Cannabinoid WIN55, 212-2 induces cell cycle arrest and inhibits the proliferation and migration of human BEL7402 hepatocellular carcinoma cells

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Abstract. Hepatocellular carcinoma (HCC) is the leading cause of cancer-associated mortality worldwide; however, only limited therapeutic treatments are currently available. The present study aimed to investigate the effects of cannabinoids as novel therapeutic targets in HCC. In addition, the mechanism underlying the effects of a synthetic cannabinoid, WIN55, 212-2, on the BEL7402 HCC cell line was investigated. The results demonstrated that WIN55, 212-2 induced cell cycle arrest of the BEL7402 cells at the G0/G1 phase via cannabinoid receptor 2 (CB2)-mediated downregulation of phosphorylated-extracellular signal-regulated kinases (ERK)1/2, upregulation of p27, and downregulation of cyclin D1 and cyclin-dependent kinase 4. Furthermore, inhibition of CB2 with the CB2 antagonist AM630 abrogated WIN55, 212-2-induced cell cycle arrest. Inhibition of ERK1/2 also resulted in cell cycle dysregulation and cell cycle arrest at the G0/G1 phase, which subsequently resulted in cell growth inhibition. In addition, the present study detected a significant reduction in matrix metalloproteinase-9, retinoblastoma protein and E2F1 expression, and migration inhibition by WIN treatment. These results suggested that cannabinoid receptor agonists, including WIN, may be considered as novel therapeutics for the treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of malignancy in China (1,2). HCC presents a serious threat to human health due to its rising incidence, high metastatic recurrence and mortality rate (3,4). Although numerous treatment options exist, including surgical resection and chemotherapy, the prognosis of HCC remains poor (5). Identifying novel molecular targets, developing novel drugs and researching mechanism-based agents are required for the improvement of HCC treatment.

Cannabis sativa has been used medically for several centuries. Cannabinoids are the major effective compound present in Cannabis sativa. Numerous previous studies have demonstrated that cannabinoids exert cell growth inhibition and antitumor effects (6-11). Furthermore, the cannabinoid receptors, which consist of seven transmembrane spanning domains, have been cloned. Two cannabinoid receptors have been identified to date: Cannabinoid receptor 1 (CB1) and 2 (CB2). A previous study demonstrated that the cannabinoid, WIN55, 212-2 (WIN), inhibited the proliferation of LNCap prostate cancer cells via cell cycle arrest at the G0/G1 phase, and elucidated the underlying mechanism (11). Furthermore, WIN has been demonstrated to inhibit cell cycle of the BEL7402 HCC cell line; however, its underlying mechanism remains to be elucidated (12). In addition, cannabinoids have been reported to inhibit the metastasis of non-small cell lung cancer (13). However, little is currently known regarding the role of synthetic cannabinoids in BEL7402 cell cycle and metastasis.

The present study demonstrated that treatment of BEL7402 HCC carcinoma cells with the cannabinoid receptor agonist, WIN, led to cell cycle arrest at the G0/G1 phase. Cell cycle arrest was associated with inactivation of extracellular signal-regulated kinases (ERK)1/2, increased expression of p27, and decreased expression of cyclin D1 and cyclin-dependent kinase (Cdk)4. Inhibiting CB2 with the CB2 antagonist, AM630, led to the inactivation of ERK1/2. Inhibition of ERK1/2 signaling by its inhibitor PD98059 also resulted in similar effects. The present study also aimed to determine the role of WIN on BEL7402 cell migration, and to explore the potential underlying mechanisms.

Materials and methods

Materials. R-(+)-[2,3-Dihydro-5-methyl-3[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-1-(naphthalenyl)methanone mesylate salt (WIN) and dimethyl sulfoxide
Flow cytometric cell cycle analysis. The cells were grown to a density of 1x10^6 cells in 100 mm culture dishes and were treated for 24 h as described above. The cells were harvested by trypsinization, re-suspended in phosphate-buffered saline (PBS) and fixed in 70% (v/v) ethyl alcohol overnight at 4°C. Cell cycle analysis was performed using a Cell Cycle kit (BestBio, Shanghai, China), and cell cycle distribution was evaluated using the FACScalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The results were analyzed using ModFit LT software (version 3.2; Verity Software House, Topsham, ME, USA).

Cell migration assay. Cell migration was detected using Transwell migration and wound healing assays. For the Transwell assay, BEL7402 cells were treated with WIN at a concentration of 0, 5 or 10 µM for 24 h. The cells were subsequently trypsinized and suspended in medium containing 2% fetal bovine serum (FBS). Cell suspensions (200 µl), containing 2x10^5 cells, were seeded into the upper chamber of a 24-well Transwell (pore size, 8 µm; Corning Incorporated, Topsham, ME, USA). The Transwells were then inserted into a 24-well plate, containing 600 µl RPMI-1640 medium, supplemented with 10% FBS and incubated at 37°C in a humidified atmosphere for 24 h, in order to allow the BEL7402 cells to migrate. Cells on the upper side of the filter (not migrated) were removed with cotton swabs. Migrated cells on the lower side of the filter were fixed and stained with DAPI (Beyotime Institute of Biochemistry and Cell Biology, Shanghai, China) and incubated in a humidified atmosphere containing 5% CO₂.

Western blot analysis. Once the cells were treated with the indicated compounds, the total protein was extracted from the cells by washing in PBS and incubating for 20 min in ice-cold lysis buffer, supplemented with a protease and phosphatase...
inhibitor cocktail (Roche Diagnostics Ltd., Shanghai, China). The cells were sonicated three times for 10 sec and the protein concentrations were subsequently determined using a bicinchoninic acid assay (Beyotime Institute of Biotechnology). Equal quantities of protein samples (80 µg/lane) were separated by 12% SDS-PAGE (SDS-PAGE kit; Beijing Dingguochangsheng Biotechnology Co. Ltd., Beijing, China) and then electrotransferred onto polyvinylidene fluoride membranes (EMD Millipore, Bedford, MA, USA). Non-specific sites on the blots were blocked by a 1 h incubation at room temperature with blocking buffer (5% nonfat dry milk, 1% Tween 20 in 20 mmol/l Tris-buffered saline; pH 7.6). The membranes were then incubated with the specific primary antibodies overnight at 4°C, followed by a 1 h incubation at room temperature with horseradish peroxidase-conjugated secondary antibodies (Proteintech Group, Inc.). The blots were developed using enhanced chemiluminescence reagents (Beyotime Institute of Biotechnology), according to the manufacturer's protocol.

**Statistical analysis.** The results are expressed as the mean ± standard deviation. The data were analyzed by Student's t-test in order to determine statistical significance. Statistical analyses were performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**WIN results in cell cycle arrest at G0/G1 phase and inhibition of proliferation.** The present study aimed to test the hypothesis that WIN inhibited the proliferation of BEL7402 cells via cell cycle arrest. A DNA cell cycle analysis was performed, in order to assess the effects of WIN treatment on cell cycle distribution. As shown in Fig. 1A, treatment with WIN resulted in a dose-dependent accumulation of cells in G1 phase of the cell cycle (66.12, 73.88% and 75.67% of cells in G1 phase following treatment with 0, 5 and 10 µM WIN, respectively), as...
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Compared with vehicle treatment. In addition, treatment with WIN led to a dose-dependent decrease in the number of cells in S phase of the cell cycle (26.08, 14.31 and 12.66% cells in S phase following treatment with 0, 5 and 10 µM WIN, respectively). As shown in Fig. 1B, WIN inhibited the proliferation of BEL7402 cells in a dose-dependent manner.

**WIN-induced cell cycle arrest is mediated via the upregulation of p27 and concomitant downregulation of cyclin D1 and Cdk4.** Since the present study demonstrated that treatment of BEL7402 cells with WIN resulted in a G1 phase arrest, the effects of WIN on the cell cycle regulatory molecules that operate in G1 phase of the cell cycle were assessed. The present study also investigated the role of the Cdk inhibitor (Cki) cyclin Cdk machinery in the WIN-mediated G1 phase cell cycle arrest of BEL7402 cells. A marked increase in the protein expression of p27 was detected following treatment with WIN at 5 and 10 µM doses (Fig. 1C). Using western blot analysis, the effects of WIN on the protein expression levels of cyclin D1 and Cdk4, which are known to be regulated by p27, were investigated. Treatment of BEL7402 cells with WIN led to a dose-dependent decrease in the protein expression levels of cyclin D1 and Cdk4 (Fig. 1C).

**WIN downregulates the levels of p-Rb and E2F1.** The present study also detected the effects of WIN on the protein expression levels of pRb and E2F1. Western blot analysis revealed that treatment of BEL7402 cells with WIN led to a significant decrease in the protein expression of pRb (Fig. 1D). Since pRb controls the cell cycle via binding to and inhibiting the E2F1 protein, the downregulation of pRb by WIN would facilitate the transcription of E2F1, resulting in the release of inhibitory effects on the cell cycle progression. This mechanism further supports the role of WIN in regulating the cell cycle through multiple regulatory pathways.

**Figure 2. Effects of CB2 on the WIN-induced cell cycle arrest of BEL7402 hepatocellular carcinoma cells.** (A) Effects of the CB2 antagonist, AM630, on the (A) cell cycle distribution and (B) protein expression levels of p-ERK1/2 in BEL7402 cells. (C) Effects of CB2 selective agonist, JWH-015, on the protein expression levels of p-ERK1/2 in BEL7402 cells. CB, cannabinoid receptor; WIN, WIN55, 212-2l p-ERK, phosphorylated-extracellular signal-regulated kinases.
transcription factor, the protein expression of E2F1 was also detected. As shown in Fig. 1D, treatment of BEL7402 cells with WIN led to a dose-dependent decrease in E2F1 expression.

**WIN-induced inactivation of ERK results in cell cycle arrest via CB2.** A significant inactivation of ERK1/2 was detected when the BEL7402 cells were treated with WIN at a dose of 5 and 10 µM (Fig. 1E). To confirm that ERK1/2 inactivation was cannabinoid receptor-mediated, the BEL7401 cells were pretreated with the CB2 antagonist, AM630, followed by treatment with both AM630 and WIN. As shown in Fig. 2B, no alteration in the activation of ERK1/2 was observed when the cells were treated with the antagonist alone, as compared with the control group. However, treatment with WIN led to a marked inactivation of ERK1/2. When the antagonist was co-administered with WIN, an increase in the protein expression of p-ERK1/2 was detected, as compared with the cells treated with WIN alone. As shown in Fig. 2A, when the cells were treated with the antagonist alone, no change in the number of cells in G1 phase (70.60%) and S phase (21.59%) was observed, as compared with the control group (G1 phase, 70.06%; S phase, 22.93%). Treatment with WIN led to a significant inhibition in cell cycle progression (G1 phase, 77.11%; S phase, 14.11%). However, when the antagonist was co-administered with WIN, the inhibition in cell cycle progression was attenuated (G1 phase, 72.55%; S phase, 18.72%). Furthermore, in the BEL7402 cells treated with the CB2 selective agonist, JWH-015, the protein expression of p-ERK1/2 was significantly decreased (Fig. 2C).

**WIN-induced inactivation of ERK1/2 results in cell growth inhibition and cell cycle arrest.** To confirm the role of p-ERK1/2 in cannabinoid receptor-induced cell growth inhibition, the BEL7402 cells were treated with 30 µM ERK1/2 inhibitor, PD98059, for 24 h. Treatment with PD98059 alone resulted in a decrease in the viability of BEL7402 cells in a dose-dependent manner; however, 85.4±0.9% cells survived following treatment with 40 µM PD98059 (Fig. 3A). The present study demonstrated that treatment of BEL7402 cells with WIN resulted in G1 phase cell cycle arrest. To determine whether the cell cycle arrest was mediated by inactivation of ERK1/2, a DNA cell cycle analysis was performed. As shown in Fig. 3B, inhibiting the activation of ERK1/2 using the inhibitor PD98059 resulted in an increase in the number of cells in G1 phase of the cell cycle (75.22%), as compared with the control group (71.32%). In addition, the number of cells in S phase of cell cycle was decreased (18.72%), as compared with the control group (21.40%). Treatment with WIN led to an accumulation of cells in G1 phase (78.83%) and a reduction
of cells in S phase (10.21%). In addition, the present study
determined the effects of ERK1/2 on the expression of p27,
a cell cycle regulatory molecule that operates in G1 phase of
the cell cycle, and cyclin D1, which is associated with cell
proliferation. Treatment with WIN increased the expression
of p27, and treatment with PD98059 also increased the expres-
sion of p27 (Fig. 3C). Furthermore, treatment with WIN or
PD98059 markedly inhibited the expression of cyclin D1
in the BEL7402 cells (Fig. 3C).

WIN inhibits BEL7402 cell migration via MMP-9 down-
regulation. The present study performed Transwell and wound healing assays using BEL7402 cells. As shown in
Fig. 4A and D, cell migration was significantly decreased
following treatment with WIN in a dose-dependent manner;
the number of migrated cells was 24.20±1.31, 19.07±0.88,
and 4.60±0.29 following treatment with 0, 5 and 10 µM WIN,
respectively. The wound healing assay demonstrated that
BEL7402 cells treated with WIN invaded the wound more
slowly compared with the control group, in a dose-dependent
manner (Fig. 4B). MMPs may be associated with the impaired
migration of WIN-treated cells. To explore this hypothesis,
the present study examined the protein expression of MMP-9.
As shown in Fig. 4C, MMP-9 was significantly reduced in the
cells treated with WIN, as compared with the control group.

Discussion

Cannabinoids and their derivatives have recently attracted
attention in the treatment of cancer due to their diverse abili-
ties, including anti-inflammation, cell growth inhibition and
antitumor properties (6,10). Previous studies have suggested
that cannabinoid receptors may be an essential target for the
treatment of cancer (14,15). Our previous study indicated
that WIN-induced apoptosis of BEL7402 HCC cells may be
mediated via the CB2 receptor; therefore, this receptor may be
considered as a potential target for the treatment of HCC (12).
A previous study suggested that cannabinoids inhibit
non-small cell lung cancer growth and metastasis; however, little is currently known regarding the effects and underlying mechanisms of cannabinoids on non-small cell lung cancer proliferation and migration (13). The present study aimed to determine the mechanism underlying the antiproliferative and antimigratory effects of the cannabinoid agonist, WIN, against HCC. The results of the present study demonstrated for the first time, to the best of our knowledge, that the treatment of BEL7402 cells with WIN led to an increase in the expression levels of p27 and a decrease in the expression levels of cyclin D1 and Cdk4. These results indicated that cell cycle dysregulation in BEL7402 cells following treatment with WIN may be regulated via the p27, cyclin D1 and Cdk4 pathway. 

It is well established that uncontrolled cellular growth and metastatic reoccurrence are responsible for the development of the majority of cancer types, including HCC. Therefore, agents that can modulate cell cycle and metastasis may be useful in the management and treatment of cancer. Consistent with this notion, developing novel targets and mechanism-based anti-proliferative and anti-migratory agents for the management of HCC is essential. One of the most promising areas of recent cannabinoid research is the ability to control cell growth (8). Numerous studies have demonstrated that the cannabinoid-mediated inhibition of growth may be cell cycle-dependent (11,17). Therefore, the present study analyzed the effects of WIN on the proportion of cells in various phases of the cell cycle. WIN was able to induce a dose-dependent accumulation of cells in G1 phase of the cell cycle, and also resulted in an inhibition of cell proliferation. Inhibition of the cell cycle has previously been suggested as a target for the management of cancer (11). The present study also investigated the role of the Cki-cyclin-Cdk machinery in the WIN-mediated G1 phase cell cycle arrest of BEL7402 cells. The eukaryotic cell cycle is regulated by protein kinase complexes, which are comprised of cyclins (the regulatory subunit), which bind to Cdk's (catalytic subunit), in order to form active cyclin-Cdk complexes. Cdk activity is additionally regulated by small proteins, known as Ckis, which include p27. It has been reported that Ckis inhibit the kinase activities associated with cyclin-Cdk complexes; therefore, modulating the phosphorylation events that have a critical role in the progression of the cell cycle (18). A previous study demonstrated that cell cycle progression through the G0/G1 phase is regulated by p27 (19). The present study revealed that treatment of BEL7402 cells with WIN led to an increase in the expression levels of p27 and a decrease in the expression levels of cyclin D1 and Cdk4. These results indicated that cell cycle dysregulation in BEL7402 cells following treatment with WIN may be regulated via the p27, cyclin D1 and Cdk4 pathway.

It has previously been reported that downregulation of Cdk4 results in phosphorylation and inactivation of pRb, which can in turn downregulate members of the E2F family and inhibit the transcription of genes required for S phase progression (20).
Progression of S phase in the cell cycle is accomplished by transcriptional activation of E2F target genes via phosphorylation of pocket proteins by Cdks (18). E2F1 is able to regulate the expression of MMP-9, and therefore induce migration (21-23). MMPs are involved in cell migration and are frequently upregulated in cancer cells (24). The present study demonstrated that treatment of BEL7402 cells with WIN downregulated the expression levels of pRb and E2F1. Protein expression levels of MMP-9 were downregulated in BEL7402 cells following treatment with WIN, which significantly impaired their migratory capability. Furthermore, Transwell and wound healing assays indicated that treatment of BEL7402 cells with WIN significantly inhibited the metastatic capability of the cells. These results indicated that metastatic inhibition of BEL7402 cells by WIN may be regulated via the Cdk4, pRb, E2F1 and MMP-9 pathway.

In conclusion, the results of the present study suggested that WIN may inhibit metastasis via the MMP-9 pathway and induce cell cycle arrest via ERK1/2 inactivation in BEL7402 HCC cells. These results provided a basis for the application of WIN in the treatment of HCC.

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