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14. ABSTRACT
In the experiments described during this funding cycle, we used respective in vivo and in vitro approaches to: 1) promote mammary tumor genesis via the carcinogen DMBA in mice and perform histology to determine the type of tumor; and 2) investigate the underlying receptor mechanisms of action of the antiproliferative actions of Δ^9-tetrahydrocannabinol (THC), the primary psychoactive constituent present in marijuana, and WIN55,212-2, a highly potent, full CB_1 receptor agonist. Female mice implanted with progesterone pellets and treated with DMBA presented with adenocarcinoma, squamous cell carcinoma, and sarcoma-like tumors. In the in vitro studies, we established that WIN55,212-2 (WIN2) produces antiproliferative effects in two human breast cell lines (MCF-7 and MDA-MB-231) and a murine mammary tumor line (4T1). The stereoisomer of WIN2, WIN55,212-3, lacked efficacy in reducing cell growth even at 40 μM. Surprisingly, the antiproliferative effects of THC were not mediated through CB_1 or CB_2 receptors. Likewise, neither Gi/Go nor Gs proteins appeared to contribute to the antiproliferative actions of WIN2. Finally, the absence of CB_1 and CB_2 receptors in each of the tumor lines indicates WIN2 and THC elicit their antiproliferative actions through a novel mechanism of action. Given the high prevalence of cannabinoid use in breast cancer patients, the knowledge gained from these experiments will have important clinical implications for breast cancer patients.

15. SUBJECT TERMS
Breast Cancer, Carcinogen, CB_1 receptor, CB_2 receptor, GPR55, Δ^9-tetrahydrocannabinol (THC), WIN55,212-2, MCF-7 cells, MDA-MB-231 cells, 4T1 cells, endogenous cannabinoid, fatty acid amide hydrolase (FAAH)

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Table of Contents

Introduction

Δ^9-tetradyrocannabinol (THC), the chief psychoactive constituent of marijuana (Gaoni and Mechoulam, 1964), as well as other naturally occurring and synthetically derived cannabinoids bind to and activate two type of cannabinoid receptors, CB_1 (Matsuda et al., 1990) and CB_2 (Munro et al., 1993) receptors. Both CB_1 and CB_2 are G-protein coupled receptors (GPCRs) and their activation results in the inhibition of adenylyl cyclase. In addition, the body manufactures two primary endogenous cannabinoids (endocannabinoids) through distinct biosynthetic pathways (Ahn et al., 2008). These endocannabinoids are *N*-arachidonoylethanolamine (i.e., anandamide; (Devane et al., 1992)) and 2 arachidonylglycerol (2-AG; (Mechoulam et al., 1995; Sugiura et al., 1995). Moreover, these endocannabinoids are tightly regulated by their respective catabolic enzymes fatty acid amide hydrolase (FAAH; (Cravatt et al., 1996)) and monoacylglycerol lipase (MAGL).

Cannabinoids have been shown to possess potential therapeutic effects related to cancer treatment, including reduction in nausea and vomiting associated with cancer chemotherapy (Kluin-Neleman et al., 1979) and pain relief in cancer patients (Noyes et al., 1975a; Noyes et al., 1975b). Interestingly, these compounds have been reported to elicit antineoplastic activity in mice (Munson et al., 1975; White et al., 1976). Endogenous cannabinoids have also been shown to have anti-cancer effects in MCF-7 breast cancer cells (Melck et al., 1999). However, there is suggestion in the literature that cannabinoids can have opposing effects on breast cancer proliferation depending on whether in vitro or in vivo models are used. Specifically, McKallip et al (2005) reported that cannabinoids elicit antiproliferative effects in 4T1 cells in vitro, but cannabinoids elicit proliferation of these breast cancer cells that were implanted via injection in mice. Thus, the thrust of this collaborative grant between the Gewirtz and Lichtman laboratories has been to use in vivo and in vitro approaches to determine whether the endogenous cannabinoid system possesses targets to treat breast cancer.

During the present funding period, we pursued both *in vivo* and *in vitro* approaches to ascertain whether the endogenous cannabinoid system possesses targets to reduce breast cancer genesis or proliferation. In the i*n vivo* studies, we employed 7,12-dimethylbenz[a]anthracene (DMBA), an established animal model of induced breast cancer (Aldaz et al., 1996), to determine whether elevation of anandamide reduces tumor genesis and proliferation. As indicated in our previous report, transgenic mice lacking FAAH, which possess elevated levels of anandamide, were compared to wild type mice for the rate of palpable mammary tumor growth and the occurrence of tumors. The mice were humanely euthanized 45 weeks after DMBA exposure, tumors were excised, and cancer types were diagnosed.

In the *in vitro* experiments, we tested the hypothesis that cannabinoid receptor agonists reduce proliferation of breast cells through a cannabinoid receptor mechanism. To this end, we evaluated THC as well as WIN55,212-2, a potent and highly efficacious synthetic cannabinoid receptor agonist originally developed as a nonsteroidal anti-inflammatory drug (Ward et al., 1991; Ward, 1992) would reduce proliferation of human (MCF-7 and MDA-MB-231) and murine (4T1) breast cancer lines. The CB_1 receptor antagonist, rimonabant, and CB_2 receptor antagonist, SR144528, were used to assess the involvement of cannabinoid receptors. In addition, we sought to determine whether the antiproliferative effects of cannabinoids involved the activation of specific G-proteins. Accordingly, we used pertussis toxin to determine the involvement of Gi/Go proteins and cholera toxin to ascertain the involvement of Gs. Collectively, the goal of this work is to establish whether the endogenous cannabinoid system offers viable targets to treat breast cancer.

Body
Methods

Subjects - Subjects consisted of female C57BL/6J (Jackson Laboratory, Bar Harbor, ME) as well as the following transgenic lines: FAAH (-/-), FAAH-NS (i.e., FAAH is exclusively expressed in the nervous system), CB_1 (-/-), and CB_2 (-/-) mice, and age-matched wild type mice from the Center Transgenic Colony at Virginia Commonwealth University. FAAH-NS have wild type levels of AEA in the neural tissues but highly elevated levels in peripheral tissue (Cravatt et al., 2004). The FAAH (-/-), FAAH-NS, and CB_1 (-/-) mice were backcrossed onto a C57BL/6J background for at least 13 generations. The CB_2 (-/-) mice were backcrossed on to a C57BL/6J background for five generations. Control mice were age and sex matched to the transgenic mice. Mice were housed in a temperature (20-22 °C) and humidity controlled, AAALAC-approved facility, with *ad libitum* access to food and water. Subjects weighed approximately 18-20 g, were housed 4-6 mice per cage, and maintained on a 12:12 light cycle. All experiments were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Tumor Induction - Prior to surgery the back of the neck of the mouse was shaved and then washed with Betadine solution and ethanol. The mice were anesthetized using isoflurane and then using sterile surgical equipment a small one inch incision was made just below the ears to prevent excessive scratching during healing. A 25 mg 60 day release progesterone pellet was inserted subcutaneously into the incision and then moved down the back. The incision was then closed using staples. The isoflurane was removed and the animals were returned to their home cages. Drinking water mixed with acetaminophen (2.5 mg/ml of water) was made available *ad libitum* for one week to reduce any post-surgical discomfort. The mice were given 21 days to recover from the progesterone pellet implantation before beginning DMBA dosing.

Gavage was performed using a syringe with a rounded tip to prevent damage to the esophagus. Each mouse was given 50 mg/kg DMBA dissolved in cottonseed oil on days 1, 8, 22, and 29 in an injection volume of 0.1 ml. Subcutaneous DMBA (5, 10 and 20 mg/kg) injections were given on day one after the 21 day recovery period from the pellet implantation.

For DMBA s.c. administration, the needle was inserted in the lower abdomen near the anus and moved parallel to the abdomen until the tip was centered under the lower right nipple. Approximately 0.1 ml of vehicle or DMBA was injected and the needle was removed slowly to prevent leakage from the site. In the first experiment female C57BL/6J mice received cottonseed oil vehicle or DMBA (20, 40, or 80 mg/kg). In the second evaluated 20 mg/kg DMBA in FAAH (-/-) and (+/+) mice, CB_1 (-/-) and (+/+) mice, and CB_2 (-/-) and (+/+) mice.

Tumor Induction. As reported during the previous funding cycle, we treated mice with progesterone pellets and 7,12-Dimethylbenzanthracene (DMBA) as a model to induce tumor formation (Aldaz et al., 1996). Pellet implantation was administered 3 weeks prior to treatment followed by 4 treatments of 50mg/kg DMBA given over 5 weeks. A small cohort of mice was given the DMBA through subcutaneous injection at various doses instead of by gavage, with the intention of optimizing the model for future attempts. Controls were given cottonseed oil alone or cottonseed oil and progesterone. The experiment was ended at 45 weeks following DMBA treatment. Each of the treatment groups of mice are listed in Table 1. Also listed are the values: 1) number of mice in each group; 2) number of deaths related to gavage technique combined with DMBA toxicity; 3) number of other deaths (i.e., DMBA-related toxicity and undetermined); 4) number of mice that survived to week 45; and 5) number of mice that developed tumors.

Tumor Tissue Removal - Animals were euthanized using CO2 asphyxiation. The tissues were surgically excised and immediately placed in a 10% formalin solution at a solution volume that was at least ten times the volume of the tissue sample. Tissue samples were also sectioned when necessary so that they were approximately 0.5 cm wide to allow rapid formalin perfusion.

Cell growth – All cell lines (4T1, MCF-7, and MDA-MB-231) were grown in a 37 ° C incubator and passed twice weekly. The cells were maintained in standard growth media (RPMI with 5% fetal bovine serum, 5% bovine calf serum, and 1% pen/strep).

Results

In vivo Studies.

At the conclusion of the previous year's funding cycle, we were in the progress of performing histological analysis on mice treated with DMBA administered via either subcutaneous injection or oral gavage. The treatment was paired with progesterone pellets implanted under the skin three weeks prior to drug administration. Due to a large number of deaths, most of which were associated with the DMBA, only 37 mice formed tumors out of 127 used in the study (see Table 1). The histology has since been completed and the surviving mice were categorized by the histology of their tumors (Figure 1).

Table 1. DMBA and Progesterone: The number of mice in each group that died because of improper gavage technique or other causes, and the number of surviving mice that developed tumors by week 45.

Injection Route	DMBA Dose (mg/kg)	Progest	Mouse Type	Initial n	# Gavage-related deaths	# Other deaths	# survived at 45 weeks	# surviving mice with tumors
s.c.	20	Yes	C57	12	0	4	8	5
s.c.	10	Yes	C57	12	0	6	6	3
s.c.	5	Yes	C57	12	0	3	9	2
gavage	0	No	C57	12	1	1	11	0
gavage	0	Yes	C57	12	2	1	9	2
gavage	0	Yes	CB_1(+/+)	5	2	1	2	1
gavage	50	Yes	CB_1(+/+)	5	3	0	2	2
gavage	50	Yes	CB_1(-/-)	12	5	5	2	2
gavage	50	Yes	CB_2(+/+)	4	2	1	1	0
gavage	50	Yes	CB_2(-/-)	11	9	1	1	1
gavage	50	Yes	FAAH(+/-)*	10	2	2	6	6
gavage	50	Yes	FAAH-NS	10	1	1	8	7
gavage	50	Yes	FAAH (-/-)	10	3	1	6	6
Total				127	30	27	71	37

* FAAH (+/-) mice were used as controls because of the husbandry used to derive FAAH-NS and FAAH (-/-) mice from the same letter. It is important to note, that although FAAH (+/-) mice express half the amount of this enzyme as wild type mice, and have wild type levels of anandamide and normal CB1 receptor function.

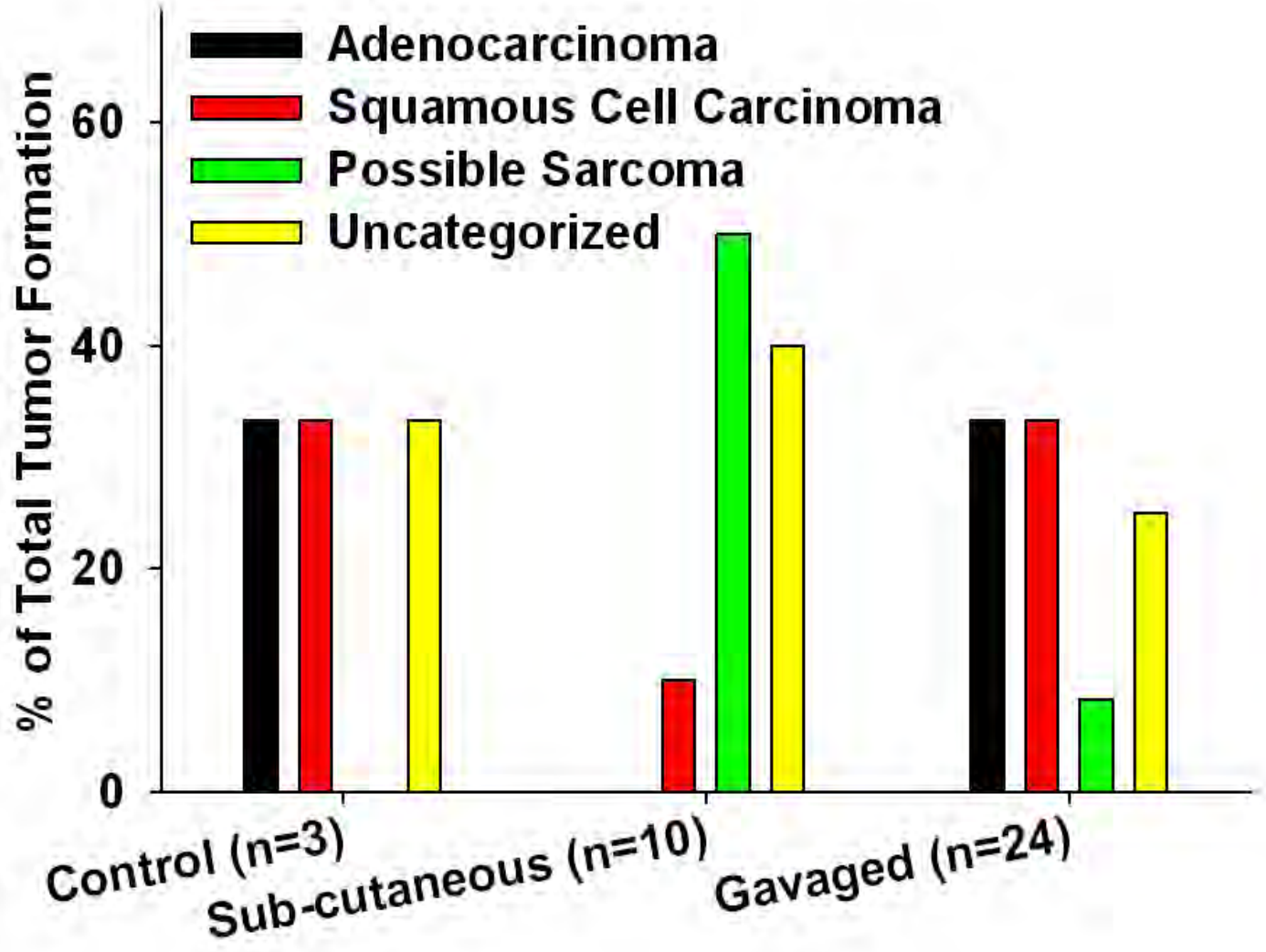

Figure 1. Histological characterization of tumors – Samples were fixed and analyzed based on marker presentation (p63, ck7, and c k5/6) and tissue morphology. A ll t umors fell i nto one o f four categories including A denocarcinoma, S quamous C ell C arcinoma, pos sible S arcoma, and unc ategorized. Uncategorized i ncluded m ice t hat h ad multiple t umors w ith di fferent hi stological c haracterization or w hen one tumor contained characteristics of more than one of the previously mentioned tumor types.

The t umors were categorized i nto one of t hree g roups based on t he types o f t umors t hat formed i ncluding adenocarcinoma, squamous cell carcinoma, and sarcoma-like. Representative H&E stains are shown in Figure 2 to show the basic tissue morphology of these tumors. Additionally, mice were assigned to a fourth category when m ultiple t umors of different phenot ypes as i ndicated abov e, o r if a tumor on a m ouse had m ultiple regions that had different tissue characteristics of more than one type of tumor. The control mice (i.e., did not receive DMBA) that presented with tumors had been implanted with progesterone pellets. Half of the tumors that formed i n t he s ubcutaneously i njected ani mals w ere sarcoma-like, which c ould pos sibly b e due t o t he route of dr ug administration. A lso i n t he s ubcutaneously i njected ani mals a l arge por tion o f t hem w as uncategorized, but many of these mice had tissue characteristics of sarcoma-like tumors. When DMBA was given by gavage there was a very low occurrence of sarcoma-like tumors that formed. The majority of tumors that formed w ere ei ther o f t he adenoc arcinoma variety or t he s quamous c ell c arcinoma phenot ype, w ith an additional and substantial portion that were of undetermined type.

A major challenge associated with the DMBA-induced mammary tumor genesis model is determining the tissue origin of the tumor. In humans, a large portion of the histological characterization comes from the knowledge of which organ or tissue the tumor was found. However, mice lack defined breast tissue because they possess very thin layers of mammary pads that cover the underside of the body. The distance between the fur, through the skin, mammary pad, muscle, and into the body cavity is in the range of a 2 mm. This small distance of tissue makes it difficult to determine by observation the exact tissue of origin. Therefore, whether these adenocarinoma tumors are derived from mammary tissue or from other glandular tissue in the skin, such as a sebaceous gland cannot easily be concluded. Additionally, squamous cell carcinomas are consistent with skin derived tumors, though they still could be mammary tumors with an abnormal phenotype.

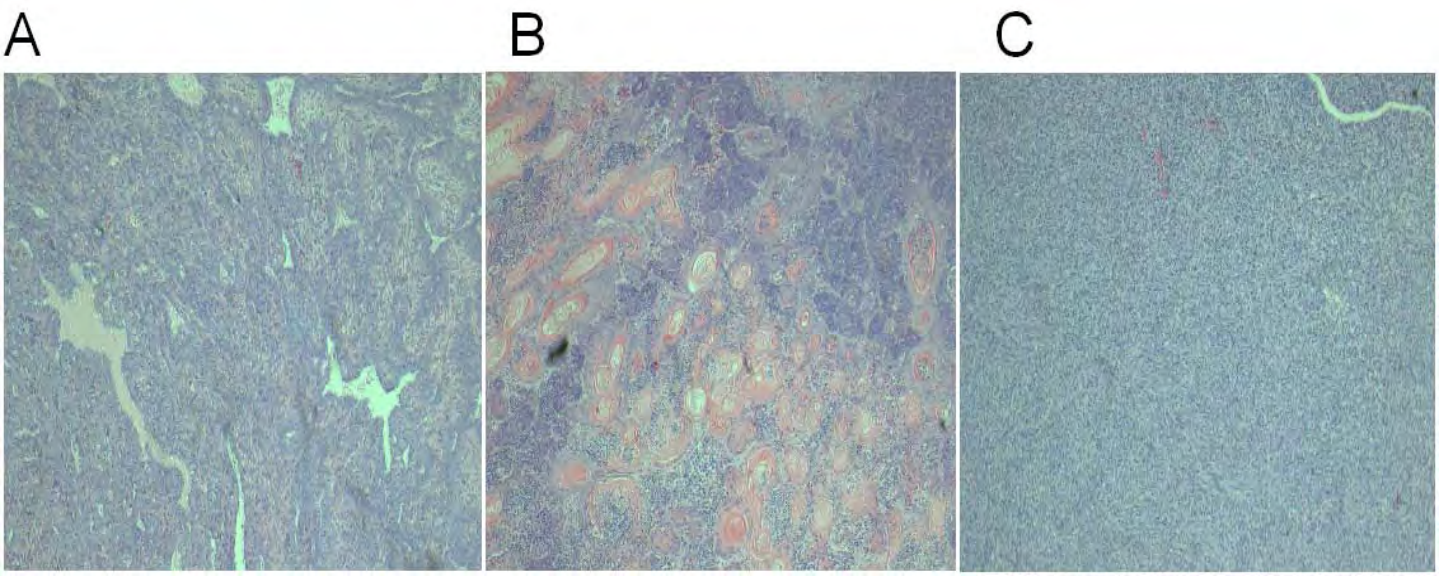

Figure 2 – Representative H&E stains – The tumors had tissue characteristics that fell into three different phenotypic categories. These included: A) Adenocarcinma, B) Squamous Cell Carcinoma, C) Sarcoma like.

In Vitro Studies

In parallel with the in vitro studies, we evaluated the effects of cannabinoid receptor agonist on proliferation of breast cells of human (MDA-MB-231 and MCF-7) and murine (4T1) origin. The bulk of these studies are presented by Dr. Gewirtz in the 2010 report of Contract W81XWH-08-1-0096. Here, we concentrate on the results of the experiments evaluating whether cannabinoid receptor agonists are indeed producing their antiproliferative effects through a cannabinoid receptor mechanism of action. A concern rising from our studies as well as those from the literature is the high concentration of cannabinoids required to elicit antiproliferative or anti-invasive effects. To address this concern, we compared the dose response curves of the full CB_1 receptor agonist WIN,212-2 (WIN2) and its inactive enantiomer WIN55,212-3 (WIN3), in all three cell lines. At a reasonable dose range, WIN2 possessed a significantly greater potency and efficacy than WIN3. This stereoselective effect suggests the existence of a specific molecular target and could be consistent with a cannabinoid receptor mechanism of action, since WIN2, but not WIN3, binds to cannabinoid receptors. WIN2 was effective in arresting growth in all three cell lines at concentrations much lower than were required for either Δ^9-tetrahydrocannabinol (THC), the primary active cannabinoid constituent present in marijuana, or cannabidiol (CBD), another marijuana constituent that does not bind to CB_1 or CB_2 receptors and does not produce THC-like effects. The antiproliferative effects of WIN2 vs. WIN3 in MDA-MB-231, MCF-7, 4T1 are shown in Figure 3 (also please see Figure 8 from the 2010 report of Contract W81XWH-08-1-0096). As can be seen, WIN2 was highly efficacious in reducing growth from each of the three breast cancer lines. In marked contrast, its stereoisomer, WIN3, failed to affect growth until excessively high concentrations (e.g.,40 μM).

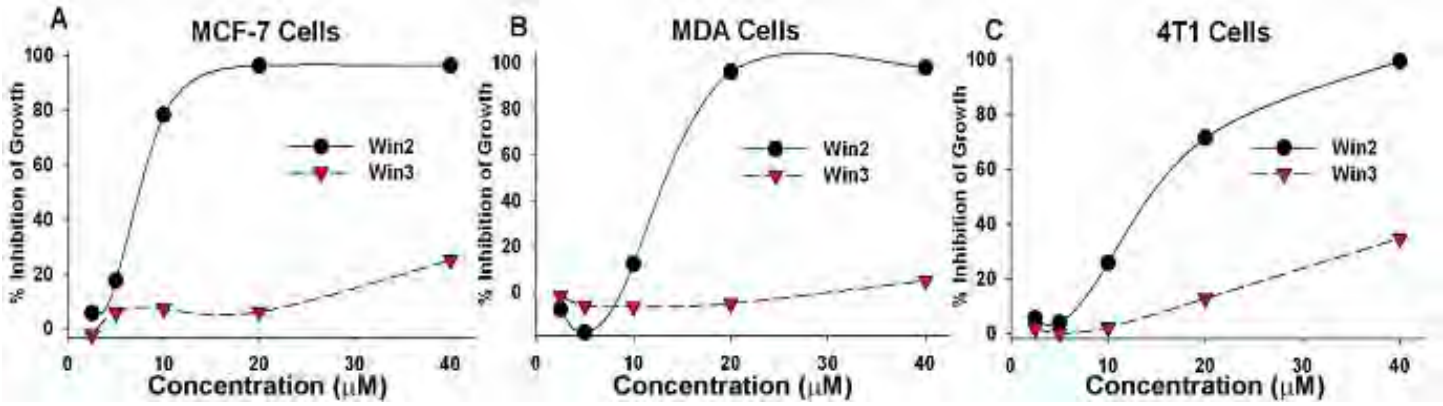

Figure 3. The aminalkylindole WIN 55,212-2 elicits a stereoselective antiproliferative action in breast cancer cellsMCF-7 (A), MDA-MB-231 (B), and 4T1 (C) breast cells were treated with the CB_1 receptor full agonist WIN55,212-2 (WIN2) or its inactive stereoisomer WIN55,212-3 (WIN3). In each of the three cell lines, the dose-response curve for WIN3 was shifted far to the right of the dose response curve of WIN2.

We next evaluated whether the antiproliferative effects of THC were mediated via a cannabinoid receptor mechanism of action. Accordingly, we assessed whether these antiproliferative effects could be blocked by selective ant agonists of CB_1 and CB_2 receptors. To this end, we evaluated whether the CB_1 receptor antagonist rimonabant or the CB_2 receptor antagonist SR144528 would antagonize the antiproliferative effects of THC in MCF-7, MDA-MB-231, and 4T1 cells. As can be seen in Figure 4, neither receptor antagonist blocked the antiproliferative effects of THC, suggesting that neither CB_1 nor CB_2 receptors appear to contribute to the antiproliferative effects of THC under the conditions in the present study.

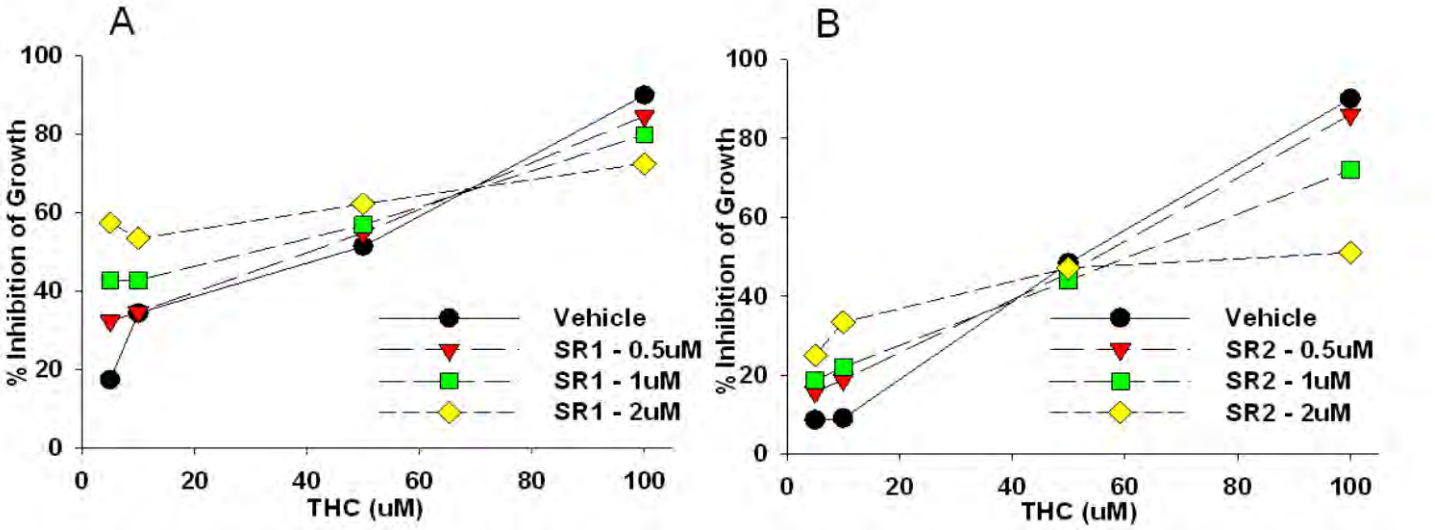

Figure 4. The antiproliferative effects of THC are mediated via a non-cannabinoid receptor mechanism of action. The antiproliferative effects of THC in MCF-7 cells were not reduced by the selective CB_1 receptor antagonist rimonabant (SR1; **Panel A**) or the CB_2 receptor antagonist SR144528 (SR1; **Panel B**).

Cannabinoid receptors are G-protein coupled receptors (GPCR) that primarily signal through either Gi/o or in some instances Gs proteins. Pertussis toxin (PTX) is used to block Gi/o proteins and cholera toxin (CTX) is used to block Gs proteins. When CTX and PTX were given in combination with a high dose of WIN2 neither of the toxins possessed efficacy to block WIN2's antiproliferative effects in MCF-7, MDA-MB231, or 4T1 cells (Fig 5). These findings strongly suggest that WIN2 is not working through either Gi/Go or Gs protein coupled

receptors. Thus, it is highly unlikely that the antiproliferative actions of these cannabinoid receptor agonists are mediated through typical cannabinoid receptor mechanism of action.

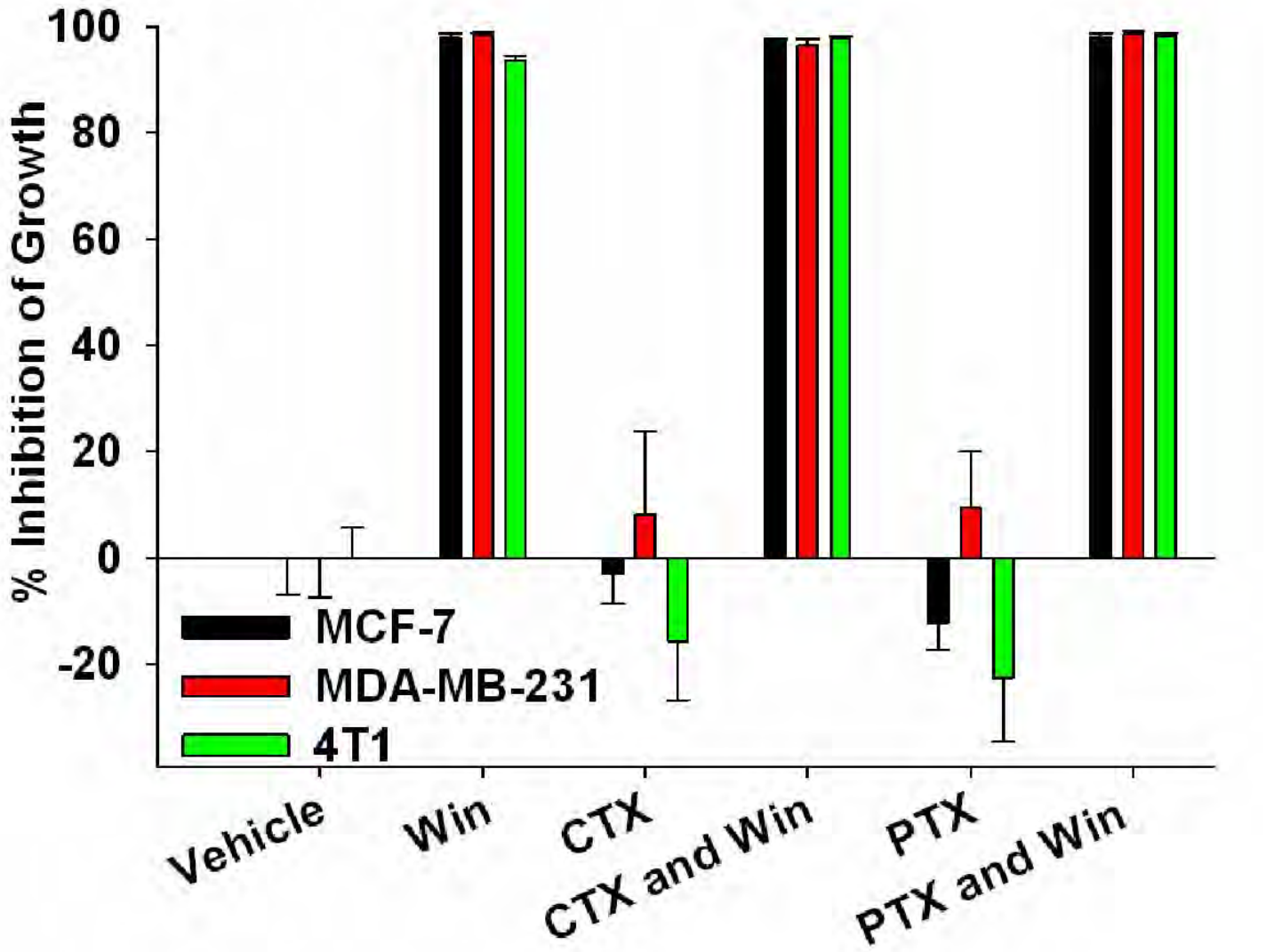

Figure 5. The antiproliferative effects of WIN55,212-2 are mediated through a mechanism other than Gi/o or Gs protein coupled receptors. Neither the Gs inhibitor cholera toxin (CTX) nor the Gi/o inhibitor pertussis toxin (PTX) attenuated the antiproliferative effects of WIN55,212-2 (20 μM; Win) in three distinct lines of breast cancers (MCF-7, MDA-MB-231, and 4T1). This dose of WIN55,212-2 produced maximal toxicity (see Figure 3).

Attempts were made to conduct Western blotting to determine the presence of CB_1 or CB_2 receptors, but we were unable to obtain repeatable and reliable results from any of the available antibodies (data not shown). Instead, we used an alternate approach using PCR to detect the presence of CB_1 or CB_2 receptor mRNA. However, we found no evidence supporting the idea that either cannabinoid receptor was present in any of our cell lines (Figure 6). During these experiments, we also tested for the presence of

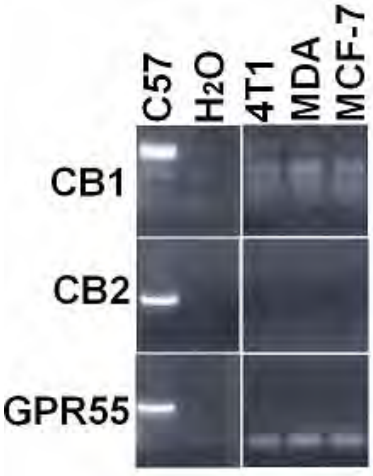

Figure 5. The breast cancer cells do not express **cannabinoid receptors or the orphan GPCR, GPR55.** PCR failed to detect the expression of CB1, CB2 or GPR55 receptors in 4T1, MDA-MB-231, or MCF-7 cells. Tail snips from C57BL/6J mice were used as a positive control and water as a negative control.

GPR55, an orphan GPCRs that has been shown to have some sensitivity to cannabinoid compounds (Henstridge et al., 2009; Ross, 2009). However, none of the cell lines expressed this orphan receptor.

Key Research Accomplishments

- Ascertained that mice implanted with progesterone and treated with the carcinogen DMBA developed several different types of cancers including adenocarcinoma, squamous cell carcinoma, and sarcoma-like.
- The phytocannabinoid THC and potent synthetic full cannabinoid agonist WIN55,212-2 produced antiproliferative effects in three distinct breast cancer lines.
- The antiproliferative effects of THC and WIN55,212-2 appear to be independent of CB_1 and CB_2 receptors, GPR55 receptor, and Gi/Go nor Gs protein coupled receptors in general.

Reportable Outcomes:

2010 International Cannabinoid Research Symposium – Poster Presentations – "Cell line specific enhancement of sensitivity to Adriamycin by phytocannabinoids in breast cancer"
2010 Virginia Academy of Science – Student Presentations – "The Full Agonist WIN55, 212-2 Exerts Growth Inhibitory Effects Through A Cannabinoid Receptor Independent Mechanism"
2010 Department of Pharmacology and Toxicology – Seminar Series – "Cannabinoids and Cancer: Tumor Development and Treatment"
2009 Department of Pharmacology and Toxicology – Seminar Series – "The Involvement of the Endocannabinoid System in the Development of Breast Cancer"

Conclusions

The in vivo studies revealed that combination of implanting progesterone pellets and treatment with the carcinogen, DMBA underwent elicited tumor genesis in female C57BL/6J mice. Several different types of tumors were identified, including adenocarcinoma, squamous cell carcinoma, and sarcoma-like. The adenocarinoma tumors could have been derived from mammary tissue or from other glandular tissue in the skin, such as a sebaceous gland. Additionally, squamous cell carcinomas are consistent with skin derived tumors, though they still could be mammary tumors with an abnormal phenotype. Consequently, the DMBA model, while possessing utility to investigate tumor genesis in rodents, possesses limitations in regard to drawing definitive claims specifically about breast cancer. Given the long duration of time required for DMBA-induced tumor genesis, this model requires a considerably long-term investigation across a significant proportion of the life span of the mouse.

The in vitro studies indicate that the phytocannabinoid THC and the potent, highly efficacious synthetic cannabinoid agonist WIN55,212-2 produce antiproliferative effects in three distinct breast cell lines (MCF-7, MDA-MB-231, and 4T1). However, our findings suggest that the effects of these drugs are not mediated through either CB_1 or CB_2 receptors. Moreover, these results suggest a lack of involvement of the major G-proteins that are activated by cannabinoid receptor stimulation (i.e., Gi/Go) as well as no apparent role of Gs proteins. Nonetheless, the observation that WIN55,212-3, the stereoisomer of WIN55,212-2, lacks efficacy in all three tumor lines, suggests that THC and WIN55,212-2 are producing specific effects at a non cannabinoid receptor target. Determining the mode of cell death will provide insights into potential mechanisms of action underlying the antiproliferative effects of THC and WIN55,212-2. Given the high prevalence of cancer patients taking legally prescribed cannabinoids or other cannabinoid remedies to treat symptoms related to the disease or to ameliorate the toxic side effects of chemotherapeutic treatments, it is important to obtain knowledge of whether cannabinoids alter the breast cancer genesis, proliferation, migration, or invasiveness, as well as whether they interfere or interact with chemotherapeutic drugs. Thus, regardless of whether the endogenous cannabinoid system is ultimately determined to possess targets to treat breast cancer, the knowledge gained from this project will have important implications for public health.

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