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COMBINED ANTIPROLIFERATIVE EFFECTS OF THE AMINOALKYLINDOLE WIN55,212-2 AND RADIATION IN BREAST CANCER CELLS

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Abstract

COMBINED ANTIPROLIFERATIVE EFFECTS OF THE AMINOALKYLINDOLE WIN55,212-2 AND RADIATION IN BREAST CANCER CELLS

By Sean Emery PhD

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2014

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The potential antitumor activity of mixed CB₁/CB₂ cannabinoid receptor agonists, such as the aminoalkylindole WIN55,212-2 (WIN2), has been extensively studied, but little information is available as to their potential interaction with conventional cancer therapies, such as ionizing radiation (IR). In the present work, we investigated the effects of WIN2 on the antiproliferative effects of radiation in human (MCF-7 and MDA-MB-231) and murine (4T1) breast cancer cells, as well as an immortalized human breast epithelial cell line (MCF-10A). WIN2 or radiation alone inhibited breast tumor growth, while the combination of WIN2 and radiation was more effective than either agent alone in breast cancer cells. WIN2 showed lower potency in MCF-10A cells than MCF-7 cells, but was still able to augment the effects of radiation at higher doses. The stereoisomer of WIN2, WIN55,212-3 (WIN3) failed to inhibit growth or potentiate the growth-inhibitory effects of radiation, indicating stereospecificity in all cell lines tested. The combination of WIN2 and IR was examined in vivo but the results were inconclusive. Interestingly, while other aminoalkylindoles, pravadoline and JWH-015, enhanced the antiproliferative effects of radiation, this was not the case for other synthetic cannabinoids (i.e., nabilone, CP55,940 and methanandamide) or phytocannabinoids (i.e., Δ^9 -tetrahydrocannabinol and cannabidiol). The

antiproliferative actions of WIN2 were not ameliorated by CB₁, CB₂, TRPV1, or PPAR receptor antagonists, suggesting the possibility of a novel site of action. Studies utilizing sphingosine-1-phosphate (S1P) agonists and estradiol suggest that WIN2 interferes with S1P signaling in cell proliferation, but agonist stimulated [³⁵S]GTPγS binding assays show that this antagonism is not occurring at the level of S1P receptors. In addition, WIN2 did not alter radiation-induced DNA damage or the rate of DNA repair based on γH2AX staining. Treatment with WIN2 and radiation promoted both autophagy and senescence, but not apoptosis or necrosis. Time course studies combined with senescence and cell death data suggest that radiation-induced senescence, while WIN2 induced classical growth arrest and the WIN2/IR combination produced parallel mechanisms of both senescent growth arrest and classical growth arrest. Taken together, these findings raise the possibility that aminoalkylindole compounds targeting a novel site of action represents a potential strategy to augment the effectiveness of radiation treatment in breast cancer.

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Section 1

General background

Breast cancer and breast cancer therapy

Breast cancer is a mammary tissue derived neoplastic disease typically beginning as a solid tumor and progressing to a malignant and metastatic disease. The surveillance, epidemiology and end result (SEER) program of the National Cancer Institute collects valuable information about cancer prevalence in the United States. According to the SEER report, an estimated 232,340 new cases of breast cancer were diagnosed in 2013, which comprised 14.1 percent of all cancer diagnoses. Breast cancer almost exclusively presents in women but 0.5-1% of all cases are diagnosed in men (Ruddy et al. 2013). The high yearly diagnosis rate translates to an estimated 2.82 million women currently living with breast cancer. With such a high rate of diagnosis, breast cancer is the most common cancer among women and the second most common cancer overall behind prostate cancer. About 12.3 percent of all women will be diagnosed with a form of breast cancer at some point in their lifetime, but fortunately breast cancer has a relatively high 5 year survival rate at 89.2 percent of cases. Despite this, the high occurrence rate for breast cancer diseases will lead to an estimated 39,620 deaths in 2013, making breast cancer the third leading cause of cancer related deaths in the United States. This high mortality rate indicates that more effective therapies need to be developed. Current treatment paradigms consist of three primary therapies, specifically including surgery, chemotherapy and radiation (Kaviani et al. 2013; Joerger et al. 2013; Yang et al. 2013; den Hollander et al. 2013), and depending on the type of breast cancer targeted therapies such as hormone based therapy can also be used (den Hollander et al. 2013).

After diagnosis of a breast tumor, a surgical consultation can lead to one of two primary options for treatment including breast conserving therapy or mastectomy. The choice of which of

these options is to be used is dependent on the state of the disease based on biopsy and/or the extent of the malignant spread. Breast conserving therapy primarily aims to eliminate the bulk of primary tumors and remaining tumor cells are treated with radiation and chemotherapy. The goal of a mastectomy is to eliminate the primary tumor and other remaining tumor cells by complete removal to the breast tissue, which eliminates the tumor bed. Chemotherapy and radiation are also used in conjunction with mastectomies to decrease relapse rates (Kaviani et al. 2013).

Chemotherapeutics function in a variety of ways depending on the drug class. Use of these therapeutic agents has evolved over the years as clinical knowledge of breast cancer has expanded. Breast cancer has primarily been treated with five drugs including, cyclophosphamide, methotrexate, 5-fluorouracil, doxorubicin and paclitaxel, but the current front line chemotherapeutic treatments are doxorubicin and paclitaxel, which are also the most recently developed of the five. Doxorubicin (Adriamycin) is a topoisomerase 2 poison capable of causing DNA damage during DNA replication by preventing DNA religation. Paclitaxel is a microtubule poison that inhibits cell division as the cancer cell goes through mitosis. Utilization of doxorubicin and paclitaxel, as well as other chemotherapeutics, is at the discretion of what the oncologist feels will be the most effective treatment (Joerger et al. 2013).

A powerful addition to the traditional adjuvant chemotherapies has been neoadjuvant therapies designed to inhibit specific signaling pathways in cancer. These treatments are also referred to as targeted therapies. The most successful targeted therapies in breast cancer include estrogen receptor antagonists, aromatase inhibitors and HER2/neu receptor antagonists. Estrogen receptor positive breast cancers depend on estrogen signaling to stimulate growth and promote tumor progression. Inhibition of the pro-cancer effects of estrogen receptor signaling is executed with estrogen receptor antagonists such as tamoxifen. Another mechanism by which breast cancers use to enhance estrogen signaling is via overexpression of

aromatase to increase local conversion of steroid precursors to estrogen. Aromatase inhibitors were developed to inhibit this process and decrease estrogen based signaling. Finally, a particularly aggressive form of breast cancer over-expresses the epidermal growth factor receptor HER2/neu, which can fuel tumor growth and survival. Recently developed antibody based antagonists of this receptor such as trastuzumab have proven to be another useful tool for improving patient survival (den Hollander et al. 2013).

In contrast to the relatively recent development of targeted therapies, radiation therapy has been a component of cancer therapy almost since the discovery in the early 1900s that ionizing radiation reduced tumor volume. Typical radiation therapy consists of 20-30 treatments of 2 gray (Gy) doses of ionizing radiation. Depending on the tumor, radiation treatments can be given pre-mastectomy, post-mastectomy or as a part of breast sparing surgery (Yang et al. 2013). The goal of radiation therapy is to inhibit tumor growth and recurrence, and these actions are linked to the DNA damaging effects of ionizing radiation. The primary mechanism for radiation-induced DNA damage is believed to be radiolysis of water leading to the formation of reactive oxygen species (ROS) (Narayanan et al. 1997). ROS formation of DNA damage is expressed as single and double strand breaks in DNA (Driessens et al. 2009).

Current cannabinoid use in cancer therapy

Cannabinoids are a class of compounds originally classified by their psychoactive effects, which are most often associated with marijuana use and abuse (Howlett et al. 2002; Pertwee et al. 2010). The cannabinoids Δ^9 -tetrahydrocannabinol (THC; Marinol) and nabilone (Cesamet) are approved by the Food and Drug Administration (FDA) for the treatment of emesis and nausea associated with cancer chemotherapy, and in the United States these remain the only federally approved cannabinoid treatments (Russo 2008). THC is the primary psychoactive

component of marijuana and nabilone is a synthetic analog of THC (Howlett et al. 2002; Pertwee et al. 2010).

The current FDA approval of Marinol and Cesamet indicates these drugs showed acceptable minimally detected negative effects on patient treatment during clinical trials, but the epidemiological literature has multiple reports that point to mixed views on cannabinoid action in the development of cancer. Three reports have linked Marijuana use to increased incidents of testicular cancer (Daling et al. 2009; Trabert et al. 2011; Lacson et al. 2012), but three other studies reported that marijuana use is not significantly correlated to lung cancer development (Sidney et al. 1997; Hashibe et al. 2006; Mehra et al. 2006). These studies suggest that marijuana smoke contains carcinogens similar to tobacco smoke that are not related to the cannabinoid compounds, and that these carcinogens could be a confounding factor of the comparisons. Resultantly, cannabinoid treatments effects on tumor development remain unresolved.

Even though the epidemiological literature has not reached a consensus on the effects of cannabinoids in tumor development, preclinical cancer treatment literature is in fairly strong agreement that cannabinoids have potential uses as anti-cancer agents. These observations begin with a 1975 report by Munson et al. where THC, Δ^8 -tetrahydrocannabinol and cannabinal inhibited growth of Lewis lung carcinoma cells in BDF mice leading to a significant increase in survival rates of the animals. Analysis of *in vitro* studies indicated THC decreases cell proliferation based on [3 H]-thymidine incorporation. Since the Munson et al. (1975) study, research has elucidated numerous mechanisms by which cannabinoids act in the body, both in non-cancerous and cancerous tissues.

The endocannabinoid system and cannabinoid sensitive receptors in preclinical cancer research

Cannabinoid receptor 1

Cannabinoid receptor 1 (CB₁) is a G protein coupled receptor (GPCR) first identified after cloning from a rat brain cDNA library. Transfection of the putative CB₁ receptor into CHO-K1 cells allowed cannabinoid agonists to inhibit forskolin stimulated cAMP production, a characteristic action of cannabinoids (Matsuda et al. 1990). CB₁ is found abundantly in the central nervous system, where its primary function is to suppress neurotransmitter release halting stimulation of postsynaptic neurons (Hoffman et al. 2000; Howlett et al. 2002; Pertwee et al. 2005). Additional research has implicated CB₁ in the antiproliferative actions of anandamide (AEA) in MCF-7 cells when the CB₁ selective antagonist SR141716 (SR1) completely blocked AEA induced growth inhibition (Melck et al. 2000). Similar findings were reported in U87-MG glioma cells, where SR1 blocked THC mediated induction of autophagy and apoptosis (Salazar et al. 2009). The Melck et al. and Salazar et al. studies exemplify that CB₁ action extends beyond mediating the psychoactive activities of cannabinoid in the CNS, and possess additional abilities to inhibit tumor growth.

Cannabinoid receptor 2

In addition to CB₁, a second cannabinoid receptor has been identified and also shown to mediate anti-tumor effects of cannabinoids. Screening in HL60 leukemia cells identified several new receptors, one of which showed 48 percent sequence homology to CB₁ receptors. Cloning of the HL60 derived receptor into COS cells allowed for binding of radiolabelled cannabinoids to identify its cannabinoid activity (Munro et al. 1993). The receptor was then identified as cannabinoid receptor 2 (CB₂), which like CB₁ receptors, is also a GPCR. CB₂ is found primarily

in immune cells circulating in the body. Primary function of CB₂ is believed to be modulation of immune function by altering immune cell migration and cytokine release (Kaminski et al. 1992; Howlett et al. 2002; Pertwee et al. 2002). CB₂ has a documented role in cancer to mediate cannabinoid based antiproliferative effects in multiple studies. Caffarel et al. (2006) showed that the CB₂ antagonist, SR144528 (SR2), but not SR1 significantly blocked the antiproliferative effects of THC in EVSA-T breast cancer cells. CB₂ involvement in cancer was later supported when the CB₂ selective cannabinoid agonist JWH-133 was shown to decrease the volume of genetically derived Erb-B2 positive mammary tumors in mice (Caffarel et al. 2010). CB₂ involvement also extends to prostate cancer where SR2, but not SR1, blocked the induction of apoptosis in PC-3 cells induced by both methanandamide and the CB₂ selective agonist JWH-015 (Olea-Herrero et al. 2009).

Cannabinoid receptor-independent effects

In contrast to the aforementioned studies, cannabinoid receptor-independent antiproliferative effects have also been reported in cancer cells. Cannabidiol (CBD), a marijuana derived cannabinoid, inhibited growth of U87 glioma cells in a manner that was not antagonizable by SR1 or SR2 (Vacca et al. 2005). Similarly, the cannabinoid selective antagonists AM251 (CB₁) and AM630 (CB₂) did not block HU-210 and AEA growth inhibition in Caco-2 colorectal cells (Gustafsson et al. 2009). Also WIN55, 212-2 (WIN2) growth inhibition was not blocked by SR1 or SR2 in Granta519 mantle cell lymphoma cells (Wasik et al. 2011), or AM251 and AM630 in OCM-1A and Colo 38 melanoma cells (Scuderi et al. 2011). Taken together, these studies demonstrate that cannabinoids inhibit tumor cell growth in a cannabinoid receptor-independent manner in certain systems. One caveat is that these studies did not assess other cannabinoid sensitive targets such as GPR55, peroxisome-proliferator activated receptors and Transient receptor potential-cation-channel subfamily V member 1 (TRPV1),

which could explain some of these effects. The observation of an as of yet unidentified third cannabinoid receptor characterized by agonist stimulated [³⁵S]GTPγS binding in CB₁ mouse knockout brains, could provide another possible explanation for the cannabinoid-independent inhibition of cancer cell growth (Breivogel et al. 2001; Nguyen et al. 2010).

GPR55

GPR55 is an orphan GPCR that has been to be shown sensitive to cannabinoids such as abnormal cannabidiol, THC, 2-arachidonoylglycerol, and anandamide by using complementary assays. These assays include: a β-arrestin based luciferase reporter assay, a GPR55 activated luciferase reporter assay and [³⁵S]GTPγS binding assays in HEK-293T (HEK) cell over expressing GPR55 (Johns et al. 2007; Yin et al. 2009). However, further research suggested that GPR55 is actually a lysophosphatidylinositol receptor with cannabinoid sensitivity (Oka et al. 2007). Unlike CB₁ and CB₂, GPR55 mediated effects on cancer were consistently linked to a pro-cancer phenotype. Andradas et al. (2011) reported that expression of GPR55 in human tumors was correlated with decreased patient survival, overexpression of GPR55 in HEK, EVSA-T and T98G cells increased growth rates, and GPR55 knockdown decreased growth rates in EVSA-T and T98G cells. The Pineiro et al. 2011 study further supports a GPR55 pro-cancer phenotype by showing that GPR55 is expressed in PC-3 (prostate) and OVCAR3 (ovarian) tumor cells, and that GPR55 downregulation with siRNA decreases cell growth compared to scrambled control siRNA. Lastly, GPR55 knockout mice showed decreased tumor formation after treatment with the carcinogen DMBA, indicating that GPR55 plays a role in tumor development (Perez-Gomez et al. 2012).

Peroxisome-proliferator activated receptors

Peroxisome-proliferator activated receptors (PPAR) are a group of three (α , δ and γ) nuclear receptors involved in metabolism and cell differentiation (Schoonjans et al. 1996; Spiegelman 1998). Activation of PPAR α by fibrate drugs treats high cholesterol (Schoonjans et al. 1996), and activation of PPAR γ by thiazolidinedione drugs treats insulin insensitivity in diabetes (Spiegelman 1998). Recent preclinical studies have found that PPAR γ is expressed in MCF-7 breast cancer cells (Nwankwo et al. 2001), and continuous treatment with the selective PPAR γ agonist troglitazone inhibited MCF-7 cell growth (Yin et al. 2001). Treatment with WIN2 in HepG2 hepatoma cells was found to induce apoptosis in a PPAR γ dependent manner as demonstrated by blockade of the effect by the PPAR γ selective antagonist GW9662 (Giuliano et al. 2009). Gene reporter (O'Sullivan et al. 2007) and antagonist studies (Mestre et al. 2009) have demonstrated that WIN2 can function as an activator of PPAR γ . Cannabinoid-induced inhibition of cancer through PPAR γ is supported by studies from Vara et al. (2013), where GW9662 also blocked the antiproliferative effects of both THC and JWH-015 in HepG2 cells.

Transient receptor potential-cation-channel subfamily V member 1

TRPV1 is a non-selective cation channel identified as the site of action for capsaicin (Caterina et al. 1997). It has also been implicated in the sensory detection of high heat stimulus (Caterina et al. 2000). AEA was later found to be a full agonist for TRPV1 based on comparison to capsaicin in electrical current measurements using HEK-293 cells transfected with TRPV1 (Smart et al. 2000). TRPV1 is expressed on some cancers but its role in growth is uncertain. For example, TRPV1 mRNA is found abundantly in MCF-7 cells (Ligresti et al. 2006), but the TRPV1 agonist capsaicin has mixed growth inhibitory effects in MCF-7 cells ranging from minimal (Tuoya et al. 2006) to significant (Thoennissen et al. 2010). Furthermore, these studies do not link actions of capsaicin to TRPV1 either genetically or pharmacologically. Conversely,

the TRPV1 antagonist capsazepine sensitizes HCT116 colon cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induced apoptosis, which argues for a protective action of TRPV1 in some cancers (Sung et al. 2012), but again capsazepine mediated actions were not linked to TRPV1 either genetically or pharmacologically.

Anandamide and fatty acid amide hydrolase

AEA was first identified as an endogenous cannabinoid by Devane et al. (1992) and AEA levels were found to be regulated by the degradative enzyme fatty acid amide hydrolase (FAAH; Deutsch et al. 1993). Treatment with AEA has been shown to inhibit the growth of numerous cancer cell lines including MCF-7 (breast), MDA-MB-231 (breast), Mz-ChA-1 (biliary), HCT116 (colon) and CaCo-2 (colorectal), (Melck et al. 2000; Laezza et al. 2006; DeMorrow et al. 2008; Patsos et al. 2010; Liao et al. 2011). Interestingly, MCF-7 cells have been shown to express FAAH. This FAAH expression might be a mechanism to protect the MCF-7 cells from the endogenously synthesized ligand AEA (Takeda et al. 2008). More work is required to prove this hypothesis however.

2-Arachidonoylglycerol and monoacylglycerol lipase

The fatty acid derivative 2-Arachidonoylglycerol (2-AG) was the second endocannabinoid discovered when Seguiria et al. (1995) used 2-AG to competitively inhibit the radiolabelled cannabinoid receptor agonist CP55,940. Later research further confirmed 2-AG as an endocannabinoid, when selective inhibition of 2-AG hydrolysis increased 2-AG levels in the brain and elicited cannabimimetic effects (Makara et al. 2005). In cancer the actions of 2-AG has not been as extensively studied as other cannabinoids, but the endocannabinoid is capable of inhibiting C6 glioma cell growth dose-dependently (Jacobsson et al. 2001). Additionally, increasing 2-AG levels by inhibiting its degradation or administering a stable 2-AG analog such

as noladin ether inhibits invasion of PC-3 and DU-145 prostate cells through matrigel in transwell plates (Nithipatikom et al. 2004).

The primary degradative enzyme responsible for regulating the levels of 2-AG is monoacylglycerol lipase (MAGL; Dinh et al. 2012), and to a lesser extent ABHD6 and ABHD12 (Blankman et al. 2007; Marrs et al. 2010). In addition to regulating 2-AG, MAGL was implicated as a regulator of a network of monoacylglycerol fatty acids in cancer cells by preventing their accumulation. The fatty acids in this network were linked to anti-cancer effects including growth and invasion inhibition (Nomura et al. 2009). Pharmacological inhibition using JZL184 and genetic knockdown using shRNA increased expression of four monoacylglycerols in C8161 melanoma cells and SKOV3 ovarian cancer cells. In both melanoma and ovarian cancers, the genetic knockdown of MAGL decreased cancer cell survival, migration and invasion *in vitro*; treatment with JZL184 decreased *in vivo* tumor growth. Overexpression of MAGL in MUM2C melanoma cells had opposite effects with decreased monoacylglycerol presence in the cells, increased migration and invasion *in vitro*, and increased *in vivo* tumor growth (Nomura et al. 2009).

Cannabinoid signaling in cancer

Decades of research have led to a robust understanding of the upstream signaling events from CB₁ and CB₂ receptors in their respective tissues, neuronal and immune. These processes are well characterized in reviews such as Howlett et al. (2002). The same level of attention, however, has not been paid to upstream cannabinoid signaling in cancer. It appears in large part that researchers assume the activation of a cannabinoid receptor in a cancer cell results in similar upstream signaling events as would be observed in a non-neoplastic tissue. While it is not necessarily incorrect to make this assumption, it cannot be stated definitively that

this is true and future research is still needed. Current research has, however, led to a number of novel findings related to downstream cannabinoid signaling events in cancer cells.

Carracendo et al. (2006) identified that CB₁ receptor activation by THC leads to modulation of ceramide signaling in U87-MG glioblastoma cells to mediate the antiproliferative actions observed with cannabinoid treatment. This ceramide signaling was later tied to the induction of endoplasmic reticulum stress, autophagy and apoptotic cell death in the same cell line by Salazar et al. (2009). Dando et al. (2013) identified that synthetic cannabinoids modulate AMPK signaling to affect cell survival through modulation of energy pathways in multiple pancreatic cancer cell lines. Additionally, in the cervical cancer model, HeLa cells, a stable analog of AEA was shown to induce apoptosis through prostaglandin production in a cyclooxygenase-2 dependent manner (Eichele et al. 2009). These examples of cannabinoid signaling in cancer are meant to exemplify that cannabinoid actions can be highly diverse in nature depending on the model and cannabinoid used, but since this document focuses on cannabinoid actions in breast cancer, the following section will explain major findings of how cannabinoid associated signaling events in breast cancer cells inhibit the growth of these breast cancer models.

Cannabinoid actions in breast cancer

Antiproliferative actions - Anandamide

In studies by De Petrocellis et al. (1998), AEA was shown to dose-dependently decrease the number of both EFM-19 and MCF-7 breast cancer cells. AEA elicited effects were attributed to growth inhibition instead of cell death in both cell lines based on time course studies and a decrease in [³H]thymidine incorporation. EFM-19 cells were shown to be capable of hydrolyzing AEA suggesting that a metabolite of AEA, instead of AEA itself, could inhibit EFM-19 cell

growth. Comparison of AEA and its primary metabolite arachidonic acid (AA) showed that AA was significantly less capable at inhibiting EFM-19 cell growth than AEA, and there was also no significant difference in growth inhibition when AEA was compared to its non-hydrolyzable form methanandamide (MAEA). Dose-dependent inhibition of EFM-19 cells by other cannabinoids, HU-210 and 2-AG, suggested that AEA inhibition of EFM-19 cell growth was cannabinoid in nature, and blockade of AEA growth inhibition by SR1 confirmed AEA mediated effects were CB₁ receptor mediated. AEA suppression of cell cycle progression was attributed to prolactin growth signaling because of dose-dependent inhibition of prolactin induced growth stimulation in EFM-19 cells by AEA. AEA antagonism of prolactin was also antagonized by SR1.

Melck et al. (2000) studied the AEA mechanism of growth inhibition in MCF-7 cells as an extension of De Petrocellis et al.'s work. As in EFM-19 cell, MCF-7 cells showed dose-dependent growth inhibition by AEA. Growth inhibition by AEA was again demonstrated to be cannabinoid in nature when the cannabinoids HU-210, methanandamide and 2-AG elicited similar levels of growth inhibition, and SR1 blocked AEA mediated antiproliferative effects. Melck et al. went on to show that the CB₂ selective antagonist, SR2, did not block AEA induced antiproliferation, further confirming a CB₁ receptor-dependent effect. Melck et al. hypothesized that AEA was antagonizing MCF-7 growth by decreasing nerve growth factor (NGF) signaling through downregulation of NGF associated Trk receptors. This hypothesis was based on a correlation between AEA growth inhibition, AEA antagonism of the growth stimulation from exogenously administered NGF and AEA induced downregulation of Trk receptors. These two papers, De Petrocellis and Melck et al., are the first demonstration that cannabinoids are capable of eliciting multiple mechanisms of action to inhibit breast cancer cell growth depending on the model.

Laezza et al. (2006; 2010) continued Melck et al.'s work in MCF-7 cells using the synthetic analog of AEA, MAEA. MAEA was shown to decrease cAMP response element

binding protein phosphorylation and the authors indicated that this is a likely explanation for the decreased expression of HMG-CoA reductase after MAEA treatment. HMG-CoA reductase is integral to the synthesis of mevalonate or mevalonic acid (MVA). Exogenous administration of MVA to MCF-7 cells rescued the cells from MAEA mediated antiproliferative effects. Radiolabelled MVA was incorporated into proteins and immunoprecipitation studies showed that proteins containing MVA were decreased after MAEA treatment, one such protein being RAS which is well known to have mitogenic effects. MVA incorporation into RAS allows it to translocate to the membrane. Under MAEA treatment, RAS levels in the membrane decreased while cytosolic RAS increased, and exogenous administration of MVA inhibited this effect. Based on the well-known RAS mediated pro-proliferative actions in cancer cells, the decrease of active RAS caused by MAEA inhibition of MVA synthesis was cited as the explanation for MAEA induced cell cycle arrested, p21 accumulation and Chk1 phosphorylation (Laezza et al. 2006; 2010).

Antiproliferative actions - THC

THC was shown to have antiproliferative actions against multiple breast tumor cell lines including MCF-7, MDA-MB231, EVSA-T and SkBr3. THC mediated inhibition of EVSA-T cells is CB₂ dependent based on SR2 but not SR1 antagonism of THC, and the expression of CB₂ but not CB₁ in EVSA-T cells. THC mediated inhibition of EVSA-T cells was attributed to G2-M cell cycle arrest quantified by propidium iodide staining, and a simultaneous occurrence of apoptosis quantified by sub-G1 population, annexin V/PI staining and caspase 3 activation. Western blotting showed an increased expression of p21 and a decrease in Cdc2, which explains the cell cycle arrest. Apoptosis was associated with decreased expression of the anti-apoptotic protein survivin which was also confirmed by western blotting. Survivin is also known to be stabilized by Cdc2 and the decrease of Cdc2 under THC treatment would explain the decrease in survivin

levels. THC invokes translocation of the transcription factor, JunD, to the nucleus of the cell. Knockdown of JunD prevents THC downregulation of Cdc2 and rescues EVSA-T cells from THC treatment; genetic overexpression of Cdc2 also rescues EVSA-T cells from THC. Together these studies show that THC activates CB₂ in EVSA-T cells to cause JunD translocation leading to downregulation of Cdc2 which results in cell cycle arrest and apoptosis (Caffarel et al 2006; 2010).

A study by von Beuren et al. (2008) showed that THC treatment in MCF-7-AR1 cells antagonized estradiol stimulated growth, but THC did not inhibit growth of MCF-7-AR1 cells alone. THC treatment in parent MCF-7 cells did however dose-dependently decrease viable cell number. This finding is partially supported by Takeda et al. (2008; 2009) where THC antagonized the growth stimulating effects of estradiol in MCF-7 cells. Surprisingly, the Takeda et al. studies also characterized a growth stimulating action of THC in MCF-7 cells, which is somewhat paradoxical when compared to growth inhibitory effects of THC in the previously mentioned study by von Beuren et al. A review of the methods used between the studies revealed no obvious differences that explain growth stimulating and growth suppressing effects in the same cell line from the same drug.

Antiproliferative actions - CBD

Ligresti et al. (2006) examined the antiproliferative effects of various phytocannabinoids across a spectrum of breast cancer cell lines. The major component of their study however focused on antiproliferative effects of cannabidiol (CBD) in MDA-MB-231 cells, which were demonstrated both *in vitro* and *in vivo*. CBD antiproliferation was attributed to a modest induction of apoptosis elicited through the increased influx of calcium and the generation of

ROS. McCallister et al. (2007; 2011) also confirmed the antiproliferative effects of CBD in MDA-MB231 cells, and that the anti-oxidant α -tocopherol protected MDA-MB231 cells from ROS produced by CBD treatment, similar to what was shown in Ligresti et al. Overexpression of the transcription factor Id-1 rescued MDA-MB231 cells from CBD treatment confirming the hypothesis that CBD downregulation of Id-1 demonstrated through western blotting mediates the inhibition of MDA-MB231 cell growth. Anti-tumor effects of CBD were extended to the whole animal using the 4T1-Balb/c syngeneic model (McCallister et al. 2007; 2011).

CBD actions in MDA-MB231 cells were again expanded in Shrivastava et al. (2011). This study confirmed ROS species involvement using the anti-oxidant α -tocopherol, which was demonstrated in both studies from Ligresti and McCallister et al. Shrivastava et al. also confirmed CBD induction of apoptosis shown by Ligresti et al. Novel observations from Shrivastava et al. highlight the induction of autophagy alongside apoptosis under CBD treatment. In a time- and dose-dependent manner, both apoptosis (annexin V staining or PARP cleavage) and autophagy (LC3 cleaved western blotting) increased in parallel under CBD treatment. Administration of the autophagic inhibitor bafilomycin or the caspase inhibitor zVAD to MDA-MB231 cells treated with CBD show modest changes in cell death. No other experiments were conducted to evaluate the interaction between apoptosis and autophagy, leaving conclusions unclear if these two processes of autophagy and apoptosis are working together or separately to mediate CBD induced antiproliferative actions. CBD inhibition of AKT phosphorylation was also hypothesized to play a role in the antiproliferative effects of CBD based on a correlation between CBD treatment and a decrease of AKT phosphorylation (Shrivastava et al. 2011).

Additional observations from the above studies show that CBD inhibited MDA-MB231 cell growth independent of cannabinoid receptors based on the use of cannabinoid receptor antagonists (Ligresti et al. 2006; Shrivastava et al. 2011). This is not surprising since CBD has

extremely poor affinity for CB₁ (K_i=4.3 μM) and CB₂ (K_i=1.2 μM) (Showalter et al. 1996). Also, Shrivastava et al. demonstrated that CBD had a lower efficacy at inhibiting growth of the non-transformed MCF-10a breast epithelial cells than the MDA-MB231 breast cancer cells, which shows a potentially cancer selective effect for CBD treatment in MDA-MB231 cells.

Antiproliferative actions - synthetic cannabinoids

Other studies have linked synthetic cannabinoids (WIN2 and JWH-133) to antiproliferative effects in cancer as well (Qamri et al. 2009; Caffarel et al. 2010). WIN2 and JWH-133 inhibited MDA-MB231 cell growth both *in vitro* and *in vivo*. *In vitro* studies found that both CB₁ and CB₂ mRNA were expressed in MDA-MB231 cells, and *in vivo* studies show that both the CB₁ selective antagonist AM251 and the CB₂ selective antagonist SR2 reduced WIN2 inhibition of tumor volume. JWH-133 is a CB₂ selective agonist, therefore only SR2 was used to antagonize its effects. This indicates that both CB₁ and CB₂ are involved in the antiproliferative actions of cannabinoids in MDA-MB231 cells. WIN2 and JWH-133 inhibition of MDA-MB231 proliferation was attributed to the induction of apoptosis quantified by a sub-G1 population from cell cycle analysis (Qamri et al. 2009).

Caffarel et al. 2010 further demonstrated the antiproliferative effects of JWH-133 using an ErbB2 based genetic model of tumor development. JWH-133 and THC suppressed growth of tumors *in vivo* as well as suppressed cell viability *in vitro* using N202.1A cells. Caffarel et al.'s histological analysis of human tumor samples showed a correlation between ErbB2 positive tumors and CB₂ receptor expression indicating a connection between the two receptors; based on this correlation, JWH-133 and THC were tested with the antagonists SR2 and SR1. Only SR2 inhibited *in vitro* tumor growth indicating CB₂ dependent antiproliferative effects. Caffarel et al. (2010) also observed a decrease in p-AKT when N202.1A breast tumor cells were treated with both THC and JWH-133. Overexpression of AKT in N202.1A cells also prevented both THC

and JWH-133 from inhibiting cell growth both *in vitro* and *in vivo*, implicating AKT signalling in the CB₂ dependent actions of THC and JWH-133.

Anti-invasive and anti-metastatic cannabinoid actions

In addition to characterizing the antiproliferative effects of synthetic cannabinoids, Qamri et al. 2009 and Caffaral et al. 2010 also characterized the anti-invasive and anti-metastatic effects of WIN2, JWH-133 and THC. WIN2 and JWH-133 decreased the *in vitro* migratory action and the *in vivo* formation of metastatic lung nodules by MDA-MB231 cells (Qamri et al. 2009). Additionally, JWH-133 and THC decreased the number of blood vessels per tumor and the number of metastatic nodules in genetically derived breast carcinomas. Decreases in number of blood vessels and metastatic nodules indicate an anti-angiogenic and anti-invasive action for cannabinoid treatment, which is likely explained by JWH-133 and THC mediated effects on matrix metalloproteases (MMP). Both JWH-133 and THC in Caffarel et al. decreased expression of the pro-invasive MMP2 and increased expression of the anti-angiogenic MMP9.

MAEA also inhibits invasion, migration and metastasis of MDA-MB231 cells both *in vivo* and *in vitro*. Antagonism of MAEA elicited anti-invasive actions with SR1 demonstrates a CB₁ component to this observation, although CB₂ antagonists were not assessed (Grimaldi et al. 2006). These anti-invasive effects were linked to MAEA-mediated decrease of RHOA, which is known to be involved in actin rearrangement for cell motility. MAEA decrease of RHOA was also shown to be CB₁ dependent using the CB₁ antagonist SR1. MVA rescued the downregulation of RHOA and anti-invasive effects demonstrated by MAEA (Laezza et al. 2008), and MVA rescue of RHOA downregulation connects MAEA associated anti-invasive actions in MDA-MB231 cells to the MAEA mediated antiproliferative effects in MCF-7 cells discussed in detail above (Laezza et al. 2006; 2010). A cannabinoid constituent of marijuana, cannabidiolic acid, was also able to inhibit MDA-MB231 invasion measured *in vitro* using transwell migration and the wound healing

assay, and like Laezza et al. (2008), this cannabidiolic acid treatment also induced a downregulation of RHOA (Takeda et al. 2012).

A unique observation by Nasser et al. (2011) showed that the CB₂ selective agonist JWH-015 inhibited CXCR4 associated migration in MCF-7/CXCR4, MDA-MB231 (SCP2) and NT 2.5 breast cancer cells, which overexpress CXCR4. Migration was quantified using the transwell migration and wound healing assays. The CXCR4 agonist CXCL12 enhanced migration in both assays compared to vehicle. Treatment with JWH-015 and CXCL12 showed less migration compared to treatment with vehicle and CXCL12 in both assays but JWH-015 effects on migration in the absence of CXCL12 were not quantified. Absence of data for the JWH-015 and vehicle control prevents the conclusion that JWH-015 is antagonizing CXCR4 mediated migration since JWH-015 might be suppressing migration in general. Regardless, JWH-015 was shown to have some effect on migration in the MCF-7/CXCR4, MDA-MB231 (SCP2) and NT 2.5 cell lines.

Summary, hypothesis and goals of the following studies

The research presented in section 1 of this document has proven that cannabinoids have the capacity to inhibit the growth of breast cancer cells through a variety of mechanisms. The goals of the following work, was to evaluate the antiproliferative effects of cannabinoids alone and in combination with established breast cancer therapies, as well as attempt to elucidate mechanism(s) for the effects of these treatments. Section 3 of the document will present further information to support the hypothesis of these studies, which was that cannabinoid agonist treatment in breast cancer cells would augment radiation and/or adriamycin treatments to enhance the antiproliferative effects of either therapeutic strategy through an autophagic mechanism.

Each section of this document will be presented with the necessary background information specifically pertinent to the aims of that section, followed by results and ending with a brief discussion. The first results section (section 3 of the document) will address cannabinoid interactions with chemotherapy and radiotherapy. The second results section (section 4) will investigate the mechanism(s) of breast cancer cell growth inhibition elicited by cannabinoid and radiation combinations, and finally the third results section (section 5) will examine receptor involvement for the cannabinoid treatment. The discussion section (section 6) presented at the end of the document aims to connect these three research aims, demonstrate the interaction of each respective project, and summarize how each contributes to the overall conclusions made from this body of research.

Section 2

Materials and Methods

Cell line maintenance

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 (Invitrogen, Grand Island, NY) with 1% penicillin/streptomycin solution, 5% fetal bovine serum and 5% bovine calf serum. MCF-10a cells were cultured in DMEM/F12 media (Invitrogen) 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 (Invitrogen) 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, glutathione, methanandamide, n-acetylcysteine, nabilone, pioglitazone, bezafibrate, capsaicin, adriamycin, paclitaxel 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). Pravastatin, JWH-015 and SEW2871 were purchased from Cayman 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 (Virginia Commonwealth University).

Drug treatments

All treatments with cannabinoids, cannabinoid antagonists, capsaicin, capsazepine, pioglitazone, GW9662, bezafibrate, S1P, SEW2871 and estradiol were initiated with a 24 h exposure period, after which the drug-containing media was aspirated, the cells were washed with phosphate-buffered solution (PBS), and replenished 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) was used at 1 μ M with an exposure time of 2 h. Paclitaxel was used at 0.5 μ M with an exposure time of 24 h. For autophagy inhibition, chloroquine (5 μ M) was administered to cells for the duration of the experiment. For ROS inhibition, N-acetylcysteine and glutathione were administered to cells 24 h before initiation of drug treatment, and maintained throughout drug treatment for a total of 48 h. H₂O₂ was administered with drug and radiation as a positive control for ROS mediated cell death. Media treated with H₂O₂ was removed after 24 h. In experiments under low serum conditions, drugs were added to the low serum media and low serum media was administered to cells for 24 h. After 24 h the low serum media and drugs were removed and replaced with regular media absent of drug. In studies involving estradiol, the cells were maintained in phenol red free 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.

Cell count methods –

Trypan blue 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 absorbances were measured at 540 nm using a microplate reader.

Flow cytometry –

Annexin V and propidium iodide - Cells were harvested at the indicated time points and washed twice with PBS prior to centrifugation at 500xg 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.

γH2AX - Both adherent and non-adherent cells were collected and pelleted at indicated time points using a 4°C 5810 R eppendorff centrifuge at 500xg. 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 bovine serum albumin (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 temperature. 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.

Cell staining –

Cell staining was used to identify senescent cells (β -galactosidase), nuclear morphology (DAPI) and autophagic vesicles (acridine orange).

β -galactosidase (pH 6.0) - As described in Biggers et al. (2013), cells were plated into 6 well plates at 10,000 cells/well. At appropriate time points, cells were washed twice with PBS and fixed with 2% formaldehyde/0.2% glutaraldehyde for 5 min. The cells were washed again with PBS and stained with a solution of 1 mg/mL 5-bromo-4-chloro-3-indolyl-b-galactosidase in dimethylformamide (20 mg/mL stock), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 40 mM citric acid/sodium phosphate, pH 6.0 and 2 mM MgCl₂. Following overnight incubation at 37°C, the cells were washed twice with PBS and the images of representative microscopic fields were captured on an Olympus 1 x 70 inverted microscope (Olympus America, Inc., Melville, NY). Senescent cells were quantified manually based on blue staining and reported as a percent of the total population.

4',6-diamidino-2-phenylindole (DAPI) - As described in Biggers et al. (2013), at the indicated time points both adherent and nonadherent cells were harvested and centrifuged at 1,500 rpm for 3 min. A dilution of 20,000 cells in 200 μ L of PBS per slide was prepared, and cells were spun at 500xg for 5 min (Shandon Cytospin 4, Thermal Electron Corp). Slides were refrigerated until ready for staining. Cells were fixed with 4 % formaldehyde in PBS for 10 min at room temperature and then washed with PBS twice for 5 min at room temperature. A 1:1,000 dilution was prepared for Vectashield:Dapi, and each slide was mounted with 10 μ L of the solution. Coverslips were sealed using clear nail polish, and photographs were taken using an Olympus 1 x 70 inverted microscope (Olympus America, Inc., Melville, NY). Slides were stored at 4°C, and three fields per condition were evaluated.

Acridine orange - As described in Biggers et al. (2013), cells were plated at a density of 2×10^5 cells per 6-well plate and allowed to adhere overnight. After drug treatment, drug was

removed and cells were washed with PBS. Cells were stained with acridine orange (1 µg/mL) for 15 min. At selected time points, cells were stained for 10 min, the stain was removed and cells were washed with PBS four times before fresh media was added to the wells. Photographs were taken using an Olympus 1 x 70 inverted microscope (Olympus America, Inc., Melville, NY). Staining was visualized at a fluorescence wavelength of 500 nm. All comparisons were made at identical magnifications.

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- GGTTTCATCAATGTGTGGGA, CB₂ forward- GACCGCCATTGACCGATACC, CB₂ reverse- GGACCCACATGATGCCCAG, TRPV1 forward- CTCACCAACAAGAAGGGAATG, TRPV1 reverse- AGGTCGTACAGCGAGGAGTG, PPAR_γ forward- ATGACAGCGACTTGGCAATA, PPAR_γ reverse- GAGGACTCAGGGTGGTTCAG, GPR55 forward- CATCTCTCAGCCCTCTCAGC, GPR55 reverse- TTCTTCCTACAACACCAACAGA, Beta actin forward- TGGGACGACATGGAGAAA, Beta actin reverse- CACAGCCTGGATAGCAACG. 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 receptors were contributed by Dr. Mary Abood of Temple University, Department of Anatomy and Cell Biology.

Agonist stimulated [³⁵S]GTPγS binding –

Activation of G proteins by sphingosine-1-phosphate (S1P) and WIN55,212-2 (WIN2) was quantified by agonist-stimulated [³⁵S]GTPγS binding assays.

Cell Harvesting - MCF7 cells were harvested and centrifuged before suspension in ice-cold membrane buffer (50 mM Tris-HCl, 3 mM MgCl₂ and 1 mM EGTA, pH 7.4). The cells were homogenized using a Polytron homogenizer for 10 seconds, then centrifuged at 50,000xg at 4°C for 10 min. Membranes were resuspended in membrane buffer, and protein was determined by the method of Bradford using 1 mg/ml bovine serum albumin (BSA) as the standard. Membrane preparations were stored in aliquots at -80°C.

Membrane preparation - Frozen membrane samples were thawed on ice and were homogenized in 50 mM Tris-HCl, 3 mM MgCl₂, 0.2 mM EGTA, 100 mM NaCl pH 7.4 (assay buffer) using a Polytron homogenizer for 10 seconds. Homogenates were centrifuged at 50,000xg at 4°C for 10 minutes and resuspended in assay buffer. Membrane protein levels were assessed via the Bradford method, using 1 mg/ml BSA as the standard (Bradford, 1976).

[³⁵S]GTPγS Binding - Concentration-effect curves were generated by incubating the appropriate concentration of membrane (10 μg protein) in assay buffer with 0.1% BSA, various concentrations of S1P and/or WIN2, 30 μM GDP and 0.1 nM [³⁵S]GTPγS in 0.5 ml total volume. Basal binding was assessed in the absence of agonist, and nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS. The reaction was terminated by filtration under vacuum through Whatman GF/B glass fiber filters, followed by three washes with cold (4°C) Tris buffer (50 mM Tris-HCl, pH 7.4). Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency for [³⁵S] after extraction overnight in Econo-Safe scintillation fluid.

In vivo studies –

Animals and maintenance - Female Balb/c mice (Jackson Laboratory, Bar Harbor, ME) weighing between 17 and 22 g (approximately 8-10 weeks of age at the start of the study) were housed 4 per cage in a temperature controlled (20-22°C) vivarium approved by the American Association for the Accreditation of Laboratory Animal Care. The mice were maintained on a 12 h light/dark cycle, with all experiments performed during the light cycle. Food and water were available *ad libitum*. All experiments were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University in accordance with the Guide for the Care and Use of Laboratory Animals.

Tumor growth - 4T1 cells were suspended into sterile PBS at 250,000 cells per ml. A 200 µl volume of the cell suspension was subcutaneously injected into the hind flank of Balb/c mice. Tumors were permitted to stabilize for 24 h prior to treatment and allowed to grow for 16-17 days, until evidence of necrosis was detected or the tumor burden exceeded 1 cm³. Tumor volume was assessed by caliper measurements by an investigator blinded to the experimental conditions; tumor volume was calculated by the formula $V=0.5(W(L^2))$. Radiation treatments were given 24 h after injection of tumor cells. Irradiated animals were anesthetized using ketamine (85 mg/kg) and xylazine (8.5 mg/kg) before being placed into a focused irradiation chamber limiting exposure to the right hind quarter. 24 h after irradiation, drug treatments were begun. Vehicle (ethanol, emulphor, and saline in a ratio of 1:1:18), WIN,212-2 (1, 5, or 10 mg/kg) or WIN,212-3 (5 mg/kg) were administered to mice twice weekly via intraperitoneal (i.p.) injection. Adriamycin (5 mg/kg), used as a positive control, was administered once per week i.p. for two total treatments.

Statistics –

In vitro tests - All experiments were performed with 3-6 replicates. Two-way repeated measures ANOVA was used to analyze radiation and drug treatments for individual treatment effects and potential interactions between treatments. All two-way ANOVA comparisons were done within time point. Paired T-test with a Bonferroni correction was used for individual comparisons and to assess interactions of combination treatments. Standard paired T-tests were used for validation of positive controls. Temporal studies utilized One way repeated measures ANOVAs for two purposes 1) to assess growth inhibition within each time point by comparison to vehicle and 2) to assess cell death by comparing an individual treatment across time points. Dunnett's post hoc test was used with One way repeated measures ANOVAs. All data are displayed as mean \pm se.

In vivo tests - All animal studies utilized 8 mice per treatment group. A Two-way ANOVA was used to analyze radiation vs. drug treatments within each time point to assess interactions and main effects of drug. A T-test with a Bonferroni correction was used to assess comparisons of combination + drug with the individual treatments. A One way ANOVA was used to assess effects of individual treatments across time points. Dunnett's post hoc test was used for comparisons to baseline. All data are displayed as mean \pm se.

Criteria for augmentation and antagonism - Interactions between treatments were evaluated using the following statistical criteria. Two way ANOVAs assess significant interactions between treatments by requiring $p < 0.05$ for interaction comparisons. If a significant interaction was found then individual comparisons with a bonferroni corrected T-tests were used to determine the type of interaction.

Using the bonferroni corrected T-test to assess augmentation. Studies will evaluate the effects of two individual treatments (cannabinoid or cancer therapeutic) and then the combination of the two treatments (cannabinoid + cancer therapeutic). If both individual

treatments are significantly different from vehicle ($p < 0.05$), then the combination treatment must be significantly different when compared to the cannabinoid treatment ($p < 0.025$) and the cancer therapeutic treatment ($p < 0.025$). If either of the individual treatments are not found significantly different from vehicle by $p < 0.05$, then the combination treatment must be significantly different from vehicle ($p < 0.0166$), cannabinoid treatment ($p < 0.0166$) and the cancer therapeutic treatment ($p < 0.0166$). To demonstrate antagonism the combination treatment need only be shown to significantly decrease antiproliferative action when compared to one or both of the individual treatments ($p < 0.05$).

Section 3

Characterization of cannabinoids in combination with ADR and radiation

Various studies have implicated a process known as autophagy in the antiproliferative action of cannabinoids (Salazar et al. 2009; Shrivastava et al. 2011; Donadelli et al. 2011, Dando et al. 2013). Autophagy was originally characterized in normal cells as a degradative process used throughout the body in which intracellular autophagosomal structures are generated that consume cellular materials such as mitochondria and endoplasmic reticulum for the purposes of nutrient recycling, energy production or management of cellular stresses. Autophagy has now been implicated in cancer cell treatment as either a novel cell death mechanism, a precursor to growth inhibitory mechanisms or protection from treatment stress. However, differences between death-promoting forms of autophagy and protective forms of autophagy are poorly understood (Yang et al 2011, Mah et al. 2012, Leone et al 2013).

Cannabinoid and cancer research clearly links autophagy to antiproliferative action in various publications. In Salazar et al. (2009), THC induced autophagy via activation of CB₁ in U87-MG glioblastoma cells. The cannabinoid receptor antagonist SR1 was used to confirm CB₁ involvement and GFP-LC3 puncta formation and electron microscopic autophagosome imaging confirmed autophagy induction. THC also induced apoptosis identified by active caspase 3 staining, annexin V/PI staining and antagonism by the pan-caspase inhibitor ZVAD. ATG1 and ATG5 knockdown inhibited autophagy induction, prevented apoptosis induction and subsequent cell death by THC treatment, demonstrating that THC induction of autophagy through CB₁ leads to the induction of apoptosis killing of glioblastoma cells. Studies further supported the hypothesis of cannabinoid induction of autophagy. In MDA-MB231 cells cannabidiol induces autophagy based on LC3 cleavage and electron microscopic autophagosome imaging. LC3 cleavage was prevented by the autophagy inhibitor bafilomycin (Shrivastava et al. 2011). In

Panc1 cells, the cannabinoid agonists GW405833 and arachidonoyl cyclopropamide treatment lead to autophagic induction quantified by LC3 cleavage, acridine orange staining and flow cytometric quantification of MDC staining. (Donadelli et al. 2011, Dando et al. 2013).

Interestingly, data from the Gewirtz laboratory supports a hypothesis termed the “autophagic switch”. This hypothesis suggests that autophagy can have multiple effects on cell fate depending on the conditions of the system. The autophagic switch concept was initially promoted in studies by Wilson et al. (2011) using radiation in ZR-75 breast cancer cells. This hypothesis was then later expanded to radiation in MCF-7 breast cancer cells (Bristol et al. 2012), and then again extended to chemotherapeutic treatment in MCF-7 breast cancer cells (Goehe et al. 2012).

The studies by Bristol et al. (2012) showed that 5x2 Gy of radiation-induced significant levels of autophagy as measured by complementary assays including acridine orange staining, RFP-LC3 puncta formation, autophagic vesicles imaged with electron microscopy and p62 degradation via western blot. Previous work has shown that senescence mediates growth inhibition by radiation (Jones et al. 2005), and the work by Bristol et al. further supported this finding by demonstrating (using cell viability and TUNEL assays) that radiation reduces proliferative capacity in the absence of apoptosis. Blockade of autophagy by pharmacological treatment with chloroquine or genetic knockdown of ATG5 further decreased viable cell number and induced apoptotic cell death, demonstrating that manipulation of autophagy leads to a transition from senescent growth arrest to cell death.

The study by Goehe et al. (2012) addressed the potential relationship between autophagy and senescence by demonstrating that manipulation of autophagy induced by adriamycin (ADR, also known as doxorubicin) treatment leads to a delay in the onset of senescence. After 72 h, ADR was shown to induce a significant amount of senescence as

measured by beta galactosidase staining, and a significant amount of autophagy as measured by acridine orange staining, RFP-LC3 puncta formation, autophagic vesicles imaged with electron microscopy and p62 degradation. Blockade of autophagy by chloroquine, 3-methyladenine and ATG5 knockdown led to a decrease in autophagic signaling and delayed senescence until 120 h post treatment. Consequently, these studies demonstrated that while senescence could occur in the absence of autophagy, there was nevertheless a clear connection between autophagy and the rate of ADR induction of senescence in MCF-7 cells.

This autophagic switch hypothesis presents the premise that autophagy is interconnected with growth inhibitory and cell death processes, and that alterations in autophagy can alter how the cell responds to various stressors. It is possible that the cited cannabinoid actions on autophagy could alter autophagic mechanisms in such a way as to enhance the antiproliferative effect as was demonstrated in Bristol et al. 2012 and Goehe et al. 2012. This previous work guided the generation of my hypothesis that cannabinoid agonist treatment in MCF-7 cells would augment radiation and/or adriamycin treatments to enhance the antiproliferative effects of either therapeutic strategy through an autophagic mechanism.

A primary aim of the studies presented in this section as well as the overall thrust of this work was to evaluate the interaction of cannabinoid agonists in combination with either radiation treatment or ADR treatment. These studies were performed in human (MCF-7 and MDA-MB-231) and murine (4T1) breast cancer cells. For the purpose of assessing selectivity in cancerous versus non-cancerous cells, the combination treatment was also evaluated in MCF-10A cells, a model of normal breast epithelial cells. WIN55, 212-2 was the primary cannabinoid evaluated in this work, and stereospecificity was determined utilizing its stereoisomer, WIN55,212-3, which does not bind to cannabinoid receptors. The impact of radiation on breast tumor cell growth was assessed in combination with a variety of cannabinoids, including THC,

nabilone, CP55,940, methanandamide, cannabidiol (CBD), JWH-015 and pravadoline. *In vivo* experiments were conducted using the 4T1-Balb/c syngeneic model to establish a WIN2 dose-response curve and to test the interaction of WIN2 and radiation.

Cell lines

Bristol et al (2012) and Goehe et al (2012) used MCF-7 cells for their studies, and cannabinoids including anandamide, cannabidiol, and THC have been shown to inhibit MCF-7 cell growth (De Petrocellis et al 1998, Legresti et al 2006, Caffarel et al 2006). These previous studies provided the rationale for studying MCF-7 cells as the primary model for evaluating cannabinoid treatment in combination with ADR and radiation and establishing and characterizing any interaction. Once interactions were established in MCF-7 cells, other models were used to assess generalization of combination treatments (MDA-MB231 and 4T1), test combination effects in an appropriate *in vivo* model (4T1) or demonstrate selectivity of the combination treatment for tumor cells (MCF-10a). A more detailed explanation and rationale for the models chosen is provided below.

MDA-MB231 cells

MDA-MB231 cells are a p53 mutant cell line compared to the p53 wild type MCF-7 (Lacroix et al. 2006). p53 is commonly mutated in cancer with 31% of all cancers and ~23% of breast cancers expressing this aberration. p53 gene mutations are also correlated with poor prognosis, due to a collection of cancer promoting effects on cell growth, DNA repair, tumorigenicity, cell death, angiogenesis and metastasis (Lacroix et al 2006, Walerych et al 2012). The frequency at which the p53 mutation is present in breast cancer and its effectiveness

at enhancing pathology, makes it important to test the cannabinoid/cancer therapy combinations against a p53 mutant model.

MDA-MB231 cells are also a triple negative breast cancer (TNBC) model, based on absence of the estrogen receptor, progesterone receptor and Her2/neu receptor. Typically, TNBC diseases are initially responsive to chemotherapy but resistant to hormone therapy. 78% of TNBC patients show a response after initial therapy; however, this 78% also have faster rates of recurrence than non-TNBC patients, show more frequent progression to metastasis than non-TNBC patients and have poorer 5 year survival rates than non-TNBC patients. Interestingly though 22% of TNBC patients given treatment show complete disease elimination (CDE) compared to the 11% CDE for non-TNBC disease. These 22% of TNBC patients also have a higher percentage of 5 year survivors compared to non-TNBC patients that have CDE after initial treatment (Liedtke et al. 2008). Based on this, combination therapies that can enhance current treatment effectiveness could also translate to higher CDE rates and potentially lead to greater 5 year survival rates in TNBC patients. Testing enhanced treatment effectiveness in a TNBC model would require the use a model that is already responsive to our selected therapies of radiation, ADR and cannabinoids.

MDA-MB231 cells have been demonstrated to be such a model. 10 gray (Gy) radiation induces a G2 cell cycle arrest at early time points followed by induction of apoptosis quantified by TUNEL staining and accumulation of a significant fraction of the cell population in the sub-G1 phase. Decreases in MDA-MB231 survival from radiation treatment persisted to 14 days (Jones et al 2005). 2 μ M ADR in MDA-MB231 cells induces delayed apoptosis as quantified by TUNEL staining (Elmore et al. 2002), leaving a small surviving fraction in senescent growth arrest quantified by β -galactosidase activity (Di et al. 2009). The cannabinoids WIN55,212-2 and JWH-133 were shown to inhibit *in vitro* cell viability and *in vivo* tumor growth of MDA-MB231 cells

(Qamri et al. 2009), and likewise the phytocannabinoid, cannabidiol (CBD), decreased cell viability via apoptosis as quantified by annexin V positive staining (Shrivastava et al. 2011).

4T1 cells

Cannabinoids and autophagy have immune related effects in tumor treatment that become relevant when studies are moved *in vivo* (McKallip et al. 2005, Michaud et al. 2011). For example, McKallip et al. (2005) showed that THC suppresses the antitumor immune response in mice, which enhanced tumor growth. When Balb/c mice were injected with non-viable 4T1 cells they developed protection from tumor growth when later challenged by injecting viable 4T1 cells, but THC suppressed this protection after the injection of non-viable 4T1 cells. Furthermore, treatment with THC increased the number and size of 4T1 metastatic nodules in lung of Balb/c mice compared to vehicle, while this did not occur in (immune-suppressed) SCID mice. If cannabinoid treatments in these studies elicit similar suppression of the anti-tumor immune response it could limit the effectiveness of a cannabinoid/cancer therapy combination approach.

Michaud et al. (2011) showed that immune function is necessary for autophagy inducing chemotherapies *in vivo*. CT26 colon cells treated with mitoxantrone (MTX) *in vivo* and *in vitro* induced autophagy quantified by LC3 cleavage. Inoculating mice with CT26 cells treated with MTX *in vitro* causes 80% protection from tumor development when mice are later challenged using healthy/untreated CT26 cells. However, knockdown of the autophagy genes ATG5 and ATG7 in CT26 cells blocks MTX mediated induction of autophagy, and when these knocked down CT26 cells are treated with MTX *in vitro* and inoculated into mice, the mice show ~15-35% decrease in protection from tumor development when challenged using healthy/untreated

CT26 cells. These studies demonstrate that autophagy is relevant to some extent in signaling the immune system after MTX treatment. If the cannabinoid/cancer therapy combination treatment interact via autophagy as hypothesized, then an immune competent animal would be required to detect this effect *in vivo*. 4T1 cells are a murine derived breast cancer model that can be implanted into an inbred Balb/c mouse with normal immune function without incurring unwanted graft host interactions (Aslakson et al. 1992).

MCF-10A cells

When augmenting growth inhibitory or cytotoxic effects of cancer therapies through the use of other agents there is always a concern that this augmentation will extend to non-cancerous tissues enhancing the toxic side-effects of these therapies. MCF-10A cells are a cell line derived from the immortalized breast epithelial cell line MCF-10 (Soule et al. 1990), and could be used for screening augmentation of toxicities in the cannabinoid/cancer therapy combination treatments. A similar idea from Shrivastava et al. (2011) used the cell viability assay to show that cannabidiol had greater growth inhibitory effects in MDA-MB231 cells than MCF-10A cells demonstrating that cannabidiol toxicities do not transfer to all cell types equally. Similar comparisons between MCF-7 and MCF-10A would offer insights into the effects of cannabinoids alone and in combination with cancer therapy in normal tissues.

Rationale for cannabinoid agonist choice

THC was shown to induce CB₁ mediated autophagic cell death in glioma cells (Salazar et al 2009), making it an appropriate candidate to be evaluated in combination with ADR and radiation, but wide ranging differences among cannabinoids in both structure and function within the endocannabinoid system provide a spectrum of tools for these studies. Structurally, the

compounds used here fall into three basic classes including a phytocannabinoid structure similar to THC (THC, CBD, nabilone, CP55,940), the fatty acid or endogenous cannabinoid structure (methanandamide (MAEA)) and the aminoalkylindole structure (WIN55,212-2, pravadoline, JWH-015) (**Fig 3.1A-H**).

THC (Marinol) and nabilone (Cesamet) are clinically approved by the Food and Drug Administration (FDA) as anti-emetics in chemotherapy treatment, although neither compound is approved for palliative use in radiation therapy. THC and nabilone are currently the only FDA approved cannabinoids in the United States. Preclinically, radiation-induced emesis in the least shrew has been shown to be attenuated by THC, WIN55,212-2 (WIN2) and CP55,940 (Darmani et al. 2007). Beyond palliative care, the cancer inhibiting properties of cannabinoids have only been assessed preclinically. CBD, THC, MAEA and WIN2 have been shown to inhibit breast cancer cell growth (Laezza et al. 2006, Legresti et al 2006, Caffarel et al 2006, Qamri et al. 2009) while CP55,940 and JWH-015 inhibit glioma cell growth (Jacobsson et al. 2001). Pravadoline was chosen for its structure, and has not yet been demonstrated to inhibit cancer cell growth.

In addition to their palliative or potential antitumor properties, all cannabinoids tested have varying efficacies and affinities for CB₁ and CB₂, both of which are G protein coupled receptors (GPCR). Agonist-stimulated [³⁵S]GTPγS binding assays have shown that both WIN2 and CP55,940 are high efficacy agonists at both CB₁ and CB₂, while THC is a relatively low efficacy agonists at both receptors (Sim et al. 1996, Showalter et al. 1996, Breivogel et al. 1998). JWH-015 was shown to have low affinity at CB₁ receptors with a 27x greater affinity for CB₂ over CB₁ (Showalter et al. 1996). Conversely MAEA, a stable analog of the rapidly hydrolyzed endogenous cannabinoid anandamide, has CB₁ selectivity with a 40x greater affinity for CB₁ over CB₂ (Abadji et al. 1994, Khanolkar et al. 1996). CBD has extremely poor affinity for CB₁ (K_i=4.3 μM) and CB₂ (K_i=1.2 μM) (Showalter et al. 1996), and likewise the stereoisomer of

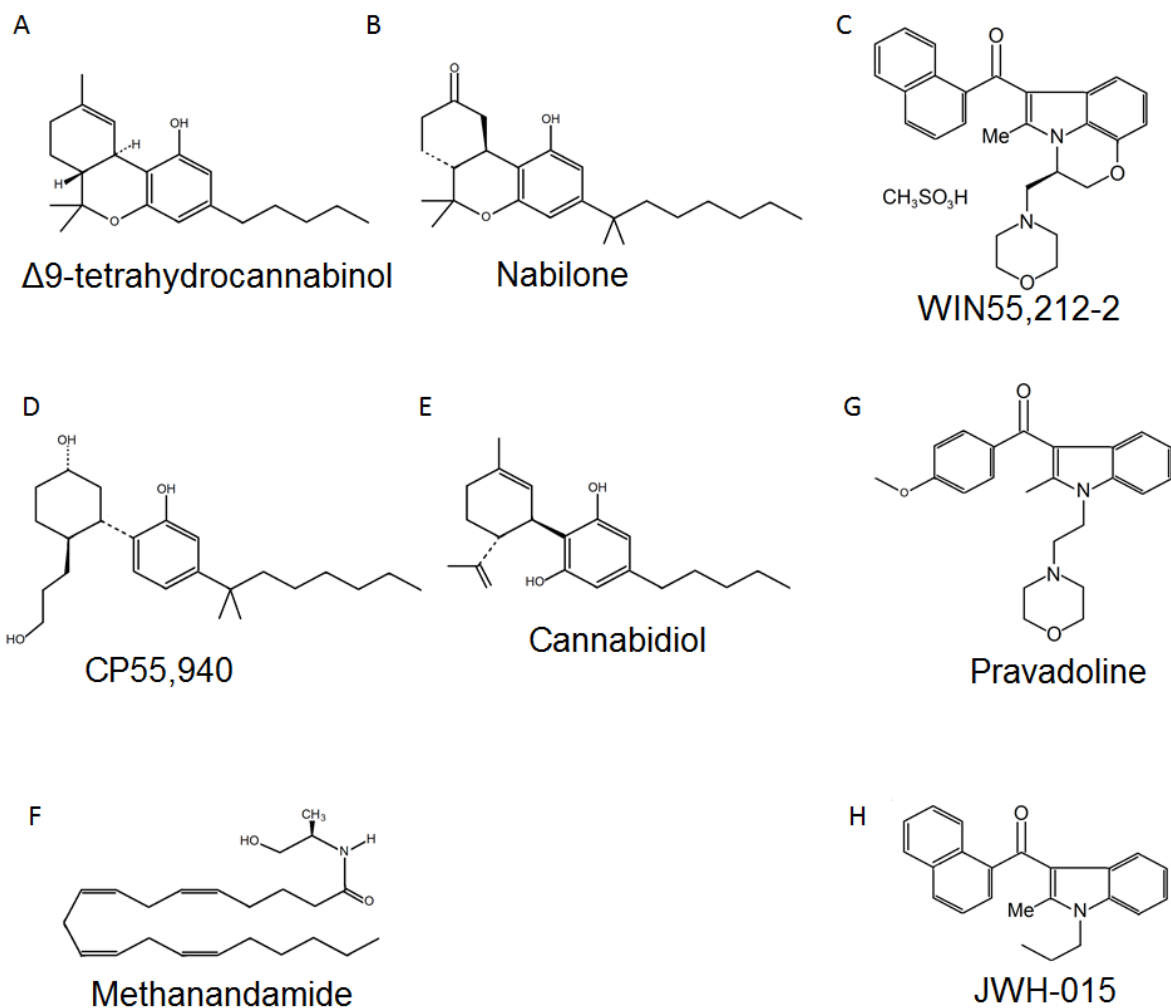


Figure 3.1 – Cannabinoid structures. (A) Δ^9 -tetrahydrocannabinol **(B)** Nabilone **(C)** WIN55,212-2 **(D)** CP55,940 **(E)** Cannabidiol **(F)** Methanandamide **(G)** Pravadoline **(H)** JWH-015

WIN2, WIN55,212-3 (WIN3), has no known agonist activity at CB₁ and CB₂ (Howlett et al 2002; Savinainen et al. 2005).

A majority of the studies presented in this document utilize the synthetic cannabinoid WIN2 due to its pharmacology (Sim et al. 1996, Showalter et al. 1996, Breivogel et al. 1998), stereochemistry (Howlett et al 2002; Savinainen et al. 2005) and efficacy as an anti-cancer agent (Giuliano et al. 2009; Qamri et al. 2009; Park et al. 2011; Scuderi et al. 2011; Wasik et al. 2011). As a high efficacy agonist for both cannabinoid receptors (Sim et al. 1996, Showalter et al. 1996, Breivogel et al. 1998), the use of WIN2 in our studies prevented us from preferentially testing one cannabinoid receptor over another. This is important, because both cannabinoid receptors have been implicated in the growth inhibition of breast cancer cells, which will be discussed in more detail in section 5 (Qamri et al. 2009). WIN2 also possesses an inactive enantiomer (WIN3) that allows for the characterization of stereospecific action for differentiation between receptor/target mediated toxicities and non-specific toxicities (Howlett et al 2002; Savinainen et al. 2005). Finally previous reports showed that WIN2 inhibited the growth of various types of cancer cells preclinically, including breast, hepatic, lymphoma, melanoma and gastric cancer models (Giuliano et al. 2009; Qamri et al. 2009; Park et al. 2011; Scuderi et al. 2011; Wasik et al. 2011).

***statistical values reported in figure legends**

Abbreviations

CB₁-cannabinoid receptor 1; **CB₂**-cannabinoid receptor 2; **WIN2**-WIN55,212-2; **WIN3**-WIN55,212-3; **IR**-ionizing radiation; **ADR**-adriamycin; **THC**- Δ^9 -tetrahydrocannabinol; **CBD**-cannabidiol; **Gy**-gray (radiation dose unit); **TNBC**-triple negative breast cancer

Results

Section 3.1 - WIN55, 212-2 stereoselectively inhibits breast cancer growth.

Figures 3.2-3.5 present dose-response curves for WIN2 in three breast cancer cell lines (MCF-7, MDA-MB231 and 4T1), and one non-cancerous breast epithelial cell line (MCF-10A). WIN3, the inactive enantiomer of WIN2, was used to assess stereoselectivity. Doses tested include 3.75, 7.5, 15, 30 and 60 μM ¹. Cell density was quantified using the crystal violet assay at 96 h. The 96 h timepoint was chosen because MCF-7 cells show significant growth arrest and autophagic induction with both ADR and radiation treatment by this time point (Jones et al. 2005, Bristol et al. 2012, Goehe et al. 2012). WIN2 dose-dependently inhibited growth of all four cell lines tested when compared to vehicle treatment. ED_{50} values for WIN2 were 11.96 ± 1.65 μM in MCF-7 cells, 17.92 ± 3.38 μM in MDA-MB231 cells, and 18.24 ± 3.00 μM in 4T1 cells. In MCF-10A cells, WIN2 achieved a maximum growth inhibition of 36% at 30 μM , preventing calculation of the ED_{50} . Significant differences were also found between WIN2 and WIN3 in each cell line confirming that WIN2 mediated growth inhibition is stereospecific in nature.

Section 3.2 - WIN55, 212-2 fails to augment adriamycin induced growth inhibition

Based on studies from Goehe et al. (2012) and Salazar et al. (2009) discussed above, ADR (100 nM, 300 nM and 1000 nM) was evaluated in combination with WIN2 (6, 12 and 18 μM) in MCF-7 cells (**Fig 3.6**). Trypan blue exclusion assessed viable cell number at 96 h after treatment. Statistical analysis showed WIN2 did not significantly augment the antiproliferative actions of ADR, even though both individual treatments dose-dependently inhibited

¹ 3.75 μM was not used in MCF-10A cells

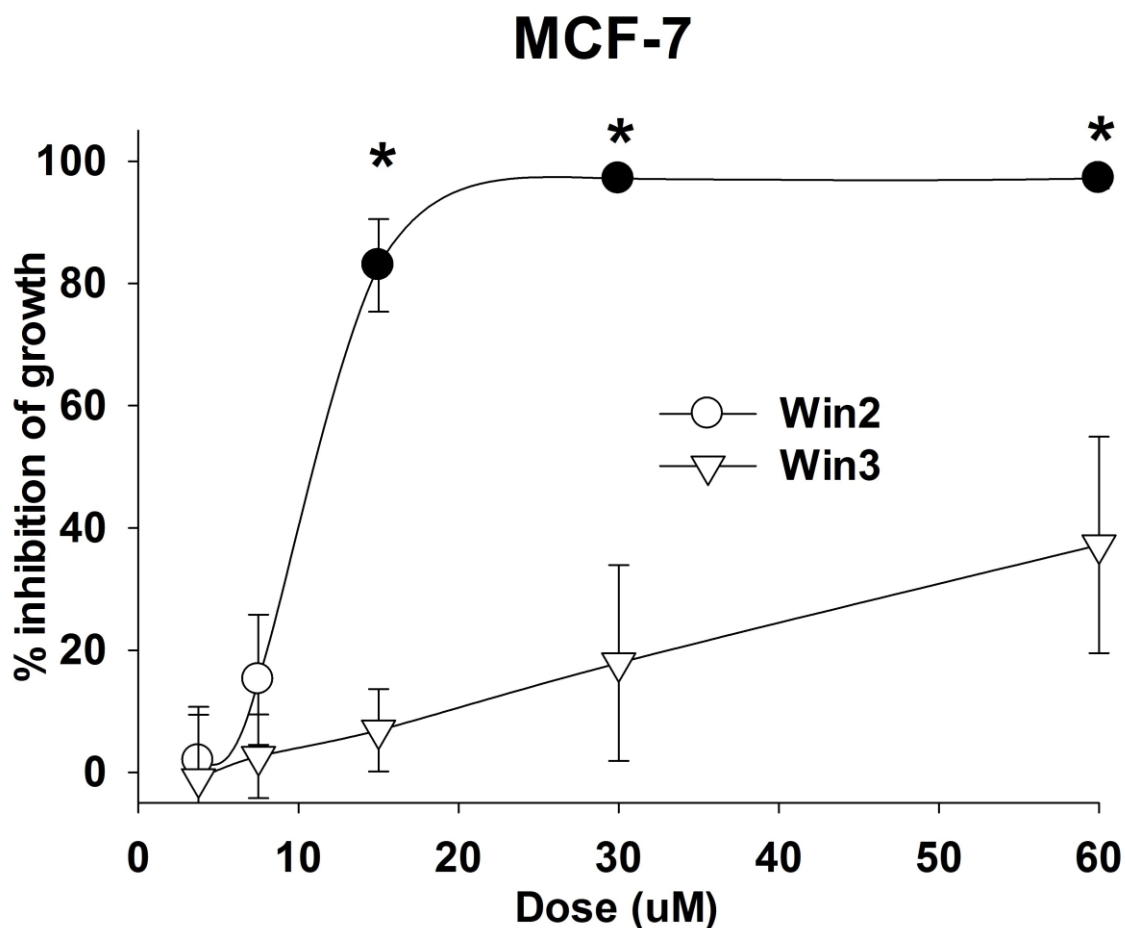


Figure 3.2 – WIN2 stereoselectively and dose-dependently inhibits the growth of MCF-7 breast cancer cells. Growth inhibition by WIN2 and WIN3 was assessed at 96 h post-treatment by the crystal violet assay in MCF-7 cells. Data presented reflect the means of 5 individual experiments \pm se; * $p < 0.05$ WIN2 vs. WIN3 within concentration; Blackened symbols $p < 0.05$ compared to vehicle.

Statistics

Two way repeated measures ANOVA : Drug-dose interacton ($F_{4,15}=7.5$, $p=0.0016$)
 Individual comparisons : Vehicle-WIN2 15-60 μ M ($p < 0.0025$). WIN2-WIN3 15-60 μ M ($p < 0.05$)

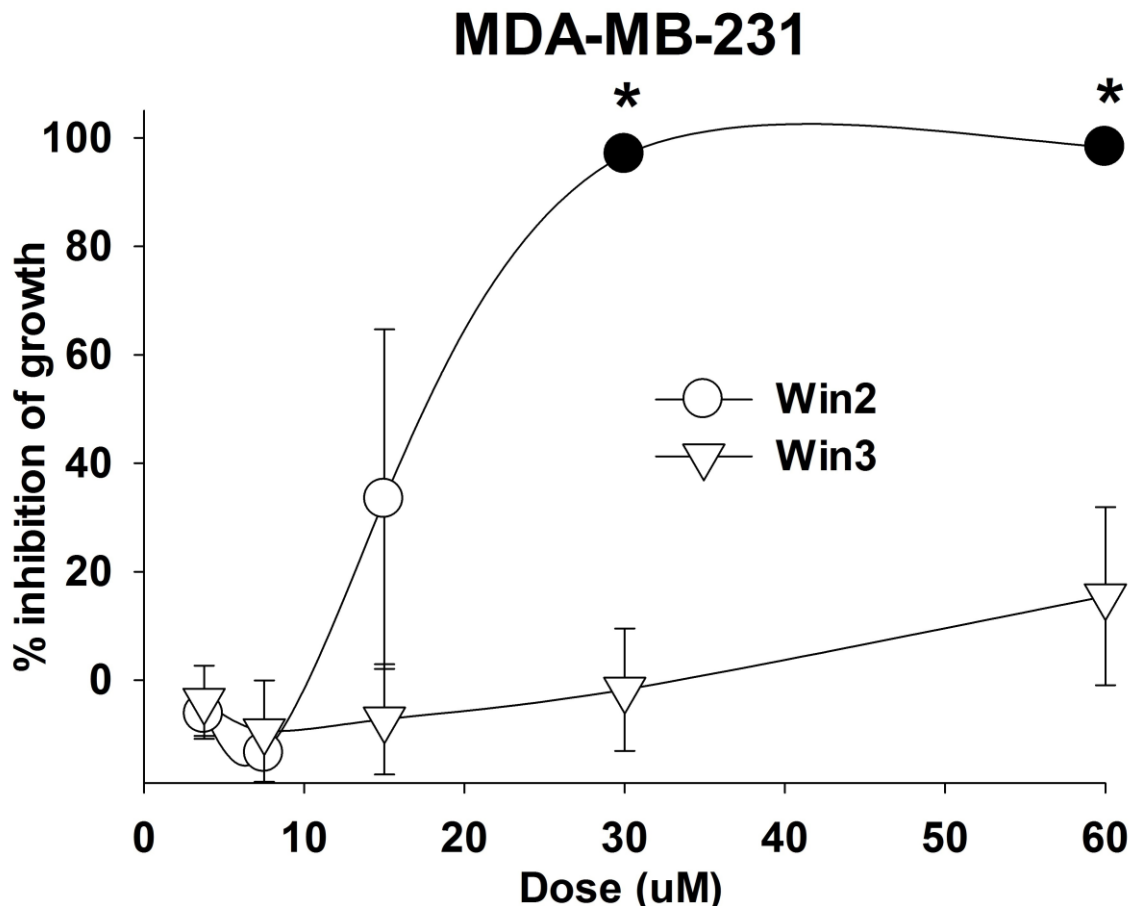


Figure 3.3 – WIN2 stereoselectively and dose-dependently inhibits the growth of MDA-MB231 breast cancer cells. Growth inhibition by WIN2 and WIN3 was assessed at 96 h post-treatment by the crystal violet assay in MDA-MB231 cells. Data presented reflect the means of 5 individual experiments \pm se; * $p < 0.05$ WIN2 vs. WIN3 within concentration; Blackened symbols $p < 0.05$ compared to vehicle.

Statistics

Two way repeated measures ANOVA : Drug-dose interacton ($F_{4,15}=7.8$, $p < 0.0013$)
 Individual comparisons : Vehicle-WIN2 30-60 μ M ($p < 0.0001$). WIN2-WIN3 30-60 μ M ($p < 0.02$)

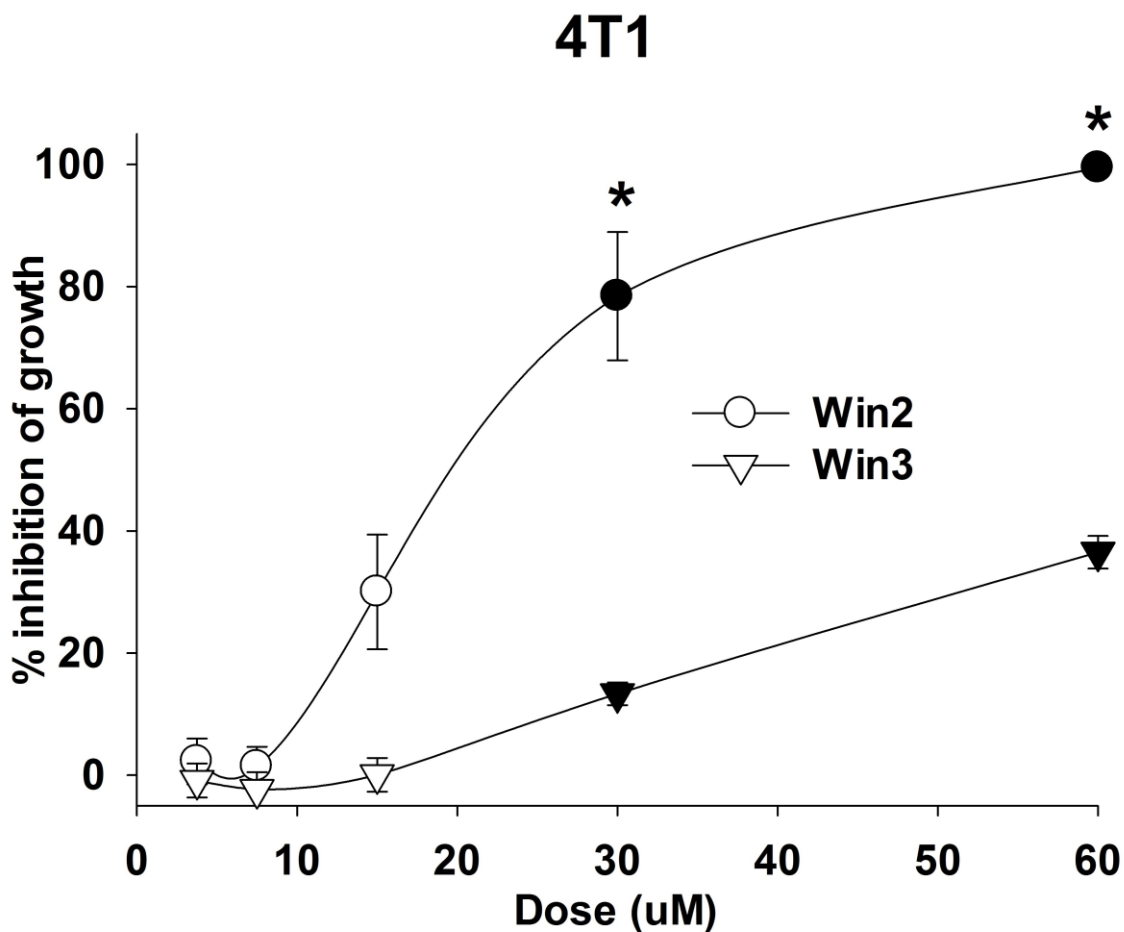


Figure 3.4 – WIN2 stereoselectively and dose-dependently inhibits the growth of 4T1 breast cancer cells. Growth inhibition by WIN2 and WIN3 was assessed at 96 h post-treatment by the crystal violet assay in 4T1 cells. Data presented reflect the means of 5 individual experiments \pm se; * $p < 0.05$ WIN2 vs. WIN3 within concentration; Blackened symbols $p < 0.05$ compared to vehicle.

Statistics

Two way repeated measures ANOVA : Drug-dose interacton ($F_{4,15}=12.8$, $p < 0.0001$)
 Individual comparisons : Vehicle-WIN2 30-60 μ M ($p < 0.0025$). Vehicle-WIN3 30-60 μ M ($p < 0.02$). WIN2-WIN3 30-60 μ M ($p < 0.02$)

MCF-10a

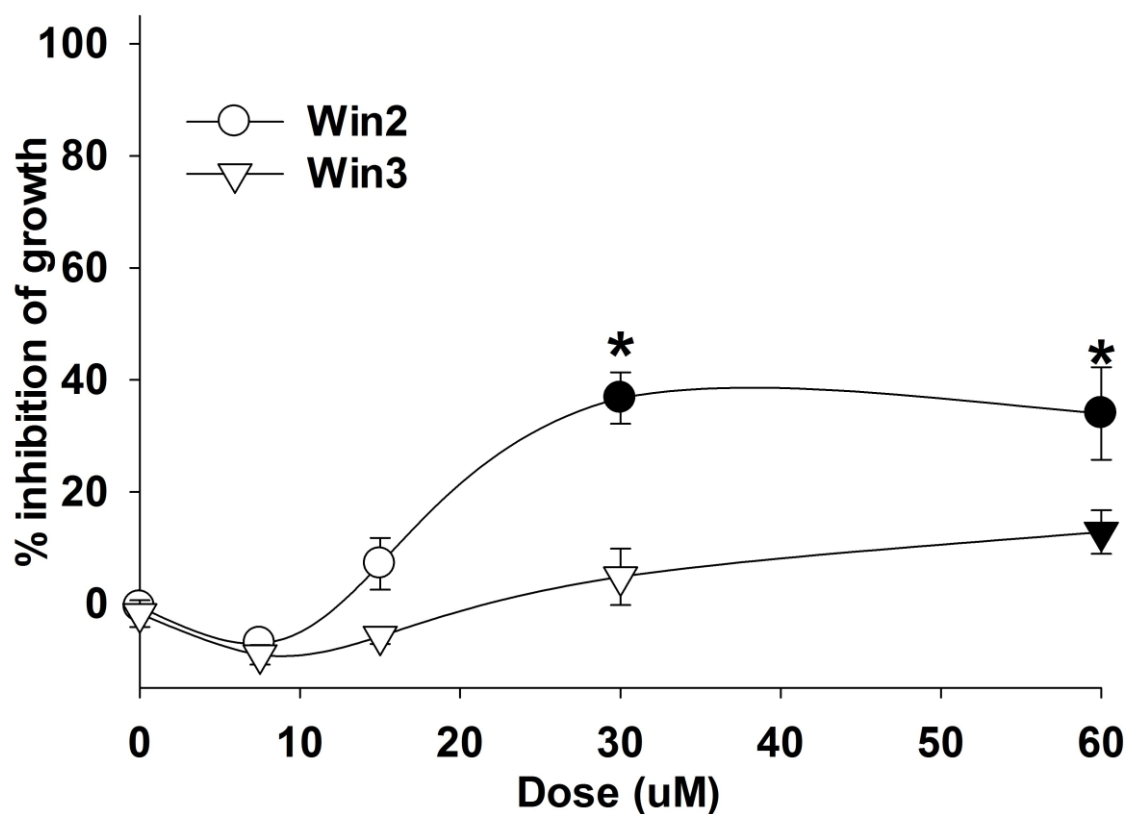


Figure 3.5 – WIN2 stereoselectively and dose-dependently inhibits the growth of non-transformed MCF-10A breast epithelial cells. Growth inhibition by WIN2 and WIN3 was assessed at 96 h post-treatment by the crystal violet assay in MCF-10A cells. Data presented reflect the means of 4 individual experiments \pm se; * $p < 0.05$ WIN2 vs. WIN3 within concentration; Blackened symbols $p < 0.05$ compared to vehicle.

Statistics

Two way repeated measures ANOVA : Drug-dose interacton ($F_{4,15}=9.8$, $p=0.0004$)

Individual comparisons : Vehicle-WIN2 30-60 μ M ($p < 0.025$). Vehicle-WIN3 60 μ M ($p < 0.02$). WIN2-WIN3 30-60 μ M ($p < 0.05$)

MCF-7 cell growth alone. WIN2 treatment also failed to antagonize ADR effects demonstrating a complete lack of interaction between the treatments.

Section 3.3 - WIN55, 212-2 stereoselectively enhances antiproliferative effects of ionizing radiation in MCF-7 cells

In addition to a cannabinoid/ADR combination it was also hypothesized that cannabinoids would augment the antiproliferative effects of radiation (Salazar et al. 2009; Bristol et al. 2012). The combination of WIN2 (6, 12 and 18 μ M) and ionizing radiation (1, 2 and 4 Gy) was assessed in MCF-7 cells using trypan blue exclusion at 96 h to measure cell viability (**Fig 3.7A**). Data analysis indicated that 12 and 18 μ M WIN2 significantly augmented the antiproliferative effects of radiation at 2 and 4 Gy. No interactions (augmentation or antagonism) were found between the lower doses used of either treatment.

WIN3 was then used to assess stereoselectivity of the WIN2/IR interaction (**Fig 3.7B**). MCF-7 cells were treated with either vehicle, WIN2 (12 μ M) or WIN3 (12 μ M). Drug treatments were given alone or in combination with 2 Gy radiation, which is a ~50% effective dose at 96 h based on results from Figure 3.7A. The combination of WIN2 and IR again elicited a significant augmentation, but WIN3 had no effect on growth either alone or in combination with IR. Together these studies show that WIN2 stereoselectively enhances the antiproliferative actions of radiation in MCF-7 cells.

Section 3.4 - MCF-10a cells require higher doses of WIN55,212-2 to augment the antiproliferative effects of radiation

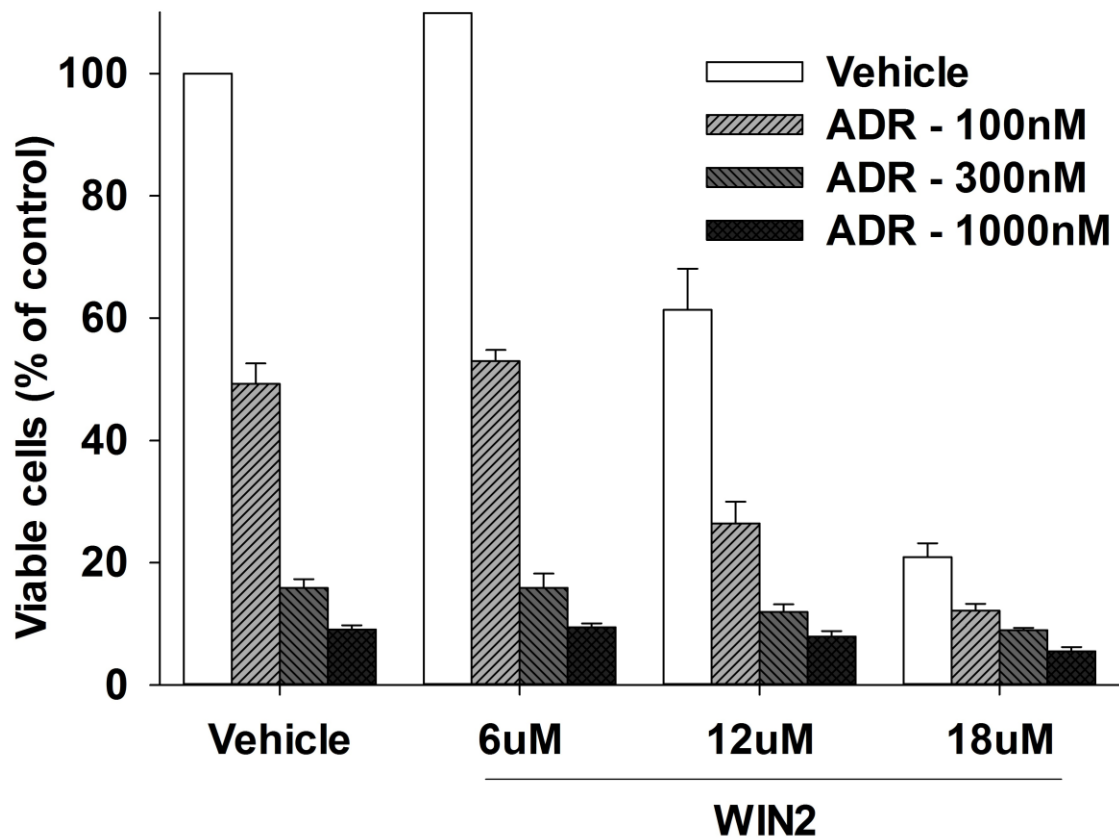


Figure 3.6 – WIN2 fails to augment ADR induced antiproliferative effects. MCF-7 cells were exposed to treatments of WIN2 (6, 12 and 18 μ M) and ADR (100, 300 and 1000nM). ADR treatments lasted 2 h. WIN2 treatments lasted 24 h. Cells were analyzed at 96 h using trypan blue exclusion. Data presented reflect the means of 3 individual experiments \pm se; no significant interactions were found.

Statistics

Two way repeated measures ANOVA: WIN2-ADR interaction ($F_{9,24}=36.6$, $p<0.0001$)

Individual comparisons for augmentation: no significant effects found (criteria described in methods)

Individual comparisons for ADR to vehicle: (100 nM $p=0.0064$; 300 nM $p=0.0004$; 1000 nM $p<0.0001$).

Individual comparisons for WIN2 to vehicle: (6 μ M $p=0.1359$; 12 μ M $p=0.0428$; 18 μ M $p=0.0012$)

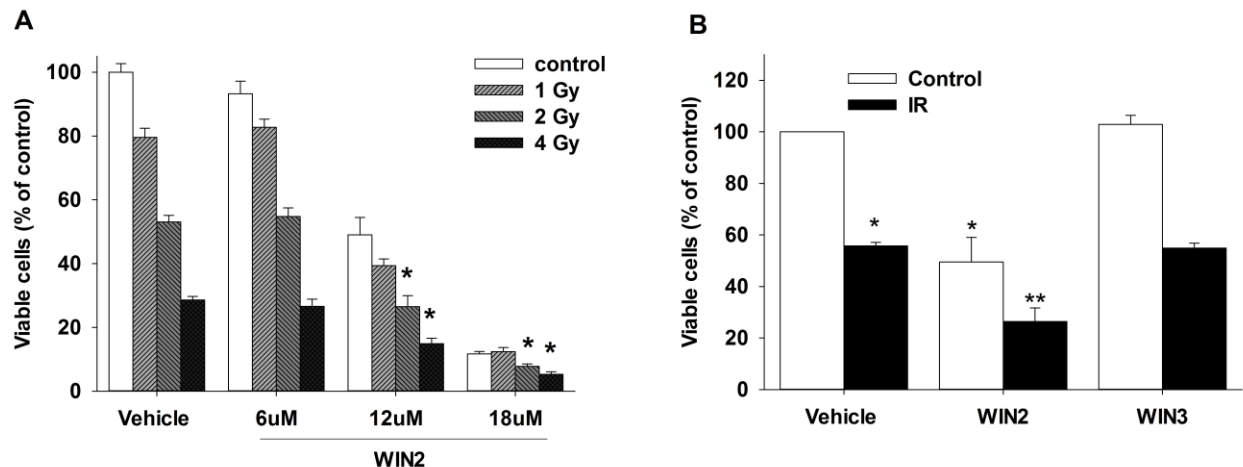


Figure 3.7 – WIN2 stereoselectively enhances the antiproliferative effect of ionizing radiation in MCF-7 cells. (A) Combination of WIN2 and radiation was evaluated. MCF-7 cells were exposed to treatments of WIN2 (6, 12 and 18 μ M) and IR (1, 2 or 4 Gy). **(B)** Stereoselectivity of the WIN2 + IR interaction was tested using WIN3. Treatments included WIN2 (12 μ M), WIN3 (12 μ M) or radiation (2 Gy). Drug treatments lasted 24 h. Cells were analyzed at 96 h using trypan blue exclusion. Data presented reflect the means of 3-4 individual experiments \pm se; **(A)** *= p <0.025 compared to WIN2 alone and IR alone; **(B)** *= p <0.05 vs vehicle and **= p <0.025 vs WIN2 alone and IR alone.

Statistics

(A) Two way repeated measures ANOVA : WIN2-IR interaction ($F_{12,32}=31.1$, p <0.0001)

(A) Individual comparisons for augmentation: 12 μ M + 2 Gy (vs WIN2 p =0.0091; vs IR p =0.0070), 12 μ M + 4 Gy (vs WIN2 p =0.0020; vs IR p =0.0175), 18 μ M + 2 Gy (vs WIN2 p =0.0083; vs IR p =0.0021) and 18 μ M + 4 Gy (vs WIN2 p =0.0008; vs IR p =0.0020).

(B) Two way repeated measures ANOVA: ($F_{2,12}=12.8$, p =0.0011)

(B) Individual comparisons with WIN2: Vehicle-WIN2 (p =0.0011). Vehicle-IR (p <0.0001). WIN2-WIN2 + IR (p =0.0024). IR-WIN2 + IR (p =0.0013).

(B) Individual comparisons with WIN3: Vehicle-WIN3 and WIN3-WIN3 + IR comparisons showed no significant differences

The effect of the WIN2/IR combination was also evaluated in MCF-10A cells, an *in vitro* model of normal breast epithelial tissue, to assess the selectivity of the WIN2/IR combination for cancer cells. MCF-10A cells were treated as in Figure 3.7B before cell viability was assessed at 96 h using trypan blue exclusion (**Fig 3.8A**). Radiation significantly inhibited MCF-10A growth but WIN2 showed no capacity to do the same, nor did WIN2 show any enhancement of the antiproliferative actions of radiation.

Higher doses of WIN2 were then tested based on the dose-response curve in Figure 3.6A, which showed growth inhibitory effects at 30 μ M WIN2. Using this higher dose of WIN2, MCF-10A cells showed significant growth inhibition for WIN2, IR and WIN2 + IR (**Fig 3.8B**). Comparisons also indicated that WIN2 significantly augmented the antiproliferative effects of radiation. This indicates that WIN2 can augment the actions of radiation in non-cancerous cells but it appears to require higher doses than those used in cancerous cells. Lastly, WIN3 showed no ability to inhibit MCF-10A growth or augment growth inhibition when combined with radiation confirming that WIN2 is acting in a stereospecific manner in MCF-10A cells as it did in MCF-7 cells.

Section 3.5 – WIN55, 212-2 augments the antiproliferative effects of radiation in other breast cancer cell lines

The combination of WIN2 and IR was evaluated in MDA-MB-231 (**Fig 3.9A**) and 4T1 cells (**Fig 3.9B**). MDA-MB-231 cells were treated with vehicle, 15 μ M WIN2, 2 Gy ionizing radiation and WIN2 + IR before being analyzed at 96 h. 4T1 cells however were analyzed at 48 h due to a faster doubling rate. Treatments for 4T1 cells included 8 Gy radiation and 30 μ M WIN2. Equivalent doses of WIN3 were used to assess stereoselectivity. Both MDA-MB231 and 4T1 cells show significant growth inhibition from treatment with WIN2 alone and IR alone. WIN2

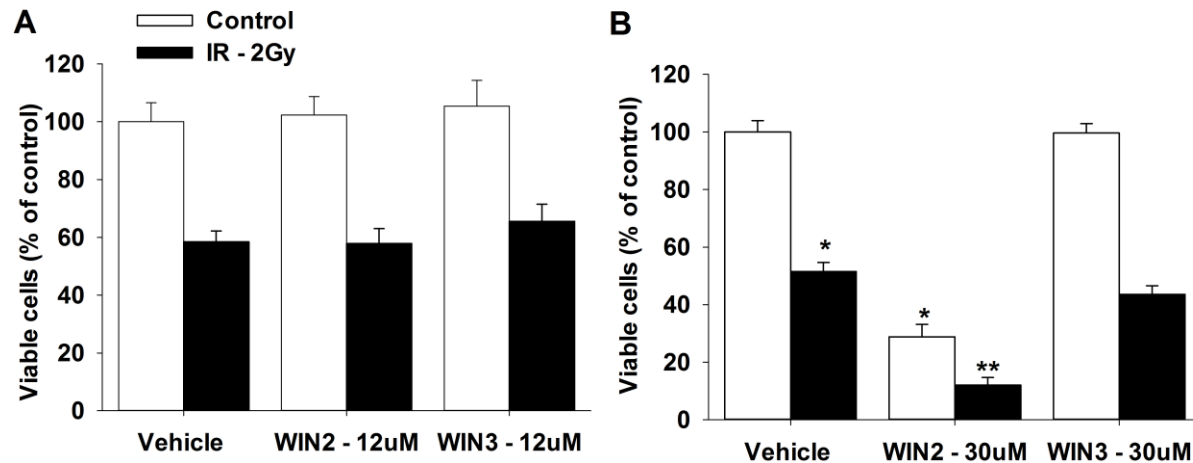


Figure 3.8 – Increased doses of WIN2 required to augment radiation in normal breast epithelial cells. MCF-10a cells were exposed to vehicle, WIN2 or WIN3 either alone or with 2 Gy radiation. WIN2 and WIN3 treatments were **(A)** 12 μ M **(B)** 30 μ M. All experiments were analyzed for cell viability by trypan blue exclusion 96 h after drug treatment. Data presented reflect the means of 3 individual experiments \pm se; *= p <0.05 vs vehicle and **= p <0.025 vs WIN2 alone and IR alone.

Statistics

- (A)** No significant interactions detected
- (B)** Two way repeated measures ANOVA: WIN2-IR interaction ($F_{2,8}=37.6$, p <0.0001)
- (B)** Individual comparisons with WIN2: Vehicle-WIN2 (p =0.0041). Vehicle-IR (p =0.0077). WIN2-WIN2 + IR (p =0.0090). IR-WIN2 + IR (p =0.0058).
- (B)** Individual comparisons with WIN3: Vehicle-WIN3 and WIN3-WIN3 + IR comparisons showed no significant differences

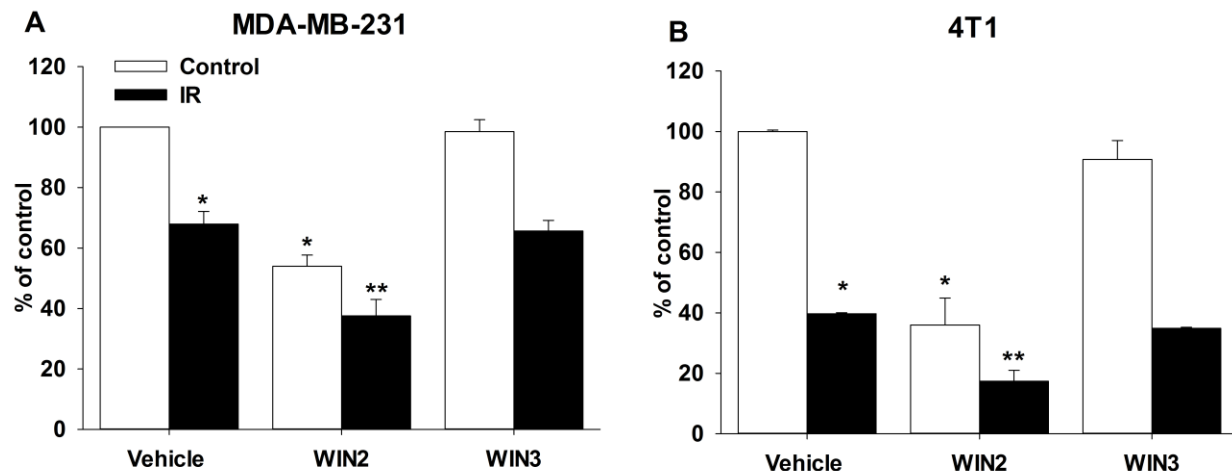


Figure 3.9 – Enhanced antiproliferation from WIN2 and IR occurs in other breast cancer cell lines. Cells were exposed to vehicle, WIN2 or WIN3 either alone or with radiation **(A)** MDA-MB231 (2 Gy) **(B)** 4T1 (8 Gy). Cells analyzed for cell viability by trypan blue exclusion: **(A)** 96 h and **(B)** 48 h (due to prohibitive growth characteristics). Data presented reflect the means of 3-4 individual experiments \pm se; *= $p < 0.05$ vs vehicle and **= $p < 0.025$ vs WIN2 alone and IR alone.

Statistics

(A) Two way repeated measures ANOVA: WIN2-IR interaction ($F_{2,16}=4.0$, $p=0.0370$)

(A) Individual comparisons with WIN2: Vehicle-WIN2 ($p < 0.0001$). Vehicle-IR ($p < 0.0001$). WIN2-WIN2 + IR ($p=0.0123$). IR-WIN2 + IR ($p=0.0237$).

(A) Individual comparisons with WIN3: Vehicle-WIN3 and WIN3-WIN3 + IR comparisons showed no significant differences

(B) Two way repeated measures ANOVA: WIN2-IR interaction ($F_{2,8}=14.6$, $p=0.0021$)

(B) Individual comparisons with WIN2: Vehicle-WIN2 ($p=0.0267$). Vehicle-IR ($p=0.0002$). WIN2-WIN2 + IR ($p=0.0090$). IR-WIN2 + IR ($p=0.0058$).

(B) Individual comparisons with WIN3: Vehicle-WIN3 and WIN3-WIN3 + IR comparisons showed no significant differences

significantly augmented the antiproliferative effects of radiation in both cell lines. WIN3 failed to inhibit cell growth or augment the effects of radiation in either cell line, which confirms that the stereospecific augmentation of radiation-induced growth inhibition generalizes to other breast cancer cell lines.

Section 3.6 - Augmentation of radiation by cannabinoids appears to be limited to the aminoalkylindoles

In addition to WIN2, other structurally diverse cannabinoids were given in combination with radiation in MCF-7 cells to screen for augmented antiproliferative effects. The following drugs were tested because of their clinical relevance, their differences in structure and/or their variable activity within the endocannabinoid system: THC (30, 50 and 70 μM), nabilone (10, 30 and 50 μM), CBD (10, 25 and 50 μM), CP55,940 (10, 20 or 30 μM), MAEA (10, 20 or 30 μM), pravadoline (15, 30 and 45 μM) and JWH-015 (15, 30 and 45 μM). Trypan blue exclusion was used to assess the number of viable cells at 96 h after treatment (**Table 3.1**). Statistical analyses of antiproliferative actions showed no significant interaction when radiation was combined with THC, nabilone, CBD, CP55,940, or MAEA. Pravadoline and JWH-015, however, were both able to significantly augment the growth inhibitory effects of radiation at the highest doses tested for each drug (45 μM).

Section 3.7 - Evaluations of WIN55, 212-2 and radiation interaction in a syngeneic tumor growth model

The syngeneic model of 4T1 cells in Balb/c mice was used to test the capacity of WIN2 to augment the established antiproliferative effects of radiation on tumor growth *in vivo*. Tumor

Drug	Control		Low dose		Medium dose		High dose	
	Vehicle	IR – 2Gy	Vehicle	IR – 2Gy	Vehicle	IR – 2Gy	Vehicle	IR – 2Gy
THC	100±0.01	57±3.93	94±2.93	55±3.92	47±4.41	33±6.02	26±7.80	21±5.77
CBD	100±0.01	60±8.04	85±7.72	59±11.26	59±4.47	45±4.77	24±7.28	16±3.95
Nabilone	100±0.01	56±4.61	88±5.41	56±5.75	66±7.56	50±5.33	32±14.88	22±8.08
CP55,940	100±0.01	70±8.12	100±1.41	77±10.58	81±4.41	59±7.13	38±7.18	37±14.72
MAEA	100±0.01	61±8.89	92±0.93	58±8.33	66±7.96	47±7.25	47±9.38	32±7.98
Pravadoline	100±0.01	53±5.67	94±1.52	43±4.95	60±5.38	37±5.64	40±5.06	25±3.85 *
JWH-015	100±0.01	53±5.67	79±6.39	45±4.04	42±7.04	31±2.61	24±2.00	17±1.25 *

Table 3.1 – Interaction of cannabinoids with radiation in MCF-7 cells. MCF-7 cells were treated with the indicated cannabinoids either alone or in combination with 2Gy radiation and cell viability was determined based on trypan blue exclusion at 96h. Drugs concentrations (μ M) were as follows: THC-30, 50,70; CBD-10,25,50; Nabilone-10,30,50; CP55,940-10,20,30; Methanandamide (MAEA)-10,20,30; Pravadoline-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; * = $p < 0.025$ vs WIN2 alone and IR alone.

Statistics

Two way repeated measures ANOVAs and individual comparisons reported no significant interactions present for THC, CBD, nabilone, CP55,940 or MAEA

Two way repeated measures ANOVA: Pravadoline-IR interaction ($F_{3,8}=29.2$, $p=0.0001$). JWH-015-IR interaction ($F_{3,8}=38.4$, $p<0.0001$)

Individual comparisons revealed no significant augmentation with the 15 and 30 μ M treatments of pravadolone and JWH-015 in combination with IR.

Individual comparisons (45 μ M pravadolone): Pravadolone-Pravadolone + IR ($p=0.0047$). IR-Pravadolone + IR ($p=0.0028$)

Individual comparisons (45 μ M JWH-015): JWH-JWH + IR ($p=0.0103$). IR-JWH + IR ($p=0.0101$).

volume was monitored using calipers, and body weight was tracked for each mouse to screen for general toxicity. 48 h after tumor implantation drug treatments were initiated including vehicle (1:1:18 of ethanol:emulphor:saline), WIN2 (1, 5 and 10 mg/kg) and ADR (5 mg/kg) as a positive control for growth inhibition (**Fig 3.10A**). This study was conducted with the assistance of Dr. Qing Tao. By day 16, comparisons showed that WIN2 dose-dependently suppressed tumor growth, but significant growth inhibition was detected as early at day 11 when compared to vehicle. ADR showed the greatest suppression of tumor growth. Body weight measurements are presented in Figure 3.10B. Significant changes in bodyweight were detected as early as day 7 but by day 16 WIN2 (10 mg/kg) and ADR were the only treatments to significantly increase and decrease bodyweight, respectively. On day 16 the maximum increase in body weight by WIN2 was 8%, and ADR had a maximum suppression of body weight at 16%.

The WIN2/IR combination was further evaluated in the 4T1-Balb/c syngeneic model by treating subjects with vehicle, WIN2 (5 mg/kg), radiation (10 Gy) or WIN2 plus radiation. WIN3 (5 mg/kg) was also administered to evaluate stereoselectivity (**Fig 3.11A**). As described previously, body weight was tracked and tumor volume was quantified using caliper measurements. Statistical comparisons revealed no significant interactions between any treatments at any time points in the study. Radiation significantly suppressed tumor growth at day 15 and 17, whether given alone or in combination with WIN2/WIN3. WIN2 alone showed no ability to significantly suppress tumor growth at any time point, which was in stark contrast to both the multiple *in vitro* studies presented above and the *in vivo* WIN2 dose-response presented in Figure 3.10A. Also in contrast to the *in vitro* studies presented above, WIN3 significantly stimulated tumor growth at days 15 and 17. These results clearly indicate that WIN2, at the dose tested, does not augment the antiproliferative effects of radiation *in vivo*, but this finding is likely confounded by the fact that WIN2 did not inhibit tumor growth in this study (**Fig 3.11A**), as it did in the dose-response study presented above (**Fig 3.10A**). Body weight

was also analyzed but at no time point was any comparison found to be significantly different from vehicle (**Fig 3.11B**).

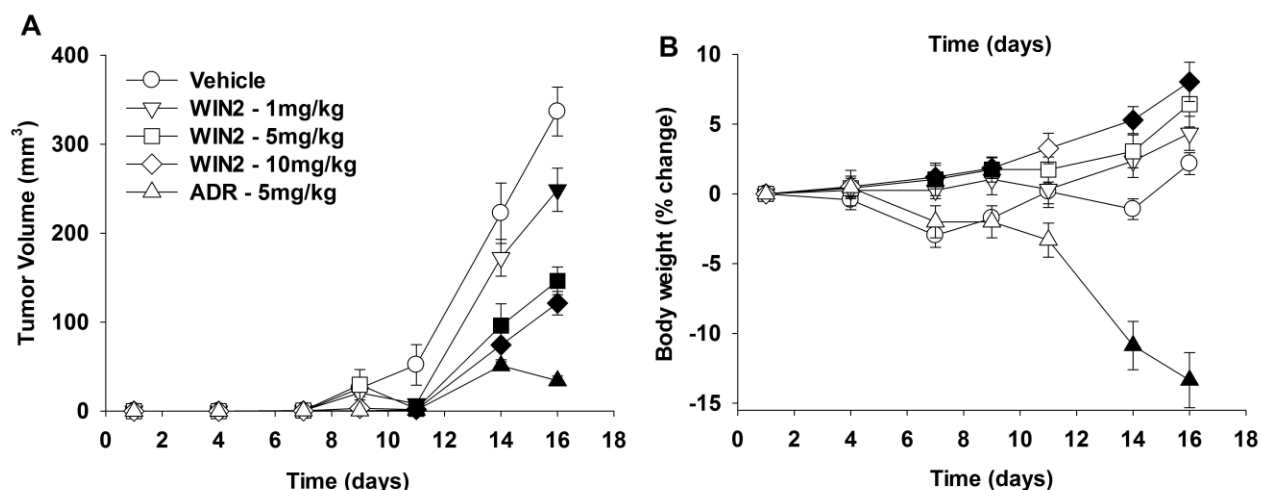


Figure 3.10 – Evaluation of WIN2 dose-dependent effects on *in vivo* tumor burden and weight change. Balb/c mice were injected with 50,000 4T1 cells and measured for **(A)** tumor volume and **(B)** body weight changes. Mice were treated with vehicle, WIN2 (1, 5 or 10 mg/kg) or ADR (5 mg/kg) and tracked for 16 days. Mean \pm se; n=8 per treatment; blackened symbols p<0.05 compared to vehicle within time points.

Statistics

(A) One way ANOVAs: day 11 (p=0.0217), day 14 (p=0.0004) and day 16 (p<0.0001)

(A) Dunnett's post hoc comparison to vehicle: 1 mg/kg (days 11 and 16 significant). 5 mg/kg (days 11, 14 and 16 significant). 10 mg/kg (days 11, 14 and 16 significant). ADR (days 14 and 16 significant).

(B) One way ANOVAs: day 7 (p=0.0235), 9 (p=0.0240), 11 (p=0.0324), 14 (p<0.0001) and 16 (p<0.0001).

(B) Dunnett's post hoc comparison to vehicle: 5 mg/kg (days 7 and 9). 10 mg/kg (days 7, 9, 14 and 16). ADR (days 14 and 16).

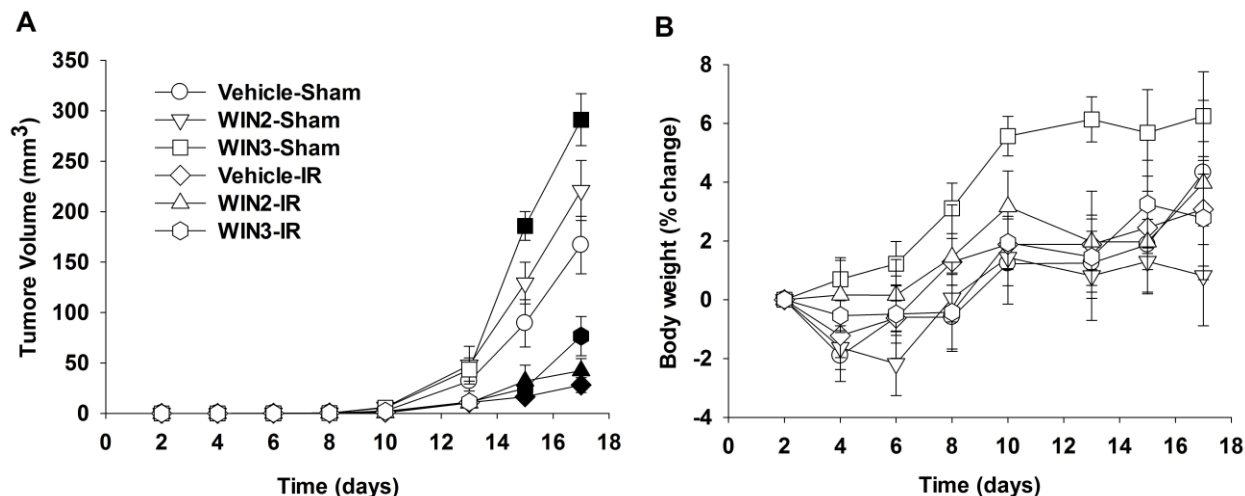


Figure 3.11 – Evaluation of WIN2 and WIN3 alone and with radiation on *in vivo* tumor burden and weight change. Balb/c mice were injected with 50,000 4T1 cells and measured for **(A)** tumor volume and **(B)** body weight changes. **(A and B)** Mice were treated with vehicle, WIN2 (5 mg/kg) and WIN3 (5 mg/kg) either alone or in combination with 2 Gy ionizing radiation, and tracked for 17 days. Mean \pm se; n=8 per treatment; blackened symbols p<0.05 compared to vehicle within time points.

Statistics

(A) Two way ANOVAs: Drug-IR interaction (none). Drug treatment main effect (day 15-p=0.0122; day 17-p=0.0028). IR treatment main effect (day 10-p=0.0215; day 13-p=0.0011; day 15-p<0.0001; day 17-p<0.0001)

(A) Individual comparisons: Vehicle-WIN2 (no significant effects). Vehicle-WIN3 (day 15-p=0.0054; day 17-p=0.0091). Vehicle-IR (day 15-p=0.0126; days 17-p=0.0006)>

(B) Two way ANOVAs reported not significant effects on any days.

Discussion

WIN2 showed no interaction (positive or negative) with ADR induced antiproliferative actions

Both ADR and radiation treatment induce autophagy and senescent growth arrest in MCF-7 cells (Jones et al. 2005, Bristol et al. 2012, Goehe et al. 2012), but WIN2 demonstrated no augmentation or antagonism with ADR in MCF-7 cells. Meanwhile, WIN2 significantly augmented the antiproliferative effects of radiation in MCF-7 cells. This difference, while unexpected, is not necessarily surprising. When autophagy is blocked in radiation treatment there is a shift from senescence to apoptosis (Bristol et al. 2012), but when autophagy is blocked in ADR treatment there is a delay in senescence initiation without a significant enhancement of antiproliferative effects of ADR (Goehe et al. 2012). Bristol et al. and Goehe et al.'s studies clearly indicate that senescence functions differently depending on how it is induced, and this difference means it cannot be assumed that WIN2 will interact with radiation and ADR in the same way.

WIN2 dose-dependently inhibits breast tumor growth and augments the antiproliferative actions of radiation stereoselectively

WIN2 dose-dependently inhibited the growth of MCF-7, MDA-MB231 and 4T1 cells, and WIN3, the inactive enantiomer of WIN2, confirmed that in all three breast cancer cell lines this dose-dependent growth inhibition was also stereospecific. This allows for the conclusion that WIN2 is eliciting its antiproliferative effects through a specific site of action, and supports the

need to elucidate a site of action for WIN2, which will be explored in later sections of this document.

WIN2 augmented the antiproliferative effects of radiation in MCF-7 cells at 12 and 18 μM . The lower dose of WIN2 tested (6 μM) did not significantly augment effects of radiation which suggests a significant level of growth inhibition is required from the WIN2 treatment to interact with radiation. This is also likely true for the reverse, because the lowest dose of IR tested (1 Gy) was not augmented by any of the higher doses of WIN2 treatments. As with the dose-dependent/stereospecific effects, augmentation of radiation-induced growth inhibition by WIN2 also generalized to MDA-MB231 and 4T1 cells, indicating that the mechanism of action for the WIN2/IR combination is not specific to one type of breast tumor cell, MCF-7s. Multiple doses of WIN2 and IR were not tested in the MDA-MB231 and 4T1 cells as they were in MCF-7 cells, which may suggest that augmentation could be observed at lower doses than was necessary in MCF-7 cells.

Finally, WIN3 was used to demonstrate that the augmentation of radiation by WIN2 acts in a stereospecific manner. Dose-response experiments with WIN2 and WIN3 provide clear evidence that showed WIN2 inhibits breast cancer cell growth in a stereospecific manner, but it cannot be concluded that this stereospecific action is relevant to the mechanism for the WIN2/IR combination. Additional studies showed that WIN3 did not produce inhibition of cell growth and was incapable of augmenting the antiproliferative effects of radiation in MCF-7, MDA-MB231 and 4T1 cells. As a result, it can be concluded from the combination of all these studies that WIN2 is acting at a specific target in three breast cancer cell lines to inhibit cell growth either alone or in an enhanced manner through its combination with radiation.

Relevance of WIN2 and radiation combination across MDA-MB231, 4T1 and MCF-10a cells

MDA-MB231 and 4T1 cells

The fact that WIN2 enhanced the antiproliferative effects of radiation in multiple breast cancer cell lines in addition to MCF-7 cells offers important insight into the generalization of the WIN2/IR combination. More importantly though, MDA-MB231 cells are a TNBC model, which is a disease known for short lived remission and poor five year survival relative to other forms of breast cancer. An advantage of TNBC treatment is that patients that show CDE (22%) after initial treatment show a better prognosis with regard to 5 year survival rates when compared to CDE patients without TNBC (11%) after initial treatment (Liedtke et al. 2008). This observation suggests that more efficacious therapies could increase 5 year survival rates by enhancing CDE rates after the initial intervention. As a result, WIN2 augmentation of radiation-induced growth inhibition in MDA-MB231 cells indicates a potential to increase patient survival after TNBC diagnosis.

Although 4T1 cells are murine derived and MCF-7 cells are human (Aslakson et al. 1992), both cell line show enhancement of radiation-induced growth inhibition by WIN2. Testing was primarily done in these cells to establish the model for future testing in whole animal syngeneic tumor growth. However, the ability of the WIN2/IR interaction to span not only species (human and mouse) but also spontaneous (MCF-7) versus carcinogen-induced (4T1) breast cancer should not be overlooked (Aslakson et al. 1992). One of the dangers of breast cancer is that it can evolve, in essence becoming more diverse than the original tumor, and this diversity can increase the likelihood of drug resistance, recurrence and death. The ability of WIN2 to effect such different tumor types, MCF-7 versus 4T1, suggests that it can also span the diversity that develops inside the patient, and potentially enhance patient outcomes.

In addition to the above mentioned factors, both MDA-MB231 and 4T1 cells have a p53 status different than MCF-7 cells. p53 mutation correlates with poor patient prognosis because of pro-cancer effects on growth, tumorigenicity, DNA repair, cell death, angiogenesis and metastasis (Lacroix et al 2006, Walerych et al 2012). Importantly, the sensitivity of mutant p53 MDA-MB231 and p53 null 4T1 cells to the WIN2 augmentation of radiation clearly shows that p53 status is not relevant (Lacroix et al 2006, Yerlikaya et al. 2012). The common nature of p53 mutations in 31% of all cancers and ~23% of breast cancers further highlight the importance of this observation.

MCF-10a

MCF-10a cells are an immortalized breast epithelial cell line that demonstrated WIN2 dose-dependently inhibited their growth and augmenting the antiproliferative actions of radiation, like was revealed in MCF-7 cells (Soule et al. 1990). Uniquely though, MCF-10A cells required higher doses of WIN2 to inhibit MCF-10A cell growth compared to MCF-7 cells, 30 versus 12 μ M, respectively. The need for higher doses in the MCF-10A cells extended to the augmentation of radiation as well, and this decreased sensitivity of MCF-10A cells to the antiproliferative effects of WIN2 alone or in combination with radiation indicates a potential selectivity for tumor cells. Shrivastava et al. (2011) had previously demonstrated that MDA-MB231 cells were more sensitive to the cannabinoid CBD than MCF-10A cells supporting the findings presented in this document regarding cannabinoid selectivity for tumor cells over non-cancerous MCF-10A cells. This selectivity supports the hypothesis that WIN2 has a therapeutic window for treatment that would be beneficial to patient outcomes.

However, as exciting as these findings could be for the patient, the MCF-10A studies are not without their limitations. First, the MCF-10A cells in culture are a proliferating population and breast epithelial cells are largely non-proliferative. Later studies will show that WIN2 elicits a

growth inhibitory mechanism in MCF-7 cells versus cell death, and a growth inhibitory mechanism is likely to have a diminished if not non-existent antiproliferative effect in a non-proliferating population. This may suggest further analysis of toxicities would show an even greater therapeutic window for WIN2 treatments. Second, breast epithelial cells are not the only cell types found within the path of ionizing radiation in the breast. Fibroblasts are a common cell type that can be analyzed, either as mouse embryonic fibroblasts or an immortalized fibroblast cell line, to assess WIN2 augmentation of IR toxicities. Finally, unlike radiation, WIN2 is a drug that will likely spread systemically via the circulation and it may have an effect on tissues outside of the breast, especially those that are rapidly dividing like that gastrointestinal epithelial cells or bone marrow. Testing for toxicities in these cell types with WIN2 treatment might prove pertinent.

Aminoalkylindole cannabinoids are more efficacious at augmenting the antiproliferative effects of radiation than other cannabinoids tested

THC, CBD, nabilone, CP55,940 and MAEA all show lack of interaction with radiation in MCF-7 cells, while JWH-015 and pravadolone augmented the effects of radiation at the high doses tested similar to WIN2. The clinical relevance of THC (Marinol) and nabilone (Cesamet) make a lack of antagonism worth noting however. Patients are given palliative treatment with Marinol and Cesamet for chemotherapy (Russo 2008), and chemotherapy is often given in combination with radiation therapy (Kaviani et al. 2013; Joeger et al. 2013; Yang et al. 2013). The lack of antagonism by THC and nabilone in the studies presented above offer preclinical evidence that these palliative treatments will not interfere with radiation therapy should they be in the patient's system during treatment.

Comparing structural differences of the cannabinoids that successfully augmented the antiproliferative effects of radiation (WIN2, JWH-015, pravadoline) versus those that did not (THC, CBD, nabilone, CP55,940, MAEA) clearly reveals that the aminoalkylindoles had a greater capacity to augment effects of radiation based on the compounds tested in these studies. The structures of each drug are presented in Figure 3.1. While it remains unclear at this time what aspect of the aminoalkylindole structure allows for the capacity to augment radiation-induced growth inhibition, future studies investigating structure-activity relationships could elucidate this structural selectivity. Augmentation of radiation by JWH-015 also poses an additional benefit, in that JWH-015 is a CB₂ specific agonist (Showalter et al. 1996). This is advantageous as CB₂ agonists are absent of the cannabimimetic effects that have blunted the potential for clinical development of WIN2 and other drugs like it (Howlett et al. 2002; Pertwee et al. 2010). Development or screening of additional aminoalkylindole analogs could provide compounds with a greater efficacy for augmentation of radiation effects without the unwanted psychoactive properties.

The ability of WIN2 to augment radiation *in vivo* cannot be determined based on current studies

WIN2 and IR were given in combination to test the ability of WIN2 to augment radiation-induced growth inhibition *in vivo*. The *in vivo* model used for this was the 4T1-Balb/c syngeneic model, but the results of these studies are inconclusive regarding the effects of the WIN2/IR combination *in vivo*. This is due to the fact that the dose of WIN2 tested did not inhibit tumor growth and *in vitro* studies using MCF-7 cells showed that effective doses of WIN2 and/or IR were required for augmentation to occur. The *in vivo* combination studies could have been repeated using higher doses of WIN2, but this approach would conflict with observations from

the dose response study. 1, 5 and 10 mg/kg WIN2 dose-dependently inhibited tumor growth in the same 4T1-Balb/c syngeneic tumor growth model. Even if 5 mg/kg hypothetically showed an unusually high level of growth inhibition in the dose-response study, 1 mg/kg WIN2 was also found to significantly inhibit tumor growth, which suggests that even accounting for variability between experiments, 5 mg/kg WIN2 should have inhibited tumor growth. Additionally, repeating the experiment until 5 mg/kg WIN2 demonstrated tumor growth inhibition was not considered because of obvious ethical considerations. It is unclear why 5 mg/kg WIN2 inhibited tumor growth in the dose-response study but failed to do so in the combination study, and to date no variables have been identified that reconcile the difference between these studies.

Since the 4T1-Balb/c model has proven unreliable in my hands it seems logical that future *in vivo* studies be moved to another *in vivo* model until the factors causing the above described variability of the 4T1 model can be identified. Options for additional models include estrogen pelleted immune-deficient SCID mice bearing MCF-7 tumors, or immune-deficient SCID mice bearing MDA-MB231 cells. It does, however, remain pertinent to evaluate the *in vivo* actions of the WIN2/IR combination in an immune competent model, based on the argument described in the introduction of this section, but MCF-7 and MDA-MB231 cells are human derived and cannot be transplanted *in vivo* without immune complications. Therefore future studies would require the *in vitro* evaluation of the WIN2/IR combination in a new murine derived tumor model.

Summary

The studies in this chapter have effectively demonstrated the proof of principle for WIN2 to augment the antiproliferative actions of radiation *in vitro*, using multiple breast cancer cell lines. This augmentation was shown to be stereospecific in nature, did not translate to other

cancer therapeutic treatments tested, demonstrated potential selectivity between cancer and non-cancerous cells and might be unique to the aminoalkylindole class of cannabinoids. The *in vivo* studies using the 4T1-Balb/c model were the first attempt to demonstrate translatability of this WIN2/IR combination, but the results were uninterpretable and the model was proven unreliable. Future studies could use a variety of *in vivo* models, but regardless of the *in vivo* model chosen it is important that these studies move beyond the *in vitro* setting, because without demonstrating the WIN2/IR combination in a whole animal model, the combination of cannabinoids and radiation can never be considered a viable therapeutic strategy.

Section 4

Evaluation of antiproliferative mechanisms governing WIN2/IR interaction

The antiproliferative effects of radiation in the treatment of cancer are caused by DNA damage which is primarily driven by radiolysis of water leading to the formation of reactive oxygen species (ROS) (Narayanan et al. 1997). This ROS formation is responsible for DNA damage expressed primarily as single and double strand breaks in DNA (Driessens et al. 2009). Radiation-induced DNA damage causes MDA-MB231 breast tumor cells to undergo apoptotic cell death demonstrated by TUNEL staining, but MCF-7 cells undergo senescent growth arrest shown by pH 6.0 dependent β -galactosidase staining (Jones et al. 2005). Artificial expression of caspase-3 in the caspase-3 deficient MCF-7 cells can induce a modest apoptotic response to radiation treatment; a robust apoptotic response requires simultaneous administration of the ATM inhibitor, caffeine and re-expression of caspase-3 in MCF-7 cells (Essmann et al. 2004). Work from Essmann et al. and Jones et al. provide evidence that the antiproliferative mechanisms of radiation are contextually dependent on the system.

Cannabinoids also have multiple antiproliferative mechanisms depending on the cannabinoid agonist used and the cancer cell type investigated. These mechanisms include autophagy (Salazar et al. 2009; Shrivastava et al. 2011, Donadelli et al. 2011, Dando et al. 2013), cell death (Giullino et al. 2009; Qamri et al. 2009; Caffarel et al. 2010) and growth arrest (Galanti et al. 2008; Park et al. 2011). However, DNA damage and senescent growth arrest have not yet been associated with cannabinoid treatment in preclinical cancer models.

Δ^9 -tetrahydrocannabinol (THC) treatment-induced autophagy in U87-MG glioblastoma cells based on GFP-LC3 puncta formation and electron microscopic autophagosome imaging, and knockdown of the autophagy genes ATG1 and ATG5 resulted in increased viable cell number (Salazar et al. 2009). Other studies have linked cannabinoid-induced autophagy to a ROS mediated mechanism (Shrivastava et al. 2011, Donadelli et al. 2011, Dando et al. 2013).

In MDA-MB231 cells, cannabidiol (CBD) induced autophagy based on LC3 cleavage and electron microscopic autophagosome imaging. LC3 cleavage was antagonized by the autophagy inhibitor bafilomycin and the ROS scavenger α -tocopherol (Shrivastava et al. 2011). In Panc1 cells the ROS scavenger N-acetyl-cysteine was shown to inhibit autophagy induction under synthetic cannabinoid treatment, GW405833 and arachidonoyl cyclopropamide. Autophagy was quantified by LC3 cleavage, acridine orange staining and flow cytometric quantification of autophagolysosomal staining by MDC (Donadelli et al. 2011, Dando et al. 2013).

Shrivastava et al. (2011) and Salazar et al. (2009) showed that CBD and THC, respectively, induced autophagic effects that were linked to the induction of apoptosis, and this finding that cannabinoids induced apoptosis is supported by numerous studies. ErbB2 positive tumors produced by MMTV-neu transgenic animals treated with THC and the synthetic cannabinoid JWH-133 express higher levels of cleaved caspase-3 when compared to vehicle (Caffarel et al. 2010). WIN55, 212-2 (WIN2) increased apoptotic markers in HepG2 cells with concomitant increases in the sub-G1 population, annexin V/propidium iodide (PI) positive staining and cleaved caspase 3 expression (Giullino et al. 2009). Qamri et al. (2009) showed that WIN2 and JWH-133 induce apoptosis in MDA-MB231 cells based on an increased sub-G1 population, TUNEL staining and imaging of apoptotic nuclei.

In addition to cell death, another common antiproliferative mechanism is growth inhibition. Park et al. (2011) showed that WIN2 treatment induced growth arrest in gastric cancer cells through downregulation of E2F1 and several cyclins and cyclin dependent kinases. Park et al. furthermore linked WIN2 growth arrest to inhibition of the survival protein pAKT. Park et al.'s observations support an earlier study by Galanti et al. (2008) where THC caused growth arrest via down regulation of E2F1 and cyclin A in both U251-MG and U87-MG human glioblastoma cell lines. Galanti et al.'s findings with THC in U87-MG cells are contradictory to

those from Salazar et al. (2009) in which THC induced autophagic cell death in U87-MG cells, but this could be explained by Salazar's use of media containing low serum, which tends to be permissive for autophagy, and Galanti's opposite use of media containing normal serum concentrations.

A primary goal of these studies was to assess the antiproliferative mechanism(s) for radiation and WIN2 alone and in combination. MCF-7 cells treated with the WIN2/IR combination were tested for ROS mediated antiproliferative actions, cell death (including apoptosis, necrosis, mitotic catastrophe and autophagy), changes in the DNA damage response and growth arrest (both classical and senescent).

***statistical values reported in figure legends**

Abbreviations

ROS-reactive oxygen species; **THC**- Δ^9 -tetrahydrocannabinol; **WIN2**-WIN55,212-2; **CQ**-chloroquine; **PI**-propidium iodide; **ADR**-adriamycin; **NAC**- N-acetyl-cysteine; **GSH**-glutathione; **IR**-ionizing radiation; **Gy**-gray (radiation dose unit); **AO**-acridine orange;

Results

Section 4.1 - Autophagy is induced in MCF-7 cells but does not appear to be relevant to WIN2 growth inhibitory mechanisms

In a previous chapter, it was hypothesized that WIN2 augmentation of the antiproliferative actions of radiation would be mediated by an autophagic mechanism based on evidence from Salazar et al. 2009 and Bristol et al. 2012. To qualitatively establish the presence of autophagy, MCF-7 cells were treated with vehicle, 12 μ M WIN2, 2 Gy radiation or the combination of WIN2 + IR. At 96 h, the treated cells were stained with acridine orange (AO) and imaged. The presence of increased numbers of orange vesicles compared to vehicle treatment confirmed the promotion of autophagy in cells exposed to WIN2, IR and WIN2 + IR (**Fig 4.1A**).

To evaluate the potential involvement of autophagy in the antiproliferative effects of the WIN2/IR combination, cells were treated with the autophagic inhibitor chloroquine (CQ) at 5 μ M in combination with the WIN2/IR combination before quantification of cell viability using trypan blue exclusion. At 96 h, CQ had no effect on viable cell number in cells treated with the vehicle, WIN2, IR or WIN2 + IR (**Fig 4.1B**). Acridine orange staining was used to qualitatively confirm that the 5 μ M CQ treatment was properly inhibiting autophagy. Markers for blockade of autophagy in AO staining include increased vesicle number (blocked degradation) and a yellow color as opposed to orange (incomplete acidification). MCF-7 cells were treated with vehicle, adriamycin (ADR; 1 μ M) or ADR + CQ before being stained with AO and imaged (**Fig 4.1C**). ADR induced autophagy compared to vehicle, shown by an increase in orange vesicles. CQ blocked autophagy compared to ADR, shown by an increase in yellow vesicle number. Similar results with ADR and CQ were previously demonstrated in Goehe et al. 2012. These combined

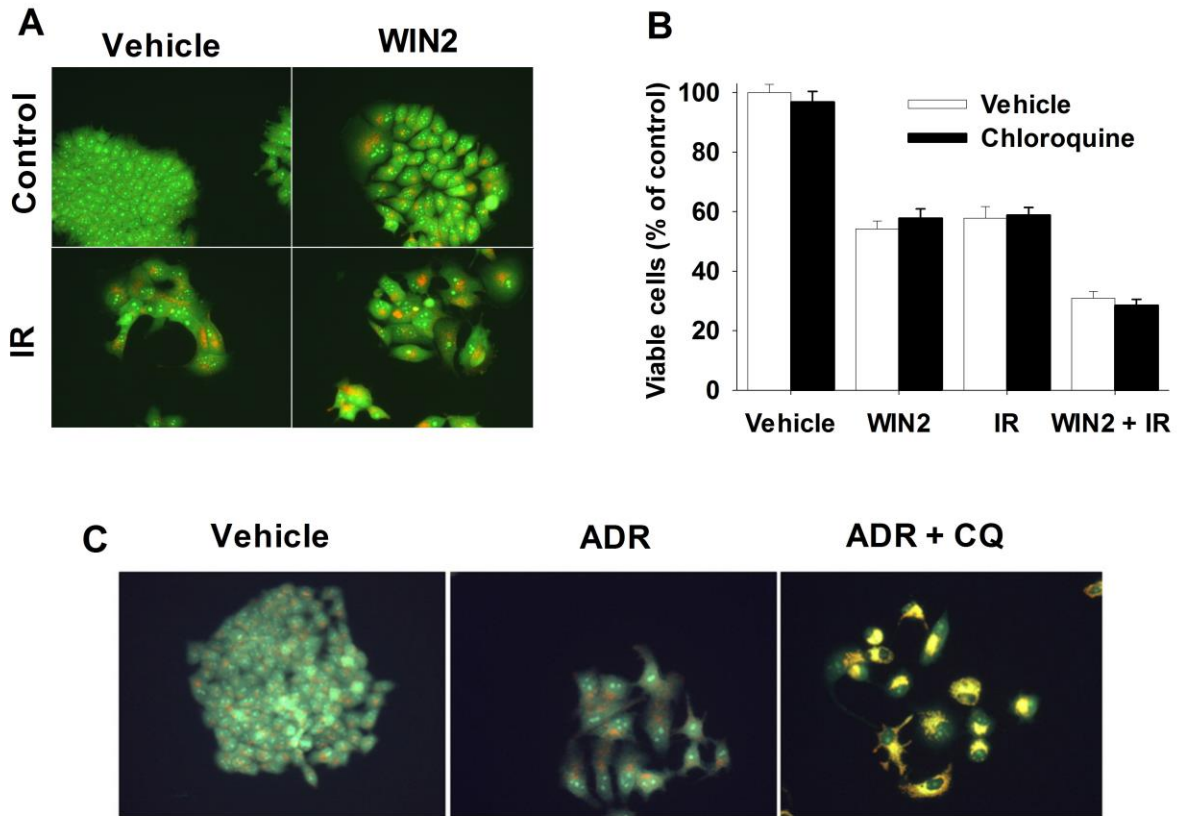


Figure 4.1 – Autophagy is induced by radiation and WIN2 but not involved in growth inhibition. Acridine orange staining was used to image autophagic vesicles in MCF-7 cells treated with vehicle, 12 μ M WIN2, 2 Gy IR or WIN2 + IR (**A**). Cell viability was quantified using trypan blue exclusion in MCF-7 cells treated as in (A) with a co-treatment of either vehicle or 5 μ M chloroquine (**B**). Acridine orange staining was used to image autophagic vesicles in MCF-7 cells treated with vehicle, 1 μ M ADR or ADR + 5 μ M chloroquine (**C**). In (**B**) data were normalized to % of control and presented as the means of 3 individual experiments \pm se; no significant differences detected.

Statistics

(**B**) Two way repeated measures ANOVA: Chloroquine-combo interaction ($p=0.8842$)

results indicate that while autophagy is clearly induced, autophagy does not appear to be relevant to the antiproliferative actions of WIN2, IR or WIN2 + IR.

Section 4.2 - ROS do not mediate antiproliferative effects of the WIN2/IR combination

ROS have been shown to mediate cannabinoid based growth inhibition (Shrivastava et al. 2011, Donadelli et al. 2011, Dando et al. 2013, Driessens et al. 2009). To assess the involvement of ROS in the antiproliferative actions of the WIN2/IR combination, MCF-7 cells were treated with the antioxidants N-acetyl-cysteine (NAC; 1 mg/ml) and glutathione (GSH; 0.5 mg/ml). NAC and GSH treatment lasted 48 h beginning 24 h before the WIN2/IR treatment (**Fig 4.2A-B**). 96 h after WIN2/IR were administered to MCF-7 cells neither NAC nor GSH demonstrated any ability to decrease viable cell number in MCF-7 cells. H₂O₂ (9.79 µM) was used as a positive control for ROS induced growth inhibition, and both NAC and GSH significantly protected MCF-7 cells from H₂O₂ insult at 96 h. These data demonstrate that ROS signaling does not mediate the antiproliferative actions of the WIN2/IR combination.

Section 4.3 - The WIN2/IR combination does not induce cell death in MCF-7 breast tumor cells

As indicated in the introduction, previous studies have documented the capacity of cannabinoids to induce apoptosis (Shrivastava et al. 2011, Salazar et al. 2009, Caffarel et al. 2010, Giullino et al. 2009, Qamri et al. 2009). In order to confirm this in our system, annexin V/PI staining was used to assess apoptosis and necrosis, respectively, at 48 h post treatment with the WIN2/IR combination (**Fig 4.3A**). Flow cytometric quantification showed no change in the percentage of healthy, apoptotic or necrotic cells treated with WIN2, IR or WIN2 + IR

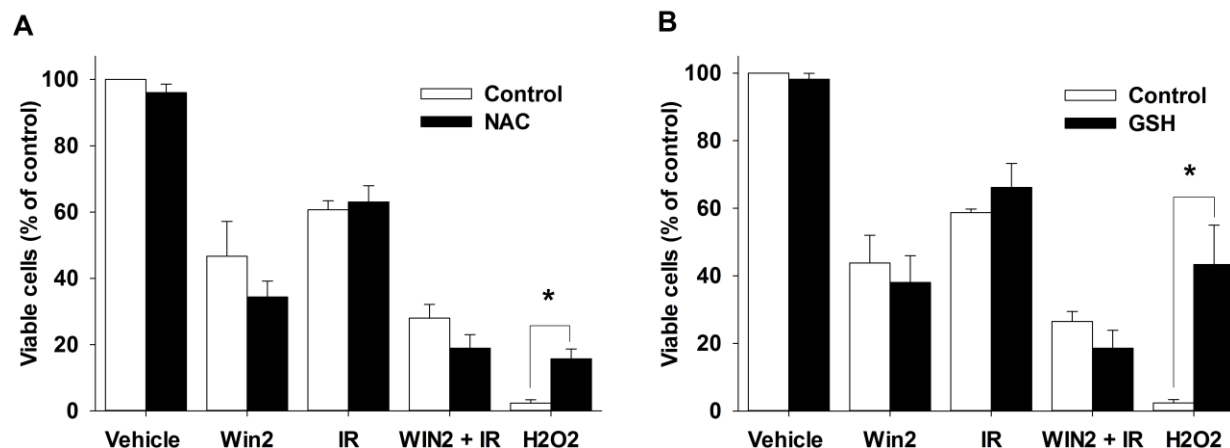


Figure 4.2 – ROS do not mediate the antiproliferative effects of the WIN2/IR combination. MCF-7 cells were treated with vehicle, WIN2 (12 μ M), IR (2 Gy), WIN2 + IR or hydrogen peroxide (9.79 μ M). Co-treatments were given of either vehicle, **(A)** NAC (1 mg/ml) or **(B)** GSH (0.5 mg/ml). Cell viability was quantified at 96 hrs using trypan blue exclusion. Data were normalized to % of control and presented as the means of 3 individual experiments \pm se; *= p <0.05.

Statistics

- (A)** Two way repeated measures ANOVA: NAC-combo (p =0.1638)
- (A)** Positive control vs. NAC paired t-test (p =0.0043)
- (B)** Two way repeated measures ANOVA: GSH-combo (p =0.1507)
- (B)** Positive control vs. GSH paired t-test (p =0.0079)

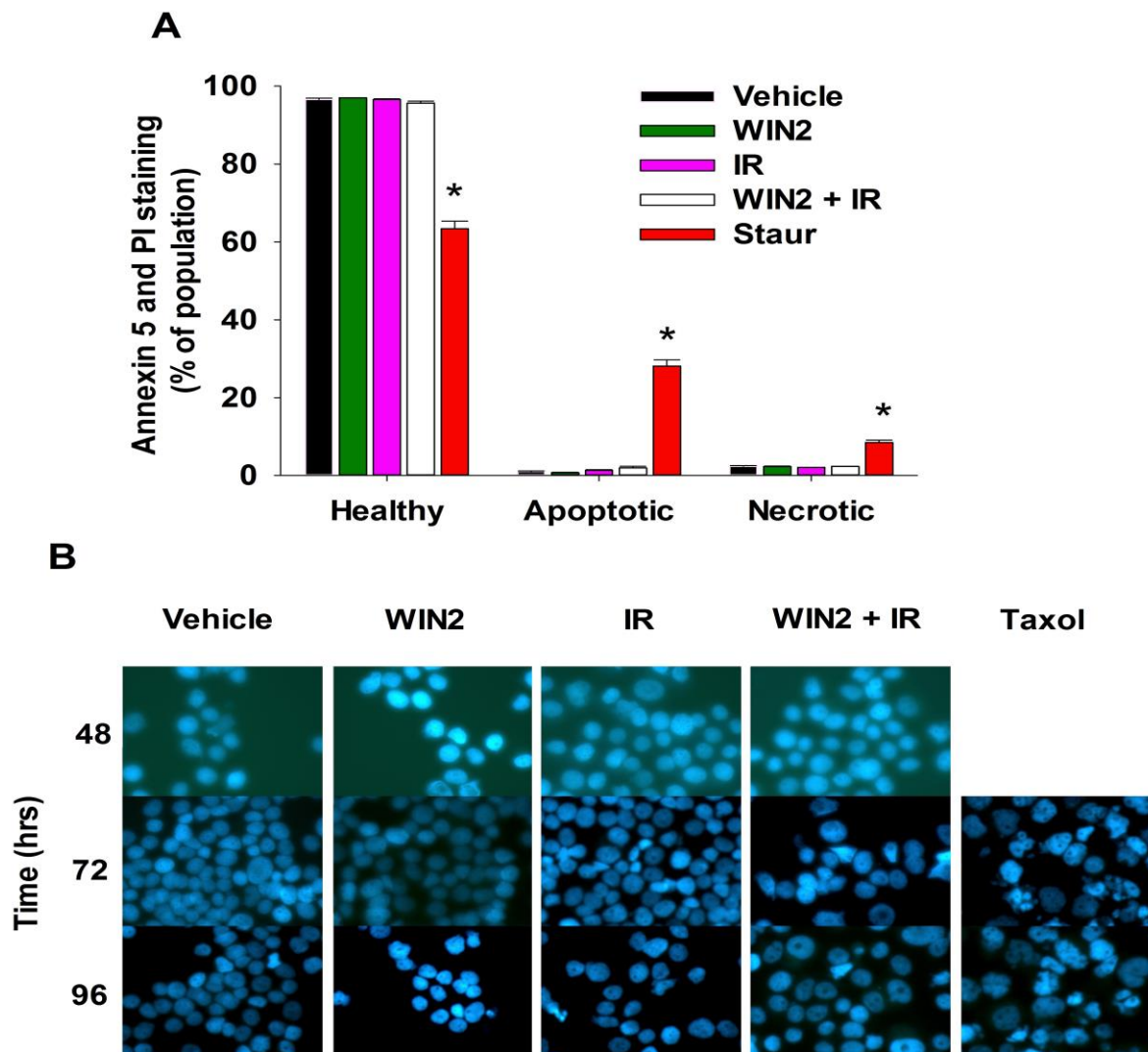


Figure 4.3 – Apoptosis, necrosis and mitotic catastrophe are not involved in the antiproliferative actions of the WIN2/IR combination. MCF-7 cells were treated with vehicle, WIN2 (12 μ M), IR (2 Gy) or WIN2 + IR. Staurosporine (1 μ M) and Paclitaxel (1 μ M) were used as positive controls. **(A)** Flow cytometry was used to quantify annexin V and PI staining at 48 hrs. **(B)** Dapi staining was used to assess nuclear morphology at 40x magnification. Data normalized to % of population in **(A)**; data presented reflect the means of 3-4 individual experiments \pm se; * $p < 0.05$ vs vehicle.

Statistics

(A) Two way repeated measures ANOVA: no significant differences for healthy, apoptotic or necrotic cells

(A) Staurosporine paired t-test: healthy cells ($p = 0.0141$). Apoptotic cells ($p = 0.0192$). Necrotic cells ($p = 0.0396$).

compared to vehicle. A 1 μ M staurosporine treatment for 24 h was used as a positive control for apoptosis and necrosis, and this treatment produced a significant decrease in healthy cells, with significant increases in both apoptotic and necrotic cells.

To confirm the absence of cell death in MCF-7 cells treated with the WIN2/IR combination, nuclear morphology was assessed at 48, 72 and 96 h using DAPI staining (**Fig 4.3B**). 0.5 μ M of the microtubule poison, paclitaxel, was used as a positive control for apoptotic nuclear morphology. DAPI staining was also used to screen for multinucleated cells, a marker of mitotic catastrophe (Jonathan et al. 1999). At 48, 72 and 96 h, no evidence of cell death was detected except in the positive control. Taken together, these results strongly argue that the antiproliferative effects of the WIN2/IR combination are not mediated by apoptosis, necrosis or mitotic catastrophe in MCF-7 cells.

Section 4.4 - Temporal effects of the WIN2/IR combination in breast cancer cells

The absence of evidence for a cell death mechanism, lead us to predict that growth inhibition was likely mediating the antiproliferative actions of the WIN2/IR combination. To test this hypothesis, trypan blue was used to assess cell viability at 24, 48, 72 and 96 h in MCF-7 cells treated with the WIN2/IR combination (**Fig 4.4**). WIN2, IR and WIN2 + IR were all significantly different from vehicle at 48, 72 and 96 h, which confirmed the growth inhibition hypothesis. Decreases in doubling times after treatment emphasize the presence of growth inhibition. Doubling time presented in hrs (mean \pm se): vehicle – 27.7 \pm 2.6, WIN2 – 44.6 \pm 7.7, IR – 36.8 \pm 4.9 and WIN2 + IR – 68.3 \pm 9.8.

A cell viability time course was also evaluated in MDA-MB231 breast tumor cells exposed to the WIN2/IR combination treatment (**Fig 4.5**). MDA-MB231 cells were treated with 15 μ M WIN2 and 2 Gy radiation. Statistical comparisons showed that growth inhibition was detected as

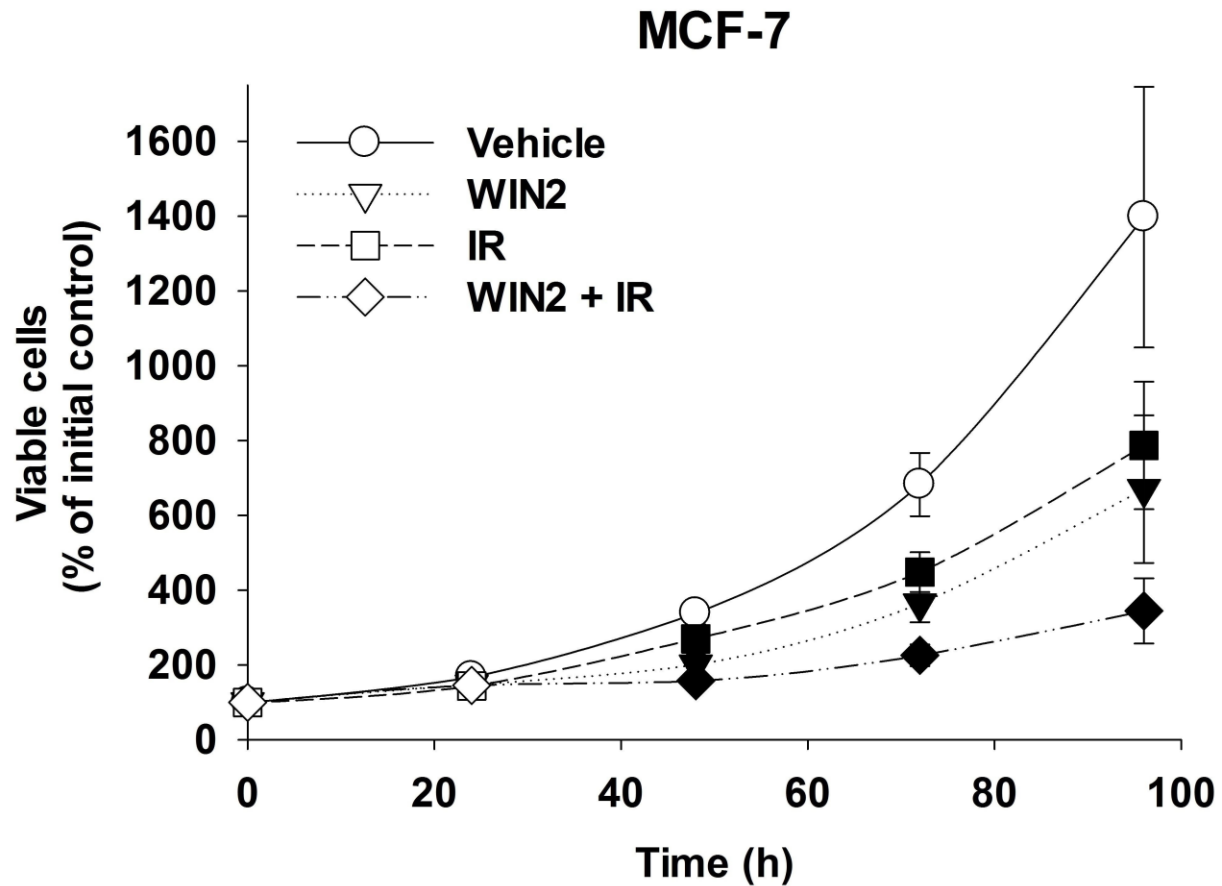


Figure 4.4 – Temporal effects of WIN2 and IR combination in MCF-7 breast cancer cells. MCF-7 cells were treated with vehicle, WIN2 (12 μ M), IR (2 Gy) or WIN2 + IR treatments. Viable cell number was monitored over a period of 96 h using the trypan blue exclusion assay. Data presented reflect the means of 5 individual experiments \pm se. Darkened symbols = $p < 0.05$ vs vehicle within time points.

Statistics

- 24 h** – No significant differences
- 48 h** – Repeated measures ANOVA ($p < 0.0001$) with Dunnett's post hoc (WIN2, IR and WIN2 + IR significant from vehicle)
- 72 h** – Repeated measures ANOVA ($p < 0.0001$) with Dunnett's post hoc (WIN2, IR and WIN2 + IR significant from vehicle)
- 96 h** – Repeated measures ANOVA ($p = 0.0006$) with Dunnett's post hoc (WIN2, IR and WIN2 + IR significant from vehicle)

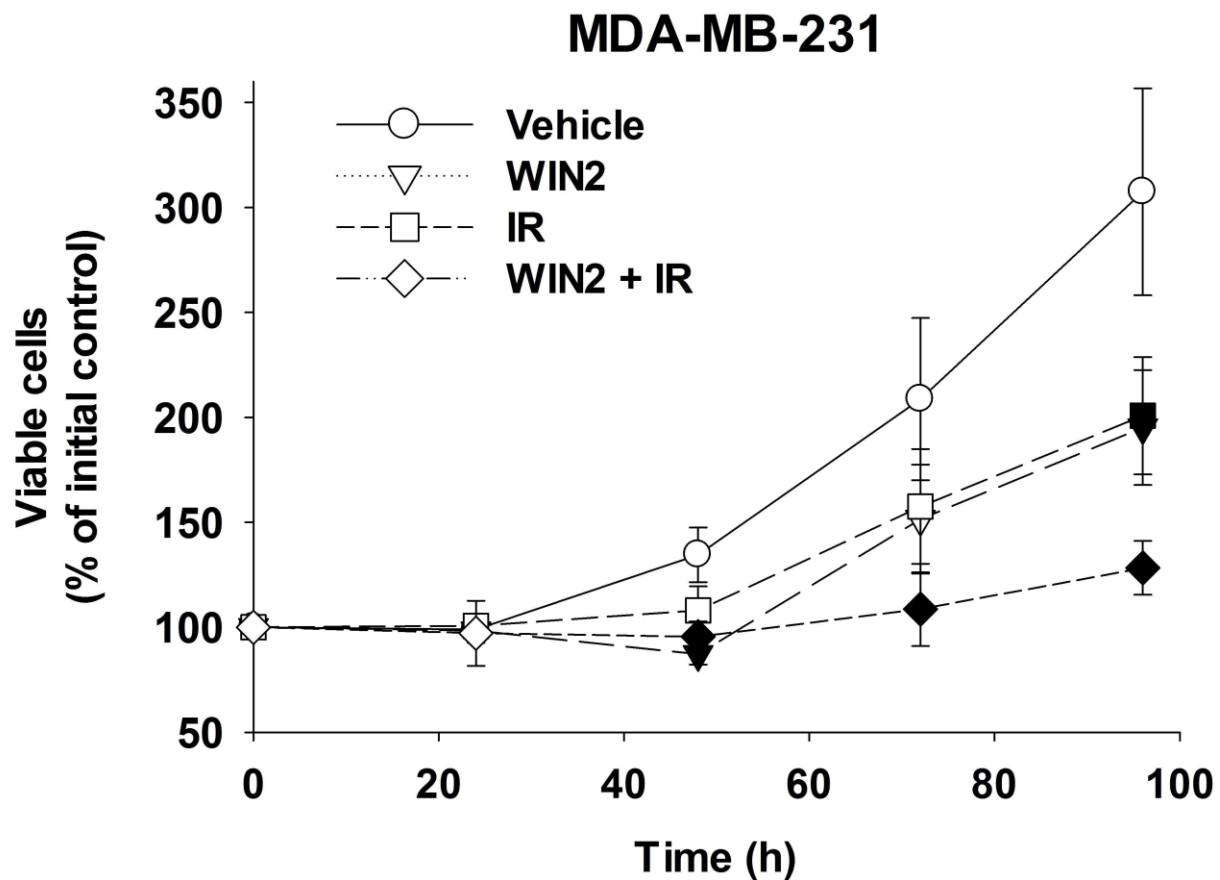


Figure 4.5 – Temporal effects of WIN2 and IR combination in MDA-MB231 breast cancer cells. MDA-MB231 cells were treated with vehicle, WIN2 (15 μ M), IR (2 Gy) or WIN2 + IR treatments. Viable cell number was monitored over a period of 96 h using the trypan blue exclusion assay. Data presented reflect the means of 5 individual experiments \pm se. Darkened symbols = $p < 0.05$ vs vehicle within time points.

Statistics

- 24 h** – No significant differences
- 48 h** – Repeated measures ANOVA ($p = 0.0062$) with Dunnett's post hoc (WIN2 and WIN2 + IR significant from vehicle)
- 72 h** – Repeated measures ANOVA ($p = 0.0011$) with Dunnett's post hoc (WIN2, IR and WIN2 + IR significant from vehicle)
- 96 h** – Repeated measures ANOVA ($p < 0.0001$) with Dunnett's post hoc (WIN2, IR and WIN2 + IR significant from vehicle)

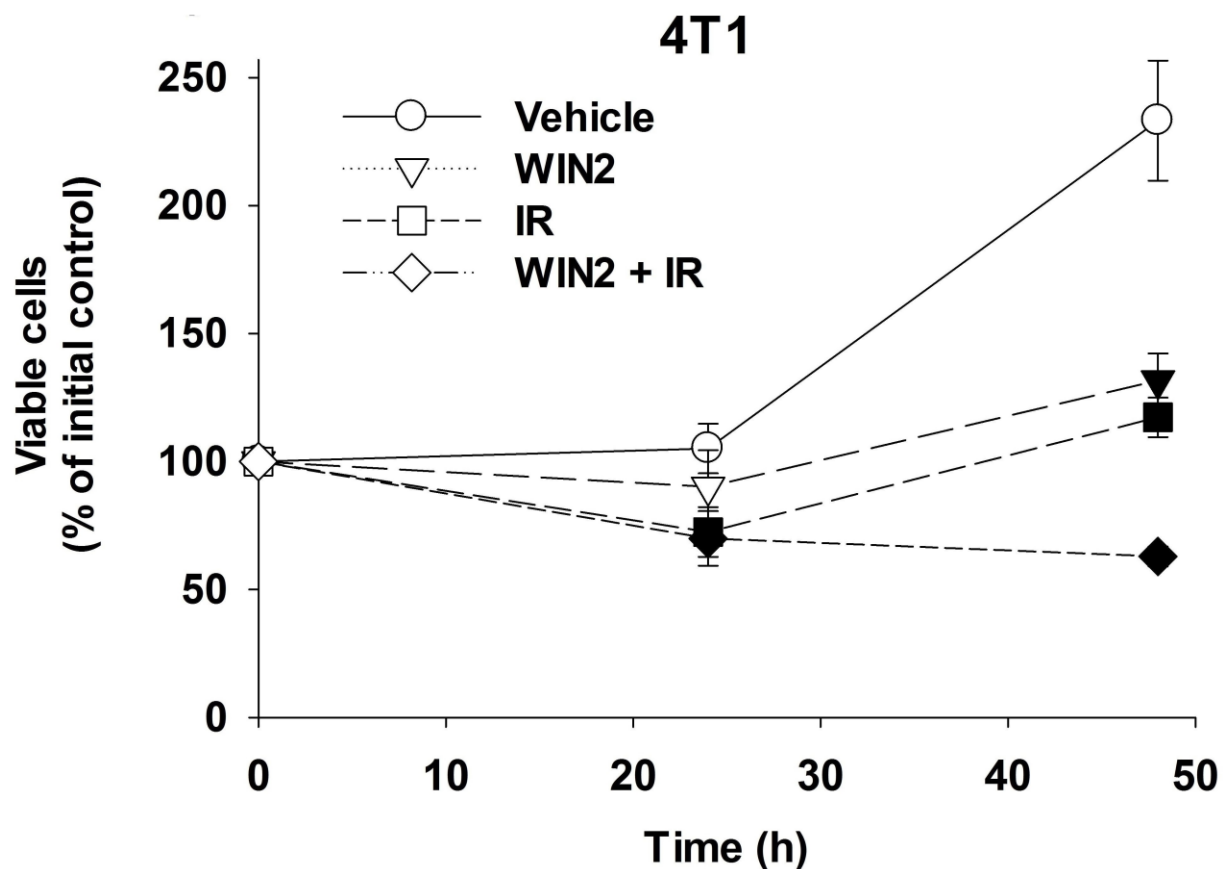


Figure 4.6 – Temporal effects of WIN2 and IR combination in 4T1 breast cancer cells. 4T1 cells were treated with vehicle, WIN2 (30 μ M), IR (8 Gy) or WIN2 + IR treatments. Viable cell number was monitored over a period of 48 h using the trypan blue exclusion assay. Data presented reflect the means of 5 individual experiments \pm se. Darkened symbols = $p < 0.05$ vs vehicle within time points.

Statistics

24 h – Repeated measures ANOVA ($p = 0.0352$) with Dunnett's post hoc (IR and WIN2 + IR significant from vehicle)

48 h – Repeated measures ANOVA ($p < 0.0001$) with Dunnett's post hoc (WIN2, IR and WIN2 + IR significant from vehicle)

WIN2 (0-24 h) – Repeated measures ANOVA ($p = 0.0438$) with Dunnett's post hoc (no treatments significantly different from time 0)

IR (0-24 h) – Repeated measures ANOVA ($p = 0.0051$) with Dunnett's post hoc (IR and WIN2 + IR significantly different from time 0)

WIN2 + IR (0-24 h) – Repeated measures ANOVA ($p = 0.0166$) with Dunnett's post hoc (WIN2 + IR significantly different from time 0)

early as 48 h in the WIN2 and WIN2 + IR groups but by 72 and 96 h, all treatment groups showed significant growth inhibition compared to vehicle. These results support those reported using MCF-7 cells.

Different than MCF-7 or MDA-MB231 cells, 4T1 cell assessment of temporal effects indicates evidence for cell death with the WIN2/IR combination. 4T1 cells were treated with 30 μ M WIN2 and 8 Gy IR before assessment at 24 and 48 h (**Fig 4.6**). Antiproliferative action was detected in the IR and WIN2 + IR group at 24 h and all treatment groups at 48 h when compared to vehicle. Interestingly, significant decreases in viable cell number compared to time 0 showed evidence for cell death in the IR treatment at 24 h and the WIN2 + IR treatment at 24 and 48 h. This suggests that WIN2 and IR may be interacting via a cytotoxic mechanism in 4T1 cells.

Section 4.5 - Radiation but not WIN2 induces DNA damage in breast cancer cells

Antiproliferative effects of IR have been linked to the induction of DNA damage (Narayanan et al. 1997). γ H2AX is a protein recruited to DNA repair complexes that is rapidly degraded after the completion of DNA repair. As a result, it is used to monitor the DNA repair response process (Rogakou et al. 1999). Changes in γ H2AX expression after radiation treatment were used to assess the potential influence of the WIN2/IR combination on DNA damage induction (1 h) and repair (24 h) in MCF-7, MDA-MB-231 and 4T1 cells (**Fig 4.7-4.9**).

In MCF-7 cells, radiation significantly increased γ H2AX expression at 1 h, while WIN2 alone had no effect on γ H2AX. Interaction comparison indicates that WIN2 had no effect on the level of γ H2AX induction by IR at 1 h, which indicates WIN2 had no effect on DNA damage induction either alone or in combination with IR. By 96 h γ H2AX levels in all treatments had

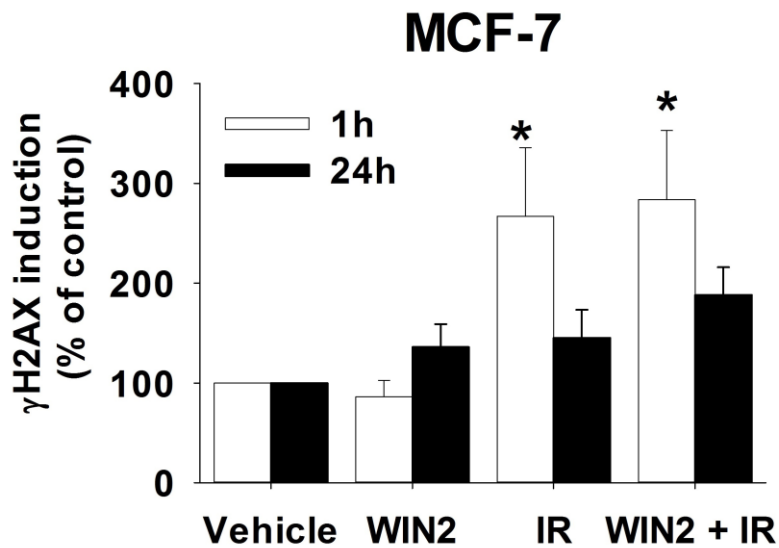


Figure 4.7 – DNA damage and repair in breast cancer cells treated with WIN2 and radiation. MCF-7 figure 4.4. γ H2AX formation analyzed by flow cytometry at 1 h and 24 h after drug treatment. Data were normalized to percent of control; data presented reflect the means of 3-5 individual experiments \pm se; * $p < 0.05$ vs vehicle.

Statistics

1 h – Two way repeated measures ANOVA: WIN2-IR interaction ($p = 0.5521$). IR treatment main effect ($p = 0.0003$).

1 h – Individual comparisons: Vehicle-IR ($p = 0.0233$). Vehicle-WIN2 + IR ($p = 0.0240$).

24 h – Two way repeated measures ANOVA: WIN2-IR interaction ($p = 0.8510$). IR treatment main effect ($p = 0.0343$).

24 h – Individual comparisons: no significant differences

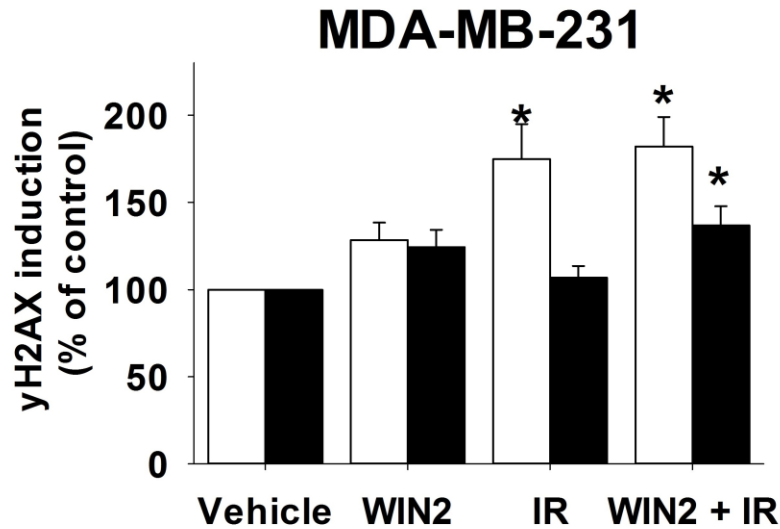


Figure 4.8 – DNA damage and repair in breast cancer cells treated with WIN2 and radiation. MDA-MB231 cells were treated as in figure 4.5. γ H2AX formation analyzed by flow cytometry at 1 h and 24 h after drug treatment. Data were normalized to percent of control; data presented reflect the means of 3-5 individual experiments \pm se; * $p < 0.05$ vs vehicle.

Statistics

1 h – Two way repeated measures ANOVA: WIN2-IR interaction ($p = 0.1189$). IR treatment main effect ($p = 0.0194$).

1 h – Individual comparisons: Vehicle-IR ($p = 0.0289$). Vehicle-WIN2 + IR ($p = 0.0126$).

24 h – Two way repeated measures ANOVA: WIN2-IR interaction ($p = 0.6982$). IR treatment main effect ($p = 0.0160$).

24 h – Individual comparisons: Vehicle-WIN2 + IR ($p = 0.0127$)

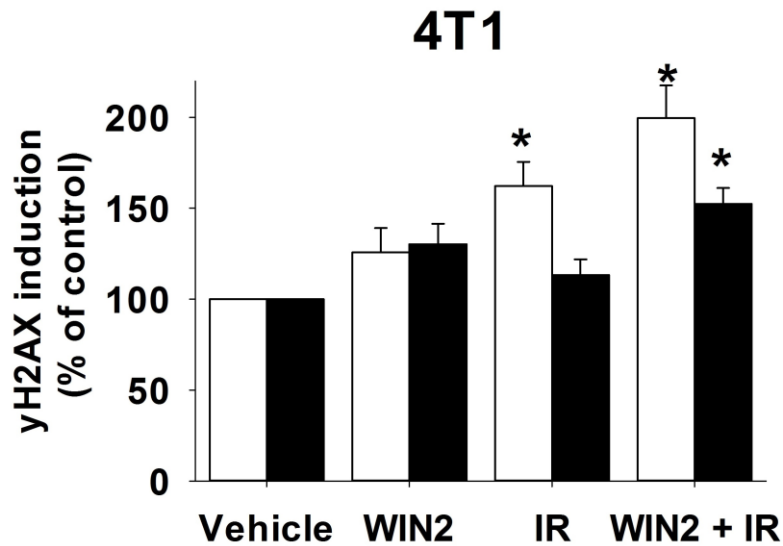


Figure 4.9 – DNA damage and repair in breast cancer cells treated with WIN2 and radiation. 4T1 cells were treated as in figure 4.6. γ H2AX formation analyzed by flow cytometry at 1 h and 24 h after drug treatment. Data were normalized to percent of control; data presented reflect the means of 3-5 individual experiments \pm se; * $p < 0.05$ vs vehicle.

Statistics

1 h – Two way repeated measures ANOVA: WIN2-IR interaction ($p = 0.5203$). IR treatment main effect ($p = 0.0063$).

1 h – Individual comparisons: Vehicle-IR ($p = 0.0138$). Vehicle-WIN2 + IR ($p = 0.0078$).

24 h – Two way repeated measures ANOVA: WIN2-IR interaction ($p = 0.7009$). IR treatment main effect ($p = 0.0313$).

24 h – Individual comparisons: Vehicle-WIN2 + IR ($p = 0.0396$)

returned to baseline levels relative to vehicle demonstrating that WIN2 had no effect on the DNA repair process in MCF-7 cells.

In MDA-MB231 and 4T1 cells radiation significantly increased γ H2AX expression at 1 h and WIN2 alone had no effect. WIN2 + IR displayed no greater induction of γ H2AX at 1 h than IR alone indicating WIN2 had no effect on DNA damage induction either alone or in combination with IR. By 96 h γ H2AX levels in WIN2 alone and IR alone treatments had returned to baseline levels, but γ H2AX in the WIN2 + IR treatment remained significantly different from vehicle. Nevertheless, statistical comparisons showed no significant interaction between the WIN2 and IR treatments indicating no presence of augmentation. These results confirm that like in MCF-7 cells, WIN2 had no effect on the induction or repair of DNA damage in MDA-MB231 or 4T1 cells.

Section 4.6 - Radiation but not WIN2 induces senescence in MCF-7 cells

Jones et al. (2005) established that radiation treatments induced growth arrest via senescence in MCF-7 cells. To test the induction of senescence, the β -galactosidase assay was used to quantify cells treated with vehicle, WIN2 (12 μ M), IR (2 Gy) or WIN2 + IR (**Fig 4.10A-B**). At 96 h, radiation significantly induced senescence, WIN2 had no ability to induce senescence and interaction comparisons confirm WIN2 had no significant effect on the level of radiation-induced senescence. These studies confirm previous reports that radiation inhibits growth via senescence (Jones et al. 2005), and in the absence of senescence it can be concluded that growth inhibition after WIN2 treatment is classical growth arrest.

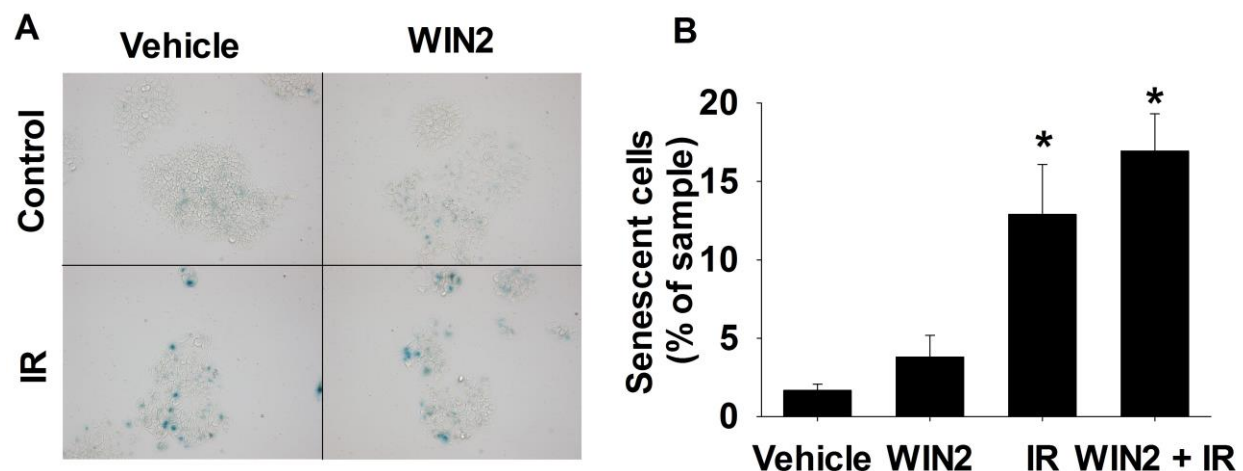


Figure 4.10 – Senescence induction by radiation ± WIN2. MCF-7 cells were treated with vehicle, WIN2 (12 μ M), (2Gy) radiation or WIN2 + radiation. **(A)** Representative images of β -galactosidase stained cells. **(B)** Quantification of β -galactosidase activity 96 h after drug treatment. Data were normalized to % of sample in **(B)**; data presented reflect the means of 3 individual experiments \pm se; * p <0.05 vs vehicle.

Statistics

(B) Two way repeated measures ANOVA: WIN2-IR interaction (p =0.6618). IR treatment main effect ($F_{1,4}=72$, p =0.0011).

(B) Individual comparisons: Vehicle-IR (p =0.0382). Vehicle-WIN2 + IR (p =0.0310)

Discussion

It was originally hypothesized that WIN2 would augment the impact of radiation in breast tumor cells through an autophagic mechanism based on observations from Salazar et al. 2009 and Bristol et al. 2012. In the studies presented above WIN2, IR and WIN2 + IR were capable of inducing autophagy in MCF-7 cells; however based on the inability of CQ to alter MCF-7 response to WIN2, IR or WIN2 + IR, it can be concluded that autophagy is not relevant to the antiproliferative mechanisms for these three treatments, and furthermore autophagy is not involved in WIN2 augmentation of radiation.

One discrepancy regarding autophagy induction in these studies and in the work of Bristol et al. (2012) is that in Bristol et al., CQ enhanced the antiproliferative effects of radiation in MCF-7 cells. A CQ induced enhancement of the antiproliferative effects of radiation was not observed in the studies presented above. This might be explained by the different doses of radiation used between the studies (1x2 Gy here; 5x2 Gy in Bristol et al.), which could lead to different autophagic mechanisms. In fact, Bristol et al. observed that autophagy had different mechanisms of action depending on the conditions of the system, which suggests that the relatively low radiation dose of 2 Gy used in the current work simply may not have been sufficient to induce the protective autophagy that was reported in Bristol et al. 2012.

Previous studies have connected the antiproliferative actions of cannabinoids to ROS induced autophagy (Shrivastava et al. 2011, Donadelli et al. 2011, Dando et al. 2013). As autophagy was apparently not directly relevant to the antiproliferative mechanism of the WIN2/IR combination, and the antioxidants NAC and GSH were unable to rescue MCF-7 cells from the antiproliferative actions of the WIN2, IR or the WIN2 + IR combination, it can be concluded that ROS do not mediate the antiproliferative effects of WIN2, IR or WIN2 + IR. On the other hand, it is well documented that ROS mediate the DNA damaging effects of radiation

therapy (Driessens et al. 2009), and NAC or GSH failed to protect MCF-7 cells from radiation might be confusing. However, this discrepancy can be explained by a report that has shown increased GSH levels from NAC treatment were unable to protect lung tumor cells from the antiproliferative effects of ionizing radiation (Wanamarta et al. 1998), and another study showed that overexpressing glutathione peroxidase in MCF-7 cells protected the cells from H₂O₂ treatment but not radiation treatment (Liebmann et al. 1995). Based on the Wanamarta et al. and Liebmann et al. studies, it is understandable that NAC and GSH can protect MCF-7 cells from the antiproliferative effects of H₂O₂ but not from radiation.

Annexin V and PI staining as well as DAPI nuclear staining showed that WIN2, IR and WIN2 + IR fail to induce apoptosis, necrosis and mitotic catastrophe in MCF-7 cells. The absence of these three cell death mechanisms as well as evidence that autophagy is not associated with the antiproliferative action of the WIN2/IR combination strongly argue that growth inhibition and not cell death is mediating the antiproliferative actions of WIN2, IR and WIN2 + IR. A growth inhibition hypothesis is also evident in the time course studies in MCF-7 cells treated with WIN2, IR and WIN2 + IR, as well as reports from the literature where WIN2 induced growth arrest as its primary mechanism of action (Park et al. 2011). Therefore, it appears likely that augmentation of IR induced growth inhibition by WIN2 is expressed in at least one of two ways, which is by the augmentation of one growth inhibitory pathway or the activation of two parallel growth inhibitory pathways. The two primary growth inhibitory pathways are senescence, involving the activation of a specific signalling process, or classical growth arrest, which is a more broad suppression of mitogenic signals (Blagosklonny et al. 2003). Previous studies have demonstrated using the β -galactosidase assay that radiation-induced growth inhibition is expressed as senescent growth arrest in MCF-7 cells (Jones et al. 2005). WIN2, however, failed to induce senescence or to augment the induction of senescence by radiation in MCF-7 cells. These combined observations demonstrate that growth inhibition by

WIN2 is mediated by classical growth arrest, either expressed as cell cycle arrest or growth delay, and the augmentation of IR treatment by WIN2 is expressed as two parallel pathways of growth inhibition, growth arrest and senescence.

DNA damage is believed to be responsible for the induction of senescence by radiation in MCF-7 cells (Jones et al. 2005). Tracking the DNA damage response by monitoring the induction and decline of γ H2AX (Rogakou et al. 1999) confirmed the presence of DNA damage by radiation in MCF-7 cells. These experiments also confirmed that WIN2 has no ability to enhance induction of DNA damage, induce DNA damage or alter the DNA repair process. Lack of interaction between WIN2 and IR treatment on γ H2AX induction was also demonstrated in the MDA-MB231 and 4T1 cells. The absence of interaction between WIN2 and IR in the DNA damage response pathway supports the hypothesis that WIN2 and IR are acting via parallel mechanisms of growth inhibition rather than by a common pathway.

The conclusion of parallel growth inhibitory pathways cannot be extended to the MDA-MB231 and 4T1 cells for multiple reasons. First, MDA-MB321 cells have previously been shown to respond to radiation therapy via an apoptotic mechanism and not senescence (Jones et al. 2005). Second, the time course viability studies for 4T1 cells shows that at 24 h radiation and WIN2 + radiation significantly decrease viable cell number compared to the 0 h controls, indicating cell death as opposed to growth arrest. These lines of evidence supporting radiation-induced cell death mechanisms in MDA-MB231 and 4T1 cells do, however, allow for the conclusion that the interaction of WIN2 and IR in MDA-MB231 and 4T1 cells is different than for MCF-7 cells. If the mechanism of growth inhibition for WIN2 in MCF-7 cells extends to MDA-MB231 and 4T1, it would be logical to hypothesize that radiation is inducing cell death in a percentage of the population and WIN2 is inducing growth arrest in the remainder. Evaluating this hypothesis of parallel mechanisms of growth inhibition and cell death in MDA-MB231 and

4T1 cells in future studies could provide a crucial insight about the interaction between WIN2 and IR in MDA-MB231 and 4T1 cells.

Summary

Section 3 demonstrated WIN2 to possess the ability to significantly augment the antiproliferative effects of radiation treatment in breast cancer cells *in vitro*. This section provided evidence that this augmentation between WIN2 and IR occurs through parallel mechanisms of growth inhibition. Studies confirmed that radiation treatment induces senescent growth arrest in MCF-7 cells, which was previously demonstrated by Jones et al. (2005). WIN2 treatment did not elicit a senescent response in MCF-7 cells, nor did it alter the extent of induction of senescence by radiation, but time course analysis showed a significant growth delay after WIN2 treatment. Cell death assays (apoptosis, necrosis, autophagy and mitotic catastrophe) confirm that a low level of cell death cannot explain the growth inhibitory actions of WIN2. In the absence of overt cell death and senescence, it was concluded that WIN2 causes a classical growth arrest event as either growth delay or cell cycle arrest. WIN2's inability to alter the induction of senescence while still demonstrating augmentation allowed for the conclusion that WIN2 and IR induce separate but parallel mechanism of growth arrest.

Section 5

Establishing the receptor mediating the antiproliferative effects of WIN2

There is well established evidence that the cannabinoid receptors 1 and 2 (CB₁ and CB₂) mediate the antiproliferative and/or cytotoxic actions of cannabinoids. CB₁ has been shown to mediate cell death in glioma cells based on the observation that the selective CB₁ antagonist SR141716 inhibited induction of cell death by Δ^9 -tetrahydrocannabinol (THC) (Salazar et al. 2009). SR141716 also antagonized the growth inhibition of MCF-7 cells by the endogenous cannabinoid anandamide (AEA) (Melck et al. 2000). This argues for a CB₁ mediated mechanism of growth inhibition by cannabinoids in MCF-7 cells. Even though Melck et al. (2000) found the expression of CB₂ mRNA in MCF-7 cells, the CB₂ selective antagonist SR144528 had no effect on AEA in MCF-7 cells further confirming CB₁ actions, but other studies have reported that CB₂ mediated growth inhibition of breast cancer cells. In MDA-MB231 breast tumor cells, both cannabinoid receptor antagonists AM251 and SR144528, CB₁ and CB₂ respectively, partially suppressed growth inhibition by WIN55,212-2 (WIN2) *in vivo*, arguing for CB₂ involvement as well as CB₁ in cannabinoid growth inhibition (Qamri et al. 2009). CB₂ involvement is supported by Caffarel et al. 2010, which reported that the CB₂ selective agonist, JWH-133, inhibits *in vivo* ErbB2 positive mammary tumor growth, and Caffarel et al. 2006, which showed that SR144528 but not SR141716 significantly antagonized the antiproliferative effects of THC in EVSA-T breast cancer cells *in vitro*. For these reasons both CB₁ and CB₂ were evaluated as potential mediators of WIN2 action in breast tumor cells.

Based on gene reporter assays (O'Sullivan et al. 2007) and antagonist studies (Mestre et al. 2009) WIN2 is an activator of the peroxisome-proliferator activated receptor γ (PPAR γ), and WIN2 activation of PPAR γ induces apoptosis in hepatoma HepG2 cells (Giuliano et al. 2009). Although WIN2 actions at PPAR γ in MCF-7 cells have not been assessed, PPAR γ has been found to be expressed in MCF-7 cells (Nwankwo et al. 2001), and continuous treatment with the

selective PPAR γ agonist troglitazone inhibited their growth (Yin et al. 2001). Both PPAR α and PPAR δ receptor mRNA have also been found in MCF-7 cells (Suchanek et al. 2002a and 2002b). WIN2 has shown activity as an agonist for PPAR α by driving luciferase transcription via a PPAR α promoter (Sun et al. 2006). WIN2 action at PPAR δ has not been determined and it remains unclear how PPAR α/δ activation would affect MCF-7.

Transient receptor potential-cation-channel subfamily V member 1 (TRPV1) is a non-selective cation channel sensitive to AEA (Smart et al. 2000), and TRPV1 mRNA is found abundantly in MCF-7 cells (Ligresti et al. 2006). However, TRPV1 involvement in MCF-7 growth is uncertain. The TRPV1 agonist capsaicin has mixed effects in MCF-7 cells ranging from minimal (Tuoya et al. 2006) to significant (Thoennissen et al. 2010) growth inhibition. Furthermore, these studies do not link capsaicin action to TRPV1 either genetically or pharmacologically. Conversely the TRPV1 antagonist capsazepine sensitizes HCT116 colon cells to TRAIL induced apoptosis, which argues for a protective action of TRPV1 in cancer (Sung et al. 2012), but again capsazepine action were not linked to TRPV1 either genetically or pharmacologically.

The primary aim of the following studies was to determine the potential receptor binding sites that may mediate the WIN2 antiproliferative action using molecular and pharmacological techniques. These included CB $_1$ and CB $_2$ receptors, peroxisome proliferator-activated receptors (PPAR α - γ), and TRPV1 receptors. RT-PCR was also used to quantify message for GPR55, a recently discovered cannabinoid sensitive target (Yin et al. 2009, Johns et al. 2007). Summary of the results presented below, will show WIN2 does not interact with CB $_1$, CB $_2$, PPAR α - γ or TRPV1; also no GPR55 message was detected. Studies, based on unpublished data from the laboratory of Dr. Dana E. Selley, were then performed to evaluate WIN2 potential as an antagonist of growth pathways activated by sphingosine-1-phosphate, SEW2871 and estradiol.

***statistical values reported in figure legends**

Abbreviations

CB1-cannabinoid receptor 1; **CB2**-cannabinoid receptor 2; **TRPV1**-transient receptor potential cation channel subfamily V member 1; **PPAR**-peroxisome proliferator-activated receptors; **AEA**-anandamide; **WIN2**-WIN55,212-2; **THC**- Δ^9 -tetrahydrocannabinol; **S1P**-sphingosine-1-phosphate; **E2**-estradiol; **GPCR**- G protein coupled receptor

Results

Section 5.1 - CB₁ and CB₂ do not mediate WIN2 elicited antiproliferative effects in MCF-7 cells

As CB₁ and CB₂ receptor-dependent growth inhibition has been reported in breast cancer cells (Melck et al. 2000, Qamri et al. 2009), RT-PCR was used to qualitatively confirm the expression of these receptors in MCF-7 cells. CB₂ receptor mRNA was clearly identified, while a weak signal was found for CB₁ (**Fig 5.1A**). The respective CB₁ and CB₂ receptor antagonists, AM251 (4 µM) and AM630 (4 µM) were evaluated for their ability to prevent WIN2-induced inhibition of cell growth (**Fig 5.1B**). Neither AM251 nor AM630 antagonized WIN2 growth suppression. Higher doses of the antagonists could not be used due to inhibition of MCF-7 cell growth. This lack of antagonism by AM251 and AM630 is strongly indicative of a CB₁ and CB₂ receptor-independent mechanism.

Section 5.2 - Members of the peroxisome-proliferator activated receptor family do not mediate WIN2 elicited antiproliferative effects in MCF-7 cells

Given the apparent lack of CB₁ and CB₂ receptor involvement in the antiproliferative effects of WIN2 (section 5.1), the contribution of other WIN2 sensitive targets were assessed. RT-PCR confirmed the presence of PPAR γ mRNA in MCF-7 cells (**Fig 5.2A**), but the PPAR γ receptor antagonist GW9662 (10 µM) did not reduce the antiproliferative effects of 12 µM WIN2 (**Fig 5.2B**). The PPAR γ receptor agonist pioglitazone (PGZ) and pan-PPAR agonist bezafibrate were tested for antiproliferative activity in MCF-7 cells (**Fig 5.2C-D**), but neither recapitulated WIN2 growth inhibition further confirming WIN2 is not inhibiting MCF-7 growth via PPAR

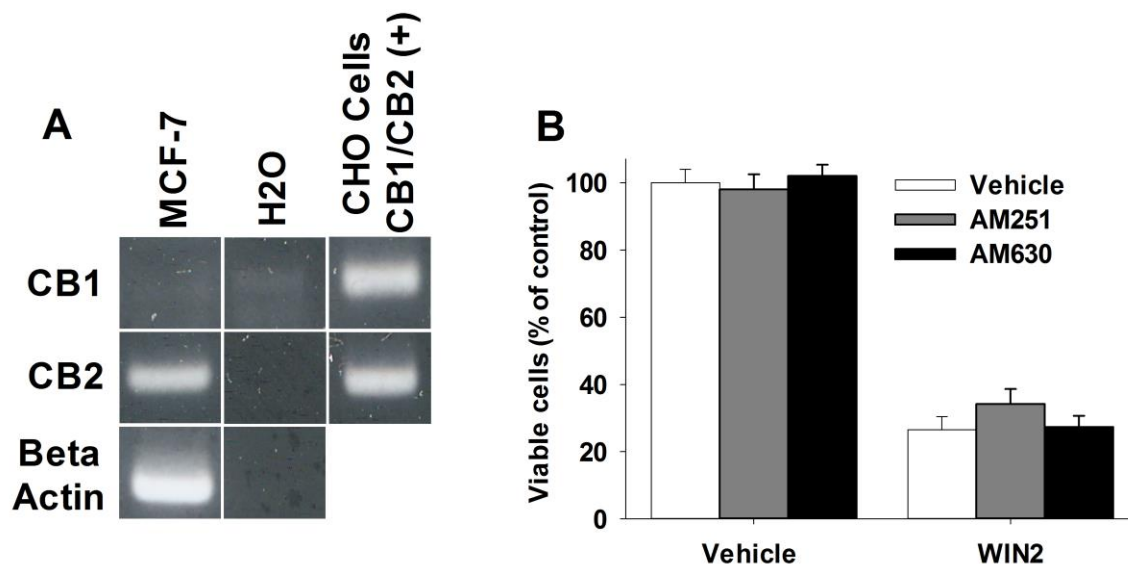


Figure 5.1 – The antiproliferative effects of WIN2 in MCF-7 cells are mediated through a non-cannabinoid receptor mechanism of action. (A) RT-PCR for the CB₁ and CB₂ receptors in MCF-7 cells. CHO cells transfected with human CB₁ or CB₂ receptors were used as a positive control. **(B)** MCF-7 cells were treated with vehicle or WIN2 (12 μ M) and vehicle, AM251 (4 μ M), or AM630 (4 μ M) for 24 h. Cell count with trypan blue was used to assess cell viability at 96 h. Data presented reflect the means of 3 individual experiments \pm se; no significant difference found. None transformed control viable cell numbers as mean \pm se - 2247442 \pm 746329

Statistics

(B) Two way repeated measures ANOVAs: AM251-WIN2 interaction - (p=0.7429); AM630-WIN2 interaction - (p=0.4901).

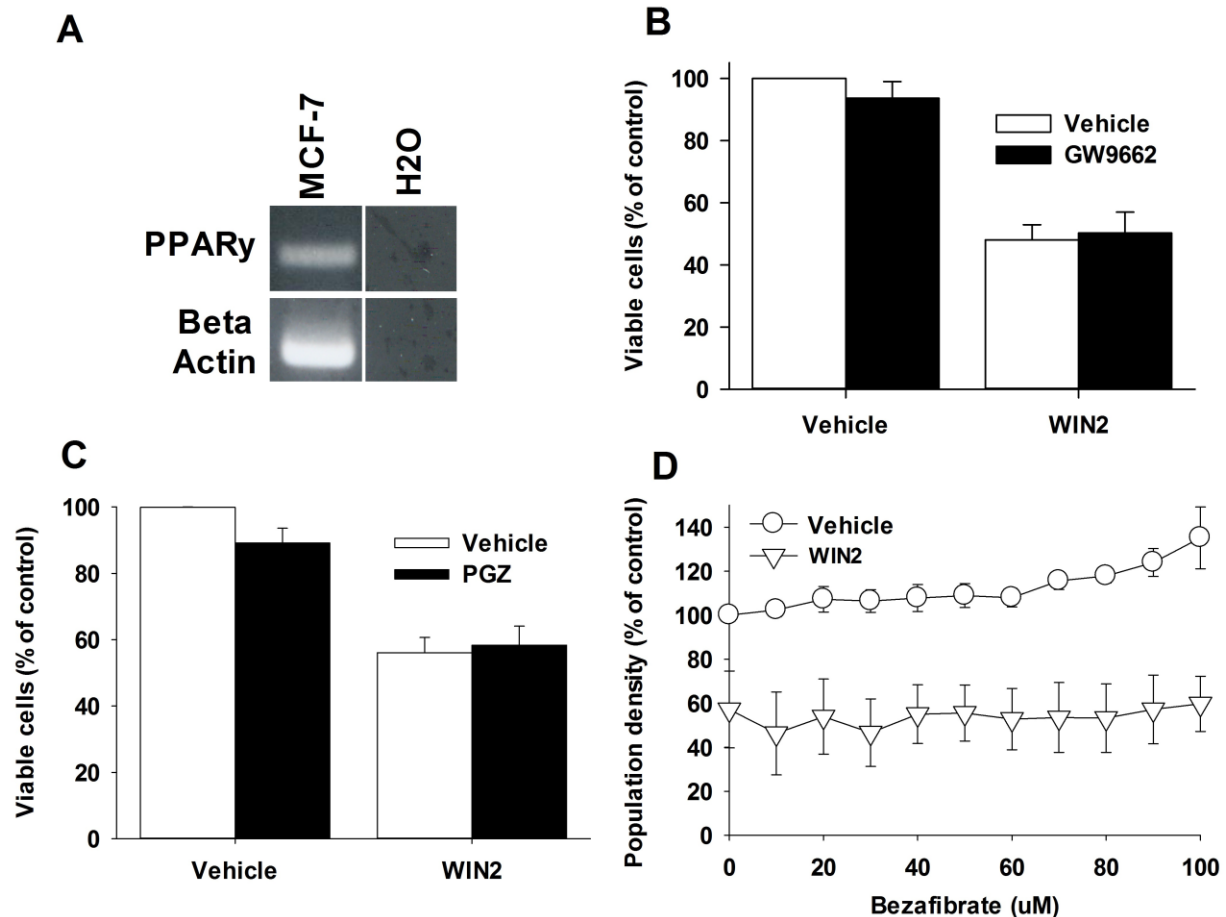


Figure 5.2 – PPAR receptor activation does not mediate WIN2 effects in MCF-7 cells. (A) RT-PCR for PPAR γ (B) MCF-7 cells were treated with vehicle or WIN2 (12 μ M) and vehicle or GW9662 (10 μ M). (C) MCF-7 cells were treated with vehicle or WIN2 (12 μ M) and vehicle or pioglitazone (40 μ M). (D) MCF-7 cells were treated with bezafibrate (0-100 μ M) and vehicle or WIN2 (12 μ M). Cells count with trypan blue was used to assess cell viability at 96 h (A, B and C). Crystal violet assessed population density at 96 h (D). Data presented reflect the means of 3 individual experiments \pm se; no significant difference found.

Statistics

(B) Two way repeated measures ANOVA: GW9662-WIN2 interaction ($p=0.3208$).

(C) Two way repeated measures ANOVA: PGZ-WIN2 interaction ($p=0.1670$). PGZ treatment main effect ($p=0.5385$).

(D) Two way repeated measures ANOVA: Bezafibrate-WIN2 interaction ($p=0.9981$). Bezafibrate treatment main effect ($p=0.8611$).

receptor activation. PGZ and bezafibrate also had no effect on the WIN2-mediated growth inhibition when given in combination (**Fig 5.2 C-D**). Together these experiments show that WIN2 fails to activate or antagonize all members of the PPAR receptor family.

Section 5.3 - TRPV1 is not involved in WIN2 mediated antiproliferative actions in MCF-7 cells

TRPV1 was evaluated as a potential target of WIN2 action after other well established WIN2 sensitive targets were experimentally eliminated. RT-PCR confirmed the presence of mRNA for TRPV1 in MCF-7 cells (**Fig 5.3A**), but the TRPV1 receptor antagonist capsazepine (10 μ M) failed to reduce the antiproliferative effects of WIN2 (**Fig 5.3 B**). Furthermore, 100 μ M of the TRPV1 agonist capsaicin (CAP) failed to elicit antiproliferative activity alone ($p=0.1410$; **Fig 5.3C**). CAP was also given in combination with WIN2 (**Fig 5.3C**), but no significant interaction between treatments was found. These studies indicate that WIN2 was unlikely to be inhibiting the growth of MCF-7 cells via interaction with the TRPV1 receptor.

Section 5.4 - GPR55 mRNA is not found in MCF-7 cells

WIN2 has not been shown to activate GPR55 (Johns et al. 2007, Yin et al. 2009), but AEA, THC and CP55,940 activated GPR55 using a β -arrestin or GPR55 activated luciferase reporter assay (Yin et al. 2009). However, depending on the study, the cannabinoid agonist abnormal-cannabidiol showed both a neutral activity (Yin et al. 2009) and agonist activity (John et al. 2007) at GPR55. This suggests a system specific effect of some cannabinoids at GPR55. Therefore, GPR55 was assessed as a potential target for the anti-proliferative actions of WIN2 in MCF-7 cells. RT-PCR qualitatively proved that no GPR55 mRNA was detectable in MCF-7

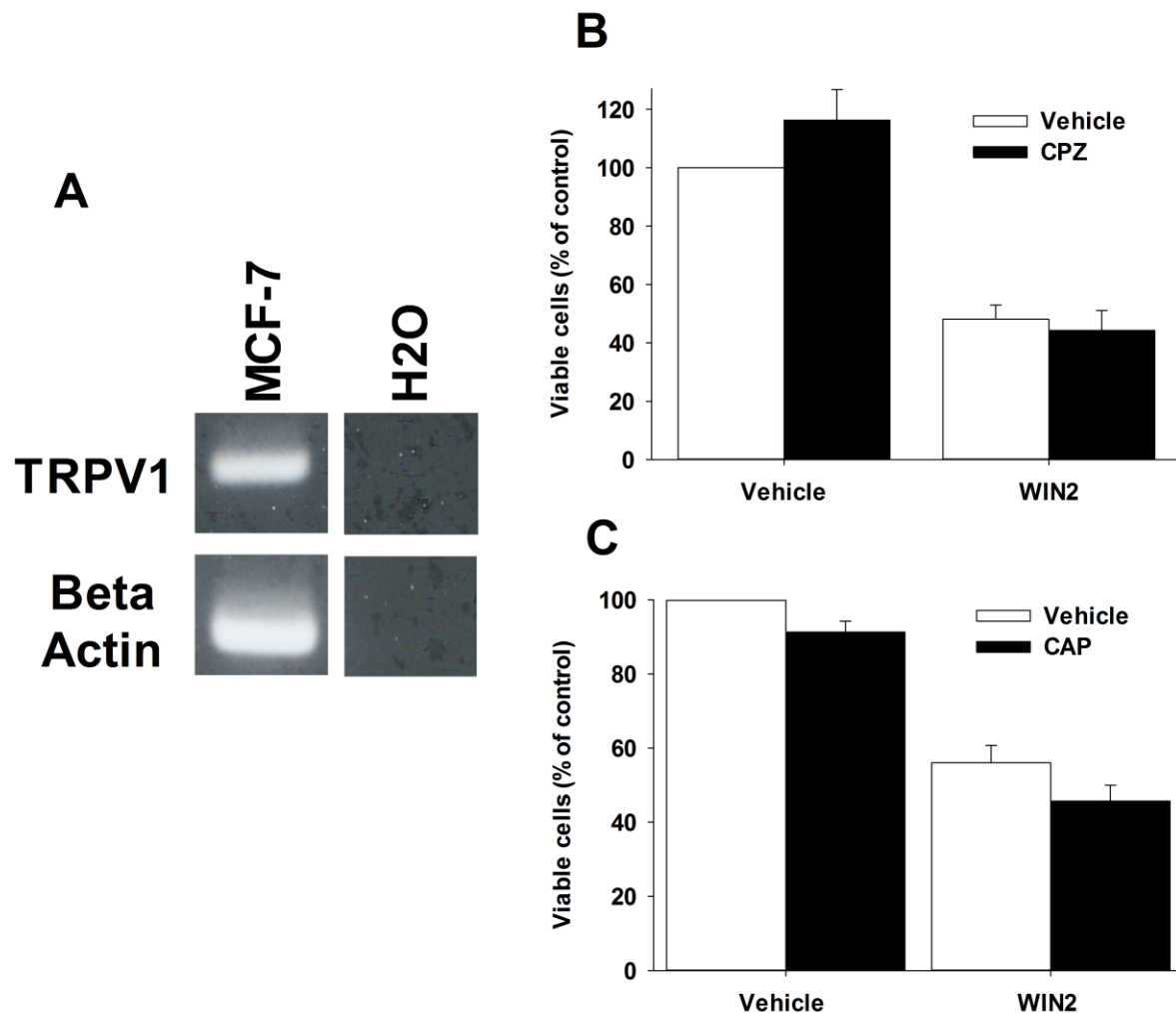


Figure 5.3 – WIN2 has no interaction with TRPV1 in spite of its mRNA expression in MCF-7 cells. (A) RT-PCR for TRPV1 (B) MCF-7 cells were treated with vehicle or WIN2 (12 μ M) and vehicle or capsazapine (10 μ M). (C) MCF-7 cells were treated with vehicle or WIN2 (12 μ M) and vehicle or capsaicin (100 μ M). Cell count with trypan blue was used to assess cell viability at 96 h. Data presented reflect the means of 3 individual experiments \pm se; no significant difference found.

Statistics

(B) Two way repeated measures ANOVA: CPZ-WIN2 interaction ($p=0.2164$)

(C) Two way repeated measures ANOVA: CAP-WIN2 interaction ($p=0.8076$). CAP treatment main effect ($p=0.1410$)

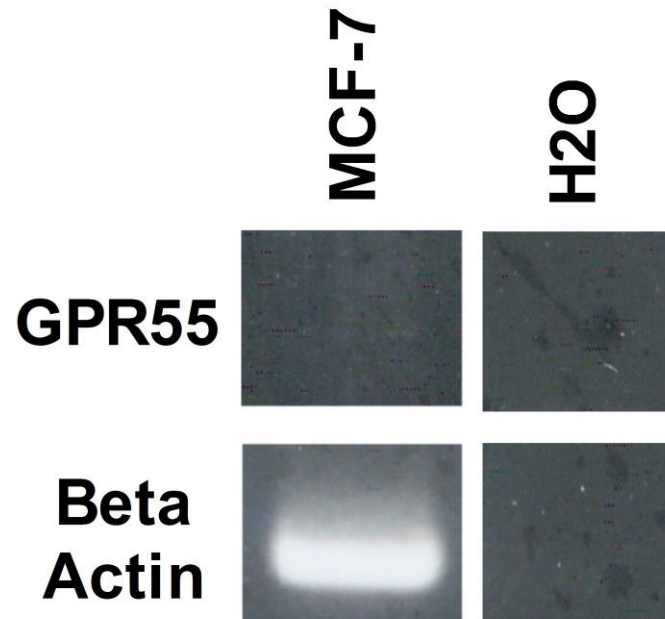


Figure 5.4 – GPR55 mRNA was not found in MCF-7 cells. Representative blot of three.

cells (**Fig 5.4**), and a lack of GPR55 expression eliminated the need for further assessment of its involvement in WIN2 mediated anti-proliferative actions.

Section 5.5 - MCF-7 cell sensitivity to growth inhibition by WIN2 is increased under serum free conditions

Studies to be presented next will utilize a low serum condition. Pertinent to this, Jacobsson et al. (2001) reported that AEA has different potencies for inhibiting C6 glioma cell growth depending on the serum concentration used in media. To assess the antiproliferative and stereospecific actions of WIN2 under low serum conditions, dose-responses for WIN2 and its inactive enantiomer WIN55,212-3 (WIN3) were compared using 0.1% serum conditions (**Fig 5.5**). Comparison of WIN2 and WIN3 show that WIN2 retains its dose-dependent and stereospecific inhibition of MCF-7 cell growth under low serum conditions, and comparisons of the ED₅₀ for WIN2 under low ($3.13 \pm 0.29 \mu\text{M}$) and normal ($11.96 \pm 1.65 \mu\text{M}$) serum also show WIN2 to be more potent under low serum with a potency ratio of 3.39 relative to normal serum conditions. The ED₅₀ for WIN2 in normal serum was reported in section 3.1.

Section 5.6 - WIN2 antagonizes growth stimulation by sphingosine-1-phosphate and SEW2871 but not estradiol

Unpublished studies by Dr. Dana E. Selley suggested WIN2 has actions at the sphingosine-1-phosphate (S1P) system, and the S1P signaling system has been shown important to the proliferation of MCF-7 cells (Sarkar et al 2005). These data suggested that WIN2 inhibited MCF-7 cell growth through activity at S1P receptors; therefore studies were designed to evaluate the S1P system as a potential site for the antiproliferative actions of WIN2

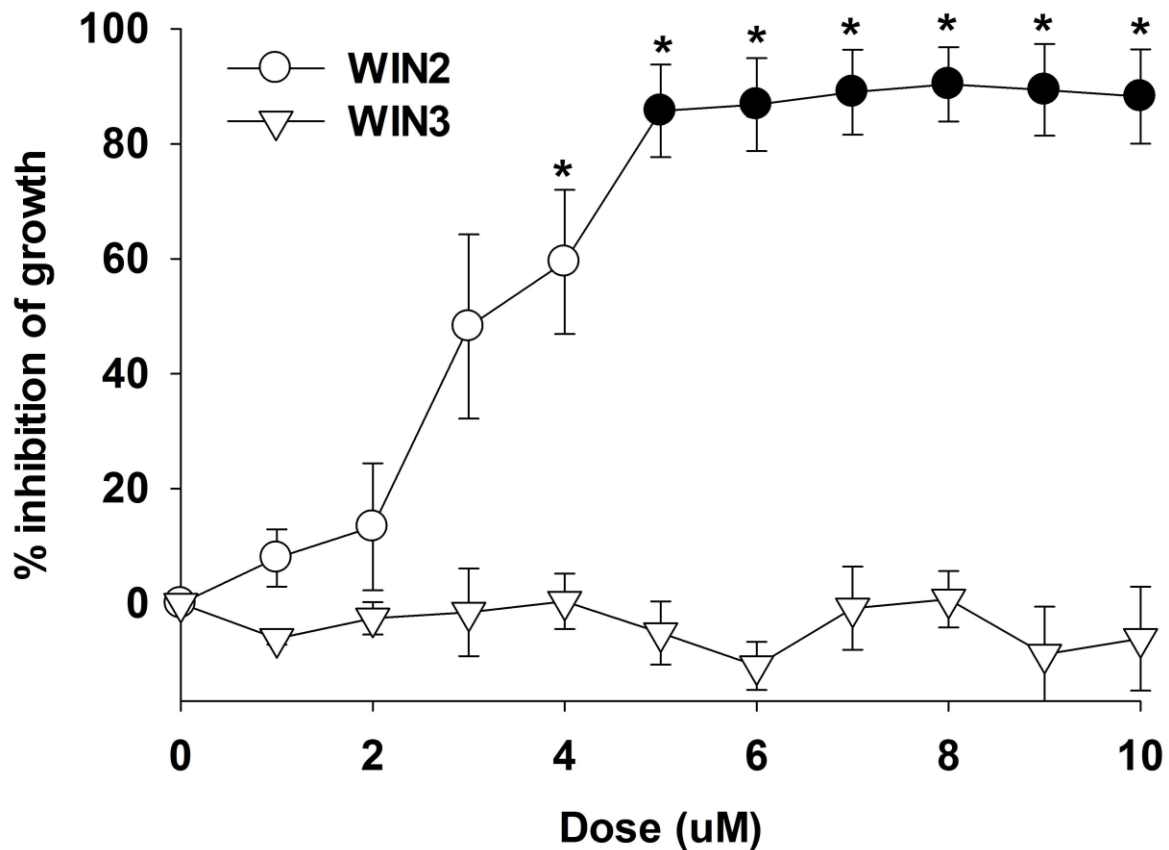


Figure 5.5 – Influence of low serum (0.1%) conditions on response of MCF-7 cells to WIN2 and WIN3. MCF-7 cells were treated with WIN2 (1-10µM) and WIN3 (1-10µM) and cell growth monitored by the crystal violet assay 96 h after treatment. Data presented reflect the means of 3 individual experiments \pm se; *= $p < 0.05$ vs WIN3 at each respective concentration of drug; darkened symbols= $p < 0.05$ vs vehicle.

Statistics

Two way repeated measures ANOVA: drug-dose interaction ($F_{10,22}=9.6$, $p < 0.0001$)

Individual comparisons: WIN2-WIN3 4-10 µM ($p < 0.025$). WIN2-vehicle 5-10 µM ($p < 0.025$). WIN3-vehicle (no significant differences)

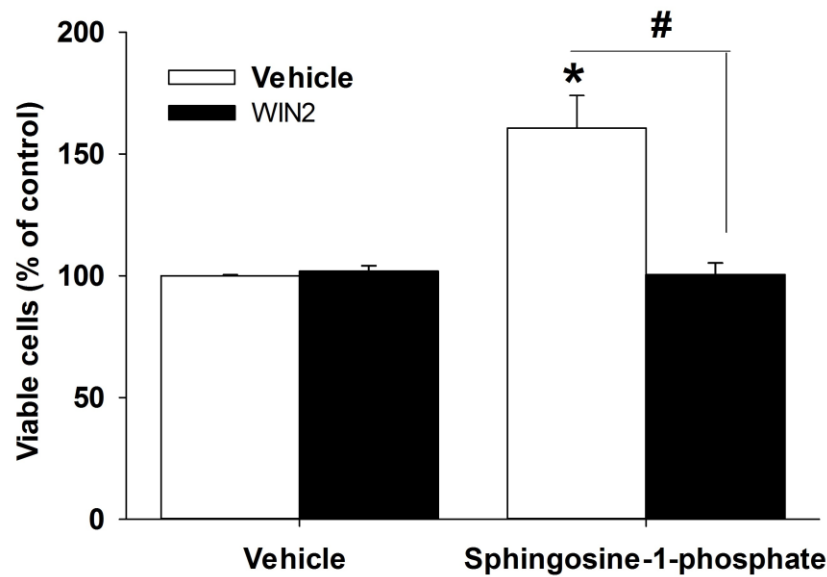


Figure 5.6 – WIN2 interferes with sphingosine-1-phosphate induced growth stimulation. MCF-7 cells were incubated under low serum conditions with 100 nM sphingosine-1-phosphate \pm WIN2 (3 μ M). Trypan blue exclusion was used to assess cell viability at 96 h post treatment. Values are presented as percent of control and represent means \pm se for 3-4 replicate experiments; * $p < 0.05$ vs vehicle; # $p < 0.05$ indicated by bars.

Statistics

Two way repeated measures ANOVA: WIN2-S1P interaction ($F_{1,4}=20.8$, $p=0.0103$)

Individual comparisons: S1P-vehicle ($p=0.0302$). Vehicle-WIN2 (no significant difference). S1P-WIN2 + S1P ($p=0.0074$).

in MCF-7 cells. Under low serum conditions of 0.1 percent serum, 100 nM S1P stimulated MCF-7 cell growth, which was subsequently suppressed by WIN2 (3 μ M); this concentration of WIN2 was not able to inhibit basal cell growth by itself (**Fig 5.6**).

In complementary studies, a sub-effective dose of WIN2 (8 μ M) under normal serum conditions prevented the growth stimulatory effects SEW2871 (5 μ M), the synthetic S1P₁ receptor-selective agonist (**Fig 5.7A**). In contrast, 25 μ M THC failed to inhibit growth stimulation by SEW2871 (**Fig 5.7B**). The differential actions of WIN2 and THC indicate that not all cannabinoids are capable of antagonizing SEW2871 induced breast tumor cell growth. To explore the possibility that WIN2 might be interfering with another growth stimulatory pathway, cells were exposed to 100 nM estradiol in the absence and in the presence of 8 μ M WIN2 (**Fig 5.8**); however, WIN2 failed to antagonize the growth stimulating effects of estradiol. In summary, these studies show that WIN2 antagonism of growth stimulation appears to be selective for the S1P signaling system.

Section 5.7 - WIN2 does not antagonize S1P-stimulated [³⁵S]GTP γ S binding

Agonist-stimulated [³⁵S]GTP γ S binding assays were used to test the ability of WIN2 to antagonize S1P stimulated G protein activation. S1P (0.1, 1 and 10 μ M) was incubated alone or in combination with WIN2 (30 μ M; **Fig 5.9**). WIN2 was unable to alter S1P-stimulated [³⁵S]GTP γ S binding, which may or may not suggest WIN2 has capacity to antagonize S1P signaling at S1P receptors (further elaboration in discussion). WIN2 stimulated [³⁵S]GTP γ S binding was also not found to be significantly greater than basal levels. The S1P receptor system has 5 receptors (Rosen et al. 2009), and Dr. Dana Selley's work has implicated WIN2 as a partial agonist at S1P₁ receptors. The inability of WIN2 to stimulate [³⁵S]GTP γ S binding in MCF-7 cells could reflect that S1P₁ receptors are not present in MCF-7 cells or that S1P₁

receptors are not present in high enough density compared to other S1P receptors to allow detection.

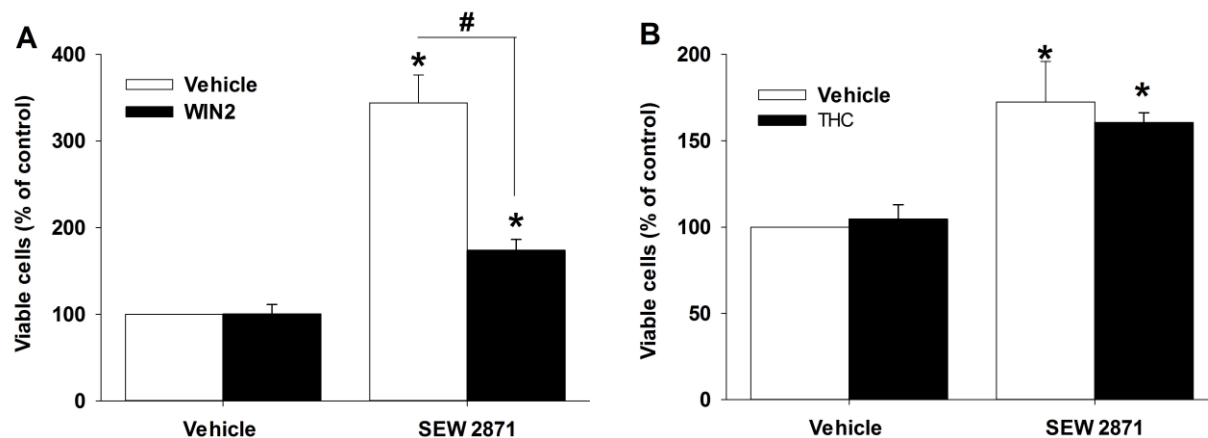


Figure 5.7 – WIN2 but not THC interferes with SEW2871 induced growth stimulation.

MCF-7 cells were incubated with (A) 5 μ M SEW2871 \pm 8 μ M WIN2 or (B) 5 μ M SEW2871 \pm 25 μ M. Trypan blue exclusion was used to assess cell viability at 96 h post treatment. Values are presented as % of control and represent means \pm se for 3-4 replicate experiments; * $p < 0.05$ vs vehicle; # $p < 0.05$ indicated by bars.

Statistics

(A) Two way repeated measures ANOVA: WIN2-SEW2871 interaction ($F_{1,4}=36.3$, $p=0.0038$)

(A) Individual comparisons: Vehicle-SEW2871 ($p=0.0254$). Vehicle-WIN2 + SEW2871 ($p=0.0416$). SEW2871-WIN2 + SEW2871 ($p=0.0038$)

(B) Two way repeated measures ANOVA: THC-SEW2871 interaction ($p=0.5969$). SEW2871 treatment main effect ($F_{1,4}=19.7$, $p=0.0113$)

(B) Individual comparisons: Vehicle-SEW2871 ($p=0.0178$). Vehicle-WIN2 + SEW2871 ($p=0.0131$).

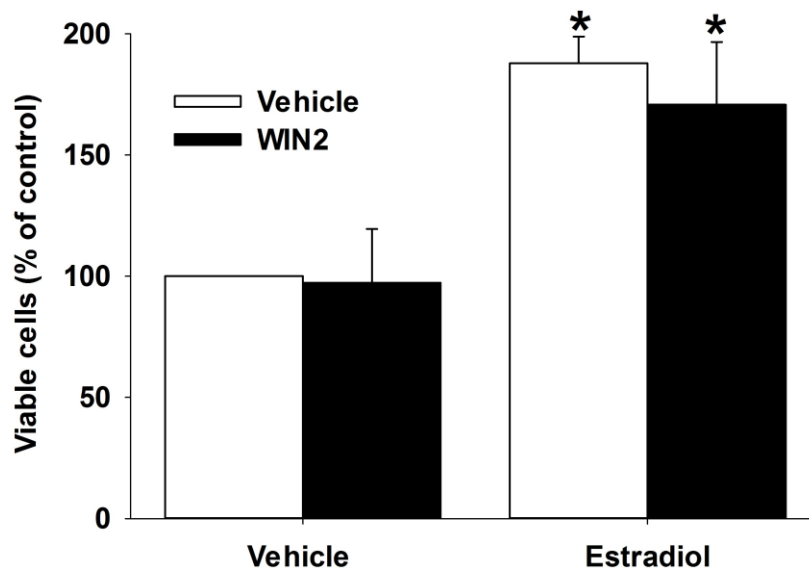


Figure 5.8 – WIN2 fails to interfere with estradiol induced growth stimulation. MCF-7 cells were incubated with 100 nM estradiol \pm 8 μ M WIN2. Trypan blue exclusion was used to assess cell viability at 96 h post treatment. Values are presented as % of control and represent means \pm se for 3-4 replicate experiments; * $p < 0.05$ vs vehicle; # $p < 0.05$ indicated by bars.

Statistics

Two way repeated measure ANOVA: WIN2-Estradiol interaction ($p = 0.7317$). Estradiol treatment main effect ($F_{1,6} = 14.4$, $p = 0.0090$)

Individual comparisons: Vehicle-estradiol ($p = 0.0062$). Vehicle-WIN2 + estradiol ($p = 0.0098$).

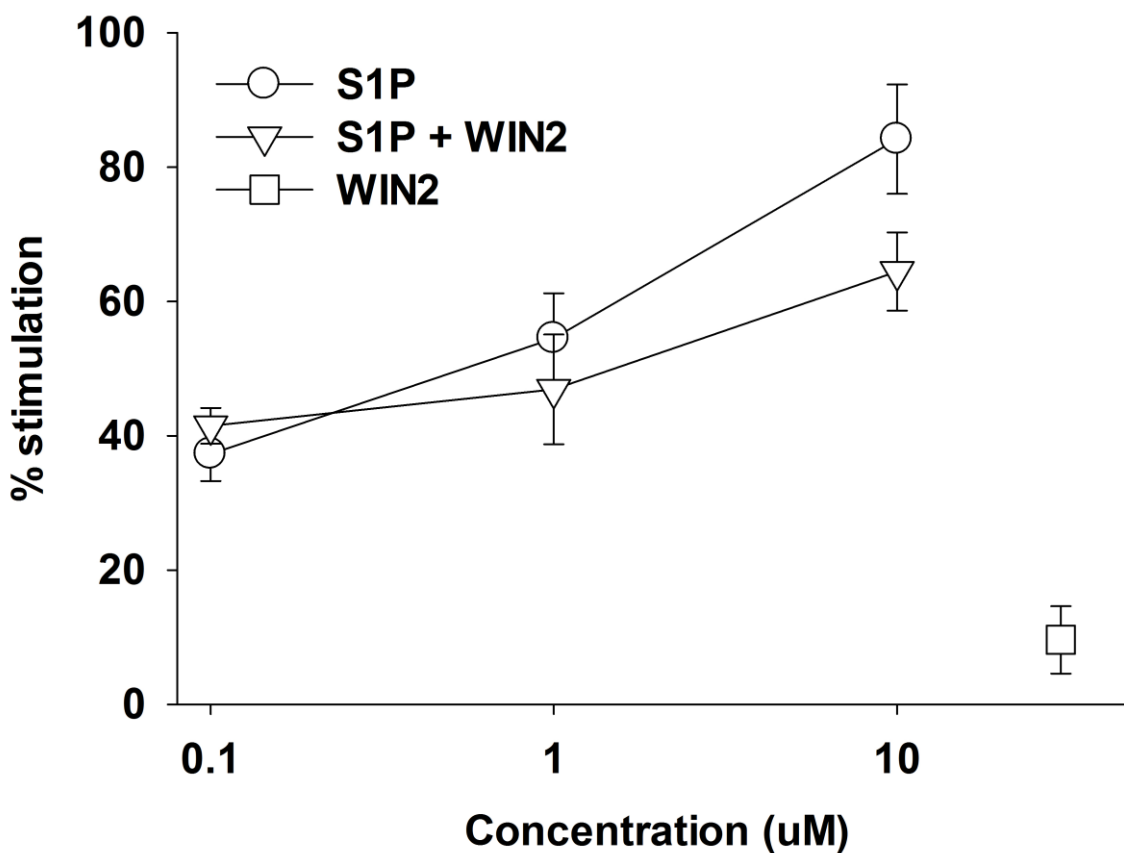


Figure 5.9 – WIN2 does not antagonize S1P stimulated G protein activation. Sphingosine-1-phosphate stimulation of [35 S]GTP γ S binding (0.1-10 μ M) \pm 30 μ M WIN2. WIN2 was also tested alone as a control. Data presented as % stimulation and represent mean \pm se for 3-7 replicate experiments. No significant differences detected between S1P and S1P + WIN2.

Statistics

Two way repeated measures ANOVA: WIN2-S1P interaction ($p=0.3580$). S1P treatment main effect ($F_{1,21}=9.5$, $p=0.0011$)

Discussion

Known cannabinoid sensitive targets are not mediating WIN2 effects

In the current studies, WIN2 was not antagonized by the CB₁ selective antagonist AM251. This is surprising because the CB₁ selective antagonist SR141716 suppressed AEA inhibition of MCF-7 cell growth in Melck et al. 2000. Both WIN2 and AEA are agonists for the CB₁ receptor and should mimic each other's actions at CB₁ (Sim et al. 1996, Showalter et al. 1996). The most likely explanation for this discrepancy is the differences between mRNA expression of CB₁ in MCF-7 cells between the studies. Melck et al. reported a clear expression of CB₁ message in MCF-7 cells while my work showed a poorly detected level of CB₁ message. This is unexpected but differences in RT-PCR results within MCF-7 cells are not unfounded. Varying RT-PCR results in MCF-7 cells include a strong signal for both CB₁ and CB₂ (Melck et al. 2000), low expression of both (Ligresti et al. 2006), no expression of either (Takeda et al. 2008), CB₁ (McKallip et al. 2005) or CB₂ alone (Caffarel et al. 2006). Differences in the RT-PCR protocol could explain the differences in CB₁ expression reported in the presented studies compared to Melck et al., but they would not explain the inability of AM251 to antagonize WIN2 here and full reversal of AEA growth inhibition by SR141716 in Melck et al. Two more likely explanations are that either WIN2 is more potent at a secondary target, which is masking WIN2 actions at those CB₁ receptors present, or the expression of CB₁ in MCF-7 cells is different between these studies due to genomic differences in the cell lines.

Published reports support the possibility of genomic differences between Melck et al.'s work and the work presented above. One study demonstrated that genomic instability can contribute to cancer progression by showing that subclones of the murine fibrosarcoma UV-2237 derived at different times after thawing of stock cells had significant differences in their

metastatic potential when injected into C3H mice (Cifone and Fidler et al. 1981). A more recent demonstration linked genetic instability to alterations in tumor population heterogeneity. Masramon et al. 2006 used DNA fingerprinting by arbitrarily primed PCR in various colon cancer cell lines (SW480, LoVo and HT116) to measure increases in heterogeneity in each population after clonal expansion when compared to parent cells. Genomic instability manifesting as changes in genetic heterogeneity could easily explain the variability in cannabinoid receptor expression between the studies described above: Melck et al. 2000, Ligresti et al. 2006, Takeda et al. 2008, McKallip et al. 2005, Caffarel et al. 2006. Nevertheless, based on the present work it can be concluded that WIN2 is not acting via CB₁ under the current experimental conditions.

In the present studies, WIN2 failed to interact with CB₂, PPAR γ and TRPV1, all of which have previously been shown to mediate potential antiproliferative roles in cancer (Qamri et al. 2009, Caffarel et al. 2010, Yin et al. 2001, Thoennissen et al. 2010). Although message for all three receptors was identified by RT-PCR, the antagonists AM630 (CB₂), GW9662 (PPAR γ) and capsazepine (TRPV1) failed to antagonize WIN2 action (Walpole et al. 1994, Ross et al. 1999, Bendixen et al. 2001). The agonists, pioglitazone (PPAR γ) and capsaicin (TRPV1), also failed to recapitulate antiproliferative effects of WIN2 when administered to MCF-7 cells with the same drug treatment protocol used for WIN2. PPAR α/δ are known to be expressed in MCF-7 cells (Suchanek et al. 2002a and 2002b), but bezafibrate, the pan-PPAR agonist, also failed to recapitulate WIN2 inhibition of MCF-7 cell growth. In the end, these studies indicate that CB₂, TRPV1 and all members of the PPAR family are not involved in the antiproliferative effects of WIN2.

Convergent lines of evidence eliminate the possibility of GPR55 involvement in WIN2 mediated effects. β -arrestin luciferase reporter assay and GPR55 activated luciferase reporter assay in transfected Hek-293 cells (Yin et al. 2009), as well as [³⁵S]GTP γ S binding assays in HEK-293T cell over expressing GPR55 (Johns et al. 2007), have shown GPR55 to be sensitive

to a variety of cannabinoids. However, WIN2 was unable to activate GPR55 in either of these studies suggesting it may not be an agonist for GPR55. In addition, GPR55 has been linked to tumor growth stimulating effects. Expression of GPR55 in human tumors was correlated with decreased patient survival, overexpression of GPR55 in HEK, EVSA-T and T98G cells increased growth rates, and GPR55 knockdown decreased growth rates in EVSA-T and T98G cells (Andradas et al. 2011). These growth stimulating effects of GPR55 were further supported by Pineiro et al. (2011) when genetic knockdown of GPR55 decreased growth rates in PC-3 and OVCAR3 cells. However, the strongest evidence for lack of GPR55 involvement in WIN2 growth inhibition is that RT-PCR indicated no GPR55 message present in MCF-7 cells utilized in these studies.

WIN2 acts through the sphingosine-1-phosphate fatty acid signaling network

S1P is present at concentrations between 0.1 μ M in fetal bovine serum used for culturing and 0.8-1 μ M in human plasma (Murata et al. 2000), and can be synthesized intracellularly by sphingosine kinase 1 (cytoplasmic/membrane) and 2 (nuclear) (Rosen et al. 2009). S1P is also known to activate the 5 known S1P G protein coupled receptors (GPCRs), S1P₁₋₅, (Rosen et al. 2009). S1P has been implicated in a host of disease processes including arthritis, asthma, atherosclerosis, cancer, diabetes and osteoporosis (Maceyka et al. 2012, Orr Grandy et al. 2012). Cancer related effects of S1P include increased proliferation, cell transformation, cell death evasion, drug resistance, inflammation, metastasis and angiogenesis (Takabe et al. 2008, Pyne et al. 2010). In MCF-7 breast cancer cells, knocking down sphingosine kinase 1 (SPK1) depresses chemotactic migration, increases apoptosis after adriamycin treatment and decreases proliferative rates (Sarkar et al. 2005). Western blotting and RT-PCR in MCF-7 cells demonstrates strong expression of S1P₃, which has been identified as a mediator of SPK1

growth stimulating properties (Wang et al. 1999, Sukocheva et al. 2006, Hadizadeh et al. 2008, Sukocheva et al. 2013). RT-PCR analysis identified S1P₂ expression in MCF-7 cells, although to a lesser extent than S1P₃, and conflicting reports suggest S1P₁ may or may not be expressed (Wang et al. 1999, Hadizadeh et al. 2008, Sukocheva et al. 2013). Unlike S1P₃, S1P₁ & 2 have not been assessed for pro-growth or survival activity in MCF-7 cells. One report also identified S1P receptor-independent effects in MCF-7 cells for S1P inhibition of motility (Wang et al. 1999), which indicates further undiscovered complexity present in the S1P signaling system.

In the current work a sub-effective dose of WIN2 antagonized growth stimulation by S1P and the synthetic S1P₁ receptor agonist SEW2871 in MCF-7 cells. Antagonism of S1P and SEW2871 indicates that WIN2 actions interact at some point along the S1P signaling pathway, although it cannot be determined if this interaction is direct or indirect. Interestingly, THC failed to antagonize SEW2871 growth stimulation demonstrating that antagonism of S1P signaling is not a function that generalizes to all cannabinoids. This lack of generalized effects for S1P action across cannabinoid agents might explain why WIN2 and the other aminoalkylindoles were unique in their ability to augment the anti-proliferative effects of radiation. Therefore, it is possible that antagonism of S1P signaling is required for augmentation of the antiproliferative actions of radiation in MCF-7 cells. Future studies should address this possibility starting with testing the other two aminoalkylindoles shown to augment the antiproliferative effects of radiation, JWH-015 and pravadoline, in combination with S1P and SEW2871. Future studies should also evaluate WIN2 interactions with S1P signaling in combination with radiation treatment, although, this will be easier to test once the interaction between WIN2 and S1P system is more fully understood.

Although a specific intersection between WIN2 actions and S1P growth signaling has not been identified by these studies, multiple lines of evidence narrow the pool of potential sites of action. WIN2 antagonism of S1P or SEW2871 growth stimulation and lack of interference with

estradiol-induced growth stimulation demonstrates that WIN2 is not antagonizing cell cycle machinery utilized by both growth stimulating pathways. Additionally, WIN2 antagonism of exogenous S1P or SEW2871 shows that WIN2 is not altering intracellular S1P synthesis or degradation. As a result, WIN2 is likely to be acting on the S1P receptors, intracellular signaling machinery that is downstream of the S1P receptor, or an alternate pathway that intersects with the S1P signaling system.

Evaluating WIN2 actions at S1P receptors is the likely place to start based on unpublished work from our collaborator, Dr. Dana Selley, which indicates that WIN2 acts as a partial agonist at S1P₁ receptors. S1P₁ is potentially expressed in MCF-7 cells, as discussed above (Wang et al. 1999, Hadizadeh et al. 2008, Sukocheva et al. 2013), and if WIN2 acts as a partial agonist at S1P₁ it could also act as a partial antagonist, explaining the observations seen with WIN2 in the studies presented in this work. However, when considering what is known about S1P receptors in MCF-7 cells, evaluating the hypothesis that WIN2 is a partial antagonist for S1P receptors becomes challenging. First, it is unknown if S1P₁ receptors are involved in growth signaling in MCF-7 cells. Second, S1P₃ receptors have been linked to growth stimulation in MCF-7 cells (Sukocheva et al. 2006, Sukocheva et al. 2013), but Dr. Dana Selley's work has not clearly addressed if WIN2 has actions at S1P₃ receptors. Additionally, those reports demonstrated S1P₃ associated growth stimulation as an intermediary of non-genomic estrogen growth signaling (Sukocheva et al. 2006, Sukocheva et al. 2013). Finally, while S1P₃ receptors might be the dominant S1P receptor in MCF-7 cells and a mediator of estrogen growth stimulation, studies presented in this document have demonstrated that WIN2 antagonism of S1P growth stimulation is estrogen independent in nature, which would suggest S1P₃ is not involved in the WIN2 mediated antagonism of S1P growth stimulation. Furthermore, WIN2 antagonized growth stimulation by the S1P₁ receptor selective agonist SEW2871 (Sanna et al. 2004), which does argue for an S1P₁ dependent mechanism.

[³⁵S]GTPγS binding studies were used to potentially answer some of these questions, but WIN2 could neither significantly stimulate [³⁵S]GTPγS binding alone, nor could it antagonize S1P stimulated [³⁵S]GTPγS binding. At first, these results suggest that WIN2 is not acting as a partial agonist/antagonist at S1P receptors but there are other possibilities to consider. The most obvious is that the duration of WIN2 treatment in the [³⁵S]GTPγS binding and cell count studies are different, 2 vs. 24 h respectively, and therefore the [³⁵S]GTPγS binding studies do not accurately mimic observations from the cell count studies. It is also possible that the kinetics for [³⁵S]GTPγS binding are different between S1P receptors, and the [³⁵S]GTPγS binding studies presented here are demonstrating a preference for one S1P receptor over the other providing an incomplete picture of WIN2 action. Another possibility is that S1P₁ receptors are expressed at considerably lower levels than S1P₃ receptors, and the [³⁵S]GTPγS binding assay is simply not sensitive enough to detect actions at these receptors. Any of these eventualities would prevent the [³⁵S]GTPγS binding assay from being able to determine if WIN2 is acting at S1P₁ as either a partial antagonist, which would inhibit S1P signaling, or as a partial agonist which would cause receptor downregulation, effectively silencing the S1P system. Future studies could address these possibilities. qRT-PCR and western blotting could identify the S1P receptors present in these MCF-7 cells and determine whether WIN2 has effects on the levels of receptor expression after treatment. Selective genetic knockout or pharmacological antagonism of S1P receptors can detect if WIN2 antagonism of S1P growth stimulation is mediated through a specific S1P receptor. Also, further [³⁵S]GTPγS binding studies using longer incubation times and either genetic knockdown or pharmacological antagonism of S1P₃ receptors could more accurately test the involvement of S1P₁ receptors in MCF-7 cells, assuming qRT-PCR and western blotting can confirm their expression.

It does also remain possible that WIN2 actions are not mediated by the S1P receptors, and WIN2 actions occur either further downstream of the receptor or in a secondary pathway

that antagonizes S1P signaling indirectly. For instance, growth stimulation by S1P occurs through RAS signaling. When SPK1 is antagonized and knocked down in T24 bladder cancer cells, RAS-GTP is decreased (Shu et al. 2001). Based on the involvement of RAS in S1P dependent cell growth, as well as WIN2 demonstration of classical growth arrest in section 4 of this document, RAS is a logical candidate for future studies examining the effects of WIN2 in MCF-7 cells. AKT is another possible target based on work from Park et al. (2011), who reported a WIN2 induced G1 cell cycle growth arrest in gastric cancer cell that was rescued by overexpression of active myristoylated-AKT. Lastly, Osawa et al. (2001) showed that S1P treatment protected hepatoma cells from apoptosis and increased p-AKT expression, while SPK1 antagonism increased apoptosis and decreased p-AKT expression. Based on these two studies AKT should also be considered a candidate for evaluation in future studies.

A substantial amount of work remains to accurately identify the site of action for WIN2 in MCF-7 cells, and whether that site of action is responsible for the observed augmentation of radiation by WIN2 presented in previous chapters. Nevertheless, it can be concluded from the work presented here that WIN2 has actions that intersect with and antagonize the S1P signaling system.

Summary

Section 3 of this document showed that WIN2 was able to augment the antiproliferative actions of radiation in MCF-7 cells, and section 4 attributed this augmentation to parallel mechanisms of growth arrest. This section, section 5, was focused on elucidating the receptor mediating WIN2 signaling in MCF-7 cells. RT-PCR and pharmacological analysis was used to exclude the involvement of known cannabinoid sensitive targets CB₁, CB₂, PPAR α - γ , TRPV1 and GPR55. WIN2 antagonism of S1P associated growth stimulation, however, indicated a

novel site of action for WIN2 in MCF-7 cells that interacts with or is present in the S1P signaling system. Future studies are still required to determine the novel site of actions and confirm that WIN2 actions at this site mediate the WIN2/IR augmentation.

Section 6

Summary, Discussion and Future Studies

The primary findings of this work are that WIN55,212-2 (WIN2) has the capacity to augment the antiproliferative effects of radiation in breast tumor cells, and that other cannabinoids that fail to have this effect do not interfere with the actions of radiation. Studies in MCF-7 cells were confirmed in MDA-MB231 cells and 4T1 cells. WIN2 augmentation of the antiproliferative effects of radiation were also shown to be stereospecific using the inactive enantiomer of WIN2, WIN55,212-3 (WIN3). These findings serve as effective proof of principle that WIN2, or similar compounds could enhance patient survival if given in combination with radiation, but due to complications with the *in vivo* studies, discussed below, it remains imperative that future studies expand this work beyond the *in vitro* models used here.

Opposite to its combination with radiation, WIN2 failed to augment the effects of doxorubicin (Adriamycin) in MCF-7 cells. As is the case with radiation, ADR induces senescence in MCF-7 cells (Jones et al. 2005; Goehe et al. 2012). One possible explanation for why WIN2 enhanced the antiproliferative effects of radiation but not doxorubicin is that senescence induced by doxorubicin is different than senescence induced by radiation. Bristol et al. (2012) demonstrated that blockade of autophagy after radiation treatment forced the cells to switch from an entirely senescent response to cell death through apoptosis, while Goehe et al. (2012) demonstrated blockade of autophagy after ADR treatment only caused a delay in the onset of senescence without a significant enhancement of the antiproliferative effects of ADR. With these differing reports of senescent responses in MCF-7 cells, it cannot be assumed that WIN2 actions will interact with radiation and ADR in the same way.

The mechanism of action for the WIN2/IR combination identified *in vitro* was studied primarily in the MCF-7 cell model. Radiation-induced senescence, assessed by β -galactosidase staining, confirmed previous findings of radiation-induced senescence (Jones et al 2005);

however, WIN2 had no effect on the extent of senescence induction. Quantification of DNA damage and repair by γ H2AX labelling also indicated that WIN2 did not increase the extent of DNA damage or interfere with cellular repair of DNA elicited by radiation treatment. Salazar et al. (2009) showed that a Δ^9 -tetrahydrocannabinol (THC) treatment in U87-MG glioblastoma induced autophagy that was toxic to tumor cells, and studies presented here show autophagy was clearly induced by both radiation and WIN2. However, pharmacological blockade of autophagy did not interfere with the effectiveness of the combination treatment, demonstrating that cellular sensitivity to radiation was not augmented by WIN2 via autophagy. Annexin V and PI staining combined with DAPI imaging of nuclear morphology demonstrated that apoptosis, necrosis and mitotic catastrophe were not induced by the WIN2/IR combination. When the effects of the WIN2/IR combination on senescence are considered in the absence of cell death, it can be concluded that WIN2 is likely to be inducing classical growth arrest, either as a growth delay or cell cycle arrest type event, and this is confirmed by temporal studies. Additionally, this classical growth arrest conclusion combined with the temporal studies indicates that the augmentation of the antiproliferative effects of radiation by WIN2 is mediated by parallel mechanisms of classical growth arrest (WIN2) and senescent growth arrest (IR).

Subsequent studies were designed to identify the receptor(s) mediating the antiproliferative actions of WIN2 in breast tumor cells. Although expression of CB₁, CB₂, PPAR γ and TRPV1 were shown in the MCF-7 cells, pharmacological experiments using various agonists and antagonists of these selected receptor pathways demonstrated that CB₁, CB₂, PPAR γ and TRPV1 were not mediating the antiproliferative effects of WIN2. Pharmacological experiments were extended to show that PPAR α - γ were also not involved in the antiproliferative mechanism of WIN2. GPR55 was excluded as a potential target based on a lack of receptor expression, as well as reports that GPR55 supports tumor growth and does not interact with WIN2 (Johns et al. 2007; Yin et al. 2009; Andradás et al. 2011; Pineiro et al. 2011; Perez-

Gomez et al. 2012). Previous work in CB₁ knockout brains has demonstrated that WIN2 has actions at a GPCR that is as of yet unidentified in the literature, which supports the possibility that WIN2 is acting at a novel site of action (Brievogel et al. 2001). With all known cannabinoid sensitive targets eliminated as potential sites of action for WIN2, studies were designed to address possible interactions with sphingosine-1-phosphate (S1P) (Selley D., unpublished data).

A sub-effective dose of WIN2 antagonized growth stimulation by S1P receptor agonists (S1P and SEW2871) in MCF-7 cells; this finding connects the antiproliferative properties of WIN2 to the S1P signaling pathway, but it does not identify a specific target of action. Several complementary lines of evidence did, however, narrow the pool of potential candidate sites. For example, WIN2 did not antagonize the growth stimulating effects of estradiol, confirming that the mechanism for WIN2 is specific to S1P signaling. Also, WIN2 antagonism of exogenous S1P and SEW2871 shows that WIN2 is not altering intracellular S1P synthesis or degradation. [³⁵S]GTPγS binding studies were attempted to confirm or refute the involvement of WIN2 actions at the S1P receptors, but limitations of the studies prevent a conclusion of this nature. Future studies are still requires assess WIN2 actions at or on the S1P receptors present within MCF-7 cells.

It is also possible that WIN2 is directly antagonizing a downstream component of the S1P signaling system, but this cannot be concluded since WIN2 could be acting outside of the S1P signaling pathways causing alterations to the S1P signaling system indirectly. One example of this type of indirect mechanism is the inhibition of MCF-7 growth by methanandamide (MAEA) reported by Laezza et al. (2006; 2010). MAEA treatment caused down regulation of HMG-CoA reductase leading to decreases in pools of mevalonic acid and prevented various proteins from trafficking to the membrane from the cytosol, one of which included the well-known growth stimulating protein RAS. This indirect down regulation of RAS by MAEA was shown to inhibit

growth of MCF-7 cells. Although WIN2 may cause down regulation of RAS in MCF-7 cells, it is unlikely that WIN2 would do so by down regulating HMG-CoA reductase. MAEA was shown to down regulate HMG-CoA via a CB₁ dependent mechanism in Laezza et al. 2006, and studies presented in this document demonstrated that CB₁ is not involved in the actions of WIN2. Nevertheless, based on the classical growth arrest mechanism associated with WIN2 in MCF-7 cells presented here, and Shu et al.'s (2001) demonstration of S1P signaling through RAS, it would be logical to evaluate RAS under WIN2 treatment. Initial studies would determine RAS levels and activity after vehicle, WIN2, IR and WIN2 + IR treatments.

A second potential target was identified in studies reported by Park et al. (2011), where WIN2 induced AKT down regulation and a G1 cell cycle growth arrest in gastric cancer cells that were rescued by overexpression of active myristoylated-AKT. Caffarel et al. (2010) also showed AKT dependent growth inhibition where overexpression of AKT in N202.1A breast cancer cells prevented both THC and JWH-133 from inhibiting cell growth. Furthermore, S1P signaling was connected to AKT in hepatoma cells where S1P administration decreased apoptosis and increased p-AKT expression, and antagonism of the kinase responsible for S1P production, sphingosine kinase 1, increased apoptosis and decreased p-AKT expression (Osawa et al. 2001). Quantifying AKT and p-AKT levels after vehicle, WIN2, IR and WIN2 + IR treatment in MCF-7 cells would evaluate potential involvement of AKT in the WIN2 mediated mechanism of growth inhibition.

There still remains a great deal of work to do before identification of the site of action for WIN2 can be elucidated conclusively. These studies may also include broader studies that utilize microarray, proteomic or metabolomic work to identify novel targets and non-canonical mechanisms that were not hypothesized here, and even when this site of action is identified it must still be connected to the WIN2 mediated augmentation of radiation. Nevertheless it can be

concluded from the work presented in this document that WIN2 has the capacity to interact with the S1P signaling system in some capacity to affect growth.

In addition to MCF-7 cells, discussed above, studies were completed using the WIN2/IR combination in the MDA-MB231 cell line where WIN2 demonstrated significant augmentation of the antiproliferative effects of radiation. Similar to the augmentation in MCF-7 cells, the augmentation in MDA-MB231 cells was also confirmed to be stereospecific in nature using WIN3. Time course studies showed that a growth inhibitory phenotype was present in MDA-MB231 cells as was present in MCF-7 cells. Mechanistic studies were not performed in MDA-MB231 cells, in part due to the differences in the reported mechanisms of action for radiation in MCF-7 and MDA-MB231 cells, senescence and apoptosis respectively (Jones et al. 2005). Identification of a target of action for WIN2 in MCF-7 cells could guide receptor evaluations in MDA-MB231 cells, but until a novel site of action is elucidated all cannabinoid sensitive targets (CB₁, CB₂, PPAR α - γ , TRPV1 and GPR55) must be systematically evaluated in MDA-MB231 cells just as they were in MCF-7 cells. This systematic evaluation would also include the interaction between WIN2 and S1P growth stimulation.

The WIN2/IR combination also augmented the effects of radiation in 4T1 cells *in vitro*. However, *in vivo* studies using the 4T1-Balb/c syngeneic tumor growth model failed to show augmentation of the antiproliferative effects of radiation by WIN2. However, this lack of augmentation was likely confounded by the fact that WIN2 did not inhibit tumor growth alone in these animals, which was entirely unexpected since the dose of WIN2 used in the combination study was based on a previous dose-response study in the 4T1-Balb/c model where WIN2 significantly inhibited tumor growth. It is unclear why WIN2 failed to replicate its inhibition of tumor growth *in vivo* between the two studies, and, to date, no variables have been identified that could explain the differences between the studies. Studies should be conducted to ascertain the effects that WIN2 and IR have in combination using a whole animal, but future

efforts utilizing the MCF-7 model in immune compromised mice implanted with estrogen pellets might prove to be a better approach.

Contrasting the breast cancer cells used (MCF-7, MDA-MB231 and 4T1 cells), MCF-10A cells are a non-transformed immortalized breast epithelial cell line that was used in these studies to test the ability of WIN2 to augment the toxicities of radiation to normal cells. While high doses of WIN2 (30 μ M) did augment radiation mediated antiproliferative actions in MCF-10A cells, the dose of WIN2 used in the MCF-7 cell studies (12 μ M) was unable to elicit growth inhibition by WIN2 alone or in combination with radiation in MCF-10A cells. This finding demonstrates that WIN2 is less potent in the non-transformed MCF-10A cells, and this lower potency suggests a therapeutic window in treatment that would create selectivity for cancer cells over normal tissue. As non-cancerous tissues in the body exist largely in a non-proliferative state, if WIN2 is acting through a classical growth arrest mechanism without inducing cell death, then adverse effects on non-cancerous tissue would be even more unlikely to occur in the whole animal. Nevertheless, future studies should be performed using proliferating non-cancerous tissues such as gastrointestinal epithelial cells to determine if WIN2 is capable of antagonizing their growth, because unlike focused irradiation, WIN2 will be distributed throughout the body by systemic blood circulation.

In addition to testing the WIN2/IR combination in multiple cells lines, multiple cannabinoid/IR combinations were tested in MCF-7 cells. These include THC, CBD, nabilone, CP55,940 and methanandamide, which all failed to interact with radiation in MCF-7 cells. The aminoalkylindoles, JWH-015 and pravadoline however, significantly augmented the effects of radiation at the highest concentrations tested (45 μ M). Later studies showed that WIN2 antagonized S1P stimulated growth but THC failed to replicate this antagonism, which may have provided some indications as to why only some cannabinoids interacted with radiation.

Pertinent future studies will include testing JWH-015 and pravadoline as antagonists of S1P stimulated growth in MCF-7 cells.

Future work should include structure-activity relationship studies to screen analogs of WIN2 for compounds that are more efficacious at inhibiting MCF-7 cell growth and/or augmenting the antiproliferative effects of radiation. Structure-activity relationship studies could be performed even in the absence of a confirmed target of action for WIN2. Identification of more efficacious analogs of WIN2 as antagonists of S1P stimulated growth, could additionally screen for compounds that possess less profound cannabimimetic effects compared to the parent compound WIN2, as these side-effects have impeded clinical development of WIN2 thus far (Howlett et al. 2002; Pertwee et al. 2010). Decreases in the cannabimimetic effects of these drugs could also enhance their likelihood of FDA approval, which is the first step to using novel drugs with radiation augmenting properties like WIN2 to prolong patient survival.

Section 7

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Section 8

Vita

EDUCATION

- Ph.D. Department of Pharmacology and Toxicology, Virginia Commonwealth University– Combined antiproliferative effects of the aminoalkylindole WIN55,212-2 and radiation in breast cancer cells – Jan, 2014
- B.S. Virginia Polytechnic Institute and State University – Double major in Biology and Biochemistry – May, 2007

PROFESSIONAL EXPERIENCE

- 2007 – 2014 Graduate Research, Virginia Commonwealth University. Dr. Gewirtz (primary mentor) and Dr. Lichtman (co-mentor)
- 2006 – 2007 Undergraduate Research, Virginia Polytechnic Institute and State University. Sible Lab

AWARDS

- 2014 Publication selected for the cover of the Journal of Pharmacology and Experimental Therapeutics – “Combined antiproliferative effects of the aminoalkylindole WIN55,212-2 and radiation in breast cancer cells”
- 2011-2014 Predoctoral Traineeship Award – Department of Defense Breast Cancer Research Program – “Involvement of the Endocannabinoid System in the Development and Treatment of Breast Cancer”
- 2011 Conference Travel Award – International Cannabinoid Research Symposium

PUBLICATIONS

- 2013 – **Emery SM**, Alotaibi MR, Tao Q, Selley DE, Lichtman AH and Gewirtz DA Combined antiproliferative effects of the aminoalkylindole WIN55,212-2 and radiation in breast cancer cells.
- JPET cover – February 2014
- 2012 – Bristol ML, **Emery SM**, Maycotte P, Thorburn A, Chakradeo S, Gewirtz DA. Autophagy inhibition for chemosensitization and radiosensitization in cancer: do the preclinical data support this therapeutic strategy?
- 2012 – Biggers JW, Nguyen T, Di X, Gupton JT, Henderson SC, **Emery SM**, Alotaibi M, White KL Jr, Brown R, Almenara J, Gewirtz DA. Autophagy, cell death and sustained

senescence arrest in B16/F10 melanoma cells and HCT-116 colon carcinoma cells in response to the novel microtubule poison, JG-03-14.

EXPERIMENTAL SKILLS

In vivo

- Animal handling and drug administration (intraperitoneal, subcutaneous, and gavage)
- Behavioral assays: Morris water maze memory task, rotorod, spontaneous activity, hot plate, inverted screen test, body temperature measurement
- Tumor growth assays: Dimethylbenzanthracene (DMBA) tumor induction in mice, xenograft implantation and animal monitoring (4T1 cells), Xenogen measurement of tumor growth using luciferin

Cell biology

- Cell lines maintenance: MCF-10a, MCF-10f, MCF-7, MDA-MB-231, and 4T1
- Development of novel cell lines from tumor tissue
- Assays: trypan blue (cell viability), crystal violet (cell growth), MTT (cell viability), boyden chamber (cell invasion), polyhema based anchorage independent growth, acini growth using matrigel based 3D cultures

Histochemistry

- Basic tumor histological analysis from *in vivo* samples
- Microscopy: β -galactosidase activity, TUNEL, DAPI (nuclear staining), acridine orange (autophagy)
- Flow cytometry analysis: Annexin V and PI, cell cycle analysis, acridine orange (autophagy), florescent antibodies labeling, β -galactosidase activity

Molecular techniques

- Bradford protein assay, western blot analysis (chemiluminescence and licor)
- RT-PCR primer design

PRESENTATIONS AND POSTERS

- 2012 - Carolina Cannabinoid Collaborative Conference – Presentation – “Enhanced Antiproliferative Actions of Combined Radiation and WIN55,212-2 on MCF-7 Breast Cancer Cells: Exploration of a Mechanism of Action” – Greenville, North Carolina
- 2012 - Virginia Academy of Science – Presentation – “The Interaction Between WIN55,212-2 and Radiation on Inhibiting the Growth of Breast Cancer Cells” – Norfolk State University, Norfolk, Virginia
- 2012 - American Association of Cancer Research – Poster – “The Cannabinoid WIN55, 212-2 Enhances the Response of Breast Cancer Cells to Radiation” – Chicago, Illinois
- 2012 - Pharmacology and Toxicology Research Retreat – Poster – “Combining Cannabinoids and Radiation in Breast Cancer” – Williamsburg, Virginia

- 2011 - Watts Day Presentation – Poster – “Combining Cannabinoids and Radiation in Breast Cancer” – Virginia Commonwealth University, Richmond, Virginia
- 2011 - Massey Cancer Center Research Retreat – Poster – “Combining Cannabinoids and Radiation in Breast Cancer” – Virginia Commonwealth University, Richmond, Virginia
- 2011 - Department of Pharmacology and Toxicology – Student Seminar Series – “The Interaction Between WIN55,212-2 and Radiation on Breast Cancer” – Virginia Commonwealth University, Richmond, Virginia
- 2011 - Pharmacology and Toxicology Research Retreat – Poster – “Combining Cannabinoids and Radiation in Breast Cancer” – Virginia Commonwealth University, Richmond, Virginia
- 2011 - Era of Hope – Poster – “Role of the Endogenous Cannabinoid System in a Murine Model of Breast Cancer” – Washington DC
- 2011 - Virginia Academy of Science – Presentation – “Combining Cannabinoids and Radiation Therapy in Breast Cancer” – University of Richmond, Richmond, Virginia
- 2011 - 7th Annual Women’s Health Research Day at VCU – Poster – “Combining Cannabinoids and Radiation Therapy in Breast Cancer” – Virginia Commonwealth University, Richmond, Virginia
- 2011 - Department of Pharmacology and Toxicology – Student Seminar Series – “Cannabinoids and Cancer Therapy” – Virginia Commonwealth University, Richmond, Virginia
- 2011 - International Cannabinoid Research Symposium – Poster – “Combining Cannabinoids and Radiation Therapy in Breast Cancer” – Chicago, Illinois
- 2010 - International Cannabinoid Research Symposium – Poster – “Cell Line Specific Enhancement of Sensitivity to Adriamycin by Phytocannabinoids in Breast Cancer” – Lund, Sweden
- 2010 - Virginia Academy of Science – Presentation – “The Full Agonist WIN55, 212-2 Exerts Growth Inhibitory Effects through a Cannabinoid Receptor Independent Mechanism” – James Madison University, Harrisonburg, Virginia
- 2010 - Department of Pharmacology and Toxicology – Student Seminar Series – “Cannabinoids and Cancer: Tumor Development and Treatment” – Virginia Commonwealth University, Richmond, Virginia
- 2009 - Department of Pharmacology and Toxicology – Student Seminar Series – “The Involvement of the Endocannabinoid System in the Development of Breast Cancer” – Virginia Commonwealth University, Richmond, Virginia

CONFERENCES

- 2012 Carolina Cannabinoid Collaborative Conference – Greenville, NC
- 2012 Virginia Academy of Science – Norfolk, VA
- 2012 American Association of Cancer Research – Chicago, IL
- 2011 International Cannabinoid Research Symposium – Chicago, IL
- 2011 Virginia Academy of Science – Richmond, VA

2010	International Cannabinoid Research Symposium – Lund, Sweden
2010	Annual NIH Career Symposium – Bethesda, MD
2010	Virginia Academy of Science – Madison, VA
2008	Carolina Cannabinoid Collaborative Conference – Williamsburg, VA

SERVICE

2012	Pharmacology and Toxicology Student Organization volunteer – St. Josephs Villa
2011	VCU graduate admissions – assistant with interviewing student dinners and lunches
2010	Pharmacology and Toxicology Student Organization graduate recruitment volunteer – interviewing students dinner coordination
2008 – 2009	Student Government Association representative for the Pharmacology and Toxicology Student Organization
2008 – 2009	Pharmacology and Toxicology graduate admissions assistant with interviewing student dinners and lunches