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Cannabinoid Receptor Agonist-induced Apoptosis of Human Prostate Cancer Cells LNCaP Proceeds through Sustained Activation of ERK1/2 Leading to G_1 Cell Cycle Arrest*

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We have recently shown that the expression levels of both cannabinoid receptors CB_1 and CB_2 are higher in human prostate cancer cells than in normal prostate epithelial cells, and treatment of LNCaP cells with WIN-55,212-2 (a mixed CB_1/CB_2 agonist) resulted in inhibition of cell growth and induction of apoptosis (Sarfaraz, S., Afaq, F., Adhami, V. M., and Mukhtar, H. (2005) *Cancer Res.* 65, 1635–1641). This study was conducted to understand the mechanistic basis of these effects. Treatment of LNCaP cells with WIN-55,212-2 (1–10 μM; 24 h) resulted in: (i) an arrest of the cells in the G_0/G_1 phase of the cell cycle; (ii) an induction of p53 and p27/KIP1; (iii) down-regulation of cyclins D1, D2, E; (iii) decrease in the expression of cdk-2, -4, and -6; (iv) decrease in protein expression of pRb; (v) down-regulation of E2F (1–4); and (vi) decrease in the protein expression of DP1 and DP2. Similar effects were also observed when androgen-independent PC3 cells were treated with WIN-55,212-2 (5–30 μM). We further observed sustained up-regulation of ERK1/2 and inhibition of PI3k/Akt pathways in WIN-55,212-2-treated cells. Inhibition of ERK1/2 abrogated WIN-55,212-2-indued cell death suggesting that sustained activation of ERK1/2 leads to cell cycle dysregulation and arrest of cells in G_0/G_1 phase subsequently leading to an induction of apoptosis. Further, WIN-55,212-2 treatment of cells resulted in a dose-dependent increase in Bax/Bcl-2 ratio in such a way that favors apoptosis. The induction of apoptosis proceeded through down-regulation of caspases 3, 6, 7, and 9 and cleavage of poly (ADP-ribose) polymerases. Based on these data we suggest that cannabinoid receptor agonists should be considered as novel agents for the management of prostate cancer.

Prostate cancer (CaP)[2] ranks as the most common noncutaneous malignancy and the second leading cause of cancer-related deaths in American males, with similar trends in many Western countries. According to an estimate of the American Cancer Society, a total of 234,460 men will be diagnosed with CaP in the United States in the year 2006 and 27,350 CaP-related deaths are predicted (1). The major cause of mortality from this disease is metastasis of hormone refractory cancer cells that fail to respond to hormone ablation therapy (2, 3). Because surgery and current treatment options have proven to be inadequate in treating and controlling CaP, the search for novel targets and mechanism-based agents for prevention and treatment of this disease has become a priority.

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 (4–9). Further interest in cannabinoid research came from the discovery of the cannabinoid system and the cloning of specific cannabinoid receptors (10). Two cannabinoid receptors have been identified: the "central" CB_1 and the "peripheral" CB_2 receptor. In a recent study, we have shown that WIN 55,212-2 (Fig. 1) a mixed CB_1/CB_2 receptor agonist imparts cell growth inhibitory effects in LNCaP cells via an induction of apoptosis. An important observation of this study was that WIN 55,212-2 treatment did not result in apoptosis of the normal prostate epithelial cell at similar doses (11).

Here, we show that treatment of human prostate cancer LNCaP cells with cannabinoid receptor agonist WIN-55,212-2 resulted in an arrest of the cells in the G_0/G_1 phase of the cell cycle, and this arrest was associated with a sustained activation of ERK1/2, induction of p27/KIP1, and inhibition of cyclin D1. Blocking of both cannabinoid receptors CB_1 and CB_2 by their specific antagonist resulted in inhibition of ERK1/2 activation. Inhibition of ERK1/2 signaling by the ERK1/2 inhibitor PD98059 and its specific siRNA abrogated these effects.

EXPERIMENTAL PROCEDURES

Materials—*R*-(+)-WIN 55,212-2 (2,3 dihydro-5-methyl-3-([morpholinyl]methyl) pyrollo (1,2,3 de)-1,4-benzoxazinyl]-[1-napthaleny]methanone, $C_{27}H_{26}N_2O_3.CH_3SO_3H$ was purchased from Sigma. CB_1 receptor antagonist SR141716 (SR1) and CB_2 receptor antagonist SR144528 (SR2) were procured from Dr. Herbert H. Seltzman (NIDA, National Institutes of Health, Division of Neuroscience and Behavioral Research, through RTI International, Research Triangle Park, NC). ERK1/2 inhibitor PD98059 was purchased from Tocris Biosciences (Ellisville, MO). Dulbecco's modified Eagle's medium and fetal bovine serum were procured from Invitrogen. Antibi-

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[2] The abbreviations used are: CaP, prostate cancer; PI3K, phosphatidylinositol 3-kinase; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; Rb, retinoblastoma.

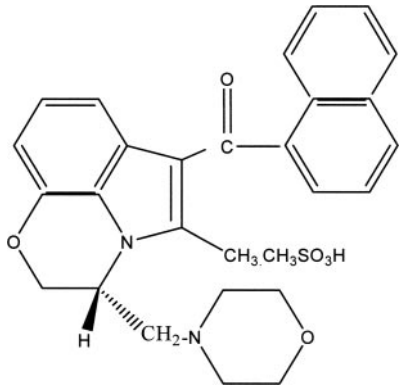

FIGURE 1. **Chemical structure of WIN-55,212-2 (2,3 dihydro-5-methyl-3 ([morpholinyl]methyl) pyrollo (1,2,3 de)-1,4-benzoxazinyl]-[1-naptha-leny] methanone.**

otics (penicillin and streptomycin) used were obtained from Cellgro Mediatech, Inc. (Herndon, VA). The mono- and polyclonal antibodies (p53, cdk2, -4, and -6, KIP1/p27, E2F-3, and DP-2) were obtained from Santa Cruz Biotechnology Inc. The human reactive monoclonal and polyclonal antibodies (cyclins D1, D2, E, pRb, E2F-1, E2F-2, E2F-4, and DP-1) were obtained from Labvision (Fremont, CA). Monoclonal and polyclonal antibodies for anti-PARP, Bcl-2 Bax were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-PARP (116 kDa) and anti-PI3K (p85) was purchased from Upstate Biotechnology and anti-PARP (p85) was purchased from Promega (Madison, WI). Anti-phospho-ERK1/2 p42/44 was purchased from Cell Signaling Technology (Beverly, MA). Anti-mouse and anti-rabbit secondary horseradish peroxidase conjugate was obtained from Amersham Biosciences. Protein was estimated using the BCA protein assay kit obtained from Pierce (Rockford, IL).

Cell Culture—LNCaP and PC3 cells obtained from ATCC (Manassas, VA) were cultured in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum and 1% antibiotic penicillin and streptomycin. PC3 cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were maintained under standard cell culture conditions at 37 °C and 5% CO_2 in a humid environment.

Treatment of Cells—WIN-55,212-2, (dissolved in Me_2SO) was used for the treatment of cells. The final concentration of Me_2SO used was 0.1% (v/v) for each treatment. For dose-dependent studies, LNCaP cells were treated with WIN-55,212-2 at 1.0, 2.5, 5.0, 7.5, 10.0 μM final concentrations for 24 h in complete cell medium. PC3 cells were treated with WIN-55,212-2 at 5.0, 10, 20, 25, and 30 μM final concentration for 24 h in complete cell medium. Control cells were treated with vehicle alone. To establish the role of CB_1 and CB_2 receptor in WIN-55,212-2 induced ERK1/2 activation cells were pretreated with 3 μM SR141716 and SR144528 alone, and in the second set, cells were pretreated with both the antagonists (3 μM each) for 3 h followed by incubation with 7.5 μM WIN-55,212-2 for 24 h. To study the role of ERK1/2 in cannabinoid receptor induced cell growth inhibition, cells were pretreated with 30 μM ERK1/2 inhibitor PD98059 for 1 h followed by incubation with 7.5 μM WIN-55,212-2 for 24 h.

Cell Viability—The cells were grown at density of 1×10^6 cells in 100-mm culture dishes and treated with WIN-55,212-2 (1–10 μM) for 24 h. The cells were trypsinized and collected in the microfuge tube. The cells were pelleted by centrifugation, and the cell pellet was resuspended in phosphate-buffered saline (PBS) (300 μl). Trypan blue (0.4% in PBS; 10 μl) was added to a smaller aliquot (10 μl) of cell suspension, and the number of cells (viable-unstained and nonviable-blue) were counted using a hemocytometer.

Quantification of Apoptosis and Cell Cycle Analysis by Flow Cytometry—The cells were grown at a 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 μM 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 the manufacturer's protocol. The labeled cells were analyzed using a FACScan benchtop cytometer (BD Biosciences, San Jose, CA) at the UWCCC Flow Cytometry Facility in the University of Wisconsin. Results were analyzed using Mod-Fit LT software (Verity Software House, Topsham, ME) for cell cycle and WinMD1 version 2.8 software for quantification of apoptosis.

Detection of Cleaved Caspase-3 by Confocal Microscopy—The cells were grown in two chambered cell culture slides (BD Biosciences), treated with WIN-55,212-2 (5.0, 7.5, 10.0 μM doses) for 24 h, washed with 1× PBS at room temperature, and were immediately fixed in cold 100% methanol at −20 °C for 10 min. Cells were blocked with blocking buffer (5.5% normal goat serum in TBST, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100) for 60 min, and were washed with TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl). Cells were then incubated with primary antibody Alexa Fluor 488 conjugate, (Cell Signaling Technology) overnight using the vendor's protocol. After incubation, cells were washed twice for 5 min with TBST and once with TBS. Coverslips were mounted using the Prolong Antifade kit obtained from Molecular Probes, (Eugene, OR). Cells were visualized with a Bio-Rad MRC1000 scan head mounted transversely to an inverted Nikon Diaphot 200 microscope at the Keck Neural Imaging Laboratory in the University of Wisconsin, Madison.

Silencing of ERK 44/42 by Small Interfering RNA—For suppressing ERK1/2 expressions, ERK1, ERK2, and control scrambled siRNA were purchased from Cell Signaling Technology (Danvers, MA). LNCaP cells were transfected with siRNAs (ERK1, 150 nmol/liter and ERK2, 80 nmol/liter, scrambled siRNA 150 nmol/liter) using the nucleofection kit R specific for LNCaP transfection from Amaxa Biosystems (Gaithersburg, MD). Cells were resuspended in a solution from nucleofector kit R following the manufacturer's guidelines. 100 μl of nucleofector solution R was mixed with 2×10^6 cells and siRNA. They were then transferred to the cuvette provided with the kit and was nucleofected with an Amaxa Nucleofector apparatus. Cells were transfected using the T-001 pulsing parameter and were transferred into 100-mm plates containing 37 °C prewarmed culture medium. After transfection, cells were cultured for 48 h, after which the medium was

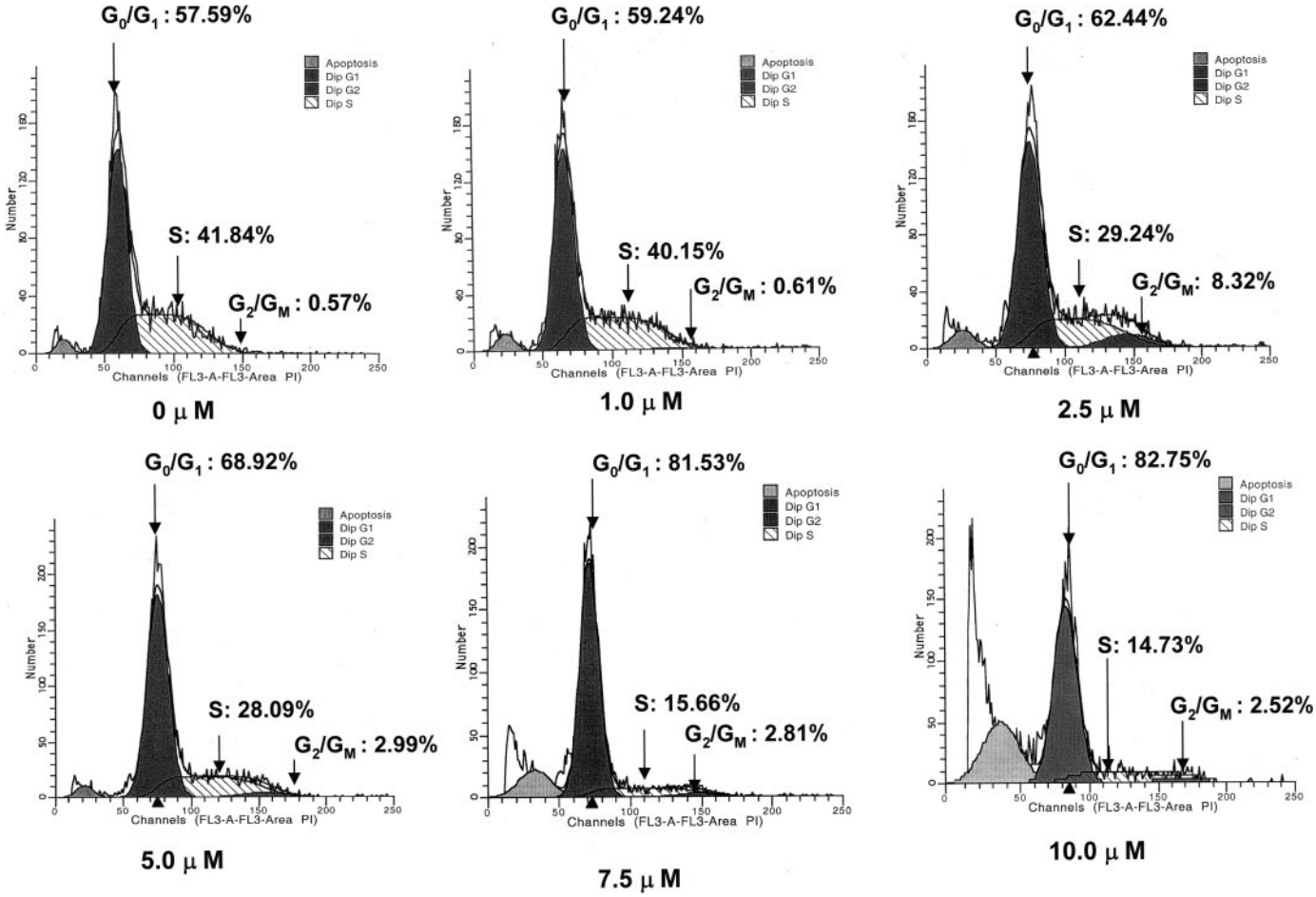

FIGURE 2. **Effect of WIN-55,212-2 treatment on cell cycle in LNCaP cells.** Cell cycle analysis was performed by flow cytometry as detailed under "Experimental Procedures." The labeled cells were analyzed using a FACScan benchtop cytometer, and the percentage of cells in the G_0–G_1, S, and G_2-M phases were calculated using ModFit LT software. The data shown here are from a typical experiment repeated three times.

replaced with fresh medium, cells were treated with 7.5 μM WIN-55,212-2 for 24 h, and protein lysates were prepared. Using 2 μg of GFP we observed 70–80% transfection efficiency with this protocol.

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/liter, pH 7.45). The cells were then incubated in ice-cold lysis buffer (50 mmol/liter Tris-HCl, 150 mmol/liter NaCl, 1 mmol/liter EGTA, 1 mmol/liter EDTA, 20 mmol/liter NaF, 100 mmol/liter Na_3VO_4, 0.5% Nonidet P-40, 1% Triton X-100, 1 mmol/liter phenylmethylsulfonyl fluoride (pH 7.4), with freshly added protease inhibitor mixture (Protease Inhibitor Mixture Set III, Calbiochem, La Jolla, CA) over ice for 20 min. The cells were scraped, and the lysate was collected in a microcentrifuge tube and passed through a 21.5-gauge needle to break up the cell aggregates. The lysate was cleared by centrifugation at 14,000 × *g* for 15 min at 4 °C, and the supernatant (total cell lysate) collected, aliquoted, and was used on the day of preparation or immediately stored at −80 °C for use at a later time. For Western blotting, 25–50 μg protein was 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/liter TBS, pH 7.6) for 1 h at room temperature, incubated with appropriate monoclonal primary antibody in blocking buffer for 90 min 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 (UK Ltd.). Densitometric measurements of the bands in Western blot analysis were performed using digitalized scientific software program UN-SCAN-IT (Silk Scientific Corporation, Orem, UT).

Statistical Analysis—Results were analyzed using a two-tailed Student's *t* test to assess statistical significance. Values of $p < 0.05$ were considered statistically significant.

RESULTS

WIN-55,212-2 Causes G_1 Phase Cell Cycle Arrest—We have earlier shown that treatment of LNCaP cells with WIN-55,212-2 (1–10 μM) for 24 h significantly decreased the cell viability and led to induction of apoptosis (11). Several studies have shown that the induction of apoptosis may be cell cycle-dependent (12–16). Therefore, in the next series of experiments, we tested the hypothesis that WIN-55,212-2-caused apoptosis of LNCaP cells is mediated via cell cycle

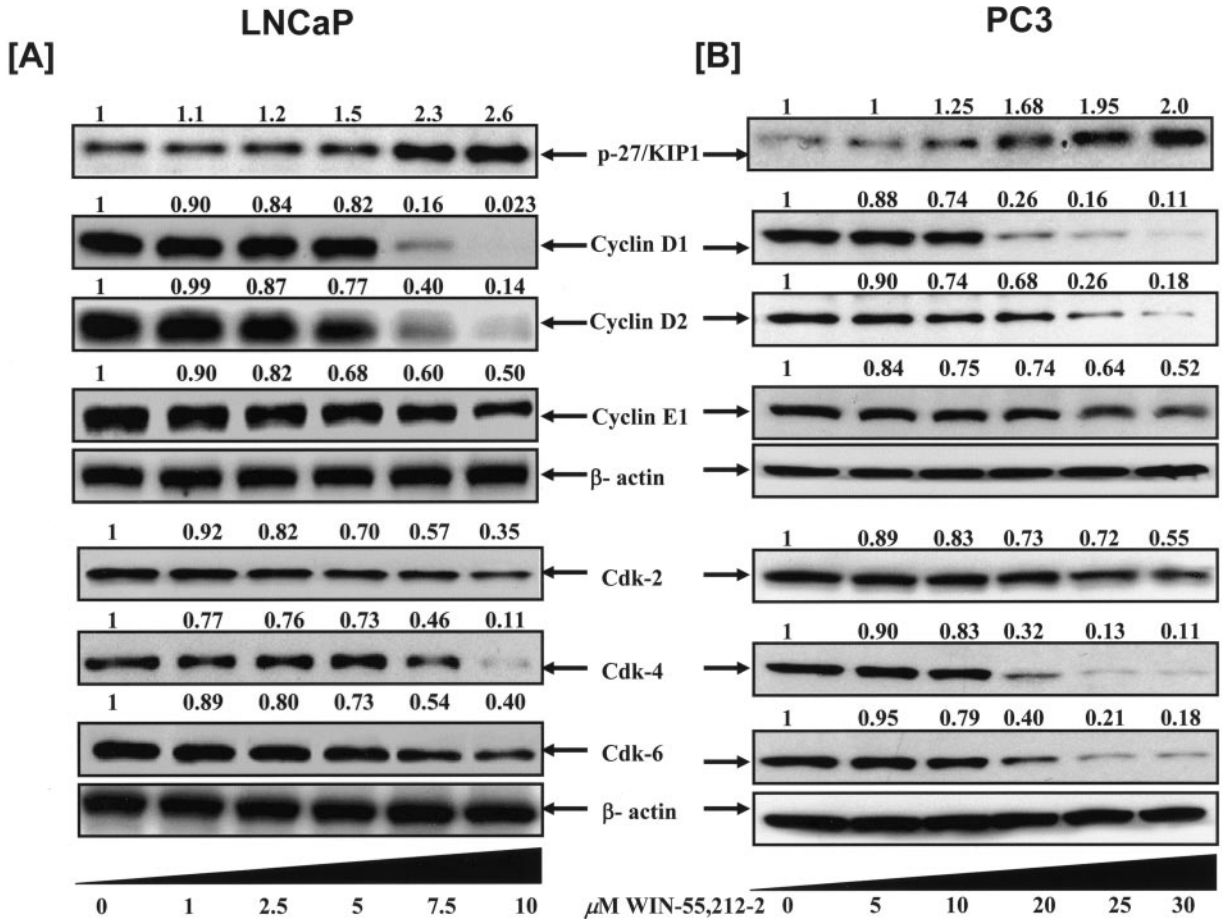

FIGURE 3. *A*, effect of WIN-55,212-2 treatment on the protein expression of KIP1/p27, cyclin D1, D2, and E and cdk 2, 4, and 6 in LNCaP cells. *B*, effect of WIN-55,212-2 treatment on the protein expression of KIP1/p27, cyclin D1, D2, and E and cdk 2, 4, and 6 in PC3 cells. As detailed under "Experimental Procedures," the cells were treated with Me_2SO alone or specified concentrations of WIN-55,212-2, and total cell lysates were prepared for immunoblot analysis. The values above the figures represent relative density of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results.

blockade. We performed DNA cell cycle analysis to assess the effect of WIN-55,212-2 treatment on the distribution of cells in the cell cycle. As shown in Fig. 2, compared with vehicle treatment, WIN-55,212-2 treatment resulted in a dose-dependent accumulation of cells in G_1 phase of the cell cycle (59, 62, 69, 81, and 83% cells in G_1 phase at 1.0, 2.5, 5.0, 7.5, and 10 μM concentrations, respectively). This observation is important because the molecular analyses of human cancers have revealed that cell cycle regulators are frequently mutated in most common malignancies (17, 18). Consistent with this observation, in recent years, inhibition of the cell cycle has been appreciated as a target for the management of cancer (19, 20).

WIN-55,212-2-induced Cell Cycle Arrest Is Mediated via an Induction of KIP1/p27 and Concomitant Inhibition in Cyclins D1, D2, E, and Cdk2, Cdk4, and Cdk6—Because our studies demonstrated that WIN-55,212-2 treatment of cells resulted in a G_1-phase cell cycle arrest and apoptosis, we examined the effect of WIN-55,212-2 on cell cycle regulatory molecules operative in G_1 phase of the cell cycle. Studies have shown a critical role of p27/KIP1 in apoptosis and cell cycle progression through G_0–G_1 phase (21–23). We observed a significant induction of p27/KIP1 by WIN-55,212-2 at 5–10 μM doses. (Fig. 3*A*). Relative density data revealed 1.5-, 2.3-, and 2.6-fold increases in the protein expression of Kip/p27 at 5.0, 7.5, and 10 μM concentrations of WIN-55,212-2, respectively. Using immunoblot analysis, we also assessed the effect of WIN-55,212-2 treatment on the protein expression of the cyclins and cdks, which are known to be regulated by KIP1/p27. WIN-55,212-2 treatment of the cells resulted in a dose-dependent decrease in protein expression of cyclin D1, cyclin D2, and cyclin E (Fig. 3*A*) as well as cdk2, cdk4, and cdk6 (Fig. 3*A*). Densitometric analysis data of cyclins revealed a significant decrease in the expression of cyclin D1 (84%, 97%), cyclin D2 (60%, 86%), and cyclin E (40%, 50%) at 7.5 and 10.0 μM concentrations of WIN-55,212-2, respectively (Fig. 3*B*). Relative density data of cdks also revealed a significant decrease in the expression of cdk2 (43%, 65%), cdk4 (54%, 89%), and cdk6 (46%, 60%) at similar doses of WIN-55,212-2. In the next series of experiments we assessed the effect of WIN-55,212-2 on p27/KIP1, cyclin and cdk in androgen-insensitive cell PC3. Cells were treated with different doses of WIN-55,212-2 (5, 10, 20, 25, 30 μM), and we found an induction in p27/KIP1 and down-regulation in the protein expression of cyclin and cdk particularly at doses of 20–30 μM (Fig. 3*B*).

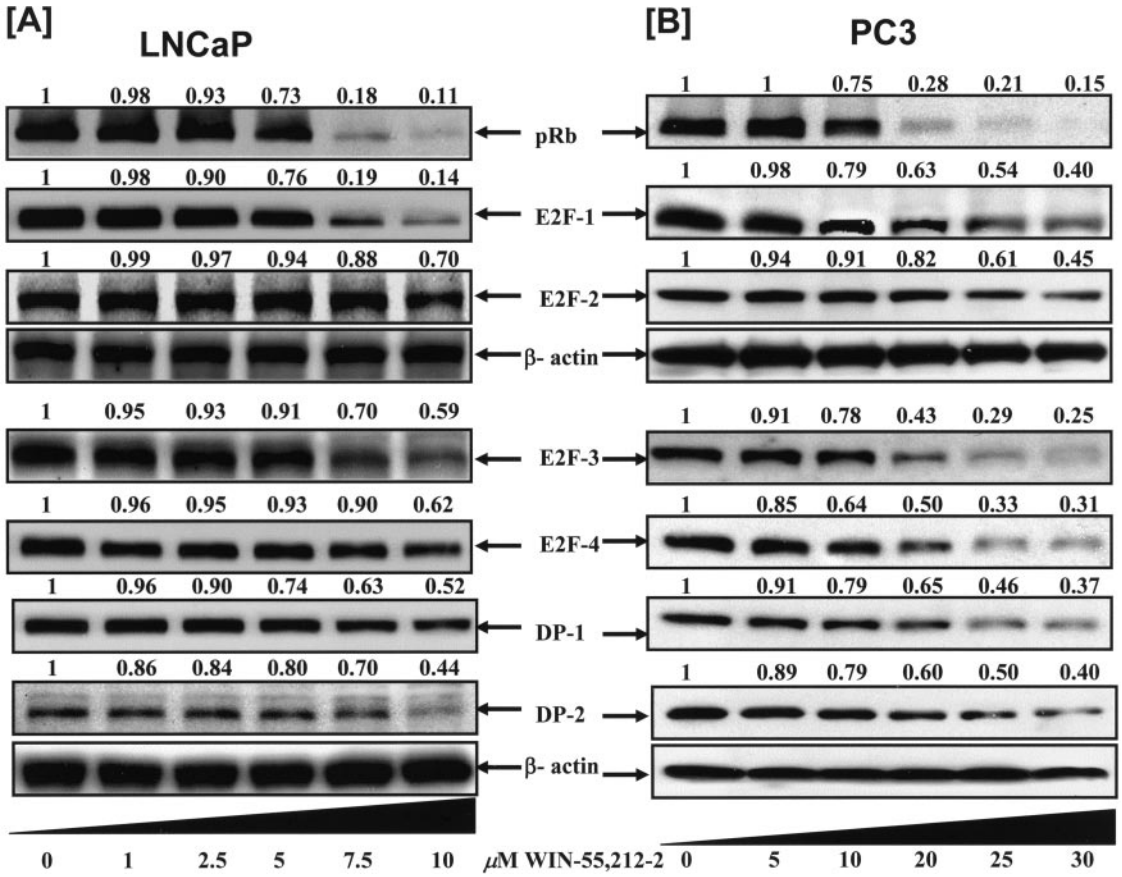

FIGURE 4. *A*, effect of WIN-55,212-2 treatment on protein expression of pRb, E2F (1–4), DP1, and DP2 in LNCaP cells. *B*, effect of WIN-55,212-2 treatment on protein expression of pRb, E2F (1–4), DP1 and DP2 in PC3 cells. As detailed under "Experimental Procedures," the cells were treated with Me_2SO alone or specified concentrations of WIN-55,212-2, and total cell lysates were prepared for immunoblot analysis. The values above the figures represent relative density of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results.

WIN-55,212-2 Inhibits Protein Expression of pRB, E2F, and DP—Down-regulation of cdk4/6 has been shown to be associated with a decrease in the expression of retinoblastoma (pRb) tumor suppressor protein a key regulator of the $G_1 \rightarrow S$ phase transition in the cell cycle (24, 25). Therefore, we next examined the effect of WIN-55,212-2 on protein expression of pRb. Immunoblot data revealed that WIN-55,212-2 treatment of cells resulted in a significant decrease in the protein expression of pRb. Densitometric analysis of immunoblots showed 27, 82, and 89% inhibition at 5.0, 7.5, and 10 μM concentrations of WIN-55,212-2 (Fig. 4*A*). Because pRb controls cell cycle by binding to and inhibiting the E2F transcription factors, we determined the protein expression of E2F (1–4) transcription factors. As shown in Fig. 4*A*, WIN-55,212-2 treatment of cells resulted in a dose-dependent decrease in E2F transcription factors. Relative density data revealed an inhibition in E2F-1 (81 and 86%), E2F-2 (12 and 30%), E2F-3 (30 and 41%), and E2F-4 (10 and 38%) at a concentration of 7.5 and 10 μM WIN-55,212-2. Because the activity of E2F is known to be dependent on its heterodimeric association with members of DP family of proteins, we also evaluated the effect of WIN-55,212-2 treatment on both members of DP family viz. DP-1 and DP-2. Immunoblot and densitometric analysis data revealed a decrease in the protein expression of DP-1 (37 and 48%) and DP-2 (30 and 56%) at 7.5 and 10 μM concentration of WIN-55,212-2 (Fig. 4*A*). In the next series of experiments we assessed the effect of WIN-55,212-2 on pRB, E2F family of proteins (1–4) and its heterodimeric partners DP-1 and DP-2 in androgen-insensitive cell PC3. Cells were treated with different doses of WIN-55,212-2 (5, 10, 20, 25, 30 μM), and we found a decrease in the protein expression of pRB, E2F (1–4), DP-1, and DP-2 at 20–30 μM doses (Fig. 3*B*).

WIN-55,212-2-induced Sustained Activation of ERK and Inhibition of PI3K/AKT Leads to Apoptosis through Cannabinoid Receptors—It has been reported that challenging gliomas with cannabinoids leads to the activation of ERK1/2 signaling and AKT inhibition (26, 4). This sustained ERK1/2 activation can mediate cell cycle arrest (8). We observed a significant and sustained activation of ERK1/2 and significant inhibition of PI3K (p85) and AKT (Thr^{308}) when LNCaP cell were treated with WIN-55,212-2 at a dose of 1–10 μM (Fig. 5*A*). To confirm that ERK1/2 activation is cannabinoid receptor-mediated, cells were pretreated with 3 μM SR141716 (CB_1 antagonist) and SR144528 (CB_2 antagonist) for 3 h followed by treatment with WIN-55,212-2. Data in Fig. 5*B* show that there was no activation of ERK1/2 when treated with the antagonists alone. WIN-55,212-2 (7.5 μM) treatment resulted in significant activation of ERK1/2. When antagonist were coadministered with WIN-55,212-2, there was a decrease in the protein expression of ERK1/2 and a significant increase in the protein expression of PARP (116) as compared with the treatment 7.5 μM WIN-55,212-2 alone (Fig. 5*B*). These data suggest that sustained

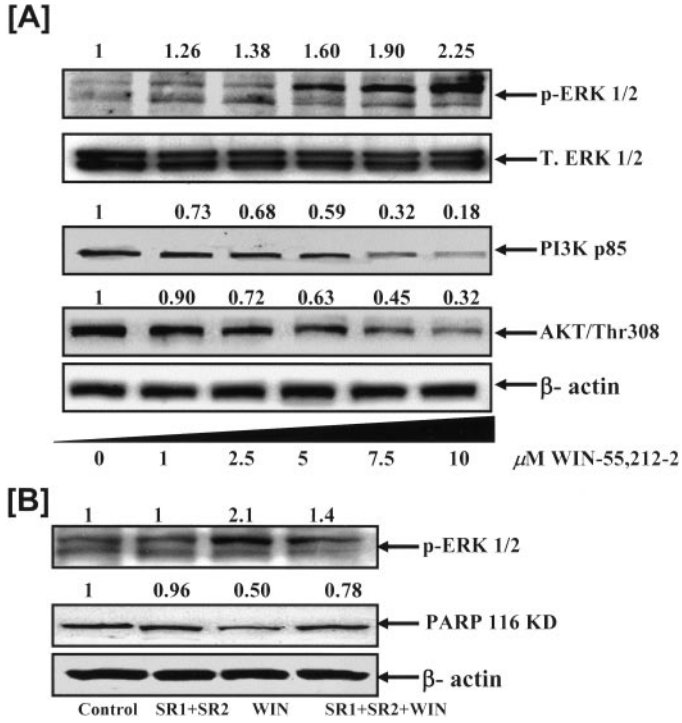

FIGURE 5. **Effect of WIN-55,212-2 treatment on protein expression.** *A*, ERK1/2 (phospho-p44/42, Thr^{202}/Tyr^{204}), anti-PI3K kinase (p85), and AKT (Thr^{308}) in LNCaP cells. *B*, effect of CB_1 (SR141716) and CB_2 (SR144528) antagonist on protein expression of ERK1/2 and PARP cleavage (116 kDa) in LNCaP cells. As detailed under "Experimental Procedures," the cells were treated with 7.5 μM concentrations of WIN-55,212-2 and 3 μM CB_1 (SR141716) and CB_2 (SR144528). Total cell lysates were prepared for immunoblot analysis. The values above the figures represent relative density of the bands normalized to β-actin using UN-SCAN-IT software (Silk Scientific, Inc.). The data shown here are from a representative experiment repeated three times with similar results.

ERK1/2 activation and subsequent apoptosis is mediated through cannabinoid receptors.

WIN-55,212-2-induced Sustained Activation of ERK1/2 Leads to Cell Growth Inhibition with the Induction of Apoptosis and Cell Cycle Arrest—To define the role of ERK1/2 in cannabinoid receptor-induced cell growth inhibition and apoptosis, LNCaP cells were pretreated with ERK1/2 inhibitor PD98059 (30 μM) for 1 h. This treatment alone resulted in no change in the morphology of the cells. However, 7.5 μM WIN-55,212-2 treatment resulted in distinct morphological changes in LNCaP cells, as cells became round and detached from the surface of the plate, whereas pretreatment of LNCaP cells with PD98059 (30 μM) prevented these morphological changes (Fig. 6*A*). WIN-55,212-2 treatment of LNCap cells results in G_1 cell cycle arrest. To assess whether cell cycle arrest is mediated via activation of ERK1/2, we next performed DNA cell cycle analysis. As shown in Fig. 6*B*, blocking of ERK1/2 activation by its inhibitor PD98059 resulted in a decrease in the number of cells in the G_1 phase of cell cycle (72%) when compared with WIN-55,212-2 treatment alone (81%). To assess whether cell cycle dysregulation leads to induction of apoptosis, we next quantified the extent of apoptosis by flow cytometric analysis. As shown in Fig. 6*C*, WIN-55,212-2 treatment of LNCaP cells at a dose of 7.5 μM resulted in 23% of apoptotic cells. Apoptosis was only 9% when WIN-55,212-2 (7.5 μM) was coadministered with PD98059 (30 μM). We next determined whether PD98059 reversed the activation of ERK1/2 by WIN-55,212-2 treatment alone, and we found that ERK1/2 protein expression was significantly decreased when WIN-55,212-2 was given in combination with PD98059 (Fig. 6*D*). We next determined the effect of PD98059 on p27/KIP1 (Fig. 6*D*), a cell cycle regulatory molecule operative in G_1 phase of the cell cycle, and cyclin D1 because of its function in influencing cell proliferation. WIN-55,212-2 treatment increased the protein expression of p27/KIP1 whereas this increase in expression was down-regulated when WIN-55,212-2 was given in combination with PD98059. WIN-55,212-2 treatment significantly inhibited the expression of cyclin D1, and this effect was significantly reversed (>55%) when WIN-55,212-2 was coadministered with PD98059 (Fig. 6*D*). We also observed a decrease (62%) in the protein expression of BCl-2, a pro-apoptotic protein when the cells were treated with WIN-55,212-2 at 7.5 μM; this effect was significantly reversed to 50% when WIN-55,212-2 was coadministered with ERK1/2 inhibitor (Fig. 6*D*).

To further validate the role of ERK1/2 in WIN-55,212-2-induced cell cycle arrest leading to apoptosis, we silenced ERK1/2 by using small interfering RNA against ERK1/2. We observed that WIN-55,212-2 did not induce ERK1/2 activation and p27 when ERK1/2 was silenced (Fig. 6*E*). Similarly, protein expression of cyclin D1 and Bcl-2 which down-regulated by WIN-55,212-2 was found to be reversed when ERK1/2 was silenced.

WIN-55,212-2 Induces Apoptosis via the Classical Apoptotic Pathway—The above data suggest that WIN-55,212-2 induces growth inhibition via cell cycle arrest in G_1 phase of the cell cycle followed by apoptosis. Because p53 is one of the major regulators of apoptosis, expression of this tumor suppressor sensitizes cells to apoptosis in response to stress. We observed a significant up-regulation in the protein expression of p53 when cells were treated with WIN-55,212-2 (Fig. 7*A*). p53-induced apoptosis results from overlapping downstream pathways that suppress mitogenic and survival signaling and promote pro-apoptotic signaling. In this context, p53 can up-regulate the pro-apoptotic Bcl-2 family member Bax and possibly transcriptionally repress the anti-apoptotic protein Bcl-2. Because Bax and Bcl-2 plays a crucial role in apoptosis, we next determined the effect of WIN-55,212-2 treatment of LNCaP cells on protein levels of Bax and Bcl-2. The Western blot analysis exhibited a significant increase in the protein expression of Bax at 7.5 and 10 μM concentrations of WIN-55,212-2 (Fig. 7*A*). In sharp contrast, the protein expression of Bcl-2 was significantly decreased by WIN-55,212-2 treatment in a dose-dependent fashion (Fig. 7*A*). A significant dose-dependent shift in the ratio of Bax to Bcl-2 was observed after WIN-55,212-2 treatment indicating the induction of apoptotic process (Fig. 7*B*). Relative density data revealed an increase in protein expression of Bax by 2.1- and 2.9-fold with concomitant decrease in Bcl-2 protein expression by 71 and 79% at a dose of 7.5 and 10 μM, respectively. A decrease in Bcl-2 expression was associated with an increase in AIF to 2.0- and 2.1-fold at the above mentioned doses of WIN-55,212-2 (Fig. 7*A*).

Alteration in Bax/Bcl-2 is known to initiate caspase signaling; therefore, we evaluated the involvement of various caspases

during WIN-55,212-2-mediated apoptotic death of LNCaP cells. As shown by the immunoblot analysis, WIN-55,212-2 treatment was found to result in a significant decrease in the pro form of caspase-3 (Fig. 7*C*), caspase-6, caspase-7, and caspase-9 (Fig. 7*D*) at a concentration of 7.5 and 10 μM. To assess possible involvement of caspase-3 activation in apoptosis, we next measured cleaved caspase-3 by immunoblot analysis and immunostaining (Fig. 7*C*). Cells were stained with Alexa Fluor 488 conjugate antibody and were viewed under confocal microscope. Intensity of the active caspase-3 staining was higher in cells treated with 7.5 and 10 μM concentrations of WIN-55,212-2 compared with that at lower concentrations of WIN-55,212-2 and control (Fig. 7*C*). The downstream signals during apoptosis are transmitted via caspases, which upon conversion from pro to active forms mediate the cleavage of PARP. We found that WIN-55,212-2 treatment caused cleavage of 116 kDa PARP to 85 kDa (Fig. 7*E*). Relative density data revealed a decrease in the protein expression of PARP (116 kDa) (49 and 81%) with a concomitant increase in its cleaved product (85 kDa) by 3.1- and 4.4-fold at concentrations of 7.5 and 10 μM, respectively.

DISCUSSION

Cannabinoids and their derivatives are drawing considerable attention in the treatment of cancer because of their diverse activities such as cell growth inhibition, anti-inflammatory effects, and tumor regression (6, 7). Accumulated evidence indicates that cannabinoid receptor(s) could be an important target for the treatment of cancer (27, 28, 29). We have earlier shown that WIN-55,212-2 induced apoptosis of

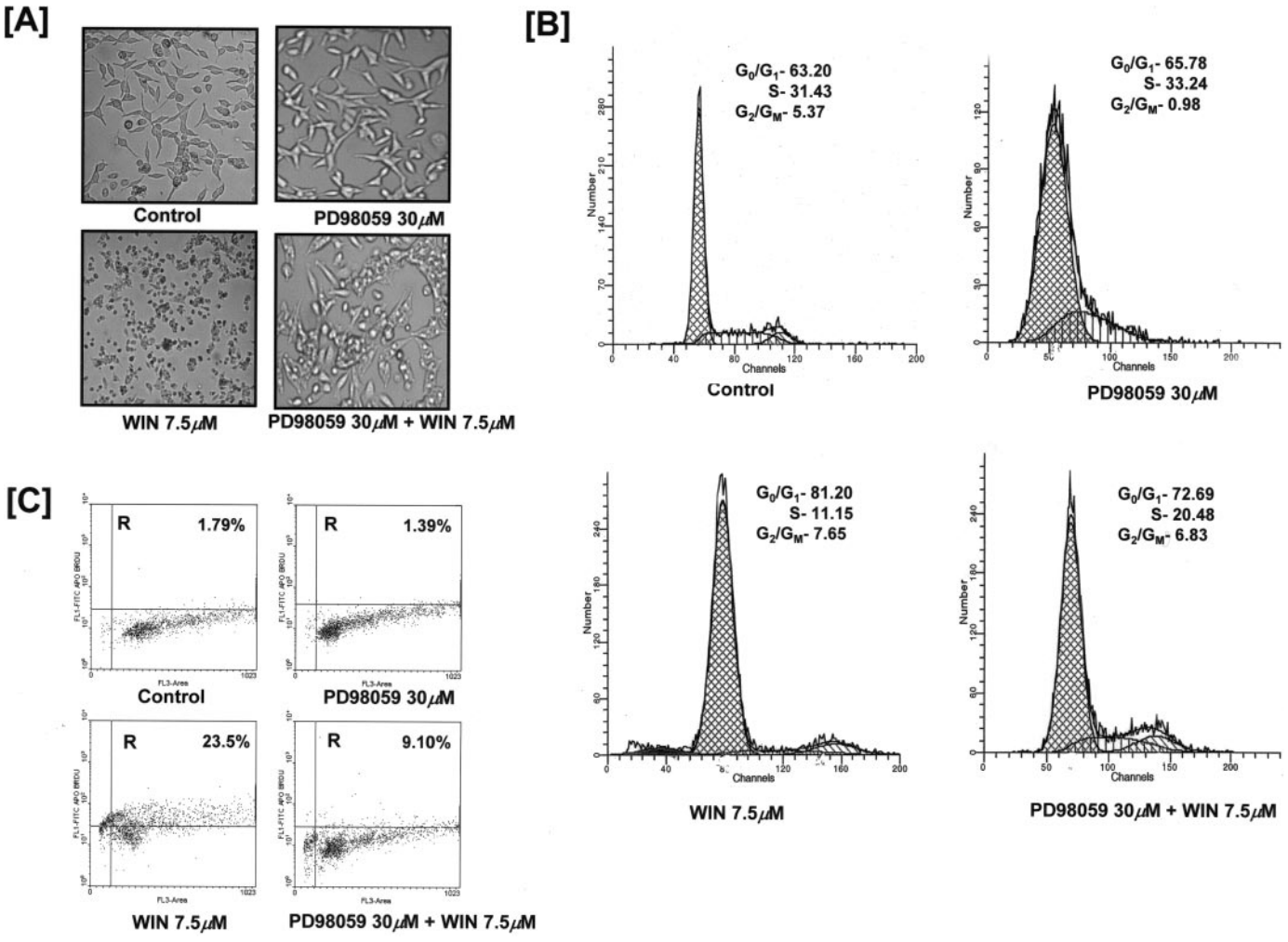

FIGURE 6. **Effect of simultaneous treatment of WIN-55,212-2 and ERK1/2 inhibitor.** *A*, morphology of LNCaP cells; *B*, cell cycle in LNCaP cells. Cell cycle analysis was performed by flow cytometry as detailed under "Experimental Procedures." The labeled cells were analyzed using a FACScan benchtop cytometer and the percentage of cells in G_0–G_1, S, and G_2-M phases were calculated using ModFit LT software. The data shown here are from a typical experiment repeated three times. *C*, quantification of apoptosis by flow cytometry. Cells showing fluorescence (*R*) are considered as apoptotic, and their percentage population is indicated. Data from representative experiments repeated thrice with similar results. *D*, protein expression of p27/KIP1, cyclin D1, and Bcl-2 in LNCaP cells. As detailed under "Experimental Procedures," the cells were treated with 7.5 μM concentrations of WIN-55,212-2 and 30 μM ERK1/2 inhibitor PD98059. Total cell lysates were prepared for immunoblot analysis. The bar diagram represent relative density of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results. *, $p < 0.01$ compared with WIN; **, $p < 0.001$ compared with control. *E*, silencing of ERK1/2 prevents activation of ERK1/2 and p27 and reverses down-regulation of cyclin D1 and Bcl-2. LNCaP cells transfected with 150 nM ERK1, 80 nM ERK2 or scrambled siRNA (150 nM) for 48 h and were then treated with 7.5 μM WIN-55,212-2 for 24 h. Cell lysates were analyzed by immunoblotting using antibodies against ERK1/2 (phospho-p44/42, Thr^{202}/Tyr^{204}), p27, cyclin D1, Bcl-2, and β-actin. The bar diagram represents relative density of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results. *, $p < 0.01$; #, $p < 0.001$ compared with WIN; **, $p < 0.001$ compared with control.

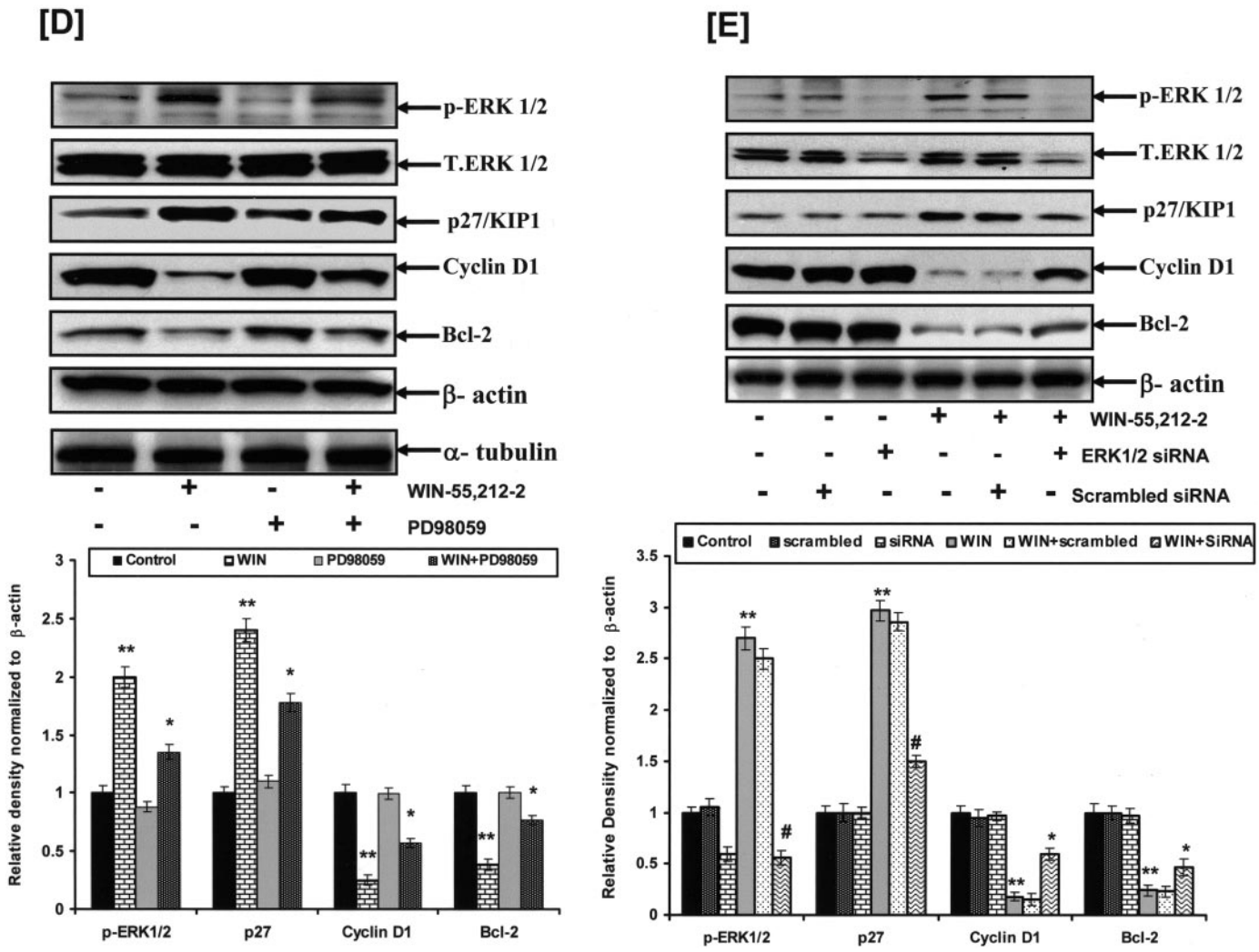

FIGURE 6—*continued*

prostate cancer LNCaP cells is mediated through CB_1 and CB_2 receptors and suggested that these receptors could be an important targets for the treatment of prostate cancer (11). The present study was designed to define the mechanism(s) of the antiproliferative and proapoptotic effects of cannabinoid receptor agonist WIN-55,212-2 against prostate cancer. We provide evidence that WIN-55,212-2 treatment of LNCaP cells activates ERK1/2 leading to cell cycle dysregulation and G_1 arrest, which in turn up-regulates the Bax/BCl-2 ratio and activates caspases resulting in an induction of apoptosis (Fig. 8).

It is well established that uncontrolled cellular growth as a consequence of defects in cell cycle and apoptotic machinery, is responsible for the development of most of the cancers including prostate cancer. Therefore those agents that 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. This notion assumes importance because in cancer a time balance between proliferation and apoptosis is lost which has been implicated in cellular mass and tumor progression. Consistent with this notion, there is a need to develop novel targets and mechanism-based apoptosis inducing 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 (8). Several studies have shown that the induction of apoptosis may be cell cycle dependent (12–16). Therefore, we determined whether WIN-55,212-2-induced apoptosis of LNCaP cells is mediated via cell cycle blockade. We therefore analyzed the effect of WIN-55,212-2 treatment on the distribution of cells in different phases of the cell cycle. As shown in Fig. 2, WIN-55,212-2 treatment was found to result in dose-dependent accumulation of cells in G_1 phase of the cell cycle. In recent years, inhibition of the cell cycle has been appreciated as target for the management of cancer (19, 20). We next studied the involvement of CKI-cyclin-CDK machinery operative in G_1-phase of cell cycle arrest in LNCaP cells by WIN-55,212-2 treatment. The cell cycle in eukaryotes is regulated by members of protein kinase complexes. Each complex is composed minimally of cyclins (regulatory subunit) that bind to cdks (catalytic subunit) to form active cyclin-cdk complexes. These complexes are activated at different checkpoints after certain intervals during the cell cycle and can also be regulated by several exogenous factors (17). Cdk activity is additionally regulated by

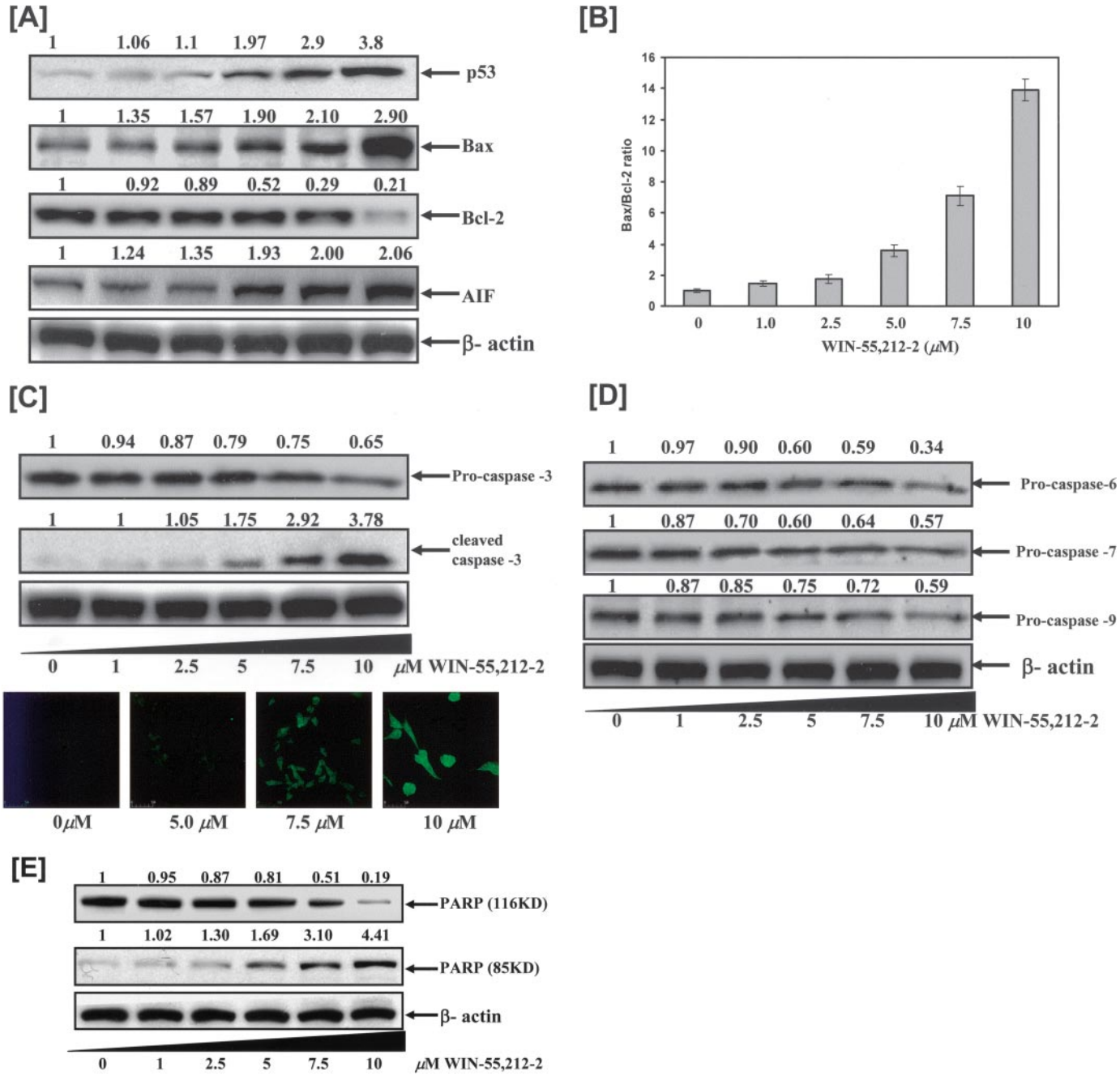

FIGURE 7. **Effect of WIN-55,212-2 treatment.** *A*, protein expression of p53, Bax, Bcl-2, and AIF; *B*, Bax/BCl-2 ratio; *C*, protein expression of pro-caspase-3 and cleaved caspase-3. *D*, protein expression of pro-caspase 6, 7, and 9. *D* and *E*, cleavage of PARP. As detailed under "Experimental Procedures," the cells were treated with Me_2SO alone or specified concentrations of WIN-55,212-2, and total cell lysates were prepared for immunoblot analysis. The values *above* the figures represent relative density of the bands normalized to β-actin. The data shown here are from a representative experiment repeated three times with similar results. The data obtained from the immunoblot analyses of Bax and Bcl-2 were used to evaluate the effect of WIN-55,212-2 on the Bax/Bcl-2 ratio. The densitometric analysis of Bax and Bcl-2 bands was performed using UN-SCAN-IT software, and the data (relative density normalized β-actin) were plotted as Bax/Bcl-2 ratio. Detection of cleaved caspase-3 by confocal fluorescence microscopy; cells were treated with WIN-55,212-2 5.0, 7.5, and 10 μM for 24 h and were stained with antibody Alexa Fluor 488 conjugate.

small proteins known as ckis. Ckis includes the p21/WAF1 and p27/KIP1 protein members. Hence, we studied the modulation in cell cycle regulatory events operational in the G_0–G_1 phase as a mechanism of WIN-55,212-2-mediated cell cycle dysregulation and apoptosis in human prostate cancer cells. It is reported that ckis inhibits the kinase activities associated with cdk-cyclin complexes, thereby modulating the phosphorylation events that play an important role in progression of the cell cycle (30–34). Recent studies have shown that cell cycle progression through G_0–G_1 phase and apoptosis is regulated by p27/KIP1 (23). We observed a significant induction of p27/KIP1 using immunoblot analysis in WIN-55,212-2-treated cells (Fig. 3*A*). These data suggest that cell cycle dysregulation in androgen-responsive LNCaP cells by WIN-55,212-2 treatment is regulated by ckis involved in G_0–G_1 phase. The progression of cell cycle is modulated via irreversible transition induced by cdks

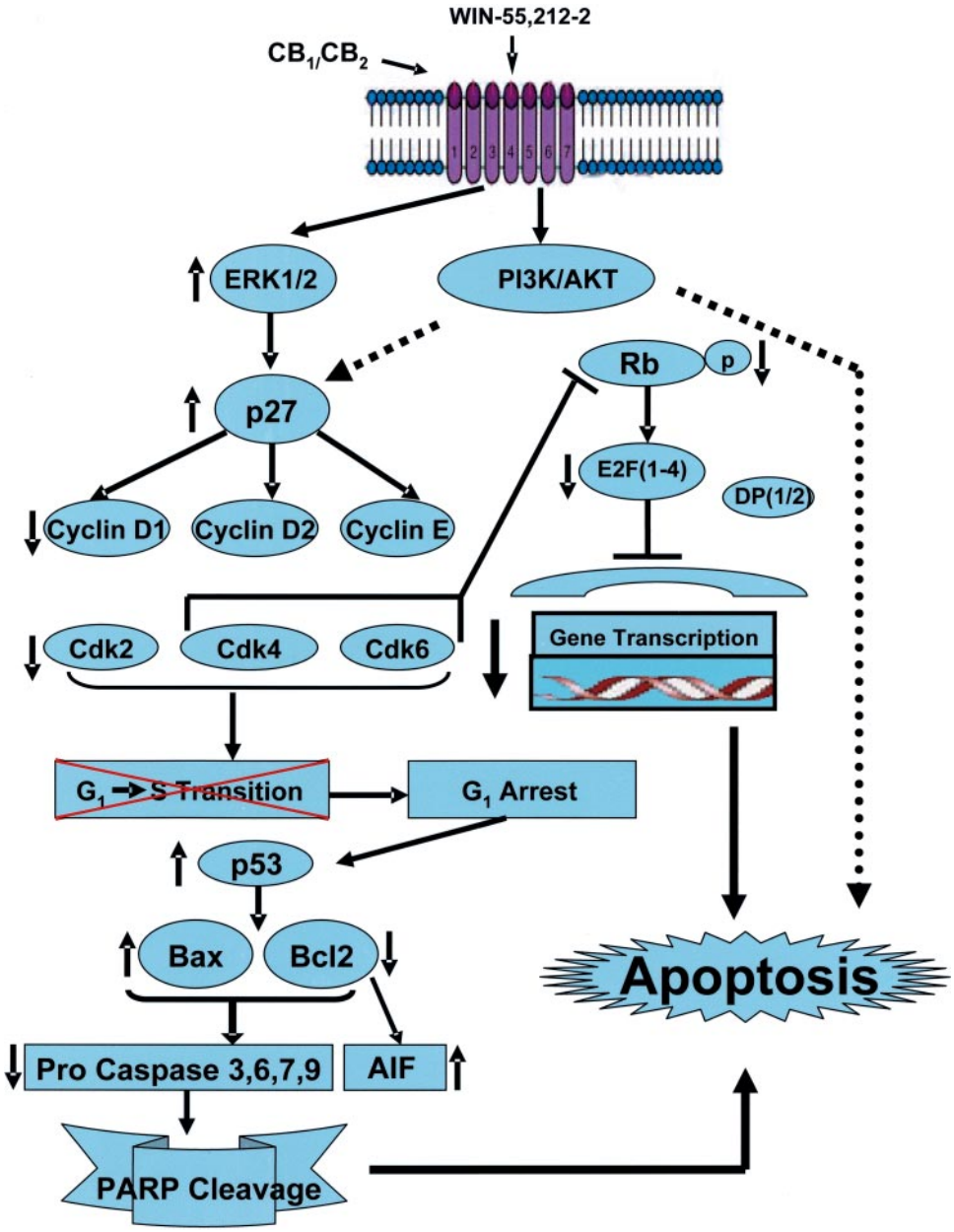

FIGURE 8. **Proposed schematic model for WIN-55,212-2-mediated cell cycle dysregulation and induction of apoptosis.**

and cyclins (35, 36). We next assessed the effect of WIN-55,212-2 treatment on the cyclins and cdks functional in the G_1 phase of the cell cycle, particularly, cyclins D1, D2, and E (Fig. 3*A*) and cdk2, cdk4, and cdk6 (Fig. 3*A*). WIN-55,212-2 treatment of the cells was found to result in significant reductions of all of these regulatory molecules. Cdk4, Cdk6, and cyclin D1 are involved in early G_1 phase and transition from G_1 to S is regulated by cdk2/cyclin E (36, 34). We observed similar results when androgen-insensitive PC3 cells were treated with WIN-55,212-2 (Fig. 3*B*).

It has been reported that down-regulation of cdk4/6 leads to phosphorylation and inactivation of pRb which then down-regulates with E2F family allowing inhibition of transcription of genes required for S phase (25). The progression of S phase in the cell cycle is accompanied by the transcriptional activation of E2F target genes through the phosphorylation of pocket proteins by cdks (37, 38, 34). Studies have established that members of retinoblastoma family are capable of exerting growth suppressive activity because of their interaction with E2F/DP heterodimers, which function to trigger the transcription of genes required for cell cycle progression (39, 37). In the present study we investigated the protein levels and the phosphorylation pattern of pRb during WIN-55,212-2-mediated cell cycle arrest and apoptosis. The immunoblot analysis demonstrated a dose-dependent decrease in the pRb and E2F (1–4) family (Fig. 4*A*) and its heterodimers DP-1 and DP-2 (Fig. 4*A*). pRb is largely found in hypophosphorylated form in the early G_1 phase. The hypophosphorylated pRb is able to bind to a subset of E2F/DP heterodimers, thereby inhibiting their transcriptional activation potential (40–43). Taken together, our data demonstrate the involvement of the pRb-E2F/DP pathway during WIN-55,212-2-mediated cell cycle arrest and apoptosis. We observed similar trend when the androgen-insensitive PC3 cell was treated with WIN-55,212-2 (Fig. 4*B*). Our findings demonstrate that treatment of human prostate cancer cell LNCaP with WIN-55,212-2 increases the protein expression of ERK1/2 and inhibits PI3K/AKT at higher doses of WIN-55,212-2 (Fig. 5*A*). The PI3K/AKT signaling pathway is a common response of cells to growth factor stimulation and is essential for survival. ERK1/2 has a dual behavior and is involved in cell proliferation as well as cell cycle arrest. ERK1/2 activation and cell death/proliferation is complex and depends on many factors, one of which is duration of stimulus. Interestingly we found that a sustained increase in ERK1/2 expression at higher doses of WIN-55,212-2 leads to cell cycle arrest and apoptosis. There was no change in the cell morphology when ERK1/2 was inhibited compared with 7.5 μM WIN-55,212-2 (Fig. 6*A*); similarly the ERK1/2 inhibitor significantly reversed the distribution of cells in G_1 phase of the cell cycle (Fig. 6*B*) and also decreased the percentage of apoptotic cells when compared with WIN-55,212-2 treatment alone. The ERK1/2 inhibitor also reversed effects of WIN-55,212-2 on p27/KIP1 and cyclin D1 proteins operative in the G_1 phase of the cell cycle and Bcl-2, an important pro-apoptotic protein (Fig. 6*D*). Similar results

were observed when ERK1/2 was silenced using small interfering RNA (Fig. 6*E*).

Members of the Bcl-2 family of proteins are critical regulators of the apoptotic pathway (44, 45) and they can be triggered by up-regulation of p53 protein. Bcl-2 is an upstream effector molecule in the apoptotic pathway and is identified as a potent suppressor of apoptosis (46). Bcl-2 is found at high levels in more than half of all human tumors and has shown to form a heterodimer complex with the pro-apoptotic member Bax, thereby neutralizing its proapoptotic effects. Therefore, alterations in the levels of Bax and Bcl-2 with shift in the ratio of Bax/Bcl-2 is considered to be a decisive factor in determining whether cells will undergo apoptosis under experimental conditions that promote cell death. In our study, a decrease in Bcl-2 protein expression was observed in LNCaP cells following WIN-55,212-2 treatment (Fig. 7*A*). Importantly, the protein expression of Bax was found to be up-regulated in these cells after 24 h of treatment (Fig. 7*A*). Therefore, the ratio of Bax to Bcl-2 observed in WIN-55,212-2-treated LNCaP cells favored apoptosis (Fig. 7*B*). Our results thus suggest that up-regulation of AIF and Bax and down-modulation of Bcl-2 may be another molecular mechanism through which WIN-55,212-2 induces apoptosis.

Caspases are cysteine proteases, which are formed constitutively in the cells and are normally present as inactive proenzymes. Caspases are activated during apoptosis in a self-amplifying cascade (47). Activation of upstream or initiator caspases, such as caspases 8, 9, and 10, by proapoptotic signals leads to the proteolytic activation of downstream or effector caspases 3, 6, and 7. The effector caspases cleave a set of vital proteins and, thus, initiate and execute the apoptotic degradation of the cell with the typical morphological and biochemical features. Two major pathways of caspase cascade activation have been characterized. One is initiated by ligation of death receptors and the activation of caspase 8. In the other pathway, cytochrome *c* is released from mitochondria in response to a variety of apoptotic stimuli. In the cytosol cytochrome *c* can bind to apaf-1 and, in the presence of dATP or ATP, activates caspase 9 (48, 47). WIN-55,212-2 treatment of cells was found to promote the activation of caspase 9 that activates caspases 3 and 6 in a dose-dependent manner. Using confocal fluorescence microscopy, cells were visualized to cleaved caspase-3 staining at varying doses (Fig. 7*C*). We observed that WIN-55,212-2 treatment caused activation of caspases 9 and 3 with concomitant cleavage of 116-kDa PARP to the 85-kDa product (Fig. 7*E*).

Based on the outcome of this study and the available literature, and as shown in the composite scheme in Fig. 8, we suggest that cannabinoid receptor agonist WIN-55,212-2 induces sustained and prolong activation of ERK1/2, which leads to induction of cyclin kinase inhibitor p27/KIP1, in turn inhibiting cell cycle regulatory molecules resulting in G_1 arrest and apoptosis. Down-regulation of cdk4/6 inhibits pRb, which inhibits protein expression of E2F family of proteins and its heterodimeric partners DP1 and DP2, leading to gene transcription and apoptosis. Because Bax and Bcl-2 play a critical role in induction of apoptosis, alteration of the Bax/Bcl-2 ratio activates caspase signaling, resulting in apoptotic cell death. Hence, we conclude that cannabinoid receptor agonist should be considered as an effective agent for the treatment of prostate cancer. If our hypothesis is supported by *in vivo* experiments, the long term implications of our study could be to develop nonhabit-forming cannabinoid agonist (s) for the management of prostate cancer.

REFERENCES

1. Jemal, A., Siegel, R. W., Ward, E., Murray, T., Xu, J., Smigal, C., and Thun, M. J. (2006) *CA Cancer J. Clin.* **56,** 106–130
2. Denmeade, S. R., Lin, X. S., and Isaacs, J. T. (1996) *Prostate* **28,** 251–265
3. Tang, D. G., and Porter, A. T. (1997) *Prostate* **32,** 284–293
4. Galve-Roperh, I., Sanchez, C., Cortes, M. L., Gomez del Pulgar, T., Izquierdo, M., and Guzman, M. (2000) *Nat. Med.* **6,** 313–319
5. Bifulco, M., Laezza, C., Portella, G., Vitale, M., Orlando, P., De Petrocellis, L., and Di Marzo, V. (2001) *FASEB J.* **15,** 2745–2747
6. Sanchez, C., de Ceballos, M. L., del Pulgar, T. G., Rueda, D., Corbacho, C., Velasco, G., Galve-Roperh, I., Huffman, J. W., Ramon, y., Cajal. S., and Guzman, M. (2001) *Cancer Res.* **61,** 5784–5789
7. Casanova, M. L., Blazquez, C., Martinez-Palacio, J., Villanueva, C., Fernandez-Acenero, M. J., Huffman, J. W., Jorcano, J. L., and Guzman, M. (2003) *J. Clin. Investig.* **111,** 43–50
8. Guzman, M. (2003) *Nat. Rev. Cancer.* **3,** 745–755
9. Klein, T. W. (2005) *Nat. Rev. Immunol.* **5,** 400–411
10. Guzman, M., Sanchez, C., and Galve-Roperh, I. (2001) *J. Mol. Med.* **78,** 613–625
11. Sarfaraz, S., Afaq, F., Adhami, V. M., and Mukhtar, H. (2005) *Cancer Res.* **65,** 1635–1641
12. Hartwell, L. H., and Kastan, M. B. (1994) *Science* **266,** 1821–1828
13. Morgan, S. E., and Kastan, M. B. (1997) *Adv. Cancer Res.* **71,** 1–25
14. King, K. L., and Cidlowski, J. A. (1998) *Annu. Rev. Physiol.* **60,** 601–617
15. Sandhu, C., and Slingerland, J. (2000) *Cancer Detect. Prev.* **24,** 107–118
16. Vermeulen, K., Berneman, Z. N., and Van Bockstaele, D. R. (2003) *Cell Prolif.* **36,** 165–175
17. Kastan, M. B., Canman, C. E., and Leonard, C. J. (1995) *Cancer Metastasis Rev.* **14,** 3–15
18. Molinari, M. (2000) *Cell Prolif.* **33,** 261–274
19. McDonald, E. R., and El-Deiry, W. S. (2000) *Int. J. Oncol.* **16,** 871–886
20. Owa, T., Yoshino, H., Yoshimatsu, K., and Nagasu, T. (2000) *Curr. Med. Chem.* **8,** 1487–1503
21. Macri, E., and Loda, M. (1998) *Cancer Metastasis Rev.* **17,** 337–344
22. Pavletich, N. P. (1999) *J. Mol. Biol.* **287,** 821–828
23. Atallah, D., Marsaud, V., Radanyi, C., Kornprobst, M., Rouzier, R., Elias, D., and Renoir, J. M. (2004) *Int. J. Hyperthermia.* **20,** 405–419
24. Nevins, J. R., Leone, G., DeGregori, J., and Jakoi, L. (1997) *J. Cell. Physiol.* **173,** 233–236
25. Deshpande, A., Sicinski, P., and Hinds, P. W. (2005) *Oncogene.* **24,** 2909–2915
26. Gomez del Pulgar, T., Velasco, G., Sanchez, C., Haro, A., and Guzman, M. (2002) *Biochem. J.* **363,** 183–188
27. Di Marzo, V., Bifulco, M., and De Petrocellis, L. (2004) *Nat. Rev. Drug Discov.* **3,** 771–784
28. Nithipatikom, K., Endsley, M. P., Isbell, M. A., Falck, J. R., Iwamoto, Y., Hillard, C. J., and Campbell, W. B. (2004) *Cancer Res.* **64,** 8826–8830
29. Bisogno, T., Ligresti, A., and Di Marzo, V. (2005) *Pharmacol. Biochem. Behav.* **81,** 224–238
30. Macleod, K. F., Sherry, N., Hannon, G., Beach, D., Tokino, T., Kinzler, K., Vogelstein, B., and Jacks, T. (1995) *Genes Dev.* **9,** 935–944
31. Sherr, C. J. (1996) *Science* **274,** 1672–1677
32. Jacks, T., and Weinberg, R. A. (1996) *Nature (Lond.)* **381,** 643–644
33. Sherr, C. J., and Roberts, J. M. (1999) *Genes Dev.* **13,** 1501–1512
34. Sanchez, I., and Dynlacht, B. D. (2005) *Semin. Cell Dev. Biol.* **16,** 311–321
35. Elledge, S. J., and Haper, J. W. (1994) *Curr. Opin. Cell Biol.* **6,** 847–852
36. Sherr, C. J. (1994) *Cell* **79,** 551–555
37. Kasten, M. M., and Giordano, A. (1998) *Cell Death Differ.* **5,** 132–140
38. Morris, L., Allen, E. K., and Thangue, N. B. L. (2000) *Nat. Cell Biol.* **2,** 232–239

39. Taya, Y. (1997) *Trends Biochem. Sci.* **22,** 14–17
40. Weinberg, R. A. (1995) *Cell* **81,** 323–330
41. Kaelin, W. G. (1999) *Bioassays* **21,** 950–958
42. Harbour, J. W., and Dean, D. C. (2000) *Nat. Cell Biol.* **2,** E65–E67
43. Masciullo, V., Khalili, K., and Giordano, A. (2000) *Int. J. Oncol.* **17,** 897–902
44. Strasser, A., Connor, L. O., and Dixit, V. M. (2000) *Annu. Rev. Biochem.* **69,** 217–245
45. Oltersdorf, T., Elmore, S. W., Shoemaker, A. R., Armstrong, R. C., Augeri, D. J., Belli, B. A., Bruncko, M., Deckwerth, T. L., Dinges, J., Hajduk, P. J., Joseph, M. K., Kitada, S., Korsmeyer, S. J., Kunzer, A. R., Letai, A., Li, C., Mitten, M. J., Nettesheim, D. G., Ng, S., Nimmer, P. M., O'Connor, J. M., Oleksijew, A., Petros, A. M., Reed, J. C., Shen, W., Tahir, S. K., Thompson, C. B., Tomaselli, K. J., Wang, B., Wendt, M. D., Zhang, H., Fesik, S. W., and Rosenberg, S. H. (2005) *Nature* **435,** 677–681
46. Hockenbery, D. M., Oltvai, Z. N., Yin, X. M., Milliman, C. L., and Korsmeyer, S. J. (1993) *Cell* **75,** 241–251
47. Saraste, A., and Pulkki, K. (2000) *Cardiovasc. Res.* **45,** 528–537
48. Nunez, G., Benedict, M. A., Hu, Y., and Inohara, N. (1998) *Oncogene.* **17,** 3237–3245