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| 14. ABSTRACT During the present funding cycle, we have focused on in vitro approaches to determine the influence of the endogenous cannabinoid system on breast cancer. Studies of transformation in cell culture have demonstrated that the combination of estrogen and benzopyrene alters the phenotype of MCF-10a cells, a normal breast epithelial cell line, to that of a tumor cell. This conclusion is based on induction of anchorage independent growth, loss of acini structure formation in 3D cultures, and the ability to proliferate in the absence of exogenous growth factors normally required for MCF-10a growth. We are now prepared to examine the influence of cannabinoid agonists and antagonists on the transformation process. We further initiated studies to evaluate the interactions of cannabinoids with conventional chemotherapy in breast tumor cell lines. Our studies are consistent with a mixture of additive and synergistic effects in the three cell lines (MCF-7, MDA-MB-231, and 4T1) and three cannabinoid combinations (THC, CBD, and Win55) in combination with Adriamycin. Our studies also strongly suggest that the interactions are most pronounced in breast tumor cells lacking functional p53. Studies are now in progress to evaluate the cannabinoids in combination with other drugs utilized in the treatment of breast cancer including paclitaxel, methotrexate and 5-fluorouracil. In addition, we have established the in vivo model of 4T1-luc cells that can be monitored in real time in an immunocompetent mouse and plan to extend the cell culture work of cannabinoids + chemotherapy to this experimental system. | | | | |
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INTRODUCTION

Δ^9 -tetrahydrocannabinol (THC), the chief psychoactive constituent of marijuana, as well as other naturally occurring and synthetically derived cannabinoids possess potential therapeutic effects related to cancer treatment, including reduction in nausea and vomiting associated with cancer chemotherapy (Kluin-Neleman et al., 1979), pain relief in cancer patients (Noyes et al., 1975), and antineoplastic activity in mice (Munson et al., 1975; White et al., 1976). These compounds are known to bind to CB₁ (Matsuda et al., 1990) and CB₂ (Munro et al., 1993) receptors. However, there is little enthusiasm for the development of mixed CB₁/CB₂ receptor agonists for therapeutic uses because of their marijuana-like and psychomimetic effects. An alternative promising approach is the indirect stimulation of cannabinoid receptors by elevating the endogenous cannabinoids *N*-arachidonoyl ethanolamine (i.e., anandamide; (Devane et al., 1992)) and 2 arachidonyl glycerol (2-AG; (Mechoulam et al., 1995; Sugiura et al., 1995)) by inhibiting their respective catabolic enzymes fatty acid amide hydrolase (FAAH; (Cravatt et al., 1996)) and monoacylglycerol lipase (MAGL; (Goparaju et al., 1999)). Accordingly, one original purpose of this project was to determine whether elevating endogenous anandamide through blockade of FAAH would prevent tumor genesis as well as produce antiproliferative and decrease tumor invasiveness. As indicated in the previous report, we encountered some difficulties with the original mouse model studies in terms of validating that the tumors generated using 7,12-dimethylbenz[a]anthracene (DMBA) were unequivocally breast derived. Consequently, in the current studies, we are working to develop a cell culture model of transformation to determine whether FAAH inhibitors and cannabinoid receptor agonists (and antagonists) will reduce (or possibly enhance) transformation as an indication of carcinogenesis. In addition, we are testing the influence of cannabinoid agonists and antagonists in combination with chemotherapy in both cell culture and animal models of breast cancer.

This is a collaborative research project between the Gewirtz and Lichtman laboratories. Although all of the work is closely coordinated, this report will cover primarily the studies relating to in vitro transformation and drug combination experiments performed in breast tumor cell lines in culture.

BODY

Methods

Cell culture conditions – MCF-7 and MDA-MB-231 breast tumor cell lines are maintained in standard growth media (RPMI with 5% fetal bovine serum, 5% bovine calf serum, and 1% pen/strep). MCF-10a breast epithelial cells are grown in specialized media: DMEM/F12, 5% horse serum, 1% pen/strep, 10ug/ml insulin, 100ng/ml cholera toxin, 20ng/ml EGF, and 500ng/ml hydrocortisone.

Transformation assays – Cells are seeded into six well plates at low density (approximately 10,000 cells per well) and allowed to grow for one week, during which drug is administered twice for 24 hrs each treatment. Treatment one is administered at 72 hours and drug is removed at 96 hours; treatment two is administered at 120 hours and drug is removed at 144 hours. Cells are then passed back into six well plates for a second week of treatment, identical to the first. At the end of the two courses of treatment, the cells are passed into culture flasks and allowed to grow to sufficient numbers for analysis. Transformation assays include measurement of anchorage independent growth, invasiveness, and growth rates under various conditions. Analysis begins by the end of week three after initiation of transformation.

Anchorage independent growth (AIG) – The bottoms of six well plates are coated with 1.5ml of 12mg/ml poly-hema dissolved in 95% ethanol. The ethanol in the plates is allowed to evaporate overnight, while under UV lights for sterilization, leaving a clear coating on the bottom of the plate that prevents cell attachment. On day one of the experiment, 100,000 cells are placed in each well with their normal growth media and allowed to grow in a 37^o incubator. At days 1, 8 and 15, the cells are counted using a hemocytometer to establish any changes in the number of cells within the well. At each time point, the cells from each condition of the transformation are seeded in triplicate and the average of the three wells is taken. MDA-MB-231 cells are used as a positive control.

Boyden chamber invasion assay – The Boyden chamber inserts are placed into 24 well plates, allowed to soak in media for 5 minutes, and then coated with matrigel diluted 1:6 using media. The matrigel is allowed to set for at least 20 minutes but must be used within 2 hours to start the assay. Trypsinized cell samples are spun down and resuspended at 1x10⁶ cells/ml in serum free media for all conditions. 200µL of the cell suspension is then placed on top of the matrigel covered insert suspended in the 24 well plate. Serum free media is also placed in the well under the matrigel insert. The cells then re-equilibrate for 24 hours in a 37^o incubator before analysis. After 24 hours, the invasive cells should have moved through the matrigel and from the top to the bottom of the boyden chamber insert. At this point, the insert is washed with PBS and the cells are fixed with glutaraldehyde. The fixed cells are then stained with toluidine blue and can be counted under a microscope. For quantification, multiple pictures are taken of the stained cells in various fields of the insert to determine the level of invasion. MDA-MB-231 cells are used as a positive control and MCF-7 cells are used as a negative control.

Crystal violet sensitivity assay – Cells grown under normal culture conditions are trypsinized and counted. Suspensions are diluted with media to concentrations of either 1.25x10⁵ cells/ml (4T1) or 2.5x10⁵ cells/ml (MCF-7 and MDA-MB-231). Using a 96 well plate, 200µl of the cell suspension is placed into each well, and cells are allowed to equilibrate for 24 hours prior to initiation of drug treatment. For analysis, the media is removed and cell are washed with PBS prior to being fixed with methanol. Crystal violet dye is added to stain the cells. After all background dye is washed away with PB, the remaining dye in the cells is solubilized with sodium citrate solution in ethanol. Absorbance values are measure on a microplate reader at 540nm.

3D acini formation assay – Assay media is normal growth media containing 2% serum (10% serum is used for routine cell growth) and the inclusion of 2% matrigel. Cells are suspended in assay media at 1x10⁵ cells/ml. Using a 48 well plate, 150µl of matrigel is placed at the bottom of each well and allowed to polymerize for 40 min. 500µl of the cell suspension is then placed on the matrigel and allowed to incubate at 37^o for the remainder of the experiment. Media is replenished every 4 days for up to 16 days.

Cell transformation

Rationale MCF-10a cells are an immortalized breast epithelial cell line that is not malignant and that has routinely been utilized for an *in vitro* model of transformation induced by various chemical compounds including carcinogens and hormones (Liu 2004, Siriwardhana 2008). We previously reported that high doses of estrogen alone were ineffective in promoting cell transformation. Treatment with other carcinogens alone (Benzopyrene and N-nitroso-N-methylurea) also met with similar lack of success. We speculated that combination treatments

might prove more effective. More specifically, the premise to be tested was that Benzo[a]Pyrene (B[a]P) would potentiate the effectiveness of estrogen as a transforming agent. B[a]P, when bound to the aryl hydrocarbon receptor (AHR), will up regulate the cytochrome p450 enzyme

that will metabolically activate both B[a]P as well as Estrogen (Kuriyama 2005, Russo 2002, Uno 2006). To make the model more relevant, we chose doses of Estrogen and B[a]P that were physiologically relevant to females and smokers respectively (Siriwardhana 2008). Two doses of Estradiol were chosen, specifically 1nM, which relates to the higher end concentration during the female estrous cycle, and 15nM, which was found in women undergoing fertility treatment (Krejza 2001, Yoldemir 2009).

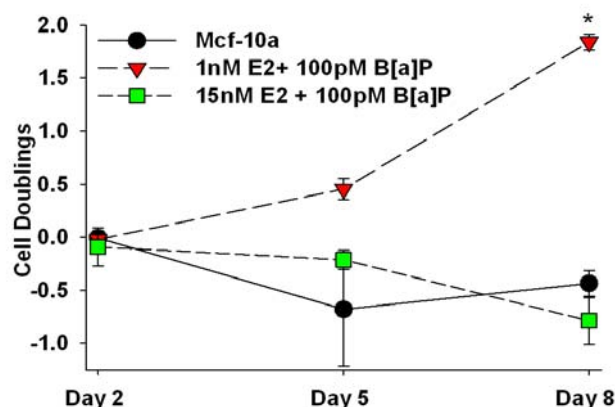


Figure 1 – Growth factor independent growth prescreening - In addition to Benzo[a]pyrene, MCF-10A cells were treated with estradiol (1nM and 15 nM). The capacity to grow in normal media when all additional growth factors were removed was used as a test of transformation. Only cells treated with 1nM Estradiol and 100pM Benzo[a]pyrene demonstrated enhanced growth (*p<0.05 compared to day 2)

Experimental Design

Cells were continuously exposed to 100pM B[a]P and either 1nM Estradiol or 15nM Estradiol. Media alone and media containing drugs was replaced daily to maintain levels of the carcinogens. Selective pressure was utilized to select for transformed cells, specifically: 1. the utilization of media lacking growth factors, which is an antiproliferative environment for normal MCF-10a cells, and 2. culture dishes coated with polyHEMA to prevent adherence to the plate,

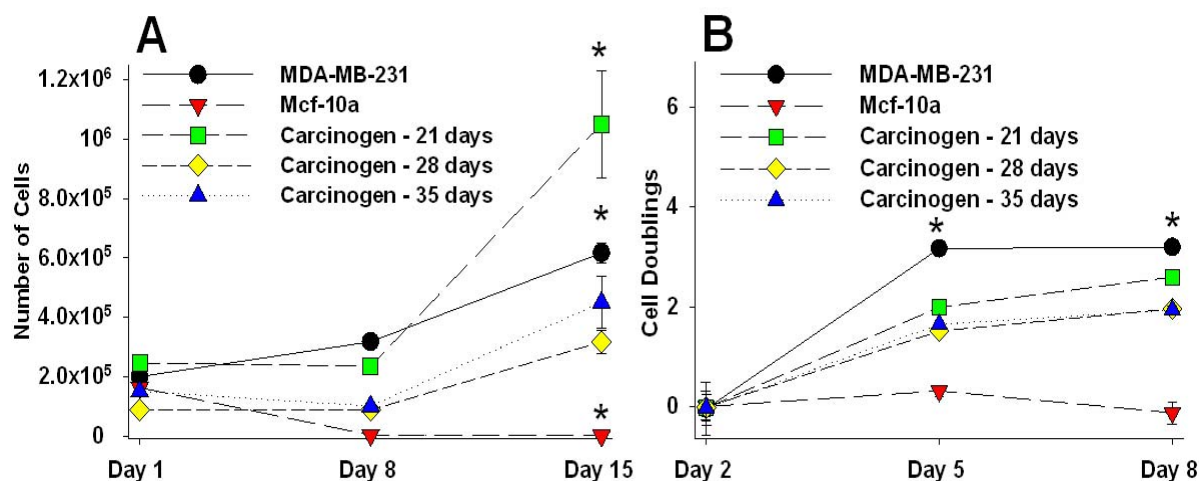


Figure 2 – Time course of MCF-10a transformation – MCF-10a cells were exposed to 1nM Estradiol and 100pM Benzo[a]pyrene for 21, 28, and 35 days to assess if shorter or longer treatments could induce varying degrees of transformation. The Anchorage Independent Growth Assay (A) and the Growth Factor Independent Growth Assay (B) showed that there were no differences in the levels of transformation with time, but that all treated cells did show transformed phenotypes when compared to MCF-10a untreated controls and MDA-MB-231 positive controls. (*p<0.05 compared to initial time point)

which is a cytotoxic environment for normal MCF-10a cells.

The cells were cycled through the compound treatment for 21, 28 and 35 days. At each time point, cell populations were split from the primary culture and grown up separately. This approach was utilized to determine whether a longer period of drug exposure would modify the transformation process or the transformed cells. Transformation was monitored based on the Growth Factor Independent Growth Assay, the Anchorage Independent Growth Assay, the 3D Acini Growth Assay, and the Boyden Chamber Invasion Assay.

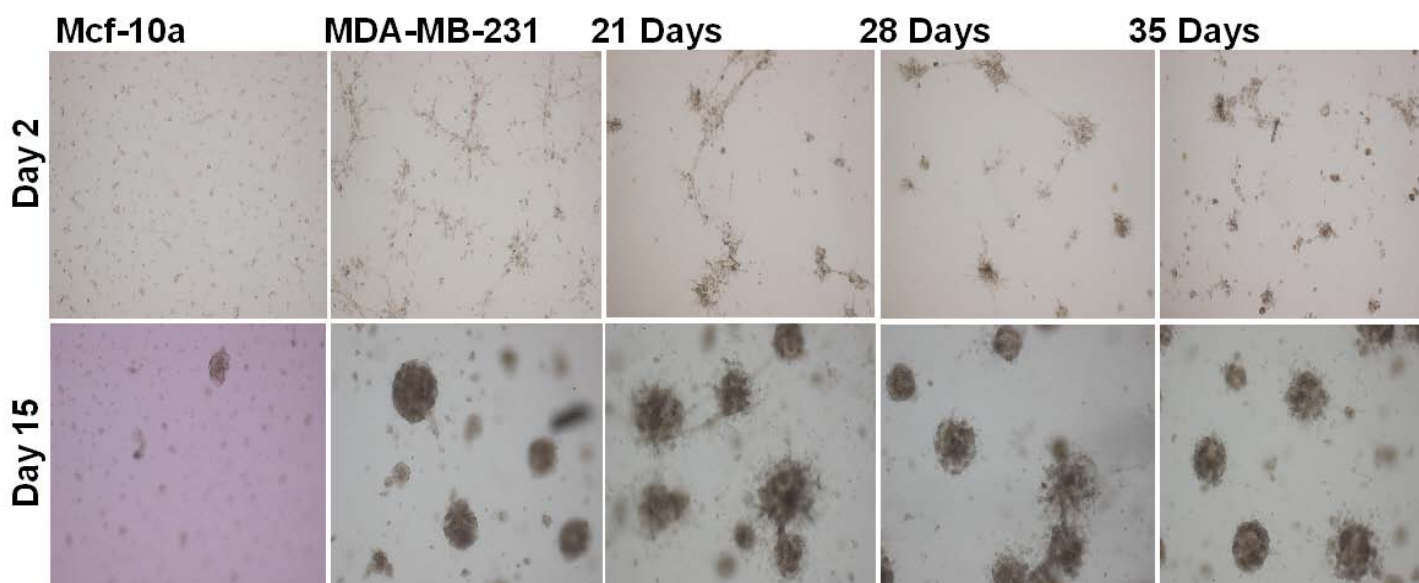


Figure 3 – 3D Acini Growth Assay –Normal MCF-10a cells were used as a positive control for the formation of Acini structures. MDA-MB-231 showed malignant characteristics and formed large cancerous growths by the end of the experiment. Cells taken from all treatment time points (days 21, 28, and 35) demonstrated a phenotype that appeared to mimic that of the MDA-MB-231 cells suggesting transformation.

Results

The Growth Factor Independent Growth Assay was used as a preliminary screen of transformation (Fig 1). Untreated MCF-10a cells were unable to grow, which can be seen by the essentially constant cell number throughout the experiment (with some degree of cell death over time). Cells treated with 1nM Estradiol and 100pM B[a]P showed a steady and significant level of growth, consistent with the possibility of transformation. Unexpectedly, cells treated with the higher dose of estradiol did not show evidence of transformation in terms of capacity to grow in the depleted medium conditions, suggesting that supra-physiological levels of estradiol may be self limiting in the transformation process.

The growth factor independent growth assay and the anchorage independent growth assay were further utilized to assess the stability of the transformation process in cells treated with 1nM Estradiol and 100pM B[a]P. MCF-10a cells were again used as the negative experimental control and the aggressive breast cancer cell line, MDA-MB-231, was used as a positive control for transformation. As before the untreated MCF-10a cells showed no capacity for growth under these conditions (Figures 2A and 2B). The MDA-MB-231 cells grew effectively

as positive controls. The data for both assays appear to indicate that the 21 day exposure was sufficient for transformation.

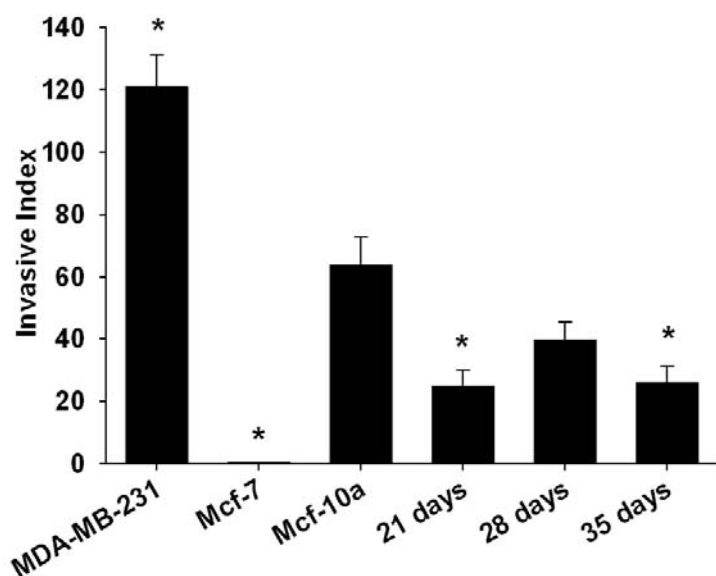


Figure 4 – Boyden Chamber Invasion Assay – MDA-MB-231 cells were used as a positive control and MCF-7 cells as a negative control. MCF-10a cells typically have a modest capacity for invasion. Transformed cells taken for all three time points (21, 28 and 35 days) all showed reduced invasive indexes compared to untreated controls (* $p < 0.05$ compared to MCF-10a cells)

Additional studies were performed utilizing the 3D Acini Growth Assay and the Boyden Chamber Invasion Assay. Normal MCF-10a cells will form small Acini like structures similar to milk forming Acini found in mammary tissue (Debnath 2003). MDA-MB-231 cells were again used as a positive control and the untreated MCF-10a cells as a negative control. Figure 3 indicates that on day 2, the MCF-10a cells are separate and isolated and by day 15 they have slowly begun to form small Acini like structures within the culture. MDA-MB-231 cells show activity by day 2 where the cells are either collecting or already beginning to spread, which can be seen by the branch formations. By day 15, the MDA-MB231 cells have formed large malignant masses and completely taken over the dish. Although this assay is difficult to quantify in terms of the level of transformation, when compared to the two controls it is clearly evident that transformation has occurred. Comparing days 21,

28, and 35 to the control provided additional evidence that the cells have developed a transformed phenotype.

Invasiveness of all treated cells was tested using the Boyden Chamber Invasion Assay (Fig 4). The untreated MCF-10a cells had a modest level of invasion, which is typical for this assay. MCF-7 cells have no invasive capacity and MDA-MB-231 cells are highly invasive, and therefore were used as negative and positive controls, respectively. Unexpectedly, calculation of the invasive index for the carcinogen treated cells was lower than that of the parental untreated MCF-10a cells. These studies suggest that the Boyden Chamber assay may not be appropriate for assessment of transformation in this experimental model systems.

Experiments are currently in progress to examine the influence of select cannabinoid agonists and antagonists on transformation. In addition, these studies are being repeated utilizing another model of transformation, MCF-10F cells (Russo 2006).

Influence of cannabinoid agonists and antagonists on response to chemotherapeutic drugs in cellular models of breast cancer

Marinol is an orally administered form of THC and currently the only cannabinoid approved for use with chemotherapy. Marinol is used primarily as an anti-emetic, but there is no information as to whether cannabinoids may modulate the response to cancer

chemotherapeutic drugs in the clinic. Although reports that cannabinoids have anti-proliferative effects in cancer suggest that the administration of cannabinoids is unlikely to compromise the effectiveness of chemotherapy (Guzman 2003), this has not been tested directly. To explore this possibility, we utilized three breast cancer cell lines, MCF-7 (human, estrogen receptor positive, and p53 wild type), MDA-MB-231 (human, estrogen receptor negative, and p53 mutant) and 4T1 (mouse, estrogen receptor negative, and p53 null). Cells were treated with THC in combination with doxorubicin (ADR), a drug commonly utilized in the treatment of breast cancer.

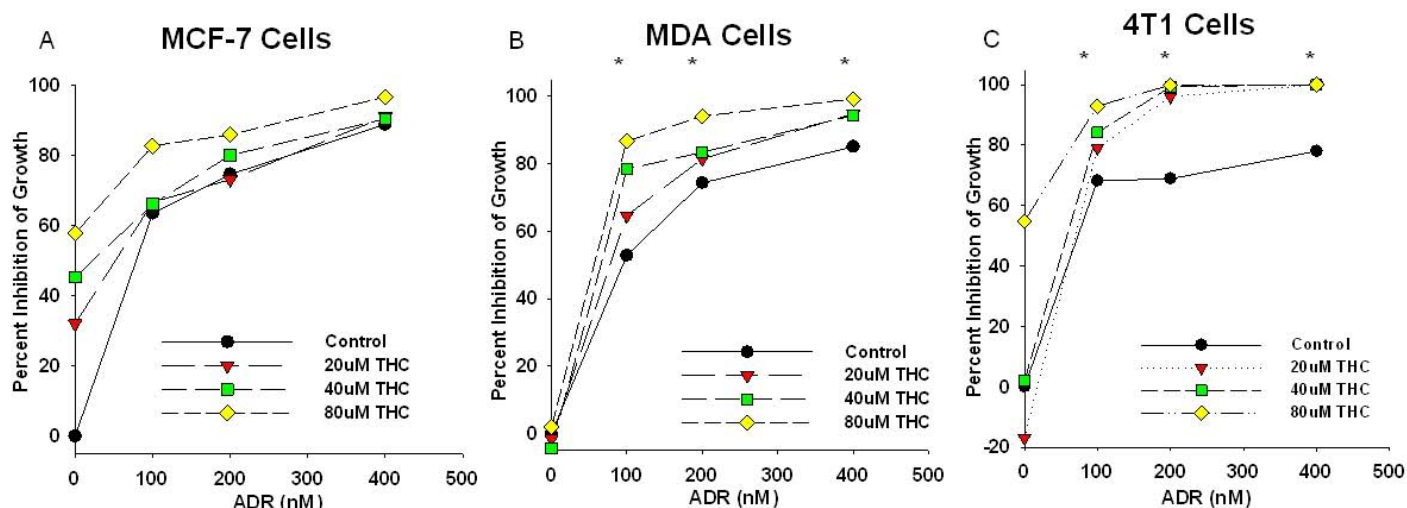


Figure 5 – THC augments the antiproliferative actions of doxorubicin (Adriamycin or ADR) in breast tumor cells

- Combinations of THC and ADR produce cell line specific antiproliferative responses. MCF-7 cells (A) showed no significant changes when treatments were given in combination even though these cells were the most sensitive to THC. The MDA-MB-231 cells (B) and 4T1 cells (C) not only showed significant enhancement of the antiproliferative effect in the combination, but they also did so with doses of THC that produced no significant effects alone. (* $p < 0.01$ vs. Control)

In MCF-7 cells, the inclusion of THC failed to produce any significant effects on sensitivity to doxorubicin (Figure 5A). While THC failed to increase the effectiveness of doxorubicin, the fact that it did not interfere with drug action is encouraging. In contrast, THC appeared to enhance sensitivity to doxorubicin in the MDA-MB231 cells (Figure 5B). The fact that enhancements occurred at doses of THC that were ineffective alone is indicative of a positive synergistic interaction; statistical analysis confirmed this (report presented in section 1 of statistical analysis). Enhancement of drug sensitivity as well as a possible synergistic interaction was also evident in the experiments utilizing the 4T1 cells (Figure 5C).

The above findings (lack of interaction in p53 wild type MCF-7 cells and apparent synergism in p53 mutant MDA-MB231 and p53 null 4T1 cells) suggest that the absence of functional p53 is permissive for drug interaction. Utilizing 4T1 cells stably transfected with a ponasterone A induced expression vector with either wild type or mutant p53, additional experiments were designed to further evaluate the involvement of p53 in drug interaction.

In p53 null naïve 4T1 cells, a THC concentration of 15uM was alone ineffective; however, this concentration of THC was able to significantly enhance the antiproliferative effects of ADR (Figure 6A). In the 4T1 cells transfected with wild type p53 the 15uM dose of THC remained ineffective, but also produced a statistically significant enhancement of sensitivity to ADR (Figure 6B); however, this effect was marginal compared to that in the p53 null wild type 4T1 cells. In the 4T1 cells transfected with mutant p53, the 15uM dose of THC was also

ineffective alone, but produced a relatively robust enhancement of sensitivity to ADR (Figure 6C. Proof of the effectiveness of the transfections is presented in the western blots in Fig 6D. Taken together these data suggest that the absence of p53 function has a significant contribution to the interaction between THC and ADR. Studies are currently planned in MCF-7 cells where p53 function has been suppressed by the viral E6 protein. We also plan to evaluate the mode(s) of cell death that occur in response to the combination treatments as well as the combination of THC with drugs such as paclitaxel and cisplatin.

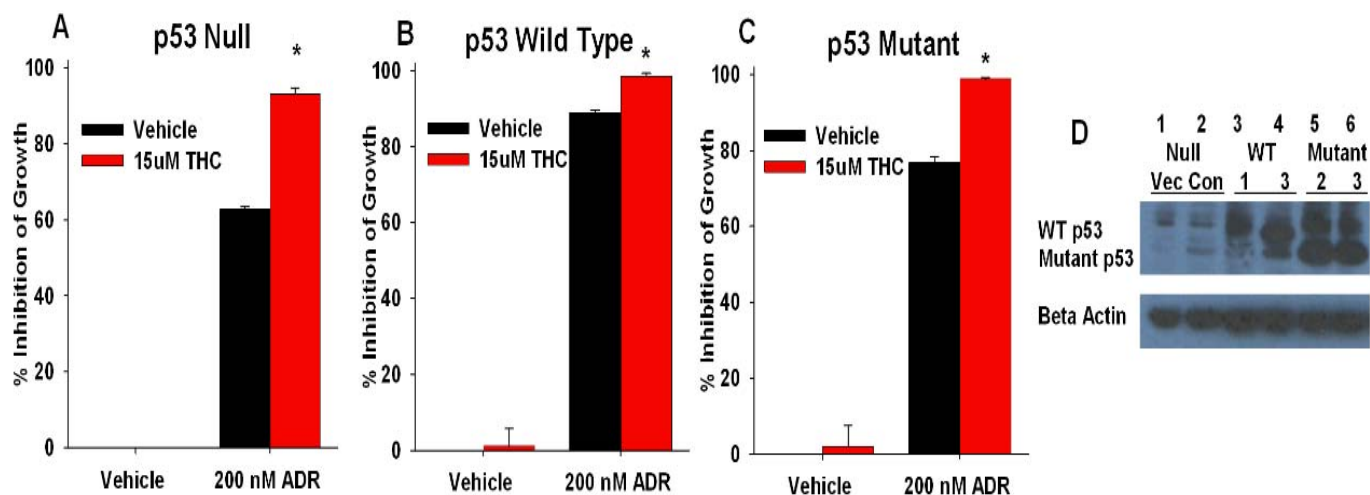


Figure 6 – The interaction between THC and ADR is at least partially p53-dependent - 4T1 parent cells are p53 null (A). 4T1 cells were stably transfected with either wild type p53 (B) or mutant p53 (C) in a Ponasterone A induced expression vector. Cells were treated with 15uM THC, alone which produced no significant response. When this treatment was combined with 200nM Adriamycin (ADR), there was a significant enhancement of the antiproliferative effect in all the variants of the 4T1 cell line. Western blotting (D) confirms success of the transfection. (*p<0.01 vs. ADR alone)

Additional studies were performed using another cannabinoid, cannabidiol, since Sativex, a 1:1 mixture of THC and Cannabidiol (CBD), is used in Canada for treatment of spasticity and neuropathic pain associated with multiple sclerosis, as well as, chronic cancer pain. As in the studies above, the three breast cancer cell lines (MCF-7, MDA-MB-231, and 4T1) were treated with cannabidiol + doxorubicin and the Crystal Violet Assay was used to assess drug interaction.

Our initial observation was that CBD appeared to be more potent than THC but still required high doses, up to 40uM, to produce antiproliferative effects alone. Unexpectedly, CBD was able to significantly increase the effects of ADR suggesting a positive interaction (Figure 7A), although further statistical analysis indicated that the interaction was additive in nature, but not synergistic. However, these findings remain encouraging as CBD is more potent than THC and has no psychoactive side effects. The combination treatment was also additive (but not synergistic) in MDA-MB-231 cells (Figure 7B). Interestingly, the enhancement of sensitivity to doxorubicin appeared to be synergistic in the 4T1 cell model (Figure 7C). The report for these interactions is in section 2 of the statistical analysis below.

One element of these studies that raised concerns is that relatively high concentration of cannabinoids were required to enhance sensitivity to doxorubicin. These concentrations would be difficult to achieve in the circulation; furthermore, at these elevated concentrations, it is likely that we are observing off-target effects that are unrelated to endocannabinoid receptor signaling. This could result in unanticipated modes of toxicity to the patient.

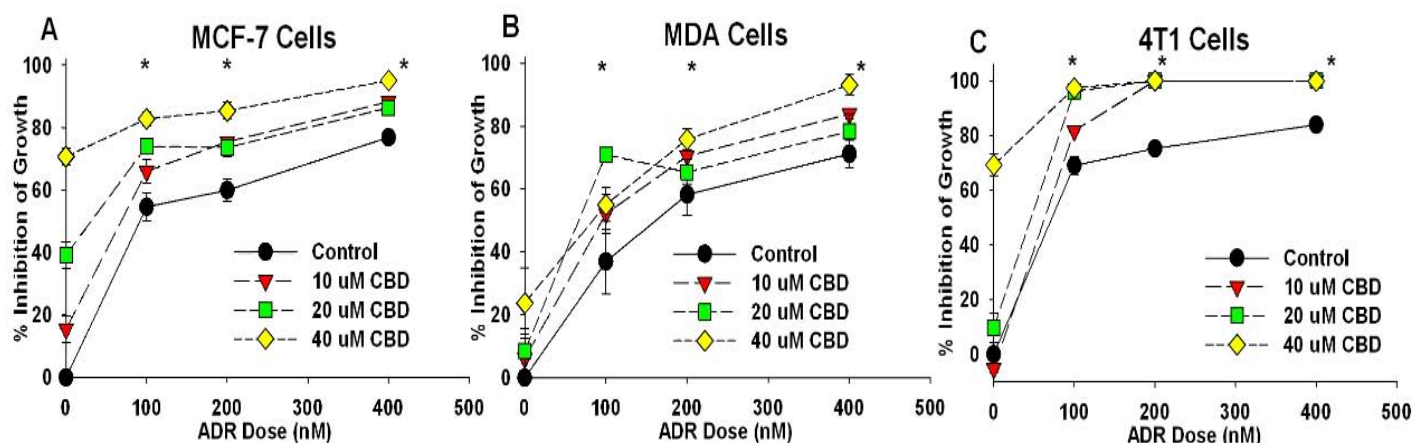


Figure 7 – CBD augments the antiproliferative actions of ADR - CBD and ADR given in combination resulted in significant increases in the antiproliferative effects occurring at doses of CBD that produced no effects alone in the MDA-MB-231 cells (B) and 4T1 cells (C). In MCF-7 cells (A) CBD had significant effects alone and the combination of CBD and ADR was able to produce significant increases in growth inhibition at all doses. (* $p < 0.01$ vs. Control)

To address this concern, we compared the dose response curves of the full cannabinoid agonist Win55,212-2 (Win2) and its inactive enantiomer Win55,212-3 (Win3) (Fig 8). In all three cell lines, we found that Win2 showed a significantly higher level of both potency and efficacy. These observations suggest that drug effects are occurring via a very selective molecular target at concentrations that would be pharmacologically relevant. This conclusion is supported by the observation that the Win3 enantiomer was relatively ineffective in these experimental models.

Given the known selectivity of Win2 and the increased potency shown in Figure 8, the impact of WIN2 on sensitivity to doxorubicin was examined, as above. Effects of the combination treatment were generally similar to those observed with both THC and CBD. In the MCF-7 cells, there was a positive interaction between the two drugs, ruling out antagonistic effects; however, statistical analysis failed to provide evidence of synergism, but only of additivity (Figure 9 A). In both the MDA-MB-231 cells and the 4T1 cells, the Win-ADR combination was found to be synergistic (Figures 9B and 9C). Detailed statistical analyses are presented below.

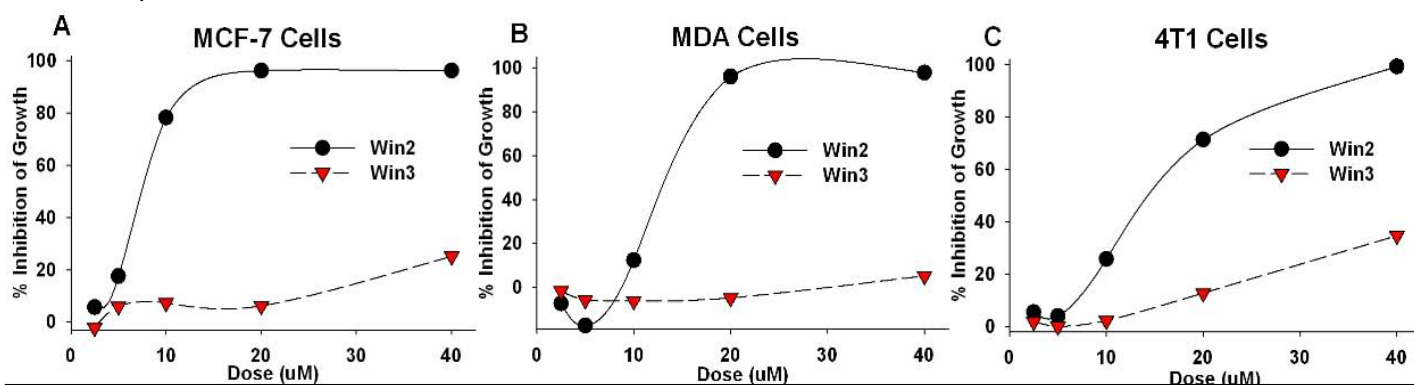


Figure 8 – Win55,212-2 elicits a stereoselective antiproliferative action in breast cancer cells MCF-7 (A), MDA-MB-231 (B), and 4T1 (C) breast cells were treated with the cannabinoid full agonist Win55,212-2 (Win2) or its inactive enantiomer Win55,212-3 (Win3).

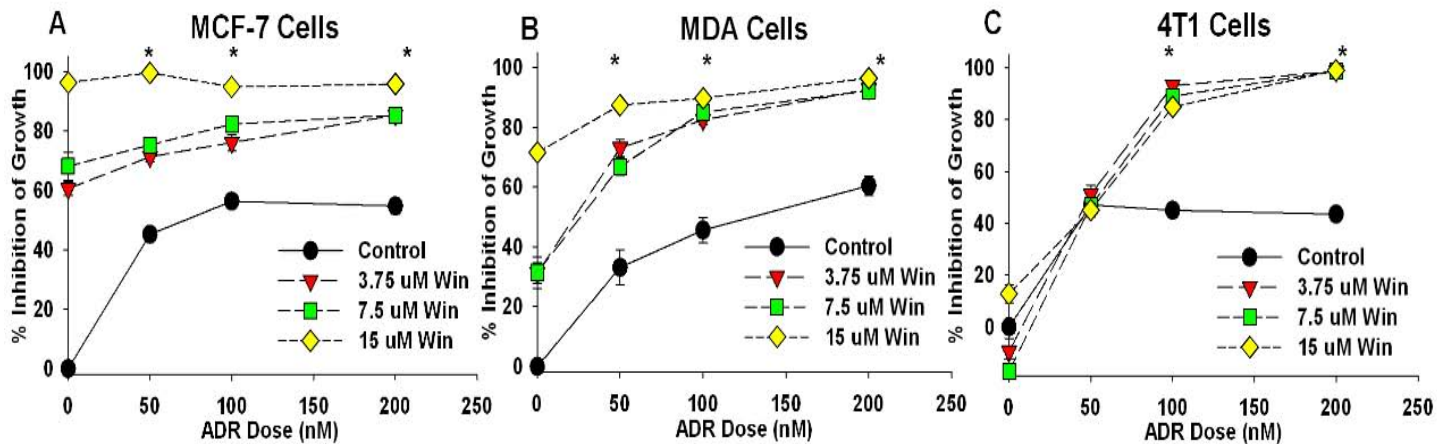


Figure 9 – Win55,212-2 augments ADR’s antiproliferative effect similarly to phytocannabinoid treatments - Win2 was administered in combination with ADR to MCF-7 cells (A), MDA-MB-231 cells (B), and 4T1 cells (C). In all three cell lines the conditions of ADR and Win2 were able to produce greater antiproliferative effects than ADR alone. (* $p < 0.01$ vs. Control)

Statistical Analysis of Drug Combinations

Section 1 – THC and ADR combination

Purpose:

The purpose of these analyses was to determine if there is a significant interaction (i.e., synergy) in combination treatment of THC and ADR for breast cancer.

Experimental data:

In vitro data was collected at varying drug dose combinations of THC (0, 20, 40, 80 μM) and ADR (0, 100, 200, 400 nM). Each of the 17 possible combinations was replicated 4 times. The measured outcome was absorbance.

Outcome variable:

The outcome of interest was percent inhibition of growth in specific cancer cell lines. This was calculated by dividing the absorbance for each dose level by the mean of the control group (0 μM THC and 0 nM ADR).

Preliminary analyses including graphical depiction of summary statistics indicated the variance of the response percent inhibition (denoted Y) decreased as Y increased. Thus, in subsequent analyses, we assume $\text{Var}(Y) = \zeta / \mu$ and use quasi-likelihood methods to estimate model parameters. All analyses were performed using iterative algorithms (e.g., Gauss-Newton) provided in PROC NLIN with LOSS and WEIGHT statements in SAS (version 9.2).

Model:

A non-linear exponential model as defined below was used to model percent inhibition of growth.

$$\mu = \gamma * (1 - \exp(-(\beta_1 * \text{ADR} + \beta_2 * \text{THC} + \beta_{12} * \text{ADR} * \text{THC})))$$

Where μ represents the estimated % inhibition for the specified dose levels and THC, ADR represent the dosage levels in μM and nM, respectively. The hypothesis of interest is:

$$H_0: \beta_{12} = 0 \text{ vs}$$

$$H_1: \beta_{12} \neq 0$$

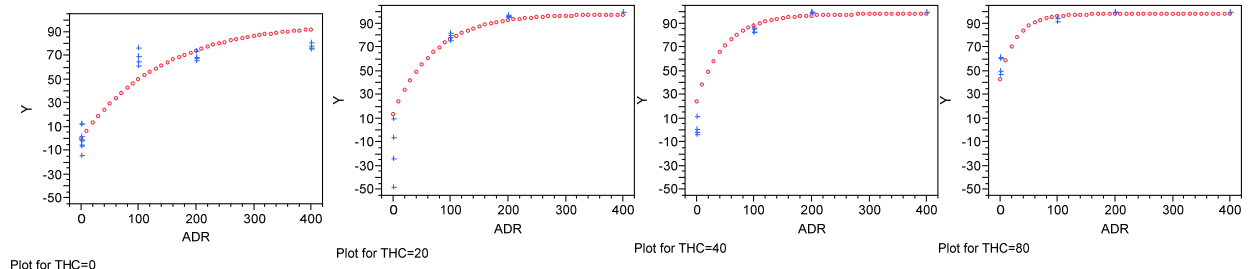
Large sample Wald-type chi-square tests were used to test for the significance of the interaction parameter. When the linear terms (i.e., β_1 and β_2) are positive and β_{12} is positive and significant, a synergy is claimed following the work of Carter et al (1988); if β_{12} is negative and significant, an antagonism is claimed; if β_{12} is not significant departure from additivity cannot be claimed.

Results:

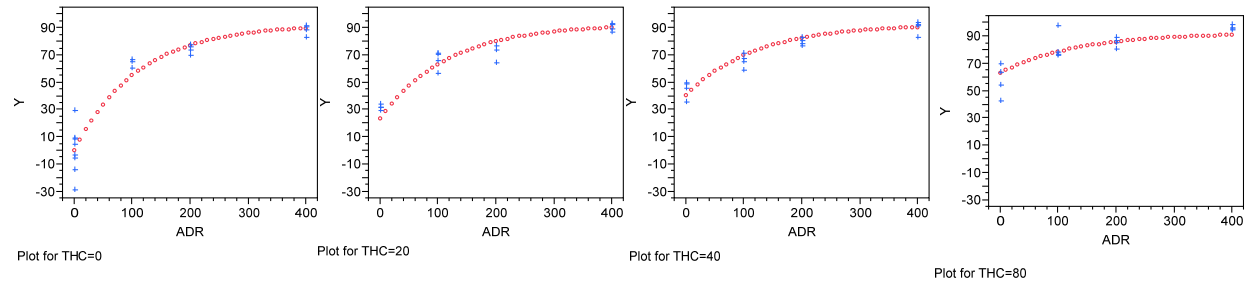
Separate analyses were conducted on each cell line. The goodness of the fit of the data by the nonlinear exponential model was assessed graphically by plotting predicted and actual percent inhibition together. These plots can be found in the figure 1 below. There was no evidence of lack of fit. These results were confirmed by chi-sq goodness of fit tests, none of which yielded significant p-values (i.e. did not indicate lack of fit).

Figure 1: Assessing Goodness of Fit

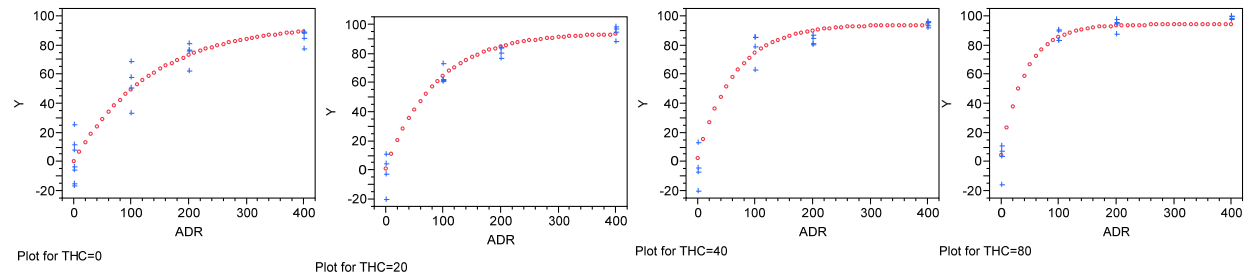
1a: 4T1 Cell line



1b: MCF_7 Cell line



1c: MDA Cell line



Y ○ Predicted Percent Inhibition + Percent Inhibition

In all three cell lines, both single drug slope parameters were positive and significant ($p < 0.05$), indicating as the drug increases there is a significant increase in the percent inhibition of cell growth. For the 4T1 and MDA cell lines, there was a significant positive interaction effect between ADR and THC dosage. In the MCF-7 cell line, the estimate for the interaction effect was negligible and not statistically significant. Therefore, there was no evidence of interaction. Model parameter estimates for each cell line can be found in Table 1.

Table 1: Parameter estimates for 4T1 Cell line

| Parameter | Estimate | Approx. SE | 95% Confidence Limits | |
|-----------------|----------|------------|-----------------------|----------|
| 4T1 Cell line | | | | |
| γ | 97.6832 | 1.7171 | 94.2430 | 101.1000 |
| β_1 | 0.0071 | 0.0007 | 0.0057 | 0.0086 |
| β_2 | 0.0072 | 0.0012 | 0.0047 | 0.0096 |
| β_{12} | 0.0003 | 0.0001 | 0.0002 | 0.0005 |
| MCF_7 Cell line | | | | |
| γ | 92.2900 | 2.0331 | 88.2170 | 96.3627 |
| β_1 | 0.0090 | 0.0008 | 0.0074 | 0.0106 |
| β_2 | 0.0143 | 0.0012 | 0.0118 | 0.0168 |
| β_{12} | -2.0E-05 | 2.4E-05 | 6.0E-05 | 3.1E-05 |
| MDA Cell Line | | | | |
| γ | 94.0211 | 1.3412 | 91.3340 | 96.7079 |
| β_1 | 0.0075 | 0.0006 | 0.0064 | 0.0086 |
| β_2 | 0.0006 | 0.0016 | -0.0030 | 0.0039 |
| β_{12} | 0.0002 | 3.4E-05 | 0.0001 | 0.0003 |

For the 4T1 and MDA cell lines, the parameter estimate for β_{12} is associated with an increase in percent inhibition of growth that is in addition to the additive effects of THC and ADR. For example, by combining a 100nM dose of ADR to a 20 μ M dose of THC would result in an estimated percent inhibition (in the MDA cell line) of over 64% while the 100nM dosage of ADR would be estimated at just under 50% and the 20 μ M of THC only 1%. The positive (synergistic) interaction effect is apparent since 64% is larger than the sum of the two effects (51%). For the 4T1 cell line, the estimated percent inhibition for the combination is 77% vs. 50% for ADR alone and 13% for THC alone. Again, the combination effect is greater than the expected additive effect, therefore there is a synergistic interaction.

Section 2 – CBD and ADR combination

Purpose:

The purpose of these analyses was to determine if there is a significant interaction (i.e., synergy) in combination treatment of CBD and ADR for breast cancer.

Experimental data:

In vitro data was collected at varying drug dose combinations of CBD (0, 10, 20, 40 μ M) and ADR (0,100,200,400 nM). Each of the 17 possible combinations was replicated 4 times. The measured outcome was absorbance.

Outcome variable:

The outcome of interest was percent inhibition of growth in specific cancer cell lines. This was calculated by dividing the absorbance for each dose level by the mean of the control group (0 μ M CBD and 0 nM ADR).

Preliminary analyses including graphical depiction of summary statistics indicated the variance of the response percent inhibition (denoted Y) decreased as Y increased. Thus, in

subsequent analyses, we assume $\text{Var}(Y) = \zeta / \mu$ and use quasi-likelihood methods to estimate model parameters. All analyses were performed using iterative algorithms (e.g., Gauss-Newton) provided in PROC NLIN with LOSS and WEIGHT statements in SAS (version 9.2).

Model:

A non-linear exponential model as defined below was used to model percent inhibition of growth.

$$\mu = \gamma * (1 - \exp(-(\beta_1 * CBD + \beta_2 * ADR + \beta_{12} * CBD * ADR)))$$

Where μ represents the estimated % inhibition for the specified dose levels and THC, ADR represent the dosage levels in μM and nM , respectively. The hypothesis of interest is:

$$H_0: \beta_{12} = 0 \text{ vs } H_1: \beta_{12} \neq 0$$

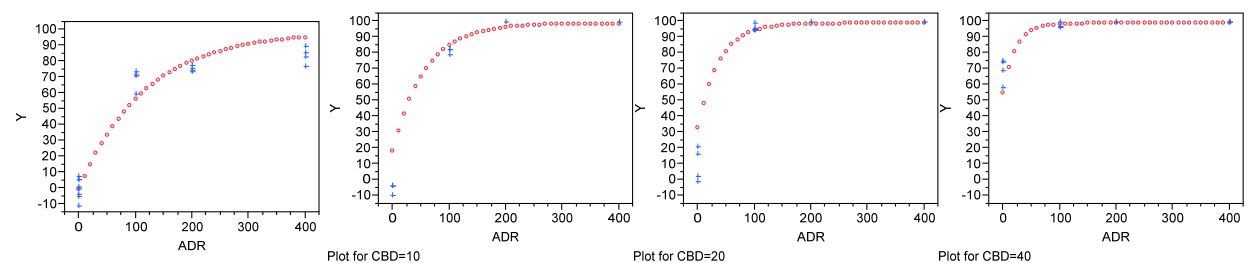
Large sample Wald-type chi-square tests were used to test for the significance of the interaction parameter. When the linear terms (i.e., β_1 and β_2) are positive and β_{12} is positive and significant, a synergy is claimed following the work of Carter et al (1988); if β_{12} is negative and significant, an antagonism is claimed; if β_{12} is not significant departure from additivity cannot be claimed.

Results:

Separate analyses were conducted on each cell line. The goodness of the fit of the data by the nonlinear exponential model was assessed graphically by plotting predicted and actual percent inhibition together. These plots can be found in the figure 1 below. There was no evidence of lack of fit. These results were confirmed by chi-sq goodness of fit tests, none of which yielded significant p-values (i.e. did not indicate lack of fit).

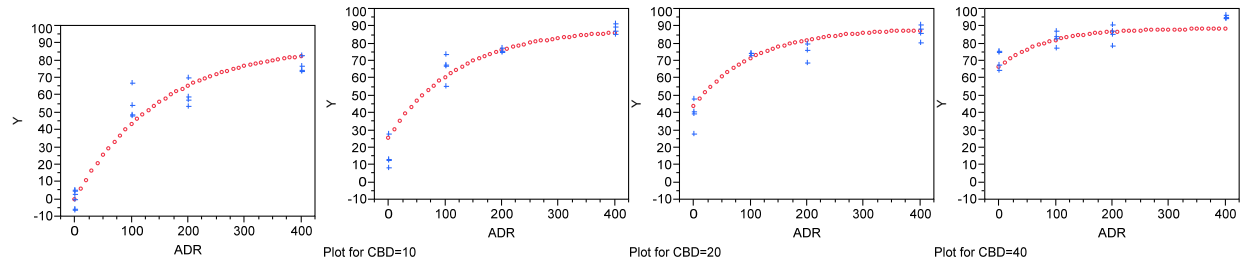
Figure 1: Assessing Goodness of Fit

1a: 4T1 Cell line



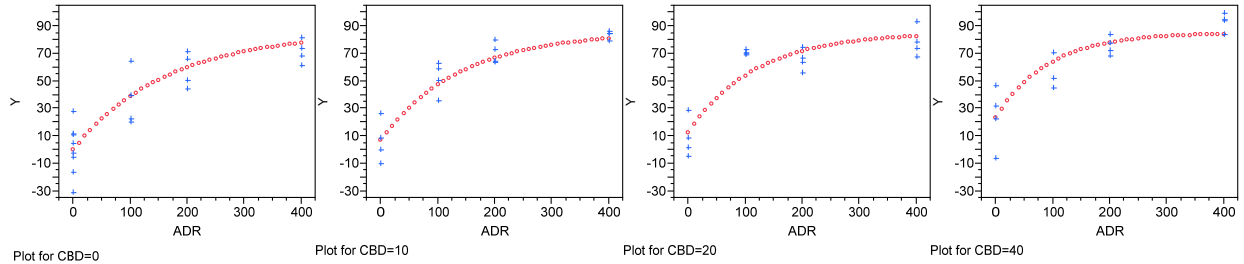
Plot for CBD=0

1b: MCF_7 Cell line



Plot for CBD=0

1c: MDA Cell line



Plot for CBD=0

Plot for CBD=10

Plot for CBD=20

Plot for CBD=40

Y ○ Predicted Percent Inhibition + Percent Inhibition

In all three cell lines, both single drug slope parameters were positive and significant ($p < 0.05$), indicating as the drug increases there is a significant increase in the percent inhibition of cell growth. For the 4T1 cell line, there was a significant positive interaction effect between ADR and CBD dosage. In the MCF-7 and MDA cell lines, the estimate for the interaction effect was negligible and not statistically significant. Therefore, there was no evidence of interaction in the MCF-7 and MDA cell lines. Model parameter estimates for each cell line can be found in Table 1.

Table 1: Parameter estimates for 4T1 Cell line

| Parameter | Estimate | Approx. SE | 95% Confidence Limits | |
|-----------------|----------|------------|-----------------------|---------|
| 4T1 Cell line | | | | |
| γ | 98.737 | 1.467 | 95.798 | 101.700 |
| β_1 | 0.020 | 0.002 | 0.016 | 0.025 |
| β_2 | 0.008 | 0.001 | 0.007 | 0.010 |
| β_{12} | 0.001 | 2.6E-04 | 3.9E-04 | 0.001 |
| MCF 7 Cell line | | | | |

| | | | | |
|----------------------|---------|---------|----------|---------|
| γ | 88.456 | 1.896 | 84.658 | 92.253 |
| β_1 | 0.007 | 0.001 | 0.005 | 0.008 |
| β_2 | 0.034 | 0.003 | 0.028 | 0.041 |
| β_{12} | 1.4E-04 | 7.7E-05 | -1.0E-05 | 3.0E-04 |
| MDA Cell Line | | | | |
| γ | 85.192 | 3.451 | 78.280 | 92.104 |
| β_1 | 0.006 | 0.001 | 0.004 | 0.008 |
| β_2 | 0.008 | 0.003 | 0.002 | 0.015 |
| β_{12} | 1.2E-04 | 6.1E-05 | -5.6E-06 | 2.4E-04 |

For the 4T1 cell line, the parameter estimate for β_{12} is associated with an increase in percent inhibition of growth that is in addition to the additive effects of CBD and ADR. For example, by combining 10 μ M of CBD and 100nM of ADR, the estimated percent inhibition for the combination is 84% vs. 56% for ADR alone and 18% for CBD alone. Since the combination effect is greater than the expected additive effect, there is a synergistic effect.

Section 3 – Win55 and ADR combination

Purpose:

The purpose of these analyses was to determine if there is a significant interaction (i.e., synergy) in combination treatment of WIN and ADR for breast cancer.

Experimental data:

In vitro data was collected at varying drug dose combinations of WIN (0, 3.75, 7.5, 15 μ M) and ADR (0, 50, 100, 200 nM). Each of the 17 possible combinations was replicated 4 times. The measured outcome was absorbance.

Outcome variable:

The outcome of interest was percent inhibition of growth in specific cancer cell lines. This was calculated by dividing the absorbance for each dose level by the mean of the control group (0 μ M WIN and 0 nM ADR).

Preliminary analyses including graphical depiction of summary statistics indicated the variance of the response percent inhibition (denoted Y) decreased as Y increased. Thus, in subsequent analyses, we assume $\text{Var}(Y) = \zeta / \mu$ and use quasi-likelihood methods to estimate model parameters. All analyses were performed using iterative algorithms (e.g., Gauss-Newton) provided in PROC NLIN with LOSS and WEIGHT statements in SAS (version 9.2).

Model:

A non-linear exponential model as defined below was used to model percent inhibition of growth.

$$\mu = \gamma * (1 - \exp(-(\beta_1 * WIN + \beta_2 * ADR + \beta_{12} * WIN * ADR)))$$

Where μ represents the estimated % inhibition for the specified dose levels and WIN, ADR represent the dosage levels in μ M and nM, respectively. The hypothesis of interest is:

$$H_0: \beta_{12} = 0 \text{ vs } H_1: \beta_{12} \neq 0$$

Large sample Wald-type chi-square tests were used to test for the significance of the interaction parameter. When the linear terms (i.e., β_1 and β_2) are positive and β_{12} is positive and significant, a synergy is claimed following the work of Carter et al (1988); if β_{12} is negative and

significant, an antagonism is claimed; if β_{12} is not significant departure from additivity cannot be claimed.

Results: 4T1 Cell line

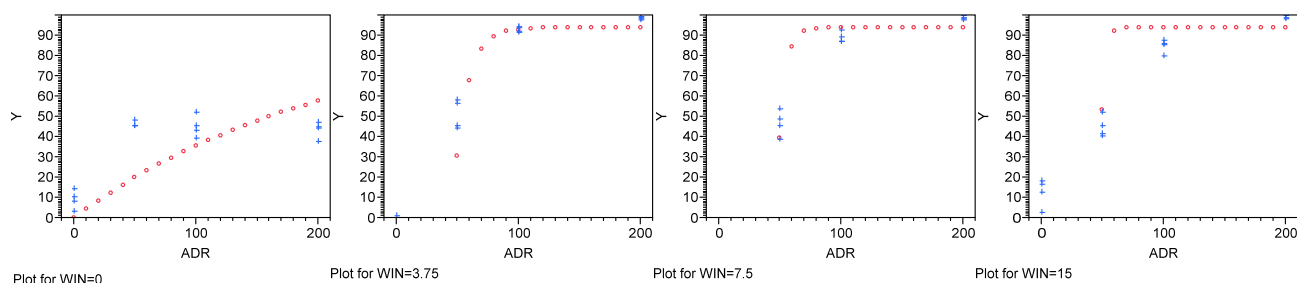
In the 4T1 cell line, the initial nonlinear exponential model including the interaction effect did not seem to accurately represent the data. The interaction effect was insignificant and there was instability in the estimate for the effect of WIN. The parameter estimates for this model can be seen in Table 1 below. A subsequent model was fit to assess the additivity of WIN and ADR. This model showed improved stability and a more sufficient fit to the data. The goodness of fit was assessed graphically (and confirmed numerically by a chi-sq test) by plotting the predicted percent inhibition of growth with the observed data. This can be seen in Figure 1. This model indicated there was not evidence of an effect attributed to WIN. The parameter estimates can be seen in Table 1.

Table 1: Parameter Estimates for 4T1 Cell Line

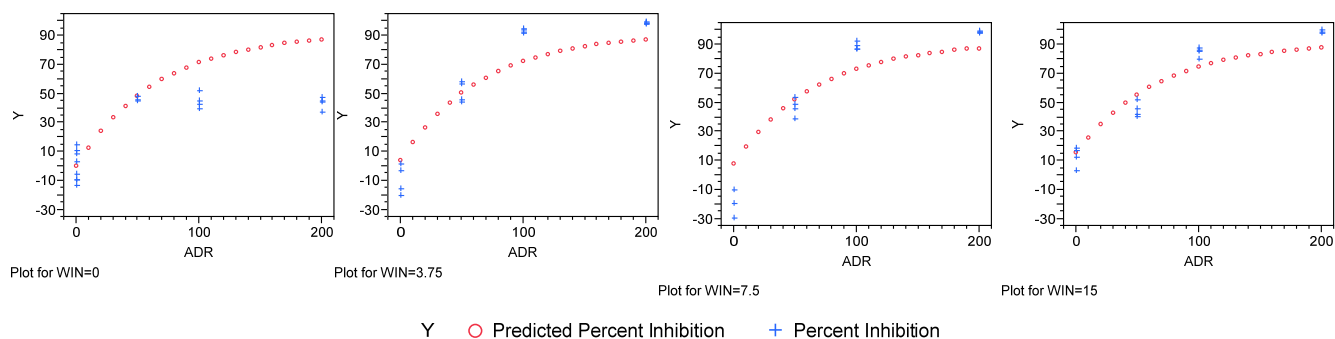
| Parameter Estimates for 411 Cell Line | | | | |
|---------------------------------------|----------|------------|-----------------------|---------|
| Parameter | Estimate | Approx. SE | 95% Confidence Limits | |
| Interaction Model | | | | |
| γ | 93.735 | 1.875 | 89.956 | 97.515 |
| β_1 | 0.005 | 0.001 | 0.004 | 0.006 |
| β_2 | -1.102 | 1.778 | -4.685 | 2.481 |
| β_{12} | 0.023 | 0.036 | -0.049 | 0.095 |
| Additive Model | | | | |
| γ | 91.628 | 6.374 | 78.865 | 104.400 |
| β_1 | 0.015 | 0.004 | 0.008 | 0.022 |
| β_2 | 0.012 | 0.012 | -0.012 | 0.037 |

Figure 1: Assessing Goodness of Fit in 4T1 Cell Line

1a: Interaction Model



1b: Additive Model



Results: MCF-7 Cell Line

In the MCF-7 cell line, there was not evidence of an interaction effect. In the reduced model, both single drug slope parameters were positive and significant ($p < 0.05$), indicating as the drug increases there is a significant increase in the percent inhibition of cell growth. The goodness of fit was assessed graphically and can be seen in Figure 2. Parameter estimates for both models can be seen in Table 2. In contrast to the 4T1 cell line in which there was not evidence of an effect for WIN, there was a very strong, positive effect in the MCF-7 cell line.

Table 2: Parameter Estimates for MCF-7 Cell Line






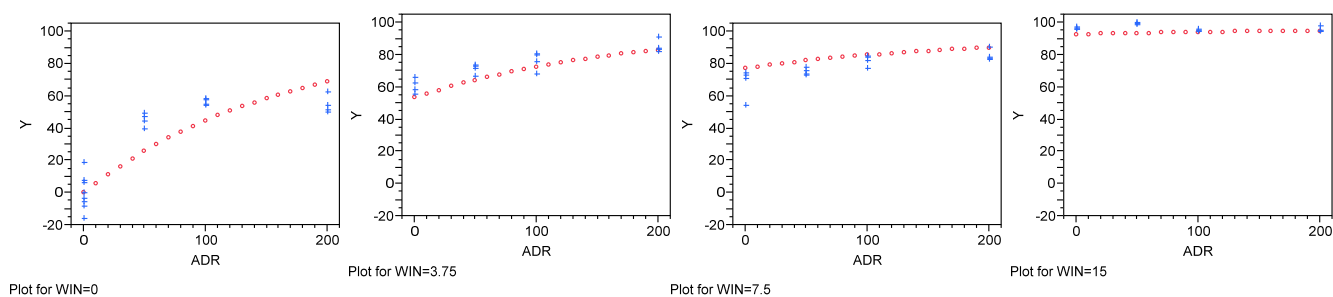
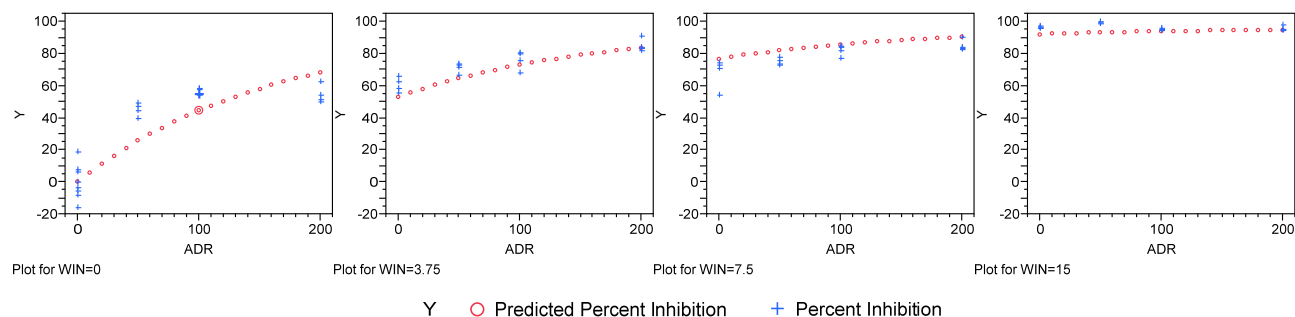
| Parameter | Estimate | Approx. SE | 95% Confidence Limits | |
|---|----------|------------|-----------------------|---------|
| Interaction Model | | | | |
| γ | 96.419 | 2.631 | 91.148 | 101.700 |
|  | 0.006 | 0.001 | 0.005 | 0.007 |
|  | 0.215 | 0.022 | 0.172 | 0.259 |
|  | -1.1E-04 | 2.9E-04 | -6.9E-04 | 4.7E-04 |
| Additive Model | | | | |
| γ | 95.901 | 2.164 | 91.567 | 100.200 |
|  | 0.006 | 0.001 | 0.005 | 0.007 |
|  | 0.215 | 0.021 | 0.173 | 0.258 |

Figure 2: Assessing Goodness of Fit in MCF_7 Cell Line

2a: Interaction Model



2b: Additive Model



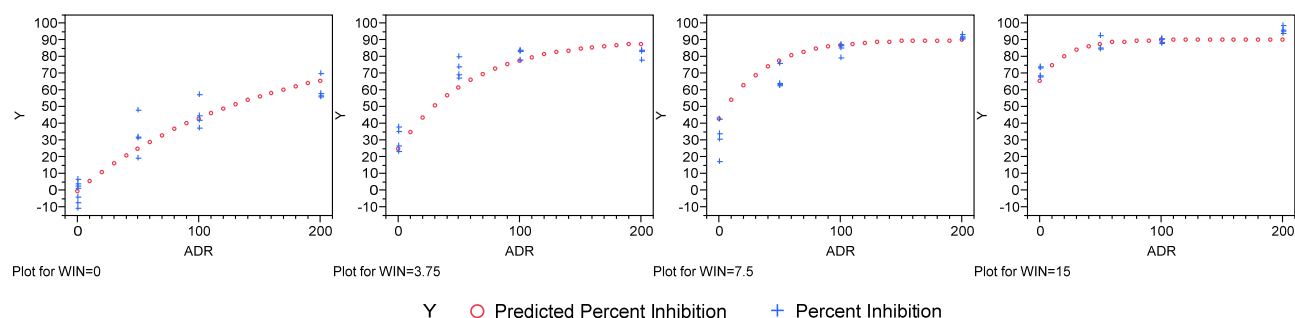
Results: MDA-MB231 Cell Line

In the MDA-MB231 cell line, there was evidence of a significant interaction. The estimate was positive, therefore indicating a synergistic interaction implying that the combination of the drug inhibits tumor growth more than would be expected under additivity. Parameter estimates are given in Table 3. Goodness of fit of the model was assessed graphically (and confirmed numerically by a chi-sq test) by plotting predicted with actual observed values (see Figure 3). The interaction is shown graphically in figure 3.

Table 3: Parameter Estimates for MDA-MB231 Cell Line

| Parameter | Estimate | Approx. SE | 95% Confidence Limits | |
|-------------------|----------|------------|-----------------------|---------|
| Interaction Model | | | | |
| γ | 90.2296 | 1.6973 | 86.8294 | 93.6297 |
| β_1 | 0.00653 | 0.000635 | 0.00526 | 0.0078 |
| β_2 | 0.0868 | 0.00845 | 0.0699 | 0.1037 |
| β_{12} | 0.00271 | 0.000543 | 0.00162 | 0.00379 |

Figure 3: Assessing Goodness of Fit in MDA Cell line



Key Research Accomplishments

- Established assays for breast tumor cell transformation in cell culture
- Demonstrated interaction between cannabinoids and adriamycin in three breast tumor cell lines.
- Identified p53 as a critical factor in potentiation of the response to adriamycin in breast tumor cells by cannabinoids.

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

Studies of transformation in cell culture have demonstrated that the combination of estrogen and benzopyrene alters the phenotype of MCF-10a cells, a normal breast epithelial cell line, to that of a tumor cell. This conclusion is based on induction of anchorage independent growth, loss of acini structure formation in 3D cultures, and the ability to proliferate in the absence of exogenous growth factors normally required for MCF-10a growth.

Studies to evaluate the interactions of cannabinoids with conventional chemotherapy in breast tumor cell lines are consistent with a mixture of additive and synergistic effects in the three cell lines (MCF-7, MDA-MB-231, and 4T1) and three cannabinoid combinations (THC, CBD, and Win55) in combination with Adriamycin.

Our studies also strongly suggest that the interactions are most pronounced in breast tumor cells lacking functional p53.

The in vivo model of 4T1-luc cells can be monitored in real time in an immunocompetent mouse to evaluate the interaction of cannabinoids + chemotherapy.

REFERENCES

- Aldaz CM, Liao QY, LaBate M, Johnston DA. Medroxyprogesterone acetate accelerates the development and increases the incidence of mouse mammary tumors induced by dimethylbenzanthracene. *Carcinogenesis* 1996; **17**, 2069-2072.
- Cravatt BF, Saghatelian A, Hawkins EG, Clement AB, Bracey MH and Lichtman AH (2004) Functional disassociation of the central and peripheral fatty acid amide signaling systems. *Proc Natl Acad Sci U S A* **101**:10821-10826.
- Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA and Gilula NB (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* **384**:83-87.
- Debnath J, Muthuswamy SK, Brugge JS. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods*, 2003; 30:256-268.
- Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A and Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* **258**:1946-1949.
- Fujii-Kuriyama F, Mimura J. Molecular mechanisms of AhR functions in the regulation of *cytochrome P450* genes. *Biochemical and Biophysical Research Communications* 2005; 338, 311–317
- Goparaju SK, Ueda N, Taniguchi K and Yamamoto S (1999) Enzymes of porcine brain hydrolyzing 2-arachidonoylglycerol, an endogenous ligand of cannabinoid receptors. *Biochem Pharmacol* **57**:417-423.
- Guzman M. Cannabinoids: Potential Anticancer Agents. *Nature Reviews* 2003; 3, 745-755
- Hill MN, McLaughlin RJ, Morrish AC, Viau V, Floresco SB, Hillard CJ and Gorzalka BB (2009) Suppression of Amygdalar Endocannabinoid Signaling by Stress Contributes to Activation of the Hypothalamic-Pituitary-Adrenal Axis. *Neuropsychopharmacology*
- Ip MM and Asch BB. *Methods in Mammary Gland Biology and Breast Cancer Research*. 2000. New York, NY: Kluwer Academic/Plenum Publishers
- Kluin-Neleman JC, Neleman FA, Meuwissen OJ and Maes RA (1979) delta 9-Tetrahydrocannabinol (THC) as an antiemetic in patients treated with

- cancer chemotherapy; a double-blind cross-over trial against placebo. *Vet Hum Toxicol* **21**:338-340.
- Krejza J, Mariak, Z, Huba M, Wolczynski S, Lewko J. Effect of Endogenous Estrogen on Blood Flow Through Carotid Arteries. *Stroke* 2001; 32, 30-36
- Liu S, Lin YC. Transformation of MCF-10A Human Breast Epithelial Cells by Zeranol and Estradiol-17. *The Breast Journal*, 2004; 10(6): 514–521
- Matsuda LA, Lolait SJ, Brownstein MJ, Young AC and Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* **346**:561-564.
- McKallip RJ, Nagarkatti M, Nagarkatti PS. Delta-9-tetrahydrocannabinol enhances breast cancer growth and metastasis by suppression of the antitumor immune response. *J Immunol*. 2005;174(6):3281-9.
- Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski N, Schatz A, Gopher A, Almog S, Martin B, Compton D, Pertwee R, Griffin G, Bayewitch M, Barg J and Vogel Z (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol.* **50**:83-90.
- Munro S, Thomas KL and Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature* **365**:61-64.
- Munson A, Harris L, Friedman M, Dewey W and Carchman R (1975) Antineoplastic activity of cannabinoids. *J. Natl. Can. Inst.* **55**:597-602.
- Noyes R, Jr., Brunk SF, Baram DA and Canter A (1975) Analgesic effect of Δ^9 -tetrahydrocannabinol. *J. Clin. Pharmacol.* **15**:139-143.
- Russo J, Tahin Q, Lareef MH, Hu Y-F, Russo IH. Neoplastic Transformation of Human Breast Epithelial Cells by Estrogens and Chemical Carcinogens. *Environmental and Molecular Mutagenesis* 2002; 39, 254–263
- Russo J, Fernandez SV, Russo PA, Fernbaugh R, Sheriff FS, Lareef HM, Garber J, Russo IH. 17-Beta-estradiol induces transformation and tumorigenesis in human breast epithelial cells. *FASEB J.* 2006; 20(10):1622-34
- Siriwardhana N, Wang HC. Precancerous carcinogenesis of human breast epithelial cells by chronic exposure to benzo[a]pyrene. *Mol Carcinog.* 2008; 47(5):338-48
- Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A and Waku K (1995) 2-Arachidonoylglycerol: A possible endogenous cannabinoid receptor ligand in brain. *Biochem. Biophys. Res. Comm.* **215**:89-97.
- Uno S, Dalton TP, Dragin N, Curran CP, Derkenne S, Miller ML, Shertzer HG, Gonzalez FJ, Nebert DW. Oral Benzo[a]pyrene in *Cyp1* Knockout Mouse Lines: CYP1A1 Important in Detoxication, CYP1B1 Metabolism Required for Immune Damage Independent of Total-Body Burden and Clearance Rate. *Mol Pharmacol* 2006; 69, 1103–1114

- White AC, Munson JA, Munson AE and Carchman RA (1976) Effects of delta9-tetrahydrocannabinol in Lewis lung adenocarcinoma cells in tissue culture. *J Natl Cancer Inst* **56**:655-658.
- Yoldemir T, Fraser IS. The effect of elevated serum estradiol levels on the day of human chorionic gonadotropin injection on pregnancy outcomes in an assisted reproduction program. *Australian and New Zealand Journal of Obstetrics and Gynaecology* 2009; **49**, 545–550