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13. ABSTRACT (Maximum 200 Words) Cannabinoids have received renewed interest in recent years due to their diverse pharmacological activities such as cell growth inhibition and tumor regression. Here we show that expression levels of both cannabinoid receptors CB ₁ and CB ₂ are significantly higher in CA-HPV-10 and other human prostate cells LNCaP, DUI45, PC3, and CWR22RV1 than in human prostate epithelial and PZ-HPV-7 cells. WIN-55,212-2 (mixed CB ₁ /CB ₂ agonist) treatment to LNCaP cells resulted in a dose (1-10 μ M) and time-dependent (24-48 h) inhibition of cell growth, blocking of CB ₁ and CB ₂ receptors by their antagonists SR141716 (CB ₁) and SR144528 (CB ₂) significantly prevented this effect. Extending this observation we found that WIN-55,212-2 treatment to LNCaP resulted in dose (1-10 μ M) and time-dependent (24-72 h) (i) induction of apoptosis, (ii) decrease in protein and mRNA expression of androgen receptor, (iii) decrease in intracellular protein and mRNA expression of prostate specific antigen (PSA), (iv) decrease in secreted PSA levels, and (v) decrease in protein expression of proliferation cell nuclear antigen and vascular endothelial growth factor. Our results suggest that WIN-55,212-2 or other non-habit forming cannabinoid receptor agonists could be developed as novel therapeutic agents for the treatment of prostate cancer.				
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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Background.....	4
Body.....	4
Experimental Details.....	5
Results.....	7
Key Research Accomplishments.....	13
Reportable Outcomes.....	13
Conclusions.....	13
References.....	15
Appendix.....	17

Background

Because prostate cancer has become the most common cancer diagnosed in men, developing novel targets and mechanism based agents for its treatment has become a challenging issue. In recent years cannabinoids, the active components of *Cannabis sativa linnaeus* (marijuana) and their derivatives are drawing renewed attention because of their diverse pharmacological activities such as cell growth inhibition, anti-inflammatory effects and tumor regression (1-5). Cannabinoids have shown to induce apoptosis in gliomas (6), PC-12 pheochromocytoma (7), CHP 100 neuroblastoma (8) and hippocampal neurons (9) *in vitro*, and most interestingly, regression of C6-cell gliomas *in vivo* (10). Further interest in cannabinoid research came from the discovery of specific cannabinoid system and the cloning of specific cannabinoid receptors (10). These diversified effects of cannabinoids are now known to be mediated by the activation of specific G protein-coupled receptors that are normally bound by a family of endogenous ligands, the endocannabinoids (11, 12). Two different cannabinoid receptors have been characterized and cloned from mammalian tissues: the "central" CB₁ receptor (13), and the "peripheral" CB₂ receptor (14).

The major purpose of this research supported by the Award W81XWH-04-1-0217 is to establish whether cannabinoid receptors could prove to be useful targets for the treatment of prostate cancer.

Body

Specific Aims: The following specific aims were proposed

1. To investigate the consequences of the activation of cannabinoid receptors in human prostate cancer cells *in vitro*.

- (a) To investigate whether the activation of cannabinoid receptors impart inhibitory effect on cell growth/cell viability in human prostate cancer cells without affecting normal cells.
- (b) To investigate whether cannabinoids selectively induces apoptosis in human prostate carcinoma cells without affecting normal cells.
- (c) To investigate whether cannabinoids is associated with inhibition of angiogenesis and PSA levels in human prostate carcinoma cells.

2. To investigate the consequences of the activation of cannabinoid receptors under *in vivo* situation, in athymic nude mice implanted with human prostate cancer cells.

Work in progress

Experimental Details

Materials. R-(+)-WIN 55,212-2 (2, 3 Dihydro-5-methyl -3 ([morpholinyl]methyl) pyrrolo (1,2,3 de)-1,4-benzoxazinyl)- [1-naphthaleny] methanone, $C_{27}H_{26}N_2O_3 \cdot CH_3SO_3H$ was purchased from Sigma (St. Louis, MO). CB_1 receptor antagonist SR141716 (SR1) and CB_2 receptor antagonist SR144528 (SR2) were procured from Dr. Herbert H. Seltzman (National Institute on Drug Abuse, Division of Neuroscience and Behavioral Research, through RTI International, Research Triangle Park, NC). Dulbecco's modified Eagle's medium and Fetal Bovine Serum (FBS) were procured from GIBCO, Invitrogen Corporation (Grand Island, N.Y). Human PSA ELISA kit from Yes Biotech laboratories (Ontario, Canada) and annexin-V-FLUOS staining kit from Roche Diagnostic Corporation, Indianapolis, IN, were procured. Antibiotic (Penicillin and Streptomycin) used were obtained from Cellgro Mediatech, Inc. (Herndon, VA). Apo-Direct kit for measuring apoptosis by flow cytometry was procured from Apo-Direct San Diego, CA. RNA isolation kit (Qiagen, Inc. Valencia, CA). Monoclonal antibodies for PSA, AR, PCNA and VEGF, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibodies for CB_1 and CB_2 were purchased from Cayman Chemical Company (Ann Arbor, Michigan). Anti-mouse secondary horseradish peroxidase conjugate was obtained from Amersham Biosciences Limited (Buckinghamshire, England). Protein was estimated using BCA Protein assay kit obtained from Pierce (Rockford, IL).

Cell Culture

The LNCaP, DU145, PC-3, CWR22Rv1, CA-HPV-10 and PZ-HPV-7 cells were obtained from ATCC (Manassas, VA.). LNCaP and DU145 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum and 1% antibiotic penicillin and streptomycin. PC-3 and CWR22Rv1 cells were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum and 1% antibiotic penicillin and streptomycin. CA-HPV-10 and PZ-HPV-7 were grown in keratinocyte-serum free medium (GIBCO-BRL 17005-042) supplemented with 5 ng/mL human recombinant EGF and 50 μ g/mL bovine pituitary extract. Human prostate epithelial cells (PrEC) were obtained from Cambrex Bioscience (Walkersville, MD) and grown in prostate epithelial basal cell medium (Cambrex Bioscience, Walkersville, MD) as per the vendors instructions. The cells were maintained under standard cell culture conditions at 37 $^{\circ}$ C and 5% CO_2 in a humid environment.

Treatment of Cells

WIN-55,212-2, (dissolved in DMSO) was used for the treatment of cells. The final concentration of DMSO used was 0.1% (v/v) for each treatment. For dose-dependent studies cells were treated with WIN-55,212-2 at 1.0, 2.5, 5.0, 7.5, 10.0 μ M final concentrations for 24 h in complete cell medium, whereas for time-dependent studies the cells (50-60% confluent) were treated with 5 μ M dose of WIN-55,212-2 for 24, 48 and 72 h. For time-dependent study, we included a control treated with DMSO for 72 h because it was the longest time-point post WIN-55,212-2 treatment in our experimental protocol. To establish the role of CB_1 and CB_2 receptors in WIN-55,212-2 induced inhibitory effects two experiments were performed. In the first experiment, cells were treated with 2 μ M of SR141716 or SR144528 alone for 24 h. In the second experiment cells

pretreated with each of these antagonists (2 μ M) for 3 h followed by incubation with 7.5 μ M WIN-55,212-2 for 24 h. In pilot experiments, it was established that DMSO (0.1% v/v) had no effects when measured at 24, 48 or 72 h.

Cell Viability

The effect of WIN-55,212-2 on the viability of cells was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) assay. The cells were plated at 1×10^4 cells per well in 200 μ l of complete culture medium containing 1.0, 2.5, 5.0, 7.5 and 10.0 μ M concentrations of WIN-55,212-2 in 96-well microtiter plates for 24 and 48 h at 37 $^{\circ}$ C in a humidified chamber. Each concentration of WIN-55,212-2 was repeated in 10 wells. After incubation for specified times at 37 $^{\circ}$ C in a humidified incubator, MTT reagent (4 μ L, 5 mg/mL in PBS) was added to each well and incubated for 2 hours. The microtiter plate containing the cells was centrifuged at 1,800 rpm for 5 minutes at 4 $^{\circ}$ C. The MTT solution was removed from the wells by aspiration and the formazan crystals were dissolved in DMSO (150 μ L). Absorbance was recorded on a microplate reader at 540 nm wavelength. The effect of WIN-55,212-2 on growth inhibition was assessed as percentage inhibition in cell growth where vehicle-treated cells were taken as 100% viable.

Western Blot Analysis

For Western blotting, 25-50 μ g proteins will be resolved over 8 -12% polyacrylamide gels and will be transferred to a nitrocellulose membrane. The blot will be probed with appropriate monoclonal or polyclonal primary antibody, followed by incubation with anti-mouse or anti-rabbit secondary antibody horse-radish peroxidase conjugate that will be obtained from Amersham Life Science Inc. (Arlington Height, IL, USA) and will be detected by chemiluminescence and autoradiography using XAR-5 film obtained from Eastman Kodak Co. (Rochester, NY, USA).

Enzyme Linked Immunoabsorbent Assay for PSA

The human PSA ELISA kit was used for the quantitative determination of PSA levels in culture medium according to the vendor's protocol. This kit uses a technique of quantitative sandwich immunoassay for determination of PSA with an estimated sensitivity of 1ng/mL PSA antigen.

Detection of Apoptosis and Necrosis by Confocal Microscopy

The Annexin-V-FLUOS staining kit was used for the detection of apoptotic and necrotic cells according to vendor's protocol. This kit uses a dual-staining protocol in which the apoptotic are stained with annexin V (green fluorescence), and the necrotic cells are stained with propidium iodide (PI; red fluorescence). LNCaP cells were grown to about 70% confluence and then treated with WIN-55,212-2 (1.0, 2.5, 5.0, 7.5, 10.0 μ M) for 24 h. The fluorescence was measured by a Zeiss 410 confocal microscope (Thornwood, NY). Confocal images of green annexin-FITC fluorescence were collected using 488 nm excitation light from an argon/krypton laser, a 560 nm dichroic mirror, and a 514–540 nm bandpass barrier filter. Images of red PI fluorescence were collected using a 568 nm excitation light from the argon/krypton laser, a 560 nm dichroic mirror,

and a 590 nm long pass filter. In a selected field, the cells stained with annexin V and PI as well as unstained cells were counted to ascertain the extent of apoptosis and necrosis.

Quantification of Apoptosis by Flow Cytometry

The cells were grown at density of 1×10^6 cells in 100 mm culture dishes and were treated with WIN-55,212-2 (1.0, 2.5, 5.0, 7.5, 10.0 μ M doses) for 24 h. The cells were trypsinized, washed with PBS, and processed for labeling with fluorescein-tagged deoxyuridine triphosphate nucleotide and propidium iodide by use of an Apo-direct apoptosis kit obtained from Phoenix Flow Systems (San Diego, CA) as per manufacturer's protocol. The labeled cells were analyzed by flow cytometry.

Quantitative Real-Time PCR for mRNA expression of AR and PSA

Total RNA was isolated from LNCaP cells using RNAeasy kit according to vendor's protocol. The ratio of optical densities of RNA samples at 260 nm and 280 nm was consistently > 1.8 . cDNA was synthesized by reverse transcription of 1 μ g of extracted RNA with 200 U MMLV reverse transcriptase (Promega, Madison, WI) in the presence of oligo dT and dNTP (Promega, Madison, WI). AR and PSA were amplified using a FailsafeTM Real-Time PCR system obtained from Epicentre, Madison, WI. Thermal cycler used for amplification was ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). Primers were designed as follows: AR Forward, 5'-AAGACGCTTCTACCAGCTCACCAA; reverse, 5'-TCCCAGAA-AGGATCTTGGGCACTT; PSA Forward, 5'-ACTCACAGCAAGGATGGAGCTGAA; reverse, 5'-TGAGGGTTGTCTGGAGGACTTCAA. Cyclex was programmed at the following conditions (a) initial denature at 94 °C for 2 min, followed by 35 cycles of (b) 94 °C for 40 sec (c) anneal the primer template at 58 °C for 40 sec d) extend at 72 °C at 40 sec.

Results

(1) Expression of Cannabinoid Receptor in Normal and Prostate Cancer Cells

We first compared the expression level of both cannabinoid receptors CB₁ and CB₂ in PrEC and a series of human prostate cancer cells. We also included a pair of cells PZ-HPV-7 (virally transformed cells, derived from normal human prostate tissue) and CA-HPV-10 (virally transformed cells, derived from adenocarcinoma of human prostate tissue) derived from the same individual. Immunoblot data shown in Fig 1 revealed that expression of both CB₁ and CB₂ receptors was significantly higher in prostate cancer cells LNCaP, DUI45, PC3, CWR22Rv1 and CA-HPV-10 as compared to normal prostate cells PZ-HPV-7 and PrEC cells.

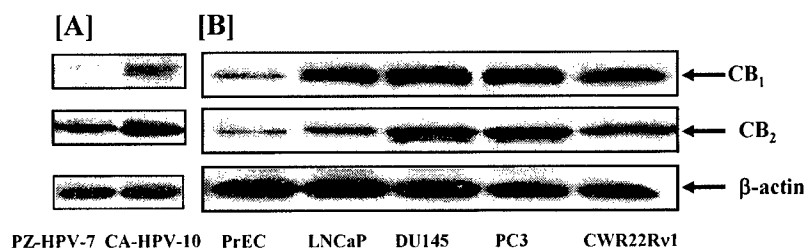


Fig 1 Western Blot analysis of CB_1 and CB_2 cannabinoid receptor expression in normal and human prostate cancer cells. (A), a pair of normal (PZ-HPV-7) and prostate cancer (CA-HPV-10) cells

obtained from same individual and (B), PrEC (normal prostate epithelial cells) and prostate cancer cells, LNCaP, DU145, PC-3 and CWR22Rv1. Total cell lysates were prepared and 30 μ g protein was subjected to SDS-PAGE, followed by immunoblot analysis and chemiluminescence detection. β -actin was used as a loading control.

(2) Effect of WIN-55,212-2 on Cell Viability of PrEC and LNCaP Cells

To evaluate the cell viability response of WIN-55,212-2 on PrEC and LNCaP cells, MTT assay was employed. Data in Fig 2 shows that treatment of PrEC cells with WIN-55,212-2 (1-10 μ M) for 24 and 48 h was without effect on cell viability (Fig 2). However, treatment of LNCaP cells with similar doses of WIN-55,212-2, in a dose-dependent manner significantly decreased the viability of LNCaP cells in 24 and 48 hr (Fig 2). The IC_{50} for inhibition of cell viability at 24 and 48 h was 6.0 μ M and 5.0 μ M respectively.

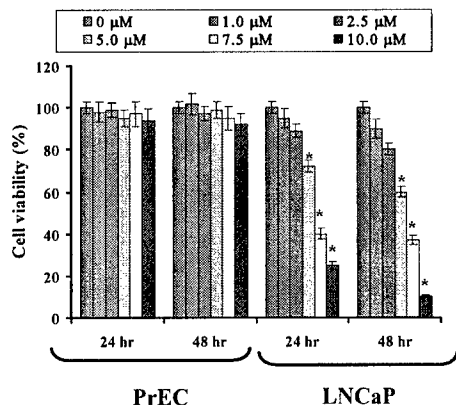


Fig 2 Effect of WIN-55,212-2 on cell viability of PrEC and LNCaP cells. As detailed in "Materials and Methods," the cells were treated with WIN-55,212-2 (1-10 μ M) for 24 h and 48 h, and their viability was determined by MTT assay. The data are expressed as percent cell viability and represent means $\pm$ SE of three separate experiments in which each treatment was performed in 10 wells. * p < 0.001 compared to control (0 μ M).

(3) CB_1 and CB_2 receptor activation signals growth inhibition in LNCaP cells

To study the possible implication of CB_1 and CB_2 receptor in WIN-55,212-2 induced cell death, the effect of their antagonists was evaluated using MTT assay. Cells pretreated with 2 μ M of SR141716 (CB_1 antagonist) or SR144528 (CB_2 antagonist) had no effect on cell viability but exhibited significant protective effect when co-administered with WIN-55,212-2 (7.5 μ M) at a molar ratio of 1:3.75 (Fig 3). These data suggest that both CB_1 and CB_2 receptors may be involved in WIN-55,212-2 mediated growth inhibition and apoptosis.

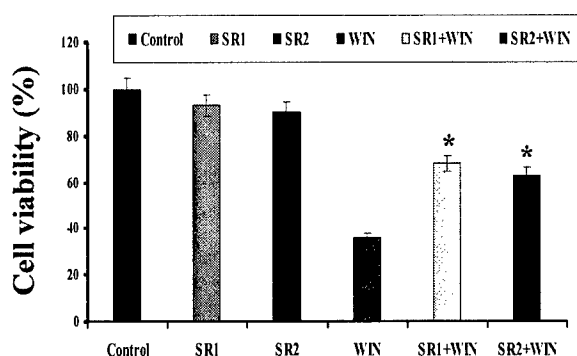


Fig 3 Effect of CB₁ receptor antagonist SR141716 (SR1) and CB₂ receptor antagonist SR144528 (SR2) on WIN-55,212-2 induced cell viability. As detailed in "Materials and Methods," cells were treated with 2 μ M of SR141716 or SR144528 alone for 24 h. In another parallel set, cells were pretreated with each of these antagonists for 3 h followed by incubation with 7.5 μ M WIN-55,212-2 for 24 h and their viability was determined by MTT assay. The data are expressed as percent cell viability and represent means $\pm$ SE of three

separate experiments in which each treatment was performed in 10 wells. * $p < 0.001$ compared to WIN.

(4) Effect of WIN-55,212-2 on Apoptosis and Necrosis of LNCaP Cells

We next assessed whether the cell growth inhibitory effect of WIN-55,212-2 was associated with induction of apoptosis. The induction of apoptosis by WIN-55,212-2 was evident from the analysis of the data obtained by confocal microscopy after labeling the cells with annexin V (Fig 4A). This method was used as it identifies the apoptotic (*green fluorescence*) as well as necrotic (*red fluorescence*) cells. As shown by the data WIN-55,212-2 treatment resulted in a dose-dependent apoptosis in LNCaP cells. We next quantified the extent of apoptosis by flow cytometric analysis of the cells labeled with deoxyuridine triphosphate and PI. LNCaP cells were treated with WIN-55,212-2 (1-10 μ M) for 24 h. As shown by data in Fig 4B, WIN-55,212-2 treatment resulted in 18.3 and 25.6% of apoptotic cells at a dose of 7.5 and 10 μ M, respectively. While the induction of apoptosis was almost negligible at the lowest dose (1.0 μ M) used, the highest dose (10 μ M) employed resulted in a massive induction of apoptosis as determined by flow cytometry.

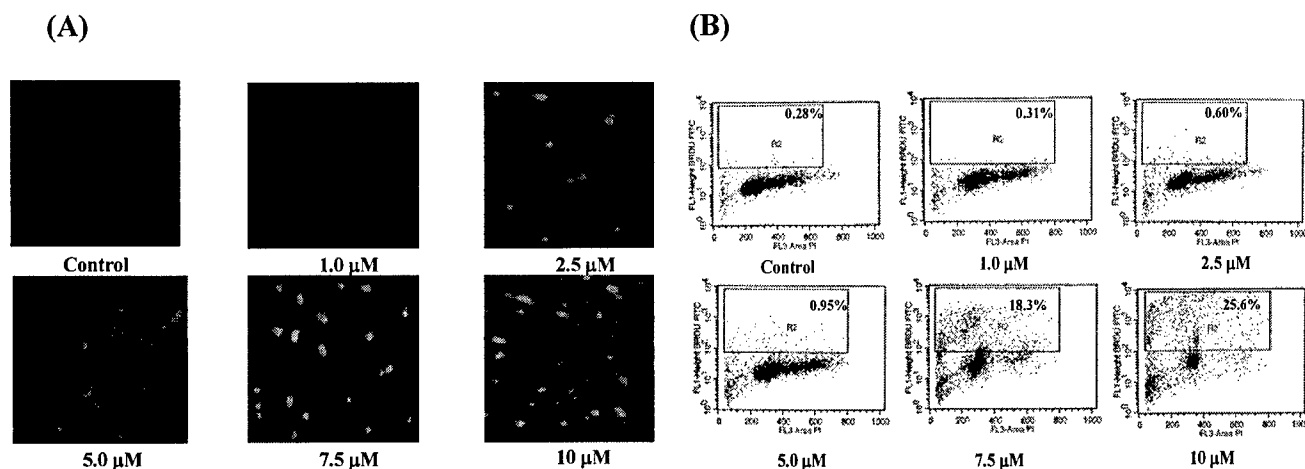


Fig 4 (A) Induction of apoptosis by confocal microscopy; cells were treated with WIN-55,212-2 1-10 μ M for 24 h for dose-dependent study. The Annexin-V-FLUOS staining kit was used for the detection of apoptotic and necrotic cells. This kit uses a dual-staining protocol in which apoptotic cells are stained with annexin V (green fluorescence) and necrotic cells are stained with PI (red fluorescence). (B), quantification of apoptosis by flow cytometry. The cells were treated with WIN-55,212-2 (1-10 μ M) for 24

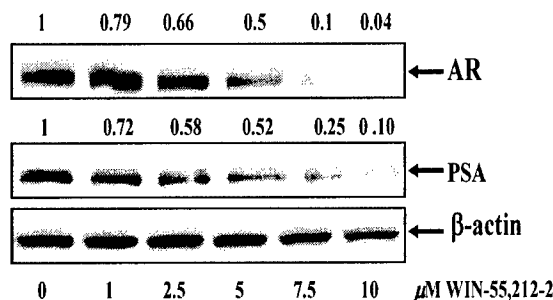
h, labeled with deoxyuridine triphosphate using terminal deoxynucleotidyl transferase and PI. Cells showing fluorescence (R2) are considered as apoptotic cells and their percentage population is shown in the figure. Data shown are from a representative experiment repeated three times with similar results.

(5) Effect of WIN-55,212-2 on AR and PSA Protein and mRNA Expression in LNCaP Cells

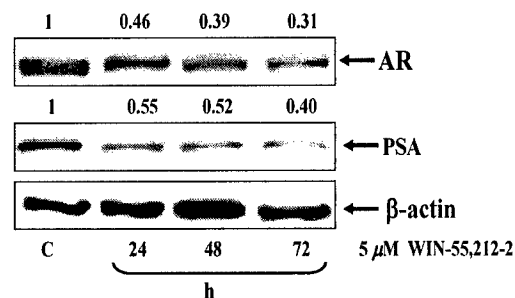
Androgens are involved in the development and progression of prostate cancer where AR is assumed to be the essential mediator for androgen action (15, 16). In the next series of experiments, we determined the effect of WIN-55,212-2 on protein and mRNA expression of AR. In dose-dependent study, we found that treatment of LNCaP cells with WIN-55,212-2 resulted in a marked decrease in AR protein expression (Fig 5A). Relative density data of these immunoblots revealed that the decrease in AR protein expression was 50% and 90 % at 5.0 and 7.5 μ M of WIN-55,212-2, respectively. In time-dependent study with 5 μ M dose of WIN-55,212-2, there was a marked decrease in AR protein expression and this corresponded with the relative density data showing a decrease of 61% and 69% at 48 h and 72 h, respectively (Fig 5B). Studies have also shown that modulation in AR leads to alteration in androgen-responsive genes (17). PSA is an androgen responsive gene and is regarded as the most sensitive biomarker and screening tool for prostate cancer in humans (18). The dose-dependent effect of WIN-55,212-2 on LNCaP cells showed a significant decrease in PSA protein expression at 5.0, 7.5 and 10 μ M concentrations when assessing at 24 h post treatment (Fig 5A). Densitometric analysis data revealed that the decrease was 48%, 75% and 90% at 5.0, 7.5 and 10.0 μ M concentrations (Fig 5A). For time-dependent study cells were treated with 5 μ M of WIN-55,212-2 for 24, 48 and 72 h. Employing western blot analysis we found a significant decrease in a time-dependent manner in PSA protein expression. Densitometric analysis revealed a decrease in PSA protein expression by 48% and 60% at 48 and 72 h respectively (Fig 5B). We also evaluated the effect of WIN-55,212-2 on mRNA levels of AR and PSA. As shown by the real time-PCR analysis data, there was an inhibition in mRNA levels of AR (Fig 5C) and PSA (Fig 5D) at 7.5 μ M and 10.0 μ M concentrations.

We next examined the effect of WIN-55,212-2 on secreted levels of PSA in LNCaP cells. Employing ELISA technique, we found that treatment of LNCaP cells with WIN-55,212-2 resulted in a dose-dependent decrease in the secreted levels of PSA by 30%, 53% and 62% at 5.0, 7.5 and 10 μ M, respectively. At similar doses of WIN-55,212-2 but varying the time-point by 48h, PSA levels decreased by 53%, 77% 80% (Fig 5E). Further at 72 h post treatment of WIN-55,212-2 secreted PSA levels decreased by 58%, 82% & 88% (Fig 5E). From these data it seems that the decrease in LNCaP cell growth was concomitant with a decrease in AR protein expression as well as decrease in both intracellular and secreted PSA levels.

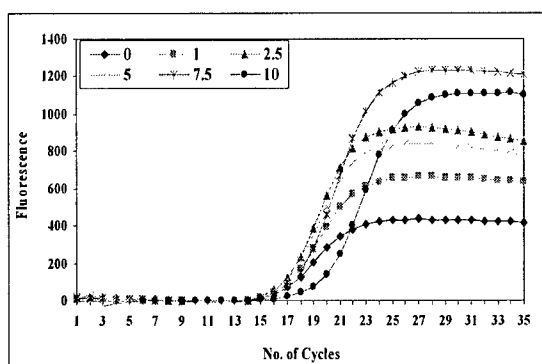
[A] Dose-dependent



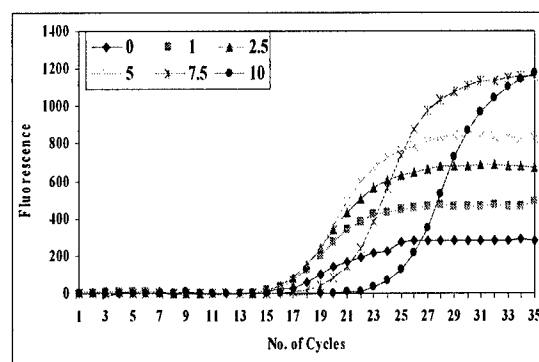
[B] Time-dependent



(C)



(D)



(E)

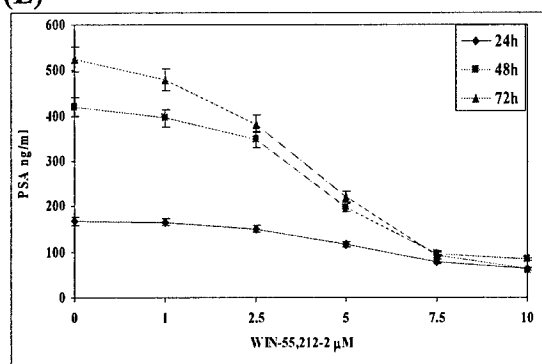


Fig 5 Effect of WIN-55,212-2 on protein and mRNA expression of AR and PSA in LNCaP cells. (A), dose-dependent effect and (B), time-dependent effect. As detailed in "Materials and Methods" the cells were treated with DMSO alone or specified concentrations of WIN-55,212-2 in DMSO and then harvested. Total cell lysates were prepared and 30 μ g protein was subjected to SDS-PAGE, followed by immunoblot analysis and chemiluminescence detection. The values above the figures represent change as compared to vehicle treatment, in protein expression of the bands normalized to β -actin. Western blot data shown here are from a representative experiment repeated three times with similar results. (C) and (D), effect of WIN-55,212-2 on mRNA expression of AR (C), and PSA (D) determined by real time-PCR. PCR data shown here are from a representative experiment repeated twice with similar results. (E), effect on secreted levels of PSA. Cells were treated with WIN-55,212-2 (1-10 μ M) for 24, 48 and 72 h and then

harvested. The PSA levels were determined by ELISA as described under "Methods". The data in E represents the mean $\pm$ SE. of three independent experiments.

(6) Effect of WIN-55,212-2 on Cell Proliferation Marker, PCNA

We next determined the effect of WIN-55,212-2 on PCNA that serves as a requisite auxiliary protein for DNA polymerase δ -driven DNA synthesis and is cell regulated (19, 20). In the dose-dependent study treatment of LNCaP cells with WIN-55,212-2 (1-10 μ M) resulted in a significant decrease in protein expression of PCNA. Western blot analysis and relative density of these bands showed that the decrease in protein expression of PCNA was 71% at 7.5 μ M WIN-55,212-2 (Fig 6A). In time-dependent study, treatment of LNCaP cells with 5 μ M WIN-55,212-2 resulted in greater than 50% inhibition in PCNA protein expression at 48 and 72 h of treatment (Fig 6B).

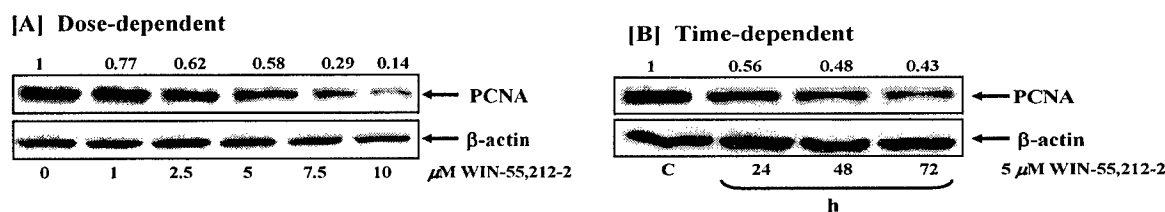


Fig 6 Effect of WIN-55,212-2 on protein expression of PCNA in LNCaP cells. (A), dose-dependent effect, (B), time-dependent effect. As detailed in "Materials and Methods" the cells were treated with DMSO alone or specified concentrations of WIN-55,212-2 in DMSO and then harvested. Total cell lysates were prepared and 30 μ g protein was subjected to SDS-PAGE, followed by immunoblot analysis and chemiluminescence detection. The values above the figures represent change as compared to vehicle treatment, in protein expression of the bands normalized to β -actin. The data shown here are from a representative experiment repeated three times with similar results.

(7) Effect of WIN-55,212-2 on Vascular Endothelial Growth Factor (VEGF)

Because VEGF is a marker for angiogenesis it is considered that blocking the angiogenic process may represent a promising way of treating tumor. Studies have shown that androgen regulates VEGF content in prostate cancer (21). As WIN-55,212-2 treatment resulted in a decrease in AR expression the effects on VEGF were also determined. It was observed that WIN-55,212-2 treatment also resulted in a decrease in VEGF protein expression (Fig 7A). Densitometric analysis data showed a decrease of 40% at 7.5 μ M concentration of WIN-5,212-2. In time-dependent study at 5 μ M WIN-55,212-2 treatment VEGF protein expression decreased in a time-dependent manner (Fig 7B).

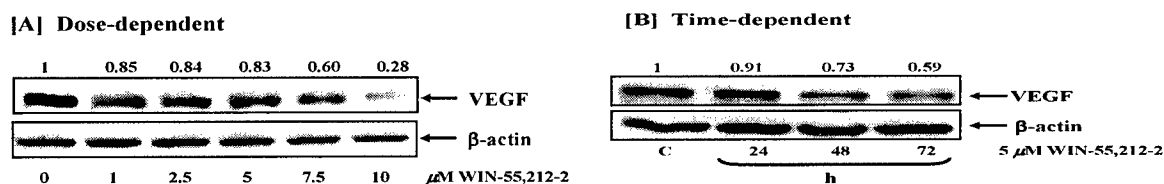


Fig 7 Effect of WIN-55,212-2 on protein expression of VEGF in LNCaP cells. (A), dose-dependent effect, (B), time-dependent effect. As detailed in "Materials and Methods" the cells were treated with DMSO alone or specified concentrations of WIN-55,212-2 in DMSO and then harvested. Total cell lysates were prepared and 30 μg protein was subjected to SDS-PAGE, followed by immunoblot analysis and chemiluminescence detection. The values above the figures represent change as compared to vehicle treatment, in protein expression of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results.

Key Research Accomplishments

- (i) We show that expression levels of both cannabinoid receptors CB₁ and CB₂ are significantly higher in CA-HPV-10 (virally transformed cells, derived from adenocarcinoma of human prostate tissue), and other human prostate cells LNCaP, DUI45, PC3, and CWR22Rv1 than in human prostate epithelial and PZ-HPV-7 (virally transformed cells, derived from normal human prostate tissue) cells.
- (ii) We show that WIN-55,212-2 (mixed CB₁/CB₂ agonist) treatment to androgen-responsive LNCaP cells resulted in a dose (1-10 μM) and time-dependent (24-48 h) inhibition of cell growth.
- (iii) We show that blocking of CB₁ and CB₂ receptors by their antagonists SR141716 (CB₁) and SR144528 (CB₂) significantly prevented the growth inhibitory effects exerted by WIN-55,212-2.
- (iv) These data suggest that both CB₁ and CB₂ receptors may be involved in WIN-55,212-2 mediated growth inhibition and apoptosis.
- (v) Our results suggest that WIN-55,212-2 or other non-habit forming cannabinoid receptor agonists could be developed as novel therapeutic agents for the treatment of prostate cancer.

Reportable Outcomes

Work described in this report is recently published in "**Cancer Research**" journal. This paper featured as a **PRIORITY REPORT** in the March 1, (65): 5, 1635-1641, 2005 issue (appendix attached).

Conclusions

It is now well accepted that uncontrolled cellular growth, which may be a result of defects in cell cycle and apoptotic machinery, is responsible for the development of most of the cancers including prostate cancer. Thus the agents which can modulate apoptosis in cancer cells may be able to affect the steady-state cell population and be useful in the management and therapy of

cancer. Consistent with this notion, there is a need to develop novel targets and mechanism-based agents for the management of prostate cancer. One of the most exciting and promising areas of current cannabinoid research is the ability of these compounds to control the cell survival/death decision

Our results suggest that treatment of androgen responsive human prostate carcinoma LNCaP cells resulted in a decrease in intracellular and secreted levels of PSA, with concomitant inhibition of AR, cell growth and induction of apoptosis. We conclude that cannabinoids should be considered as agents for the management of prostate cancer. If our hypothesis is supported by in vivo experiments then long term implications of our work could be to develop non-habit forming cannabinoid agonist (s) for the management of prostate cancer.

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Appendix 1

Cannabinoid Receptor as a Novel Target for the Treatment of Prostate Cancer

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Abstract

Cannabinoids, the active components of *Cannabis sativa* Linnaeus (marijuana) and their derivatives have received renewed interest in recent years due to their diverse pharmacologic activities such as cell growth inhibition, anti-inflammatory effects and tumor regression. Here we show that expression levels of both cannabinoid receptors, CB₁ and CB₂, are significantly higher in CA-human papillomavirus-10 (virally transformed cells derived from adenocarcinoma of human prostate tissue), and other human prostate cells LNCaP, DU145, PC3, and CWR22Rv1 than in human prostate epithelial and PZ-HPV-7 (virally transformed cells derived from normal human prostate tissue) cells. WIN-55,212-2 (mixed CB₁/CB₂ agonist) treatment with androgen-responsive LNCaP cells resulted in a dose- (1-10 μ mol/L) and time-dependent (24-48 hours) inhibition of cell growth, blocking of CB₁ and CB₂ receptors by their antagonists SR141716 (CB₁) and SR144528 (CB₂) significantly prevented this effect. Extending this observation, we found that WIN-55,212-2 treatment with LNCaP resulted in a dose- (1-10 μ mol/L) and time-dependent (24-72 hours) induction of apoptosis (a), decrease in protein and mRNA expression of androgen receptor (b), decrease in intracellular protein and mRNA expression of prostate-specific antigen (c), decrease in secreted prostate-specific antigen levels (d), and decrease in protein expression of proliferation cell nuclear antigen and vascular endothelial growth factor (e). Our results suggest that WIN-55,212-2 or other non-habit-forming cannabinoid receptor agonists could be developed as novel therapeutic agents for the treatment of prostate cancer. (Cancer Res 2005; 65(5): 1635-41)

Introduction

Because prostate cancer has become the most common cancer diagnosed in men, developing novel targets and mechanism-based agents for its treatment has become a challenging issue. In recent years cannabinoids, the active components of *Cannabis sativa* Linnaeus (marijuana) and their derivatives have drawn renewed attention because of their diverse pharmacologic activities such as cell growth inhibition, anti-inflammatory effects, and tumor regression (1-5). Cannabinoids have been shown to induce apoptosis in gliomas (6), PC-12 pheochromocytoma (7), CHP 100 neuroblastoma (8), and hippocampal neurons (9) *in vitro*, and most interestingly, regression of C6-cell gliomas *in vivo* (10). Further interest in cannabinoid research came from the discovery

of specific cannabinoid systems and the cloning of specific cannabinoid receptors (10). These diversified effects of cannabinoids are now known to be mediated by the activation of specific G protein-coupled receptors that are normally bound by a family of endogenous ligands, the endocannabinoids (11, 12). Two different cannabinoid receptors have been characterized and cloned from mammalian tissues: the "central" CB₁ receptor (13), and the "peripheral" CB₂ receptor (14).

In the present study, we show for the first time that expression levels of both cannabinoid receptors, CB₁ and CB₂, are higher in human prostate cancer cells than in normal cells. Importantly, we also show that WIN-55,212-2 (CB₁/CB₂ agonist) treatment with androgen-responsive LNCaP cells results in a dose- and time-dependent inhibition of cell growth with a concomitant induction of apoptosis, decrease in protein and mRNA expression of androgen receptor and prostate-specific antigen (PSA), decrease in secreted PSA levels, protein expression of proliferating cell nuclear antigen (PCNA), and vascular endothelial growth factor (VEGF). We suggest that cannabinoid receptor agonists may be useful in the treatment of human prostate cancer.

Materials and Methods

Materials. R-(+)-WIN-55,212-2 (2,3 dihydro-5-methyl-3 [(morpholinyl)methyl]pyrrolo (1,2,3 de)-1,4-benzoxazinyl]-[1-naphthalenyl]methanone, C₂₇H₂₆N₂O₃·CH₃SO₃H was purchased from Sigma (St. Louis, MO). CB₁ receptor antagonist SR141716 (SR1) and CB₂ receptor antagonist SR144528 (SR2) were procured from Dr. Herbert H. Seltzman (National Institute on Drug Abuse, Division of Neuroscience and Behavioral Research, through RTI International, Research Triangle Park, NC). DMEM and fetal bovine serum were procured from Life Technologies, Invitrogen Corporation (Grand Island, NY). Human PSA ELISA kit from Yes Biotech Laboratories (Ontario, Canada) and annexin-V-FLUOS staining kit were from Roche Diagnostic Corporation (Indianapolis, IN). Antibiotics (penicillin and streptomycin) used were obtained from Cellgro Mediatech, Inc. (Herndon, VA). Apo-Direct kit for measuring apoptosis by flow cytometry was procured from Apo-Direct (San Diego, CA). RNA isolation kit was from Qiagen, Inc. (Valencia, CA). Monoclonal antibodies for PSA, androgen receptor, PCNA, and VEGF, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-mouse secondary horseradish peroxidase conjugate was obtained from Amersham Biosciences Limited (Buckinghamshire, United Kingdom). Protein was estimated using bicinchoninic acid protein assay kit obtained from Pierce (Rockford, IL).

Cell Culture. The LNCaP, DU145, PC-3, CWR22Rv1, CA-HPV-10, and PZ-HPV-7 cells were obtained from American Type Culture Collection (Manassas, VA). LNCaP and DU145 cells were cultured in DMEM supplemented with 5% heat-inactivated fetal bovine serum and 1% antibiotic penicillin and streptomycin. PC-3 and CWR22Rv1 cells were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum and 1% antibiotic penicillin and streptomycin. CA-HPV-10 and PZ-HPV-7 were grown in keratinocyte serum-free medium (17005-042, Life Technologies) supplemented with 5 ng/mL human recombinant EGF and 50 μ g/mL bovine pituitary extract. Human prostate epithelial cells (PrEC) were obtained from Cambrex Bioscience (Walkersville, MD) and grown in prostate epithelial basal cell medium (Cambrex Bioscience)

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according to the manufacturer's instructions. The cells were maintained under standard cell culture conditions at 37°C and 5% CO₂ in a humid environment.

Treatment of Cells. WIN-55,212-2 (dissolved in DMSO), was used for the treatment of cells. The final concentration of DMSO used was 0.1% (v/v) for each treatment. For dose-dependent studies, cells were treated with WIN-55,212-2 at final concentrations of 1.0, 2.5, 5.0, 7.5, and 10.0 $\mu\text{mol/L}$ for 24 hours in complete cell medium, whereas for time-dependent studies, the cells (50-60% confluent) were treated with 5 $\mu\text{mol/L}$ dose of WIN-55,212-2 for 24, 48, and 72 hours. For time-dependent study, we included a control treated with DMSO for 72 hours because it was the longest time point post-WIN-55,212-2 treatment in our experimental protocol. To establish the role of CB₁ and CB₂ receptors in WIN-55,212-2-induced inhibitory effects, two experiments were done. In the first experiment, cells were treated with 2 $\mu\text{mol/L}$ of SR141716 or SR144528 alone for 24 hours. In the second experiment, cells pretreated with each of these antagonists (2 $\mu\text{mol/L}$) for 3 hours followed by incubation with 7.5 $\mu\text{mol/L}$ WIN-55,212-2 for 24 hours. In pilot experiments, it was established that DMSO (0.1% v/v) had no effects when measured at 24, 48, or 72 hours.

Cell Viability. The effect of WIN-55,212-2 on the viability of cells was determined by (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) (MTT) assay. The cells were plated at 1×10^4 cells per well in 200 μL of complete culture medium containing 1.0, 2.5, 5.0, 7.5, and 10.0 $\mu\text{mol/L}$ concentrations of WIN-55,212-2 in 96-well microtiter plates for 24 and 48 hours at 37°C in a humidified chamber. Each concentration of WIN-55,212-2 was repeated in 10 wells. After incubation for specified times at 37°C in a humidified incubator, MTT reagent (4 μL , 5 mg/mL in PBS) was added to each well and incubated for 2 hours. The microtiter plate containing the cells was centrifuged at 1,800 rpm for 5 minutes at 4°C. The MTT solution was removed from the wells by aspiration and the formazan crystals were dissolved in DMSO (150 μL). Absorbance was recorded on a microplate reader at 540 nm wavelength. The effect of WIN-55,212-2 on growth inhibition was assessed as the percentage of inhibition in cell growth where vehicle-treated cells were taken as 100% viable.

Preparation of Cell Lysates and Western Blot Analysis. Following treatment of cells with WIN-55,212-2, the medium was aspirated and the cells were washed with cold PBS [10 mmol/L (pH 7.45)]. The cells were then incubated in ice-cold lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na₃VO₄, 0.5% NP40, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (pH 7.4)), with freshly added protease inhibitor cocktail (protease inhibitor cocktail set III, Calbiochem, La Jolla, CA) over ice for 20 minutes. The cells were scraped and the lysate was collected in a microfuge tube and passed through a 21.5-gauge needle to break up the cell aggregates. The lysate was cleared by centrifugation at $14,000 \times g$ for 15 minutes at 4°C, and the supernatant (total cell lysate) was collected, aliquoted, and used on the day of preparation or immediately stored at -80°C for use at a later time. For Western blotting, 25 to 50 μg of protein were resolved over 12% polyacrylamide gels and transferred onto a nitrocellulose membrane. The nonspecific sites on blots were blocked by incubating in blocking buffer [5% nonfat dry milk/1% Tween 20 in 20 mmol/L TBS (pH 7.6)] for 1 hour at room temperature, incubated with appropriate monoclonal primary antibody in blocking buffer for 90 minutes to overnight at 4°C, followed by incubation with anti-mouse or anti-rabbit secondary antibody horseradish peroxidase conjugate and detected by chemiluminescence and autoradiography using Hyperfilm obtained from Amersham Biosciences. Densitometric measurements of the bands in Western blot analysis were done using digitalized scientific software program UN-SCAN-IT purchased from Silk Scientific Corporation (Orem, UT).

ELISA for PSA. The human PSA ELISA kit was used for the quantitative determination of PSA levels in culture medium according to the vendor's protocol. This kit uses a technique of quantitative sandwich immunoassay for determination of PSA with an estimated sensitivity of 1 ng/mL PSA antigen.

Detection of Apoptosis and Necrosis by Confocal Microscopy. The annexin-V-FLUOS staining kit was used for the detection of apoptotic and necrotic cells according to vendor's protocol. This kit uses a dual-staining protocol in which the apoptotic cells are stained with annexin-V (green fluorescence), and the necrotic cells are stained with propidium iodide (PI; red fluorescence). LNCaP cells were grown to about 70% confluence and then treated with WIN-55,212-2 (1.0, 2.5, 5.0, 7.5, and 10.0 $\mu\text{mol/L}$) for 24 hours. The fluorescence was measured by a Zeiss 410 confocal microscope (Thornwood, NY). Confocal images of green annexin-FITC fluorescence were collected using 488 nm excitation light from an argon/krypton laser, a 560 nm dichroic mirror, and a 514 to 540 nm bandpass barrier filter. Images of red PI fluorescence were collected using a 568 nm excitation light from the argon/krypton laser, a 560 nm dichroic mirror, and a 590-nm-long pass filter. In a selected field, the cells stained with annexin-V and PI as well as unstained cells were counted to ascertain the extent of apoptosis and necrosis.

Quantification of Apoptosis by Flow Cytometry. The cells were grown at density of 1×10^6 cells in 100 mm culture dishes and were treated with WIN-55,212-2 (1.0, 2.5, 5.0, 7.5, and 10.0 $\mu\text{mol/L}$ doses) for 24 hours. The cells were trypsinized, washed with PBS, and processed for labeling with fluorescein-tagged dUTP nucleotide and PI by use of an Apo-Direct apoptosis kit obtained from Phoenix Flow Systems (San Diego, CA) and was used according to the manufacturer's protocol. The labeled cells were analyzed by flow cytometry.

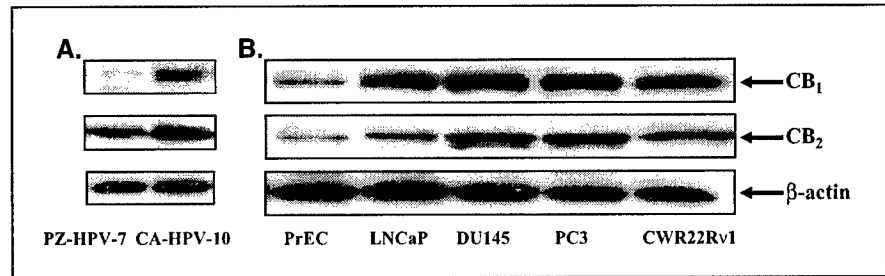
Quantitative Real-time PCR for mRNA Expression of Androgen Receptor and PSA. Total RNA was isolated from LNCaP cells using RNeasy kit according to the vendor's protocol. The ratio of optical densities of RNA samples at 260 and 280 nm was consistently >1.8. cDNA was synthesized by reverse transcription of 1 μg of extracted RNA with 200 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) in the presence of oligo dT and deoxynucleotide triphosphate (Promega). Androgen receptor and PSA were amplified using a Failsafe real-time PCR system obtained from Epicentre (Madison, WI). The thermal cycler used for amplification was an ABI-PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). Primers were designed as follows: androgen receptor forward, 5'-AAGACGCTTCTACAGCTCAC-CAA; reverse, 5'-TCCCAGAAAGGATCTTGGGCACTT; PSA forward, 5'-ACTCACAGCAAGGATGGAGCTGAA; reverse, 5'-TGAGGGTTGTCTGAGGACTTCAA. The cycler was programmed with the following conditions (a) initial denaturation at 94°C for 2 minutes, followed by 35 cycles of (b) 94°C for 40 seconds, (c) annealing of the primer template at 58°C for 40 seconds, and (d) extension at 72°C for 40 seconds.

Results

Expression of Cannabinoid Receptor in Normal and Prostate Cancer Cells. We first compared the expression levels of both cannabinoid receptors CB₁ and CB₂ in PrEC and a series of human prostate cancer cells. We also included a pair of cells, PZ-HPV-7 (virally transformed cells, derived from normal human prostate tissue) and CA-HPV-10 (virally transformed cells, derived from the adenocarcinoma of human prostate tissue) derived from the same individual. Immunoblot data shown in Fig. 1 revealed that expression of both CB₁ and CB₂ receptors was significantly higher in prostate cancer cells LNCaP, DU145, PC3, CWR22Rv1, and CA-HPV-10 as compared with normal prostate cells PZ-HPV-7 and PrEC cells. To establish the specificity of the cannabinoid receptor antibodies used in the blotting experiments, antigen preabsorption experiments were carried out. The peptides blocked anti-CB₁ and anti-CB₂ antibody binding in all cells (data not shown).

Effect of WIN-55,212-2 on Cell Viability of PrEC and LNCaP Cells. To evaluate the cell viability response of WIN-55,212-2 on PrEC and LNCaP cells, MTT assay was employed. Data in Fig. 2A shows that treatment of PrEC cells with WIN-55,212-2 (1-10 $\mu\text{mol/L}$) for 24 and 48 hours had no effect on cell viability (Fig. 2A). However,

Figure 1. Western blot analysis of CB₁ and CB₂ cannabinoid receptor expression in normal and human prostate cancer cells. **A**, a pair of normal (PZ-HPV-7) and prostate cancer cells (CA-HPV-10) obtained from the same individual; **B**, PrEC (normal prostate epithelial cells) and prostate cancer cells, LNCaP, DU145, PC-3, and CWR22Rv1. Total cell lysates were prepared and 30 μ g of protein were subjected to SDS-PAGE, followed by immunoblot analysis and chemiluminescence detection. β -Actin was used as a loading control. For other details, see Materials and Methods.



treatment of LNCaP cells with similar doses of WIN-55,212-2 in a dose-dependent manner significantly decreased the viability of LNCaP cells at 24 and 48 hours (Fig. 2A). The IC₅₀ for inhibition of cell viability at 24 and 48 hours was 6.0 and 5.0 μ mol/L, respectively.

CB₁ and CB₂ receptor activation signals growth inhibition in LNCaP cells. To study the possible implication of CB₁ and CB₂ receptors in WIN-55,212-2-induced cell death, the effect of their antagonists were evaluated using MTT assay. Cells pretreated with 2 μ mol/L of SR141716 (CB₁ antagonist) or SR144528 (CB₂ antagonist) had no effect on cell viability but exhibited significant protective effect when coadministered with WIN-55,212-2 (7.5 μ mol/L) at a molar ratio of 1:3.75 (Fig. 2B). These data suggest that both CB₁ and CB₂ receptors may be involved in WIN-55,212-2-mediated growth inhibition and apoptosis.

Effect of WIN-55,212-2 on Apoptosis and Necrosis of LNCaP Cells. We next assessed whether the cell growth inhibitory effect of WIN-55,212-2 was associated with induction of apoptosis. The induction of apoptosis by WIN-55,212-2 was evident from the analysis of the data obtained by confocal microscopy after labeling the cells with annexin-V (Fig. 2B). This method was used because it identifies the apoptotic (green fluorescence) as well as necrotic (red fluorescence) cells. As shown by the data, WIN-55,212-2 treatment resulted in a dose-dependent apoptosis in LNCaP cells. In a time-dependent study with a 5 μ mol/L dose of WIN-55,212-2, there was an increasing trend of apoptotic cells at 72 compared with 48 hours after treatment (Fig. 2C). We next quantified the extent of apoptosis by flow cytometric analysis of the cells labeled with DUTP and PI. LNCaP cells were treated with WIN-55,212-2 (1-10 μ mol/L) for 24 hours. As shown by the data in Fig. 2D, WIN-55,212-2 treatment resulted in 18.3% and 25.6% of apoptotic cells at a dose of 7.5 and 10 μ mol/L, respectively. Whereas the induction of apoptosis was almost negligible at the lowest dose (1.0 μ mol/L) used, the highest dose employed (10 μ mol/L) resulted in a massive induction of apoptosis as determined by flow cytometry. A similar trend was evident when apoptosis was measured by ladder formation on agarose gel electrophoresis (data not shown).

Effect of WIN-55,212-2 on Androgen Receptor and PSA Protein and mRNA Expression in LNCaP Cells. Androgens are involved in the development and progression of prostate cancer where androgen receptor is assumed to be the essential mediator for androgen action (15, 16). In the next series of experiments, we determined the effect of WIN-55,212-2 on protein and mRNA expression of androgen receptor. In a dose-dependent study, we found that treatment of LNCaP cells with WIN-55,212-2 resulted in a marked decrease in androgen receptor protein expression (Fig. 3A). Relative density data of these immunoblots revealed that the decrease in androgen receptor protein expression was 50% and 90% at 5.0 and 7.5 μ mol/L of WIN-55,212-2, respectively.

In a time-dependent study with 5 μ mol/L dose of WIN-55,212-2, there was a marked decrease in androgen receptor protein expression and this corresponded with the relative density data showing a decrease of 61% and 69% at 48 and 72 hours, respectively (Fig. 3B). Studies have also shown that modulation in androgen receptor leads to alteration in androgen-responsive genes (17). PSA is an androgen-responsive gene and is regarded as the most sensitive biomarker and screening tool for prostate cancer in humans (18). The dose-dependent effect of WIN-55,212-2 on LNCaP cells showed a significant decrease in PSA protein expression at 5.0, 7.5, and 10 μ mol/L concentrations when assessing at 24 hours post-treatment (Fig. 3A). Densitometric analysis data revealed that the decrease was 48%, 75%, and 90% at 5.0, 7.5, and 10.0 μ mol/L concentrations (Fig. 3A). For time-dependent studies, cells were treated with 5 μ mol/L of WIN-55,212-2 for 24, 48, and 72 hours. Employing Western blot analysis, we found a significant decrease in a time-dependent manner in PSA protein expression. Densitometric analysis revealed a decrease in PSA protein expression by 48% and 60% at 48 and 72 hours, respectively (Fig. 3B). We also evaluated the effect of WIN-55,212-2 on mRNA levels of androgen receptor and PSA. As shown by the real time-PCR analysis data, there was an inhibition in mRNA levels of androgen receptor (Fig. 3C) and PSA (Fig. 3D) at 7.5 and 10.0 μ mol/L concentrations.

We next examined the effect of WIN-55,212-2 on secreted levels of PSA in LNCaP cells. Employing ELISA technique, we found that treatment of LNCaP cells with WIN-55,212-2 resulted in a dose-dependent decrease in the secreted levels of PSA by 30%, 53%, and 62% at 5.0, 7.5, and 10 μ mol/L, respectively. At similar doses of WIN-55,212-2, but varying the time point by 48 hours, PSA levels decreased by 53%, 77%, and 80% (Fig. 3E). Furthermore, at 72 hours post-treatment of WIN-55,212-2, secreted PSA levels decreased by 58%, 82%, and 88% (Fig. 3E). From these data, it seems that the decrease in LNCaP cell growth was concomitant with a decrease in androgen receptor protein expression as well as a decrease in both intracellular and secreted PSA levels.

Effect of WIN-55,212-2 on Cell Proliferation Marker, PCNA. We next determined the effect of WIN-55,212-2 on PCNA which serves as a requisite auxiliary protein for DNA polymerase δ -driven DNA synthesis and is cell-regulated (19, 20). The dose-dependent study treatment of LNCaP cells with WIN-55,212-2 (1-10 μ mol/L) resulted in a significant decrease in protein expression of PCNA. Western blot analysis and relative density of these bands showed that the decrease in protein expression of PCNA was 71% at 7.5 μ mol/L WIN-55,212-2 (Fig. 4A). In a time-dependent study, treatment of LNCaP cells with 5 μ mol/L WIN-55,212-2 resulted in >50% inhibition in PCNA protein expression at 48 and 72 hours of treatment (Fig. 4B).

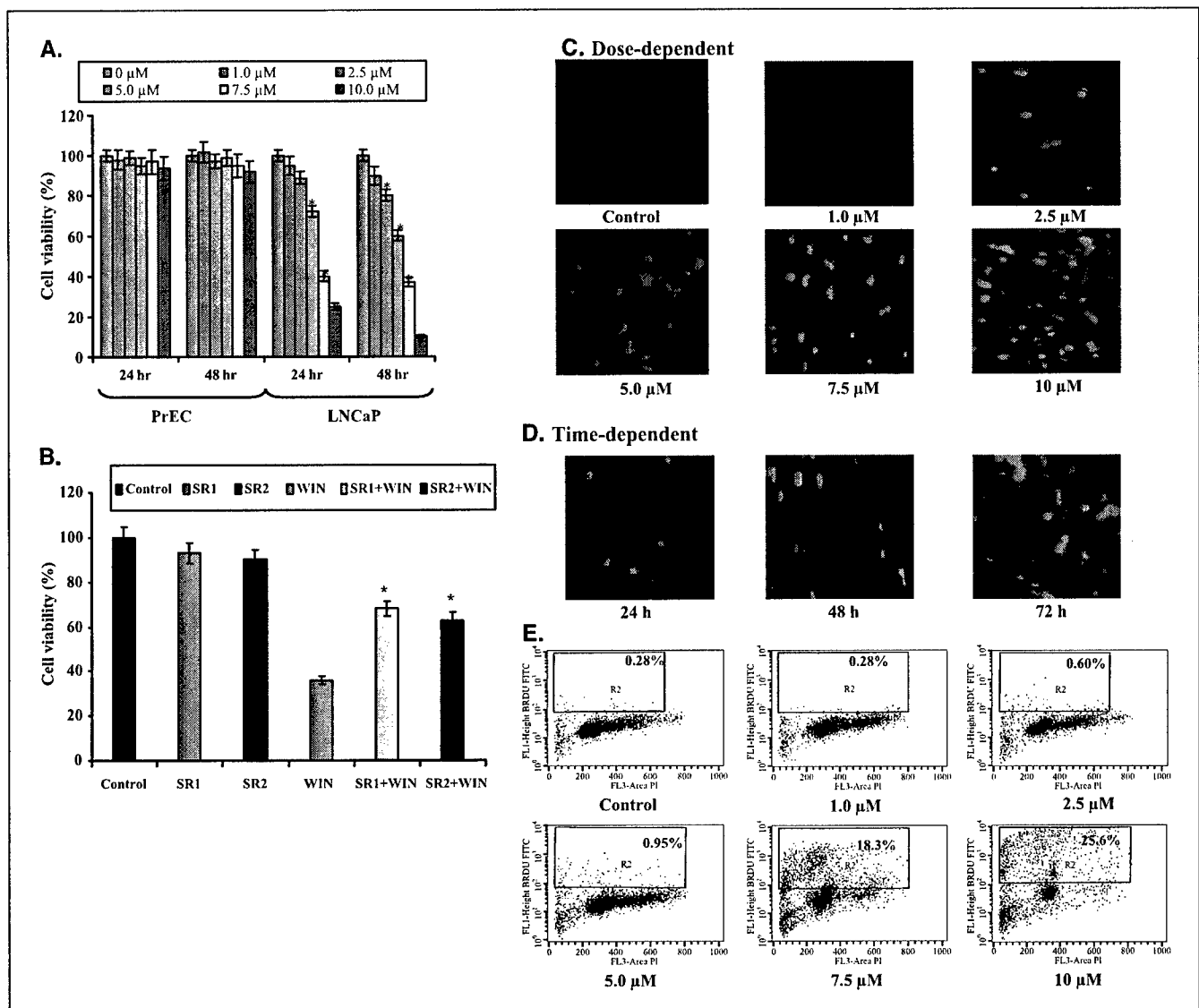


Figure 2. Effect of WIN-55,212-2 on cell viability and apoptosis in LNCaP cells. *A*, effects on the viability of PrEC and LNCaP cells. As detailed in Materials and Methods, the cells were treated with WIN-55,212-2 (1–10 $\mu\text{mol/L}$) for 24 and 48 hours, and their viability was determined by MTT assay. Columns, means; bars, $\pm$ SE of three separate experiments in which each treatment was done in 10 wells; *, $P < 0.001$ compared with control (0 $\mu\text{mol/L}$). *B*, effects of CB₁ receptor antagonist SR141716 and CB₂ receptor antagonist SR144528 on WIN-55,212-2-induced cell viability. As detailed in Materials and Methods, cells were treated with 2 $\mu\text{mol/L}$ of SR141716 or SR144528 alone for 24 hours. In another parallel set, cells were pretreated with each of these antagonists for 3 hours, followed by incubation with 7.5 $\mu\text{mol/L}$ WIN-55,212-2 for 24 hours and their viability was determined by MTT assay. Columns, means; bars, $\pm$ SE of three separate experiments in which each treatment was done in 10 wells; *, $P < 0.001$ compared with WIN. *C* and *D*, induction of apoptosis by confocal microscopy, cells were treated with WIN-55,212-2 (1–10 $\mu\text{mol/L}$) for 24 hours for the dose-dependent study (*C*), and WIN-55,212-2 (5 $\mu\text{mol/L}$) for 24, 48, and 72 hours for the time-dependent study (*D*). The annexin-V-FLUOS staining kit was used for the detection of apoptotic and necrotic cells. This kit uses a dual-staining protocol in which apoptotic cells are stained with annexin-V (green fluorescence) and necrotic cells are stained with PI (red fluorescence). *E*, quantification of apoptosis by flow cytometry. The cells were treated with WIN-55, 212-2 (1–10 $\mu\text{mol/L}$) for 24 hours, labeled with dUTP using terminal deoxynucleotidyl transferase and PI. Cells showing fluorescence (R2) are considered as apoptotic cells and their percentage population is indicated. Data from representative experiments repeated thrice with similar results.

Effect of WIN-55,212-2 on VEGF. Because VEGF is a marker for angiogenesis, blocking the angiogenic process may represent a promising way of treating the tumor. Studies have shown that androgen regulates VEGF content in prostate cancer (21). As WIN-55,212-2 treatment resulted in a decrease in androgen receptor expression, the effects on VEGF were also determined. It was observed that WIN-55,212-2 treatment also resulted in a decrease in VEGF protein expression (Fig. 4A). Densitometric analysis data showed a decrease of 40% at 7.5 $\mu\text{mol/L}$ concentration of WIN-55,212-2. In a time-dependent study at

5 $\mu\text{mol/L}$ WIN-55,212-2 treatment, VEGF protein expression decreased in a time-dependent manner (Fig. 4B).

Discussion

It is now well accepted that uncontrolled cellular growth, which may be a result of defects in cell cycle and apoptotic machinery, is responsible for the development of most of the cancers including prostate cancer. Thus, the agents which can modulate apoptosis in cancer cells may be able to affect the

steady-state cell population and may be useful in the management and therapy of cancer. Consistent with this notion, there is a need to develop novel targets and mechanism-based agents for the management of prostate cancer. One of the most exciting and promising areas of current cannabinoid research is the ability of these compounds to control the cell survival/death decision (1). In this study, we found that compared with PrEC and PZ-HPV-7 cells, the expression levels of both cannabinoid receptors CB₁ and CB₂ were significantly higher in CA-HPV-10 and other human prostate cells LNCaP, DU145, PC-3, and CWR22Rv1. These data suggest that CB₁ and CB₂ receptors could be a target for novel treatment options for prostate cancer. We also found that mixed CB₁/CB₂ agonist WIN-55,212-2 treatment of LNCaP cells resulted in a decrease of cell viability as determined by MTT assay at varying doses and time points (Fig. 2A), suggesting the involvement of both CB₁ and CB₂ in the antiproliferative action

of cannabinoids (Fig. 2B). It is widely recognized that apoptosis is an ideal way of elimination of cancer cells and that selective apoptotic events could provide suitable targets for cancer treatment and prevention. In this study, we also observed an increase in apoptosis of LNCaP cells by treatment with WIN-55,212-2. This observation was confirmed by employing confocal microscopy (Fig. 2C and D) and flow cytometry (Fig. 2E). This could be an important observation which might be useful for devising strategies for the management of human prostate cancer because apoptosis is a physiological and discrete way of cell death different from necrotic cell death and is regarded to be an ideal way of cell elimination.

Androgens are essential for the growth, differentiation, and functioning of the prostate as well as in increasing prostate cancer development (22, 23). Many molecular mechanisms have been suggested for the development of recurrent hormone refractory

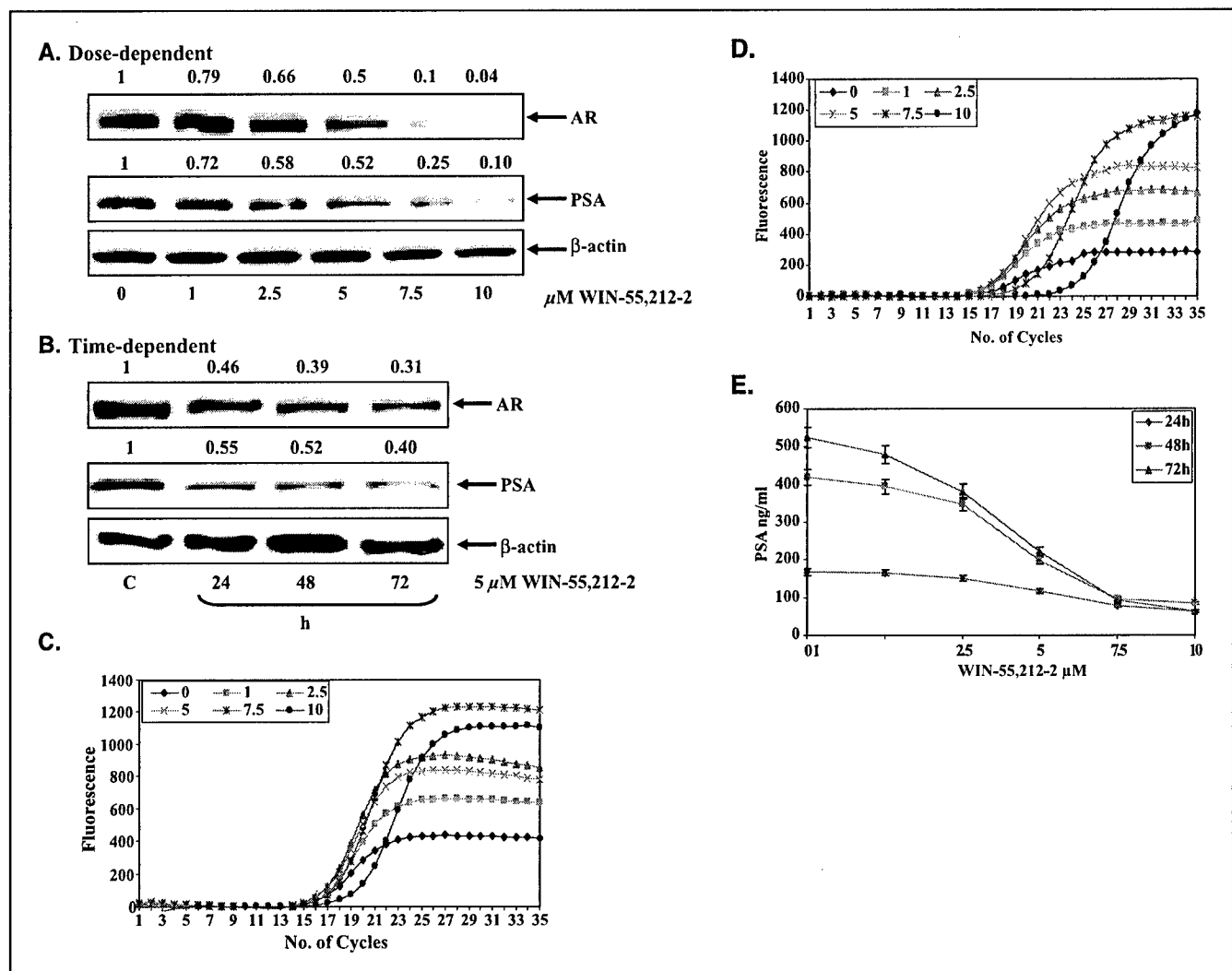


Figure 3. Effect of WIN-55,212-2 on protein and mRNA expression of androgen receptor and PSA in LNCaP cells. *A*, dose-dependent effect; and *B*, time-dependent effect. As detailed in Materials and Methods, the cells were treated with DMSO alone or with specified concentrations of WIN-55,212-2 in DMSO and then harvested. Total cell lysates were prepared and 30 μg of protein were subjected to SDS-PAGE, followed by immunoblot analysis and chemiluminescence detection. The values above the blots represent change as compared with vehicle treatment in protein expression of the bands normalized to β-actin. Western blot data from a representative experiment repeated thrice with similar results. *C* and *D*, effects of WIN-55,212-2 on mRNA expression of androgen receptor (*C*) and PSA (*D*) determined by real time-PCR from representative experiments repeated twice with similar results. *E*, effect on secreted levels of PSA. Cells were treated with WIN-55,212-2 (1-10 μmol/L) for 24, 48, and 72 hours and then harvested. The PSA levels were determined by ELISA as described under Materials and Methods. Points, means; bars, ± SE of three independent experiments.

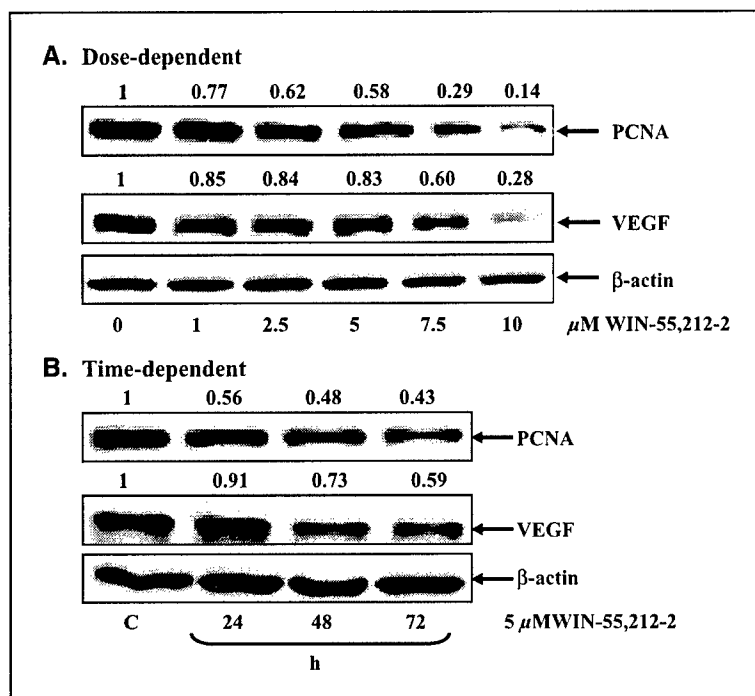


Figure 4. Effect of WIN-55,212-2 on protein expression of PCNA and VEGF in LNCaP cells. **A**, dose-dependent effect; **B**, time-dependent effect. As detailed in Materials and Methods, the cells were treated with DMSO alone or specified concentrations of WIN-55,212-2 in DMSO and then harvested. Total cell lysates were prepared and 30 μg of proteins were subjected to SDS-PAGE, followed by immunoblot analysis and chemiluminescence detection. The values above the blots represent change as compared with vehicle treatment in protein expression of the bands normalized to β-actin. Data from a representative experiment repeated thrice with similar results.

tumors. Most of these mechanisms postulate an alteration in the function of the androgen receptor and its signaling pathways (23). The overexpression of androgen receptor in prostate cancer may promote cell growth. Hence, elimination or reducing the androgen receptor in prostate cancer should help in treating this neoplastic disease. As most of the molecular mechanism for the development of prostate cancer involves modulation in the function of androgen receptor and its signaling pathways, we further studied the effect of WIN-55,212-2 on androgen receptor protein and mRNA expression and its subsequent effect on PSA production. Our results indicate that WIN-55,212-2 treatment significantly decreases androgen receptor protein (Fig. 3A) and mRNA expression (Fig. 3C) in LNCaP cells.

PSA belongs to the kallikrein family (17), is a serine protease with highly prostate-specific expression, and is the most widely employed marker in the detection of early prostate cancer. For these reasons, it is considered that agents which could reduce PSA levels may have important clinical implications for prostate cancer. Earlier studies reported that PSA is primarily regulated by androgens (24). This observation was based on the fact that the antiandrogen, cyproterone acetate, had the ability to induce PSA, and that hydroxyflutamide could block androgen and progesterone induction of PSA glycoprotein, thus suggesting that PSA glycoprotein expression is influenced predominantly by androgens via its receptor, and the mutation of the receptor can affect the expression of this gene by steroids other than androgens (24). Recent studies have established that androgen receptor functions as a transcriptional regulator via its binding to androgen response elements within promoter and enhancer regions of PSA. PSA is currently the most accepted marker for assessment of prostate cancer progression in humans and is being detected in the serum of patients with prostate diseases including prostatitis, benign prostatic hypertrophy, and prostate cancer (18). It is reported that in LNCaP cells, androgens regulate PSA glycoprotein expression and mRNA via androgen receptor (25, 26). Our studies show a significant decrease

in intracellular, mRNA (Fig. 3D), as well as secreted levels of PSA by WIN-55,212-2 treatment of cells, suggesting that cannabinoid receptor agonists may be exploited to prevent prostate cancer progression.

PCNA recognizes nuclear antigens and its overexpression is associated with increase in PSA serum levels (27). PCNA expression has significant prognostic value and it seems to be a significant biomarker in prognosis and treatment of prostate cancer (27). Our results also suggest that concomitant with the decrease in PCNA protein expression (Fig. 4A), there was a decrease in PSA serum levels following WIN-55,212-2 treatment (Fig. 3E).

VEGF is a ubiquitous cytokine that regulates embryonic vasculogenesis and angiogenesis. Normal prostate epithelium expresses low levels of VEGF, whereas premalignant lesions have increased VEGF expression, which is additionally increased in prostate carcinoma (28). Studies have shown that cannabinoid treatment markedly reduced the expression of VEGF in gliomas, the most potent proangiogenic factor and also of angiopoietin 2, which contributes to the angiogenic process by preventing vessel maturation (29). Our results showed that treatment of LNCaP with WIN-55,212-2 inhibits growth and VEGF protein expression (Fig. 4A and B).

Recently, cannabinoids have received considerable attention due to their diverse pharmacologic activities such as cell growth inhibition, anti-inflammatory effects, and tumor regression. Our results suggest that treatment of androgen-responsive human prostate carcinoma LNCaP cells resulted in a decrease in intracellular and secreted levels of PSA, with concomitant inhibition of androgen receptor, cell growth, and induction of apoptosis. We conclude that cannabinoids should be considered as agents for the management of prostate cancer. If our hypothesis is supported by *in vivo* experiments, then the long-term implications of our work could be to develop non-habit-forming cannabinoid agonist(s) for the management of prostate cancer.

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