Cannabinoid WIN 55,212-2 induces cell cycle arrest and apoptosis, and inhibits proliferation, migration, invasion, and tumor growth in prostate cancer in a cannabinoid-receptor 2 dependent manner

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Background: Cannabinoids have demonstrated anticarcinogenic properties in a variety of malignancies, including in prostate cancer. In the present study, we explored the anti-cancer effects of the synthetic cannabinoid WIN 55,212-2 (WIN) in prostate cancer.

Methods: Established prostate cancer cells (PC3, DU145, LNCaP) were treated with varying concentrations of WIN. Cell proliferation was determined by the MTS assay. The anti-migration and anti-invasive potential of WIN was examined by the wound healing assay and the matrigel invasion assay. Cell cycle analysis was performed by flow cytometry, and mechanistic studies were performed by Western blot. Athymic mice (n = 10) were inoculated with human PC3 cells. Once tumors reached 100 mm\(^3\), animals were randomized into two groups: saline control and WIN (5 mg/kg), delivered by intraperitoneal injection three times per week for 3 weeks.

Results: WIN significantly reduced prostate cancer cell proliferation, migration, invasion, induced apoptosis, and arrested cells in Go/G1 phase in a dose-dependent manner. Mechanistic studies revealed these effects were mediated through a pathway involving cell cycle regulators p27, Cdk4, and pRb. Pre-treatment with a CB\(_2\) antagonist, AM630, followed by treatment with WIN resulted in a reversal of the anti-proliferation and cell cycle arrest previously seen with WIN alone. In vivo, administration of WIN resulted in a reduction in the tumor growth rate compared to control (P < 0.05).

Conclusions: The following study provides evidence supporting the use of WIN as a novel therapeutic for prostate cancer.

KEYWORDS
- cannabinoid
- cannabinoid receptor 2
- cell cycle
- prostate cancer
- WIN 55,212-2

1 INTRODUCTION

Prostate cancer (PCa) is the second most commonly diagnosed cancer in American men and the second leading cause of cancer death, behind lung cancer. It is estimated that one in nine men will develop prostate cancer over the course of their lifetime, and that one in 41 men will die from the disease in Canada and the United States.\textsuperscript{1} Prostate cancer in its late stages is aggressive and incurable. Therefore, strategies that effectively target PCa in its early stages are likely to improve the quality of life and overall survival of men with prostate cancer.
Cannabis has been used for medicinal purposes, with its origin dating back more than 5000 years. Since the discovery of the cannabinoid receptors and their endogenous ligands, the amount of research on the physiology and therapeutic benefit of cannabinoids has grown. Studies have demonstrated that cannabinoids contribute to maintaining homeostasis in cell proliferation, and thereby inhibit the growth of a variety of cancers, including breast, brain, skin, thyroid, prostate, and colorectal cancers.

Cannabinoids exert their effects by binding to two G-protein coupled receptors: cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2). The CB2 receptor is responsible for analgesic activity and other effects, including locomotion and temperature control. The CB2 receptor is involved in the mediation of anti-inflammatory effects. WIN is a synthetic cannabinoid and binds with high affinity to the CB2 receptor. A previous study demonstrated that WIN inhibited the proliferation of LNCaP cells via induction of phosphatase dependent apoptosis. In hepatocellular carcinoma cells, HepG2, WIN treatment sensitized cells to TRAIL-induced apoptosis, mediated by ER stress proteins. Studies using AGS and MKN-1 human gastric cancer cells demonstrated that WIN caused cells to arrest in the G0/G1 phase via activation of the MAPK pathway and inhibition of pAKT. However, little is known regarding the role of WIN and its mechanism of action in prostate cancer.

We have investigated the anti-cancer effects of the synthetic cannabinoid WIN in both in vitro and in vivo models of prostate cancer. We observed that treatment with WIN reduced cell proliferation, migration, invasion, induced apoptosis, and led to cell cycle arrest at the Go/G1 phase. Cell cycle arrest was associated with increased expression of p27Kip1 and decreased expression of cyclin dependent kinase 4 (Cdk4) and phosphorylated retinoblastoma protein (pRb). Inhibition with the CB2 antagonist, AM630, led to an increase in the proliferation of cells and the promotion of cell cycle progression. We also found that in the PC3 xenograft model, animals treated with WIN experienced a significant delay in tumor growth compared to control animals.

2 | MATERIALS AND METHODOLOGY

2.1 | Cell culture

Three human prostate cancer cell lines, DU145, LNCaP, and PC3 were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). LNCaP cells were cultured in RPMI 1640 medium (Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), 0.3 mg/mL L-glutamine and 100 IU/mL penicillin and 100 mg/mL streptomycin (Invitrogen). PC3 and DU145 cells were cultured in Dulbecco’s minimal essential medium/F12 (Invitrogen) with 10% FBS supplemented with 0.3 mg/mL L-glutamine and 100 IU/mL penicillin and 100 mg/mL streptomycin. All cells were maintained at 37°C in a 5% CO2 incubator under sterile conditions.

2.2 | Chemicals

Health Canada approval was obtained for the use of controlled substances for research purposes. WIN 55,212-2 and AM630 were obtained from Cayman Chemical (Ann Arbor, MI) and were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) to create a stock concentration of 10 mM and stored at −20°C. All compounds were prepared and stored with minimal exposure to light to avoid oxidation. All other chemicals were purchased from Sigma unless otherwise specified.

2.3 | Cell proliferation assay

Cell proliferation was assessed using the NTS assay as previously described. Cells were plated in 96-well plates at a density of 4000 cells per well. Cells were then allowed to adhere for 24 h and dose standardization was performed using WIN ranging from 1 to 30 μM. Optical density recordings at 490 nm were obtained at 24, 48, and 72 h.

2.4 | Wound healing assay

Cell migration was assessed in PC3 and DU145 cells using a wound-healing assay. A total of 5 × 10⁴ cells per well were plated in a 24-well plate and grown until cells reached 100% confluence. A vertical scratch across the well was made with a 100 μL pipette tip, followed by two washes with PBS. WIN (5-20 μM) was then added to each well and left for 24 h. Microscopy images were taken at 0 and 24 h to visually assess cell migration. Each experiment was carried out in duplicate wells and experiments were repeated three times.

2.5 | Matrigel invasion assay

The effect of WIN on PC3 and DU145 cell invasion was determined using BD BioCoat™ Matrigel™ Invasion Chamber 8.0 Micron, obtained from BD Biosciences (Mississauga, ON, Canada). PC3 (5 × 10⁴ cells/well) cells were seeded into the upper chamber using six-well plates and cultured for 24 h at 37°C. WIN (1-5 μM) was added to the bottom wells, and cells were left to invade for 24 h. After 24 h, the non-migrating cells were removed from the upper chamber with a cotton swab. The inserts were fixed with methanol and stained with 0.1% crystal violet solution. The number of invading cells was counted using a microscope. Each experiment was carried out in duplicate wells and repeated three times.

2.6 | Annexin V

The proportion of apoptotic to live cells was assessed using the FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen, cat# V133242). PC3 and DU145 cells (1 × 10⁵) were plated in 10 cm Petri dish and treated with WIN (10-20 μM) for 24 h. Control plates were treated with vehicle alone (0.01% DMSO). After treatment, cells were trypsinized, washed with cold PBS, and centrifuged at 1200 rpm for 5 min. The supernatant was discarded, and the cells were resuspended in 1 mL of cold PBS. Cells were diluted in binding buffer to a density of 1 × 10⁶
cells/mL. Cells were labeled with 2 μL FITC Annexin V and 1 μL of 100 μg/mL PI and incubated at room temperature for 15 min. After the incubation period, 400 μL binding buffer was added to each cytophytum test tube, samples were filtered through a nylon mesh, and cell analysis was performed with the FACScalibur flow cytometer using the Cell Quest Pro software package (Becton-Dickinson, San Jose, CA).

2.7 Flow cytometry

Cell cycle distribution was determined by flow cytometry in PC3 and DU145 cells labeled with anti-bromodeoxyuridine (BrdU) fluorescein isothiocyanate (FITC) and propidium iodide (PI) as per the published manufacturer protocol. Briefly, asynchronously growing cells were plated at a density of 1 x 10^6 cells per 10 cm Petri dish and treated with WIN (10-20 μM) for 24 h. Control plates were treated with vehicle alone (0.01% DMSO). Cells were pulse labeled with BrdU for 2 h prior to harvesting. A no-BrdU control was included as a negative control. Cells were trypsinized, fixed in ice-cold 70% ethanol, and stored at −20°C until further analysis. Cells were then washed in PBS buffer with 0.5% Tween-20 and treated with 2 N HCl for 20 min. Cells were incubated in the dark on ice for 1 h with anti-BrdU conjugated FITC (DAKO, Burlington, ON, Canada). Cells were then washed, centrifuged and resuspended in 10 μg/μL PI and incubated for 30 min in the dark on ice. Samples were filtered through a nylon mesh and cell cycle analysis was performed on the FACScalibur flow cytometer using Cell Quest Pro software package. Ten thousand events were counted for each experiment.

2.8 Western blot analysis

Western Blot analyses of lysates from PC3 treated cells were carried out as described. Briefly, PC3 cells were plated at a density of 1 x 10^6 cells per 10 cm plate. Twenty-four hours after plating, adherent cells were treated with WIN (10-20 μM). Control plates were treated with vehicle alone (0.01% DMSO). After treatment for 24 h, the cells were lysed using NP-40 lysis buffer containing inhibitors (leupeptin, pepstatin, aprotinin, and phenylmethylsulfonyl fluoride), sodium dodecyl sulfate (SDS), deoxycholate, and ethylenediaminetetraacetic acid (EDTA). Protein concentration was quantified using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Rockford, IL) prior to loading into SDS polyacrylamide gels for electrophoresis. Antibodies for p27, Cdk4, and p27 were purchased from Cell Signaling Technology (Beverly, MA), and antibodies for β-actin were purchased from Abcam (Cambridge Science Park, UK). ImageJ software (US National Institute of Health, Bethesda, MA) was used to semi-quantitatively determine protein expression levels, relative to β-actin.

2.9 Effect of WIN on prostate cancer in vivo

Ethical approval for the in vivo component of this work was obtained from the Sunnybrook Research Institute Ethics Board and all work was conducted in accordance with established guidelines and protocols approved by the Canadian Council on Animal Care (CCAC). Six-week old male nu/nu athymic nude mice (Charles River Laboratories, Wilmington, MA) were used in this study. 1 x 10^6 PC3 cells resuspended in 100 μL matrigel solution (BD Biosciences, CA) were inoculated subcutaneously into the flank of each mouse. Mice were monitored each day for tumor growth. When tumors achieved an average volume of 100 mm³, mice were randomized into two treatment groups; control (n = 5) and WIN (n = 5). WIN was administered to mice 3 days per week by intraperitoneal injection at a dose of 5 mg/kg body weight over the course of 3 weeks. Mice were weighed thrice weekly with simultaneous tumor measurement. Mice with tumors exceeding 17 mm diameter were culled in accordance with CCAC guidelines. At experimental termination (day 30), serum samples were collected by direct cardiac puncture. Liver samples were also obtained at the termination of the study and fixed for histological analysis by a pathologist for any toxicities associated with the study.

2.10 In vitro mitogenicity assay

For measurement of cell growth, the MTS method was employed as previously described. PC3 cells (4 x 10^5 cells/well) were plated in 96-well plates. After 24 h, cells were washed twice with PBS and treated with serum-free media for an additional 24 h. Following this, serum-free media was removed, and cells were cultured for up to 72 h in animal serum (100 μL, filtered through a 0.2 μm syringe filter) obtained from mice in the control and treatment groups.

2.11 Statistics

Statistical analysis was completed using Microsoft Excel 2016. All in vitro experiments were assessed using Student’s t-testing. Analysis of the in vivo results were performed using either Student’s t-testing or repeated measures One-Way Analysis of Variance (ANOVA) techniques. Statistical analysis was performed using SAS software, version 8 (SAS Institute Inc., Cary, NC). Results were considered significant at the 5% level (P < 0.05). The data shown represent the mean ± standard deviation (SD).

3 RESULTS

3.1 WIN 55,212-2 inhibits proliferation of prostate cancer cells

The MTS cell proliferation assay was carried out at 24, 48, and 72 h time points on various prostate cancer cells treated with a range of doses (1-30 μM). In each of the cell lines tested, increasing concentrations of WIN treatment significantly reduced proliferation of the cancer cells. In the PC3 cell line, cells were treated with 5, 10, and 20 μM of WIN, and resulted in a 50%, 55%, and 64% reduction in cell proliferation, respectively (Figure 1A, P < 0.05). Treating the DU145 cell line with the same concentrations of WIN resulted in a 46%, 51%, and 65% reduction in cell proliferation, a similar dose-response to PC3 cells (Figure 1B, P < 0.05). The LNCaP cell line was treated with 20 and 30 μM of WIN, a slightly higher dose compared to
the other two cell lines and resulted in a 69% and 66% reduction in proliferation (Figure 1C, *P < 0.05).

3.2 | WIN 55,212-2 treatment reduces the migration and invasion capacity of prostate cancer cells

The wound-healing assay was performed to test whether WIN treatment could alter the migration of PC3 and DU145 cells. Results of this assay indicate that WIN has a considerable capacity to reduce the migration of both PC3 and DU145 cells in a dose-dependent manner. There were no significant reductions at 10 μM of WIN, however, there were significant reductions in PC3 cell migration by 59% and 63% at 15 and 20 μM, respectively (Figures 2A and 2B, *P < 0.05). In DU145 cells, treatment with 15 and 20 μM resulted in a 64% and 72% reduction in cell migration (Figures 2D and 2E, *P < 0.05). The matrigel invasion assay was used to assess the ability of a cell to penetrate an extracellular matrix-like environment. We observed that as little as 1.5 μM of WIN significantly inhibited the invasion of PC3 cells by 34% and 39% (Figure 2C, *P < 0.05) and inhibited the invasion of DU145 cells by 36% and 41%, respectively (Figure 2F, *P < 0.05).

3.3 | WIN 55,212-2 treatment induces apoptosis in prostate cancer cells

A FITC Annexin V assay was used to analyze the pro-apoptotic effect of WIN treatment on prostate cancer cells. In both DU145 and PC3 cells, the percentage of cells in the apoptotic state significantly increased in a dose-dependent manner after treatment with 10-20 μM WIN. In PC3 cells, there was a significant increase in the number of apoptotic cells by 15% and 22% (Figure 3A, *P < 0.05), and in DU145 cells, there was a significant increase in the number of apoptotic cells by 5% and 19% (Figure 3B, *P < 0.05).

3.4 | WIN 55,212-2 treatment causes cell cycle arrest in prostate cancer cells

To determine whether WIN treatment causes any alterations in the cell cycle, cells were treated with 10-20 μM WIN, collected, fixed, and assessed for cell cycle alterations. In both DU145 and PC3 cells, we observed significant reductions in the percentage of cells in the S-phase of the cell cycle when cells were treated with WIN compared to controls. In addition, we saw significant increases in the percentage of cells in G1 when cells were treated with WIN compared to controls (Figures 3C and 3D, *P < 0.05). These data are consistent with G0/G1 cell cycle arrest, suggesting that WIN is inducing cell cycle arrest in prostate cancer cells.

3.5 | WIN 55,212-2 alters expression of key cell cycle regulator proteins

To better understand the mechanism driving changes in cell cycle distribution associated with WIN treatment, we examined key cell cycle regulation proteins. Analysis of the PC3 cell lysates after treatment revealed a reduction in phosphorylated
FIGURE 2  WIN 55,212-2 Inhibits cell migration and invasion. A and D, Morphological representation of wound healing assay, where cells were treated with WIN 55,212-2 (5-20 μM) for 24 h. B and E, Quantification of wound healing assay. C and F, Quantification of Matrigel invasion assay. Significance (P < 0.05) is denoted as (*) and P < 0.001 (**) relative to control. A-C, PC3 cells; D-F, DU145 cells

retinoblastoma (pRb) and Cdk4 expression in a dose-dependent manner, and an increase in p27 expression compared to controls. Representative Western blots of p27, Cdk4, and pRb are depicted in Figure 3E with the corresponding densitometric analysis depicted in Figure 3F-H, supporting the inhibitory effects of WIN on cell cycle progression.

3.6 | WIN 55,212-2’s anti-proliferative effects are mediated through the cannabinoid receptor 2

To determine whether WIN’s effects in vitro are mediated by the cannabinoid receptor 2, cells were pre-treated with the CB2 antagonist, AM630, and analyzed for changes in cell proliferation.

FIGURE 3  WIN 55,212-2 induces apoptosis and cell cycle arrest in a dose-dependent manner and alters expression of cell cycle regulator proteins. A and B, Apoptosis as determined by Annexin V: Cells were treated with WIN for 24 h, labeled with fluorescein isothiocyanate (FITC) and propidium iodide (PI), and then subsequently analyzed by flow cytometry to determine the percentage of live versus apoptotic cells. C and D, Cell cycle analysis: Cells were treated with WIN, labeled with anti-bromodeoxyuridine (BrdU) fluorescein isothiocyanate (FITC) and propidium iodide (PI), and fixed at 24 h, and then subsequently analyzed by flow cytometry to determine the percentage of cells in each phase of the cell cycle. E-H, Western blotting with corresponding densitometric analyses: Analyses demonstrate the changes in expression of p27, Cdk4, and pRb in PC3 cells treated with WIN. Significance (P < 0.05) is denoted as (*) relative to control. A,C,E-H, PC3 cells; B,D, DU145 cells
and cell cycle distribution to assess whether the effects of WIN could be reversed with AM630 treatment. Pre-treatment with AM630 alone on prostate cancer cells resulted in no changes in cell viability (data not shown). In PC3 cells, pre-treatment with AM630, followed by treatment with WIN resulted in a significant increase in cell proliferation by 165% compared to WIN alone (Figure 4A, P < 0.05). In addition, cell cycle analysis by flow cytometry showed that pre-treatment with AM630 followed by treatment with WIN resulted in an increase in cells in S phase and a decrease in cells in G1 (Figure 4B, P < 0.05). This is consistent with a reversal of WIN's inhibitory effect on cell cycle progression. Taken together, these data suggest that WIN's anti-proliferative effects are partially mediated through the CB2 receptor.

3.7 WIN 55,212-2 treatment reduces tumor growth in a mouse xenograft model

The effect of WIN was tested in vivo using the PC3 xenograft model. Tumor size and animal weight was measured thrice weekly during the duration of the study. Mice in the WIN treatment group had significant reductions in tumor size compared to the control group (Figure 5A, P < 0.05). At Day 21, mice in the control group had tumors reaching a mean volume of 335 mm³. In contrast, mice in the WIN treatment group had tumors reaching an average volume of 104 mm³, resulting in a 31% reduction compared to controls. Mice had no significant change in body weight or food consumption (data not shown). WIN was well-tolerated with no liver toxicities as assessed histologically (data not shown).

3.8 Serum from mice treated with WIN 55,212-2 reduces mitogenicity of prostate cancer cells

To investigate if serum from mice treated with WIN alters the growth of human prostate cancer cells in vitro, mitogenicity studies were completed. PC3 cells were treated with serum obtained from mice in the control and WIN treatment groups. The MTS method was used to measure cell proliferation at 24 h. PC3 cells treated with 10% serum from mice treated with WIN showed reductions in proliferation by 55% compared to cells treated with serum from mice in the control group (Figure 5B, P < 0.05).

4 DISCUSSION

In addition to the well-known palliative effects of cannabinoids on cancer-associated symptoms, evidence suggests these molecules inhibit the growth of tumor cells in culture and animal models. This effect is mediated by several key signaling pathways. Previous studies have suggested that cannabinoid receptors may be targetable, as their expression is upregulated with increasing disease severity in several cancer sites. Cannabinoids are known to exert anti-proliferative actions in a variety of cancer cells, including prostate, breast, brain, skin, thyroid, and colorectal cancers. Administration of cannabinoids to nude mice slows tumor growth rate in lung, glioma, thyroid epithelium, skin carcinoma, and lymphoma xenograft models. Despite evidence indicating the anti-proliferative effect of cannabinoids in other tumor types, there is very limited evidence for the use of WIN as a therapeutic agent in prostate cancer. The present study aims to address this gap in knowledge by exploring the in vitro and in vivo inhibitory effects of WIN in prostate cancer and the mechanism underlying these effects.

Several studies have demonstrated the anti-proliferative and pro-apoptotic effect of a variety of cannabinoids in prostate cancer, including the endocannabinoids anandamide and 2-arachidonoylglycerol, and phytocannabinoids cannabidiol and tetrahydrocannabino. We have shown that treatment with the synthetic cannabinoid WIN significantly inhibits cell proliferation and induces apoptosis in human prostate cancer cells in a dose-dependent manner (Figures 1, 3A, and 3B, respectively).

Our study has shown that WIN treatment significantly inhibits the migration and invasion of both PC3 and DU145 human prostate cancer cells (Figure 2). The mechanism mediating these effects has yet to be explored. Metalloproteinasises (MMPs), specifically MMP2 and MMP9, promote tumor invasion through the proteolytic degradation of major basement membrane components. In a previous study, WIN inhibited invasion of BEL7402 hepatocellular carcinoma cells through downregulation of MMP9. The expression and activity of MMPs is increased in almost every type of human cancer including prostate.
Figure 5. WIN 55,212-2 significantly reduces tumor growth, and cells cultured in serum obtained from WIN 55,122-2 treated animals have a reduced proliferation rate. A, In vivo study: Variation in tumor volume (mm\(^3\)) measured over time in two groups: Control (vehicle alone) and WIN 55,212-2 (5 mg/kg body weight). WIN 55,212-2 was administered over a 3-week period on days 17-35 post-inoculation. Tumor growth was monitored over time. Error bars represent standard deviation (SD). B, In vitro mitogenicity: Cells were cultured in serum obtained from mice in control or WIN 55,212-2 group. Optical density was recorded at 490 nm. Significance (P < 0.05) is denoted as (*) relative to control.

In prostate cancer, overexpression of MMP2 and MM9 have been correlated with worse outcome and higher recurrence of disease in patients.\(^{25}\) Hence, further studies are required to explore the MMP9 pathway as a possible mechanism through which WIN is modulating metastatic inhibition. Additionally, the use of a xenograft model in this study limits our ability to explore potential anti-metastatic effects in vivo. Future studies utilizing transgenic models to elucidate potential anti-metastatic effects and to provide better insight as to optimal time point for maximum treatment effectiveness are warranted.

Regulation of the cell cycle involves processes that are crucial to the survival of a cell, including the detection and repair of genetic damage and the prevention of uncontrolled cell division. Loss of this regulatory cell-cycle control is a hallmark of cancer cells.\(^{24}\) Most studies propose a pathway by which expression of key regulators of the cell cycle are altered. In this pathway, cyclin-dependent kinase inhibitor, p27\(^{kip1}\), is activated and binds to cyclin D, inhibiting the catalytic activity of Cdk4. This prevents Cdk4 from phosphorylating retinoblastoma protein, thereby, preventing pRb from releasing transcription factors necessary for the progression of cells through the G1 and S phase.\(^{25}\)

Targeting the cell cycle pathway is a powerful strategy in cancer therapy, as cancer development has been associated with dysregulation of cell cycle machinery. Cell cycle arrest represents a survival mechanism that provides the tumor cell with the opportunity to repair its damaged DNA or can activate the apoptotic cascade, leading to cell death.\(^{26}\) Previous studies have shown that WIN causes alterations in the distribution of cell cycle phases, and/or cell cycle arrest in various cancer cell lines. In human gastric cancer cells (AGS and MKN-1), WIN caused cells to arrest in the G0/G1 phase.\(^{7}\) A previous study demonstrated that cell cycle progression was mediated by the ERK1/2 and p27 pathway in LNCaP prostate cancer cells.\(^{27}\) Therefore, we determined whether the anti-proliferative effects of WIN were mediated via cell cycle blockade. We analyzed the effect of WIN treatment on cell cycle distribution of PC3 and DU145 cells and demonstrated a dose-dependent accumulation of cells in G1 phase of the cell cycle and a dose-dependent decrease of cells in S phase (Figures 3C and 3D). We next explored the involvement of key regulators of the cell cycle. Passage through the cell cycle involves the activation of several cyclin-dependent protein kinases.\(^{28}\) The activity of these protein kinases is partially regulated by associations with Cdk Inhibitory subunits (Ciks), one of which includes p27. Hence, we explored the modulation of cell cycle regulators as a mechanism of WIN-mediated cell cycle dysregulation and inhibition of proliferation in prostate cancer cells. We observed a significant increase in the expression of p27 in WIN-treated cells and a decrease in the expression of Cdk4, suggesting that cell cycle dysregulation by WIN treatment is mediated through the Go/G1 phase Cdk Inhibitory subunit. In addition, as the downregulation of Cdk4 results in the phosphorylation and inactivation of pRb, we investigated the protein levels of pRb in cells treated with WIN. Our Western blot analyses show a dose-dependent decrease in the levels of pRb with 10-20 μM WIN (Figure 3E-H). Taken together, our results demonstrate the involvement of the p27-Cdk4-pRb pathway in the cell cycle dysregulation and inhibition of proliferation associated with WIN treatment. This proposed pathway is depicted in Figure 6, whereby cannabinoid receptor 1 inhibits cell cycle progression via inhibition of transcription factors necessary for the progression of cells through G1 phase.

The cannabinoid receptors are members of the G protein-coupled receptor family. They inhibit adenyl cyclase and activate mitogen-activated protein kinase (MAPK) by signaling through G\(_{\alpha}\) proteins.\(^{29}\) Previous studies have focused on CB\(_1\) mediated anti-carcinogenic potential of WIN; however, few have explored the CB\(_2\) receptor.\(^{30,31}\) To determine whether WIN exerts its anti-proliferative and cell cycle deregulatory effects through CB\(_2\) receptor activation, we used the CB\(_2\) receptor antagonist, AM630. We observed that cells pretreated with AM630 and subsequently treated with WIN resulted in a significant increase in cell proliferation. Cell cycle analysis revealed an increase in cells in S phase and a decrease in cells in G1 phase, consistent with an attenuation of WIN’s effect on cell cycle blockade (Figure 4). These
data suggest that WIN's anti-proliferation and cell cycle deregulation is partially regulated through the CB$_2$ receptor. It is also likely that WIN may be activating other cannabinoid receptors, including CB$_1$. Further analysis with other cannabinoid receptor antagonists or knock out models are required to understand how cannabinoid receptors affect the anti-cancer potential of WIN in prostate cancer.

To study the effect of WIN in vivo, we used a P3 xenograft model. Results of our study revealed that intraperitoneal administration of WIN thrice weekly over a 3-week period significantly reduces the tumor growth rate and was not associated with any significant toxicities (Figure 5A). Previous studies using the androgen-sensitive prostate cancer cell CWR22R v1 xenograft model has demonstrated that i.p. administration of WIN significantly inhibited tumor growth and significantly reduced PSA secretions in the serum.$^{27}$ Subsequent mitogenicity studies using serum collected from mice showed significant reductions in human prostate cancer cell proliferation, confirming our in vivo findings.

The bioavailability of WIN in mice is not well documented. Limited clinical studies have been conducted on the metabolism of WIN in humans. The first study discussing the metabolism of WIN in humans reported that WIN is extensively metabolized in the liver, with a predicted human clearance rate of 16 mL/min/kg, suggesting a fast and nearly complete metabolism in vivo, as well as a short half-life of the drug.$^{32}$ Serum concentrations and metabolism of WIN were not detailed in our study. Although, we have not assessed the levels of WIN in mice post treatment, our in vitro mitogenicity studies could likely indicate that there are detectable levels of WIN in the blood, as demonstrated by reduced cell proliferation (Figure 5B). In addition, there was no indication of toxicity following intraperitoneal administration of 5 mg/kg of WIN. Taken together, our results suggest that WIN is a well-tolerated and bioavailable treatment agent. Experiments analyzing the precursors or metabolites of WIN in media or mice serum would help us to better understand concentrations and time points for optimal treatment. Thus, more in-depth analysis on the bioavailability and pharmacokinetics of WIN in vivo is warranted through serum analysis of xenograft mice models.

In conclusion, we report for the first time that the synthetic cannabinoid WIN reduces the growth, migration, and invasion of prostate cancer cells, and induces apoptosis. Furthermore, WIN induces cell cycle arrest in Go/G1 phase and alters expression levels of key regulatory proteins in the cell cycle pathway. The in vivo xenograft studies revealed that intraperitoneal administration of a dose as low as 5 mg/kg WIN is well-tolerated and significantly reduces tumor growth, without significant impact on body weight. Based on these findings, further studies to assess the anti-cancer effects of WIN and to determine its potential as a treatment option for prostate cancer through future clinical trials are warranted.

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CONFLICTS OF INTEREST

There are no conflicts of interest to disclose.

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REFERENCES


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