Combined Antiproliferative Effects of the Aminoalkylindole WIN55,212-2 and Radiation in Breast Cancer Cells

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ABSTRACT

The potential antitumor activity of cannabinoid receptor agonists, such as the aminoalkylindole WIN55,212-2 (WIN2), has been studied extensively, but their potential interaction with conventional cancer therapies, such as radiation, remains unknown. In the present work, the influence of WIN2 on the antiproliferative activity of radiation in human (MCF-7 and MDA-MB231) and murine (4T1) breast cancer cells was investigated. The antiproliferative effects produced by combination of WIN2 and radiation were more effective than either agent alone. The stereoisomer of WIN2, WIN55,212-3 (WIN3), failed to inhibit growth or potentiate the growth-inhibitory effects of radiation, indicative of stereospecificity. Two other aminoalkylindoles, pravadoline and JWH-015 ([2-methyl-1-propyl-1H-indol-3-yl]-1-naphthalenyl-methanone), also enhanced the antiproliferative effects of radiation, but other synthetic cannabinoids [i.e., nabilone, CP55,940 ([1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone; WIN3 (WIN55,212-3), failed to inhibit growth or potentiate the growth-inhibitory effects of radiation. Although the antiproliferative actions of WIN2 were mediated through noncannabinoid receptor-mediated pathways, the observation that WIN2 interfered with growth stimulation by sphingosine-1-phosphate (SIP) implicates the potential involvement of SIP/ceramide signaling pathways. In addition to demonstrating that aminoalkylindole compounds could potentially augment the effectiveness of radiation treatment in breast cancer, the present study suggests that THC and nabilone are unlikely to interfere with the effectiveness of radiation therapy, which is of particular relevance to patients using cannabinoid-based drugs to ameliorate the toxicity of cancer therapies.

Introduction

The cannabinoids Δ^9-tetrahydrocannabinol (THC; Marinol; Solvay Pharmaceuticals, Marietta, GA) and nabilone (Cesamet; Meda Pharmaceuticals, Somerset, NJ) are approved by the Food and Drug Administration for the treatment of emesis and nausea associated with cancer chemotherapy (Russo, 2008). The results of preclinical trials suggest that these agents may prove to be of use for patients experiencing nausea and vomiting due to radiation therapy (Darmani et al., 2007). Cannabinoids are also known to suppress growth or promote cell death in a variety of cancer cell lines, including glioma, pancreatic, melanoma, lymphoma, lung, and breast (Carracedo et al., 2006; Qamri et al., 2009; Salazar et al., 2009; McAllister et al., 2011; Preet et al., 2011; Scuderi et al., 2011; Wasik et al., 2011). Given that cannabinoid-based drugs are used for suppression of nausea and for appetite stimulation in patients with cancer, as well as their potential utility as adjunctive treatments along with conventional therapies such as radiation, the present studies were initiated to determine whether cannabinoids might augment the antiproliferative actions of radiation in breast tumor cells.

The aminoalkylindole derivative WIN55,212-2 (WIN2) has been extensively used to investigate the endogenous...
WIN2 were examined, which included selective CB1 and CB2 binding sites that may mediate the antiproliferative actions of mitotic catastrophe (Jonathan et al., 1999). Potential receptor agonists (Goehe et al., 2012), apoptosis (Vermes et al., 1995), and doline. Growth arrest and cell death were evaluated by monosized counter (Invitrogen, Grand Island, NY). Senescent cells were quantified manually and reported as a percent of the total population.

Washing twice with PBS followed by centrifugation at 500 g H2AX Quantified by Flow Cytometry. Both adherent and nonadherent cells were collected and pelleted at indicated time points using a 4°C 5810 R Eppendorf centrifuge at 500 g. Samples were fixed in formaldehyde (3.7%) in PBS for 10 minutes at 37°C before being permeabilized using methanol, the methanol was removed, and cells were washed with 0.5% solution of crystal violet in 25% methanol. Samples were solubilized with a 0.1M sodium citrate solution in 50% ethanol before absorbance was measured at 540 nm using a microplate reader.

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\text{H2AX} \to \text{fluorescein isothiocyanate conjugated antibody}
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Materials and Methods

Cell Lines. MCF-7, MDA-MB231, and MCF-10A cells were obtained from ATCC (Manassas, VA). Luciferase transfected 4T1 cells were obtained from Caliper (Hopkinton, MA). MCF-7, MDA-MB231, and 4T1 cells were cultured in RPMI 1640 media with 1% penicillin/streptomycin solution, 5% fetal bovine serum, and 5% bovine calf serum. MCF-10A cells were cultured in Dulbecco’s modified Eagle’s medium/F12 media supplemented with 1% penicillin/ streptomycin solution, 10% horse serum, 10 μg/ml insulin, 100 ng/ml cholera toxin, 20 ng/ml EGF, and 500 ng/ml hydrocortisone. For studies under low serum conditions, cells were cultured in RPMI 1640 medium with 1% penicillin/streptomycin, 0.05% fetal bovine serum, and 0.05% bovine calf serum. For studies using estradiol, MCF-7 cells were cultured in phenol red free improved minimum essential medium supplemented with 1% penicillin/streptomycin solution and 10% fetal bovine serum.

Drugs and Reagents. WIN55,212-2, WIN55,212-3, chloroquine diphosphate salt, staurosporine, CP55,940, methanandamide, nabilone, pioglitazone, bezafibrate, capsaicin, adriamycin, AM251, capsazepine, GW9662, and estradiol were purchased from Sigma-Aldrich (St. Louis, MO). CBD and THC were generously provided by the National Institute on Drug Abuse (Bethesda, MD). AM630 was purchased from Enzo Life Sciences (Farmingdale, NY). Pravastatin, JWH-015, and SEW2871 were purchased from Cayman Chemical (Ann Arbor, MI). S1P was a gift from the laboratory of Dr. Sarah Spiegel (Department of Biochemistry, Virginia Commonwealth University, Richmond, VA).

Drug Treatments. All treatments with cannabinoids, capsaicin, pioglitazone, S1P, SEW2871, and estradiol were initiated with a 24-hour exposure period, after which the drug-containing media were aspirated and the cells were washed and replaced with fresh media. Radiation was administered at the same time as drug, unless otherwise indicated. Exposure to drug antagonists was coincidental with the receptor agonists. Adriamycin (doxorubicin), as a positive control for select studies, was used at 1 μM with an exposure period of 2 hours. For autophagy inhibition, chloroquine (5 μM) was administered to cells for the entirety of the experiment. In experiments under low serum conditions, drugs were added to the low serum media for the first 24 hours and then removed and replaced with regular media. In studies involving estradiol, the cells were maintained in improved minimum essential medium through the course of the experiment. All experimental results were analyzed at 96 hours, unless otherwise indicated. Cell counts for 4T1 cells were determined at 48 hours because of their rapid growth rate.

Determination of Viable Cell Number. Cells were plated into six-well plates (MCF-7 and MDA-MB231 cells, 50,000 cells/well; 4T1 cells, 100,000 cells/well). Viability was determined based on trypan blue exclusion using a hemocytometer or Invitrogen Countess automated counter (Invitrogen, Grand Island, NY).

Crystal Violet Assay. Cells were plated into 96-well plates and allowed to adhere overnight (MCF-7 and MDA-MB231 lines, 5,000 cells; 4T1 cells, 10,000 cells). After 96 hours, cells were washed with phosphate-buffered saline (PBS), fixed with methanol and stained with a 0.5% solution of crystal violet in 25% methanol. Samples were solubilized with a 0.1M sodium citrate solution in 50% ethanol before absorbance was measured at 540 nm using a microplate reader.

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\beta\text{-Galactosidase Activity Assay. Cells were plated into six-well plates at 10,000 cells/well. At appropriate time points, samples were fixed and histochemically stained as previously described (Biggers et al., 2013) using 5-bromo-4-chloro-indolyl-β-D-galactopyranoside as a substrate. Images of representative microscopic fields were captured on an Olympus 1 x 70 inverted microscope (Olympus America, Inc., Melville, NY) and senescent cells were quantified manually and reported as a percent of the total population.}
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Flow Cytometry for Annexin V and Propidium Iodide Staining. Cells were harvested at the indicated time points and washed twice with PBS followed by centrifugation at 500 g in a 4°C
were performed using media containing 10% serum. Accordingly, in contrast, the experiments presented in the present work (Salazar et al., 2009; McAllister et al., 2011; Wasik et al., 2011). Formed under low-serum conditions (Carracedo et al., 2006; 5810 R Eppendorf centrifuge. Annexin V and PI were obtained from BD Biosciences (San Jose, CA) and diluted in binding buffer according to the manufacturer’s instructions before being added to cells. Samples were analyzed by flow cytometry at 520 nm for fluorescein isothiocyanatelabeled annexin V and 677 nm for PI.

Cell Staining. Cells were stained using DAPI and acridine orange as previously reported (Biggers et al., 2013).

Reverse Transcription-Polymerase Chain Reaction. Total RNA was extracted from cells by using TRIzol Reagent (Invitrogen, Grand Island, NY) and reverse-transcribed with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The cDNA obtained from each sample was used as template for PCR using Mouse Genotyping Kit (KAPA Biosystems, Wilmington, MA). The primer was synthesized in Invitrogen, and primer sequences were as follows: CB1 forward, 5'-GACCATAGCCATTTGATCG-3'; CB1 reverse, 5'-GTTGCTACATTCTGGGA-3'; CB2 forward, 5'-GACCGCGATTGACCCGATACC-3'; CB2 reverse, 5'-GGACCCACATGATGCCCCAG-3'; TRPV1 forward, 5'-CTCACCACACAGAAGGGAATG-3'; TRPV1 reverse, 5'-AGGGTTGACAGCGAAGTGG-3'; PPARγ forward, 5'-ATGACACGGACTTGGAATAA-3'; PPARγ reverse, 5'-GGGACTACGGTGTTGAA-3'; β-actin forward, 5'-TGGAGCAGCTGAAGAAA-3'; β-actin reverse, 5'-CAGACGCTTGATAGCAACG-3'. In addition, the PCR program was as follows: 95°C for 3 minutes, 35 cycles of 95°C for 15 seconds, 58°C for 15 seconds, 72°C for 20 second, and 72°C for 2 minutes. Primer sequences for CB1 and CB2 were contributed by Dr. Mary Abood of Temple University (Department of Anatomy and Cell Biology, Philadelphia, PA).

Statistics. All experiments were performed with three to six replicates. Each experiment included vehicle, WIN2, radiation and WIN2 + radiation, unless otherwise stated. Two-way repeated-measures analysis of variance (ANOVA) was used to analyze radiation versus drug treatments. One-way repeated-measures ANOVA was used to assess overall significance for dose-response experiments. The Tukey-Kramer test was used for post hoc comparisons when appropriate (P < 0.05). Paired t test with a Bonferroni correction was used to assess comparisons of combination + drug with the individual treatments (P < 0.0156). All data are displayed as mean ± S.E.

Results

Effect of the Combination of WIN55,212-2 With Radiation in Breast Cancer Cells. Initial studies were performed to determine sensitivity to WIN2 in two human and one murine breast tumor cell lines, specifically p53 wild-type breast tumor cell lines, specifically p53 wild-type MCF-7 cells, p53 mutant ER negative MDA-MB231 cells, and p53 null ER negative 4T1 cells. Figure 1, A–C, shows that WIN2 dose dependently inhibited growth of each breast cancer cell line. The ED50 values for WIN2 were 11.96 ± 3.31 μM in MCF-7 cells, 17.92 ± 6.75 μM in MDA-MB231 cells, and 18.24 ± 4.15 μM in 4T1 cells. To test whether the growth-inhibitory effects of WIN2 were stereospecific, the antiproliferative activity of its stereoisomer WIN55,212-3 (WIN3), which does not bind to cannabinoid receptors (Howlett et al., 2002), was also evaluated. As shown in Fig. 1, A–C, WIN3 lacked efficacy in all three cell lines, with no significant effects even at concentrations up to 60 μM. These findings establish stereoselectivity and support the premise that WIN2 likely interferes with breast tumor cell growth through its actions at a specific target.

A preponderance of studies in the literature investigating the effects of cannabinoids on cancer cells have been performed under low-serum conditions (Carracedo et al., 2006; Salazar et al., 2009; McAllister et al., 2011; Wasik et al., 2011). In contrast, the experiments presented in the present work were performed using media containing 10% serum. Accordingly, to rule out the possibility that the relative activities of WIN2 and WIN3 might be a consequence of nonspecific serum binding, the capacity of WIN2 and its stereoisomer WIN3 to inhibit growth of MCF-7 cells was also assessed under serum-free conditions. Supplemental Figure 1 indicates that the absence of serum markedly increased the potency of WIN2 (a more than 3-fold reduction in the ED50 from ~10 to ~3 μM). However, WIN3 was entirely inactive, indicating that stereoselectivity was maintained under low-serum conditions.

Fig. 1. WIN2 stereoselectively and dose dependently inhibits the growth of breast cancer cells. Growth inhibition by WIN2 and WIN3 was assessed at 96 hours post-treatment by the crystal violet assay in MCF-7 (A), MDA-MB231 (B), and 4T1 breast tumor cells (C). Data presented reflect the means of 4 individual experiments ± S.E.; *P < 0.05 versus WIN3 at each respective concentration of drug.
Subsequent studies were focused on determining whether WIN2 would alter the antiproliferative effects of radiation, one of the most frequently used therapies in the treatment of breast cancer. Figure 2, A–C, presents the effects of combined radiation and WIN2 (at a concentration that alone inhibits breast tumor growth by ~50%) in each of the three breast tumor cell lines. The combination treatment was more effective than either treatment alone in all three breast tumor cell lines. WIN3 had no effect on sensitivity to radiation, again establishing the stereoselective action of WIN2.

To evaluate whether the enhanced antiproliferative effects of the WIN2-radiation combination might extend to non-cancerous cells, the combination was tested in MCF-10A cells, which are considered to be a model of normal breast epithelial cells (Tait et al., 1990). Figure 2D demonstrates that a concentration of WIN2 (12 μM) that enhanced the effects of radiation in MCF-7 cells failed to alter MCF-10A cell growth or to augment the antiproliferative effects of radiation.

To determine whether the combination of WIN2 with radiation promoted growth arrest and/or cell death, a time course study was performed to monitor viable cell number after treatment with radiation or WIN2 alone and the combination of WIN2 and radiation. Figure 3A shows that exposure of MCF-7 cells to either radiation (2 Gy) or WIN2 (12 μM) results in growth inhibition. As in the studies presented in Fig. 2A, the combination treatment was more effective than either WIN2 or radiation alone in inhibiting breast tumor growth. Furthermore, the combination treatment of WIN2 with radiation reduced the recovery of proliferative capacity observed with either radiation alone or WIN2 alone. A similar pattern of effects (enhanced growth inhibition and suppression of proliferative recovery) was evident in the MDA-MB231 and 4T1 cells (Fig. 3, B and C).

**Induction of DNA Damage.** It is well established that radiation acts through the induction of DNA damage, which can be monitored by γH2AX formation (Rogakou et al., 1999). The capacity of radiation alone, WIN2 alone, or the combination to affect DNA damage (1 hour) and repair (24 hour) in MCF-7 cells was evaluated based on γH2AX levels. As shown in Fig. 4A, radiation induced γH2AX foci formation was elevated at 1 hour and declined over a 24-hour period. However, WIN2 neither increased the induction of DNA damage nor interfered with the rate of repair (the latter based on the reduction of γH2AX staining). γH2AX foci formation and

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**Fig. 2.** Enhanced antiproliferative effects of the combination of WIN2 and radiation. Cells were exposed to vehicle, WIN2, or WIN3 either alone or with 2 Gy radiation in MCF-7 (A), MDA-MB231 (B), 4T1 (8 Gy) (C), and MCF-10A (D) cells. Cells were treated with equieffective doses of WIN2 based on the concentration effect curves in Fig. 1 (12 μM for MCF-7 cells, 15 μM for MDA-MB231 cells, 30 μM for 4T1 cells, and 12 μM for MCF-10A cells). All experiments were analyzed for cell viability by trypan blue exclusion 96 hours after drug treatment (4T1 cells were analyzed 48 hours after treatment because of rapid growth rate). Data presented reflect the means of three or four individual experiments ± S.E.; *P < 0.05 versus vehicle and **P < 0.0156 compared with vehicle, drug treatment alone, and radiation alone. Two-way ANOVA reports: MCF-7 [F(2,12) = 12.8, P < 0.05]; MDA-MB231 [F(2,16) = 4.1, P < 0.05]; 4T1 [F(2,8) = 14.7, P < 0.01]; MCF-10A (P = 0.95).
decline was also evaluated for radiation alone and for the WIN2 and radiation combination in MDA-MB231 and 4T1 cells (Fig. 4, B and C). As was the case with the MCF-7 cells, WIN2 did not alter the extent of DNA damage induced by radiation. However after 24 hours, residual DNA damage was slightly increased for the WIN2 + radiation combination compared with radiation alone, whereas WIN2 alone did not appear to promote senescence. Adriamycin-induced senescence (Goehe et al., 2012) was used as a positive control (data not shown).

Radiation has been shown to induce a cytoprotective form of autophagy (Wilson et al., 2011; Bristol et al., 2012, 2013), whereas THC was reported to induce autophagic cell death in glioma cells (Salazar et al., 2009). Consequently, studies were designed to investigate whether WIN2 or the WIN2 and radiation combination would promote autophagy in this experimental system. Acridine orange staining clearly indicates that both WIN2 and radiation induced autophagy in MCF-7 cells (Fig. 6A). To determine whether autophagy was playing either a cytoprotective or cytotoxic function in the effects of radiation and/or WIN2, autophagy was inhibited utilizing chloroquine. However, chloroquine showed no evidence of altering the actions of either treatment alone or in combination when administered to MCF-7 cells (Fig. 6B). Inhibition of autophagy by chloroquine was validated in experiments where autophagy was induced by adriamycin, as demonstrated in Goehe et al. (2012) (data not shown).

The Combination of WIN2 and Radiation Fails to Induce Apoptosis or Necrosis. As shown in Fig. 3, the combination treatment of WIN2 + radiation promoted a prolonged growth arrest with limited proliferative recovery. To investigate the possibility that a low level of cell death might have contributed to the growth inhibition observed, the induction of apoptosis and/or necrosis were determined by staining with annexin V and PI (Vermes et al., 1995). However, neither apoptosis nor necrosis was detected in response to treatment (Fig. 6C). In contrast, apoptosis was clearly detected with staurosporine treatment as a positive control (Belmokhtar et al., 2001) (Fig. 6C). This observation was confirmed qualitatively using DAPI staining to assess nuclear morphology (Fig. 6D) where paclitaxel (Saunders et al., 1997) was used as positive control for apoptosis and cell death (data not shown). The lack of change in nuclear morphology was confirmed at 72 and 96 hours post-treatment (data not shown). The DAPI staining experiments also failed to indicate the induction of mitotic catastrophe, which is characterized by multinucleated cells containing micronuclei (Jonathan et al., 1999).

Interaction of Other Cannabinoids with Radiation. Although WIN2 behaves as a cannabinoid receptor agonist, other cannabinoid agonists were also tested for their capacity to interact with radiation in MCF-7 cells. As shown in Table 1, the highest concentrations of JWH-015 and pravadoline augmented the growth inhibitory effects of radiation. These two compounds belong to the class of aminoalkylindoles and share structural similarities to WIN2. In contrast, cannabinoids outside this class, including CBD, methanandamide, CP55,940, nabilone, and THC failed to enhance the antiproliferative effects of radiation alone.

Assessment of Potential Cannabinoid Receptor Targets of WIN55,212-2. Cannabinoids have been reported to act at CB1 or CB2 receptors to inhibit the growth of tumor cells (Qamri et al., 2009; Salazar et al., 2009). RT-PCR analysis
clearly showed that MCF-7 cells express CB2 receptor mRNA, whereas an extremely faint band was found for CB1 (Fig. 7A). G-protein activation studies in rat brain tissue have shown that WIN2 acts as a full agonist at CB1 and CB2 receptors (Sim et al., 1996; Breivogel et al., 1998). Therefore, the respective CB1 and CB2 receptor antagonists, AM251 (4 μM) and AM630 (4 μM), were evaluated for the ability to prevent WIN2-induced inhibition of cell growth (Lan et al., 1999; Ross et al., 1999). Neither AM251 nor AM630 significantly inhibited the growth inhibitory effects of WIN2 (Fig. 7B), suggesting that CB1 and CB2 receptor signaling may not be necessary for the antiproliferative actions of WIN2.

Given the apparent lack of involvement of CB1 and CB2 receptors in the antiproliferative effects of WIN2, the contribution of other potential receptor targets of WIN2, including PPARγ and TRPV1 was considered. Both have been shown to be activated by various cannabinoids (O’Sullivan, 2007; Pertwee et al., 2010). RT-PCR confirmed the presence of mRNA of both TRPV1 and PPARγ in MCF-7 cells (Fig. 7C). However, neither the TRPV1 receptor antagonist capsazepine nor the PPARγ receptor antagonist GW9662 (Fig. 7, D and E) reduced the antiproliferative effects of WIN2 (Doherty et al., 2005; Willson et al., 2000). Furthermore, the observations that the PPARγ receptor agonist pioglitazone and TRPV1 agonist capsaicin failed to elicit antiproliferative activity alone (data not shown) further argues against the function of these receptors in the breast tumor cells. Similarly, the pan-PPAR agonist, bezafibrate, which is used to screen for the potential involvement of other PPAR receptors, did not inhibit the growth of MCF-7 cells or interfere with the antiproliferative activity of WIN2 (data not shown). Taken together, these experiments indicate that WIN2 does not appear to be acting through known receptor targets in MCF-7 breast tumor cells.

WIN2 Antagonizes S1P-Associated Growth Stimulation. As the ceramide/S1P signaling system has been shown to stimulate the proliferation of MCF-7 cells (Sarkar et al., 2005), studies were designed to evaluate the S1P system as a potential site for the antiproliferative actions of WIN2 in MCF-7 cells. Under low-serum conditions, in which 100 nM S1P stimulated MCF-7 cell growth, a 3 μM concentration of WIN2 that did not inhibit basal cell growth effectively suppressed growth stimulation by S1P (Fig. 8A). In complementary studies under normal serum conditions, a subeffective dose of WIN2 (8 μM) also reversed the growth stimulatory effects of a 5 μM treatment with the synthetic S1P1 receptor-selective agonist SEW2871 (Fig. 8B). In contrast, 25 μM THC failed to reverse growth stimulation by SEW2871 (Fig. 8C). To explore the possibility that WIN2 might have interfered with another growth stimulatory pathway, cells were exposed to 100 nM estradiol in the absence and presence of WIN2 (8 μM); however, WIN2 failed to antagonize the growth stimulatory effects of estradiol (Fig. 8D).

**Discussion**

The current studies indicate that the aminoalkylindole, WIN2, has the capacity to inhibit growth in two human breast cancer cell lines (MCF-7 and MDA-MB-231) and a murine breast tumor cell line (4T1). In addition, WIN2 augmented the antiproliferative effects of radiation in all three breast cancer

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**Fig. 4.** DNA damage induction and repair by radiation ± WIN2. MCF-7 (A), MDA-MB231 (B), and 4T1 (C) cells were treated as in Fig. 2. γH2AX formation analyzed by flow cytometry at 1 and 24 hours after drug treatment. Data were normalized to percentage of control; data presented reflect the means of 3–5 individual experiments ± S.E.; *P < 0.05 versus vehicle.

**Fig. 5.** Senescence induction by radiation ± WIN2. MCF-7 cells were treated with vehicle, WIN2 (12 μM), (2 Gy) radiation or WIN2 + radiation. (A) Representative images of β-galactosidase stained cells. (B) Quantification of β-galactosidase activity 96 hours after drug treatment. Data were normalized to percentage of sample in (B); data presented reflect the means of three individual experiments ± S.E.; *P < 0.05 versus vehicle.
cell lines. Experiments comparing WIN2 with its stereoiso-
mer WIN3 support the conclusion that the effects of WIN2 are
stereoselective. Studies in MCF-10A cells suggest that the
antiproliferative effects of WIN2 are selective to tumor cells.
Time course studies in all three breast tumor cell lines indi-
cate that WIN2, either alone or in combination with radiation,
promotes growth arrest rather than tumor cell killing. This
conclusion is supported by experiments in MCF-7 cells showing
the absence of significant apoptosis or necrosis by WIN2 alone
or in combination with radiation. Both autophagy and senescence
induction are evident, but neither response appears to play
a central role in the antiproliferative effects of this compound.
Furthermore, the increased antiproliferative activity of the
WIN2 + radiation combination does not appear to be a con-
sequence of an increase in DNA damage or decreased DNA
repair compared with radiation alone.

WIN2 is known to be an agonist with high efficacy at both
CB1 and CB2 receptors (Sim et al., 1996; Breivogel et al., 1998;
Govaerts et al., 2004), and the expression of CB2 receptor
mRNA and possibly low levels of CB1 receptor mRNA was

**TABLE 1**

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<thead>
<tr>
<th>Drug</th>
<th>Control Low Dose</th>
<th>Medium Dose</th>
<th>High Dose</th>
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<td>Vehicle, IR, 2 Gy</td>
<td>Vehicle, IR, 2 Gy</td>
<td>Vehicle, IR, 2 Gy</td>
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<td>THC</td>
<td>100 ± 0.01, 57 ± 3.93</td>
<td>94 ± 2.93, 55 ± 3.92</td>
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<td>CBD</td>
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<td>Nabilone</td>
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<td>88 ± 5.41, 56 ± 5.75</td>
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<td>CP-55,940</td>
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<td>100 ± 1.41, 77 ± 10.58</td>
<td>81 ± 4.41, 59 ± 7.13</td>
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<td>Methanandamide</td>
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<td>92 ± 0.93, 58 ± 8.33</td>
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<td>Provadoline</td>
<td>100 ± 0.01, 53 ± 5.67</td>
<td>94 ± 1.52, 43 ± 4.95</td>
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<tr>
<td>JWH-015</td>
<td>100 ± 0.01, 53 ± 5.67</td>
<td>79 ± 6.39, 45 ± 4.04</td>
<td>42 ± 7.04, 31 ± 2.61</td>
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*P < 0.0156 compared with vehicle, drug treatment alone, and radiation alone.

**Fig. 6.** Assessment of apoptosis, necrosis, and autophagy induction by WIN2. MCF-7 cells were treated as in Fig. 3. (A) Autophagy induction by acridine
orange staining. Images were taken at 96 hours and 40× magnification. (B) Autophagy induction in the absence and presence of chloroquine. (C) Flow
cytometry at 48 hours for Annexin V and propidium iodide staining. Staurosporine (1 μM, 24 hours) was used as a positive control. (D) DAPI staining for
nuclear morphology at 48 hours using 40× magnification. Data were normalized to percentage of control in (B) and percentage of population in (C); data
presented reflect the means of three or four individual experiments ± S.E.; *P < 0.05 versus vehicle.
confirmed in MCF-7 cells. However, the antiproliferative activity of WIN2 was not inhibited by the respective CB1 or CB2 antagonists, AM251 and AM630. In efforts to identify potential receptors for WIN2 action, the involvement of TPRV1 and PPAR nuclear receptors that are known to be sensitive to cannabinoids (O'Sullivan, 2007; Pertwee et al., 2010) was also assessed. RT-PCR confirmed mRNA expression for both receptors, but selective antagonists for these receptors did not reduce the antiproliferative effects of WIN2, arguing against a role for TRPV1 and PPARγ in the activity of WIN2. Consistent with these observations, TRPV1 and PPARγ agonists failed to reduce cell growth. The failure of the pan-PPAR agonist bezafibrate to affect proliferation of MCF-7 cells argues against the involvement of other PPARs in breast tumor cell proliferation under the present experimental conditions. Taken together, these data suggest that the antiproliferative actions of WIN2 in MCF-7 breast cancer cells are not mediated by conventional receptor targets of WIN2. This conclusion is further supported by the observation that WIN2 was also active in 4T1 cells that do not express either the CB1 or CB2 receptors (McKallip et al., 2005). Likewise, in studies in melanoma cells and mantle cell lymphoma, WIN2 was shown to act through a noncannabinoid receptor mechanism (Scuderi et al., 2011; Wasik et al., 2011), and all three of these cell lines were shown to be sensitive to the growth inhibitory effects of WIN2. WIN2 was also shown to antagonize growth stimulation in MCF-7 cells by S1P and the synthetic S1P1 receptor agonist SEW2871, but not by estradiol, suggesting some degree of specificity relating to S1P signaling pathways. The fact that THC failed to augment the antiproliferative effects of radiation or to antagonize growth stimulation by SEW2871 indicates that WIN2's inhibition of S1P signaling cannot be generalized to other cannabinoids, further suggesting that WIN2 interferes with the S1P pathway through a noncannabinoid mechanism.

A perhaps critical difference between the current work and other studies in the literature of cannabinoid action in tumor cells is the concentration of serum used in the media. Most studies use low-serum media, whereas in the present study, the media contained 10% serum. In this context, the breast tumor cells were markedly more sensitive to WIN2 under low-serum conditions. The decreased potency of WIN2 in high-serum is likely to be a consequence of its sequestration by serum-binding proteins. Additionally, the increased potency of WIN2 in low serum could be related to the fact that reduced serum conditions are likely to make the cells fragile and susceptible to injury by exogenous stressors (Pirkmajer and Chibalin, 2011). Regardless, an important finding in the present study was that the stereoselectivity of WIN2 was sustained under both low- and high-serum conditions.
Several other synthetic cannabinoids and plant-derived cannabinoids were evaluated for their effectiveness to augment the antiproliferative effects of radiation in breast cancer cells. JWH-015 and pravadoline are aminoalkylindole compounds that are structurally similar to WIN2. THC and nabilone were selected for their clinical relevance to cancer because these are the active ingredients of the respective U.S. Food and Drug Administration-approved cannabinoid-based medications THC (Marinol) and nabilone (Cesamet) to treat cancer chemotherapy-induced nausea and emesis. Cannabidiol is a major cannabinoid found in marijuana, which does not bind to CB1 or CB2 receptors, and is a component of nabiximol (Sativex; Bayer Healthcare, Berkshire, UK), a medication prescribed in Canada and several European countries (Oreja-Guevara, 2012) for the treatment of spasticity due to multiple sclerosis. Methanandamide, a stable analog of anandamide, was used in lieu of the endogenous cannabinoid, which is rapidly hydrolyzed. Finally, CP55,940 possesses high potency and efficacy at CB1 and CB2 receptors but is structurally distinct from WIN2 (Pertwee et al., 2010). It is significant that of all of the compounds tested, only two compounds structurally similar to WIN2 (i.e., JWH-015 and pravadoline) modestly augmented the effects of radiation. It is also noteworthy that in no case did any of the agents reduce the antiproliferative effects of radiation, indicating that cannabinoid-based medications are unlikely to interfere with the effectiveness of radiation therapy.

Nevertheless, the combination treatment of WIN2 + radiation was found to be significantly more effective than radiation alone in arresting the cells for an extended period of time and suppressing proliferative recovery. Although the profound cannabimimetic effects of WIN2 (Compton et al., 1992) have impeded its clinical development, drugs with a similar structure and/or mechanism of action could represent potential therapeutic agents to enhance the antiproliferative effects of ionizing radiation. The observed, albeit modest, effectiveness of other aminoalkylindoles, JWH-015 and pravadoline, in enhancing the antiproliferative effects of radiation suggests that other aminoalkylindole derivatives might ultimately have utility as adjunctive cancer treatments without the limitations imposed by the cannabimimetic effects of WIN2.

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Authorship Contributions

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References


Supplementary figure 1 – Influence of low serum (0.1%) conditions on response of MCF-7 cells to WIN2 and WIN3. MCF-7 cells were treated with WIN2 (1-10µM) and WIN3 (1-10µM) and cell growth monitored by the crystal violet assay 96 h after treatment. Data presented reflect the means of 3 individual experiments ± se; *p<0.05 vs WIN3 at each respective concentration of drug.

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