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Award Number: W81XWH-11-1-0163

TITLE: Involvement of the Endocannabinoid System in the Development and Treatment of Breast Cancer

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REPORT DATE: ~~April~~ 2012

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
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1. REPORT DATE (DD-MM-YYYY) 01-02-2012		2. REPORT TYPE Annual Summary		3. DATES COVERED (From - To) 15 Jan 11-14 Jan 12	
4. TITLE AND SUBTITLE Involvement of the Endocannabinoid System in the Development and Treatment of Breast Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-11-1-0163	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Sean Emery E-Mail: emerysm@vcu.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Virginia Commonwealth University Richmond, VA 23219				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The synthetic cannabinoids, Win55,212-2 (Win2), enhanced the growth-inhibitory response to ionizing radiation in three different (MCF7, MDA-MB-231 and 4T1) experimental breast tumor cell lines. Studies using an inactive analog of Win-2 demonstrated that this effect was structure-specific. This finding was confirmed in an immune-competent (4T1) rodent model of tumor growth. Unexpectedly, Win2 failed to improve the response to a variety of cancer chemotherapeutic agents such as Adriamycin and Paclitaxel; studies in vivo also failed to demonstrate that the combination of Win-2 + Adriamycin was more effective than Adriamycin alone. However, in view of the fact that cannabinoids are currently used to improve the quality of life for cancer chemotherapy patients, the absence of antagonistic effects suggests that Win2 could be used in combination with radiation and chemotherapy without interfering with the effectiveness of chemotherapeutic agents as antitumor drugs.					
15. SUBJECT TERMS Cannabinoids, Breast Cancer, Radiation, Chemotherapy, Win55,212-2, Drug Interaction					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code)
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Introduction

I am currently a predoctoral candidate at Virginia Commonwealth University working toward a Ph.D. degree in the Department of Pharmacology and Toxicology under the guidance of mentors Dr. David Gewirtz (primary) and Dr. Aron Lichtman (secondary). This grant is supporting my current research on a project that was initiated through the Department of Defense Breast Cancer Research Program to evaluate the influence of cannabinoids on the effectiveness of chemotherapy and radiation in the treatment of breast cancer. A closely related goal is to determine whether the use of cannabinoids might interfere with the effectiveness of breast cancer therapies. My training involves the development of an in-depth understanding of current and proposed treatments for breast cancer, focusing on both chemotherapeutic drugs and ionizing radiation, as well as the nature of tumor cells response to treatment, encompassing growth arrest (cell cycle checkpoint arrest and senescence) and cell death (apoptosis, autophagy, mitotic catastrophe and necrosis). Additional studies involve efforts to elucidate receptor mediated effects as well as receptor-linked signaling pathways. I am also developing expertise in use of the scientific method for experimental design and technical execution at the bench. In addition, the training also focuses on the development of necessary scientific career skills through literature review, the writing of grants and manuscripts as well as formal oral presentations through both poster and slideshow based presentations.

Body

Specific Aim 1 of my statement of work indicated that we would “Evaluate the interaction between various phytocannabinoids and common chemotherapeutics in established breast cancer cell lines.” We proposed to pursue this aim via three separate tasks including establishing drug interaction, determining changes in mechanism (i.e. mode of cell death), and confirming that the observed interactions would transfer to established in vivo models of tumor development. Our initial experiments were directed at establishing the interaction between the cannabinoids Δ^9 -tetrahydrocannabinol (THC) and Cannabidiol (CBD) and the chemotherapeutic drugs Adriamycin (ADR) and Paclitaxel (Taxol), all of which were proposed in the narrative. Preliminary experiments that combined THC and ADR, CBD and ADR, and THC and Taxol showed no significant enhancements of drug sensitivity in MCF-7 breast tumor cells (fig 1A, B and C). Nevertheless, these

studies clearly demonstrated that the cannabinoid compounds did not interfere or attenuate the effectiveness of these therapeutic agents; these findings indicate that when cannabinoids are used for suppression of nausea or other side effects of chemotherapy, their use would be unlikely to have a negative impact on the clinical treatment of breast cancer.

We expanded the cannabinoid of interest to include Nabilone and Win55,212-2 (Win2). Nabilone was chosen for its clinical relevance in the treatment of emesis associated with chemotherapy, while Win2 was chosen because it is a more potent and maximally efficacious synthetic cannabinoid that would ensure full activation of the cannabinoid receptors (1,2). We also expanded the spectrum of chemotherapeutic agents to include Methotrexate (MTX), 5-Fluorouracil (5-FU) and Cisplatin (CP), all of which are conventionally used in the treatment of

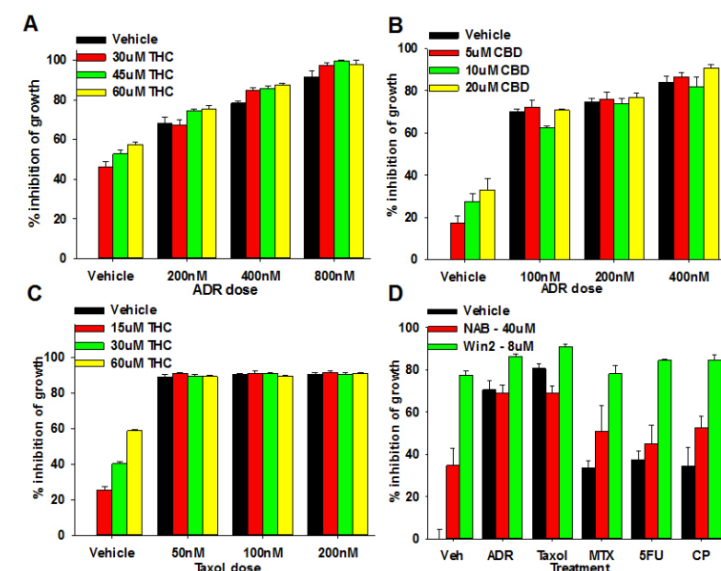


Figure 1 – Cannabinoid combination with chemotherapeutics demonstrate no significant enhancements in growth inhibition. MCF-7 cells plated in the 96 well plate crystal violet assay were subjected to various combinations of chemotherapeutics and cannabinoids. The data was then normalized as % inhibition of growth. Individual drug comparisons were performed using various doses for the following combinations: (A) ADR/THC (B) ADR/CBD (C) Taxol/THC. Multiple drug comparisons were done using single doses for each drug with the following: Vehicle, ADR, Taxol, MTX, 5FU, or CP vs. Vehicle Win2 or NAB (D). No significant enhancements were evident.

breast cancer. As with the initial studies, when compared to treatment with the individual cancer chemotherapeutic agents, none of the combinations showed any detectable enhancement in the inhibition of MCF-7 cell growth (fig 1D). As before though, the cannabinoids were shown not to attenuate the effectiveness of cancer therapeutic drugs.

One important point needs to be emphasized here. All of our studies were performed in the presence of 10% serum in the cell culture media while most of the current literature relating to cannabinoids in cancer involves studies in serum-free medium (3,4). We consider this to be a non-physiological condition that raises questions as to the clinical applicability of these observations; even so, in order to provide a more thorough evaluation of the nature of drug interaction, we plan to repeat selected studies of cannabinoids in combination with chemotherapeutic drugs (and radiation, see below) in serum-free media to determine the impact of this approach on drug-chemotherapy and drug-radiation interaction.

Specific Aim 2 of my statement of work dictated that we would “Evaluate the capacity of cannabinoid compounds to antagonize cell transformation using an in vitro model.” In the narrative of the predoctoral proposal, we were able to provide data showing that carcinogen treatment of the spontaneously immortalized breast epithelial cell line, MCF-10A, was able to induce a neoplastic transformation. However, subsequent experiments failed to confirm these initial observations and our

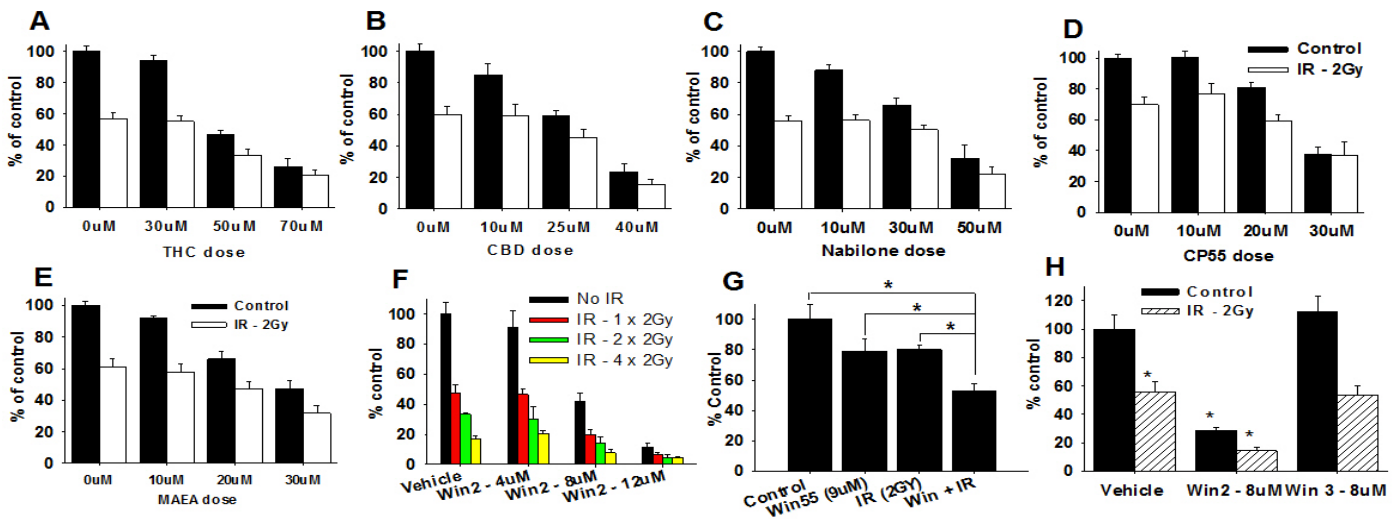


Figure 2 – Win55,212-2 additively interacts with radiation to inhibit the growth of MCF-7 cells. Experiments A-F and H done using cell count and experiment G was done using clonogenic survival. One dose of 2Gy radiation was given in combination with the cannabinoids (A) THC, (B) CBD, (C) Nabilone, (D) CP55, and (E) MAEA for the purpose of assessing interactions between the two treatments. (F) Individual or multiple treatments of 2Gy radiation were given in combination with various concentration of Win2 to assess interaction of the treatments. (G) Vehicle, Win2, radiation, or Win2+radiation were given to cells in the clonogenic survival assay to confirm interactions. (H) Cells were treated with both Win2 and its inactive enantiomer Win3 either alone or in combination with radiation to assess specificity. (*= $p < 0.05$ compared to vehicle unless otherwise indicated. No relevant difference detected in A-E. F displayed significant differences in all relevant comparisons.)

initial protocol using estradiol and benzopyrene was deemed unreliable. We further attempted to modify the procedure used for transformation by altering the carcinogen used, doses used and duration of exposure to the carcinogens; however, despite our best efforts, we were unable to reliably promote a transformed phenotype

Given the critical role of radiation treatment in the therapy of breast cancer, we initiated studies to evaluate the influence of cannabinoids on the antiproliferative effects of radiation in breast cancer cells. Experiments were initiated using cell count assays to test the clinically relevant cannabinoids THC, CBD and Nabilone in combination with one dose of 2Gy ionizing radiation administered to MCF-7 cells. These combinations generally appeared to demonstrate positive interactions; however, analysis of the data failed to demonstrate significance (fig 2A, B and C). We then expanded these experiments to the synthetic cannabinoid CP55,940 (CP55) and the endogenous cannabinoid analog, Methanandamide (MAEA), both of which have higher potencies than THC, CBD and nabilone, which again resulted in generally positive interactions in MCF-7 cells but again with less than statistically significant levels of interaction (fig 2D and E). When Win2 was tested in combination with radiation, this interaction proved to be more promising. Therefore, we expanded the study to include not only multiple doses of Win2 (4, 8 and 12uM) but also multiple 2Gy treatments of radiation (1, 2 and 4). This approach demonstrated clearly additive interactions to suppress breast tumor growth that exceeded the effects observed with other cannabinoids tested (fig 2F). These findings were further confirmed using the clonogenic survival assay (fig 2G). The selectivity of this interaction was established using the

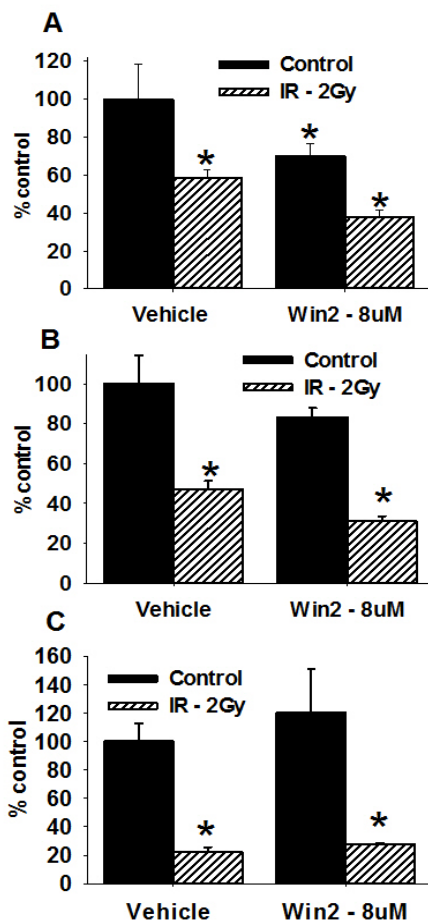


Figure 3 – Interaction between Win2 and radiation appropriately transfer to other cell lines. Using cell count various cell lines were treated with one of four treatments: Vehicle, Win2-8uM alone, 1x2Gy radiation alone, or Win2 + radiation. Cell lines used included MDA-MB-231 (A), 4T1 (B), and MCF-10a (C). (*= $p < 0.05$ vs vehicle)

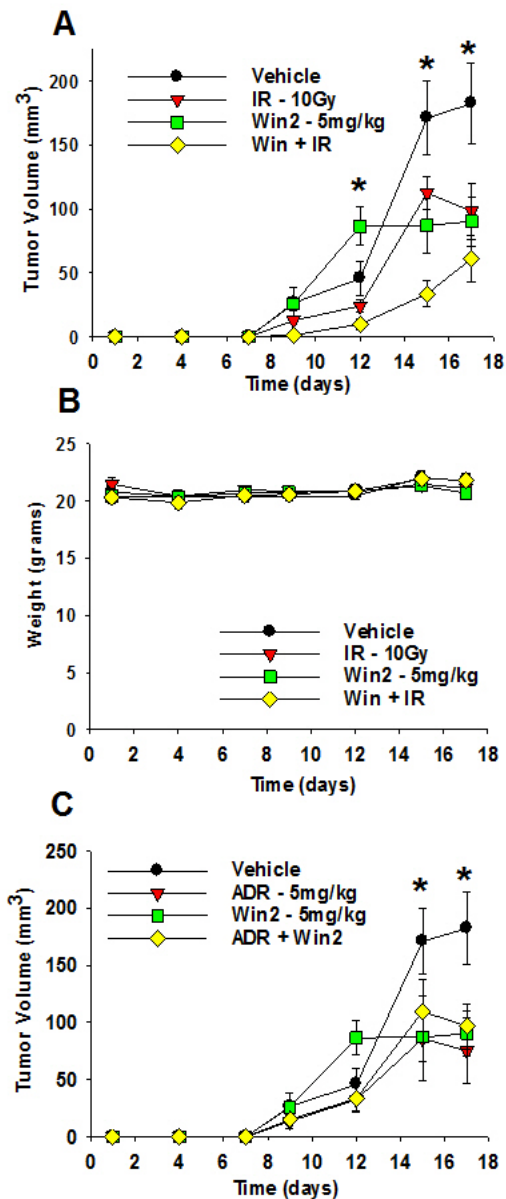


Figure 4 – In rodents Win2 enhances radiations ability to inhibit tumor growth but not ADR's. 4T1 cells were implanted into the right flank of balb/c mice as a tumor growth model. (A) Tumor volume was measure for mice given vehicle, 5mg/kg Win2, 10Gy radiation or Win2 + radiation, and (B) body weights were measured to assess toxicity. (C) Tumor volume was also measure for mice given vehicle, 5mg/kg ADR, 5mg/kg Win2, or Win2 + ADR. (* $p < 0.05$ for individual time points)

steady tumor growth. Win2 alone and radiation alone both produced minor growth delays that led to significantly smaller tumors by the end of the study at day 17. The mice treated with Win2 and radiation had not only significantly smaller tumors than all the other groups but were also the first groups to show significant separation in tumor size from the vehicle treatment (fig 4A). Together these observations indicate that the combination of Win2 + radiation also is effective in vivo in an immune competent animal, which should be highly relevant to clinical protocols. As an overall indication of the animal's health, we determined that body weights of the mice showed no significant changes during the course of the study (fig 4B).

We also took this opportunity to reconfirm that our in vitro observation regarding Win2's inability to interact with ADR was not solely a consequence of the use of an in vitro model. Mice were

inactive enantiomer of Win2, Win55,212-3 (Win3); figure 2H indicated that Win 3 had no ability to inhibit the growth of MCF-7 cells or enhance radiations effects.

In the original statement of work, we proposed using additional breast cancer cell lines in our evaluation of the drug combinations. These cell lines included MDA-MB-231 cells (a model of triple negative metastatic breast cancer) and 4T1 cells (a murine metastatic breast cancer model). When the combination of Win2 + radiation was administered to MDA-MB-231 cells the same additive enhancement was evident as in MCF-7 cells. This effect was also evident in studies using the 4T1 cells (fig 3A and B). The ability of Win2 to enhance the actions of radiation in multiple breast cancer cell lines provides support for potential translation of these findings. However, with any therapeutic strategy, there is the concern and risk of also enhancing the toxic effects of radiation to normal tissue. To address this question, we utilized the MCF-10a cell line as a model of normal breast epithelial cells. Treatment of MCF-10a cells with similar concentrations of Win2 as in the studies with MCF-7, MDA-MB-231, and 4T1 cells showed no effects alone, but more importantly failed to augment radiations effects (fig 3C). This indicates the possibility that there would be little enhancement of unwanted side effects in the patient.

Studies using in vivo models of breast cancer were initiated to establish the validity of the combination treatment effects. A possible concern with these studies was that cannabinoids have been shown to have immune suppressive effects; one report has shown that high doses of THC treatment could suppress the anti-tumoral immune response (5). To address this aspect of immune modulation, instead of using MCF-7 cells in vivo, which require the use of an immune deficient mouse, studies were performed with 4T1 murine breast tumor cells which were originally derived from immune competent Balb/c mice and can subsequently be injected back into the mice without concerns for immune rejection (5).

After injection of the tumor cells, the mice were subjected to one of four treatments: twice weekly injection of saline, twice weekly injections of 5mg/kg Win2, one dose of 10Gy radiation, or Win2 and radiation treatments. Tumor volumes were assessed by caliper measurements throughout the study. Mice treated with vehicle showed a

subjected to one of four treatments: twice weekly injection of vehicle, twice weekly injection of 5mg/kg Win2, two total injections of 5mg/kg ADR, or ADR and Win2 in combination. When tumor volumes were analyzed, all treatment groups reached a significantly lower volume by day 17 when compared to vehicle treatment alone; however, the treatment of Win2 + ADR was not significantly different from Win2 alone or ADR alone (fig 4C). This confirms that our observation in vitro were valid and not dependent on the model used.

In summary we have been able to demonstrate that the combination of Win2 and radiation treatment in various breast cancer cell lines has the capacity to enhance the inhibition of tumor growth. Marijuana and its related compounds are known to be quite safe, a fact that would only be enhanced when administered by a medical professional (6). As an added benefit, Win2 is likely to share the same characteristics of promoting increased food consumption and anti-nausea properties for which cannabinoids are currently used in combination with cancer therapies (1). Finally, there is accumulating evidence that cannabinoid compounds are able to alleviate cancer associated pain that could translate to the patient and increase quality of life (7).

In future studies, we plan to develop these observation relating to the interaction of Win2 with radiation in terms of elucidating the nature of this interaction at the mechanistic level. Our first aim is to look at the upstream signaling events mediating the observed effects, beginning with the identification of the cannabinoid receptor that might be activated by Win2. We have access to pharmacological inhibitors that can be used in conjunction with molecular techniques including western blotting and RT-PCR to accurately determine the cannabinoid receptors are expressed and/or activated in our cells. Our second aim is to determine the mode of cell death that is causing the decrease in cell numbers observed with the combination treatment strategy. Finally, we would plan to identify the signaling pathways leading from receptor mediated effects to the growth arrest/cell death outcome of treatment.

As a final note, although the studies of Win2 in combination with radiation were not considered in the original statement of work, overcoming unforeseen challenges of this project has, I believe, provided an extremely useful learning opportunity that has contributed to my research training under this grant.

Key research accomplishments

Cannabinoid and chemotherapeutic interactions

- Tested cannabinoids do not interact negatively with commonly used chemotherapeutics in preclinical cell culture models.
- Lack of antagonism between Win2 and ADR confirmed using an immune competent in vivo model.

Cannabinoid and radiation interaction

- Established the interaction between Win2 and ionizing radiation in human breast tumor cell line MCF-7 cells.
- Proved selectivity of the interaction between Win2 and IR using the inactive enantiomer of Win2, Win3.
- Determined that other cannabinoid currently used in the clinical for palliative measures do not interfere with IR's effects in the absence of an enhancing effect.
- Demonstrated that the interaction between Win2 and IR transfers to other breast cancer cell lines using MDA-MB-231 and 4T1 cells
- Provided data indicating that Win2 will not enhance the toxic effect IR has on normal cells using MCF-10a cells.
- Showed in vivo data that offers proof that Win2 and IR will positively interact to inhibit the growth of a tumor in a whole animal environment.

Reportable outcomes

Abstracts submitted to

- American Association of Cancer Research (AACR) – for 2012 conference
- International Cannabinoid Research Symposium (ICRS) – for 2011 conference
- Virginia Academy of Science (VAS) – for 2011 conference
- Era of Hope meeting – for 2011 conference

Presentations

- Watts Day Presentation – Poster Presentation – “Combining Cannabinoids and Radiation in Breast Cancer”
- Massey Cancer Center Research Retreat – Poster Presentation – “Combining Cannabinoids and Radiation in Breast Cancer”
- Pharmacology and Toxicology Research Retreat – Poster Presentation – “Combining Cannabinoids and Radiation in Breast Cancer”
- Era of Hope meeting – Poster Presentation – “Role of the Endogenous Cannabinoid System in a Murine Model of Breast Cancer”
- Virginia Academy of Science – Student Presentations - “Combining Cannabinoids and Radiation Therapy in Breast Cancer”
- 7th Annual Women’s Health Research Day at VCU – Poster Presentations – “Combining Cannabinoids and Radiation Therapy in Breast Cancer”
- Department of Pharmacology and Toxicology – Seminar Series – “The Interaction Between Win55,212-2 and Radiation on Breast Cancer”
- International Cannabinoid Research Symposium – Poster Presentations – “Combining Cannabinoids and Radiation Therapy in Breast Cancer”

Conclusion

The proposed studies of the potential role of the endocannabinoid system in the development of breast cancer were unsuccessful due to the lack of reliable and reproducible results in the in vitro model of tumor transformation. Studies of cannabinoids in combination with conventional cancer chemotherapeutic drugs indicate that cannabinoids are unlikely to enhance the efficacy of current treatment strategies. However, in view of the fact that cannabinoids are used palliatively to improve the quality of life during treatment, the observation that these drugs did not antagonize chemotherapeutic activity is likely to be relevant to the continued use of these drugs. Alternatively, the redirected focus of our studies towards the interaction of cannabinoids with ionizing radiation, one of the primary treatments for breast cancer, does show therapeutic promise. Studies both in cell culture and an immune-competent animal model are consistent with the conclusion that Win2 could potentially improve the effectiveness of radiation therapy in breast cancer treatment.

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