out in three replicates.

Preparation of cell extracts. The cells were plated in 100-mm culture dishes and then treated with WIN 55,212-2 (1, 3.4, or 5 μM) or an equivalent amount of DMSO for 48 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, USA), supplemented with protease inhibitors (Sigma-Aldrich) and centrifuged at 14,000 ×*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, USA). For western blotting, equal amounts of protein samples were loaded onto 10% sodium dodecyl sulfate polyacrylamide electrophoresis gels and subjected to electrophoresis. Proteins were then transferred onto nitrocellulose membranes at 350 mA for 1 h at room temperature. The membranes were subsequently 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 washed 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, USA). Antibody binding detection and intensities of the different proteins were quantified using a western blotting analysis system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). All experiments were repeated at least three times and yielded similar results. Antibodies to phospho- extracellular-signal-regulated kinases (ERK) 1/2, ERK1/2, phospho-Akt (Ser473), B-cell lymphoma-2 (BCL2), BCL2-associated X (BAX), and cleaved poly (ADP-ribose) polymerase (PARP) were from Cell Signaling Technology. Cleaved caspase-3 antibody was from Abcam (Cambridge, UK). β-Actin antibody (loading control) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Statistical analysis. Statistical analysis was performed with SAS software (SAS Institute, Cary, NC, USA). Data were analyzed using the two-tailed Student's *t*-test. A value of $p<0.05$ was considered significant. Data are expressed as the mean±standard error of the mean (SEM).

Results

WIN 55,212-2 inhibits the proliferation of 5-FU-resistant gastric cancer cells. We confirmed that SNU-620-5FU/1000 cells were resistant to 5-FU by treatment of 5-FU for 24 h and 48 h. Cell viability of SNU-620-5FU/1000 cells was not significantly reduced by concentration of 5-FU up to 10 μM (Figure 1). In order to evaluate the effect of WIN 55,212-2 on viability of the 5-FU-resistant gastric cancer cells, we treated SNU-620-5FU/1000 cells with different concentrations of WIN 55,212-2 for 24 h and 48 h. As shown in Figure 1B, WIN 55,212-2 reduced cell survival dose-dependently. Specifically, the 24 h half-maximal inhibitory concentration (IC_{50}) value was 3.5 μM and the 48 h IC_{50} value was 3.4 μM of WIN 55,212-2.

WIN 55,212-2 induced apoptosis in 5-FU-resistant gastric cancer cells. To ascertain whether the cannabinoid agonist reduces 5-FU-resistant gastric cancer cell survival by inducing apoptosis, we treated SNU-620-5FU/1000 cells with WIN 55,212-2 for 48 h and measured the proportion of apoptotic

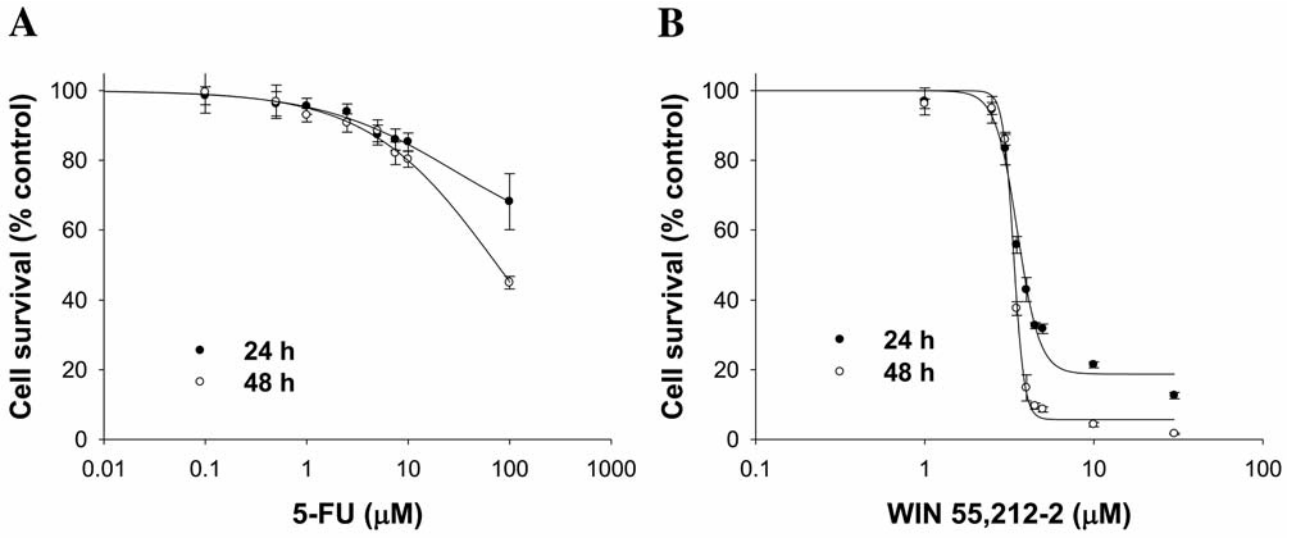


Figure 1. *Anti-proliferative effect of 5-fluorouracil (5-FU) (A) and cannabinoid agonist WIN 55,212-2 (B) in SNU-620-5FU/1000 cells, as assessed by CCK-8 assay. Cells were seeded in 96-multiwell plates and the drugs were added at the indicated concentrations. Cell growth assessment was carried out by CCK-8 assay after 24 or 48 h of treatment (see Materials and Methods). Results are shown as the percentage cell survival relative to that of controls. The data are expressed as mean ± SEM of six experiments.*

cells by flow cytometry. Treatment of WIN 55,212-2 was shown to significantly induce apoptosis of SNU-620-5FU/1000 cells, as indicated by the dose-dependently increased proportion of PE-Annexin V-positive/7-AAD-negative cells (Figure 2). The proportion of cells which were double-positive for PE-Annexin V and 7-AAD was also observed to increase dose-dependently after treatment of WIN 55,212-2 (Figure 2).

Cell-cycle arrest with WIN 55,212-2 treatment in 5-FU-resistant cells. Next, we analyzed the cell cycle to characterize the inhibition of 5-FU-resistant gastric cancer cell growth by WIN 55,212-2 and to relate this with cell-cycle progression. Compared with the vehicle-treated cells, WIN 55,212-2 treatment resulted in a dose-dependent accumulation of SNU-620-5FU/1000 cells in the sub-G_0/G_1 phase of the cell cycle (Figure 3). When the concentration of WIN 55,212-2 was increased to 3.4 μM, the accumulation of SNU-620-5FU/1000 cells in the sub-G_0/G_1 phase was 14.1±1.4% compared to that of the controls ($p<0.001$). After 5 μM treatment, the accumulation of cells in the sub-G0/G1 phase was 42.0±0.7% compared to the control ($p<0.001$).

Phospho-ERK1/2 and phospho-AKT Expression decreased in 5-FU-resistant cells with WIN 55,212-2 treatment. To investigate the mechanism of WIN 55,212-2-induced apoptosis, firstly, we studied its effects on signal transduction through the ERK/mitogen-activated protein kinase (MAPK) pathway and phospho-AKT. SNU-620-5FU/1000 cells were treated with different concentrations of WIN 55,212-2 for 48 h, which led to a dose-dependent decrease in the phospho-ERK1/2 and phospho-AKT (Ser473) protein levels (Figure 4). The phospho-ERK1/2 and phospho-AKT protein levels were significantly reduced after WIN 55,212-2 treatment in SNU-620-5FU/1000 cells (Figure 4). Densitometric analysis revealed a significant decrease in SNU-620-5FU/1000 cells in the expression of phospho-ERK1/2 to 54.3±6.7% and 32.1±8.5% at 3.4 and 5 μM of WIN-55,212-2 (*vs*. controls), respectively (both $p<0.001$). The expression of phospho-AKT was reduced to 31.5±2.9% at 5 μM of WIN-55,212-2, compared to controls ($p<0.001$).

Effect of WIN 55,212-2 on the expressed levels of BAX, BCL2, cleaved caspase-3 and cleaved PARP. Next, we studied the effect of WIN 55,212-2 on the expression of BAX and BCL2, and cleaved caspase-3 and PARP. The BAX and BCL2 protein levels in SNU-620-5FU/1000 cells significantly decreased after treatment with 5 μM of WIN 55,212-2 (Figure 4B). Densitometric analysis revealed a significant decrease in the expression of BAX and BCL2 in SNU-620-5FU/1000 cells to 28.6±1.8% and 25.9±15.0% of control values at 5 μM of WIN-55,212-2, respectively (both $p<0.001$). The expression of cleaved caspase-3 and PARP proteins significantly increased after 5 μM of WIN 55,212-2 treatment in these cells. Densitometric analysis revealed a significant increase in the expression of cleaved caspase-3 and cleaved PARP in SNU-620-5FU/1000 cells to 598.4±110.4% and 631.7±78.0% of control values at 5 μM of WIN-55,212-2, respectively (both $p<0.001$).

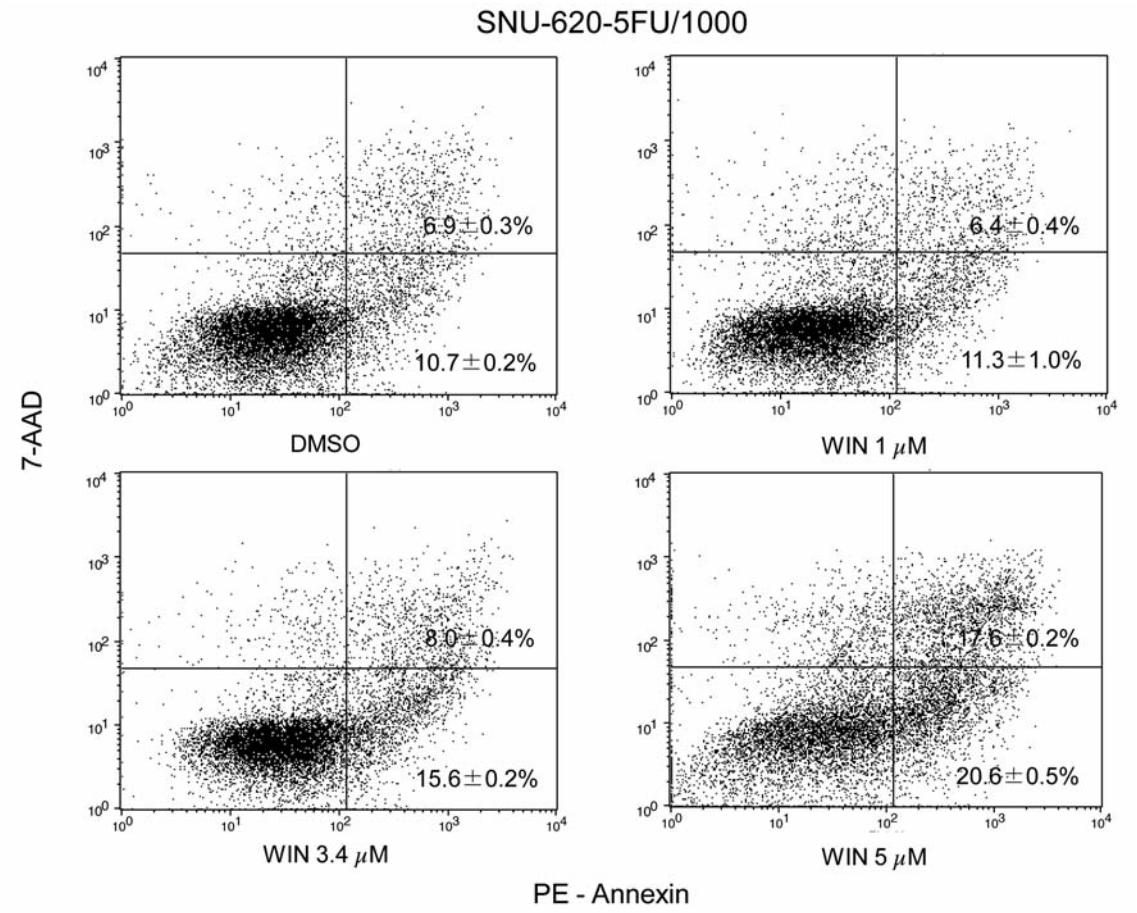


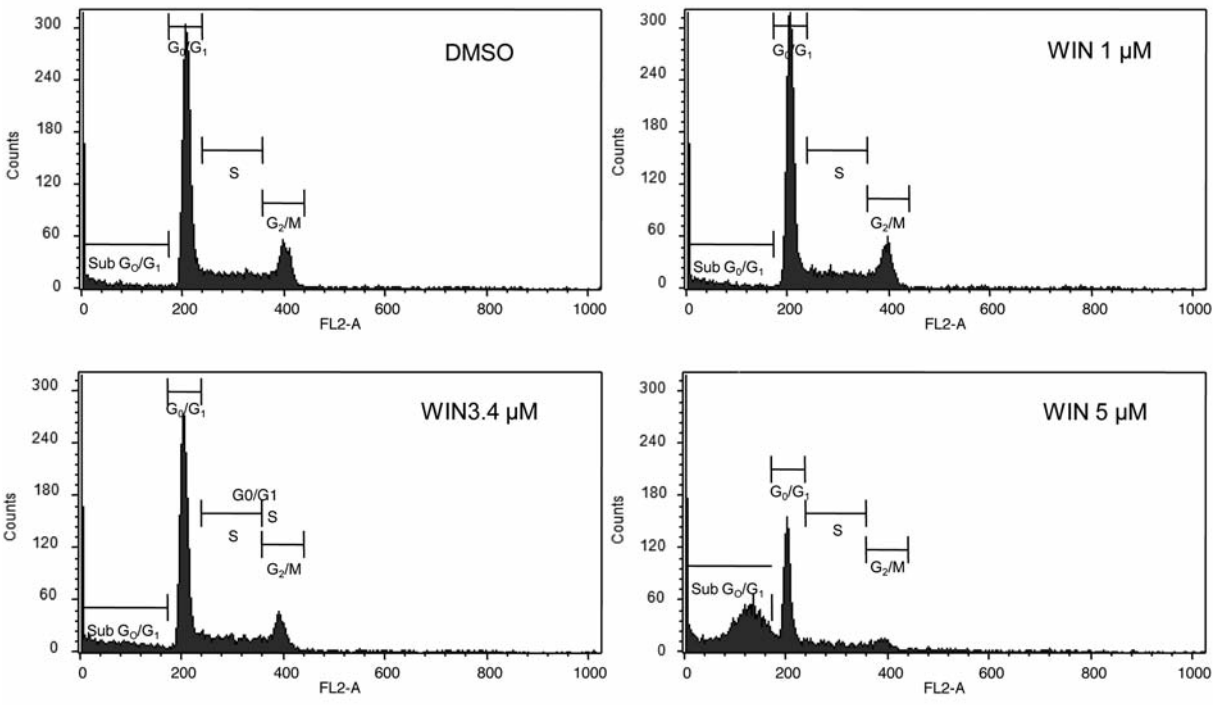
Figure 2. *Apoptosis induced by WIN 55,212-2 treatment of SNU-620-5FU/1000 cells. Apoptosisl was quantified by flow cytometry after Phycoerythrin (PE)-annexin V staining. Here, 7-amino-actinomycin D (7-AAD) was employed as a plasma membrane-impermeable vital dye, which allows for distinguishing dying and dead cells in the bi-parametric analysis with annexin V. The dual parametric dot plots combining PE-annexin V and 7-AAD fluorescence show the viable cell population in the lower left quadrant (both PE-annexin V and 7-AAD negative), the early apoptotic cells in the lower right quadrant (positive for PE-annexin V and negative for 7-AAD), and the late apoptotic cells in the upper right quadrant (both PE-anexin V and 7-AAD positive). SNU-620-5FU/1000 cells were treated with WIN 55,212-2 (1, 3.4, and 5 μM) or an equivalent amount of dimethyl sulfoxide (DMSO) for 48 h. Data from representative experiments repeated thrice with similar results are shown.*

Discussion

In previous studies, we reported that treatment of gastric cancer cells with the cannabinoid agonist WIN-55,212-2 significantly reduces cell proliferation, and induces apoptosis and G_0/G_1 phase cell-cycle arrest (17, 18). To further delineate the effect of this cannabinoid agonist on gastric cancer, we treated fluorouracil-resistant gastric cancer cells with WIN 55,212-2 and assessed cell viability, apoptosis and ERK/MAPK pathway. The present study showed that the cannabinoid agonist WIN-55,212-2 has anti-neoplastic effects even towards 5-FU-resistant gastric cancer cells, indicated by the inhibition of proliferation and induction of apoptosis. These results suggest that this cannabinoid agonist has potential as an alternative treatment regimen in gastric cancers with 5-FU resistance.

We first evaluated the effects on cell viability by the treatment of WIN 55,212-2 of 5-FU-resistant cells. WIN 55,212-2 treatment reduced the survival of 5-FU-resistant gastric cancer cells dose-dependently. The anti-proliferative efficacy on these cells was similar to the findings of the previous study using gastric cancer cells without 5-FU resistance, shown by similar IC_{50} values for WIN 55,212-2 treatment between 5-FU-resistant and -sensitive cells (18).

Uncontrolled cellular growth is a consequence of defects in cell cycle and apoptotic machinery, which is responsible for the development and progression of most cancers. Those agents that can modulate apoptosis in cancer cells may be able to affect the steady-state cell population and be useful in cancer treatment. Previous studies have shown that cannabinoid agonists such as Δ^9-tetrahydrocannabinol and anandamide inhibit cancer cell proliferation and induce



WIN 55,212-2 (μM)	0	1	3.4	5
Sub G_0/G_1%	7.6±0.2	7.7±0.3	14.1±1.4**	42.0±0.7**
G_0/G_1%	48.8±0.4	50.0±0.7	44.9±1.1**	23.7±0.5**
S%	17.2±0.9	17.3±0.5	16.6±0.2	9.0±0.2**
G_2/M%	15.1±0.5	14.8±0.6	12.0±0.6*	5.4±0.2**

Figure 3. *Effects of WIN 55,212-2 on the cell cycle in SNU-620-5FU/1000 cells. SNU-620-5FU/1000 cells were treated with WIN 55,212-2 (1, 3.4, and 5 μM) or equivalent concentrations of the vehicle DMSO for 48 h. Cell-cycle analysis was conducted using flow cytometry as described in Materials and Methods. Results are from three independent experiments and the data are expressed as the mean±SEM. Statistical analyses used comparisons with vehicle controls (*p<0.01, **p<0.001).*

apoptosis (10, 16). In the present study, we also showed that WIN-55,212-2 induced apoptosis of 5-FU-resistant cells, which was consistent with our previous works using non-5-FU-resistant cell lines (18). However, SNU-620-5FU/1000 cells exhibited cell-cycle arrest in the sub-G_0/G_1 phase with WIN 55,212-2, which was different from the previous observation that WIN 55,212-2 resulted in a dose-dependent accumulation of MKN-1 and AGS cells in the G_0/G_1 phase (17). Although we do not know exactly what caused this different phase arrest, we speculate that different cell types may raise this discrepancy.

We studied the potential mechanisms that could induce these effects by examining the effects of WIN 55,212-2 treatment on signal transduction through the AKT and ERK/MAPK pathway. Recent studies reported that cannabinoids activate ERK1/2 signaling and inhibit AKT in glioma and prostate cancer (11, 19). In our previous data, the expression of phospho-AKT (Ser473) and phosphor-ERK1/2 in AGS and MKN-1 cells were decreased and increased dose-dependently, respectively, with WIN 55,212-2 treatment. However, the present results showed that WIN 55,212-2 treatment reduced phospho-ERK1/2 and phospho-AKT expression in 5-FU-resistant gastric cancer cells. This reduction of phospho-ERK1/2 may be due to the different cell types used in our study.

The cleaved form of caspase-3 is a key effector of cell apoptosis. As shown in the previous study using human neuroglioma cells (20), caspase-3 and PARP cleavage was dose-dependently increased after treatment of WIN 55,212-2 in 5-FU-resistant gastric cancer cells.

Members of the BCL2 family proteins are critical regulators of the apoptotic pathway. BCL2, which forms a

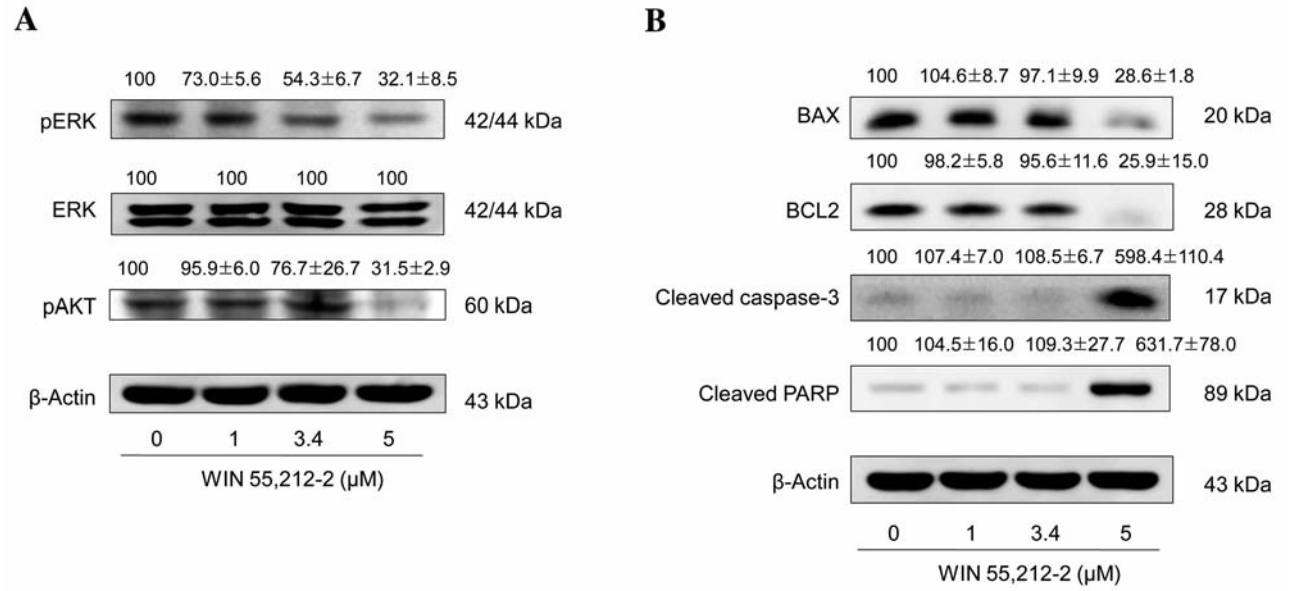


Figure 4. *Effects of WIN 55,212-2 on the expression of extracellular-signal-regulated kinases (ERK) 1/2, phospho-AKT, B-cell lymphoma-2 (BCL2), BCL2-associated X (BAX), cleaved caspase-3, and cleaved poly-(ADP-ribose) polymerase (PARP) in SNU-620-5FU/1000 cells. A: Western blot analysis of total ERK1/2, phospho-ERK1/2, phospho-AKT expression in SNU-620-5FU/1000 cells. B: Western blot analysis of BAX, BCL2, cleaved caspase-3, and cleaved PARP expression in SNU-620-5FU/1000 cells. SNU-620-5FU/1000 cells were treated with WIN 55,212-2 (1, 3.4, and 5 μM) or an equivalent concentration of DMSO vehicle for 48 h; β-actin was used as a loading control. Densitometric analyses were obtained from three independent experiments and the data are shown as the mean±SEM.*

heterodimer with the pro-apoptotic protein BAX and neutralizes its pro-apoptotic effect, is found at high levels in more than half of all human tumors (21). Previous studies showed that BCL2 protein levels decreased and BAX protein levels increased in human prostate cancer cells and hepatoma HepG2 cells after treatment with WIN 55,212-2 (11, 22). However, our results showed that both BCL2 and BAX protein levels decreased after treatment of 5-FU-resistant-gastric cancer cells with WIN 55,212-2. This reduction of BAX may be due to a translocation of cytosolic BAX to mitochondria and activation of caspase-3 (23).

In conclusion, we found that the cannabinoid agonist WIN 55,212-2 can induce cytotoxicity in 5-FU-resistant gastric cancer cells by up-regulation of cleaved caspase-3 and cleaved PARP, and down-modulation of phospho-ERK1/2, phospho-AKT, BCL2 and BAX. Our results open new possibilities for the use of cannabinoids as a therapy for 5-FU-resistant gastric cancer.

Competing Interests

None.

Acknowledgements

This study was funded by the Korean government (MEST), grant number: 2009-0066500.

References

1 Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics, 2011. CA Cancer J Clin *61*: 59-90, 2011.
2 Ajani JA: Standard chemotherapy for gastric carcinoma: Is it a myth? J Clin Oncol *18*: 4001-4003, 2000.
3 Pasini F, Fraccon AP and DE Manzoni G: The role of chemotherapy in metastatic gastric cancer. Anticancer Res *31*: 3543-3554, 2011.
4 Toffoli G and Cecchin E: Pharmacogenetics of stomach cancer. Suppl Tumori *2*: S19-22, 2003.
5 Thomas DM and Zalcberg JR: 5-Fluorouracil: A pharmacological paradigm in the use of cytotoxics. Clin Exp Pharmacol Physiol *25*: 887-895, 1998.
6 Guzman M: Cannabinoids: potential anticancer agents. Nat Rev Cancer *3*: 745-755, 2003.
7 Carracedo A, Gironella M, Lorente M, Garcia S, Guzman M, Velasco G and Iovanna JL: Cannabinoids induce apoptosis of pancreatic tumor cells via endoplasmic reticulum stress-related genes. Cancer Res *66*: 6748-6755, 2006.
8 Carracedo A, Lorente M, Egia A, Blazquez C, Garcia S, Giroux V, Malicet C, Villuendas R, Gironella M, Gonzalez-Feria L, Piris MA, Iovanna JL, Guzman M and Velasco G: The stress-regulated protein p8 mediates cannabinoid-induced apoptosis of tumor cells. Cancer Cell *9*: 301-312, 2006.
9 Gustafsson K, Christensson B, Sander B and Flygare J: Cannabinoid receptor-mediated apoptosis induced by R(+)-methanandamide and Win55,212-2 is associated with ceramide accumulation and p38 activation in mantle cell lymphoma. Mol Pharmacol *70*: 1612-1620, 2006.

10 Ligresti A, Moriello AS, Starowicz K, Matias I, Pisanti S, De Petrocellis L, Laezza C, Portella G, Bifulco M and Di Marzo V: Antitumor activity of plant cannabinoids with emphasis on the effect of cannabidiol on human breast carcinoma. J Pharmacol Exp Ther *318*: 1375-1387, 2006.
11 Sarfaraz S, Afaq F, Adhami VM, Malik A and Mukhtar H: Cannabinoid receptor agonist-induced apoptosis of human prostate cancer cells LNCaP proceeds through sustained activation of ERK1/2 leading to G1 cell cycle arrest. J Biol Chem *281*: 39480-39491, 2006.
12 Greenhough A, Patsos HA, Williams AC and Paraskeva C: The cannabinoid delta(9)-tetrahydrocannabinol inhibits RAS-MAPK and PI3K-AKT survival signalling and induces BAD-mediated apoptosis in colorectal cancer cells. Int J Cancer *121*: 2172-2180, 2007.
13 Ligresti A, Bisogno T, Matias I, De Petrocellis L, Cascio MG, Cosenza V, D'Argenio G, Scaglione G, Bifulco M, Sorrentini I and Di Marzo V: Possible endocannabinoid control of colorectal cancer growth. Gastroenterology *125*: 677-687, 2003.
14 Patsos HA, Hicks DJ, Dobson RR, Greenhough A, Woodman N, Lane JD, Williams AC and Paraskeva C: The endogenous cannabinoid, anandamide, induces cell death in colorectal carcinoma cells: A possible role for cyclooxygenase 2. Gut *54*: 1741-1750, 2005.
15 Sreevalsan S, Joseph S, Jutooru I, Chadalapaka G and Safe SH: Induction of apoptosis by cannabinoids in prostate and colon cancer cells is phosphatase dependent. Anticancer Res *31*: 3799-3807, 2011.
16 Miyato H, Kitayama J, Yamashita H, Souma D, Asakage M, Yamada J and Nagawa H: Pharmacological synergism between cannabinoids and paclitaxel in gastric cancer cell lines. J Surg Res *155*: 40-47, 2009.
17 Park JM, Xian XS, Choi MG, Park H, Cho YK, Lee IS, Kim SW and Chung IS: Antiproliferative mechanism of a cannabinoid agonist by cell cycle arrest in human gastric cancer cells. J Cell Biochem *112*: 1192-1205, 2011.
18 Xian XS, Park H, Cho YK, Lee IS, Kim SW, Choi MG, Chung IS, Han KH and Park JM: Effect of a synthetic cannabinoid agonist on the proliferation and invasion of gastric cancer cells. J Cell Biochem *110*: 321-332, 2010.
19 Galve-Roperh I, Sanchez C, Cortes ML, Gomez del Pulgar T, Izquierdo M and Guzman M: Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. Nat Med *6*: 313-319, 2000.
20 Eichele K, Weinzierl U, Ramer R, Brune K and Hinz B: R(+)-methanandamide elicits a cyclooxygenase-2-dependent mitochondrial apoptosis signaling pathway in human neuroglioma cells. Pharm Res *23*: 90-94, 2006.
21 Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, Bruncko M, Deckwerth TL, Dinges J, Hajduk PJ, Joseph MK, Kitada S, Korsmeyer SJ, Kunzer AR, Letai A, Li C, Mitten MJ, Nettesheim DG, Ng S, Nimmer PM, O'Connor JM, Oleksijew A, Petros AM, Reed JC, Shen W, Tahir SK, Thompson CB, Tomaselli KJ, Wang B, Wendt MD, Zhang H, Fesik SW and Rosenberg SH: An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature *435*: 677-681, 2005.
22 Giuliano M, Pellerito O, Portanova P, Calvaruso G, Santulli A, De Blasio A, Vento R and Tesoriere G: Apoptosis induced in HepG2 cells by the synthetic cannabinoid WIN: involvement of the transcription factor PPARgamma. Biochimie *91*: 457-465, 2009.
23 Espada L, Meo-Evoli N, Sancho P, Real S, Fabregat I, Ambrosio S and Tauler A: ROS production is essential for the apoptotic function of E2F1 in pheochromocytoma and neuroblastoma cell lines. PloS one *7*: e51544, 2012.

Received March 10, 2013
Revised April 18, 2013
Accepted April 22, 2013