The Cannabinoid WIN 55,212-2 Decreases Specificity Protein Transcription Factors and the Oncogenic Cap Protein eIF4E in Colon Cancer Cells

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Abstract

2,3-Dihydro-5-methyl-3-[(morpholinyl)methyl]pyrollo(1,2,3-de)-1,4-benzoazinyl]-[1-naphthaleny]methanone [WIN 55,212-2, (WIN)] is a synthetic cannabinoid that inhibits RKO, HT-29, and SW480 cell growth, induced apoptosis, and downregulated expression of survivin, cyclin D1, EGF receptor (EGFR), VEGF, and its receptor (VEGFR1). WIN also decreased expression of specificity protein (Sp) transcription factors Sp1, Sp3, and Sp4, and this is consistent with the observed downregulation of the aforementioned Sp-regulated genes. In addition, we also observed by RNA interference (RNAi) that the oncogenic cap protein eIF4E was an Sp-regulated gene also downregulated by WIN in colon cancer cells. WIN-mediated repression of Sp proteins was not affected by cannabinoid receptor antagonists or by knockdown of the receptor but was attenuated by the phosphatase inhibitor sodium orthovanadate or by knockdown of protein phosphatase 2A (PP2A). WIN-mediated repression of Sp1, Sp3, and Sp4 was due to PP2A-dependent downregulation of microRNA-27a (miR-27a) and induction of miR-27a–regulated ZBTB10, which has previously been characterized as an “Sp repressor.” The results show that the anticancer activity of WIN is due, in part, to PP2A-dependent disruption of miR-27a:ZBTB10 and ZBTB10-mediated repression of Sp transcription factors and Sp-regulated genes, including eIF4E. Mol Cancer Ther; 12(11); 2483–93. ©2013 AACR.

Introduction

There are three major classes of cannabinoids, which include plant-derived compounds such as Δ[9]-tetrahydrocannabinol (THC) and cannabidiol, endogenous cannabinoids (anandamide and 2-arachidonoylglycerol), and synthetic compounds that mimic the effects of cannabinoids (1, 2). Cannabinoids bind the CB1 and CB2 receptors, which are differentially expressed in some but not all tissues, and there is also evidence that some cannabinoids also interact with other G-protein–coupled receptors (GPCR) including the receptor transient receptor potential vanilloid type I (TRPV1) and GPR55 (3–5). THC is the main psychoactive cannabinoid found in the marijuana plant Cannabis sativa L; however, in addition to the psychoactive effects of THC, endocannabinoids and synthetic cannabinoids also play a role in energy metabolism, pain and inflammation, cardiovascular, musculoskeletal and respiratory disorders, and cancer, and opportunities for cannabinoid-based pharmacotherapies are extensive (2).

The anticancer activities of cannabinoids have been known for over 3 decades, and clinical trials for treatment of gliomas with cannabinoids have been reported (6). Cannabinoids inhibit growth, induce apoptosis, and exhibit antimetastatic and antiangiogenic activities in multiple cancer cell lines and inhibit tumor growth in vivo mouse models (7–10). Cannabinoids are active in the tumor microenvironment and the effects of cannabinoids are complex and dependent on ligand structure and cell context, and the responses can also be cannabinoid (CB; CB1 and/or CB2) receptor–dependent or -independent (7–10). For example, the synthetic mixed CB1 and CB2 receptor agonist 2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrollo(1,2,3-de)-1,4-benzoazinyl]-[1-naphthaleny]methanone [WIN 55,212-2, (WIN)] inhibited gastric cancer cell growth and decreased VEGF expression and these responses were blocked in cells cotreated with WIN plus CB receptor antagonists (11), and similar CB receptor–dependent responses were observed in mantle cell lymphoma (12). In contrast, WIN induced phosphatase-dependent apoptosis in SW480 colon and LNCaP prostate cancer cells and these responses were inhibited by the phosphatase inhibitor sodium orthovanadate (SOV) but not by CB receptor antagonists or by CB receptor knockdown (13).
Treatment of cancer cells with cannabinoids activates or inactivates various kinases, ceramide synthesis, and ceramide-mediated proapoptotic and stress-related genes, and downregulates expression of EGFR receptor (EGFR), survivin, cyclin D1, bcl-2, and VEGF (11–26). Previous RNA interference (RNAi) studies have shown that these genes are also regulated by one or more of the specificity protein (Sp) transcription factors Sp1, Sp3, and Sp4, which are overexpressed in colon and other cancer cell lines and tumors; moreover, knockdown of Sp proteins by RNAi results in growth inhibition and induction of apoptosis (27–39). Therefore, we hypothesized that the anticancer activity of cannabinoids such as WIN in colon cancer cells may also be due, in part, to downregulation of Sp genes and pro-oncogenic Sp-regulated genes and this could also be related to the reported activation of phosphatases by cannabinoids in these cells (13). Results of this study show that WIN induced protein phosphatase 2A (PP2A)-dependent downregulation of Sp1-, Sp3-, Sp4-, and Sp-regulated genes including the pro-oncogenic cap protein elf4e in colon cancer cells. These responses were CB receptor-independent and involved disruption of microRNA-27a (miR-27a)–mediated suppression of the zinc finger transcriptional repressor ZBTB10, which acts as an Sp repressor (31, 33–35).

Materials and Methods

**Chemicals, antibodies, and reagents**

WIN 55,212-2 mesylate (1038), AM251 (1117), and AM630 (1120) were purchased from Tocris Bioscience. Tyrosine phosphatase inhibitor, SOV, was purchased from Calbiochem. Dithiothreitol (DTT) was obtained from Boehringer Mannheim Corp. Gliotoxin was kindly provided by Dr. Alan Taylor (National Research Council of Canada, Halifax, NS, Canada). p-Elf4e (S209), elf4e (9742S), and Cleaved poly (ADP-ribose) polymerase (PARP; 9541) antibodies were obtained from Cell Signaling Technology. Lactacystin, glutathione (GSH), and antibodies for β-actin (A1978), CB1 (C1233), and CB2 (WH0001269M1) receptors were purchased from Sigma-Aldrich. Antibodies for EGFR (1005), Sp3 (D-20), and Sp4 (V-20) were purchased from Santa Cruz Biotechnology. PP2A Immunoprecipitation Phosphatase Assay Kit (17-313), Immobilon Western Chemiluminescent HRP substrate (WBKLS0500), and antibodies for Sp1 (07-645) and PP2A (05421) were purchased from EMD Millipore. Cyclin D1 (2261-1) and survivin (2463-1) antibodies were purchased from Epitomics. VEGFR1 (ab32152) antibody was purchased from Abcam. Antibody for VEGF (209-403-B99) was purchased from Rockland Antibodies and Assays. ZBTB10 (A303 258A) antibody was purchased from Bethyl Laboratories. Apoptotic, Necrotic and Healthy Cells Quantification Kit (30018) was purchased from Biotium. An In situ cell death detection POD kit (1164817910) was obtained from Roche. Dulbecco’s Modified Eagle Medium (DMEM) and siRNAs for CB1 and CB2 receptors, PP2A, and ZBTB10 were obtained from Sigma-Aldrich. Lipofectamine 2000 was purchased from Life Technologies.

**Cell lines**

Human SW480 colon carcinoma cell line was provided by Dr. Stanley Hamilton (M.D. Anderson Cancer Center, Houston, TX) and nontransformed CCD-18Co colon cells were provided by Dr. Susanne Talcott (Texas A&M University, College Station, TX). RKO and HT-29 colon carcinoma cell lines were obtained from American Type Culture Collection. Cells were maintained at 37°C in the presence of 5% CO2 as described previously (13).

**Cell proliferation assay**

Colon cancer cells (1 × 10^5 per well) were seeded in 12-well plates and allowed to attach for 24 hours. The medium was then changed to DMEM/Ham F-12 medium containing 2.5% charcoal-stripped FBS, and either vehicle (dimethyl sulfoxide, DMSO) or varying concentrations of WIN was added. Cells were then trypsinized and counted after 24 and 48 hours using a Coulter Z1 cell counter. Each experiment was carried out in triplicate, and results were expressed as the mean ± SE for each set of experiments.

**Annnexin V staining assay**

SW480 and RKO cells were seeded in 2-well Nunc Lab-Tek chambered coverglass plates from Thermo Scientific and were allowed to attach for 24 hours. The medium was then changed to DMEM/Ham F-12 medium containing 2.5% charcoal-stripped FBS, and either DMSO or WIN was added. Cells were stained after 9 hours with fluorescein isothiocyanate (FITC)–Annexin V, propidium iodide, and DAPI. The proportion of apoptotic cells was determined by the amount of green fluorescence observed.

**Terminal deoxyribonucleotide transferase-mediated nick-end labeling assay**

HT-29 (7 × 10^6) were seeded in four-chambered glass slides and left overnight to attach. After treatment with WIN for 48 hours, the in situ cell death detection POD kit was used for the terminal deoxyribonucleotide transferase-mediated nick-end labeling (TUNEL) assay according to the manufacturer’s protocol. The percentage of apoptotic cells was determined by counting stained cells from eight fields, each containing 50 cells.

**Western blot analyses**

Colon cancer cells were seeded in DMEM/Ham F-12 medium and were allowed to attach for 24 hours. Cells were treated with either DMSO or WIN for indicated time periods or pretreated with the proteasome inhibitors, antioxidants, phosphatase inhibitors, and CB receptor inhibitors. Western blots were analyzed using the Odyssey infrared imaging system.
antagonists, and then treated with WIN. Cells were lysed and analyzed by Western blot analysis as described previously (13).

**siRNA interference assay**

Colon cancer cells were seeded (2 × 10^5 per well) in six-well plates in DMEM/Ham F-12 medium supplemented with 2.5% charcoal-stripped FBS without antibiotics and left to attach for 24 hours. siRNAs specific for the CB1 and CB2 receptors, PP2A, and ZBTB10 along with iLamin/iGL2 as control were transfected using Lipofectamine 2000 reagent according to the manufacturer’s protocol.

**Quantitative real-time PCR**

SW480 colon cancer cells were plated (2 × 10^5) and left to attach for 24 hours. Cells were transfected with siRNAs for PP2A and then treated with either DMSO or WIN for 24 and 48 hours. miRNA was isolated using the mirVana miRNA isolation kit from Ambion-Life Technologies according to the manufacturer’s protocol. cDNA was prepared using the Taqman MicroRNA Reverse Transcription Kit and was subjected to quantitative real-time PCR (qRT-PCR) with specific primers for miR-27a using the Taqman Universal PCR Master Mix from Applied Biosystems in the CFX384 Real-Time PCR Detection System from Bio-Rad.

**PP2A phosphatase assay**

Cells were seeded (3 × 10^5) and left to attach for 24 hours. Cells were treated with DMSO, okadaic acid, and 5 and 7.5 μmol/L WIN. Cells were harvested and lysed using the high salt lysis buffer. The lysates were then subjected to buffer exchange using the Zeba Desalt Spin Columns (8981) from Thermo Scientific to eliminate any contaminating phosphates that could skew the experimental results. The lysates were then immunoprecipitated with anti-PP2A, C subunit, antibody, and were incubated in a mixture of diluted phosphopeptidase and serine/threonine assay buffer for 10 minutes at 30°C in a shaking incubator. The phosphatase activity was determined by addition of malachite green dye and comparing the absorbance between controls and treatment at 650 nm in a plate reader.

**Results**

Treatment of SW480, RKO, and HT-29 colon cancer cells with 2.5, 5.0, and 7.5 μmol/L WIN for 24 or 48 hours significantly inhibited cell proliferation at the two higher concentrations (Fig. 1A–C), whereas minimal growth inhibition was observed for nontransformed CCD-18Co colon cells (Supplementary Fig. S1A). In addition, WIN also induced Annexin V staining in SW480 and RKO cells within 9 hours after treatment, indicating rapid induction of early apoptosis, whereas Annexin V was not induced in HT-29 cells at the early time-point (data not shown). In contrast, TUNEL staining was increased in HT-29 cells treated with WIN for 48 hours, indicating differences between the timing of WIN-induced apoptosis in SW480/
knockdown of Sp1 (by RNAi) also enhances PARP cleavage (31). A recent study showed that \( \alpha \)-tocopherol succinate–induced downregulation of Sp1 in prostate cancer cells was PP2A-dependent (40) and Western blot analysis of whole-cell lysates from SW480 cells transfected with a nonspecific oligonucleotide (iCtrl) or a specific oligonucleotide that targets PP2A (iPP2A) and treated with 7.5 \( \mu \)mol/L WIN or DMSO showed that WIN-mediated downregulation of Sp1, Sp3, and Sp4 proteins was significantly attenuated in cells transfected with iPP2A (Fig. 4B). Similar results were also observed in RKO cells with some differences in the relative effectiveness of PP2A knockdown (Supplementary Fig. S1B). PP2A knockdown also attenuated WIN-induced downregulation of cyclin D1 and VEGFR1 but had minimal effect on EGFR protein levels (Fig. 4C), suggesting that decreased expression of EGFR in SW480 cells treated with WIN was phosphatase- and Sp-independent.

We also used an in vitro assay to confirm that WIN induced PP2A activity because previous studies did not observe induction of PP2A mRNA levels in SW480 cells treated with WIN (13). PP2A activity in SW480 was measured using the PP2A Immunoprecipitation Phosphatase Assay Kit from Millipore. Treatment of SW480 cells with 7.5 \( \mu \)mol/L of WIN for 48 hours and whole-cell lysates were examined for expression of cleaved PARP by Western blot analysis. \( \beta \)-actin served as a loading control for all Western blot analyses.

![Figure 1](image-url). WIN inhibits colon cancer cell growth, induces apoptosis and PARP cleavage. SW480 (A), RKO (B), and HT-29 (C) cells were treated with DMSO and 2.5 to 7.5 \( \mu \)mol/L WIN for 24 and 48 hours and counted or treated with DMSO and 7.5 \( \mu \)mol/L of WIN for 9 (A and B) and 48 (C) hours and effects on Annexin V (SW480 and RKO) and TUNEL (HT-29) staining were determined. Results are expressed as the mean ± SE for at least three separate determinations, and significant (\( P < 0.05 \)) growth inhibition or induction of apoptosis by WIN is indicated (*)}. D, cells were treated with DMSO and 2.5 to 7.5 \( \mu \)mol/L of WIN for 48 hours and whole-cell lysates were examined for expression of cleaved PARP by Western blot analysis. \( \beta \)-actin served as a loading control for all Western blot analyses.
significantly inhibited PP2A activity in SW480 cells (positive control). Cell lysates used for the phosphatase activity assays were then subjected to Western blot analysis, and expression of Sp1, Sp3, and Sp4 protein was quantitated (C) relative to β-actin (levels in the DMSO group were set at 1.0). Results are expressed as the mean ± SE (three replicate experiments); and significantly ($P < 0.05$) decreased proteins are indicated (*). Data in Figures 2A and 2B are from the same experiment.

Cannabinoids Target Sp Transcription Factors

![Graph showing expression of Sp proteins (Sp1, Sp3, Sp4) with concentrations of WIN (0, 2.5, 5, 7.5 μmol/L) in SW480, RKO, and HT-29 cells.](https://www.aacrjournals.org)

Figure 2. WIN downregulates Sp-regulated proliferative, survival (A), and angiogenic (B) gene products and Sp proteins (C) in SW480, RKO, and HT-29 cells. Cells were treated with 2.5 to 7.5 μmol/L of WIN for 48 hours, whole-cell lysates were analyzed by Western blot analysis, and expression of Sp1, Sp3, and Sp4 protein was quantitated (C) relative to β-actin (levels in the DMSO group were set at 1.0). Results are expressed as the mean ± SE (three replicate experiments); and significantly ($P < 0.05$) decreased proteins are indicated (*). Data in Figures 2A and 2B are from the same experiment.
 Whereas Sp4 knockdown had minimal effects, suggesting that in SW480 cells, eIF4E expression is primarily regulated by Sp1. The knockdown of the Sp transcription factors was specific for Sp4 and Sp3; however, iSp1 also decreased expression of Sp4 as previously reported, indicating that Sp4 is an Sp1-regulated gene in SW480 cells (43). In contrast, knockdown of eIF4E by RNAi decreased expression of the targeted protein and slightly increased expression of Sp1, Sp3, or Sp4 proteins, suggesting that cross-regulation between Sp transcription factors and eIF4E is unidirectional. A comparable experiment was carried out in RKO cells; iSp1, iSp3, and iSp4 decreased eIF4E protein expression and the combination of all 3 oligonucleotides caused a marked decrease of eIF4E protein (Supplementary Fig. S1C), indicating that eIF4E is an Sp-regulated gene in colon cancer cells.

Drug-induced proteasome-independent downregulation of Sp1, Sp3, and Sp4 has been related to transcriptional repression because of downregulation of miR-27a and miR-20a/miR-17-5p, which regulate the “Sp repressors” ZBTB10 and ZBTB4 (34, 44). Treatment of SW480 cell with 7.5 μmol/L WIN induced ZBTB10 protein within 12 hours and induction was high after 24 hours but markedly decreased after 36 and 48 hours (Fig. 6A). In contrast, induction of ZBTB4 protein was not observed (data not shown). SW480 cells were treated with 7.5 μmol/L WIN and also transfected with siRNAs against CB1 and CB2 receptors, followed by treatment with WIN for 48 hours, and whole-cell lysates were analyzed by Western blot analysis. D, cells were transfected with siRNAs against CB1 and CB2 receptors, followed by treatment with WIN for 48 hours, and whole-cell lysates were analyzed by Western blot analysis. iLamin was used as a control oligonucleotide.

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These results show that WIN-mediated downregulation of Sp proteins in SW480 cells is due to activation of PP2A and PP2A-dependent disruption of the miR-27a:ZBTB10, which results in induction of ZBTB10 and ZBTB10-dependent repression of Sp proteins and Sp-regulated genes. The miR-27a promoter contains binding sites for both c-Myc and NF-κB (45), which are Sp-regulated genes in some cell lines (30, 31, 33); however, knockdown of Myc or p65 by RNAi did not decrease miR-27a expression (Supplementary Fig. S2) and, currently, we are investigating the mechanism of phosphatase-induced downregulation of miR-27a.

Discussion

Sp1, Sp3, and Sp4 proteins are highly expressed in multiple cancer cell lines and tumors, whereas levels of these transcription factors are low to nondetectable in...
nontumor tissue (29–33), and these observations are consistent with reports that Sp1 expression in rodent and human tissues decreases with age (46, 47). The pro-oncogenic activity of Sp proteins is primarily due to Sp-regulated genes, which include several genes that play pivotal roles in cancer cell proliferation (cyclin D1, c-Met, EGFR), survival (survivin, bcl-2), angiogenesis [VEGF and its receptors (VEGFR1 and VEGFR2)], and inflammation (NF-κB, p65). Thus, Sp transcription factors are an excellent example of nononcogene addiction by cancer cells (48) and therefore an important target for mechanism-based anticancer drugs. Reports from different laboratories have identified diverse agents that decrease expression of Sp transcription factors and include various phytochemical anticancer compounds including betulinic acid and their synthetic analogs, NSAIDs, bortezimib,
α-tocopherol succinate, arsenic trioxide, and other ROS inducers (29–40, 49).

Betulinic acid decreases expression of Sp1, Sp3, and Sp4 in prostate, colon, bladder, and breast cancer cell lines (32, 35, 37, 38). Results illustrated in Figs. 1 and 2 confirm that the cannabinoid WIN also inhibited growth and induced apoptosis in SW480, RKO, and HT-29 colon cancer cells and downregulated Sp1-, Sp3-, Sp4-, and Sp-regulated genes. Because knockdown of Sp1 or multiple Sp transcription factors alone in cancer cells inhibits growth and induces apoptosis (29–32), the anticancer activity of WIN is due, in part, to downregulation of Sp proteins. However, in contrast with betulinic acid and other agents that downregulate Sp transcription factors, WIN-induced Sp downregulation was proteasome- and ROS-independent and knockdown or inhibition of CB1 or CB2 receptors did not affect this response in SW480 cells (Fig. 3). The receptor-independent effects of WIN were consistent with previous studies showing that the anticancer activity of WIN was both receptor-dependent and -independent (11–13).

We recently reported that WIN-induced apoptosis in SW480 and LNCaP cells is inhibited by the phosphatase inhibitor SOV, but not by CB receptor knockdown, and WIN also induces multiple phosphatase mRNAs (13). These results, coupled with a report that PP2A plays a role in α-tocopherol succinate–induced downregulation of Sp1 in prostate cancer cells (40), prompted us to investigate the effects of SOV and PP2A knockdown on WIN-induced repression of Sp proteins. Both SOV and PP2A
knockdown attenuated WIN-mediated downregulation of Sp1, Sp3, Sp4, and Sp-regulated gene products (Fig. 4), confirming a role for PP2A and possibly other identified phosphatases in mediating the anticancer activities of WIN. An important proteasome-independent pathway for drug-induced downregulation of Sp1, Sp3, and Sp4 involves induction of the transcriptional repressors ZBTB10 and ZBTB4, which exhibit low expression in cancer cell lines due to their regulation (repression) by miR-27a and miR-20a/miR-17-5p, respectively (38, 39, 44, 49). WIN did not induce ZBTB4 in SW480 cells; however, a time-dependent induction of ZBTB10 protein was observed (Fig. 6A) and this response was abrogated after knockdown of PP2A (Fig. 6B), and these results were paralleled by PP2A-dependent repression of miR-27a (Fig. 6C). Moreover, because knockdown of ZBTB10 by RNAi blocks WIN-mediated downregulation of Sp1, Sp3, and Sp4 proteins (Fig. 6D) and because miR-27a antagonists and ZBTB10 expression decrease Sp protein expression in colon cancer cells (49), it is clear that disruption of miR-27a:ZBTB10 is critical for the observed responses, and results of c-Myc and p65(NF-kB) silencing did not decrease miR-27a expression (Supplementary Fig. S2). The mechanism of PP2A-mediated effects on miR-27a:ZBTB10 is unknown and are currently being investigated.

The induction of PP2A activity by WIN suggested that WIN may also regulate phosphorylation of the important cap protein eIF4E as it has been reported that PP2A dephosphorylates p-eIF4E, which has been characterized as a pro-oncogenic phosphoprotein (42). WIN clearly decreased expression of p-eIF4E; however, this was unexpected accompanied by downregulation of elf4E (total protein). Because elf4E and Sp transcription factors regulate expression of several genes in common (e.g., cyclin D1 and VEGF), we used RNAi to show for the first time that elf4E is an Sp-regulated gene in colon cancer cells (Fig. 5D). This observation extends the number of pro-oncogenic factors that are regulated by Sp1, Sp3, and Sp4 in cancer cells, emphasizing the potential clinical applications of drugs that target Sp proteins. Previous studies show that other cannabinoids such as cannabidiol, other synthetic cannabinoids, and endocannabinoids inhibit colon cancer cell and tumor growth (22–26), and in vivo studies show that loss of the CB1 receptor enhances intestinal adenoma growth in Apcmin/+ mice (23). The effects of these compounds on Sp transcription factors and elf4E expression and the role of the CB receptors in mediating these responses are currently being investigated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: S. Sreevalsan, S. Safe
Development of methodology: S. Sreevalsan, S. Safe
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Sreevalsan, S. Safe
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Sreevalsan, S. Safe
Writing, review, and/or revision of the manuscript: S. Sreevalsan, S. Safe
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Safe
Study supervision: S. Safe

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References

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