Combined antiproliferative effects of the aminoalkylindole WIN55,212-2 and radiation in breast cancer cells

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Abbreviations – ADR - adriamycin, AEA - anandamide, CB1 – cannabinoid receptor 1, CB2 – cannabinoid receptor 2, CBD - cannabidiol, GPCR – G protein couple receptor, IR – ionizing radiation, MAEA - methanandamide, PPAR – peroxisome proliferator-activated receptor, THC – Δ9-tetrahydrocannabinol, TRPV1 – transient receptor potential cation channel subfamily V member 1, WIN2 – WIN55,212-2, WIN3 – WIN55,212-3

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1. Chemotherapy, Antibiotics, and Gene Therapy
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, also enhanced the antiproliferative effects of radiation, but other synthetic cannabinoids (i.e., nabilone, CP55,940 and methanandamide) or phytocannabinoids (i.e., Δ⁹-tetrahydrocannabinol (THC) and cannabidiol) did not. The combination treatment of WIN2 + radiation promoted both autophagy and senescence, but not apoptosis or necrosis. WIN2 also failed to alter radiation-induced DNA damage or the apparent rate of DNA repair. While the antiproliferative actions of WIN2 were mediated through non-cannabinoid receptor mediated pathways, the observation that WIN2 interfered with growth stimulation by sphingosine-1-phosphate (S1P) implicates the potential involvement of S1P/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.
The cannabinoids Δ⁹-tetrahydrocannabinol (THC; Marinol) and nabilone (Cesamet) 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 possess utility 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 (Salazar et al. 2009, Carracedo et al. 2006, Scuderi et al. 2011, Wasik et al. 2011, Preet et al. 2011, Qamri et al. 2009, McAllister et al. 2011). Given that cannabinoid-based drugs are utilized for suppression of nausea and for appetite stimulation in cancer patients, 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 employed to investigate the endogenous cannabinoid system (Compton et al. 1992). WIN2 binds with high affinity at both identified cannabinoid G-protein coupled receptors (GPCR), CB₁ and CB₂, and is considered to be a high efficacy agonist based on agonist-stimulated [³⁵S]GTPγS binding assays (Sim et al. 1996, Breivogel et al. 1998), though it also stimulates non-cannabinoid GPCRs (Breivogel et al. 2001, Nguyen et al. 2010) and the nuclear protein receptors peroxisome proliferator-activated receptors α-γ (PPARα-γ) (O’Sullivan 2007). Of note, WIN2 suppresses the growth of melanoma, mantle cell lymphoma, non-small cell lung cancer, and breast cancer cell lines (Scuderi et al. 2011, Wasik et al. 2011, Preet et al. 2011, Qamri et al. 2009), as well as suppressing radiation-induced emesis in the least shrew (Darmani et al. 2007) and producing antinociceptive actions in rodent models of cancer pain (Guerrero et al. 2008). Despite its prevalent use as a cannabinoid pharmacological tool, WIN2 has yet to be examined for potential interactions with radiation in terms of tumor growth effects, despite the fact that radiation has been a mainstay of cancer therapy for decades.
The purpose of the present study was to determine the effect of WIN2 on the antiproliferative actions of radiation in breast cancer cells. Mechanistic studies were conducted on human MCF-7 cells, while human MDA-MB231 and murine 4T1 cells lines were employed to assess the generalizability of combined administration of WIN2 and radiation to multiple types of breast cancer lines. For the purpose of assessing selectivity, the combination treatment was also assessed in MCF-10A cells, a model of normal breast epithelial cells. Stereospecificity was determined utilizing the stereoisomer, WIN55,212-3, which does not bind to either CB1 or CB2 receptors. The effect of radiation on breast tumor cell growth was also assessed in combination with a variety of structurally distinct cannabinoids, including THC, nabilone, CP55,940, methanandamide, cannabidiol (CBD), JWH-015 and pravadoline. Growth arrest and cell death were evaluated by monitoring senescence (Debacq-Chainiaux et al. 2009), autophagy (Goehe et al. 2012) apoptosis (Vermes et al. 1995) and mitotic catastrophe. Potential receptor binding sites that may mediate WIN2’s antiproliferative actions were examined, which included selective CB1 and CB2 receptors, peroxisome proliferator-activated receptors (PPARs) (O’Sullivan 2007), and TRPV1 receptors (Pertwee et al. 2010). Finally, studies were performed evaluating WIN2’s potential for antagonizing growth stimulation induced by sphingosine-1-phosphate (S1P), the S1P agonist SEW2871, and estradiol.
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 media with 1% pen/strep solution, 5% fetal bovine serum and 5% bovine calf serum. MCF-10a cells were cultured in DMEM/F12 media supplemented with 1% pen/strep solution, 10% horse serum, insulin 10 ug/ml, cholera toxin 100 ng/ml, EGF 20 ng/ml, and hydrocortisone 500 ng/ml. For studies under low serum conditions, cells were cultured in RPMI with 1% pen/strep, 0.05% fetal bovine serum, and 0.05% bovine calf serum. For studies utilizing estradiol, MCF-7 cells were cultured in phenol red free IMEM media supplemented with 1% pen/strep 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 (St. Louis, MO). CBD and THC were generously provided by NIDA (Bethesda, MD). AM630 was purchased from Enzo Life Sciences (Farmingdale, NY). Pravadoline, JWH-015 and SEW2871 were purchased from Caymen Chemical (Ann Arbor, Michigan). Ketamine and xylazine were obtained from Butler Schein Animal Health. S1P was a gift from the laboratory of Dr. Sarah Spiegel.

Drug treatments All treatments with cannabinoids, capsaicin, pioglitazone, S1P, SEW2871 and estradiol were initiated with a 24 h exposure period, after which the drug-containing media was aspirated, 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 h. 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 h, and then removed and replaced with regular media. In studies involving estradiol, the cells were maintained in IMEM media through
the course of the experiment. All experimental results were analyzed at 96 h, unless otherwise indicated. Cell counts for 4T1 cells were determined at 48 h due to 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.

**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 h, cells were washed with PBS, fixed with methanol and stained with a 0.5% solution of crystal violet in 25% methanol. Samples were solubilized with a 0.1M Na-Citrate solution in 50% ethanol before absorbance were measured at 540 nm using a microplate reader.

**γH2AX quantified by flow cytometry** Both adherent and non-adherent cells were collected and pelleted at indicated time points using a 4°C 5810 R eppendorff centrifuge at 500 g. Samples were fixed in formaldehyde (3.7%) in PBS for 10 min at 37°C before being chilled on ice and re-pelleted. Fixative was removed, cells were permeabilized using methanol, the methanol was removed and cells were washed twice with 5 mg/ml BSA in PBS, and then blocked using the BSA solution for 10 min at room temperature. γH2AX-FITC conjugated antibody was added at a dilution of 1:200 in 200 µl per sample followed by incubation for 60 min at room temp. Cells were washed with BSA solution twice more before being resuspended in PBS. Measurements were performed by flow cytometry at a wavelength of 520 nm.

**β-galactosidase activity assay** Cells were plated into 6 well plates at 10,000 cells/well. At appropriate time points, samples were fixed and histochemically stained as previously described (Biggers et al. 2012) 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.

**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 5810 R eppendorf centrifuge. Annexin V and PI were obtained from BD Bioscience 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 FITC labeled annexin V and 617 nm for PI.

**Cell staining** Cell were stained using DAPI and acridine orange as previously reported (Biggers et al. 2012).

**RT-PCR** Total RNA was extracted from cells by using Trizol Reagent (Gibco BRL Technologies, USA), and reverse-transcribed with iScript cDNA Synthesis Kit (BIO-RAD, USA). The cDNA obtained from each sample was used as template for PCR using KAPA Mouse Genotyping Kit (KAPA Biosystems, USA). The primer was synthesized by Invitrogen (USA) and primer sequences were as follows: CB1 forward- GACCATAGCCATTGTGATCG, CB1 reverse- GTTTCATCAATGTGTGGGA, CB2 forward- GACCGCCATTGACCAGATACC, CB2 reverse- GACGGGACATCATCTATCCAA, TRPV1 forward- CTACCAACAAGGAGGAATG, TRPV1 reverse- AGGTCGTACAGCGAGGAGTG, PPARγ forward- ATGACAGCGACTTGGAATA, PPARγ reverse- GAGGACTCAGGGGTTCAG, Beta actin forward- TGGGACGACATGGAGAAA, Beta actin reverse- CACAGCCTGGGATGCAACG. Additionaly the PCR program was as follows: 95 °C for 3 min; 35 cycles of 95 °C for 15 s, 58 °C for 15 s and 72 °C for 20 s; 72 °C for 2 min. Primer sequences for CB1 and CB2 were contributed by Dr. Mary Abood of Temple University, Department of Anatomy and Cell Biology.
Statistics All experiments were performed with 3-6 replicates. Each experiment included vehicle, WIN2, radiation and WIN2 + radiation, unless otherwise stated. Two-way repeated measures ANOVA was used to analyze radiation vs. 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±se.
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 ER positive MCF-7 cells, p53 mutant ER negative MDA-MB231 cells and p53 null ER negative 4T1 cells. Figures 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. In order 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 Figures 1 A-C, WIN3 lacked efficacy in all three cell lines, with no significant effects even at a concentration of 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, in order to rule out the possibility that the relative activities of WIN2 and WIN3 might be a consequence of non-specific serum binding, the capacity of WIN2 and its stereoisomer WIN3 to inhibit growth of MCF-7 cells was also assessed under serum-free conditions. Supplementary Figure 1 indicates that the absence of serum markedly increased the potency of WIN2 (a more than three-fold reduction in the ED50 from ~10 µM to ~3 µM). However, WIN3 was entirely inactive, indicating that stereoselectivity was maintained under low serum conditions.
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. **Figures 2A-2C** present 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.

In order 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.

In order 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 **Figure 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 (**Figures 3B and C**).

**Induction of DNA damage** It is well established that radiation acts through the induction of DNA damage, which can be monitored by γ-H2AX formation (Rogakou et al. 1999). The capacity of radiation alone, WIN2
alone or the combination to affect DNA damage (1 h) and repair (24 h) in MCF-7 cells was evaluated based on γ-H2AX levels. As shown in Figure 4A, radiation induced γ-H2AX foci formation was elevated at 1 h and declined over a 24 h period. However, WIN2 neither increased the induction of DNA damage nor interfered with the rate of repair (the latter based on the reduction of γ-H2AX staining). γ-H2AX foci formation and decline was also evaluated for radiation alone and for the WIN2 and radiation combination in MDA-MB231 and 4T1 cells (Figure 4B 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 h, residual DNA damage was slightly increased for the WIN2 + radiation combination compared to radiation alone while the number of γ-H2AX foci had declined to background levels for radiation alone. WIN2 alone had no significant effect on γ-H2AX foci formation.

**Senescent growth arrest and autophagy induced by IR or WIN2 + IR** Based on our previous finding that senescence represents the primary antiproliferative response to radiation in p53 wild-type breast tumor cells (Jones et al. 2005), senescence induction was evaluated based on β-galactosidase staining (Debacq-Chainiaux et al. 2009). A representative image of staining for each treatment condition is shown in Figure 5A. Quantification of senescence indicated that β-galactosidase activity was significantly elevated by radiation and WIN2 + radiation but there was no increase with WIN2 + radiation compared to radiation alone (Figure 5B), while WIN2 alone did not appear to promote senescence. Adriamycin (ADR)-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, Bristol et al. 2013) while THC was reported to induce autophagic cell death in glioma cells (Salazar et al. 2009). Consequently, it was investigated 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 (Figure 6A). In order 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 (Figure 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 Figure 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 (Figure 6C). In contrast, apoptosis was clearly detected with staurosporine treatment as a positive control (Belmokhtar et al. 2001) (Figure 6C). This observation was confirmed qualitatively using DAPI staining to assess nuclear morphology (Figure 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 h 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 While 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 (Salazar et al. 2009, Qamri et al. 2009). RT-PCR analysis clearly showed that MCF-7 cells express CB₂ receptor mRNA, while an extremely faint band was found for CB₁ (Figure 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. Neither AM251 nor AM630 significantly inhibited the growth inhibitory effects of WIN2 (Figure 7B), suggesting that CB₁ and CB₂ receptor signaling may not be necessary for 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 (Figure 7C). However, neither the TRPV1 receptor antagonist capsazepine nor the PPARγ receptor antagonist GW9662 (Figures 7D and 7E) reduced the antiproliferative effects of WIN2. Furthermore, the observations that the PPARγ receptor agonist pioglitazone (PGZ) and TRPV1 agonist capsaicin (CAP) 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 (Figure 8A). In complementary studies under normal serum conditions a sub-effective dose of WIN2 (8 μM) also reversed the growth stimulatory effects a 5 μM treatment with the synthetic S1P₁ receptor-selective agonist SEW2871 (Figure 8B). In contrast, 25 μM THC failed to reverse growth stimulation by SEW2871 (Figure 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 (Figure 8D).
Discussion

The current studies indicate that the aminoalklyindole, WIN2, has the capacity to inhibit growth in two human breast cancer cell lines (MCF-7 and MDA-MB231) and a murine breast tumor cell line (4T1). In addition, WIN2 augmented the antiproliferative effects of radiation in all three breast cancer cell lines. Experiments comparing WIN2 with its stereoisomer, 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 indicate 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 consequence of an increase in DNA damage or decreased DNA repair compared to 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 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 TRPV1 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). Similarly, in studies in melanoma cells and mantle cell lymphoma, WIN2 was shown to act through a non-cannabinoid receptor mechanism (Wasik et al. 2011, Scuderi et al. 2011). Our findings are somewhat distinct from those of Qamri et al (2009) in which CB1 and CB2 antagonists prevented WIN2’s antiproliferative effects in MDA-MB231, and MDA-MB468 breast cancer cells. However, Qamri et al performed their study under low serum conditions and not in MCF-7 cells, which was the focus of our experiments relating to cannabinoid receptor action.

Knockdown of sphingosine kinase has implicated the S1P system in MCF-7 breast tumor cell growth (Sarkar et al 2005). S1P associated growth signaling has also been demonstrated in MDA-MB231 and 4T1 cells (Wang et al 1999, Nagahashi et al 2012) 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 sphingosine-1-phosphate (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 non-cannabinoid 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 employ low serum media, while 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 due to 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 et al. 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 as these are the active ingredients of the respective FDA-approved cannabinoid-based medications Marinol and 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 Sativex, 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 the compounds tested, only the two compounds structurally similar to WIN2 (ie., 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. While WIN2’s profound cannabimimetic effects (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 suggest
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

Conducted experiments – Emery, Tao, Alotaibi

Experimental design – Emery, Lichtman, Gewirtz, Selley

Data analysis – Emery, Lichtman, Gewirtz

Writing and editing of manuscript – Emery, Lichtman, Gewirtz, Selley
References


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Footnotes

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Figure Legends

**Figure 1 – WIN2 stereoselectively and dose-dependently inhibits the growth of breast cancer cells.** Growth inhibition by WIN2 and WIN3 was assessed at 96 h post-treatment by the crystal violet assay in (A) MCF-7, (B) MDA-MB231 and (C) 4T1 breast tumor cells. Data presented reflect the means of 4 individual experiments ± se; *p<0.05 vs. WIN3 at each respective concentration of drug.

**Figure 2 – Enhanced antiproliferative effects of combination of WIN2 and radiation.** Cells were exposed to vehicle, WIN2 or WIN3 either alone or with 2 Gy radiation (A) MCF-7 (B) MDA-MB231 (C) 4T1 (8 Gy) and (D) MCF-10A cells. Cells were treated with equi-effective doses of WIN2 based on the concentration effect curves in figure 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 h after drug treatment (4T1 cells were analyzed 48 h after treatment due to rapid growth rate). Data presented reflect the means of 3-4 individual experiments ± se; *=p<0.05 vs vehicle and **=p<0.0156 when compared to 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).

**Figure 3 – Temporal response to the combination of WIN2 + radiation.** (A) MCF-7, (B) MDA-MB231 and (C) 4T1 cells were treated as in figure 2. Viable cell number was monitored over a period of 96 h using the trypan blue exclusion assay. Data presented reflect the means of 5 individual experiments ± se. Darkened symbols = p<0.05 vs vehicle within time points.

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

**Figure 5 – Senescence induction by radiation ± WIN2.** MCF-7 cells were treated with vehicle, WIN2 (12µM), (2Gy) radiation or WIN2 + radiation. (A) Representative images of β-galactosidase stained cells. (B) Quantification of β-galactosidase activity 96 h after drug treatment. Data were normalized to % of sample in (B); data presented reflect the means of 3 individual experiments ± se; *p<0.05 vs vehicle.

**Figure 6 – Assessment of apoptosis, necrosis and autophagy induction by WIN2.** MCF-7 cells were treated as in figure 3. (A) Autophagy induction by acridine orange staining. Images were taken at 96 h and 40x magnification. (B) Autophagy induction in the absence and presence of chloroquine. (C) Flow cytometry at 48 h for Annexin V and Propidium Iodide staining. Staurosporine (1 µM, 24 h) was used as a positive control. (D) DAPI staining for nuclear morphology at 48 h using 40x magnification. Data were normalized to % of control in (B) and % of population in (C); data presented reflect the means of 3-4 individual experiments ± se; *p<0.05 vs vehicle.

**Figure 7 – The antiproliferative effects of WIN2 in MCF-7 cells are mediated through a non-cannabinoid receptor mechanism of action** (A) RT-PCR for the CB₁ and CB₂ receptor in MCF-7 cells. CHO cells transfected with human CB₁ or CB₂ receptors were used as a positive control. (B) MCF-7 cells were treated with vehicle or WIN2 (12 µM) and vehicle, AM251 (4 µM), or AM630 (4 µM) for 24 h. (C) RT-PCR for TRPV1 and PPARγ (D) MCF-7 cells were treated with vehicle or WIN2 (12 µM) and vehicle or GW9662 (10 µM). (E) MCF-7 cells were treated with vehicle or WIN2 (12 µM) and vehicle or capsazapine (10 µM). Cell count with trypan blue was used to assess cell viability at 96 h. Data presented reflect the means of 3 individual experiments ± se; no significant difference found.
Figure 8 – WIN2 interferes with sphingosine-1-phosphate induced growth stimulation. (A) MCF-7 cells were incubated under low serum conditions with 100 nM sphingosine-1-phosphate ± WIN2 (3 µM) or under normal serum with (B) 5 µM SEW2871 ± 8 µM WIN2 (C) 5 µM SEW2871 ± 25 µM THC or (D) 100 nM estradiol ± 8 µM WIN2. Trypan blue exclusion was used to assess cell viability at 96 h post treatment. Values are presented as % of control and represent means±se for 3-4 replicate experiments; * p<0.05 vs vehicle; #p<0.05 indicated by bars. Two way ANOVA reports: (A) F(1,4) = 10.9, P<0.05 (B) F(1,4) = 36.3, P<0.01 (C) P=0.60 (D) P=0.73
Table 1 – Interaction of cannabinoids with radiation in MCF-7 cells

MCF-7 cells were treated with the indicated cannabinoids either alone or in combination with 2 Gy radiation and cell viability was determined based on trypan blue exclusion at 96 h. Drugs concentrations (µM) were as follows: THC-30, 50, 70; CBD-10, 25, 50; Nabilone-10, 30, 50; CP55,940-10, 20, 30; MAEA-10, 20, 30; Provadoline-15, 30, 45; JWH-015-15, 30, 45. All data normalized to % of control; sample size n=3-5 experiments/study; values expressed as mean±se; *= p<0.0156 when compared to vehicle, drug treatment alone and radiation alone.
Figure 3

A

MCF-7

- Vehicle
- WIN2
- IR
- WIN2 + IR

Viable cells (% of initial control)

Time (h)

B

MDA-MB-231

Viable cells (% of initial control)

Time (h)

C

4T1

Viable cells (% of initial control)

Time (h)
Figure 4

A) MCF-7

\[
\gamma H2AX \text{ induction (\% of control)}
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B) MDA-MB-231

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C) 4T1

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Figure 5

A

Vehicle
WIN2

Control
IR

B

Senescent cells (% of sample)

Vehicle WIN2 IR WIN2 + IR

*