

Modulation of breast cancer cell viability by a cannabinoid receptor 2 agonist, JWH-015, is calcium dependent

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Introduction: Cannabinoid compounds, both nonspecific as well as agonists selective for either cannabinoid receptor 1 (CB₁) or cannabinoid receptor 2 (CB₂), have been shown to modulate the tumor microenvironment by inducing apoptosis in tumor cells in several model systems. The mechanism of this modulation remains only partially delineated, and activity induced via the CB₁ and CB₂ receptors may be distinct despite significant sequence homology and structural similarity of ligands.

Methods: The CB₂-selective agonist JWH-015 was used to investigate mechanisms downstream of CB₂ activation in mouse and human breast cancer cell lines in vitro and in a murine mammary tumor model.

Results: JWH-015 treatment significantly reduced primary tumor burden and metastasis of luciferase-tagged murine mammary carcinoma 4T1 cells in immunocompetent mice in vivo. Furthermore, JWH-015 reduced the viability of murine 4T1 and human MCF7 mammary carcinoma cells in vitro by inducing apoptosis. JWH-015-mediated reduction of breast cancer cell viability was not dependent on G_α_i signaling in vitro or modified by classical pharmacological blockade of CB₁, GPR55, TRPV1, or TRPA1 receptors. JWH-015 effects were calcium dependent and induced changes in MAPK/ERK signaling.

Conclusion: The results of this work characterize the actions of a CB₂-selective agonist on breast cancer cells in a syngeneic murine model representing how a clinical presentation of cancer progression and metastasis may be significantly modulated by a G-protein-coupled receptor.

Keywords: cannabinoid receptor-2, CB₂, breast cancer, JWH-015, MAPK/ERK, apoptosis, calcium

Introduction

Breast cancer is the leading cause of cancer-related deaths among women aged 34–50 worldwide,¹ and is the most commonly diagnosed metastasizing tumor in women of all ages.² The staggering prevalence of breast cancer has made it a central focus of investigative efforts over the last 70 years. The results of such expansive research efforts are encouraging: not only have there been tremendous advances in understanding the etiology of the disease but significant improvements have also been made in detection and diagnosis of breast cancer. These advances have aided in increasing the 10-year survival rates of metastatic breast cancer from 3.3% in 1944 to 22.2% in 2004, with even greater life expectancy in patients with localized disease (76.5%).³ Despite advances in understanding breast cancer as a disease, there remains a critical need for novel disease-modifying therapeutics. The possible genetic mutations occurring in breast tumors vary widely from patient to patient,⁴ making protein-targeted therapies

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applicable to specific subsets of patients and increasing the occurrence of therapeutic resistance.⁵ In addition, there is an inverse relationship between disease progression and efficacy of analgesics, contributing to the 75%–95% of breast cancer patients experiencing chronic debilitating pain that is predominantly associated with metastasis.⁶

Nonspecific cannabinoids,^{7–9} cannabinoid receptor 2 (CB₂)-selective,^{10,11} as well as cannabinoid receptor 1 (CB₁)-selective compounds^{12,13} have yielded similar antitumor results in several tumor models. The lack of neuronal expression of CB₂ receptors precludes CB₂ selective compounds from inducing the psychotropic effects that typically accompany CB₁ activation.¹⁴ Our group and others have shown that CB₂ agonists displaying selectivity for the CB₂ receptor can decrease tumor cell viability and significantly attenuate cancer-induced bone pain without displaying psychoactive or addictive properties.^{15,16} Additionally, CB₂ receptors are markedly upregulated in many breast tumors,¹⁷ which is a curious finding given that many of these tumor cells are of epithelial origin, while in normal adults, CB₂ receptor expression is primarily limited to cells of the immune system and bone maintenance cells.¹⁸

While the antitumor effects of cannabinoids have been demonstrated in a variety of tumor models, the intracellular pathway(s) by which cannabinoid antitumor activity occurs remain unclear. Several cannabinoid mechanisms have been reported, some conflicting, that identify cannabinoid activity through G α_p ,¹⁹ G α_q ,²⁰ mTORC1,²¹ AKT/PI3K inhibition,¹⁷ AKT/PI3K stimulation,²² MAPK/ERK modulation,²³ p38/MAPK modulation,²⁴ ceramide accumulation,²⁵ induction of reactive oxygen species,²⁶ modification of matrix metalloproteinases,¹⁰ Id-1 inhibition,⁹ and the involvement of extraneous receptors including TRPV1²⁷ and GPR55.²⁸ To further complicate matters, it has also been suggested that the action of cannabinoids on tumor cells alone is not sufficient to produce *in vivo* antitumor effects and also requires immune system interaction to achieve full efficacy.⁷ Here, we focus on the direct effects and mechanism of the CB₂-selective agonist JWH-015 on breast cancer cells. We show that JWH-015 inhibits tumor cell viability and induces apoptosis of breast cancer cell lines (murine) 4T1 and (human) MCF7 *in vitro* and attenuates primary tumor growth and metastasis *in vivo*. In addition, we show that the MAPK/ERK pathway is modified by JWH-015. Finally, we demonstrate that these effects occur through a Ca²⁺-dependent mechanism that is unlikely to lie downstream of the traditional G α_i pathway previously associated with cannabinoid receptor activity. The antiproliferative effects of a CB₂ agonist along with our previous work demonstrating significant efficacy in inhibiting

bone cancer pain and slowing bone loss in a murine model of advanced breast cancer strongly suggest that CB₂ agonists should be investigated in humans as adjunct therapy for advanced stages of breast cancer.

Methods

In vitro

Cell culture

4T1 and MCF7 cell lines were obtained from American Type Culture Collection Biological Resource Center (Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum, 100 IU mL⁻¹ penicillin and 100 μ g/mL streptomycin. Cells were plated in 10 cm tissue culture dishes and allowed to grow exponentially.

No ethical approval was sought or required for experiments involving commercially purchased cell lines, as per guidelines of the University of Arizona human subjects protection program.

Stable expression of 4T1-Luc cells

4T1 cells stably expressing the pGL4.17 (luciferase) vector (Promega, Fitchburg, WI, USA) were developed via transfection with Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and selected with neomycin with expression being verified prior to each assay according to the manufacturer's instructions.

CB₂ small hairpin RNA knockdown

CB₂ and control scramble small hairpin RNA (shRNA) DNA lentiviral particles (Santa Cruz Biotechnology, Dallas, TX, USA) were used to transiently knockdown CB₂ receptor expression in 4T1 cells according to the manufacturer's instructions.

Compounds

The National Institute on Drug Abuse provided Cannabidiol, SR144528, and SR141716. Pertussis toxin (PTX) and capsazepine were purchased from Sigma-Aldrich (St Louis, MO, USA). JWH-015, o-1602, nifedipine, ω -conotoxin MVIIC, and HC-030031 were purchased from Tocris (Ellisville, MO, USA). All compounds were dissolved in dimethyl sulfoxide (DMSO) and added to fresh culture media such that the effective DMSO concentration was 0.1% per well.

Cellular viability assay

Cellular viability was measured using the sulforhodamine B (SRB; Sigma-Aldrich) assay as previously described.²⁹

Immunoblotting

Cells were lysed, protein extracts were separated electrophoretically, and then transferred to a polyvinylidene fluoride membrane as previously described.³⁰ The membrane was incubated with either polyclonal anti-CB₂ (Cayman Chemical, Ann Arbor, MI, USA; item number 101550) or monoclonal phospho-ERK1/2 (Cell Signaling Technology, Danvers, MA, USA) antibody followed by horseradish peroxidase-conjugated secondary IgG antibody (Cell Signaling Technology) and developed using a chemiluminescent system (Amersham Biosciences, Piscataway, NJ, USA). α -Tubulin and glyceraldehyde phosphate dehydrogenase (Cell Signaling Technology) were used as loading controls. Quantification of Western blot was performed using gel analysis in ImageJ (National Institutes of Health, Bethesda, MD, USA).

Ca²⁺ imaging

4T1 cells were cultured to 50% confluence in dual-chamber slides in complete media. On the day of imaging, cells were switched to serum-low, antibiotic-free Opti-minimal essential medium (Opti-MEM) media (Thermo Fisher Scientific) and equilibrated for 1 hour in a 5% CO₂ incubator at 37°C. Lyophilized fura-2 acetoxymethyl (Fura-2 AM) (Thermo Fisher Scientific) dissolved at 1 mg/mL in DMSO constituted stock solution, which was then diluted to 5 mg/mL in Opti-MEM. Cells were incubated for 1 hour in 5 μ g/mL Fura-2 AM for loading, followed by 30-minute incubation in Opti-MEM without Fura-2 AM to allow de-esterification of the fluorescent dye. Cells were imaged on a Nikon fluorescent microscope (Nikon Instruments, Inc., Melville, NY, USA) equipped with a charge-coupled device camera. Absorbance at 340 and 380 nm were simultaneously recorded, and the absorbance ratio (340 nm [Ca²⁺-bound Fura-2] over 380 nm [free Fura-2]) was plotted for a period of 60 minutes before spiking the media with potassium chloride for positive control.

Caspase 3/7 activity

Caspase-Glo 3/7 assay (Promega) was used to measure caspase 3 and 7 activities in 4T1 cells and MCF7 cells (which express caspase 7 but not caspase 3) according to the manufacturer's instructions. Results were normalized to total protein and expressed as a percentage of control.

Immunohistochemistry

4T1 cells were allowed to grow to 50% confluence in dual-chamber slides before replacing media with serum-low Opti-MEM. Cells were treated with JWH-015 for 24 hours and fixed with 10% formalin for 20 minutes at room

temperature. Cells were permeabilized with 0.25% Triton X-100 (Sigma-Aldrich) for 30 minutes and blocked with 5% bovine serum albumin for 30 minutes at room temperature. Cells were incubated in polyclonal Ki67 antibody (Abcam, Cambridge, MA, USA) diluted 1/200 in 5% bovine serum albumin overnight at 4°C. Slides were incubated for 2 hours at room temperature with Alexa Fluor 488 (Thermo Fisher Scientific) anti-rabbit IgG secondary antibody, mounted with 4',6-diamidino-2-phenylindole (Abcam), and visualized on a fluorescent microscope (Zeiss, Oberkochen, Germany).

Cyclic adenosine monophosphate

A cyclic adenosine monophosphate (cAMP) complete enzyme immunoassay kit (Enzo Life Sciences, Farmingdale, NY, USA) was used to measure forskolin-induced cAMP formation in vitro in response to JWH-015. Assay was conducted per manufacturer instructions and normalized to total protein.

In vivo

Animals

All procedures were approved by the University of Arizona Animal Care and Use Committee and conformed to the Guidelines of the National Institutes of Health and the International Association for the Study of Pain. Female BALB/cfC3H mice (Harlan, Indianapolis, IN, USA) weighed 15–20 g prior to initiation of study. Mice were maintained in a climate-controlled room on a 12-hour light/dark cycle and allowed food and water ad libitum.

Mammary fat pad injections

Mice were injected with 4T1-Luc cells (5×10^5) into the c-9 mammary fat pad as previously described.³¹

Treatment

The CB₂ receptor agonist JWH-015 with affinity at receptor (K_i) at CB₂=13.8 nM and 30-fold selectivity over CB₁ was dissolved in a vehicle solution of 10% DMSO, 10% Tween-80, and 80% saline. All injections were made at a volume of 10 mL/kg and consisted of once-daily intraperitoneal (ip) injections for 21 days (8–28). Randomly assigned animals were treated with JWH-015 (6 mg/kg ip) or vehicle.

Bioluminescence imaging

Animals inoculated into the mammary fat pad with 4T1-Luc cells were live-imaged on a weekly basis as previously described.³¹ Briefly, 4T1-Luc tumor-bearing animals were anesthetized with inhaled isoflurane and injected ip with K+ D-luciferin (Molecular Imaging Products, Bend, OR, USA) 5 minutes before imaging

(5-minute exposure in a dark chamber). Dark images were assigned intensity map coloring in ImageJ and overlaid on 100 ms light exposures of the same animals.

Ex vivo

Primary tumor and metastasis analysis

Twenty-eight days after tumor inoculation, animals were sacrificed, primary tumors excised, and tumor mass recorded. Common sites of mammary tumor metastasis were harvested (brain, lung, femur, kidney, and spleen) and homogenized in the presence of protease inhibitor cocktail (Sigma-Aldrich) for ex vivo detection of metastasis using a dual-luciferase reporter assay (Promega) performed according to the manufacturer's instructions. Data are represented as % of total samples in treatment group testing positive for luciferase activity.

Statistical analysis

SRB assays, quantified Western blots, caspase 3/7 assays, cAMP assays, and primary tumor mass were analyzed using

one-way analysis of variance followed by Student–Neuman–Kuels testing for multiple comparisons in FlashCalc.³² Differences were considered to be significant if $P \leq 0.05$. When possible, potencies (or A_{50}) were determined by regression analysis of dose–response curve (log dose [x] vs response [y]) using a 95% confidence interval (CI) according to the method of analysis of the graded dose–response.³³ For the calculations of A_{50} s, the minimal possible response was set to 0%.

Results

Breast cancer cells functionally express the CB₂ receptor

Cellular expression of CB₂ was positively identified in cell lysate from several immortalized mouse and human breast cancer cell lines (Figure 1A). To ascertain whether JWH-015 stimulation of the CB₂ receptor engages the traditionally described G α_1 pathway, cAMP was assayed in the presence of varying concentrations of JWH-015 after forskolin stimulation

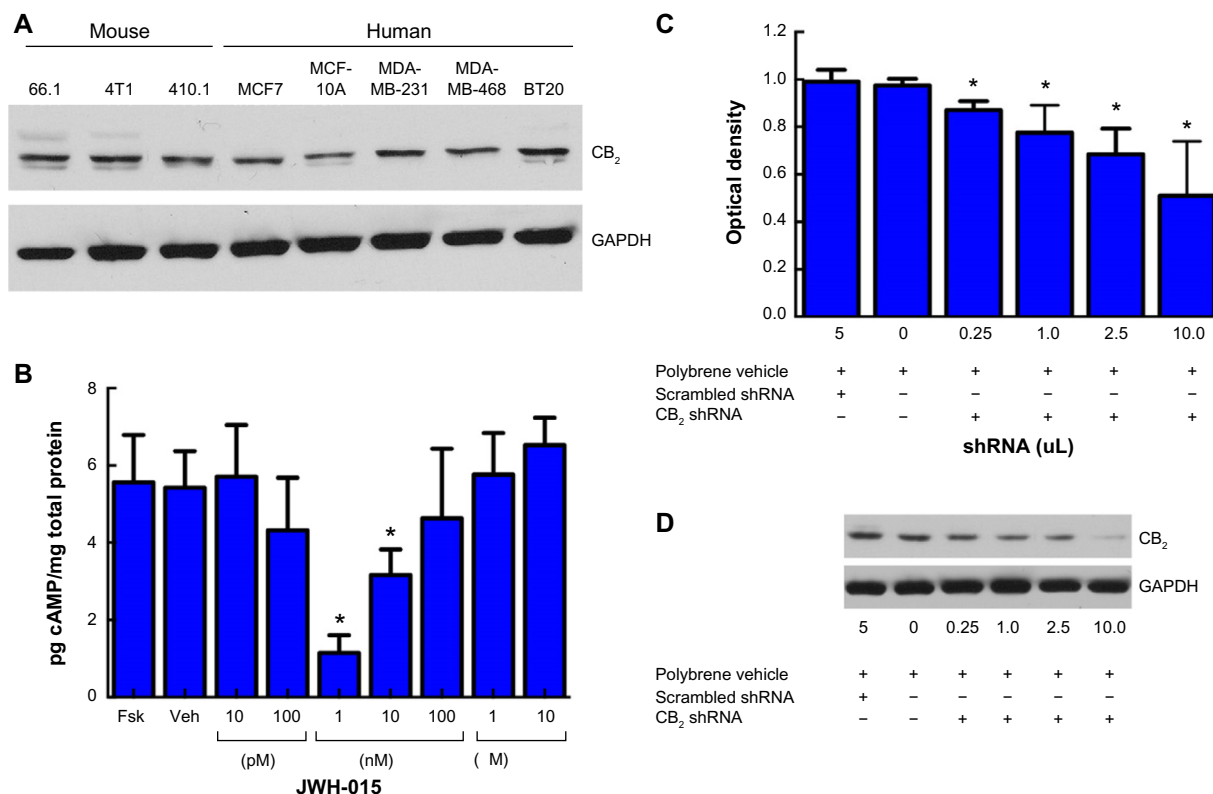


Figure 1 Breast cancer cells functionally express the CB₂ receptor.

Notes: (A) Western blot of cell lysates from mouse and human breast cancer cell lines showing expression of the CB₂ receptor (top) and loading control α -tubulin (bottom). (B) In vitro cAMP ELISA performed on 4T1 cells after 30-minute incubation with phosphodiesterase inhibitor IBMX and 30-minute stimulation with either positive control Fsk, control Veh, or JWH-015 (range 10 pM to 10 μ M) ($*P < 0.05$). (C) Sulforhodamine B-cell viability assays after shRNA DNA lentiviral knockdown of CB₂ receptor. Cell viability 60 hours after a 12-hour incubation period with indicated concentrations of anti-CB₂ shRNA shown ($*P < 0.05$). (D) Western blot showing degree of CB₂ knockdown after incubation with shRNA DNA lentiviral particles.

Abbreviations: CB₂, cannabinoid receptor 2; GAPDH, glyceraldehyde phosphate dehydrogenase; cAMP, cyclic adenosine monophosphate; shRNA, small hairpin RNA; ELISA, enzyme-linked immunosorbent assay; IBMX, 3-isobutyl-1-methylxanthine; Fsk, forskolin; Veh, vehicle.

in 4T1 cells (Figure 1B). We found that 4T1 cells demonstrate high baseline adenylate cyclase activity that is not increased by incubation with forskolin ($P>0.05$) and can be blocked by low nanomolar concentrations of JWH-015. Peak inhibition of cAMP occurs at concentrations of 1 nM ($P<0.05$) and 10 nM ($P<0.05$) of JWH-015, while inhibition of cAMP accumulation is abolished at JWH-015 concentrations higher than 100 nM ($P>0.05$). To evaluate the functionality of CB₂ expression in 4T1 mammary carcinoma cells, we performed knockdown of the CB₂ receptor in 4T1 murine mammary carcinoma cells in vitro via shRNA DNA lentiviral particles (Figure 1C, D). shRNA knockdown of the CB₂ receptor reduced cancer cell viability in a manner consistent with degree of knockdown ($P<0.05$). These data suggest the

expression of the CB₂ receptor as part of the vitality of the cancer cell line.

JWH-015 reduces primary tumor burden and decreases metastasis in vivo

4T1 cells were transfected with luciferase, and the resulting stable 4T1-Luc cell line was injected into the c-9 mammary fat pad of immunocompetent female BALB/cfC3H mice. The reported data are combined from three separate experiments in which tumors were allowed to establish for 7 days postinoculation and mice were then treated with either CB₂ agonist JWH-015 6 mg/kg ip (n=19) or vehicle (n=12) for 21 days. Mice were imaged on a weekly basis (Figure 2A) beginning on day 7 through day 28 to track primary tumor growth and sites

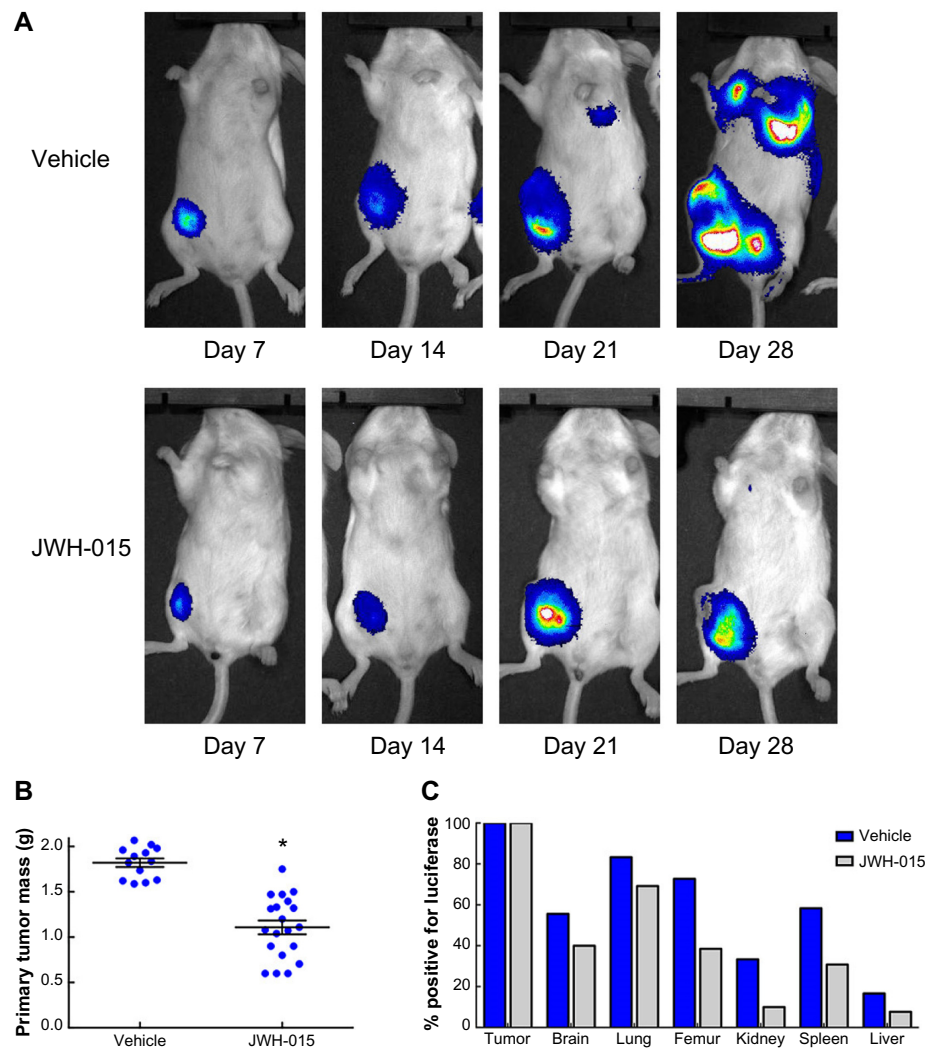


Figure 2 In vivo administration of JWH-015 attenuates primary mammary tumor growth.

Notes: (A) Weekly in vivo bioluminescence imaging representative of tumor progression in immunocompetent mice receiving either vehicle (top) or JWH-015 (6 mg/kg) for 21 days beginning 7 days after tumor inoculation (1×10^6 4T1 cells via orthotopic injection into the c-9 mammary fat pad) in the 4T1-Luc murine mammary carcinoma model. (B) Day 28 mass of resected primary tumors from vehicle- (left) or JWH-015-treated mice ($*P<0.05$). (C) Mice were evaluated for metastasis via luminometer analysis of cell lysates isolated from common metastatic sites on day 28.

of metastasis. On day 28, mice were sacrificed, primary tumors were immediately resected, and common metastatic site tissues were harvested. Chronic administration of JWH-015 (6 mg/kg) significantly reduced primary tumor mass ($P < 0.05$; Figure 2B). Tissue samples from common metastatic sites were harvested for ex vivo analysis with a Promega dual-luciferase reporter assay. Detectable metastases (defined by the presence of luciferase expressing cells in the tissue sample) were reported as a percentage of the total number of the respective tissue samples per experimental group (Figure 2C). Comparison of samples isolated from either vehicle- or JWH-015 (6 mg/kg)-treated animals revealed a trend toward reduced metastasis in tissues isolated from brain, femur, kidney, spleen, lung, and liver; however, since this assay evaluated the presence or absence of luciferase-expressing cells within each tissue sample without quantification, only trends in CB_2 agonist-induced reduction in metastasis can be reported along with the statistically significant reduction in primary tumor burden.

The CB_2 inverse agonist SR144528 does not block JWH-015-mediated reduction of breast cancer cell viability

The SRB assay was used to determine the viability of mouse and human (4T1 and MCF7, respectively) breast

tumor cells in vitro in the presence of the CB_2 -specific agonist JWH-015, the CB_2 -specific inverse agonist SR144528, or a combination thereof (Figure 3). Treatment with JWH-015 over a 48-hour period reduced the viability of 4T1 cells ($A_{50} = 2.8 \mu\text{M}$; 95% CI = 2.56–3.07) and MCF7 cells ($A_{50} = 4.16 \mu\text{M}$; 95% CI = 3.24–5.34) in a dose-dependent manner. Pretreatment with the CB_2 inverse agonist SR144528 1 hour prior to administration of JWH-015 did not block the viability reduction by JWH-015 in either 4T1 cells ($A_{50} = 2.475 \mu\text{M}$; 95% CI = 1.77–3.46) or MCF7 cells ($A_{50} = 5.78 \mu\text{M}$; 95% CI = 5.04–6.52). When SR144528 was administered alone in either 4T1 cells ($A_{50} = 4.88 \mu\text{M}$; 95% CI = 4.12–5.77) or MCF7 cells ($A_{50} = 7.01 \mu\text{M}$; 95% CI = 3.53–13.92), cellular viability was also reduced, suggesting that CB_2 -activity-modifying compounds significantly decrease breast cancer proliferation.

JWH-015-induced apoptosis is independent of cell cycle

To determine whether loss of breast cancer cell viability is the direct result of cell killing or is an effect secondary to senescence, Ki67 imaging was done in vitro using a Ki67 primary antibody and an fluorescein isothiocyanate-conjugated secondary antibody (Figure 4A). No observable difference was seen between the number of 4T1 cell nuclei

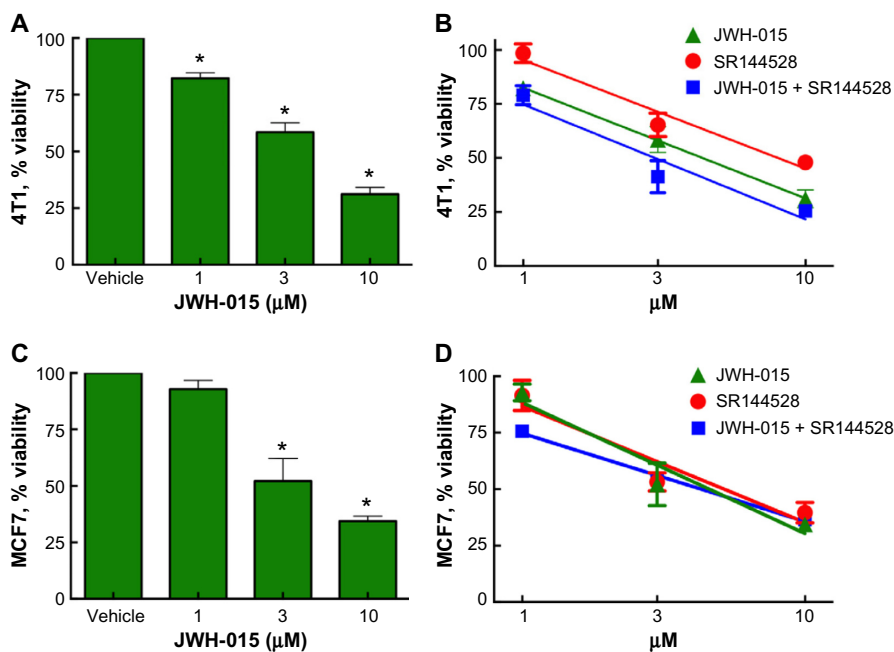


Figure 3 CB_2 inverse agonist SR144528 does not attenuate cell viability reduction induced by JWH-015 in vitro.

Notes: Sulforhodamine B assays for cell viability were performed on murine 4T1 (A, B) and human MCF7 (C, D) breast cancer cells after administration of either vehicle, JWH-015 alone, SR144528 alone, or JWH-015 and SR144528 in combination. Cell viability in response to 48-hour incubation with JWH-015 is shown (A, C; * $P < 0.05$). Dose-response curves in response to all treatment groups are depicted in panels (B) and (D) for comparison.

Abbreviation: CB_2 , cannabinoid receptor 2.

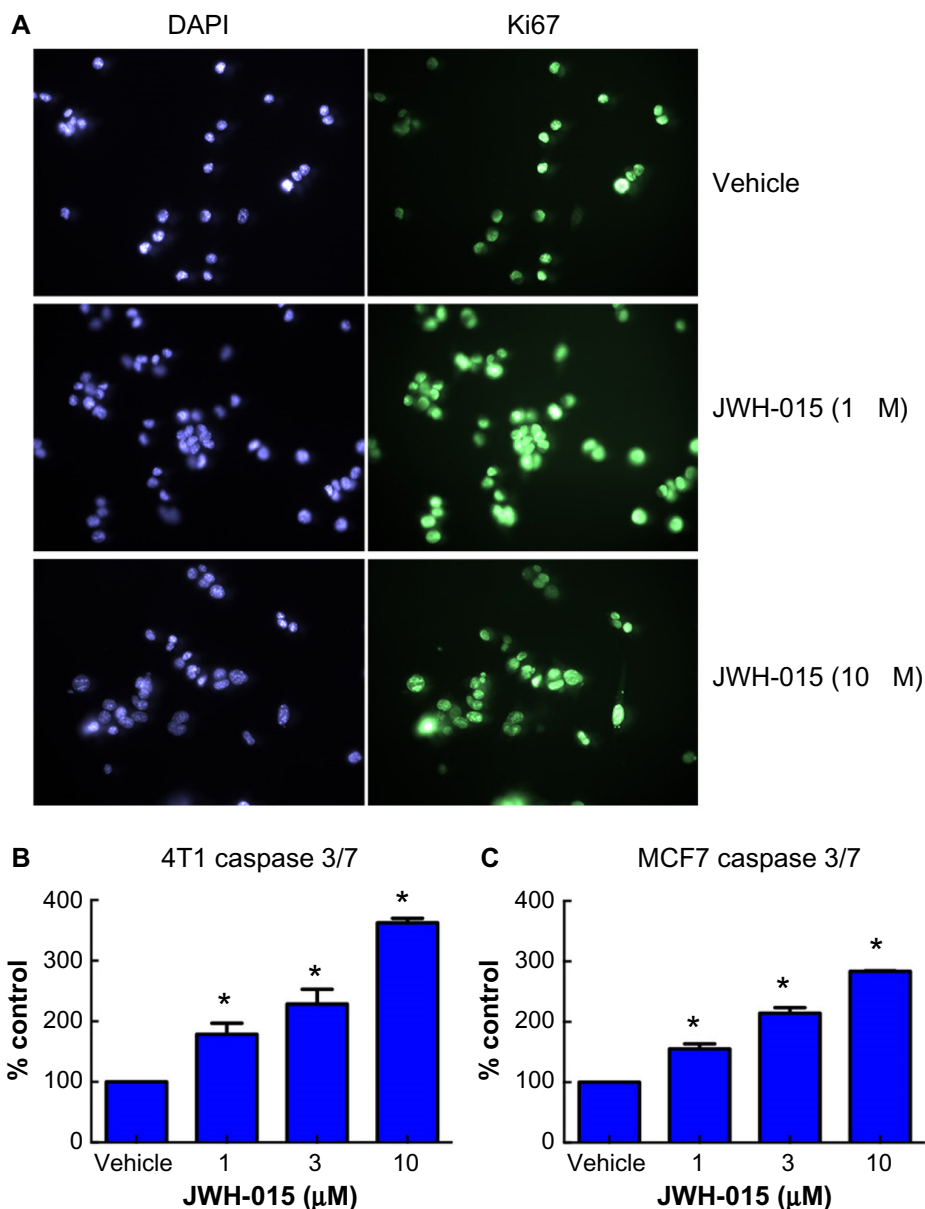


Figure 4 In vitro administration of JWH-015 induces apoptosis of breast cancer cells independent of cell cycle.

Notes: (A) 40× magnification. Ki67 imaging studies were performed on 4T1 cells in response to vehicle (upper panels), 1 μM JWH-015 (middle panels) or 10 μM JWH-015 (lower panels). DAPI nuclear staining (left) is consistent with Ki67 staining (right) in all treatment conditions. Early caspases 3 and 7 were evaluated via ELISA in 4T1 cells (B) and MCF7 cells (which express caspase 7 but not caspase 3); (C) after 24 hours of exposure to vehicle or 1, 3, or 10 μM JWH-015. Samples were normalized to caspase levels in untreated control samples (* $P < 0.05$).

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay.

taking up 4',6-diamidino-2-phenylindole stain and those expressing fluorescein isothiocyanate fluorescence in naïve cells, media-treated cells, or JWH-015 (10 μM)-treated cells over a 24-hour period.

To verify that, consistent with other cannabinoid investigations,^{8,34} JWH-015 attenuates cellular viability in 4T1 and MCF7 breast cancer cells by inducing apoptosis, caspase 3/7 activity was assessed. JWH-015 produced activation of early caspases in a dose-dependent manner in vitro, 12 hours posttreatment (Figure 4B and C) in 4T1 cells and MCF7 cells

($P < 0.05$). These data suggest a CB₂ drug-induced apoptosis and not changes in the cell cycle.

JWH-015 induces calcium flux and changes in ERK phosphorylation

To determine whether JWH-015 induces calcium flux in breast cancer cells, Fura-2 AM staining was performed in vehicle- and JWH-015-treated 4T1 cells (Figure 5). Graphical representation of calcium flux was created by plotting the A340/A380 ratio of recorded cells with respect to time (Figure 5A and C),

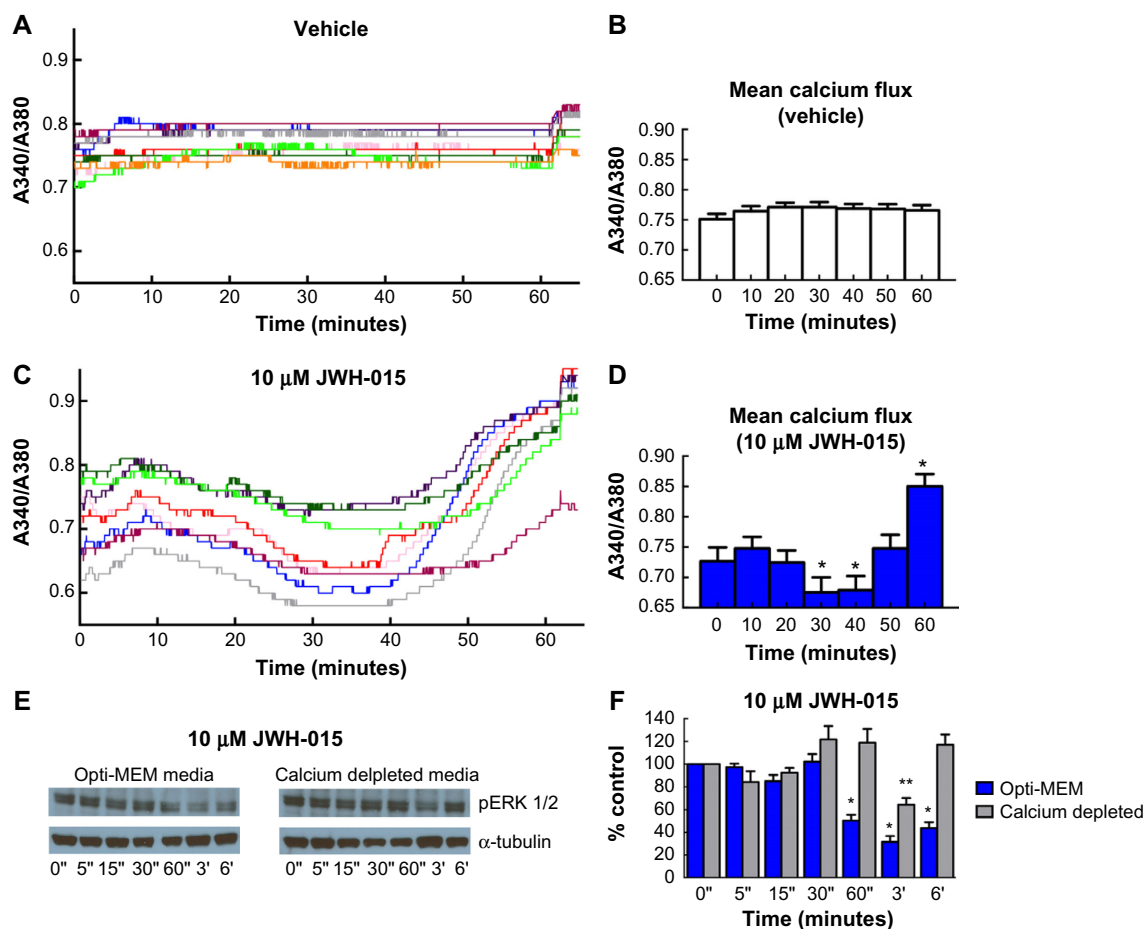


Figure 5 JWH-015 induction of calcium flux and modulation of MAPK/ERK phosphorylation is calcium dependent.

Notes: Fura 2-AM signal was recorded every 30 seconds for a period of 60 minutes in 4T1 cells treated with vehicle (**A**) or 10 μM JWH-015 (**C**). Graphs are depicted as ratio of recordings at 340 over 380 nm vs time. Quantification of signal (**B**, **D**) shows an initial inhibition of calcium efflux followed by a marked increase in efflux 60 minutes postadministration of JWH-015 ($*P < 0.05$). Western blot analysis of phospho-ERK1/2 (**E**) demonstrates JWH-015 inhibition of the MAPK/ERK pathway that is attenuated when culture media is replaced with calcium-depleted media 20 minutes prior to administration of 10 μM JWH-015. ImageJ quantification of the blots normalized to α -tubulin loading control is depicted in panel (**F**) ($*P < 0.05$ in Opti-MEM cultured cells and $**P < 0.05$ in calcium-depleted cultures).

Abbreviations: Opti-MEM, Opti-minimal essential medium; Fura 2-AM, Fura 2-acetoxymethyl.

and statistical analyses were performed on individual cell recordings (Figure 5B, D; $n=9$ per group; $P < 0.05$).

It has been shown previously that cannabinoids exert effects on the MAPK/ERK intracellular signaling cascade.^{23,35} To verify JWH-015-induced attenuation of ERK1/2 phosphorylation and to determine whether this phenomenon is downstream of JWH-015-induced calcium flux, Western blots were performed after treatment of 4T1 cells in vitro with JWH-015 for the indicated time periods in both normal or calcium-depleted media (Figure 5D). Blots were reprobbed with α -tubulin as a loading control. ImageJ was used to evaluate relative density of scanned blot images and the data were normalized to relative density of control (data are averaged from three separate experiments; Figure 5F). JWH-015 (10 μM) reduced phosphorylation of ERK1/2 at 1, 3, and 6 hours postadministration ($P < 0.05$ as compared to vehicle-treated cell lysates). JWH-015 did not reduce

phosphorylation of ERK1/2 in calcium-depleted cultures ($P > 0.05$) when compared to vehicle-treated cell lysates.

JWH-015 induction of apoptosis is dependent on cellular calcium flux

JWH-015 failed to induce caspase 3/7 activation in 4T1 cells cultured in calcium-depleted media (Figure 6A and B; $P < 0.05$). To establish whether the loss of JWH-015 effect occurs due to intracellular or extracellular calcium channel activity, further inquiry was made by pharmacological blockade of specific classes of calcium channels during JWH-015 stimulation. SRB assays were performed on 4T1 cells in the presence of JWH-015 and either the L-type calcium-channel blocker nifedipine (Figure 6C) ($A_{50}=1.89 \mu\text{M}$; 95% CI = 0.96–3.72) or the N/P/Q-type calcium-channel inhibitor ω -conotoxin MVIIC (Figure 6C) ($A_{50}=0.41 \mu\text{M}$; 95% CI = 0.21–0.79). Neither cotreatment restored JWH-015-

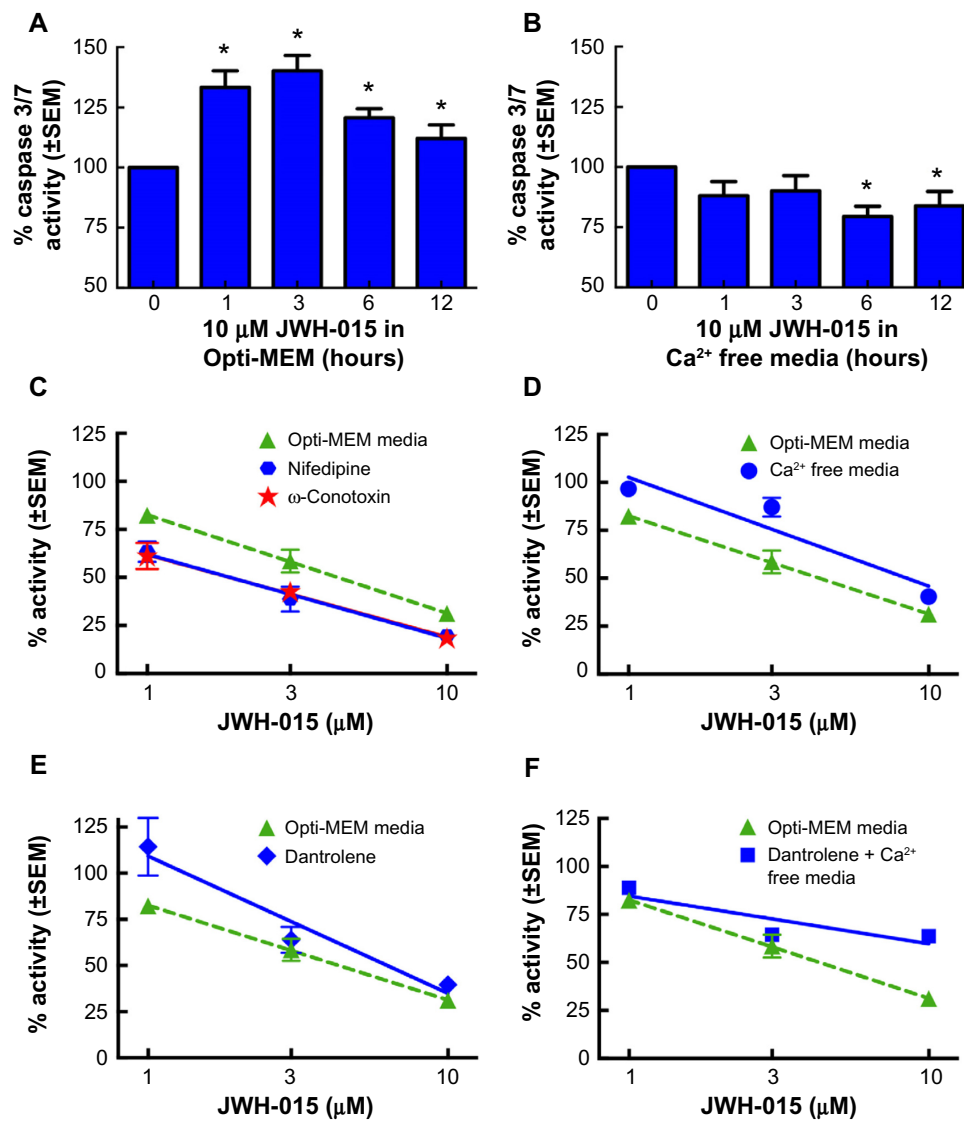


Figure 6 Inhibition of calcium flux attenuates JWH-015-induced apoptosis in 4T1 cells.

Notes: Timeline analysis of caspase 3/7 protein expression in 4T1 cells in response to 10 μM JWH-015 in normal media (A) or calcium-depleted media (B) demonstrates ablation of JWH-015 effects when performed in calcium-depleted media ($*P < 0.05$). Relevance of extracellular calcium flux vs intracellular calcium flux was evaluated pharmacologically by performing SRB assays on 4T1 cells in response to JWH-015 in the presence of L-type calcium-channel blocker nifedipine, N/P/Q-type calcium-channel blocker ω -Conotoxin MIIVC (C), normal vs calcium-depleted media (D), intracellular ryanodine receptor antagonist dantrolene (E), and calcium depletion concurrent with dantrolene (F).

Abbreviations: SEM, standard error of mean; Opti-MEM, Opti-minimal essential medium; SRB, sulforhodamine B.

attenuated viability of breast cancer cells. Similarly, blockade of the transient receptor potential channels TRPV1 and TRPA1 did not alter the effects of JWH-015 on tumor cell viability. The TRPV1 antagonist capsazepine did not block JWH-015 activity ($A_{50} = 2.65 \mu\text{M}$; 95% CI = 2.18–3.21), nor did pretreatment with the TRPA1 antagonist HC-030031 ($A_{50} = 0.32 \mu\text{M}$; 95% CI = 0.14–0.69), suggesting the CB_2 -mediated effects are not from extracellular calcium sources.

We next assessed JWH-015's ability to modulate intracellular calcium flux induced by activation of the ryanodine receptor. SRB assays were carried out in the presence of JWH-

015 and either the ryanodine receptor antagonist dantrolene, Ca^{2+} -depleted media (as a mechanism to block extracellular calcium influx), or a combination thereof. Cells cultured in Ca^{2+} -free media for 16 hours prior to treatment with JWH-015 experienced a significant dose–response curve shift ($A_{50} = 9.80 \mu\text{M}$; 95% CI = 7.57–12.69) (Figure 6D). Similarly, blockade of intracellular calcium flux by pretreatment with dantrolene shifted the JWH-015 dose–response curve in 4T1 cells significantly rightward compared to JWH-015 alone ($A_{50} = 18.77 \mu\text{M}$; 95% CI = 10.56–33.00) (Figure 6E), supporting an intracellular CB_2 -mediated calcium hypothesis.

Synergistic effects were observed when SRB assays were performed in Ca^{2+} -free media with dantrolene pretreatment (Figure 6F) as evidenced by the dose–response curve's rightward shift of approximately 40-fold ($A_{50}=112 \mu\text{M}$; 95% CI =60–211).

Finally, investigations were made to rule out the involvement of non- CB_2 receptors or alternative G-protein coupling at relevant concentrations of JWH-015. The CB_1 inverse agonist SR141716a, the GPR55 antagonist cannabidiol, or GPR55 agonist O-1602 was administered to cultures 1 hour prior to JWH-015 in SRB assays on 4T1 cells. Pretreatment with SR141716a did not alter JWH-015's effects on cellular viability ($A_{50}=3.0 \mu\text{M}$; 95% CI =1.82–4.94). Similarly, pretreatment with either cannabidiol or O-1602 did not block JWH-015 activity on 4T1 cells ($A_{50}=2.81 \mu\text{M}$; 95% CI =2.21–3.43) ($A_{50}=3.21 \mu\text{M}$; 95% CI =2.37–3.79), respectively. Treatment with either cannabidiol or O-1602 (concentrations from 0.1 to 10 μM) alone had no effect on cell viability. To determine whether the $\text{G}\alpha_i$ pathway is responsible for the reduction in cancer cell viability, 4T1 cells were pretreated with PTX prior to administration of JWH-015. We found that PTX inhibition of $\text{G}\alpha_i$ does not prevent JWH-015 reduction in 4T1 cancer cell viability ($A_{50}=3.82 \mu\text{M}$; 95% CI =3.22–4.94).

Discussion

Despite marked advances in chemotherapeutics for early-stage breast cancer, there are few new therapies effective in slowing the progression of advanced-stage disease. Genotypic profiling of human breast cancers over the last several years has led to broad phenotypic subclassification of breast tumors, shedding light on the complexity of the disease, and demonstrating some of the genetic intratumoral differences responsible for the mixed outcomes of protein-specific therapeutics. There is a clear need for novel tumor-specific therapeutics that do not result in severe toxic side effects for the treatment of late-stage and chemotherapy-resistant breast cancer.

Cannabinoid receptors are classically defined as members of the seven transmembrane-spanning G-protein-coupled receptor family. Two distinct cannabinoid receptors have been cloned: CB_1 ,³⁶ which is one of the most abundantly expressed G-protein-coupled receptors on neurons, and CB_2 ,³⁷ found primarily in cells of the immune system and on bone maintenance cells.¹⁴ In physiologically normal systems, CB_1 and CB_2 were first reported to couple predominantly with $\text{G}\alpha_i$,³⁸ yet several recent studies have demonstrated CB_2 receptor signaling via $\text{G}\alpha_q$ and other alternative coupling pathways.^{39,40}

Several groups have shown that both nonselective cannabinoid and CB_2 -specific compounds decrease breast cancer viability in vitro and in vivo: Δ^9 -tetrahydrocannabinol and CB_2 -selective agonist, JWH-133, have been demonstrated to exert considerable antitumoral effects in the MMTV-neu mouse model of breast cancer, which overexpresses the EGF receptor HER2.¹⁷ Similarly, CB_2 -selective JWH-133 has been shown to retard growth and metastasis of aggressive spontaneous breast tumors occurring in PyMT transgenic mice, implying the cyclooxygenase-2/prostaglandin E2 signaling pathway.³⁴ Cannabidiol inhibited human breast cancer cell line MDA-MB-231 growth in vitro by inducing apoptosis and autophagy.⁸ Anandamide, an endogenous cannabinoid CB_1 agonist, has been shown to inhibit breast cancer cell growth and migration in multiple models,^{41,42} and several other studies have demonstrated CB_2 agonist efficacy in breast cancer systems;^{43–45} however, the mechanism(s) of how cannabinoids achieve these effects is incompletely defined.

Here we demonstrate that CB_2 receptors are functionally expressed on breast cancer cells and that low nanomolar concentrations of JWH-015 reduce cAMP generation, yet results in no change in cAMP levels at the higher concentrations required for in vitro cancer cell growth modification. This suggests a concentration-dependent mechanism where higher concentrations may induce activation of an alternative-coupling pathway. This effect may be due to Ca^{2+} modulation of adenylate cyclase.^{46,47} Additionally, we were unable to augment intracellular cAMP activity with forskolin stimulation, suggesting that basal cAMP formation is elevated in 4T1 murine mammary carcinoma cells. Deregulated intracellular pathway activity, particularly in growth-promoting pathways, has been repeatedly documented in cancerous cells.⁴⁸ Interestingly, we also show decreased persistence of cancer cell viability following shRNA knockdown of the CB_2 receptor, indicating a dependence on basal cannabinoid receptor activity, though further studies are needed to explore this phenomenon.

Several previous studies by our laboratory and others have demonstrated that CB_2 receptor-mediated biological effects including analgesia,^{49–51} antiallodynia,^{52,53} and osteoclast inhibition^{14,54} are attenuated by the CB_2 inverse agonist SR144528. However, here we present evidence that the expression level of CB_2 and associated production of cAMP are substantially elevated in 4T1 mammary carcinoma cells, which may indicate a change in receptor response to pharmacological modulators including agonists and inverse agonists. Indeed, a recent study demonstrated the ability of SR144528 to act as a CB_2 agonist in activated microglia.⁵⁵ It is therefore likely that cell type, basal CB_2 activity, and

intracellular protein expression dictate whether an inverse agonist behaves like an antagonist or an agonist. We have verified here that JWH-015 reduces cellular viability by inducing cell-cycle-independent apoptosis and were unable to block the activity of JWH-015 *in vitro* with coadministration of the CB₂ inverse agonist SR144528. Interestingly, our data also show that SR144528 attenuates proliferation of breast cancer cells, indicating that the inverse agonist may act as an agonist in this model.

We have established that the apoptosis of breast tumor cells induced by JWH-015 does not occur through a G α_i -coupled mechanism in two distinct assays: by blocking the G α_i pathway with PTX prior to treatment with JWH-015 and by direct measurement of cellular cAMP in response to treatment with JWH-015. Furthermore, we have ruled out the interaction of JWH-015 with two other G α_i -coupled receptors, CB₁ and GPR55, using selective antagonists for each of the receptors. Because studies have also demonstrated that the endogenous cannabinoid anandamide can act as a low-efficacy agonist at the transient receptor potential TRPV1 calcium channels,⁴⁵ we also show independence of our effect from TRPA1 and TRPV1 channels as the target for JWH-015 antitumoral effects by blocking these channels with HC-030031 and capsazepine, respectively. Finally, we have ruled out the possibility that JWH-015's effects are mediated via L-type and N/P/Q-type calcium channels using nifedipine and ω -conotoxin MVIIC, respectively.

Although the effect of JWH-015 on breast cancer cells *in vitro* is not mediated by TRPA1, TRPV1, L-type, or N/P/Q-type calcium channels, we have shown that it is a calcium-dependent process. We demonstrate that by blocking either intracellular calcium flux with the ryanodine receptor antagonist dantrolene, or depleting extracellular calcium using media devoid of Ca²⁺, a significant rightward shift (19- and 10-fold, respectively) of the JWH-015 viability reduction dose–response curve is observed. Notably, when both intracellular and extracellular calcium fluxes are restricted simultaneously, the rightward shift (40-fold) of the JWH-015 dose–response curve observed is synergistic. Further, we show that *in vitro* treatment with JWH-015 induces cytoplasmic calcium flux by first depletion beginning 25 minutes postadministration and later influx beginning 40 minutes postadministration of the compound in 4T1 cells. Immediate calcium flux is not observed, as would be expected in the case that JWH-015 was primarily acting through integral membrane calcium channels. This data is supported by the work done by Felder et al⁵⁶ suggesting that CB₂ receptors do not modulate ion channels directly. Taken together, these data suggest that intracellular calcium flux is

a secondary event necessary for induction of apoptosis that is stimulated by another primary event activated by administration of JWH-015.

Consistent with the findings on the intracellular activity of cannabinoids by other groups,^{7,22,57–59} we have demonstrated that CB₂-selective JWH-015 reduces phosphorylation of ERK1/2 at time points relevant to peak measured caspase 3/7 activity, suggesting that the apoptosis induced by JWH-015 in breast cancer cells may be mediated by MAPK/ERK activity. Such findings are also in line with recent reports demonstrating CB₂ modulation of the PI3K/AKT and MAPK/ERK pathways in hippocampal neurons.²¹

Our study characterizes the actions of the CB₂-selective agonist JWH-015 on breast cancer cells *in vitro* and *in vivo* in a syngeneic murine model of late-stage breast cancer. Importantly, our model does not compromise immune function or produce oncogenic mutation in the host, yielding a model that bears resemblance to the clinical presentation of advanced breast cancer progression and metastasis. Additionally, because we transfected 4T1 cells with a luciferase reporter prior to host inoculation, the model utilized allows noninvasive *in vivo* tracking of primary tumor growth and metastasis over the entire duration of tumor establishment and treatment. In the studies presented herein, we show a reduced profile of metastasis on treatment with JWH-015 as well as reduced primary tumor burden *in vivo*.

Conclusion

We have demonstrated that the CB₂ agonist JWH-015 induces apoptosis in a non-G α_i -mediated, calcium-dependent, and cell-cycle-independent mechanism accompanied by attenuation of activated MAPK/ERK intracellular pathway. These are findings that should be considered with our previous studies indicating that JWH-015 significantly modifies the bone microenvironment in metastatic disease⁵⁴ by decreasing bone wasting in advanced stages of cancer and by significantly reducing spontaneous and evoked pain while simultaneously reducing fracture occurrence.⁵⁴ Given these findings, JWH-015 and other CB₂-selective agonists demand attention as adjuvant therapies, particularly in patients experiencing bone metastasis where CB₂-selective compounds may not only improve disease outcome but also increase the patient's quality of life.

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Disclosure

The authors report no conflicts of interest in this work.

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