Potentiation of the antitumor activity of adriamycin against osteosarcoma by cannabinoid WIN-55,212-2

FENG NIU[1], SONG ZHAO[1], CHANG-YAN XU[2], HUI SHA[1], GUI-BIN BI[1], LIN CHEN[1], LONG YE[1], PING GONG[1] and TIAN-HONG NIE[1]

Departments of [1]Spine Surgery and [2]Medical Records, The First Hospital of Jilin University, Changchun, Jilin 130021, P.R. China

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Abstract. Osteosarcoma is the most frequent primary malignant bone tumor that occurs in children and adolescents. The present study aimed to identify novel therapeutic strategies for osteosarcoma, by assessing the antitumor activity of the cannabinoid WIN-55,212-2 and its combined effect with adriamycin (ADM) against the MG-63 human osteosarcoma cell line. To evaluate the antiproliferative action of these molecules, a Cell Counting kit-8 (CCK-8) assay was used. The ability of cannabinoid to inhibit the migration, invasion and angiogenic activity of MG-63 cells were assessed by scratch, Transwell® chamber and angiogenesis assays, respectively, *in vitro*. To examine the alterations in expression of targeted genes, quantitative polymerase chain reaction and western blot analysis were used. The administration of cannabinoid combined with ADM was demonstrated to inhibit the growth of MG-63 cells, resulting in a cell viability of 32.12±3.13%, which was significantly lower ($P<0.05$) compared with the cell viability following treatment with cannabinoid (70.86±7.55%) and ADM (62.87±5.98%) alone. Greater antimetastasis and antiangiogenic activities were also observed following the coadministration of the two agents compared with individual treatments and controls. In addition, the expression levels of Notch-1, matrix metalloproteinase-2 (MMP-2) and vascular endothelial growth factor (VEGF) in MG-63 cells were downregulated following the treatments with cannabinoid alone or in combination with ADM. In conclusion, the present findings demonstrated that cannabinoid WIN-55,212-2 may significantly potentiate the antiproliferative, antimetastasis and antiangiogenic effects of ADM against MG-63 cells via the downregulation of Notch-1, MMP-2 and VEGF. These findings may offer a novel strategy for the treatment of osteosarcoma.

Correspondence to: Dr Song Zhao, Department of Spine Surgery, The First Hospital of Jilin University, 71 Xinmin Street, Changchun, Jilin 130021, P.R. China
E-mail: zaosongh@163.com

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Introduction

Osteosarcoma is a bone malignancy that predominantly affects children and adolescents, and exhibits high invasion and metastasis rates. It has been reported that the five-year survival rate of patients who suffer from this disease remains at only 20% due to a high rate of systemic spread at the early phase and the strong chemotherapy resistance of osteosarcoma (1). Although adriamycin (ADM) is an effective benchmark agent for the management of osteosarcoma, it also results in harmful side-effects including toxicity and chemoresistance that substantially affect the quality of life of patients (2-4). Therefore, novel therapeutic approaches and drugs must be sought for the treatment of osteosarcoma.

Natural products which have potential antitumor activities have become a focus of attention for study in previous years (5,6). Cannabinoids, the active components naturally derived from the marijuana plant *Cannabis sativa* L., have been reported as potential antitumor drugs based on their ability to limit inflammation, cell proliferation and cell survival (7). To date, several cannabinoids have been identified and characterized, including Δ(9)-tetrahydrocannabinol (THC), cannabidiol, cannabinol (CBN) and anandamide, as well as synthetic cannabinoids, including WIN-55,212-2, JWH-133 and (R)-methanandamide (7). In the early 1970s, THC and CBN were shown to inhibit tumor growth in Lewis lung carcinoma (8). Subsequently, cannabinoids were found to induce apoptosis and inhibit the proliferation of various cancer cells, including those of glioma (9) and lymphoma (10), and prostate (11-13), breast (14), skin (15) and pancreatic cancer (13,16).

Previous studies have speculated about the possible mechanisms responsible for the physiological and behavioral effects of cannabinoids. There is a view that cannabinoids exert their diversified activities via the activation of G-protein-coupled receptors which are normally bound by endocannabinoids (8). Two types of receptors, CB1 and CB2, have been cloned and characterized from mammalian tissues. CB1 and CB2 are primarily expressed in the brain and immune system, respectively (17,18). Another viewpoint states that cannabinoids suppress tumor cell growth and induce apoptosis by modulating different cell signaling pathways and the expression of associated molecules, such as downregulating

phosphoinositide 3-kinase (PI3K), protein kinases, extracellular signal-regulated kinase (ERK) signaling pathways and cell division cycle gene 2 (Cdc2), coupled with an activation of the proapoptotic function of Bad protein and small guanosine triphosphatase (9,19,20). An effect on the stress-regulated protein p8 and its downstream targets has also been identified as a mechanism of the antitumor action of THC (21).

At present, it is believed that the development of novel combinational therapies may contribute to the enhancement of the therapeutic effect on tumors. In line with this hypothesis, trials have been conducted to investigate the synergistic antitumor effect of multiple drugs. It has been reported that the combined administration of cannabinoids and temozolomide (TMA) exert a strong antitumor action in glioblastoma multiforme (22). Combinations of thymoquinone and diosgenin have also shown potent antiproliferative and apoptotic effects on squamous cell carcinoma (20). Therefore, the present study aimed to investigate the antitumor activity of cannabinoid WIN-55,212-2 and its synergistic effects with ADM against osteosarcoma cells *in vitro*. In addition, the present study explores the possible mechanisms of these antitumor actions, in order to set the basis for novel strategies for the management of osteosarcoma.

Materials and methods

Cell culture and reagents. The MG-63 human osteosarcoma cell line was obtained from Nanjing KeyGen Biotechnology, Co., Ltd. (Nanjing, China). The human umbilical vein endothelial cells (HUVECs) were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The two cell lines were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) (Gibco-BRL, Gaithersburg, MD, USA) at 37˚C in an incubator with 5% CO_2. Cannabinoid WIN-55,212-2 was purchased from Sigma-Aldrich (St. Louis, MO, USA) and ADM was purchased from Pharmacia (Milan, Italy).

Cell proliferation assay. The cell proliferation assay was performed using a Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instructions. Briefly, exponentially growing MG-63 cells ($6x10^3$ cells/well, 100 μl) were seeded into 96-well plates and cultured overnight using the conditions described above for the cell culture. Five experimental groups were established: 'Cannabinoid,' 'ADM,' 'Cannabinoid + ADM,' 'Control' and 'Blank.' The adherent cells in 'Cannabinoid' and 'ADM' were exposed to 100 μl per well of 20 μM cannabinoid WIN-55,212-2 and 20 μM ADM, respectively, dissolved in RPMI-1640 fresh medium (Gibco-BRL). Cells in 'Cannabinoid + ADM' were exposed to a mixture of the above cannabinoid and ADM solutions at a ratio of 1:1 of cannabinoid:ADM (100 μl/well). Fresh medium without drugs (100 μl/well) were added into 'Control' as the control group. The wells for 'Blank' which contained medium without either drugs or cells (100 μl/well) served as the zero adjustment. The plates were then incubated for 23 h. CCK-8 (10 μl) was subsequently added to each well and the cells were further incubated at 37˚C for 1 h. Absorbance at 450 nm was measured using a microplate reader (Infinite® M200 PRO, Tecan GmbH, Grödig, Austria). The viable rate of cells in each group was calculated using the following equation:

$$\text{Viable rate}=\frac{\text{(OD (treated)-OD (adjustment))}}{\text{(OD (control)-OD (adjustment))}}\times 100(\%)$$

In which optical density (OD) (control) was obtained from 'Control,' OD (treated) was obtained from the groups with cells in the presence of drugs, and OD (adjustment) was obtained from 'Blank.' The assay was performed in triplicate.

Scratch assay. The cell migration was assessed by scratch assay. MG-63 cells were seeded at a density of $5x10^5$ cells per well in a 6-well plate and grown overnight to confluence on the plates. A single scrape was made in the confluent monolayer using a sterile pipette tip. The monolayer was washed with PBS [Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China] and 2 ml serum-free medium containing 20 μM cannabinoid WIN-55,212-2, 20 μM ADM or 'Cannabinoid + ADM' (Gibco-BRL) was added. Fresh medium without any drugs was used as the control. Serial photographic images of the same scraped section were captured at 0, 12, 24 and 36 h using a Leica TCS 4D microscope (Leica Camera AG, Wetzlar, Germany).

Invasion assay. The invasive capacity of MG-63 cells was measured using a Transwell® chamber (Corning, NY, USA). All reagents and the Transwell® chamber were pre-incubated at 37˚C. The suspension of exponentially growing MG-63 cells ($2x10^5$ cells/ml) was placed into the upper compartment in serum-free medium. Subsequently, 20 μM cannabinoid WIN-55,212-2, 20 μM ADM or 'Cannabinoid + ADM' were separately added into the upper compartments. The complete medium containing 10% FBS was placed into the lower compartment and served as chemoattractant. After 48 h of incubation at 37˚C with 5% CO_2, non-invading cells were removed using cotton swabs. Invading cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 40 min, stained with 0.1% crystal violet (Sigma-Aldrich) for 30 min and counted in six random image fields by microscopy (TCS 4D; Leica Camera AG). The invasion rates were calculated as follows:

$$\text{Invasion rates}=\frac{\text{N (treated)}}{\text{N (Control)}}\times 100\%$$

In which N (control) was the number of invading cells in the control group and N (treated) was the number of invading cells in the groups with cannabinoid and/or ADM treatment.

Angiogenesis assays in vitro. The angiogenic properties of MG-63 cell-conditioned medium were assessed using the angiogenesis assay with HUVECs *in vitro*. Exponentially growing MG-63 cells ($1x10^5$ cells/ml) were seeded in a 24-well plate and incubated with 20 μM cannabinoid WIN-55,212-2, 20 μM ADM or 'Cannabinoid + ADM' for 24 h at 37˚C with 5% CO_2. Conditioned medium was obtained by collecting the supernatant following centrifugation at 1,000 x g for 10 min. Exponentially growing HUVECs were cultured in the mixed medium containing equal amounts of conditioned and serum-free medium, and then seeded into 12-well plates

Table I. Primers used in the quantitative polymerase chain reaction.

Target gene	Accession number	Primer	Sequence	Annealing length (bp)	Temperature (˚C)
Notch-1	NM_017617.3	F	5'-TCAGCGGGATCCACTGTGAG-3'	104	65
		R	5'-ACACAGGCAGGTGAACGAGTTG-3'		
MMP-2	NM_004530.4	F	5'-CAGGAATGAATACTGGATCTACT-3'	173	65
		R	5'-GCTCCAGTTAAAGGCGGCATCCAC-3'		
VEGF	NM_001025370.1	F	5'-GAGCCTTGCCTTGCTGCTCTAC-3'	148	50
		R	5'-CACCAGGGTCTCGATTGGATG-3'		
GAPDH	NM_002046.3	F	5'-GCACCGTCAAGGCTGAGAAC-3'	138	58
		R	5'-TGGTGAAGACGCCAGTGGA-3'		

MMP-2, matrix metalloproteinase-2; VEGF, vascular endothelial growth factor; F, forward; R, reverse.

precoated with Matrigel™ at a density of $5x10^4$ cells/ml. Plates were incubated at 37˚C with 5% CO_2 for 8 h. The tube formation of HUVECs was observed by microscopy (TCS 4D; Leica Camera AG) and counted in five random image fields. The assay was performed in triplicate.

Total RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-qPCR). Following treatment with 20 μM cannabinoid (WIN-55,212-2), 20 μM ADM or 'Cannabinoid + ADM' and incubation for 24 h at 37˚C with 5% CO_2, MG-63 cells were harvested. MG-63 cells without treatment served as the control group. The total RNA was extracted from each sample of $1x10^6$ cells using TRIzol® (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. M-MLV reverse transcriptase (Takara Bio Inc., Otsu, Japan) and oligo(dT) were used to synthesize the first-strand cDNA from the total RNA. The mixture was then incubated at 37˚C for 2 h, terminated by heating at 95˚C for 5 min and stored at -20˚C. The transcript levels of Notch-1, matrix metalloproteinase-2 (MMP-2) and vascular endothelial growth factor (VEGF) were detected by RT-qPCR using a Light Cycler 2.0 (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) using SYBR® Green Master Mix (Toyobo, Osaka, Japan). The RT-PCR program was as follows: 95˚C for 10 sec, followed by 40 cycles of 95˚C for 10 sec, 56-65˚C for 5 sec and 72˚C for 10 sec. The GAPDH gene, which served as the reference gene for the internal standard, was amplified with forward and reverse primers. The PCR primers used to detect each gene were synthesized by SBS Genetech Co., Ltd. (Beijing, China) and the sequences and information are listed in Table I. The dissociation curve analysis of amplification products was performed at the end of each PCR reaction to confirm the specificity of the amplification. The data were then analyzed by the $2^{-\Delta\Delta Ct}$ method (23) to obtain the relative gene expression levels compared with that of the controls.

Western blot analysis. The expression levels of Notch-1, MMP-2 and VEGF proteins in MG-63 cells were detected by western blot analysis, and β-actin was used as the loading control. As described for the total RNA extraction, MG-63 cells with or without drug treatments were lysed in ice-cold lysing buffer consisting of 50 mM Trizma base (pH 7.4; Sigma-Aldrich), 1% Triton X-100, 150 mM NaCl, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 2 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 1 mM EDTA, 0.5 μg/ml leupeptin and 1 mM sodium vanadate. All reagents were purchased from Sigma-Aldrich. Cell lysates were centrifuged at 13,000 x g for 5 min at 4˚C. The supernatant was collected and the protein expressions were measured as follows. The whole cell extracts (50 μg/lane) were separated on 12% SDS-PAGE gels and then transferred to polyvinylidene difluoride membranes. Subsequent to blocking in Tris-buffered saline with 5% (w/v) non-fat dried milk, the membranes were probed with a primary mouse monoclonal anti-human antibodies for Notch-1, MMP-2 or VEGF (Abcam, Cambridge, UK) diluted in blocking buffer to a concentration of 1:1,000 at 4˚C overnight. Subsequent to washing three times with Tris-buffered saline with Tween (TBST), membranes were incubated with diluted secondary HRP-labeled goat anti-mouse IgG antibody (Abcam), at a concentration of 1:5,000, conjugated with horseradish peroxidase and detected by enhanced chemiluminescence reagent [Sangon Biotech (Shanghai) Co., Ltd.]. Photographic images of the bands were taken and analyzed by National Institutes of Health Image software (Bethesda, MD, USA).

Statistical analysis. Statistical analysis was performed with a Student's t-test using SPSS 20.0 (IBM SPSS, Armonk, NY, USA). All data are presented in the form of the mean ± standard deviation. Qualitative data were analyzed using the χ^2 test. Differences between the treatment and control groups were considered significant at $P<0.05$.

Results

Cannabinoid and ADM inhibit the proliferation of MG-63 cells. Cannabinoid and ADM were tested individually and as a combined treatment to ascertain whether these agents inhibited the proliferation of MG-63 cells. The groups, 'Cannabinoid,' 'ADM,' and 'Cannabinoid + ADM,' in which cells were incubated with 20 μM cannabinoid (WIN-55,212-2), 20μM ADM and or the two agents for 24 h, were shown to effectively inhibit the growth of MG-63 cells. The viability rates of cells in the 'Cannabinoid,' 'ADM' and 'Cannabinoid + ADM' treatment groups were 70.86±7.55, 62.87±5.98 and 32.12±3.13%,

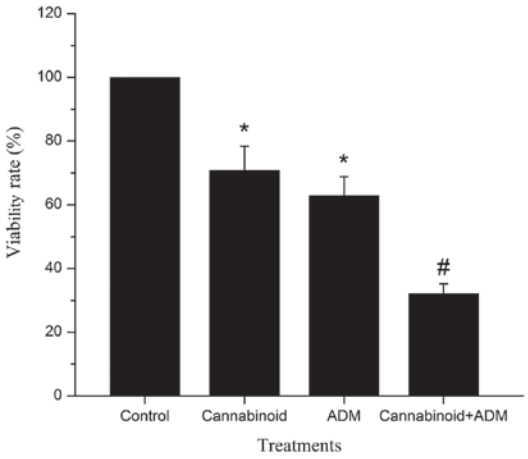

Figure 1. Cell viability of MG-63 cells treated with cannabinoid and/or ADM. Vertical bars represent the mean ± standard deviation (n=3). *P<0.05 compared with 'Control'; #P<0.05 compared with 'Cannabinoid' and 'ADM'. ADM, adriamycin.

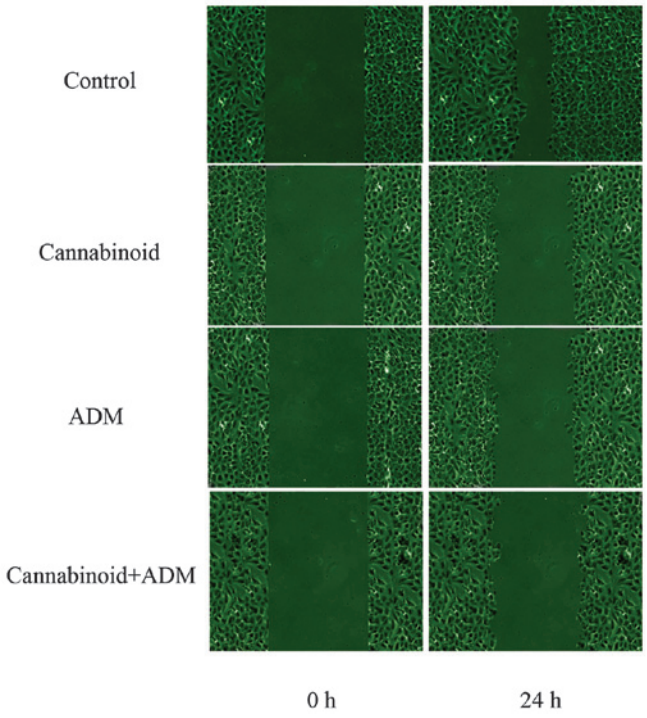

Figure 2. Effect of cannabinoid and/or ADM on the migration of MG-63 cells. Cells were plated to confluence on 6-well plates. A single scratch was made in the confluent monolayer. The scratch was monitored and photographed at 0 and 24 h post-treatment. ADM, adriamycin.

respectively, which were all significantly reduced (P<0.05) compared with those in 'Control' (100%). Notably, the viability rate of cells in the 'Cannabinoid + ADM' group was significantly lower (P<0.05) than those in 'Cannabinoid' and 'ADM' groups (Fig. 1).

Cannabinoid and ADM inhibit the migration and invasion of MG-63 cells. MG-63 cells were treated with 20 μM cannabinoid (WIN-55,212-2) and ADM alone or combined, and cell migration was measured by scratch assay. The results demonstrated that the individual and combined treatments of

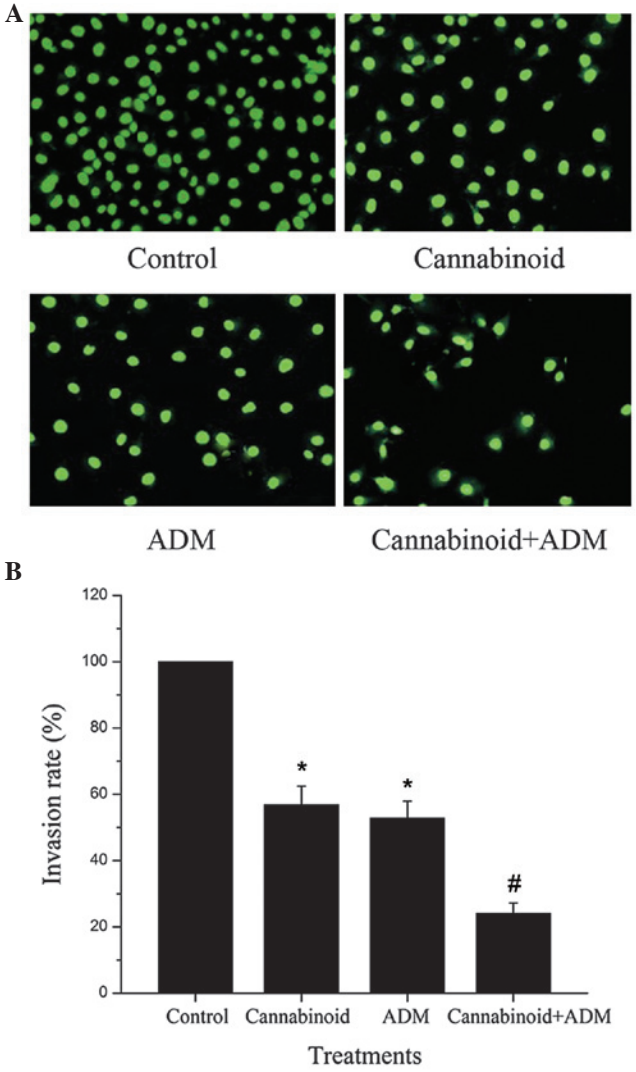

Figure 3. Effect of cannabinoid and/or ADM on the invasion of MG-63 cells. Invading cells were fixed, stained and counted by microscopy at 48 h post-treatment. (A) Microscopic images of fields and (B) the proportion of invading cells following the different treatments. Vertical bars represent the mean ± standard deviation (n=3). *P<0.05 compared with 'Control'; #P<0.05 compared with 'Cannabinoid' and 'ADM'. ADM, adriamycin.

cannabinoid and ADM had time-dependent inhibition effects on the migration of cells. This inhibition was enhanced as time progressed. Compared with the control treatment, the number of cells that had migrated after 24 h to heal the wound was fewer in the cells with cannabinoid and/or ADM treatment, and the scraped sections in these treatment groups were also wider (Fig. 2). Notably, these phenomena were observed most markedly in 'Cannabinoid + ADM'. In the cell invasion assay, as shown in Fig. 3A, the number of invading cells in the treatment groups with cannabinoid and ADM alone or combined was reduced compared with the control group, and the number of invading cells in 'Cannabinoid + ADM' was the lowest in all the three treatment groups. Consistently, the invasion rate of cells in 'Cannabinoid + ADM' was significantly lower than those in the groups with the individual treatments of either cannabinoid or ADM (Fig. 3B).

Cannabinoid and ADM inhibit the angiogenic activity of HUVECs treated with MG-63 cell-conditioned medium. The

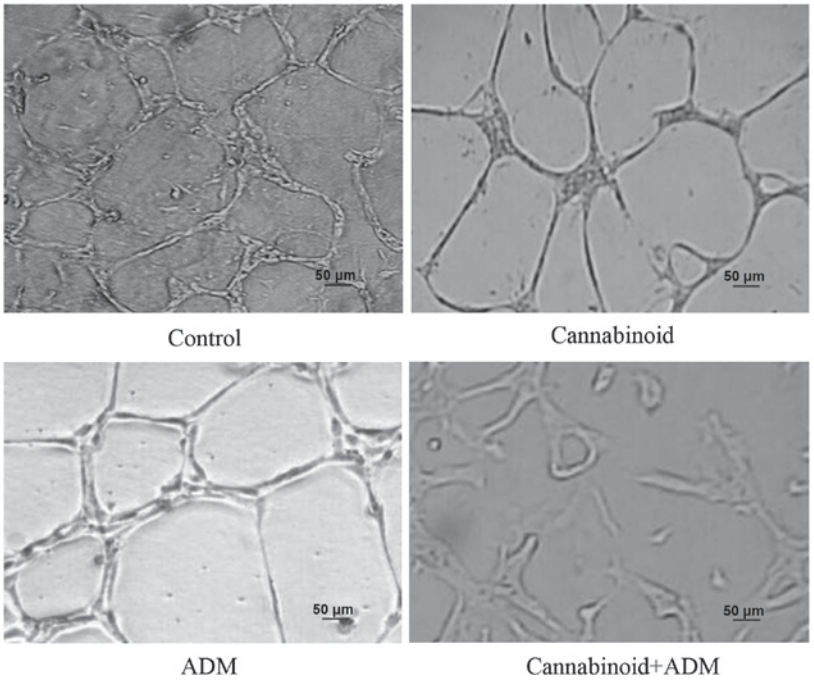

Figure 4. Effect of cannabinoid and/or ADM on the angiogenic activity of MG-63 cells for HUVECs. The tube formation of HUVECs in different treatments was observed by Olympus phase-contrast microscopy (magnification, x200). HUVEC, human umbilical vein endothelial cells; ADM, adriamycin.

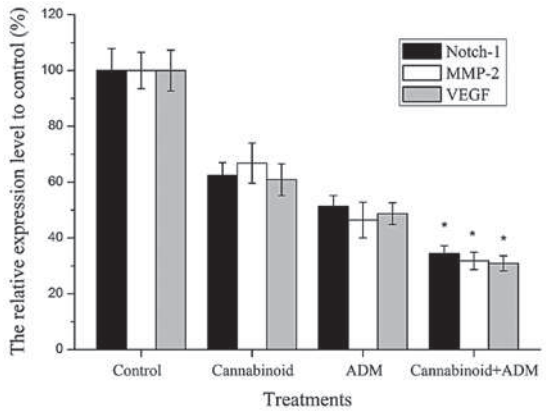

Figure 5. The expression levels of Notch-1, MMP-2 and VEGF genes in MG-63 cells with cannabinoid and/or ADM treatments. Vertical bars represent the mean ± standard deviation (n=3). Significant differences between the 'Cannabinoid + ADM' and the other groups were identified (*P<0.05). MMP-2, matrix metalloproteinase-2; VEGF, vascular endothelial growth factor; ADM, adriamycin.

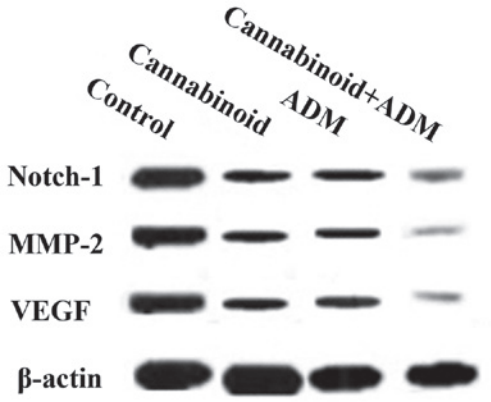

Figure 6. Western blot analysis of Notch-1, MMP-2 and VEGF proteins in MG-63 cells with cannabinoid and/or ADM treatments. β-actin was used as loading control. MMP-2, matrix metalloproteinase-2; VEGF, vascular endothelial growth factor; ADM, adriamycin.

angiogenic properties of MG-63 cell-conditioned medium were assessed using the angiogenesis assay with HUVECs *in vitro*. The results showed that tube formation of HUVECs was observed with the drug-free MG-63 cell-conditioned medium. However, tube formation was significantly reduced when the HUVECs were cultured with the cannabinoid and ADM alone, or the combined conditioned medium. The combined treatment reduced the angiogenesis to the greatest extent (Fig. 4).

Notch-1, MMP-2 and VEGF expression levels were downregulated by cannabinoid and ADM. The expression levels of Notch-1, MMP-2 and VEGF genes were measured by RT-qPCR (Fig. 5). The results showed that the mRNA expression levels of these genes in MG-63 cells with cannabinoid and/or ADM treatment reduced significantly (P<0.05) compared with those in the control group without any drug treatment. Furthermore, the expression levels of Notch-1, MMP-2 and VEGF in cells with the combined treatment of cannabinoid and ADM were significantly lower (P<0.05) compared with cells that received individual treatment of either cannabinoid or ADM. Western blotting further confirmed these downregulation effects (Fig. 6).

Discussion

Despite its potent antitumor activity, ADM, the conventional medicine against osteosarcoma, has side-effects. Therefore,

there is a requirement to search for novel therapeutic drugs and approaches aimed at improving the poor prognosis of patients with osteosarcoma. The results obtained in the present study demonstrated that WIN-55,212-2, the synthetic cannabinoid from the main active component of marijuana, reduced the growth of osteosarcoma MG-63 cells. This is consistent with reports of the suppressive effects of cannabinoids on lung, prostate, breast, skin and pancreatic cancer cells (8-16,24). These data together indicate that cannabinoids may have potential as antitumor drugs for osteosarcoma therapy. Notably, the present data also demonstrated that the combined administration of ADM and cannabinoid exerted an enhanced antiproliferation effect on MG-63 cells. Similar synergistic effects of drugs were also observed with cannabinoids and TMA against glioblastoma multiforme (22). Cannabinoids have been shown to be devoid of the strong side-effects associated with other chemotherapeutic agents (25,26) and no overt toxic effects of cannabinoids in patients have been reported in clinical trials for various applications (8,27). Thus, the synergistic effects of cannabinoid and ADM in the present study indicated a novel strategy that may possibly reduce the side-effects and also enhance the antitumor activity of ADM, while requiring a lower dosage.

Metastasis has been shown to be the main cause of mortality in patients with osteosarcoma, which must be suppressed for improved prognosis (28). The present results demonstrated that cannabinoid (WIN-55,212-2) and/or ADM could inhibit the migration and invasion of MG-63 cells. This was consistent with the defined role of ADM in the migration of osteosarcoma cells (3). Suppression of metastasis by THC was also reported in severe combined immunodeficient mice (29). The results implied that cannabinoid may enhance the antimetastastic effect of ADM for osteosarcoma, since the combined treatment with cannabinoid and ADM inhibited the migration and invasion of MG-63 cells to a significantly higher extent than the individual treatment with either of these two agents.

In terms of the mechanism of the antimetastatic activities, it was shown that the expression levels of Notch-1 and MMP-2 were significantly downregulated following the cannabinoid (WIN-55,212-2) and/or ADM treatments in the present study. Previous studies have demonstrated that the Notch signaling pathway is critical in cell proliferation and apoptosis (30,31). Among the Notch genes, Notch-1 has been reported to be involved in the migration and invasion of cancer cells (32) and to exhibit crosstalk with nuclear factor κB (NF-κB), another important regulatory pathway in the processes of tumor cell invasion and metastasis (33-37). The MMPs, a family of associated enzymes that degrade the extracellular matrix, are also considered to be important in facilitating tumor invasion (38). The level of active MMP-2 is considered to be a cancer metastasis indicator (39). A previous study also revealed that the invasion and metastasis of human breast cancer cells is inhibited by the knockdown of Notch-1, coupled with the inactivation of MMP expression (32). The present results showed the downregulation of Notch-1 and MMP-2 in MG-63 cells following treatments with cannabinoid and ADM alone or in combination, indicating that cannabinoid and/or ADM may inhibit the metastasis and invasion of osteosarcoma cells via the suppression of the Notch signaling pathway and MMPs. Similar observations were reported in human cervical cancer cells following cannabinoid treatment, in which the expression level of MMP-2 was downregulated (40).

In the present study, cannabinoid WIN-55,212-2 was also shown to suppress the angiogenic activity of MG-63 cells and to enhance the antiangiogenic effect of ADM as well. Similar suppressive effects of cannabinoid were also observed against gliomas *in vitro* (41) and human colon carcinoma xenografts in nude mice (42). The present results supported the view that cannabinoid exhibits highly effective antiangiogenic action and has a synergetic effect in combination with ADM. Additionally, previous studies revealed that VEGF is vital for tumor-associated microvascular invasion as an angiogenic factor (43,44) and its expression was shown to be associated with the distant metastasis of tumor cells (45,46). The present study also showed the inhibition of VEGF expression in MG-63 cells following cannabinoid and/or ADM treatment. Thus, the present results indicated that cannabinoid and/or ADM may exert their antiangiogenic and antimetastatic activities partially through the downregulation of VEGF expression. Furthermore, combination therapy 'Cannabinoid + ADM' was shown to be more effective in the downregulation of Notch-1, MMP-2 and VEGF expression levels compared with treatments of cannabinoid or ADM alone, which further supports the findings of synergistic antitumor effects of the two drugs against osteosarcoma.

In conclusion, the present study indicated that cannabinoid WIN-55,212-2 is antiproliferative, antimetastatic and antiangiogenic against MG-63 cells *in vitro*, and presented evidence that cannabinoid WIN-55,212-2 may result in synergistic antitumor action in combination with ADM against osteosarcoma. The present data also indicated that the mechanism of cannabinoid and/or ADM antitumor activity may be based on the inactivation of the Notch signaling pathway and the downregulation of MMP-2 and VEGF, which may be useful for the development and exploration of potent, nontoxic and novel therapeutic strategies for osteosarcoma. However, further in-depth studies are required to investigate the precise and comprehensive molecular mechanisms of these antitumor activities, and whether this coadministration of cannabinoid and ADM could be effectively applied in practice.

References

1. Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, Kotz R, Salzer-Kuntschik M, Werner M, Winkelmann W, *et al*: Prognostic factors in high-grade osteosarcoma of the extremities or trunk: An analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 20: 776-790, 2002.
2. Friedman MA and Carter SK: The therapy of osteogenic sarcoma: Current status and thoughts for the future. J Surg Oncol 4: 482-510, 1972.
3. Jaffe N: Recent advances in the chemotherapy of metastatic osteogenic sarcoma. Cancer 30: 1627-1631, 1972.
4. Cortes EP, Holland JF, Wang JJ, Sinks LF, Blom J, Senn H, Bank A and Glidewell O: Amputation and adriamycin in primary osteosarcoma. N Engl J Med 291: 998-1000, 1974.
5. Clark AM: Natural products as a resource for new drugs. Pharm Res 13: 1133-1144, 1996.
6. Potterat O and Hamburger M: Drug discovery and development with plant-derived compounds. Prog Drug Res 65: 47-118, 2008.
7. Sarfaraz S, Adhami VM, Syed DN, Afaq F and Mukhtar H: Cannabinoids for cancer treatment: Progress and promise. Cancer Res 68: 339-342, 2008.

8. Guzmán M: Cannabinoids: Potential anticancer agents. Nat Rev Cancer 3: 745-755, 2003.
9. Ellert-Miklaszewska A, Kaminska B and Konarska L: Cannabinoids down-regulate PI3K/Akt and Erk signalling pathways and activate proapoptotic function of Bad protein. Cell Signal 17: 25-37, 2005.
10. Flygare J, Gustafsson K, Kimby E, Christensson B and Sander B: Cannabinoid receptor ligands mediate growth inhibition and cell death in mantle cell lymphoma. Febs Lett 579: 6885-6889, 2005.
11. Ruiz L, Miguel A and Diaz-Laviada I: Delta (9)-tetrahydrocannabinol induces apoptosis in human prostate PC-3 cells via a receptor-independent mechanism. FEBS Lett 458: 400-404, 1999.
12. Nithipatikom K, Endsley MP, Isbell MA, Falck JR, Iwamoto Y, Hillard CJ and Campbell WB: 2-arachidonoylglycerol: A novel inhibitor of androgen-independent prostate cancer cell invasion. Cancer Res 64: 8826-8830, 2004.
13. Carracedo A, Gironella M, Lorente M, Garcia S, Guzmán 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.
14. 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.
15. Blázquez C, Carracedo A, Barrado L, Real PJ, Fernández-Luna JL, Velasco G, Malumbres M and Guzmán M: Cannabinoid receptors as novel targets for the treatment of melanoma. Faseb J 20: 2633-2635, 2006.
16. Fogli S, Nieri P, Chicca A, Adinolfi B, Mariotti V, Iacopetti P, Breschi MC and Pellegrini S: Cannabinoid derivatives induce cell death in pancreatic MIA PaCa-2 cells via a receptor-independent mechanism. Febs Lett 580: 1733-1739, 2006.
17. Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, *et al*: International union of pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacol Rev 54: 161-202, 2002.
18. Fernández-Ruiz J, Romero J, Velasco G, Tolón RM, Ramos JA and Guzmán M: Cannabinoid CB2 receptor: A new target for controlling neural cell survival? Trends Pharmacol Sci 28: 39-45, 2007.
19. Caffarel MM, Sarrió D, Palacios J, Guzman M and Sánchez C: Delta9-tetrahydrocannabinol inhibits cell cycle progression in human breast cancer cells through Cdc2 regulation. Cancer Res 66: 6615-6621, 2006.
20. Takeda S, Okajima S, Miyoshi H, Yoshida K, Okamoto Y, Okada T, Amamoto T, Watanabe K, Omiecinski CJ and Aramaki H: Cannabidiolic acid, a major cannabinoid in fiber-type cannabis, is an inhibitor of MDA-MB-231 breast cancer cell migration. Toxicol Lett 214: 314-319, 2012.
21. Carracedo A, Lorente M, Egia A, Blázquez C, García S, Giroux V, Malicet C, Villuendas R, Gironella M, González-Feria L, *et al*: The stress-regulated protein p8 mediates cannabinoid-induced apoptosis of tumor cells. Cancer Cell 9: 301-312, 2006.
22. Torres S, Lorente M, Rodríguez-Fornés F, Hernández-Tiedra S, Salazar M, García-Taboada E, Barcia J, Guzmán M and Velasco G: A combined preclinical therapy of cannabinoids and temozolomide against glioma. Mol Cancer Ther 10: 90-103, 2011.
23. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C (T)) Method. Methods 25: 402-408, 2001.
24. 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.
25. Mackie K: Cannabinoid receptors as therapeutic targets. Annu. Rev Pharmacol Toxicol 46: 101-122, 2006.
26. Pertwee RG: Emerging strategies for exploiting cannabinoid receptor agonists as medicines. Br J Pharmacol 156: 397-411, 2009.
27. Hall W, Christie M and Currow D: Cannabinoids and cancer: Causation, remediation and palliation. The lancet oncology 6: 35-42, 2005.
28. Wang YH, Han XD, Qiu Y, Xiong J, Yu Y, Wang B, Zhu ZZ, Qian BP, Chen YX, Wang SF, *et al*: Increased expression of insulin-like growth factor-1 receptor is correlated with tumor metastasis and prognosis in patients with osteosarcoma. J Surg Oncol 105: 235-243, 2012.
29. Preet A, Ganju R and Groopman J: Delta9-Tetrahydrocannabinol inhibits epithelial growth factor-induced lung cancer cell migration in vitro as well as its growth and metastasis in vivo. Oncogene 27: 339-346, 2008.
30. Berx G, Nollet F and Van Roy F: Dysregulation of the E-cadherin/catenin complex by irreversible mutations in human carcinomas. Cell Adhes Commun 6: 171-184, 1998.
31. Artavanis-Tsakonas S, Rand MD and Lake RJ: Notch signaling: Cell fate control and signal integration in development. Science 284: 770-776, 1999.
32. Wang J, Fu L, Gu F and Ma Y: Notch1 is involved in migration and invasion of human breast cancer cells. Oncol Rep 26: 1295-1303, 2011.
33. Curran S and Murray G: Matrix metalloproteinases: Molecular aspects of their roles in tumour invasion and metastasis. Eur J Cancer 36: 1621-1630, 2000.
34. John A and Tuszynski G: The role of matrix metalloproteinases in tumor angiogenesis and tumor metastasis. Pathology oncology research 7: 14-23, 2001.
35. Nagakawa Y, Aoki T, Kasuya K, Tsuchida A and Koyanagi Y: Histologic features of venous invasion, expression of vascular endothelial growth factor and matrix metalloproteinase-2 and matrix metalloproteinase-9 and the relation with liver metastasis in pancreatic cancer. Pancreas 24: 169-178, 2002.
36. Xiong HQ, Abbruzzese JL, Lin E, Wang L, Zheng L and Xie K: NF-kappaaB activity blockade impairs the angiogenic potential of human pancreatic cancer cells. Int J Cancer 108: 181-188, 2004.
37. Dong Z, Bonfil RD, Chinni S, Deng X, Trindade Filho JC, Bernardo M, Vaishampayan U, Che M, Sloane BF, Sheng S, *et al*: Matrix metalloproteinase activity and osteoclasts in experimental prostate cancer bone metastasis tissue. Am J Pathol 166: 1173-1186, 2005.
38. Wang Z, Banerjee S, Li Y, Rahman KM, Zhang Y and Sarkar FH: Down-regulation of notch-1 inhibits invasion by inactivation of nuclear factor-kappaB, vascular endothelial growth factor and matrix metalloproteinase-9 in pancreatic cancer cells. Cancer Res 66: 2778-2784, 2006.
39. Jezierska A and Motyl T: Matrix metalloproteinase-2 involvement in breast cancer progression: A mini-review. Med Sci Monit 15: RA32-RA40, 2009.
40. Ramer R and Hinz B: Inhibition of cancer cell invasion by cannabinoids via increased expression of tissue inhibitor of matrix metalloproteinases-1. J Natl Cancer Inst 100: 59-69, 2008.
41. Machado Rocha FC, Dos Dos Santos Júnior JG, Stefano SC and da Silveira DX: Systematic review of the literature on clinical and experimental trials on the antitumor effects of cannabinoids in gliomas. J Neurooncol 116: 11-24, 2014.
42. Kogan NM, Blázquez C, Alvarez L, Gallily R, Schlesinger M, Guzmán M and Mechoulam R: A cannabinoid quinone inhibits angiogenesis by targeting vascular endothelial cells. Molecular Pharmacology 70: 51-59, 2006.
43. Joo YE, Sohn YH, Lee WS, Park CH, Choi SK, Rew JS, Park CS and Kim SJ: Expression of vascular endothelial growth factor and p53 in pancreatic carcinomas. Korean J Intern Med 17: 153-159, 2002.
44. Zeng H, Datta K, Neid M, Li J, Parangi S and Mukhopadhyay D: Requirement of different signaling pathways mediated by insulin-like growth factor-I receptor for proliferation, invasion and VPF/VEGF expression in a pancreatic carcinoma cell line. Biochem Biophys Res Commun 302: 46-55, 2003.
45. Fujioka S, Sclabas GM, Schmidt C, Niu J, Frederick WA, Dong QG, Abbruzzese JL, Evans DB, Baker C and Chiao PJ: Inhibition of constitutive NF-kappa B activity by I kappa B alpha M suppresses tumorigenesis. Oncogene 22: 1365-1370, 2003.
46. Wey JS, Fan F, Gray MJ, Bauer TW, McCarty MF, Somcio R, Liu W, Evans DB, Wu Y, Hicklin DJ, *et al*: Vascular endothelial growth factor receptor-1 promotes migration and invasion in pancreatic carcinoma cell lines. Cancer 104: 427-438, 2005.