ARTICLE
Journal of Cellular Biochemistry 110:321–332 (2010)

Journal of Cellular Biochemistry

Effect of a Synthetic Cannabinoid Agonist on the Proliferation and Invasion of Gastric Cancer Cells

Xiang-Shu Xian,[1] Hyeyeon Park,[1] Yu Kyung Cho,[1] In Seok Lee,[1] Sang Woo Kim,[1] Myung-Gyu Choi,[1] In-Sik Chung,[1] Ki-Hwan Han,[2] and Jae Myung Park[1*]

[1] *Division of Gastroenterology, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, Seoul, Korea*

[2] *Department of Anatomy, College of Medicine, Ewha Woman's University, Seoul, Korea*

ABSTRACT

Although cannabinoids are associated with antineoplastic activity in a number of cancer cell types, the effect in gastric cancer cells has not been clarified. In the present study, we investigated the effects of a cannabinoid agonist on gastric cancer cell proliferation and invasion. The cannabinoid agonist WIN 55,212-2 inhibited the proliferation of human gastric cancer cells in a dose-dependent manner and that this effect was mediated partially by the CB_1 receptor. We also found that WIN 55,212-2 induced apoptosis and down-regulation of the phospho-AKT expression in human gastric cancer cells. Furthermore, WIN 55,212-2 treatment inhibited the invasion of gastric cancer cells, and down-regulated the expression of MMP-2 and VEGF-A through the cannabinoid receptors. Our results open the possibilities in using cannabinoids as a new gastric cancer therapy. J. Cell. Biochem. 110: 321–332, 2010.

KEY WORDS: CANNABINOID; GASTRIC CANCER; PROLIFERATION; INVASION; APOPTOSIS

Cannabinoids are the active components of *Cannabis sativa* (marijuana) and its derivatives. Cannabinoids act by activating specific cell surface G-protein-coupled cannabinoid receptors [Di Marzo et al., 2004]. 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]. CB_1 is expressed abundantly in the brain and at many peripheral sites, and CB_2 is expressed almost exclusively in the immune system [Howlett et al., 2002].

Synthetic cannabinoids are used clinically. Synthetic Δ^9-tetrahydrocannabinol (Marinol™) and its analogue nabilone (Cesamet™) are licensed in the USA, Canada, and the UK for their palliative effects on chemotherapy-induced nausea and vomiting [Guzmán, 2003; Hall et al., 2005]. Rimonabant is also used in treating obesity and related cardiometabolic disease [Pi-Sunyer et al., 2006].

Since the first report of the antineoplastic activity of cannabinoids [Munson et al., 1975], it is now known that cannabinoids can inhibit tumor cell growth by modulating different cell signaling pathways in diverse cancer cells, such as lymphoma, prostate cancer, breast cancer, pancreatic cancer, and skin cancer cells [Carracedo et al., 2006a; Casanova et al., 2003; Gustafsson et al., 2006; Ligresti et al., 2006; Sarfaraz et al., 2006]. In cancers of the gastrointestinal tract, cannabinoids inhibit cancer cell growth, migration, and induce apoptosis in colorectal cancer cells [Ligresti et al., 2003; Joseph et al., 2004; Patsos et al., 2005; Greenhough et al., 2007].

Although the antineoplastic activity of cannabinoids has been reported in a wide spectrum of cancer cells, this effect on human gastric cancer cells has been investigated in only a few reports. Recent studies pointed that Δ^9-tetrahydrocannabinol or anandamide inhibited gastric cancer cell proliferation [Ligresti et al., 2006; Miyato et al., 2009]. However, the mechanism leading to the induction of apoptosis and decreasing the invasiveness of gastric cancer cells exposed to cannabinoids has not been clarified.

To better understand the inhibitory mechanism of cannabinoids on gastric cancer progression, we studied whether a cannabinoid agonist inhibits human gastric cancer cell proliferation and induces cancer cell apoptosis. We also studied whether a cannabinoid agonist can inhibit invasions by human gastric cancer cells.

Grant sponsor: Korean Government; Grant number: KRF-2008-331-E00102; Grant sponsor: Catholic Cancer Center of Korea.

*Correspondence to: Dr. 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 137-701, Korea.
E-mail: parkjerry@catholic.ac.kr

Received 17 April 2009; Accepted 19 January 2010 • DOI 10.1002/jcb.22540 • © 2010 Wiley-Liss, Inc.
Published online 24 March 2010 in Wiley InterScience (www.interscience.wiley.com).

MATERIALS AND METHODS

DRUGS

The mixed CB_1/CB_2 agonist (R)-(+)-WIN 55,212-2 ((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate, $C_{27}H_{26}N_2O_3CH_3SO_3H$), CB_1-selective receptor antagonist AM251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3 carboxamide, $C_{22}H_{21}Cl_2IN_4O$), and CB_2-selective receptor antagonist AM630 (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl(4-methoxyphenyl) methanone, $C_{23}H_{25}IN_2O_3$) were purchased from Tocris Bioscience (Bristol, UK). Dimethyl sulfoxide (DMSO) was purchased from Sigma (St. Louis, MO).

CELL CULTURE

The human gastric cancer cell lines AGS and MKN-1 as well as additional human colorectal carcinoma cell line HT-29 were obtained from Korean Cell Line Bank (Seoul, Korea). RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were purchased from WelGENE (Daegu, Korea), and penicillin–streptomycin was purchased from Sigma. AGS and MKN-1 cells were cultured in RPMI 1640 medium, HT-29 cell was cultured in DMEM, and all cells were 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.

MTT ASSAY

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to determine cell viability. WIN 55,212-2, AM251, and AM630, all of which were dissolved in DMSO, were added to the cell culture medium with serum. The final concentration of DMSO was 0.1% (v/v) for each treatment. Five thousand cells were seeded in 96-well plates and stimulated with different concentrations of the agents indicated, and control cells were treated with an equivalent amount of vehicle (DMSO). After incubation for the specified times at 37°C in a humidified incubator, MTT reagent (50 μl, 2 mg/ml in phosphate-buffered saline (PBS)) (Sigma) was added to each well and incubated for 3 h. After incubation, the MTT solution was removed from the wells by aspiration, and the formazan crystals were dissolved in DMSO (100 μl). Absorbance was recorded on a microplate reader (Spectra Max 250, Molecular Devices, Sunnyvale, CA) at 550 nm. Six wells were used for each treatment condition.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION ANALYSIS (RT-PCR)

RNA was isolated using Trizol reagent (Sigma). cDNA was obtained using *amfiRivert* cDNA synthesis Premix (GenDEPOT, Barker, TX). The following sense and antisense primers were used to amplify human CB_1 (CGTGGGCAGCCTGTTCCTCA and CATGCGGGCTTGGTCTGG, 408-bp product); human CB_2 (CGCCGGAAGCCCTCATACC and CCTCATTCGGGCCATTCCTG, 522-bp product); human vascular endothelial growth factor-A (VEGF-A, GGGGGATCCGCCTCCGAAACCATGAACTT and CCCGAATTCTCCTGGTGAGAGATCTGGTT, 786-bp product); and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, AATGCATCCTGCACCACCAA and GTAGCCATATTCATTGTCATA, 520-bp product). PCR reactions were performed using the following parameters: 95°C for 5 min, 94°C for 30 s, 57°C CB_1, 57°C CB_2 or 60°C VEGF-A or 55°C GAPDH for 30 s, and 72°C CB_1, 72°C CB_2 for 1 min or VEGF-A and GAPDH for 30 s, followed by a final extension step of 72°C for 5 min. The number of cycles (37 cycles for CB_1 and CB_2, 35 cycles for VEGF-A, 30 cycles for GAPDH) was adjusted to allow detection in the linear range. Finally, PCR products were separated on 1.0% agarose gels.

TISSUE PROTEIN PREPARATION

The rat brain and spleen, mouse brain and spleen were quickly dissected and homogenized in lysis buffer (Cell Signaling Technology, Beverly, MA) supplemented with protease inhibitors (Sigma) on ice. Tissue homogenates were disrupted on ice for 15 min. Later, the lysates were centrifuged at 14,000*g* for 15 min at 4°C. The supernatants were used for Western blot analysis.

CELL PROTEIN PREPARATION

The cells were incubated with different concentrations of the agents indicated or an equivalent amount of DMSO for the times indicated. After incubation, cells were washed, harvested, disrupted on ice for 5 min using lysis buffer (Cell Signaling Technology) supplemented with protease inhibitors (Sigma), and centrifuged at 14,000*g* for 15 min at 4°C. The supernatants were used for Western blot analysis.

WESTERN BLOT ANALYSIS

The cell or tissue protein concentration was determined using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein were separated by 10% sodium dodecyl sulfate polyacrylamide electrophoresis (SDS–PAGE) and transferred onto nitrocellulose membranes, immunoblotted with specific primary and secondary antibodies, and visualized using a West-Q Chemiluminescent Substrate Plus Kit (GenDEPOT). CB_1 and CB_2 antibodies were from Cayman (Ann Arbor, MI). Antibodies to activated (cleaved) caspase-3, MMP-2, MMP-7, MMP-9, and p8 were from Abcam (Cambridge, UK). Antibodies to phospho-AKT (Ser473) and AKT were from Cell Signaling Technology. Actin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

FLOW CYTOMETRY

The phycoerythrin (PE)-annexin V apoptosis detection kit was from BD Biosciences (Bedford, MA) and 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 24 h. The cells were then washed twice with cold PBS and resuspended cells 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, San Jose, CA).

MATRIGEL MIGRATION AND INVASION ASSAYS

Cell migration and invasion assays were performed in 24-well plates containing either 8 μm pore size BioCoat control inserts (migration assays) or Matrigel-coated inserts (28.4 μg Matrigel, invasion assays) according to the manufacturer's instructions (BD Biosciences). Briefly, 2.5×10^5 cells in 0.5 ml of serum-free RPMI 1640 culture medium were added to each insert and WIN 55,212-2 (0.1, 1, or 2 μM) or the equivalent amount of DMSO was added. To address the role of CB_1 and CB_2, cells were pretreated for 1 h with 1 μM of AM251, AM630, or equivalent amount of DMSO, followed by incubation with 2 μM WIN 55,212-2 or DMSO for 48 h. RPMI 1640 containing 10% FBS was used as a chemoattractant in the companion plate. The cells were incubated in a humidified incubator at 37°C in 5% CO_2 for 48 h, and after the noninvading cells on the upper surface of the membrane were removed with a cotton swab, viable cells on the lower surface of membrane were stained with crystal violet (Sigma). The chambers were washed twice with water and allowed to air dry. The membranes were then removed and mounted in immersion oil, and the number of invading cells was counted in five microscopic fields (200×) per membrane. Invasion is expressed as the invasion index, which is calculated as the number of cells that invaded through the Matrigel-coated inserts divided by the number of cells that migrated through the uncoated control inserts with equal treatment ([invasion/migration]×100%).

IMMUNOCYTOCHEMISTRY

The cells were treated with WIN 55,212-2 (1 or 10 μM) or an equivalent amount of DMSO for 48 h. The cells were washed, harvested, centrifuged at 4°C for 10 min at 1,500*g*, and fixed in periodate-lysine-2% paraformaldehyde. The resuspended aliquots were cytocentrifuged (Cytospin 2, Shandon, Frankfurt, Germany) onto microscope slides for 5 min at 500*g* and processed for immunocytochemistry. After endogenous peroxidase quenching and serum blocking, microscope slides were incubated with antibodies to cleaved caspase-3 (Abcam) at 4°C overnight. The slides were washed, incubated with peroxidase-conjugated donkey anti-rabbit IgG Fab fragment (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h, and then incubated with peroxidase substrate solution (0.05% DAB and 0.01% H_2O_2) for 5 min at room temperature. Slides were sealed with coverslips, and observations and photography were performed using an Axio Imager A1 (Zeiss, Germany).

STATISTICAL ANALYSIS

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

RESULTS

HUMAN GASTRIC CANCER CELL LINES EXPRESSED THE CANNABINOID RECEPTORS

We examined the expression of the cannabinoid receptors, CB_1 and CB_2, in human gastric cancer cell lines. HT-29 cell, rat brain and spleen, mouse brain and spleen were used as appropriate positive controls for cannabinoid receptors expression. RT-PCR (Fig. 1A) and Western blot analysis (Fig. 1B,C) showed that both human gastric cancer cell lines AGS and MKN-1 expressed the CB_1 and CB_2.

WIN 55,212-2 INHIBITED THE PROLIFERATION OF HUMAN GASTRIC CANCER CELLS AND THIS EFFECT OCCURRED THROUGH THE CB_1 RECEPTOR

To evaluate the effect of WIN 55,212-2 on viability of the human gastric cancer cell lines, we treated AGS and MKN-1 cells with

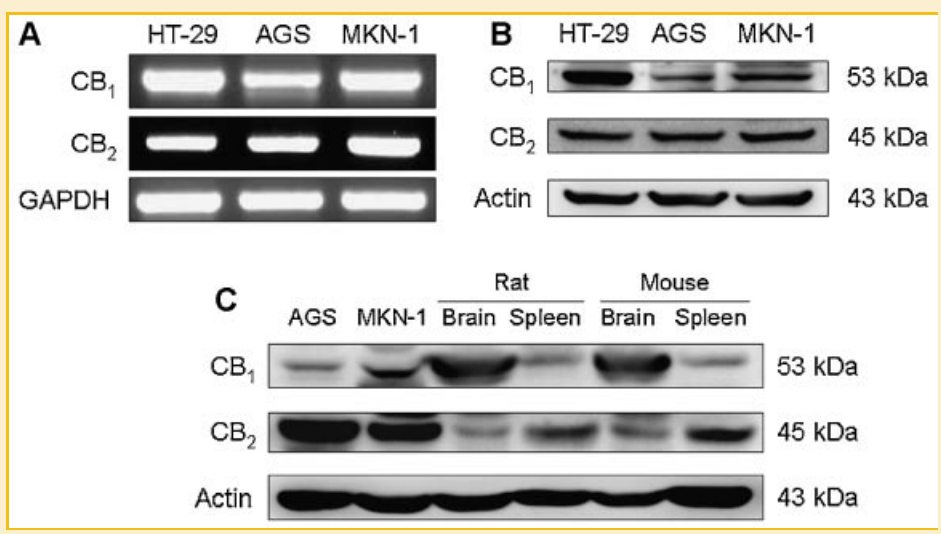

Fig. 1. The cannabinoid receptors expressed in human gastric cancer cell lines. A: RT–PCR analysis of the cannabinoid receptors expressed in gastric cancer cell lines AGS, MKN-1, and colorectal carcinoma cell line HT-29. B: Western blot analysis of the cannabinoid receptors expressed in AGS, MKN-1, and HT-29 cells. HT-29 cell was taken as positive control for the cannabinoid receptors expression. C: Western blot analysis of the cannabinoid receptors expressed in AGS, MKN-1, rat brain and spleen, mouse brain and spleen. Rat brain and spleen, mouse brain and spleen were taken as positive controls for the cannabinoid receptors expression. GAPDH and actin were used as loading control. Here, representative images from three independent experiments with similar results are shown.

different concentrations of WIN 55,212-2 for 72 h. As shown in Figure 2A, WIN 55,212-2 dose-dependently decreased cell survival. When the concentration of WIN 55,212-2 was increased to 2.5 μM, percentages of surviving cells relative to control values were 72.7 ± 1.8% in AGS cells ($P < 0.01$) and 78.6 ± 1.7% in MKN-1 cells ($P < 0.01$). Increasing the concentration of WIN 55,212-2 to 10 μM decreased the percentages of surviving cells to 15.6 ± 0.4% in AGS cells ($P < 0.001$) and 20.4 ± 0.5% in MKN-1 cells ($P < 0.001$).

We next examined whether WIN 55,212-2 can reduce human gastric cancer cell survival through the CB_1 or CB_2. As shown in Figure 2B, the reduction in cell survival induced by WIN 55,212-2

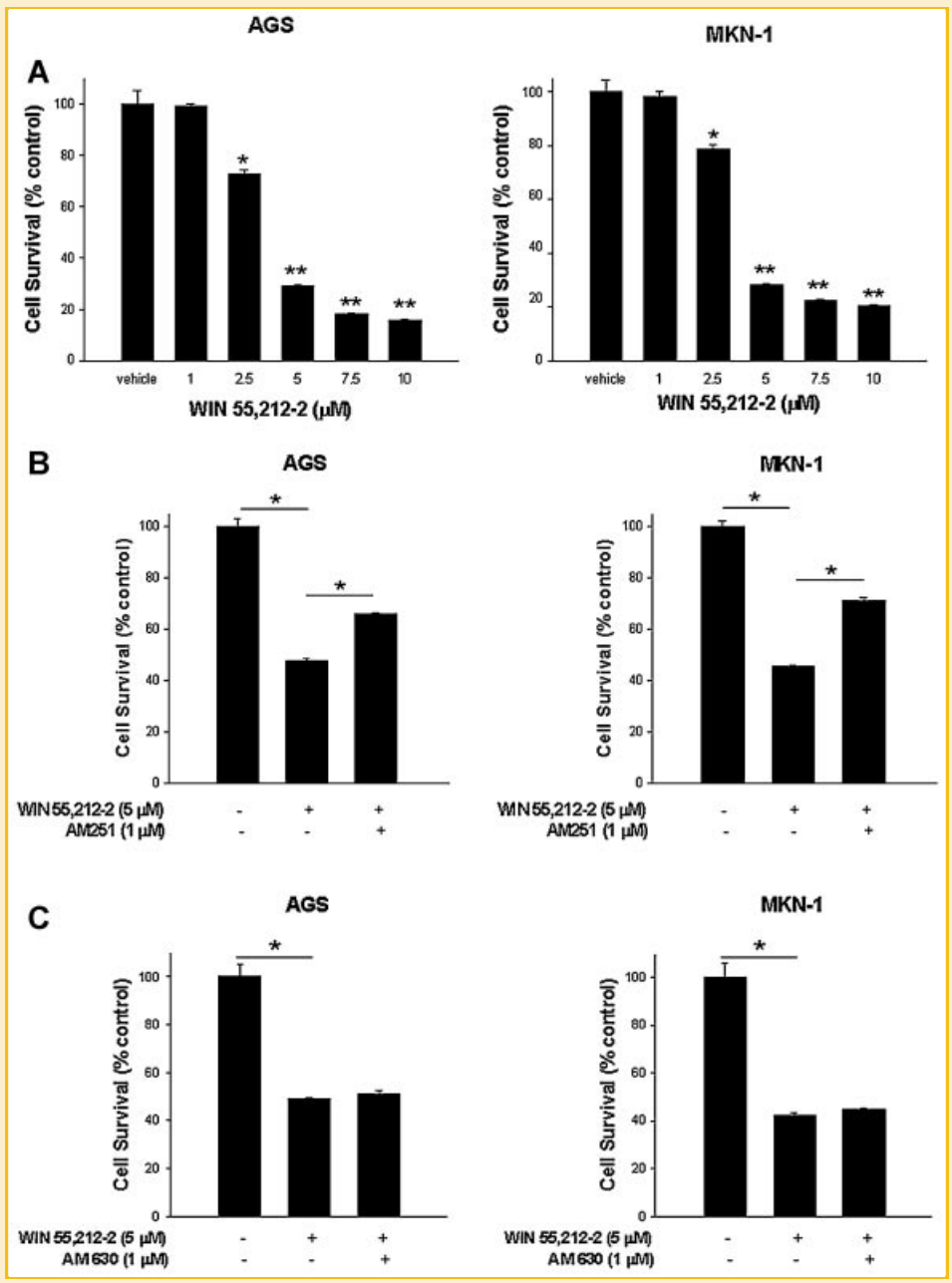

Fig. 2. Effect of WIN 55,212-2 on human gastric cancer cell proliferation. A: AGS and MKN-1 cells were treated with WIN 55,212-2 (1, 2.5, 5, 7.5, and 10 μM) or equivalent DMSO as indicated for 72 h. Cell survival was determined using the MTT assay. Results are shown as percentage cell survival relative to controls. The data are expressed as mean ± SEM of six experiments. Statistical analyses were conducted by comparisons with vehicle values ($^{*}P < 0.01$, $^{**}P < 0.001$). B: AGS and MKN-1 cells were pretreated for 1 h with 1 μM of AM251 or equivalent DMSO, followed by incubation with 5 μM WIN-55,212-2 or equivalent DMSO for 48 h. Cell survival was determined using the MTT assay. Results are shown as percentage cell survival relative to controls. The data are expressed as mean ± SEM of six experiments. Statistical analyses were conducted by comparisons with vehicle or WIN 55,212-2 alone values ($^{*}P < 0.001$). C: AGS and MKN-1 cells were pretreated for 1 h with 1 μM of AM630 or equivalent DMSO, followed by incubation with 5 μM WIN-55,212-2 or equivalent DMSO for 48 h. Cell survival was determined using the MTT assay. Results are shown as percentage cell survival relative to controls. The data are expressed as mean ± SEM of six experiments ($^{*}P < 0.001$).

was rescued partially by blocking the CB_1 receptor with CB_1 receptor antagonist AM251. The percentages of surviving cells relative to the control values were $47.7 \pm 0.9\%$ for WIN 55,212-2 treatment and $66.0 \pm 0.4\%$ for WIN 55,212-2 treatment after AM251 pretreatment in AGS cells ($P < 0.001$); the respective values were $45.6 \pm 0.6\%$ and $71.0 \pm 1.3\%$ in MKN-1 cells ($P < 0.001$). By contrast, cell survival did not be rescued by WIN 55,212-2 in the presence of CB_2 receptor antagonist AM630 (Fig. 2C).

WIN 55,212-2 INDUCED APOPTOSIS IN HUMAN GASTRIC CANCER CELLS

To ascertain whether WIN 55,212-2 can reduce human gastric cancer cell survival by inducing apoptosis, we treated human gastric cancer cells with WIN 55,212-2 and measured the proportion of apoptotic cells by flow cytometry. Figure 3A shows the flow cytometry results with dual parameters, including PE-annexin V and 7-AAD. In AGS cells treated with vehicle, 7.5% of the cells were PE-annexin V positive/7-AAD negative, and 15.5% of cells were both PE-annexin V and 7-AAD positive. In cells treated with 1 μM of WIN 55,212-2, 8.5% of cells were PE-annexin V positive/7-AAD negative, and 16.9% of cells were both PE-annexin V and 7-AAD positive. In cells treated with 5 μM of WIN 55,212-2, 10.2% of cells were PE-annexin V positive/7-AAD negative and 14.0% of cells were both PE-annexin V and 7-AAD positive. The percentages of PE-annexin V positive/7-AAD-negative and double-positive cells increased to 26.1% and 28.6%, respectively, after treatment with 10 μM of WIN 55,212-2 for 24 h.

In MKN-1 cells treated with vehicle, 5.3% of cells were PE-annexin V positive/7-AAD negative, and 7.3% of cells were both PE-annexin V and 7-AAD positive. In cells treated with 1 μM of WIN 55,212-2, 3.7% of cells were PE-annexin V positive/7-AAD negative and 8.6% of cells were both PE-annexin V and 7-AAD positive. In cells treated with 5 μM of WIN 55,212-2, 6.9% of cells were PE-annexin V positive/7-AAD negative and 9.6% of cells were both PE-annexin V and 7-AAD positive. The percentages of PE-annexin V positive/7-AAD-negative and double-positive cells increased to 20.7% and 18.5%, respectively, after treatment with 10 μM of WIN 55,212-2 for 24 h.

The cleaved activated form of caspase-3, a key effector of apoptosis, was detected by Western blotting (Fig. 3B) and immunocytochemistry (Fig. 3C). Treatment of AGS and MKN-1 cells with different concentrations WIN 55,212-2 for 24 h led to a dose-dependent increase in the cleaved of caspase-3 protein products (Fig. 3B). As shown in Figure 3B, cleaved caspase-3 level was increased after treatment with WIN 55,212-2 at a concentration 5 μM in AGS and MKN-1 (mean expression levels relative to vehicle, $257.6 \pm 16.6\%$ and $141.2 \pm 11.1\%$, respectively; both $P < 0.05$). As shown in Figure 3C, treatment of AGS and MKN-1 cells with WIN 55,212-2 at concentrations of 1 and 10 μM for 48 h increased the cleaved of caspase-3, as determined by immunostaining.

WIN 55,212-2 TREATMENT DOWN-REGULATED PHOSPHO-AKT (Ser473) IN HUMAN GASTRIC CANCER CELLS

We next examined the effect of WIN 55,212-2 on protein expression of p8 and AKT. As shown in Figure 4, phospho-AKT (Ser473) protein level was decreased after treatment with WIN 55,212-2 at a concentration of 5 μM in AGS and MKN-1 (mean expression levels relative to vehicle, $73.6 \pm 2.0\%$ and $69.5 \pm 3.1\%$, respectively; both $P < 0.01$).

WIN 55,212-2 INHIBITED HUMAN GASTRIC CANCER CELL INVASION AND THIS EFFECT OCCURRED THROUGH THE CANNABINOID RECEPTORS

We examined the effect of WIN 55,212-2 on human gastric cancer cell invasion after treatment with WIN 55,212-2 at concentrations of 0.1, 1, and 2 μM for 48 h. As shown in Figure 5A, in AGS cells, the invasion index relative to the vehicle was $82.0 \pm 2.7\%$ in cells treated with 0.1 μM of WIN 55,212-2 ($P < 0.01$), $73.6 \pm 3.7\%$ in cells treated with 1 μM of WIN 55,212-2 ($P < 0.001$), and $57.5 \pm 7.2\%$ in cells treated with 2 μM of WIN 55,212-2 ($P < 0.001$). In MKN-1 cells, the invasion index relative to vehicle was $76.5 \pm 5.5\%$ in cells treated with 1 μM of WIN 55,212-2 ($P < 0.01$) and $56.0 \pm 4.4\%$ in cells treated with 2 μM of WIN 55,212-2 ($P < 0.001$).

Next, we investigated which cannabinoid receptor was involved in the gastric cancer cell invasiveness. As shown in Figure 5B, the reduction in cell invasion induced by WIN 55,212-2 was rescued by treating AM251 and AM630 either simultaneously or separately. In AGS, inhibition of cell invasion by WIN 55,212-2 was rescued using AM251, AM630, or both AM251 and AM630 (invasion indices relative to vehicle, $99.1 \pm 3.1\%$, $101.4 \pm 3.1\%$, and $100.3 \pm 2.4\%$, respectively; all $P < 0.001$). These rescue was also observed in MKN-1 cells with AM251, AM630, and both with AM251 and AM630 (invasion indices relative to vehicle, $99.6 \pm 2.1\%$, $103.8 \pm 3.3\%$, and $109.4 \pm 4.7\%$, respectively; all $P < 0.001$, respectively).

WIN 55,212-2 DECREASED THE EXPRESSION OF MMPs AND VEGF-A IN HUMAN GASTRIC CANCER CELLS AND THIS EFFECT OCCURRED THROUGH THE CANNABINOID RECEPTORS

Because MMPs and VEGF-A play important roles in tumor invasion and metastasis, we analyzed the changes in MMP-2, MMP-7, MMP-9, and VEGF-A expression after treatment with WIN 55,212-2. As shown in Figure 6A, MMP-2 protein level decreased after treatment with WIN 55,212-2 at a concentration as low as 0.1 μM in AGS cells (mean expression levels relative to vehicle, $90.1 \pm 2.9\%$; $P < 0.05$) and 1 μM in MKN-1 cells (mean expression level relative to vehicle, $79.7 \pm 1.5\%$; $P < 0.001$). By contrast, MMP-7 and MMP-9 protein levels did not change. Next, we investigated the role of cannabinoid receptors in MMP-2 pathway. As shown in Figure 6B, the reduction in MMP-2 protein level induced by WIN 55,212-2 was rescued by blocking with AM251 and AM630 either alone or together. In AGS cells, inhibited MMP-2 protein level by WIN 55,212-2 was rescued with using AM251, AM630, and both AM251 and AM630 (invasion indices relative to vehicle, $118.5 \pm 2.8\%$, $118.2 \pm 5.9\%$, and $109.4 \pm 3.9\%$, respectively; all $P < 0.001$). In MKN-1 cells, the change of MMP-2 protein level was also observed with AM251, AM630, and both (invasion indices relative to vehicle, $112.7 \pm 4.8\%$, $112.6 \pm 1.6\%$, and $114.3 \pm 5.1\%$, respectively; all $P < 0.001$).

As shown in Figure 7, VEGF-A mRNA level decreased after treatment with 2 μM of WIN 55,212-2 in AGS cells (mean expression level relative to vehicle, $28.8 \pm 5.0\%$; $P < 0.001$) and with 2 μM WIN 55,212-2 in MKN-1 cells (mean expression level relative to vehicle, $49.3 \pm 3.6\%$; $P < 0.001$). Next, we investigated the role of

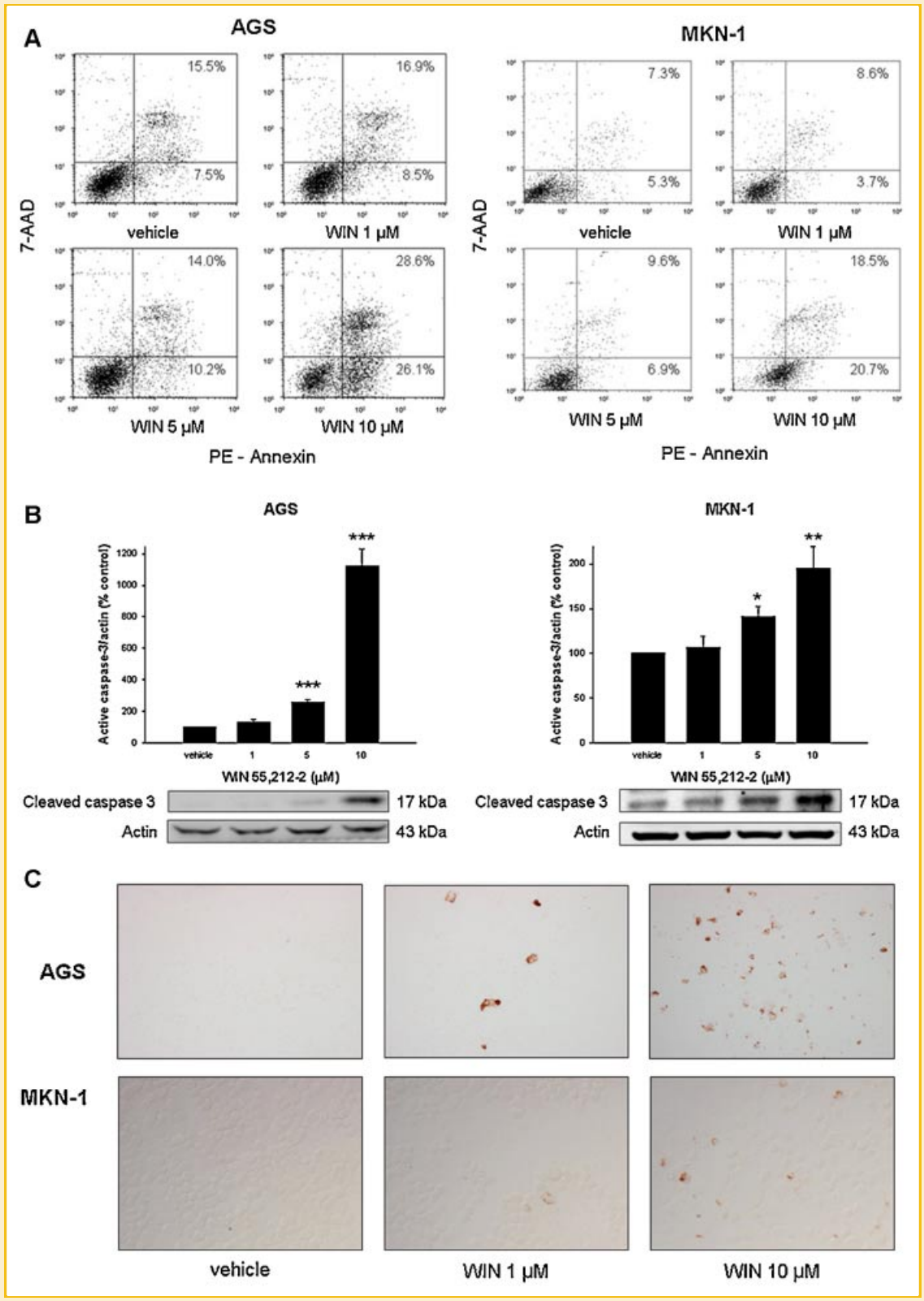

Fig. 3. WIN 55,212-2 induced apoptosis in human gastric cancer cells. A: Quantification of apoptosis by flow cytometry, with PE-annexin V staining. Here, 7-AAD has been employed as a plasma membrane-impermeable vital dye, which allows for distinguishing dying and dead cells in the biparametric analysis with annexin V. Therefore, 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 24 h. Data from representative experiments repeated thrice with similar results. B: Western blot analysis of cleaved caspase-3. AGS and MKN-1 cells were treated with WIN 55,212-2 (1, 5, and 10 μM) or equivalent DMSO for 24 h. Actin was used as a loading control. Densitometric analyses were obtained from four independent experiments. Data reflect the mean ± SEM. Statistical analyses were conducted by comparisons with vehicle values ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). C: Immunocytochemistry for cleaved caspase-3 in gastric cancer cells. AGS and MKN-1 cells were treated with WIN 55,212-2 (1 and 10 μM) or equivalent DMSO for 48 h. Data from representative experiments repeated thrice with similar results (original magnification, 200×).

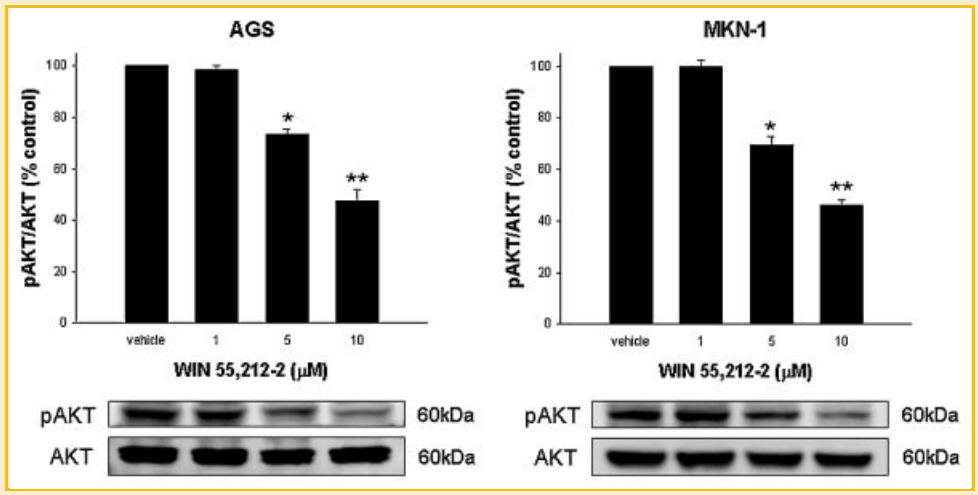

Fig. 4. Effect of WIN 55,212-2 on the expression of AKT in human gastric cancer cells. Western blot analysis of AKT and phospho-AKT (Ser473) expressed in AGS and MKN-1 cells. AGS and MKN-1 cells were treated with WIN 55,212-2 (1, 5, and 10 μM) or equivalent of DMSO for 24 h. Densitometric analyses were obtained from three independent experiments. Data reflect the mean ± SEM. Statistical analyses were conducted by comparisons with vehicle values ($^{*}P < 0.01$, $^{**}P < 0.001$).

cannabinoid receptors in VEGF-A expression. The reduction in VEGF-A mRNA level induced by WIN 55,212-2 was rescued by blocking with AM251, AM630, and both AM251 and AM630. In AGS cells, inhibited VEGF-A mRNA level by WIN 55,212-2 was rescued with AM251, AM630, and both AM251 and AM630 (invasion indices relative to vehicle, 98.3 ± 3.4%, 92.9 ± 2.9%, and 92.5 ± 3.4%, respectively; all $P < 0.001$). In MKN-1 cells, the change of VEGF-A mRNA level was also observed with AM251, AM630, and both AM251 and AM630 (invasion indices relative to vehicle, 100.5 ± 0.8%, 99.1 ± 1.4%, and 98.9 ± 5.0%, respectively; all $P < 0.001$).

DISCUSSION

This study showed that the cannabinoid agonist inhibited the proliferation of human gastric cancer cells and that this effect was mediated partially by the CB_1 receptor. We also found that the cannabinoid agonist induced apoptosis and down-regulated phospho-AKT expression in human gastric cancer cells. Cannabinoid treatment inhibited invasion of gastric cancer cells, down-regulated MMP-2 and VEGF-A expression, and these effects occurred through the cannabinoid receptors CB_1 and CB_2.

The presence of CB_1 receptors was reported in the parietal cells of human gastric mucosa and in human gastric cancer cell HGC-27 [Pazos et al., 2008; Miyato et al., 2009]. A previous study reported that there was no CB_1 and CB_2 using the RT-PCR method in AGS cell line, which is the same cell line used in this study [Ligresti et al., 2006]. On the contrary, we could confirm the expression of the cannabinoid receptors, CB_1 and CB_2, in AGS and MKN-1 gastric cancer cell lines by RT-PCR and Western blot analysis.

We also evaluated the effects on cell viability of the cannabinoid agonist WIN 55,212-2 in human gastric cancer cell lines. As shown in Figure 2A, AGS and MKN-1 cells treated with WIN 55,212-2 (1, 2.5, 5, 7.5, and 10 μM) showed a dose-dependent decrease in cell survival, which was significant in AGS and MKN-1 cells tested at concentration of 2.5 μM and above. Although a different cannabinoid agonist, Δ^9-tetrahydrocannabinol was used in a previous study of a colon cancer cell line, the concentration of this agonist also reduced cell survival at similar doses [Greenhough et al., 2007]. WIN 55,212-2 at a concentration of 1 μM had no discernable effect on cell proliferation in our study. This result is similar to a previous result showing that cell viability did not decrease in hepatoma HepG2 cells incubated with 1 μM of WIN 55,212-2 [Giuliano et al., 2009]. As shown in Figure 2B, the reduction in cell survival induced by WIN 55,212-2 was rescued partially by blocking the CB_1 receptor with AM251, but not by blocking the CB_2 receptor antagonist AM630 (Fig. 2C). This might indicate that the CB_1 receptor is involved in inhibition of gastric cancer cell growth. However, the reversal of cell survival was incomplete and the percentage of surviving cells did not recover to the baseline value. Possible explanations are as follows. First, AM251 may be toxic to gastric cancer cell lines. We assessed cell viability after treatment with AM251 alone, which decreased cell number in a dose-dependent manner (data are not shown). This finding is consistent with previous studies [Fogli et al., 2006; Lee et al., 2008].

Second, the concentrations needed for WIN55,212-2 to inhibit proliferation were too high, considering that the Ki for CB_1 and CB_2 receptors for this compound in the nmolar range. A possible explanation for this is that the compound is inhibiting proliferation in part via CB_1 receptors and in part via other mechanisms, as shown by the fact that the authors can only reverse in part its effect using a relatively high concentration of AM251 (Fig. 2B) Experimental data suggest that the WIN 55,212-2 at high μM concentrations possibly interact with CB_2 receptors and transient receptor potential vanilloid 1 (TRPV1) receptors, which may induce cellular apoptosis [Contassot et al., 2004; Akopian et al., 2009]. However, our study revealed that the CB_2 receptor was not involved in the cell survival, as shown in Figure 2C, which could explain the importance of CB_1 receptor pathway in determining the action of cannabinoid agonist.

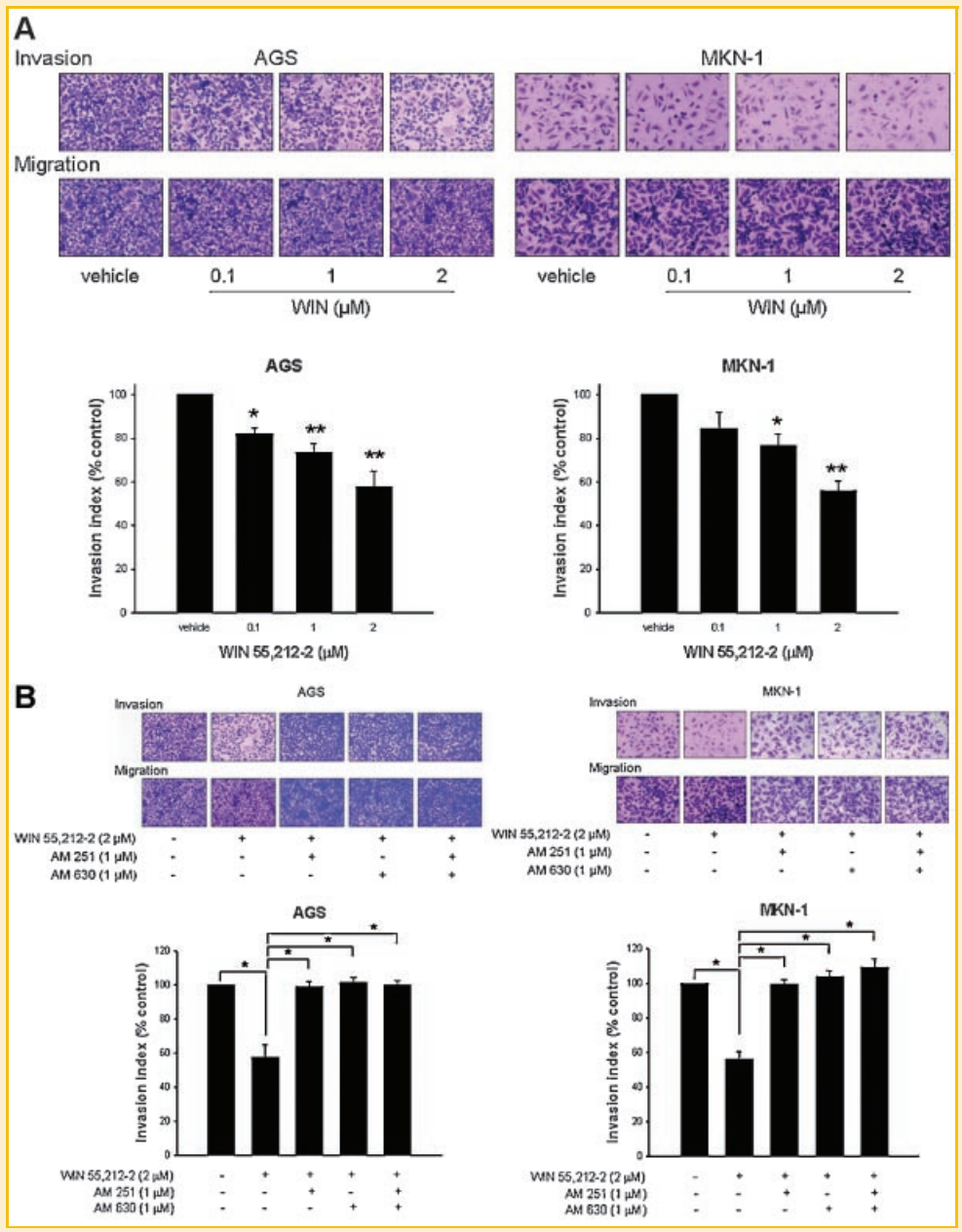

Fig. 5. Effect of WIN 55,212-2 on cell invasion in human gastric cancer cells. A: Effect of WIN 55,212-2 on invasion of AGS and MKN-1 cells. Cell migration and invasion assays were conducted as described in the Materials and Methods Section. AGS and MKN-1 cells were treated with WIN 55,212-2 (0.1, 1, and 2 μM) or equivalent DMSO for 48 h. Results are obtained from four independent experiments. Data reflect the mean ± SEM. Statistical analyses were conducted by comparisons with vehicle values ($^{*}P < 0.01$, $^{**}P < 0.001$) (original magnification, 200×). B: Effect of a 1-h pretreatment with the AM251 (1 μM), AM630 (1 μM) on invasiveness of cells that were treated with WIN 55,212-2 (2 μM) or equivalent DMSO for 48 h. Results are obtained from three independent experiments. Data reflect the mean ± SEM. Statistical analyses were conducted by comparisons with vehicle or WIN 55,212-2 alone values ($^{*}P < 0.001$) (original magnification, 200×).

Other reports suggest that other pathways might be responsible for the cannabinoid effects [Hinz et al., 2004; Eichele et al., 2006; Fogli et al., 2006], and this was also viewed by the data of Ligresti et al. [2006], which suggested non-CB_1 and non-CB_2 dependent pathways for THC inhibition of proliferation. Furthermore, it is possible that high concentrations of WIN55,212-2 are needed because the expression levels of CB_1 are relatively low as shown in Figure 1C and depending on cell passages, culture media, etc. as discussed by the previous article [Ligresti et al., 2006].

Experimental reports have suggested that cannabinoids could inhibit cell growth and induce apoptosis by modulating several signaling pathways in cancer cells. Recently, accumulation of de novo synthesized ceramide, up-regulation of the stress protein p8 and inhibition of AKT have been proposed to mediate cannabinoid antitumoral action in rhabdomyosarcoma, pancreatic cancer, and gliomas tumor cells [Carracedo et al., 2006a,b; Oesch et al., 2009]. We could show the inhibition of phospho-AKT also in the gastric cancer cells. AKT phosphorylation is well documented as one of the

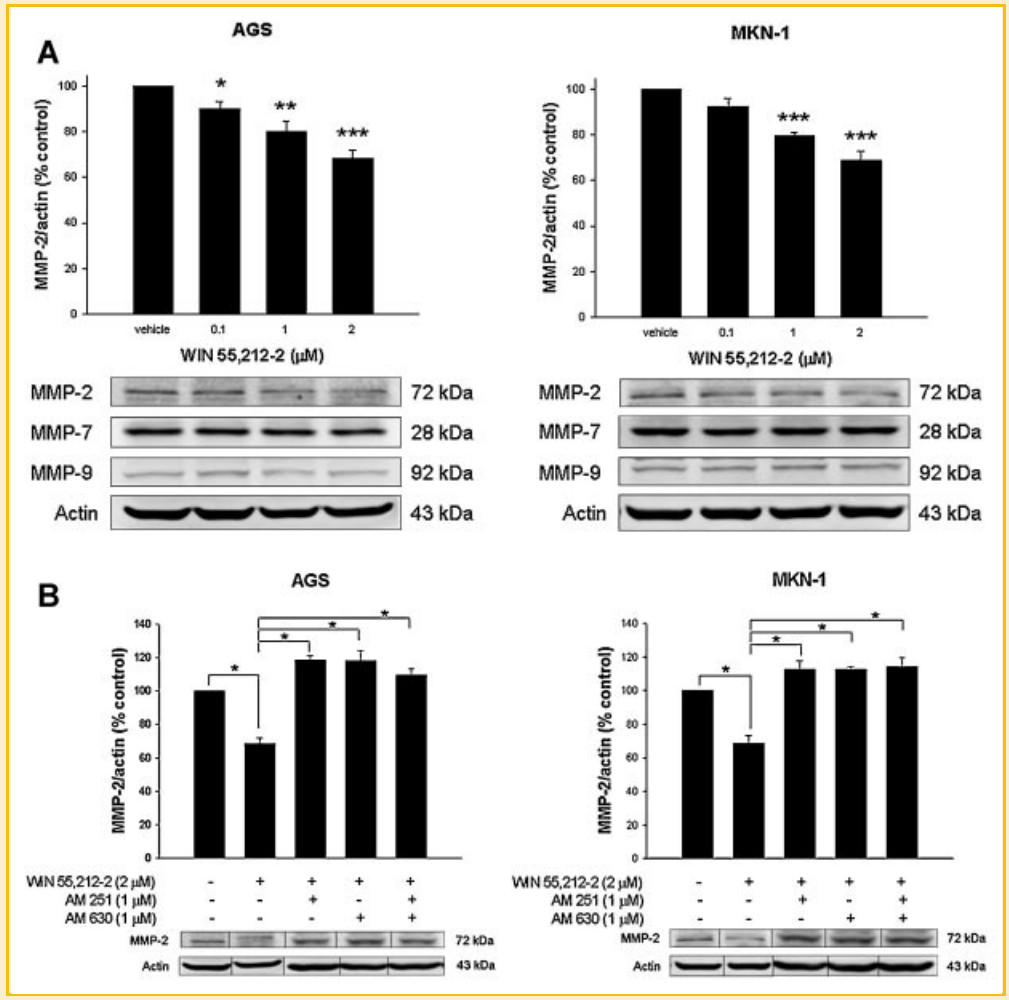

Fig. 6. Effect of WIN 55,212-2 on the expression of MMPs in human gastric cancer cells. A: Western blot analysis of MMP-2, MMP-7, and MMP-9. AGS and MKN-1 cells were treated with WIN 55,212-2 (0.1, 1, and 2 μM) or equivalent DMSO for 48 h. Actin was used as a loading control. Densitometric analyses were obtained from four independent experiments. Data reflect the mean ± SEM. Statistical analyses were conducted by comparisons with vehicle values ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). B: Western blot analysis of MMP-2. Effect of a 1-h pretreatment with the AM251 (1 μM), AM630 (1 μM) on MMPs levels of cells incubated with WIN 55,212-2 (2 μM) or equivalent DMSO for 48 h. Actin was used as a loading control. Densitometric analyses were obtained from three independent experiments. Data reflect the mean ± SEM. Statistical analyses were conducted by comparisons with vehicle or WIN 55,212-2 alone values ($^{*}P < 0.001$).

signaling pathways involved in controlling cell survival, apoptosis, and migration [Brunet et al., 1999; Cardone et al., 1998; Cheng et al., 2005]. Cannabinoids down-regulate phosphoinositide 3-kinase (PI3K)/AKT signaling pathway leading to the induction of apoptosis [Ellert-Miklaszewska et al., 2005] Thus, this result suggested that AKT phosphorylation could mediate cannabinoid antitumoral action in the gastric cancer. However, we could not detect p8 in the gastric cancer cell lines with or without WIN 55,212-2. There has been no report on the presence of p8 in the gastric cancer. Thus, we postulated that gastric cancer cell lines we used may not have the p8 pathway or that the cannabinoid may not affect the p8 pathway.

Although the apoptotic and antiproliferative actions of cannabinoids have been studied, there are only a few reports on the anti-invasive properties of the cannabinoids [Casanova et al., 2003; Portella et al., 2003; Nithipatikom et al., 2004, 2005; Grimaldi et al., 2006; Blázquez et al., 2008; Ramer and Hinz, 2008]. In our study, WIN 55,212-2 treatment led to significant dose-dependent decreases in cancer cell invasion (Fig. 5A). Cancer cell invasion is one of the crucial events in local spreading, growth, and metastasis of tumors. MMPs are a group of enzymes that exert an important function during tumor invasion and metastasis by degrading extracellular matrix components such as collagens and proteoglycans. The expression and activity of MMPs such as MMP-2, MMP-7, and MMP-9 are increased in almost every type of human cancer including gastric cancer [Egeblad and Werb, 2002]. Furthermore, in cancer cells, overexpression of MMPs is linked closely with invasion and metastasis of malignancy [Rundhaug, 2005]. In particular, MMP-2 expression is up-regulated in almost all human cancers, and the observation for tumor cells expressing MMP-2 provides direct evidence for a role of MMP-2 in tumor progression [Egeblad and Werb, 2002; Overall and Kleifeld, 2006]. In gastric cancer, MMP-2 also plays an important role in tumor invasion and metastasis [Nomura et al., 1996]. Our data showed that WIN 55,212-2 treatment

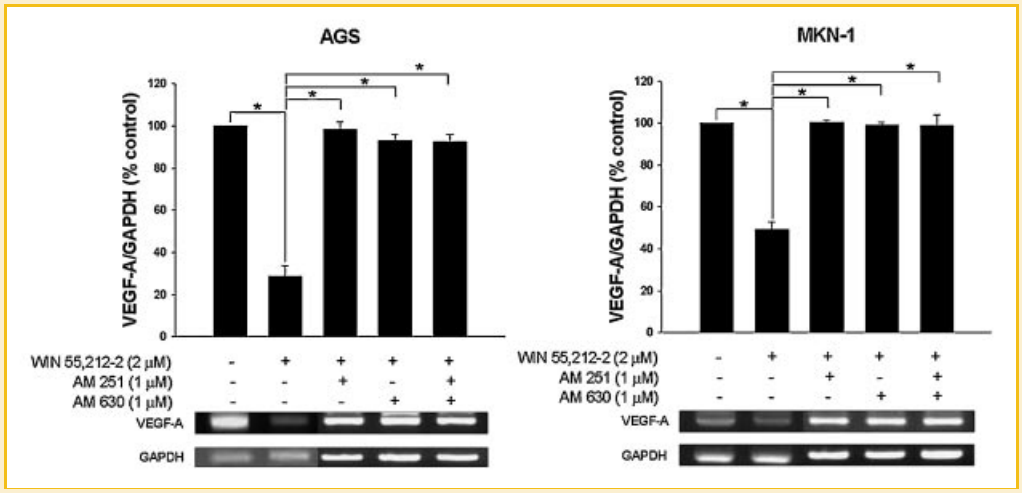

Fig. 7. Effect of WIN 55,212-2 on the expression of VEGF-A in human gastric cancer cells. RT-PCR analysis of VEGF-A mRNA. AGS and MKN-1 cells were 1-h pretreatment with the AM251 (1 μM), AM630 (1 μM) on VEGF-A levels of cells incubated with WIN 55,212-2 (2 μM) or equivalent DMSO for 48 h. GAPDH was used as a loading control. Densitometric analyses were obtained from three independent experiments. Data reflect the mean ± SEM. Statistical analyses were conducted by comparisons with vehicle or WIN 55,212-2 alone values ($^*P < 0.001$).

resulted in the dose-dependent down-regulation of MMP-2 expression in gastric cancer cells, but not in the levels of MMP-7 and MMP-9 protein. This result is similar to that of a previous study on cervical cancer cells [Ramer and Hinz, 2008] and glioma cells [Blázquez et al., 2008]. However, the role of MMPs in tumor invasion is a complex issue, and further studies are needed.

Cancer cells invade either the blood or lymphatic vessels to access the general circulation and establish themselves in other tissues. VEGF and its receptors play the most important role in vessel formation, and VEGF is expressed abundantly by a wide variety of human tumors. Furthermore, VEGF stimulation can enhance the motility of targeted tumor cells in cooperation with MMPs and urokinase plasminogen activator (uPA)-mediated pathways [McCawley and Matrisian, 2001]. Inhibition of VEGF activity by immunoneutralizing antibody has been reported to inhibit primary tumor growth and metastasis in fibrosarcoma [Asano et al., 1995], colon, and gastric carcinoma [Kanai et al., 1998]. VEGF-A is the most prominent and biologically active member of the VEGF family. VEGF-A is accepted as the important molecule in angiogenesis, which is crucial for metastasis in solid tumors. In gastric cancer, the overexpression of VEGF-A is positively correlated with metastasis and prognosis [Saito et al., 1999; Karayiannakis et al., 2002; Kondo et al., 2007]. We found that WIN 55,212-2 treatment down-regulated VEGF-A expression in gastric cancer cells, this effect might be useful in preventing cancer metastasis. This is similar to the observed effects of WIN 55,212-2 in prostate cancer cells [Sarfaraz et al., 2005]. However, tumor cell invasion and metastasis is a complex process, and further studies are needed on the effects of cannabinoids on metastasis in gastric cancer.

In our study, decreased expression of MMP-2 and VEGF-A, which was induced by WIN 55,212-2, was rescued by blocking with AM251, AM630, or both AM251 and AM630. These results could suggest that the CB_1 and CB_2 receptors are involved in inhibition of gastric cancer cell invasion and metastasis. However, further evaluation will be needed to confirm this hypothesis.

We studied the influences of WIN 55,212-2 on human gastric cancer cells in vitro. Clinical relevance will be increased and should be followed by in vivo experiments in tumor xenografts, in which the ability of WIN 55,212-2 to reduce tumor growth, inhibit invasion of tumor cells or reduce tumor angiogenesis is investigated.

In conclusion, we found that the cannabinoid agonist can induce apoptosis and inhibit invasion of human gastric cancer cells. Our results open new possibilities for the use of cannabinoids as a therapy for gastric cancer.

ACKNOWLEDGMENTS

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (KRF-2008-331-E00102) and in part by the Catholic Cancer Center of Korea.

REFERENCES

Akopian AN, Ruparel NB, Jeske NA, Patwardhan A, Hargreaves KM. 2009. Role of ionotropic cannabinoid receptors in peripheral antinociception and antihyperalgesia. Trends Pharmacol Sci 30:79–84.

Asano M, Yukita A, Matsumoto T, Kondo S, Suzuki H. 1995. Inhibition of tumor growth and metastasis by an immunoneutralizing monoclonal antibody to human vascular endothelial growth factor/vascular permeability factor 121. Cancer Res 55:5296–5301.

Blázquez C, Salazar M, Carracedo A, Lorente M, Egia A, González-Feria L, Haro A, Velasco G, Guzmán M. 2008. Cannabinoids inhibit glioma cell invasion by down-regulating matrix metalloproteinase-2 expression. Cancer Res 68:1945–1952.

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. 1999. Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. Cell 96:857–868.

Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC. 1998. Regulation of cell death protease caspase-9 by phosphorylation. Science 282:1318–1321.

Carracedo A, Gironella M, Lorente M, Garcia S, Guzmán M, Velasco G, Iovanna JL. 2006a. Cannabinoids induce apoptosis of pancreatic tumor cells via endoplasmic reticulum stress-related genes. Cancer Res 66:6748–6755.

Carracedo A, Lorente M, Egia A, Blázquez C, García S, Giroux V, Malicet C, Villuendas R, Gironella M, González-Feria L, Piris MA, Iovanna JL, Guzmán M, Velasco G. 2006b. The stress-regulated protein p8 mediates cannabinoid-induced apoptosis of tumor cells. Cancer Cell 9:301–312.

Casanova ML, Blázquez C, Martínez-Palacio J, Villanueva C, Fernández-Aceñero MJ, Huffman JW, Jorcano JL, Guzmán M. 2003. Inhibition of skin tumor growth and angiogenesis in vivo by activation of cannabinoid receptors. J Clin Invest 111:43–50.

Cheng JQ, Lindsley CW, Cheng GZ, Yang H, Nicosia SV. 2005. The Akt/PKB pathway: Molecular target for cancer drug discovery. Oncogene 24:7482–7492.

Contassot E, Tenan M, Schnuriger V, Pelte MF, Dietrich PY. 2004. Arachidonyl ethanolamide induces apoptosis of uterine cervix cancer cells via aberrantly expressed vanilloid receptor-1. Gynecol Oncol 93:182–188.

Di Marzo V, Bifulco M, De Petrocellis L. 2004. The endocannabinoid system and its therapeutic exploitation. Nat Rev Drug Discov 3:771–784.

Egeblad M, Werb Z. 2002. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2:161–174.

Eichele K, Weinzierl U, Ramer R, Brune K, Hinz B. 2006. R(+)-methanandamide elicits a cyclooxygenase-2-dependent mitochondrial apoptosis signaling pathway in human neuroglioma cells. Pharm Res 23:90–94.

Ellert-Miklaszewska A, Kaminska B, Konarska L. 2005. Cannabinoids down-regulate PI3K/Akt and Erk signalling pathways and activate proapoptotic function of Bad protein. Cell Signal 17:25–37.

Fogli S, Nieri P, Chicca A, Adinolfi B, Mariotti V, Iacopetti P, Breschi MC, Pellegrini S. 2006. Cannabinoid derivatives induce cell death in pancreatic MIA PaCa-2 cells via a receptor-independent mechanism. FEBS Lett 580:1733–1739.

Giuliano M, Pellerito O, Portanova P, Calvaruso G, Santulli A, De Blasio A, Vento R, Tesoriere G. 2009. Apoptosis induced in HepG2 cells by the synthetic cannabinoid WIN: Involvement of the transcription factor PPARgamma. Biochimie 91:457–465.

Greenhough A, Patsos HA, Williams AC, Paraskeva C. 2007. 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.

Grimaldi C, Pisanti S, Laezza C, Malfitano AM, Santoro A, Vitale M, Caruso MG, Notarnicola M, Iacuzzo I, Portella G, Di Marzo V, Bifulco M. 2006. Anandamide inhibits adhesion and migration of breast cancer cells. Exp Cell Res 312:363–373.

Gustafsson K, Christensson B, Sander B, Flygare J. 2006. Cannabinoid receptor-mediated apoptosis induced by R(+)-methanandamide and Win 55, 212-2 is associated with ceramide accumulation and p38 activation in mantle cell lymphoma. Mol Pharmacol 70:1612–1620.

Guzmán M. 2003. Cannabinoids: Potential anticancer agents. Nat Rev Cancer 3:745–755.

Hall W, Christie M, Currow D. 2005. Cannabinoids and cancer: Causation, remediation, and palliation. Lancet Oncol 6:35–42.

Hinz B, Ramer R, Eichele K, Weinzierl U, Brune K. 2004. Up-regulation of cyclooxygenase-2 expression is involved in R(+)-methanandamide-induced apoptotic death of human neuroglioma cells. Mol Pharmacol 66:1643–1651.

Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R, Pertwee RG. 2002. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacol Rev 54:161–202.

Joseph J, Niggemann B, Zaenker KS, Entschladen F. 2004. Anandamide is an endogenous inhibitor for the migration of tumor cells and T lymphocytes. Cancer Immunol Immunother 53:723–728.

Kanai T, Konno H, Tanaka T, Baba M, Matsumoto K, Nakamura S, Yukita A, Asano M, Suzuki H, Baba S. 1998. Anti-tumor and anti-metastatic effects of human-vascular-endothelial-growth-factor-neutralizing antibody on human colon and gastric carcinoma xenotransplanted orthotopically into nude mice. Int J Cancer 77:933–936.

Karayiannakis AJ, Syrigos KN, Polychronidis A, Zbar A, Kouraklis G, Simopoulos C, Karatzas G. 2002. Circulating VEGF levels in the serum of gastric cancer patients: Correlation with pathological variables, patient survival, and tumor surgery. Ann Surg 236:37–42.

Kondo K, Kaneko T, Baba M, Konno H. 2007. VEGF-C and VEGF-A synergistically enhance lymph node metastasis of gastric cancer. Biol Pharm Bull 30:633–637.

Lee YM, Uhm KO, Lee ES, Kwon J, Park SH, Kim HS. 2008. AM251 suppresses the viability of HepG2 cells through the AMPK (AMP-activated protein kinase)-JNK (c-Jun N-terminal kinase)-ATF3 (activating transcription factor 3) pathway. Biochem Biophys Res Commun 370:641–645.

Ligresti A, Bisogno T, Matias I, De Petrocellis L, Cascio MG, Cosenza V, D'argenio G, Scaglione G, Bifulco M, Sorrentini I, Di Marzo V. 2003. Possible endocannabinoid control of colorectal cancer growth. Gastroenterology 125:677–687.

Ligresti A, Moriello AS, Starowicz K, Matias I, Pisanti S, De Petrocellis L, Laezza C, Portella G, Bifulco M, Di Marzo V. 2006. Antitumor activity of plant cannabinoids with emphasis on the effect of cannabidiol on human breast carcinoma. J Pharmacol Exp Ther 318:1375–1387.

Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI. 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346:561–564.

McCawley LJ, Matrisian LM. 2001. Tumor progression: Defining the soil round the tumor seed. Curr Biol 11:R25–27.

Miyato H, Kitayama J, Yamashita H, Souma D, Asakage M, Yamada J, Nagawa H. 2009. Pharmacological synergism between cannabinoids and paclitaxel in gastric cancer cell lines. J Surg Res 155:40–47.

Munro S, Thomas KL, Abu-Shaar M. 1993. Molecular characterization of a peripheral receptor for cannabinoids. Nature 365:61–65.

Munson AE, Harris LS, Friedman MA, Dewey WL, Carchman RA. 1975. Antineoplastic activity of cannabinoids. J Natl Cancer Inst 55:597–602.

Nithipatikom K, Endsley MP, Isbell MA, Falck JR, Iwamoto Y, Hillard CJ, Campbell WB. 2004. 2-arachidonoylglycerol: A novel inhibitor of androgen-independent prostate cancer cell invasion. Cancer Res 64:8826–8830.

Nithipatikom K, Endsley MP, Isbell MA, Wheelock CE, Hammock BD, Campbell WB. 2005. A new class of inhibitors of 2-arachidonoylglycerol hydrolysis and invasion of prostate cancer cells. Biochem Biophys Res Commun 332:1028–1033.

Nomura H, Fujimoto N, Seiki M, Mai M, Okada Y. 1996. Enhanced production of matrix metalloproteinases and activation of matrix metalloproteinase 2 (gelatinase A) in human gastric carcinomas. Int J Cancer 69:9–16.

Oesch S, Walter D, Wachtel M, Pretre K, Salazar M, Guzmán M, Velasco G, Schäfer BW. 2009. Cannabinoid receptor 1 is a potential drug target for treatment of translocation-positive rhabdomyosarcoma. Mol Cancer Ther 8:1838–1845.

Overall CM, Kleifeld O. 2006. Tumour microenvironment-opinion: Validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. Nat Rev Cancer 6:227–239.

Patsos HA, Hicks DJ, Dobson RR, Greenhough A, Woodman N, Lane JD, Williams AC, Paraskeva C. 2005. The endogenous cannabinoid, anandamide, induces cell death in colorectal carcinoma cells: A possible role for cyclooxygenase 2. Gut 54:1741–1750.

Pazos MR, Tolón RM, Benito C, Rodríguez CF, Gorgojo JJ, Nevado M, Alvarez M, Arias F, Almodóvar F, Fernández MT, Lledó JL, González S, Fernández-

Ruiz JJ, Romero J. 2008. Cannabinoid CB1 receptors are expressed by parietal cells of the human gastric mucosa. J Histochem Cytochem 56: 511–516.

Pi-Sunyer FX, Aronne LJ, Heshmati HM, Devin J, Rosenstock J. 2006. Effect of rimonabant, a cannabinoid-1 receptor blocker, on weight and cardiometabolic risk factors in overweight or obese patients: RIO-North America: A randomized controlled trial. JAMA 295:761–775.

Portella G, Laezza C, Laccetti P, De Petrocellis L, Di Marzo V, Bifulco M. 2003. Inhibitory effects of cannabinoid CB1 receptor stimulation on tumor growth and metastatic spreading: Actions on signals involved in angiogenesis and metastasis. FASEB J 17:1771–1773.

Ramer R, Hinz B. 2008. Inhibition of cancer cell invasion by cannabinoids via increased expression of tissue inhibitor of matrix metalloproteinases-1. J Natl Cancer Inst 100:59–69.

Rundhaug JE. 2005. Matrix metalloproteinases and angiogenesis. J Cell Mol Med 9:267–285.

Saito H, Tsujitani S, Kondo A, Ikeguchi M, Maeta M, Kaibara N. 1999. Expression of vascular endothelial growth factor correlates with hematogenous recurrence in gastric carcinoma. Surgery 125:195–201.

Sarfaraz S, Afaq F, Adhami VM, Malik A, Mukhtar H. 2006. Cannabinoid receptor agonist-induced apoptosis of human prostate cancer cells LNCaP proceeds through sustained activation of ERK1/2 leading to G_1 cell cycle arrest. J Biol Chem 281:39480–39491.

Sarfaraz S, Afaq F, Adhami VM, Mukhtar H. 2005. Cannabinoid receptor as a novel target for the treatment of prostate cancer. Cancer Res 65:1635–1641.