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ORIGINAL ARTICLE

The cannabinoid WIN 55,212-2 prevents neuroendocrine differentiation of LNCaP prostate cancer cells

C Morell[1], A Bort[1], D Vara[2], A Ramos-Torres[1], N Rodríguez-Henche[1] and I Díaz-Laviada[1]

BACKGROUND: Neuroendocrine (NE) differentiation represents a common feature of prostate cancer and is associated with accelerated disease progression and poor clinical outcome. Nowadays, there is no treatment for this aggressive form of prostate cancer. The aim of this study was to determine the influence of the cannabinoid WIN 55,212-2 (WIN, a non-selective cannabinoid CB1 and CB2 receptor agonist) on the NE differentiation of prostate cancer cells.

METHODS: NE differentiation of prostate cancer LNCaP cells was induced by serum deprivation or by incubation with interleukin-6, for 6 days. Levels of NE markers and signaling proteins were determined by western blotting. Levels of cannabinoid receptors were determined by quantitative PCR. The involvement of signaling cascades was investigated by pharmacological inhibition and small interfering RNA.

RESULTS: The differentiated LNCaP cells exhibited neurite outgrowth, and increased the expression of the typical NE markers neuron-specific enolase and βIII tubulin (βIII Tub). Treatment with 3 μM WIN inhibited NK differentiation of LNCaP cells. The cannabinoid WIN downregulated the PI3K/Akt/mTOR signaling pathway, resulting in NE differentiation inhibition. In addition, an activation of AMP-activated protein kinase (AMPK) was observed in WIN-treated cells, which correlated with a decrease in the NE markers expression. Our results also show that during NE differentiation the expression of cannabinoid receptors CB1 and CB2 dramatically decreases.

CONCLUSIONS: Taken together, we demonstrate that PI3K/Akt/AMPK might be an important axis modulating NE differentiation of prostate cancer that is blocked by the cannabinoid WIN, pointing to a therapeutic potential of cannabinoids against NE prostate cancer.

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INTRODUCTION

Prostate cancer is one the most common prevalent cancer among men worldwide and the second cause of cancer-induced deaths in western countries.[1]

A distinctive feature of prostate cancer is the occasional appearance within the prostate tumor mass of a large number of single or clustered neuroendocrine (NE) cells, a situation called NE prostate cancer. NE cells secrete neuropeptides that induce mitogenic effects on prostate cancer cells.[2] The NE cells are defined immunohistochemically by the presence of cytoplasmic markers, such as, chromogranin A, neuron-specific tubulin 3 (βIII tubulin) and neuron-specific enolase (NSE).[3] NE prostate cancers become rapidly growing and highly aggressive,[4] as NE cells might contribute to the regrowth of prostate cancer cells that have adapted to the hormone-deprived environment or the absence of androgen receptor stimulation.[5] In fact, NE prostate cancer usually occurs as a recurrent tumor in men who have received hormonal therapy for prostatic adenocarcinoma, and its presence correlates with tumor progression and poor prognosis.[6,7]

The origin of NE cells in prostate cancer is under discussion. It is thought that NE-like cells come from a 'epithelial-to-neuroendocrine' transition process of prostate cancer cells, known as NE differentiation, as they differ in some aspects from NE cells present in the normal prostate. NE differentiation is a well-recognized phenotypic change by which prostate cancer cells transdifferentiate into NE-like cells. Nevertheless, the mechanism underlying NE differentiation remains still unclear, and the management of patients with NE prostate cancer is a challenge for oncologists. Therefore, novel therapies are needed for this clinically significant and defiant variant of prostate cancer.[8]

Over the last decade, several research groups including ours have proposed that cannabinoid receptor agonists exert a direct antitumor activity in a variety of aggressive cancers. In prostate cancer cells, natural and synthetic cannabinoids have been shown to inhibit cell growth in culture and in experimental animal models.[9–11] Numerous investigations demonstrate the ability of cannabinoids to inhibit prostate cancer cells' viability/proliferation, as well as invasion and metastasis.[12,13] The expression of cannabinoid receptors in prostate cancer cells is higher than that in corresponding non-malignant tissues,[14] and also the enzymes responsible for cannabinoid degradation, suggesting that the endocannabinoid system has a role in prostate growth.[10,15] Two cannabinoid receptors, CB1 and CB2, have been identified to date and belong to a Gi/o family of receptors presenting seven transmembrane domains.[16,17] The mechanisms by which activation of cannabinoid receptors affect prostate cancer cell survival are quite diverse and a matter of current research. Moreover, receptor-independent effects also mediate

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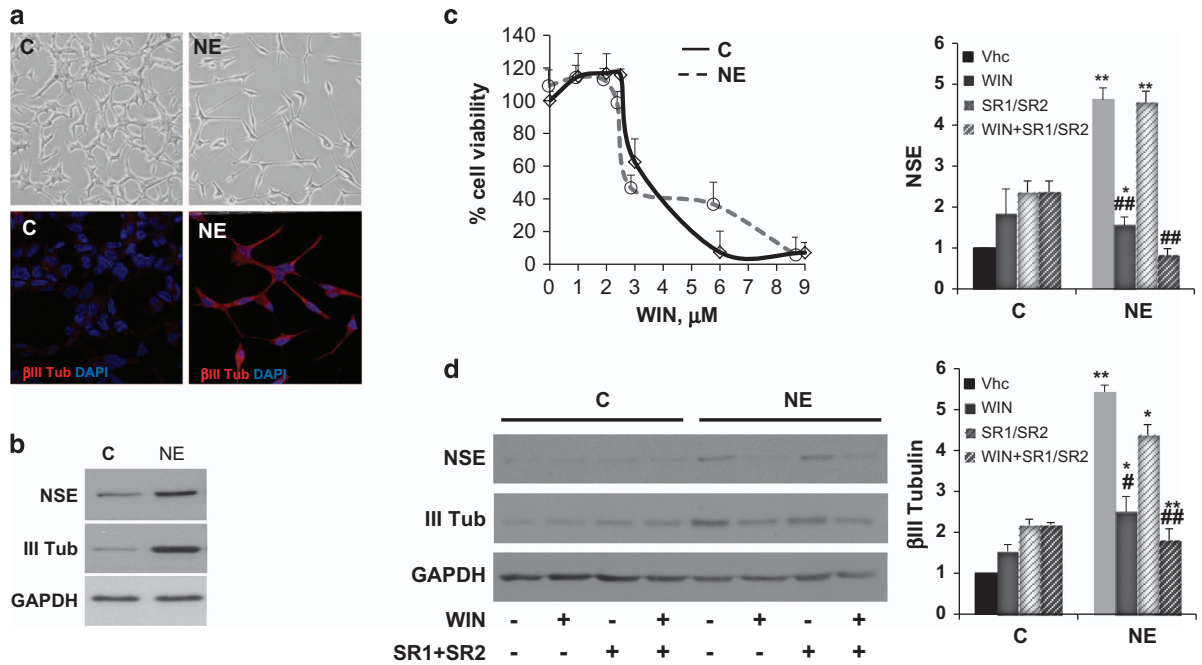

Figure 1. The synthetic cannabinoid WIN 55,212-2 (WIN) inhibits NE differentiation of LNCaP cells. (**a**) NE differentiation cells was induced by serum deprivation of LNCaP for 6 days. Cell morphology of control LNCaP cells (control, C) and serum-deprived cells (NE cells) was monitored by phase-contrast microscopy (upper panel) and immunofluorescence of class III β Tubulin (βIII Tub, red) counterstained with DAPI (blue) (lower panel). (**b**) Expression of the NE markers neuron-specific enolase (NSE) and βIII Tub in control and NE cells. GADPH was probed as a loading control. (**c**) LNCaP control cells (C) and NE cells were incubated with increasing concentrations of WIN for 48 h and cell viability was monitored by MTT. (**d**) LNCaP control cells and NE cells were treated with vehicle (Vhc), 3 μM of WIN (WIN) or 1 μM of the CB1inverse agonist SR1 and 1 μM of the CB2 inverse agonist SR2 (SR1/SR2) for 6 days. Levels of the NSE and βIII Tub were determined by western blot. GADPH was probed as a loading control. The image is representative of other four experiments. Densitiometric analysis of the western blot bands is shown on the right. The data shown are the means ± s.d. of four different experiments ($^{*}P < 0.05$ and $^{**}P < 0.01$ versus control cells, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus NE, compared by the Student's *t*-test). NE, neuroendocrine.

many of the antiproliferative actions of cannabinoid ligands on prostate tumor cells.[18]

Herein, we explored the potential role of the synthetic cannabinoid WIN 55,212-2 (WIN) on serum deprivation-induced NE differentiation of prostate LNCaP cells.

MATERIALS AND METHODS

Materials

The cannabinoid WIN 55-212,2 (WIN) was purchased at Sigma (St Louis, MO, USA). The CB1 antagonist SR-141716 and the CB2 antagonist SR-144528 were kindly provided from Sanofi-Synthelabo (Montpellier, France). The anti-p-S6, p-p70S6K, p-AKT-ser473, p-mTOR, p-AMPKα1-thr172, p-ACC-ser79 and the antibodies against the corresponding total forms were obtained from Cell Signaling Technology (Danvers, MA, USA). The anti-βIII Tub polyclonal antibody was obtained from Covance (Princeton, NJ, USA). The anti-NSE monoclonal antibody was obtained from Dako (Glostrup, Denmark). Recombinant human interleukin-6 (IL-6) was purchased at Prepotech (New York, NY, USA) All the other chemicals were obtained from Sigma.

Cell culture

Human prostate epithelial LNCaP (ATCC CRL-1740) and PC-3 (ATCC CRL-1435) cells were purchased frozen from American Type Culture Collection (Rockville, MD, USA). All the experiments were performed with cells at passages 3–15. Cells were routinely grown in RPMI 1640 medium supplemented with 100 IU ml^{-1} penicillin G sodium, 100 μg ml^{-1} streptomycin sulfate, 0.25 μg ml^{-1} amphotericin B (Invitrogen, Paisley, UK) and 10% fetal calf serum. For androgen deprivation-induced NE differentiation, the medium was replaced for serum-free RPMI 1640 and then incubated for 6 days. For NE differentiation induced by long treatment with IL-6, 20 ng ml^{-1} of human recombinant IL-6 was added to the medium and the cells were incubated for 6 days.

Immunofluorescence staining

Cells were plated on glass coverslips and immunofluorescence was performed as previously described.[19]

Proliferation assay

Cells were seeded in a 12-well plate at 5000 cells per well, maintained for 24 h and then treated with increasing concentrations of WIN with or without 10% fetal bovine serum for 6 days, according to the experiment. MTT cell viability assay was performed as previously described.[20]

Immunoblotting assay

LNCaP cells were washed with phosphate-buffered saline and lysed in a lysis buffer (50 mM Tris pH 7.4, 0.8 M NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100) containing Protease Inhibitor and Phosphatase inhibitor Cocktails (Roche, Basel, Switzerland), and cleared by microcentrifugation. Western blotting was performed as previously described.[19]

Real-time quantitative PCR

Complementary DNA was obtained from cells using Transcriptor (Roche Applied Science, Indianapolis, IN, USA). Real-time quantitative PCR assays were performed using the FastStart Universal Probe Master mix with Rox (Roche Applied Science), and probes were obtained from the Universal ProbeLibrary Set (Roche Applied Science); CB1 sense primer 5′-CATTA AGACGGTGTTTGCATTCT-3′; CB1 antisense primer 5′-CGTGTCGCAGGTCC TTACTC-3′; CB2 sense primer 5′-GACACGGACCCCTTTTTGCT-3′; CB2 antisense primer 5′-CCTCGTGGCCCTACCTATCC-3′ or from Ambion (Life Technologies, Carlsbad, CA, USA). Amplifications were run in a 7900

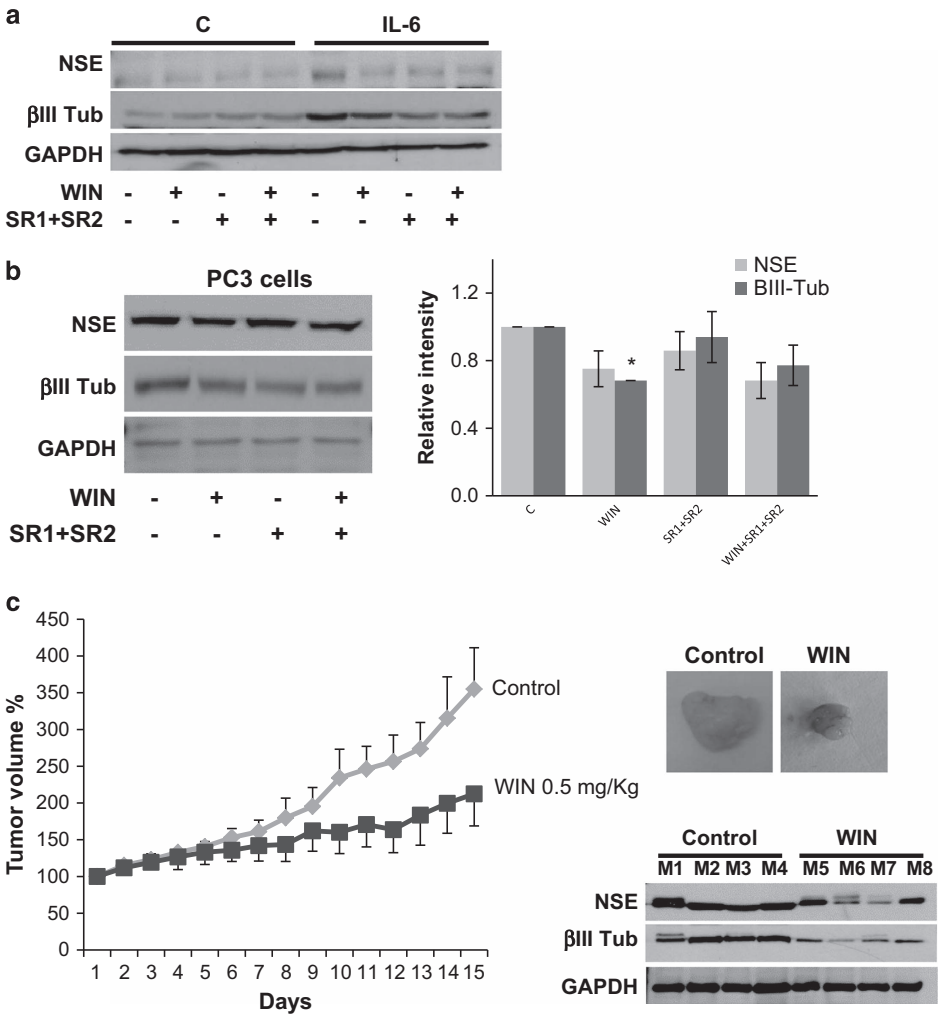

Figure 2. Inhibition of NE differentiation by WIN 55,212-2 (WIN) in IL-6 long treated LNCaP cells and in PC-3 cells. (**a**) NE differentiation of LNCaP cells was induced by incubation with IL-6 (20 ng ml^{1}) -supplemented medium for 6 days. Cells were treated with vehicle (Vhc), 3 μM of WIN (WIN) or 1 μM of the CB1inverse agonist SR1 and 1 μM of the CB2 inverse agonist SR2 (SR1/SR2) for 6 days. Levels of the NE markers neuron-specific enolase (NSE) and βIII tubulin (βIII Tub) were determined by western blot. (**b**) NE markers NSE and βIII Tub in PC-3 cells treated as above. The image is representative of other three experiments. GADPH was probed as a loading control. Densitiometric analysis of the western blot bands is shown on the right. The data shown are the means ± s.d. of three different experiments (*$P < 0.05$ versus control cells, compared by the Student's *t*-test). (**c**) Effect of WIN on PC-3 growth and βIII Tub expression *in vivo*. PC-3 xenografts were generated by subcutaneously. injection in athymic mice ($n = 8$). When tumors reached 100 mm^3 volume, mice were randomly divided into two groups and treated with 0.5 mg kg^1 WIN or vehicle. Graph represents tumor growth from the first day of treatment and results are expressed as the mean ± s.e.m. of the size of the tumor. A representative image of the tumor at the end of the treatment is shown on the right. Down, levels of βIII Tub in the dissected tumors. IL, interleukin; NE, neuroendocrine.

HT-Fast Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Each value was adjusted by using 18S RNA levels as a reference.

Animal study

Eight male athymic nude-Foxn1 (nu/nu) mice aged 4 weeks were purchased from Envigo RMS (Barcelona, Spain), and housed under specific pathogen-free conditions in a 12-hour light–dark cycle at 21–23 °C and 40–60% humidity with access to food pellets and tap water *ad libitum*. All animal studies were conducted in accordance with the Spanish institutional regulation (Decree 53/2013) for the housing, care and use of experimental animals with the approval of the Institutional Animal Care and Use Committee of Alcala University and by Comunidad de Madrid (PROEX 241/15). This study met the European Community directives regulating animal research. Recommendations made by the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) have been kept carefully.

After 1-week adaptation period, mice were injected subcutaneously in the right flank with 5×10^6 PC-3 cells in 0.1 ml of phosphate-buffered saline+0.5% BSA to induce prostate cell tumors. Tumor sizes were measured everyday and calculated using the formula V (mm^3) = 1/2 (length × width^2). When tumors reached a volume of 100 mm^3 (~2 weeks after transplantation), the mice were randomly divided into two groups ($n = 4$) and daily treated subcutaneously. with vehicle, or 0.5 mg kg^{-1} WIN 55,212-2 for 15 days. At the end of the study, the mice were killed by placing them in a CO_2 gas-filled chamber, and the excised tumors were recovered and homogenized in lysis buffer for protein quantification and western blotting.

Statistical analysis

All experiments were executed at least three times and performed in triplicate. The statistical significance of differences between the means was evaluated using the unpaired Student's *t*-test. The level of significance was set at $P \leqslant 0.05$. Calculations were performed using Instat (Graphpad Software, San Diego, CA, USA).

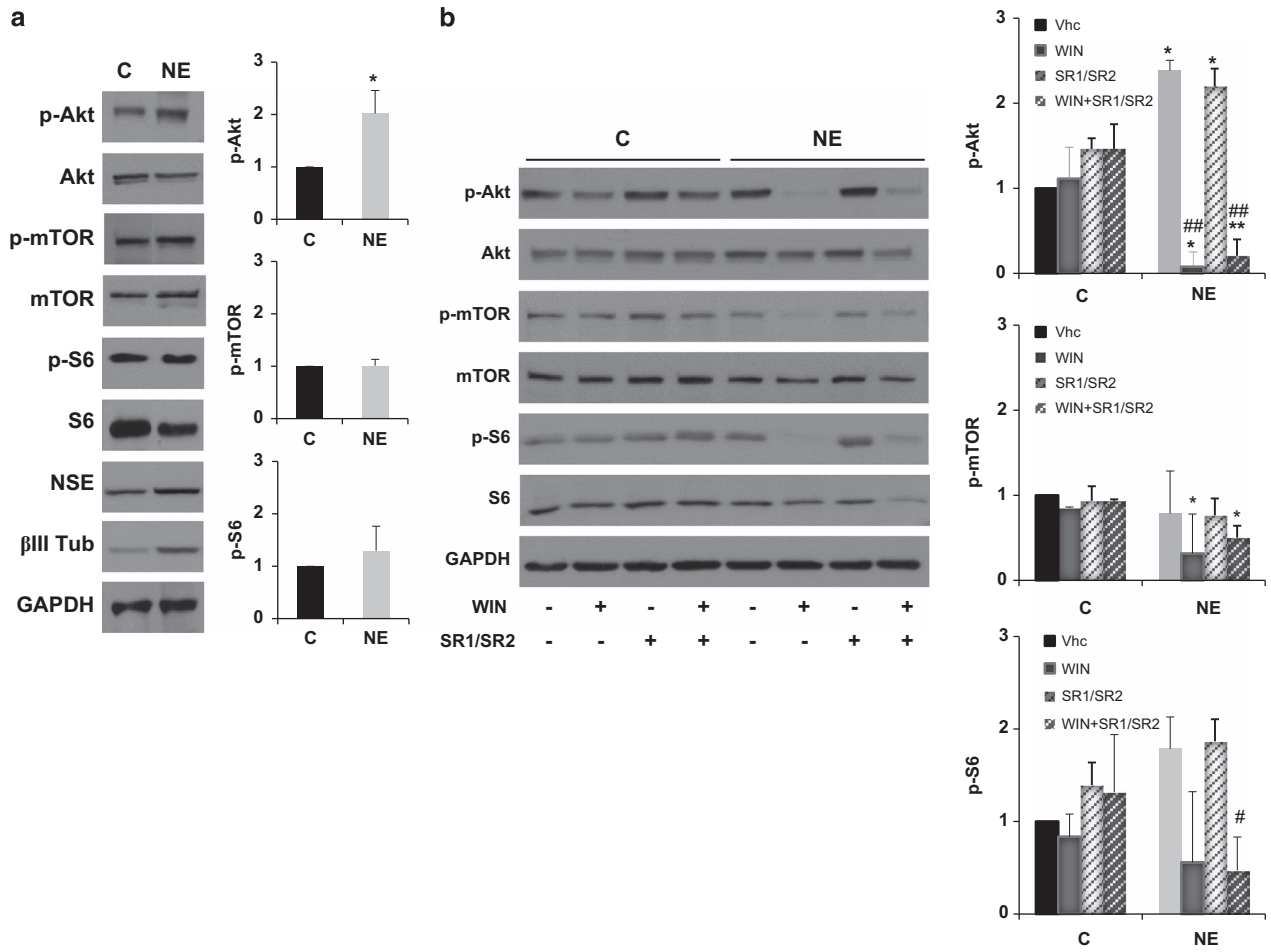

Figure 3. The synthetic cannabinoid WIN 55,212-2 (WIN) blocks the PI3K/Akt/mTOR axe activation produced in NE differentiation of LNCaP cells. (**a**) Phosphorylation profile of key proteins of the PI3K/Akt pathway in LNCaP cells (control, C) and in NE cells (NE). Expression of neuron-specific enolase (NSE) and βIII tubulin (βIII Tub) were monitored as a NE differentiation control. GADPH was probed as a loading control. (**b**) LNCaP control and NE cells were treated with vehicle (Vhc), 3 μM of WIN, or 1 μM of the CB1inverse agonist SR1 and 1 μM of the CB2 inverse agonist SR2 (SR1/SR2) for 6 days. Levels of the phosphorylated and total forms of Akt, mTOR and S6 proteins were determined by western blot. GADPH was probed as a loading control. The image is representative of other three experiments. Densitiometric analyses of the western blot bands are shown on the right of the WB. The data shown are the means ± s.d. of three different experiments. (*$P < 0.05$ and **$P < 0.01$ versus control cells, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus NE cells, compared by the Student's *t*-test). NE, neuroendocrine.

RESULTS

The synthetic cannabinoid receptor ligand WIN prevents NE differentiation induced by serum-deprivation of prostate LNCaP cells

Different cell models have been used for understanding the molecular mechanisms of NE differentiation. The most widely used is the experimental manipulation of the androgen-sensitive prostate cancer cell line LNCaP to induce a transition into a NE phenotype. This phenotypic switch has been seen especially when cells are grown in hormone-depleted medium.[21] In order to mimic the situation of androgen removal, prostate LNCaP cells were incubated in the absence of serum for 6 days. Cells grown in the absence of serum stop division and stay quiescent (Supplementary Figure 1). As seen in Figure 1a, serum deprivation of LNCaP cells for 6 days induced a NE-like phenotype corroborated by the appearance of many features of NE cells, like neurite outgrowth (cell body prolongation longer than twice the cell body diameter), and the expression of the highly specific markers for NE cells class III β tubulin (βIII-Tub). The expression of βIII-Tub as well as the dominant enolase-isoenzyme found in NE tissues, the NSE,[22] were also determined by western blot (Figure 1b).

In order to study the effect of cannabinoids on prostate LNCaP cells' NE differentiation, we choose the non-selective CB1 and CB2 agonist WIN 55,212-2 (WIN). WIN is a synthetic cannabinoid that has been shown to act on both CB1 and CB2 and one of the most widely used analogs of endocannabinoids designed to activate cannabinoid receptors. In the LNCaP prostate cancer cell line, as well as in the NE differentiated cells, WIN dose-dependently decreased cell viability, with an overall IC_{50} value of 3 μM (Figure 1c). We then used this dose to investigate whether the cannabinoid WIN had any effect on the neuroendocrine differentiation (NED) of LNCaP cells. As shown in Figure 1d, incubation of LNCaP cells with WIN notably and significantly reduced the NE markers NSE and βIII Tub expressed by NE cells. In order to investigate the involvement of the cannabinoid receptors, we used the CB1 inverse agonist SR1 and the CB2 inverse agonist SR2. These compounds when added together did not modify the effect of WIN.

To reinforce the notion that the cannabinoid WIN abrogated NE differentiation of prostate LNCaP cells, we used another model of NE differentiation. As stated previously, NE differentiation of LNCaP cells can also be induced by long-treatment with IL-6.[23] We then incubated LNCaP cells with 20 ng ml^{1} of human recombinant IL-6 for 6 days in the presence or not of 3 mM WIN. IL-6-treated

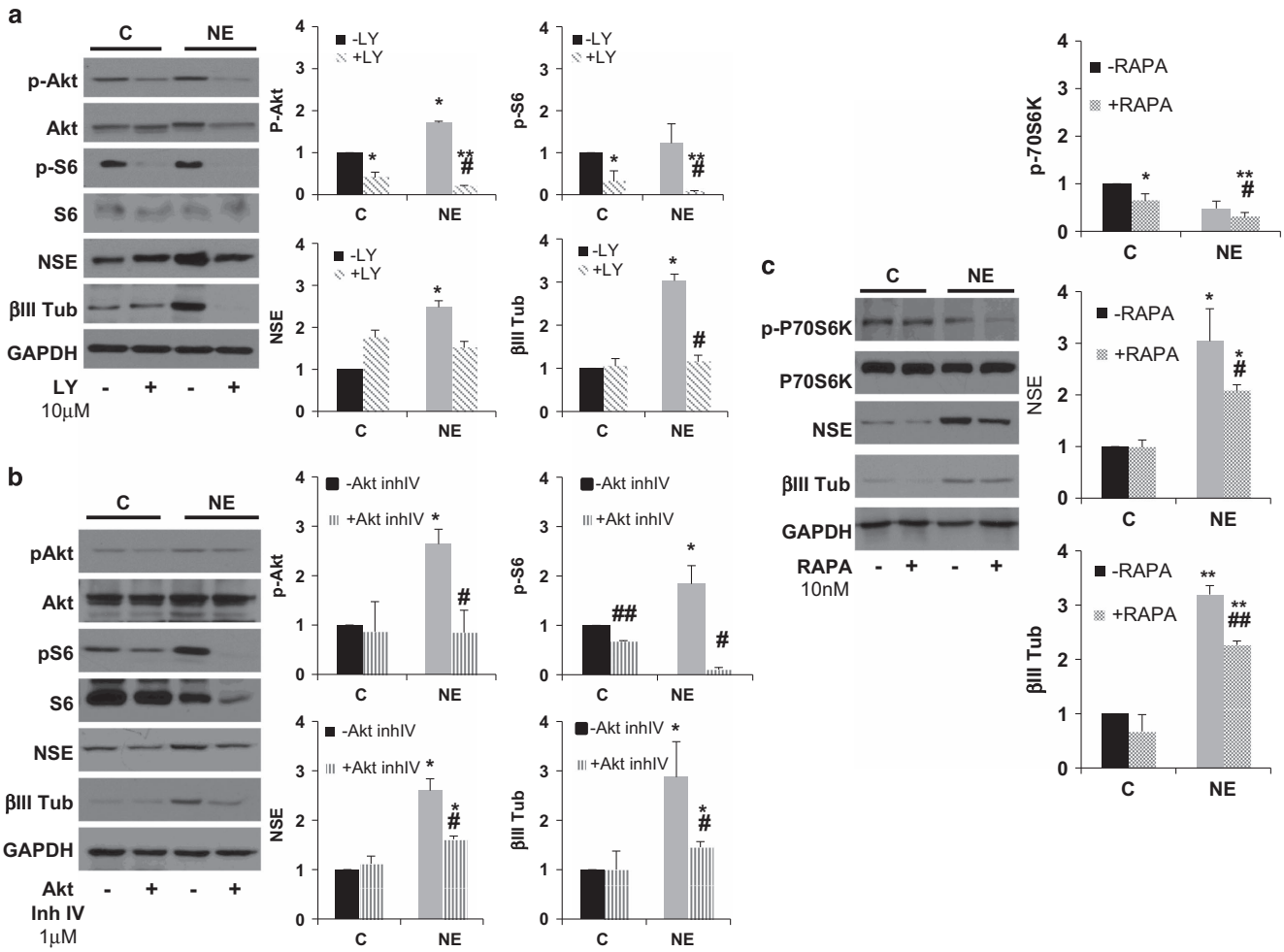

Figure 4. Pharmacological inhibition of the PI3K/Akt/mTOR pathway prevents NE differentiation of LNCaP cells. LNCaP (Control, C) and NE cells were incubated either with the PI3K inhibitor LY 294002 (**a**) the Akt inhibitor AKT Inhibitor IV (**b**) or the mTOR inhibitor rapamycin (**c**) during serum-deprivation. Levels of phosphorylated and total forms of the signaling proteins as well as of the NE markers neuron-specific enolase (NSE) and βIII tubulin (βIII Tub) were determined by western blot. GADPH was probed as a loading control. The image is representative of other four experiments. Densitiometric analysis of the western blot bands are shown on the right. The data shown are the means ± s.d. of four different experiments ($^{*}P < 0.05$ and $^{**}P < 0.01$versus control cells, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus NE cells, compared by the Student's *t*-test). NE, neuroendocrine.

cells displayed morphological characteristics of NE cells (not shown) and increased the expression of NSE and βIII Tub, confirming the acquisition of a NE phenotype (Figure 2a). When NE cells were treated with 3 nM WIN a downregulation of NSE and βIII Tub expression was observed (Figure 2a).

NE differentiation of LNCaP cells induced by serum deprivation or IL-6 treatment was reversible, and the cells turned back to control LNCaP when serum was replenished or IL-6 was removed (not shown). Therefore, to validate the inhibitory effect of WIN *in vivo*, we moved to PC-3 cells as they may be considered as a cell line characteristic of prostatic small cell NE carcinoma.[24] In fact, PC-3 cells express high levels of NE markers such as NSE, Chromogranin A and βIII Tub, and they do not express AR and PSA. Moreover, PC-3 cells, usually maintain NE characteristics along an *in vivo* experiment even in the presence of the animal serum. We first tested the effect of WIN on PC-3 cells *in vitro*. Cells were treated with 3 nM WIN for 6 days and levels of NSE and βIII Tub were determined. As shown in Figure 2b, the expression of both markers diminished after 6 days of WIN treatment. Next, PC-3 tumors were generated in male athymic nude mice and treated with 0.5 mg kg^{1} WIN for 15 days. WIN-treated PC-3 xenografts grew slower and the size of the tumors at the end of the experiment was smaller compared with vehicle-treated PC3 xenografts (Figure 2b). Likewise, levels of βIII Tub decreased in WIN-treated tumors compared with non-treated tumors (Figure 2b).

The cannabinoid WIN inhibits the PI3K/AKT/mTOR signaling pathway in NE differentiated LNCaP cells

To investigate the role of the PI3K/Akt/mTOR pathway in the NE differentiation of the prostate cancer cell line LNCaP, cells were deprived of serum for 6 days and phosphorylation levels of Akt and its downstream signaling proteins mTOR and S6 were determined by western blotting. Consistent with previous observations,[25,26] the PI3K/Akt signaling pathway was hyper-activated as deduced by the significantly increased levels of phosphorylated Akt in Ser473, compared with control LNCaP cells (Figure 3a). Levels of phosphorylated downstream signaling proteins, mTOR and S6, although not significantly, were also increased.

In order to gain insight into the mechanism whereby the cannabinoid WIN prevented NE differentiation of prostate LNCaP cells, we analyzed the PI3K/Akt/mTOR pathway. Addition of 3 μM WIN during serum deprivation, markedly reduced the increase in phosphorylated Akt, phosphorylated mTOR and phosphorylated

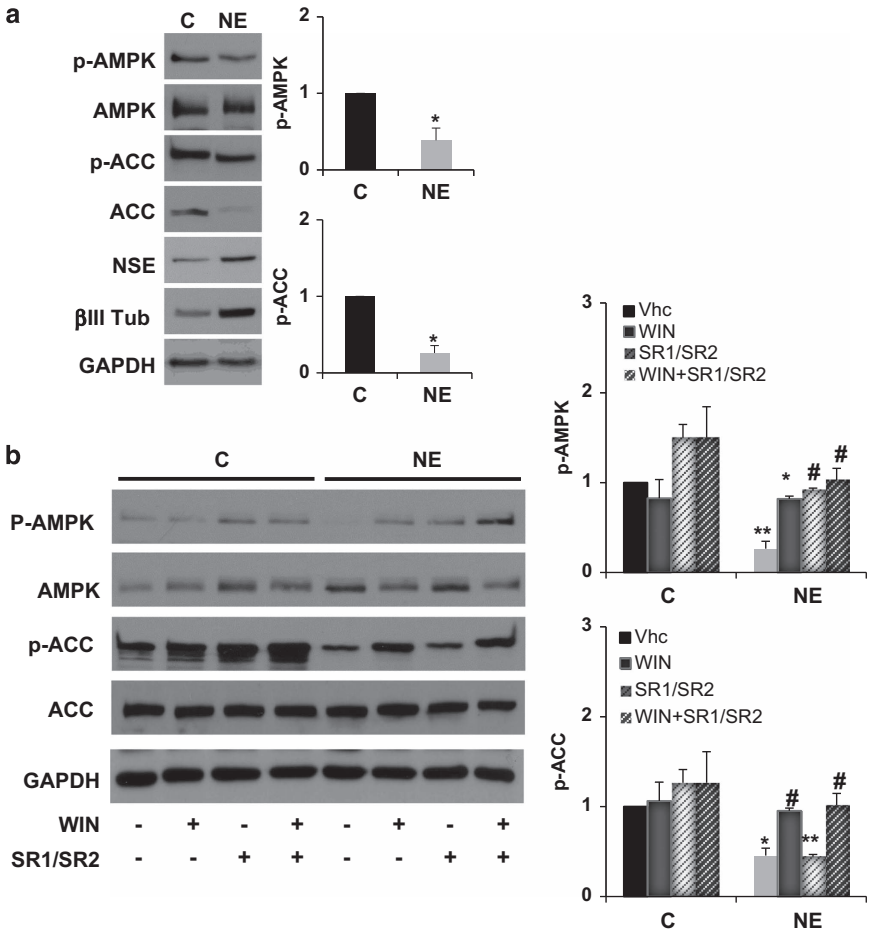

Figure 5. The synthetic cannabinoid WIN 55,212-2 (WIN) prevents AMPK inhibition produced in NE differentiation of LNCaP cells. (**a**) LNCaP cells were serum-deprived for 6 days and levels of phosphorylated and total forms of AMPK and acetyl CoA carboxylase (ACC), as well as of the NE markers neuron-specific enolase (NSE) and βIII tubulin (βIII Tub) were determined by western blot. GADPH was probed as a loading control. Densitiometric analysis of the western blot bands are shown on the right. The data shown are the means ± s.d. of four different experiments (*$P < 0.05$ versus control cells compared by The Student's *t*-test). (**b**) LNCaP control and NE cells were treated with vehicle (Vhc), 3 μM of WIN 55-212,2 (WIN), 1 μM of the cannabinoid receptor inverse agonists SR1 and SR2 (SR1/SR2) or combined together for 6 days. Levels of the phosphorylated and total forms of AMPK and ACC proteins were determined by western blot. GADPH was probed as a loading control. The image is representative of other four experiments. Densitiometric analysis of the western blot bands are shown on the right. The data shown are the means ± s.d. of three different experiments. (*$P < 0.05$ and **$P < 0.01$ versus control cells, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus NE cells, compared by the Student's *t*-test). ACC, acetyl CoA carboxylase; AMPK, AMP-activated protein kinase; NE, neuroendocrine.

S6 produced in NE cells, implying a WIN-induced blocking of the PI3K/Akt pathway (Figure 3b). It is worthy to note that in these conditions, WIN did not have any effect on PI3K/Akt/mTOR axe in control cells (Figure 3b).

We then compared the effect of WIN with that produced by the widely used PI3K/Akt/mTOR signaling cascade inhibitors. Treatment of cells either with the PI3K inhibitor LY 294002, the Akt selective inhibitor Inhibitor IV (Inh IV) or the mTOR inhibitor rapamycin, resulted in a decrease of the NE markers NSE and βIII Tubulin expression in serum-deprived cells (Figure 4). These results were comparable to those produced by WIN (Figure 3b). Interestingly, whereas LY and Akt Inh IV almost totally blocked NSE and βIII Tubulin expression, the mTOR inhibitor rapamycin, had a lesser effect. This result indicates that Akt may regulate the differentiation process by other mTOR-independent mechanisms.

The cannabinoid WIN inhibits NE differentiation by activation of AMPK

In addition to its energy sensor function, the AMP-activated protein kinase (AMPK) signaling system has recently emerged as a modulator of cell proliferation and survival.[27] Therefore, AMPK has been revealed as a novel target for anticancer therapy.[28] We then investigated the effect of WIN treatment on AMPK and the role of this kinase in NE differentiation. To investigate the role of AMPK in the NE differentiation of prostate LNCaP cells, cells were serum-deprived for 6 days and phosphorylation levels of AMPK in Thr172 and its well-established substrate acetyl coenzyme A carboxylase (ACC) were monitored by western blot. Figure 4a shows that during NE differentiation there is a decrease in Thr172 phosphorylation of AMPK and in ser 79 of ACC, which indicates an inhibition of the AMPK pathway. Interestingly, levels of total ACC decrease during NED, suggesting that AMPK is also regulating ACC expression. Levels of the NE markers NSE and βIII Tub are shown as a NE differentiation control (Figure 5a).

When 3 μM WIN was added to the serum-deprived medium, the inhibition of AMPK observed in NE cells was prevented. This effect was independent of cannabinoid receptors, as SR1 and SR2 when combined together did not modify the effect of WIN (Figure 5b).

To further analyze the function of AMPK on NE differentiation, LNCaP cells were treated with the AMPK pharmacological activator AICAR during the 6 days of serum deprivation. As shown

in Supplementary Figure 2, AICAR increased phosphorylation of ACC in both control and NE cells, indicating that AMPK is active. Moreover, AICAR treatment rescued the decrease of ACC expression observed in NE, confirming that AMPK regulates its expression. Interestingly, as observed with the treatment with WIN, AMPK activation with AICAR notably reduced the expression of NED markers NSE and βIII Tub (Supplementary Figure 2).

The effect of Win on the PI3K/Akt pathway and AMPK in NE cells was further corroborated in IL-6 long-term-treated LNCaP cells. As shown in Figure 6, in IL-6-induced NE differentiation, a decrease in p-AMPK and p-ACC is observed, which is in consonance with serum withdrawal-induced NE differentiation, suggesting that AMPK inhibition is a general phenomenon of NE differentiation. As was the case in NE differentiation induced by serum depletion, treatment with 3 nM WIN increased both p-AMPK and p-ACC in NE cells.

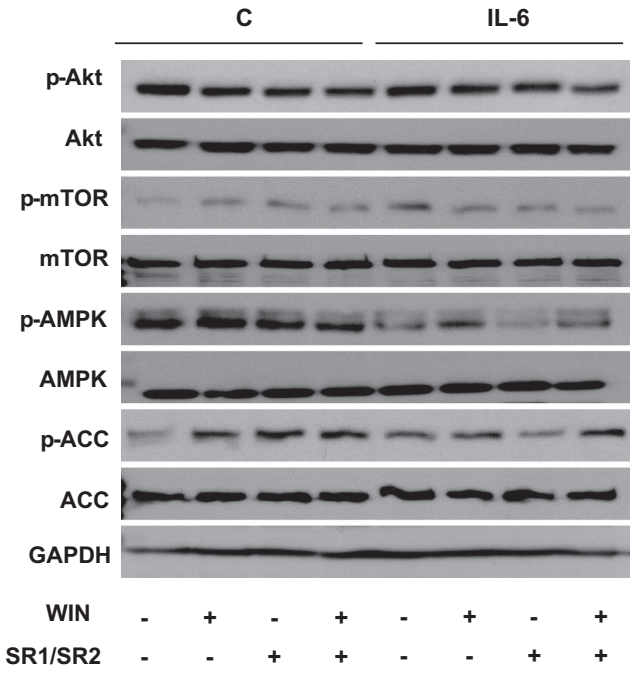

Figure 6. Effect of WIN 55,212-2 on IL-6-induced NE differentiation. NE differentiation of LNCaP cells was induced by incubation with IL-6 (20 ng ml^{-1}) -supplemented medium for 6 days. Cells were treated with vehicle (Vhc), 3 μM of WIN (WIN) or 1 μM of the CB1inverse agonist SR1 and 1 μM of the CB2 inverse agonist SR2 (SR1/SR2) for 6 days. Levels of the phosphorylated and total forms of Akt, AMPK and acetyl CoA carboxylase (ACC) proteins were determined by western blot. GADPH was probed as a loading control. The image is representative of other three experiments. IL, interleukin; NE, neuroendocrine.

Suppression of PI3K/Akt/mTOR pathway prevents AMPK inhibition and reduces NE differentiation

In order to establish a relationship between the two pathways studied, we determined the effect of PI3K/Akt/mTOR pathway inhibition on AMPK phosphorylation and activity. When cells were incubated either with the PI3K inhibitor LY 294002 or with the Akt inhibitor IV, the decrease of phosphorylated AMPK observed in NE cells was abrogated (Figure 7). This indicates that inhibition of PI3K or Akt prevents inhibition of AMPK and, therefore, the PI3K/Akt pathway modulates AMPK in NE cells. According to this idea, the NE differentiation-induced diminution in phosphorylated ACC was prevented in LY 294002 and inhibitor IV-treated cells (Figure 7). In these conditions, the NE markers NSE and βIII Tubulin also decreased (Figure 7).

The cannabinoid receptors CB1 and CB2 expression decrease during NE differentiation of prostate LNCaP cells

We then sought to study whether WIN-induced inhibition of LNCaP differentiation was linked to CB1 or CB2. The cannabinoid receptors CB1 and CB2 are constitutive components of the endocannabinoid system and mediate many of the effects induced by cannabinoids. Although the effect of the cannabinoid receptor ligand WIN on signaling cascades of NE cells was not prevented by the cannabinoid receptors, inverse agonists SR1 and SR2, we decided to analyze the expression levels of both receptors. Quantitative PCR analyses showed that in NE cells there

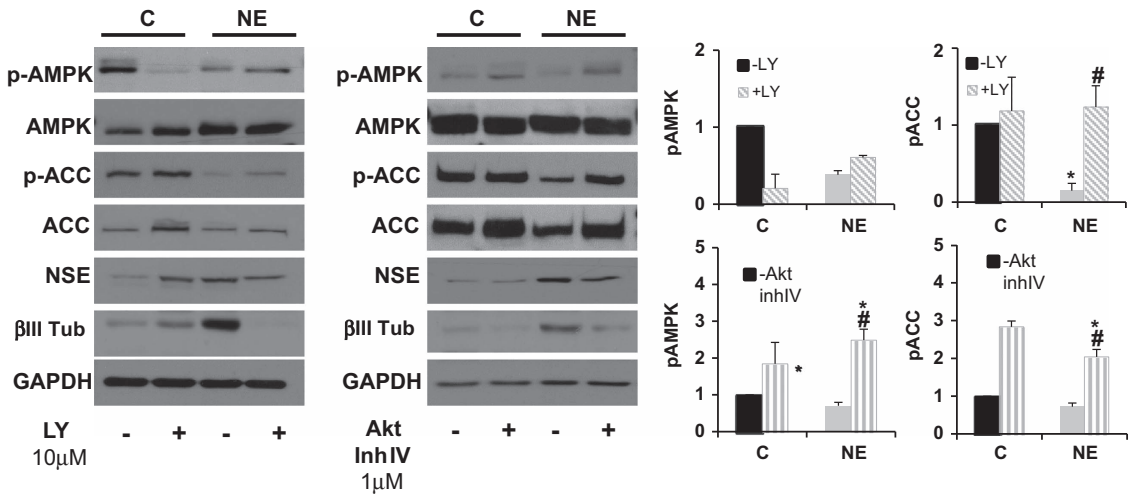

Figure 7. Suppression of PI3K/Akt activation prevents AMPK inhibition. LNCaP control and NE cells were treated with the PI3K inhibitor LY 294002 (LY) or the Akt inhibitor IV (AKT InhIV) for 6 days. Levels of phosphorylated and total forms of AMPK and acetyl CoA carboxylase (ACC), as well as of the NE markers neuron-specific enolase (NSE) and βIII tubulin (βIII Tub) were determined by western blot. GADPH was probed as a loading control. The image is representative of other three experiments. Densitiometric analysis of the western blot bands are shown on the right. The data shown are the means ± s.d. of three different experiments (*$P < 0.05$ versus non-treated cells and $^{\#}P < 0.05$ versus NE cells, compared by the Student's *t*-test). AMPK, AMP-activated protein kinase; NE, neuroendocrine.

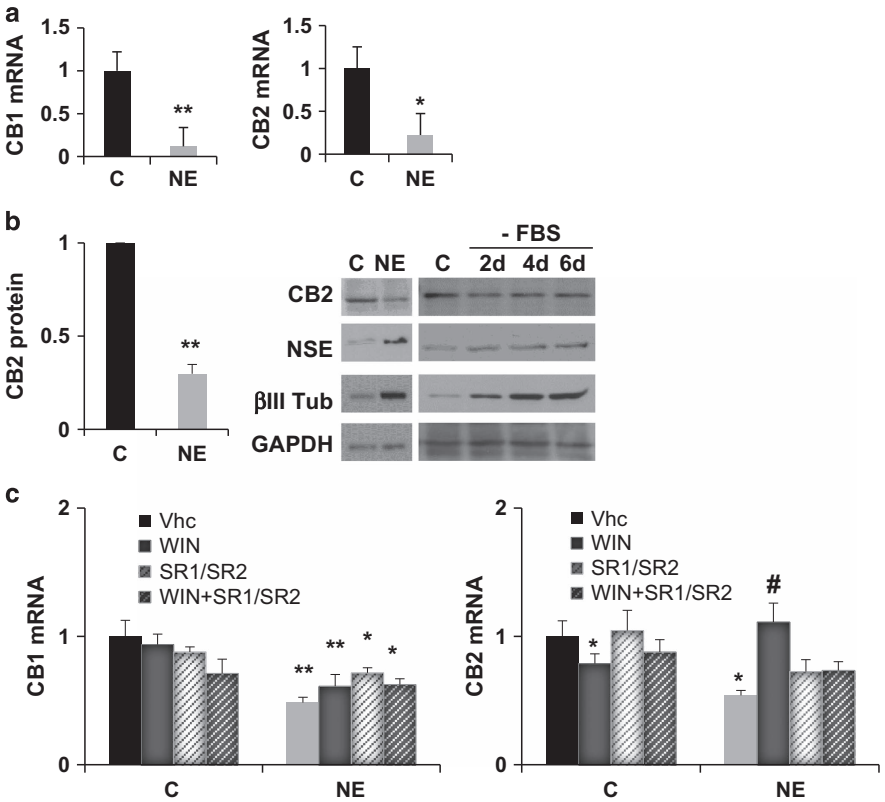

Figure 8. NE differentiation of LNCaP cells induces a decrease of cannabinoid receptors CB1 and CB2 expression. (**a**) Cannabinoid receptors CB1 and CB2 mRNAs levels in LNCaP control and NE cells analyzed by quantitative PCR according to the Materials and Methods section. (**b**) LNCaP cells were serum-deprived for 2, 4 and 6 days and levels of the cannabinoid receptor CB2 protein and NE markers neuron-specific enolase (NSE) and βIII tubulin (βIII Tub) were analyzed by western blot. GADPH was probed as a loading control. The image is representative of other three experiments. Densitiometric analysis of the western blot bands of CB2 is shown on the left. (**c**) LNCaP control and NE cells were treated with vehicle (Vhc), 3 μM of WIN 55-212,2 (WIN) 1 μM of the cannabinoid receptor inverse agonists SR1 and SR2 (SR1/SR2) or combined together for 6 days. Levels of CB1 and CB2 mRNA were analyzed by quantitative PCR according to the Materials and Methods section. The data shown are the means ± s.d. of three different experiments ($^{*}P < 0.05$; $^{**}P < 0.01$ versus control cells and $^{\#}P < 0.05$ versus NE cells, compared by the Student's *t*-test). NE, neuroendocrine.

was a dramatic decrease in CB1 and CB2 expression (Figure 8a). Immunoblotting revealed that serum withdrawal reduced the expression of CB2 protein in a time-dependent manner (Figure 8b). Then we treated cells with 3 μM WIN during serum deprivation and found that NE cells treated with WIN not only preserved but increased CB2 mRNA levels compared with control cells (Figure 8c). These results indicate that WIN treatment prevents CB2 decay during NE differentiation, which could mediate the NE differentiation inhibition induced by WIN.

DISCUSSION

The purpose of this study was to analyze the signaling pathway involved in NE differentiation of prostate LNCaP cells and the influence of WIN 55,212-2 on this process. Compelling evidence has implicated the endocannabinoid system in the regulation of cell fate. Natural cannabinoids as well as synthetic cannabinoid receptor ligands have been shown to reduce tumor cell proliferation, including prostate tumor cells.[9,29] However, little is known about the role of cannabinoids on the NE differentiation of tumors. Here, we demonstrate that the cannabinoid WIN prevents NE differentiation of LNCaP cells by inhibition of PI3K/Akt/mTOR activation and stimulation of AMPK, providing a promising therapeutic opportunity for NE differentiated prostate cancer (Figure 9).

The PI3K/Akt/mTOR axe controls many functions in prostate cells including cell survival, growth and proliferation. Moreover, preclinical studies reveal a dynamic interplay between PI3K and androgen receptor during the development of androgen-deprivation therapy resistance.[30] Therefore, it seems like the suppression of androgen signaling is one of the stimulus that induces activation of PI3K/Akt. According to this, it has been recently demonstrated that androgen receptor negatively regulates PI3K through repression of the regulatory subunit p85α protein.[31] Our results are in line with this idea as serum deprivation of LNCaP cells increased AKT phosphorylation at Ser473 as well as the phosphorylation of its downstream signaling protein S6, which correlated with the increase in NE markers expression. Similar results showed by Qi *et al.*[32] indicate that androgen-depletion increased AR protein level and Akt phosphorylation at Ser473 and Thr308 in LNCaP cells. The importance of the PI3K/Akt signaling pathway in NE differentiation of LNCaP cells has been also reported by other groups.[25,33] Long-term exposure of LNCaP cells to the cytokine IL-6 causes irreversible NE differentiation and activation of the PI3K/Akt cascade.[26,34] Our results are in line with this notion as IL-6 treatment of LNCaP cells induced an increase of NE markers and p-mTOR. Activation of Akt has also been observed in other models of NE differentiation. For instance, NE differentiation of LNCaP cells by treatment with the neuropeptide VIP induces cAMP increase and activation of PKA and PI3K.[33] These results indicate that the activation of the PI3K/Akt route is a general mechanism associated with NE differentiation of prostate cells at least *in vitro*. Our results showing an inhibition of this pathway by the cannabinoid WIN indicate that

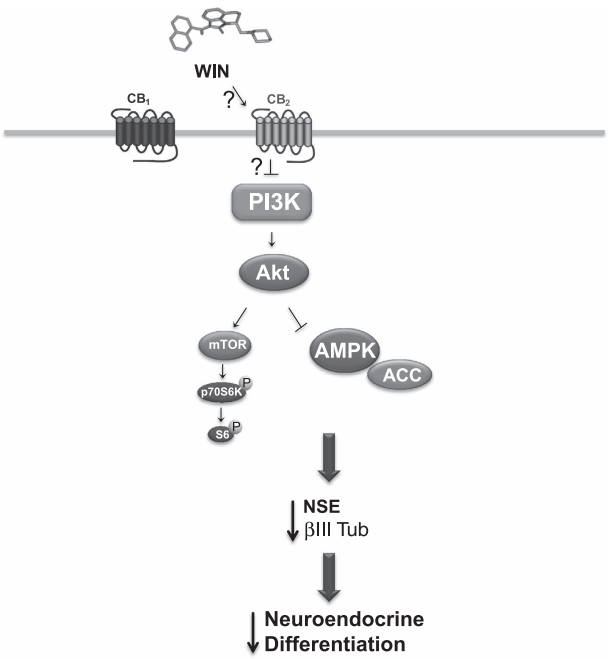

Figure 9. Proposed mechanism for WIN-induced inhibition of prostate LNCaP cells NE differentiation. The synthetic cannabinoid agonist WIN 55,212-2 (WIN) preserves the levels of the cannabinoid receptor CB2. This results in inhibition of the PI3K/Akt pathway. Akt signals through two pathways, activation of mTOR and inhibition of AMPK. The inhibition of Akt by the cannabinoid WIN produces inhibition of mTOR and activation of AMPK. Both phenomena cause inhibition of NE differentiation, although mTOR has a minor contribution than AMPK. ACC, acetyl CoA carboxylase; AMPK, AMP-dependent protein kinase; NE, neuroendocrine.

WIN is probably acting at the beginning of the process and that this cannabinoid may be useful for the treatment of NE cancers.

We believe our findings also demonstrate for the first time that AMPK is inhibited in NE cells. Similar to our findings demonstrating an inhibitory effect of AICAR on NED, activation of AMPK with metformin reduced the aggressiveness of NE tumors of the gastro–entero–pancreatic system.[35] Recent clinical studies on patients with well-differentiated pancreatic NE tumors, showed that the survival of metformin-treated patients was twice that observed in the control group.[36] To our knowledge, our work is the first study regarding the role of AMPK on NE differentiation of prostate cells. We also found that Akt activation is involved in the AMPK inactivation, and such AMPK inhibition contributes to NE differentiation of prostate LNCaP cells. Recent findings shown by other authors demonstrated that AKT activation induced AMPK Ser485 phosphorylation, which negatively regulated AMPK activity by inhibiting the Thr172 phosphorylation.[37] In line with this, results by Lin *et al.*[38] recently demonstrated that AKT activation in basophils could rapidly inhibit AMPK activation through increased AMPK phosphorylation at the inhibitory Ser485/491 residues, and this was accompanied by a reduction of ACC phosphorylation. In addition, data shown by Kim *et al.*[39] revealed that cortical neurons chronically treated with insulin underwent Akt activation, which provoked an increase of AMPK phosphorylation in Ser485 and inhibition of AMPK activity. Although we have not determined the AMPK phosphorylation in Ser485, our results show that inhibition of PI3K as well as Akt, abrogated the inhibition of AMPK in NE cells, providing a signaling link between Akt and AMPK in NED of prostate cells. Therefore, inhibition of PI3K/Akt by the cannabinoid WIN would remove the inhibitory phosphorylation of AMPK resulting in AMPK activation and prevention of NE differentiation (Figure 9).

A major finding of our study is the spectacular decrease in cannabinoid receptors during the NE-differentiation process. The cannabinoid receptors would exert a tonic inhibition of NE differentiation in prostate cells even in the absence of ligand and, therefore, a decrease of cannabinoid receptors may be concomitant to NE differentiation.

In line with our results, data shown by Sugawara *et al.*,[40] demonstrated that CB1 knockdown increased the differentiation of mast cells from resident progenitor cells and treatment with the endogenous cannabinoid ligand anandamide counteracted the activation of mast cells. The role of cannabinoid receptors in NE differentiation is not surprising as those receptors change their expression pattern along neuronal differentiation,[41] and NE differentiation of prostate tumors resembles features of neurons. Further research with endocannabinoids and its receptors will throw additional light on the actions of these compounds on prostate tumors differentiation and growth.

In perspective, cannabinoids could represent a viable strategy to prevent or delay the progression of advanced prostate cancer with NE differentiation features.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Prostate Cancer and Prostatic Diseases website (http://www.nature.com/pcan)