# Developing Novel Chemotherapeutics:

# A Structure-Activity Study of Anandamide Analogs and their Cytotoxic Profiles

By: Andrew Morris

January, 2020

Director of Dissertation: Dr. Colin Burns

Department of Chemistry

Many epithelial cancers have been shown to overexpress the enzyme cyclooxygenase-2 (COX-2), an enzyme responsible for both the metabolism of arachidonic acid (AA) to prostaglandins and arachidonoyl ethanolamine (AEA) to prostaglandin-ethanolamides (prostamides). AEA has demonstrated cytotoxicity in COX-2 overexpressing cancers via its metabolism to the novel J-series prostamide, 15d-$PMJ_2$. Using what is known about how AEA induces cell death, derivatives of AEA were synthesized to investigate COX-2 metabolism, PGDS metabolism, FAAH degradation resistance, and the inherent cytotoxicity of their J-series prostaglandin analogs.

A structure-activity relationship study was conducted with ten AEA derivatives to determine what modifications to the ethanolamide moiety improve anti-cancer activity in COX-2 overexpressing JWF2 tumorigenic keratinocytes and HCA-7 colorectal cancer cells. Important cytotoxic characteristics of AEA and AEA analogs were identified from the SAR study. Hydrogen bond accepting or donating ability seems to be the most important characteristic retained in

cytotoxic AEA analogs. Distance between the amide bond and terminal hydroxyl moiety seems to be important, but no trend as to whether a shorter or longer carbon chain is apparent. Addition of an aryl group does not interfere with cytotoxicity and in fact improves it as long as a terminal hydroxyl group is retained.

Two derivatives which displayed cytotoxicity similar to or greater than that of AEA, NAGly and arvanil, were selected for further studies exploring their cytotoxic and metabolic profiles. Arvanil ($LC_{50}$ = 6.03 μM) demonstrated greater cytotoxicity than NAGly ($LC_{50}$ = 9.54 μM) and our positive control AEA ($LC_{50}$ = 9.39 μM). Metabolism by COX-2 and PGDS to D-series and J-series prostaglandin analogs were responsible for arvanil's apoptotic anti-cancer activity. The novel J-series prostamide analog, 15d-$PMJ_2$-arvanil, was identified for the first time as well as another metabolite, possibly an A-series prostamide analog, with identical mass-to-charge ratios and fragmentation patterns in the mass spectrum. These molecules will be synthesized, and their anti-cancer activity investigated in future studies. Collectively, these results suggest that structural modification of the ethanolamide moiety of AEA provides a means for improving both the selectivity and cytotoxicity of prodrugs resulting in formation of novel J-series prostamides.

# Developing Novel Chemotherapeutics:

# A Structure-Activity Study of Anandamide Analogs and Their Cytotoxic Profiles

A Dissertation

Presented to

The Faculty of the Department of Chemistry

Thomas Harriot College of Arts and Sciences

East Carolina University

In Partial Fulfillment

Of the Requirements for the Degree of

Doctor of Philosophy in Chemistry

By: Andrew Morris

January, 2020

# Developing Novel Chemotherapeutics:

# A Structure-Activity Study of Anandamide Analogs and Their Cytotoxic Profiles

By: Andrew Morris

Approved by:

Director of Dissertation: _____

(Colin Burns, Ph.D.)

Committee Member: _____

(Rukiyah Van Dross-Anderson, Ph.D.)

Committee Member: _____

(Allison Danell, Ph.D.)

Committee Member: _____

(William Allen, Ph.D.)

Committee Member: _____

(Kennth Soderstrom, Ph.D.)

Chair of the Department: _____

(Andrew Morehead Ph.D.)

Dean of the Graduate School: _____

(Paul J. Gemperline Ph.D.)

Dedicated to my incredible wife, Emily, who has believed in me every step of this journey.

Without your endless support, this would not have been possible.

AND

To my family

Who have provided me with wisdom and courage to accomplish anything.

# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| **$\Delta^9$THC** | $\Delta^9$ tetrahydrocannabinol |
| **15d-PGJ$_2$** | 15-deoxy $\Delta^{12.14}$ PGJ$_2$ |
| **15d-PMJ$_2$** | 15-deoxy $\Delta^{12.14}$ PGJ$_2$-EA |
| **5-FU** | 5-fluorouracil |
| **AA** | Arachidonic Acid |
| **AC** | Arachidonoyl 2'-chloroethylamide |
| **ADA** | Arachidonoyl Diethanolamine |
| **AEA** | Arachidonoyl Ethanolamine |
| **AM251** | 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide |
| **AM404** | N-arachidonoylaminophenol |
| **AMG9810** | 3-(4-t-butylphenyl)-N_(2,3-dihdrobenzo(b)(1,4)dioxin-6-yl)acrylamide |
| **AMT** | Anandamide Membrane Transporter |
| **ANOVA** | Analysis of Variance |
| **A-Pro** | Arachidonoyl Propanolamine |
| **Arvanil D1** | Arachidonoyl 3-hydroxybenzylamine |
| **Arvanil D2** | Arachidonoyl 3,4-dimethoxybenzylamine |
| **AS** | Arachidonoyl Serinol |
| **CapeOx** | Capecitabine and oxaliplatin |
| **CB$_1$** | Cannabinoid receptor 1 |
| **CB$_2$** | Cannabinoid receptor 2 |
| **COX-2** | Cyclooxygenase-2 |
| **DIEA** | N,N-diisopropylethylamine |
| **Endocannabinoids** | Endogenous cannabinoids |

| | |
|---|---|
| **ER** | Endoplasmic Reticulum |
| **FAAH** | Fatty Acid Amide Hydrolase |
| **FBS** | Fetal Bovine Serum |
| **FOLFOX** | 5-FU, leucovorin, and oxaliplatin |
| **HOBt** | 1-hydroxybenotriazole |
| **NAGly** | Arachidonoyl Glycine |
| **NMR** | Nuclear Magnetic Resonance |
| **NMSC** | Non-Melanoma Skin Cancer |
| **PBS** | Phosphate Buffer Saline |
| **PGDS** | Prostaglandin-D Synthase |
| **PGES** | Prostaglandin-E Synthase |
| **PGFS** | Prostaglandin-F Synthase |
| **$PGD_2$** | Prostaglandin-$D_2$ |
| **$PGE_2$** | Prostaglandin-$E_2$ |
| **$PGF_{2\alpha}$** | Prostaglandin-$F_{2\alpha}$ |
| **$PGG_2$** | Prostaglandin-$G_2$ |
| **$PGH_2$** | Prostaglandin-$H_2$ |
| **$PMD_2$** | Prostamide-$D_2$ |
| **$PME_2$** | Prostamide-$E_2$ |
| **$PMF_{2\alpha}$** | Prostamide-$F_{2\alpha}$ |
| **$PMJ_2$** | Prostamide-$J_2$ |
| **Prostamide** | Prostaglandin-Ethanolamide |
| **SAR** | Structure-Activity Relationship |
| **TBTU** | N,N,N’,N’-tetramethyluronium tetrafluoroborate |

| | |
|---|---|
| **TRPV1** | Transient Receptor Potential Cation Channel Subfamily V Member 1 |

# CHAPTER ONE: INTRODUCTION

## 1.1: Cancer

Cancer accounts for almost a quarter of all deaths in the United States annually. Cancer is the global term for a disease with two characteristics, uncontrollable proliferation and metastasis of malignant cells (American Cancer Society, National Cancer Institute). Cancer is caused by genetic changes or mutations in DNA which normally control cell function, growth, division, and apoptosis or programmed cell death. Cancer can initiate in any tissues of the body, but some forms of cancer are more common than others, such as non-melanoma skin cancer, or more deadly than others, such as melanoma or colorectal cancer.

### 1.1.1: Non-Melanoma Skin Cancer (NMSC)

NMSC is the most commonly diagnosed cancer in the United States with an estimated two million cases each year (American Cancer Society). The rate of incidence is due to environmental factors such as UV radiation as well as an increasing average age shift (Diffey and Langtry 2005, Kricker et al. 1995). NMSC is not as deadly as melanoma, but there are still an estimated 2,000 deaths attributed to NMSC every year in the United States (American Cancer Society). NMSC puts a monetary strain on the health care system with an estimated cost of $4.8 billion per year (US Surgeon General, 2014). Current treatments for NMSC include surgical removal, a costly but effective treatment, and topical treatment with 5-fluorouracil (5-FU), which produces severe adverse effects (Metterle et al. 2016). Because of the prevalence of NMSC, there is a need for effective, convenient, and economical chemotherapeutic treatments (Guy and Ekwueme 2011).

### 1.1.2: Colorectal Cancer

Colorectal cancer is the third most commonly diagnosed cancer in the United States, behind only NMSC and melanoma skin cancer, with an estimated 145,000 new cases in the United States in 2019 (American Cancer Society). Unlike NMSC, colorectal cancer is a deadly cancer and is expected to cause 50,000 deaths in 2019. Evidence suggests colorectal cancer can be prevented by regular colonoscopy screening and removal of adenomatous polyps which progress to adenocarcinoma (Winawer et al. 1993). Even with regular colonoscopy screenings, the incidence of colorectal cancer is on the rise due to the same age-related factors as NMSC as well as dietary factors. Current treatments include surgical removal of the tumor or chemotherapy with FOLFOX (5-FU, leucovorin, and oxaliplatin), CapeOX (capecitabine and oxaliplatin), or Irinotecan (Schrag 2004, American Cancer Society). Unlike NMSC, chemotherapeutic treatments for colorectal cancer are systemic, leading to widespread side effects and tissue damage. Because of the prevalence and mortality rate of colorectal cancer, there is a need for effective, convenient, and economical treatments.

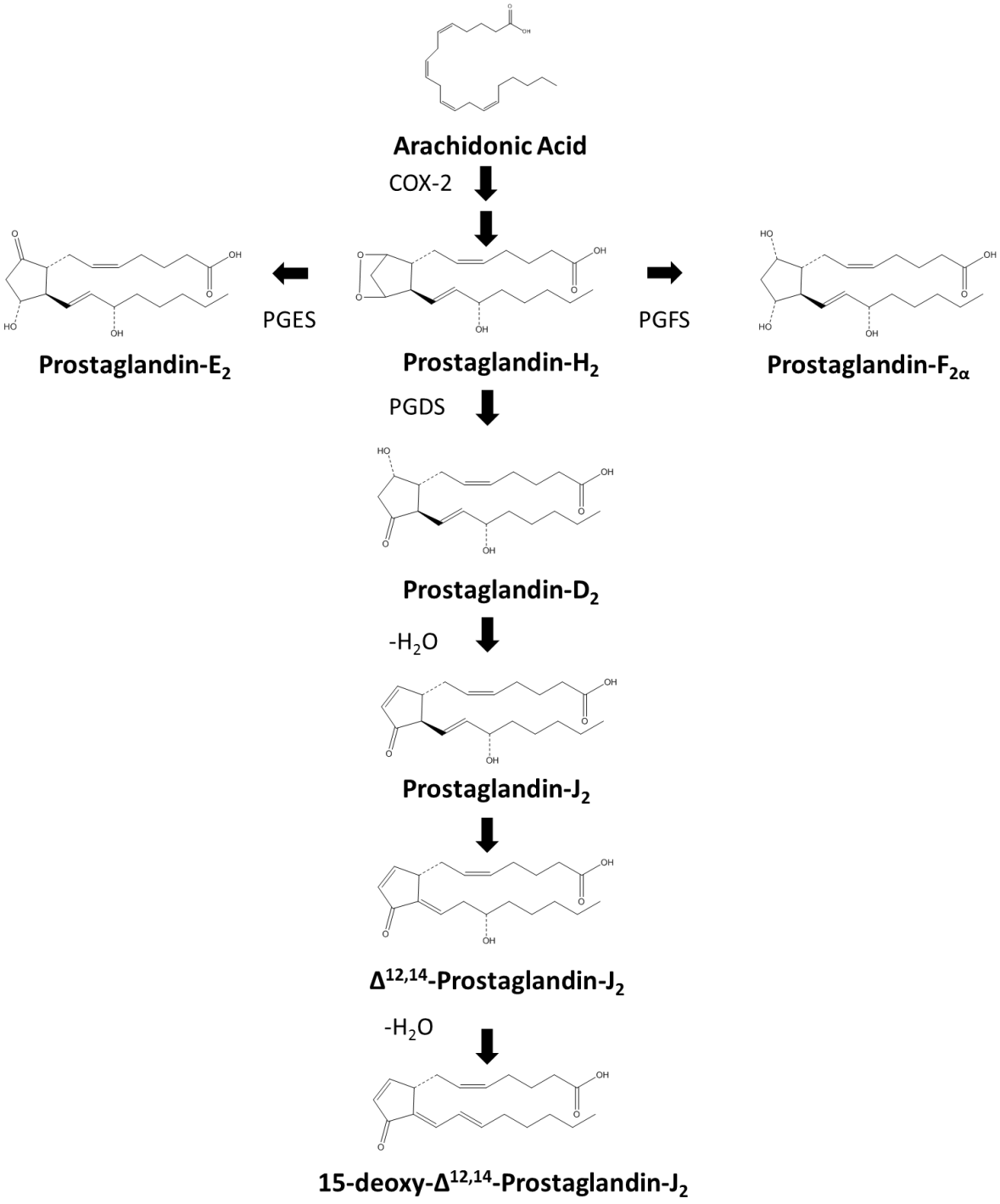
## 1.2: Cyclooxygenase-2 (COX-2) and Cancer

The primary function of COX-2 is to synthesize polyunsaturated fatty acids called prostaglandins from the 20-carbon arachidonic acid (AA) (Scheme 1.1). COX-2 also produces ethanolamide-conjugated prostaglandins (prostamides) from anandamide (AEA). There are several types of prostaglandins and prostaglandin-ethanolamides (prostamides) including E-series, F-series, and D-series. These series of prostaglandins are distinguished by the substituents of the cyclopentane ring, specifically the placement of the hydroxyl or ketone substituents (Scheme 1.1). These lipids regulate diverse processes including inflammation, platelet function, and pain (Funk

2001, Matias et al. 2004). It has been also determined that prostaglandins play a prominent role in carcinogenesis. Numerous studies have shown that COX-2 and its metabolic product, prostaglandin-$E_2$ ($PGE_2$), are overexpressed in many epithelial cancers including NMSC and colon cancer (Boukamp, P. 2005, Buckman et al. 1998, Armstrong and Kricker 2001). $PGE_2$ binding to E-type prostaglandin receptors activates a signal transduction cascade that leads to cancer cell proliferation (Fischer et. al 2000, Kiraly et al. 2016). In contrast, both the inhibition of COX-2 activity and the antagonism of EP receptors blocks epithelial tumor growth, particularly in NMSC and colorectal cancer (Sheng et al. 1997, Kiraly et al. 2016).

D- and J-series prostaglandins are formed when AA is metabolized by COX-2 to $PGH_2$. $PGH_2$ is enzymatically converted by PGDS to $PGD_2$ and $PGD_2$ is spontaneously dehydrated to prostaglandin-$J_2$ ($PGJ_2$), $\Delta^{12.14}$-$PGJ_2$, and 15-deoxy-$\Delta^{12.14}$-$PGJ_2$ (15d-$PGJ_2$). Furthermore, we discovered that COX-2 also metabolizes AEA to the cytotoxic molecules prostamide-$J_2$ ($PMJ_2$), $\Delta^{12.14}$-$PMJ_2$, and 15-deoxy-$\Delta^{12.14}$-$PMJ_2$ (15d-$PMJ_2$). D- and J-series prostaglandins and prostamides have demonstrated anti-proliferative effects on a variety of cancers (Van Dross et. al. 2009, Kuc et al. 2012, Clay et. al 1999, Soliman et al. 2016a, Ladin et al. 2017).

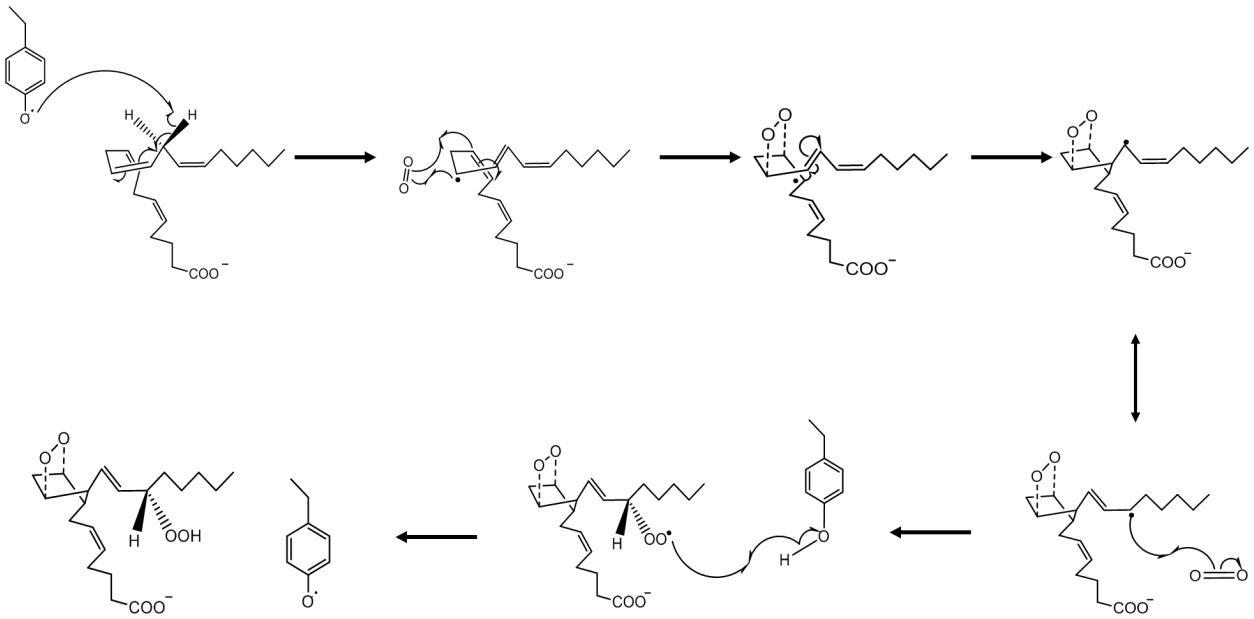
**Scheme 1.1: COX-2 Metabolism of Arachidonic Acid to Prostaglandins**



### 1.2.1: Mechanism of Arachidonic Acid Oxygenation by COX-2

A series of radical reactions converts arachidonic acid to prostaglandin-$G_2$ ($PGG_2$) followed by reduction of $PGG_2$ to prostaglandin-$H_2$ ($PGH_2$). This series of radical reactions is initiated by the removal of the 13-*pro*(S)-hydrogen of arachidonic acid by the tyrosyl radical of Tyr385 followed by the trapping of a pentadienyl radical by $O_2$ at C-11 (Scheme 1.2, steps 1 and 2) (Dietz et al. 1988). This peroxyl radical then cyclizes at C-9 and the ensuing carbon-centered radical at C-8 cyclizes at C-12 resulting in an endoperoxide (Scheme 1.2, step 3). The resonance-stabilized allylic radical at C-15 is trapped by $O_2$ forming the 15-(S)-peroxyl radical which is then reduced by hydrogen atom abstraction from tyrosine to prostangladin-$G_2$ (Scheme 1.2, steps 4 and 5) (Hamberg and Samuelsson 1967).

**Scheme 1.2: Conversion of Arachidonic Acid to Prostaglandin-$G_2$ by COX-2 Mechanism**



### 1.3: Endogenous Cannabinoid (Endocannabinoid) System

The endocannabinoid system consists of endocannabinoids, which are lipid neurotransmitters, cannabinoid receptor 1 ($CB_1$), cannabinoid receptor 2 ($CB_2$), and enzymes responsible for the synthesis and inactivation of cannabinoid and endocannabinoid ligands (Di Marzo et al. 1994, Di Marzo et al. 2004, Bisogno et al. 2005, Di Marzo 2009). Endocannabinoids regulate pathological and physiological processes including inflammation, analgesia, learning, cancer, and appetite through the activation of the endocannabinoid system (Di Marzo et al. 2000, Bisogno et al. 2005). This system was originally discovered due to investigation of the receptors responsible for the effects of the phytocannabinoid $\Delta^9$ tetrahydrocannabinol ($\Delta^9$THC), the psychoactive ingredient of marijuana. Endocannabinoids have the ability to promote tumor cell death by initiating growth arrest and apoptosis (Fowler et al. 2003, Linsalata et al. 2010). These cytotoxic actions occur by both cannabinoid receptor dependent and independent pathways (Melck et al. 1999a, Melck et al. 2000, De Petrocellis et al. 1998, Grimaldi et al. 2006, Gustafsson et al. 2009, Patsos et al. 2010, Alpini and Morrow. 2009, Brown et al. 2010, Van Dross et al. 2013, Soliman and Van Dross 2016b).

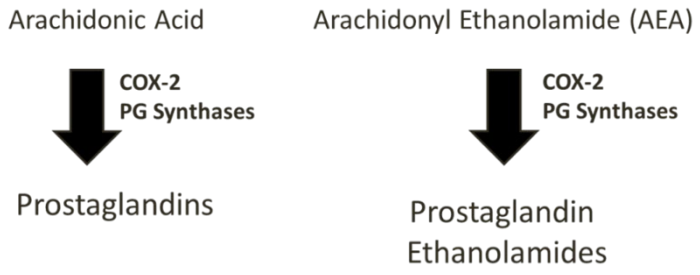
#### 1.3.1: Arachidonoyl Ethanolamine (Anandamide) (AEA)

AEA was the first endocannabinoid discovered in the endocannabinoid system (Devane et al. 1992). To initiate its biological response, AEA binds to the cannabinoid receptors and then it is transported into the cells via the anandamide membrane transporter (AMT). AEA is inactivated by fatty acid amide hydrolase (FAAH), an enzyme that converts AEA to AA and ethanolamine (Deutsch et al. 1993, Cravatt et al. 1996).

### 1.3.2: COX-2 Metabolism of AEA

COX-2 not only metabolizes AA, but also its ethanolamine-conjugated counterpart AEA at a rate of metabolism 27% that of AA (Scheme 1.3) (Kozak et al. 2001, Kozak et al. 2002). AEA is an endocannabinoid produced in the brain, which plays a role in a variety of biological processes including pain, inflammation, depression, and pleasure. (Di Marzo 2009, Matias et al. 2004). In cancer cell lines that overexpress COX-2, AEA is cytotoxic (Di Marzo 2009, Soliman et al. 2016a). AEA is metabolized by COX-2 and then by PGE-synthase (PGES), PGD-synthase (PGDS), or PGF-synthase (PGFS) to prostamide-$E_2$ ($PME_2$), prostamide-$D_2$ ($PMD_2$), and prostamide-$F_{2\alpha}$ ($PMF_{2\alpha}$) respectively (Matias et al. 2004). Prostamide-$D_2$ ($PMD_2$) is spontaneously dehydrated to prostamide-$J_2$ ($PMJ_2$) and $\Delta^{12,14}$-$PMJ_2$, which are spontaneously dehydrated to 15-deoxy-$\Delta^{12,14}$-$PGJ_2$-EA (15d-$PMJ_2$). In previous studies we determined 15d-$PMJ_2$ demonstrates selective toxicity via endoplasmic reticulum (ER) stress induced apoptosis in NMSC and melanoma *in vitro* as well as inducing potent tumor cell apoptosis *in vivo* (Ladin et al. 2017). Selective toxicity is a crucial characteristic of 15d-$PMJ_2$, which is a requirement for novel chemotherapeutics to reduce side effects.

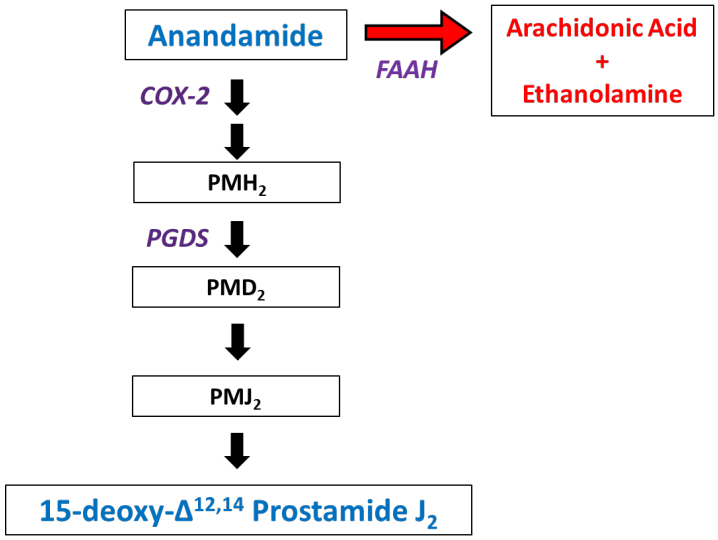
**Scheme 1.3: COX-2 Metabolism of AA and AEA**



### 1.4: Fatty Acid Amide Hydrolase (FAAH)

AEA is a known substrate for another enzyme, FAAH, a membrane protein belonging to the serine hydrolase family. The hydrolase activity of FAAH regulates the biological responses of AEA by controlling its intracellular concentration (Cravatt et al. 1996, Deutsch et al. 1993, Bisogno et al. 1997, Bifulco et al. 2004, Siegmund et al. 2006). FAAH degrades AEA into AA and ethanolamine, and once degraded, AA is sequestered in the phospholipid membrane (Scheme 1.4). This knowledge can be used to design AEA derivatives that resist degradation by FAAH and thereby exhibit greater activity than AEA. Specifically, we hypothesize that the addition of functional groups to AEA that inhibit its inactivation by FAAH will make more of the AEA analog created available for metabolism by COX-2 to cytotoxic J-series prostaglandin analogs.

**Scheme 1.4: The Role of FAAH in the Metabolism of AEA to 15d-$PMJ_2$**



### 1.5: Arvanil

Arvanil is a structural hybrid of the endocannabinoid, AEA, and the potent transient receptor potential cation channel subfamily V member 1 (TRPV-1) agonist, capsaicin. Arvanil demonstrates affinity for the $CB_1$ receptor comparable to AEA, activates TRPV-1 more potently than capsaicin, inhibits the anandamide membrane transporter (AMT), and inhibits FAAH's ability to hydrolyze endocannabinoids (Melck et al. 1999b, Glaser et al. 2003). Sancho et al. demonstrated arvanil-induced apoptosis in the Jurkat cell line via a FADD/caspase-8 dependent pathway (Sancho et al. 2009). Similar to AEA, arvanil can be metabolized by COX-2, but the rate of metabolism is 5.6% that of AA (Prusakiewicz et al. 2007). Interestingly, COX-2 metabolites of arvanil, including J-series metabolites, have not been reported.

### 1.6: Arachidonoyl Glycine (NAGly)

NAGly is a carboxylic analog of the endocannabinoid AEA. It was identified as an endogenous conjugate of arachidonic acid and glycine that is produced in response to inflammatory pain (Huang et al. 2001). NAGly also inhibits the hydrolytic activity of FAAH on AEA, effectively serving as an endogenous regulator of AEA concentrations (Burstein et al. 2002). NAGly was the first charged species identified to be metabolized selectively by COX-2 to $PGH_2$-Gly and is metabolized at 40% the rate of AA (Prusakiewicz et al. 2002, Prusakiewicz et al. 2007). However, it is unclear if J-series metabolites of NAGly are produced by the COX-2 metabolic pathway.

### 1.7: COX-2 and FAAH Expression Levels Regulate the Cytotoxicity of AEA

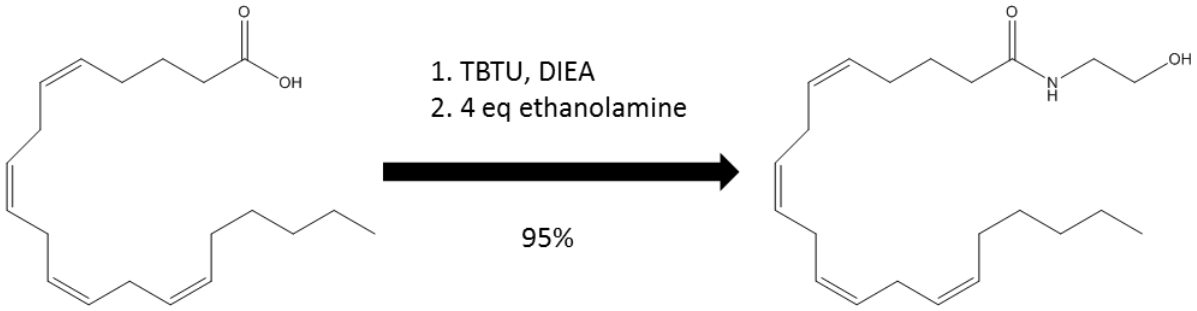
NMSC, colorectal cancer, and other epithelial cancers overexpress COX-2, creating a potential target for chemotherapeutics (Buckman et al., 1998). As stated earlier, AEA's cytotoxicity relies on the overexpression of COX-2 which metabolizes AEA to 15dPMJ$_2$, the primary cancer-killing metabolite (Soliman et al. 2016a, Ladin et al. 2017). If the expression of COX-2 is low in cancer cells, the metabolism of AEA to J-series prostamides will be reduced. FAAH's activity is also crucial because high levels of FAAH will lower the concentration of AEA thereby reducing the production of J-series prostamides. To identify an appropriate model for our studies, we screened a series of NMSC and colon cancer cell lines. We found that both murine JWF2 NMSC cells and human colon cancer HCA-7 cells overexpressed COX-2 and expressed low levels of FAAH (Soliman 2014). Hence, JWF2 and HCA-7 cells were used for the studies described in this dissertation.

### 1.8: Synthesis of AEA

AEA can be chemically synthesized from its base components, arachidonic acid and ethanolamine via an amide coupling reaction in a "one-pot" synthesis (Scheme 1.5). This synthesis is optimized to produce derivatives in a simple yet efficient manner. The uronium amide coupling reagent, N,N,N',N'-tetramethyuronium tetrafluoroborate (TBTU), is most commonly used in peptide syntheses, but in this instance works efficiently to couple AA and ethanolamine. The solvent, acetonitrile, is a crucial choice as it is polar enough to bring the ethanolamine into solution, but nonpolar enough to dissolve the arachidonic acid which contains a significantly non-polar chain. Once AA is activated via TBTU, the ethanolamine is added in excess to assure all AA, which is the more costly reagent, is converted to AEA to maximize efficiency and for ease of

purification. This synthesis typically yields greater than 95% AEA. This amide coupling reaction is a general synthesis which can be used to make any number of AEA derivatives with the expectation of the same purity and yield

**Scheme 1.5: Synthesis of AEA from AA using the coupling reagent TBTU**



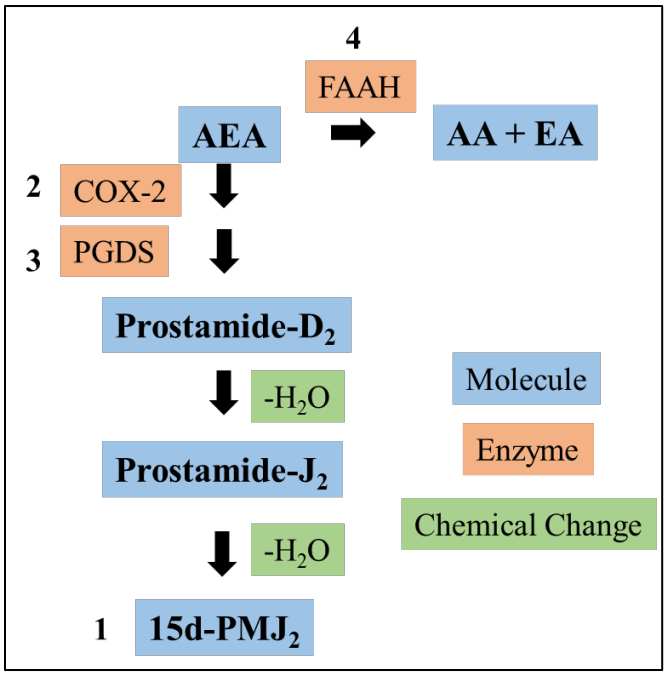
# SPECIFIC AIMS

AEA is an endocannabinoid that demonstrates cytotoxicity in cancer cell lines that overexpress the enzyme, COX-2. We have previously demonstrated AEA's anti-cancer activity is due to its metabolism by COX-2, followed by PGDS, and subsequent dehydrations to 15d-$PMJ_2$ (Kuc et al. 2012, Soliman et al. 2016a, Ladin et al. 2017). This metabolic pathway and resulting cytotoxicity can be disrupted by the hydrolase activity of FAAH, which degrades AEA to AA and ethanolamine, thereby reducing its anti-cancer activity. This system of metabolic and cytotoxic pathways provides an opportunity to improve the anti-cancer activity of AEA, and more specifically, the cytotoxicity of the J-series prostamide, 15d-$PMJ_2$.

Since AEA is a prodrug requiring metabolic processing for production of the pharmacologically active drug, and there is a competing pathway for degradation of the prodrug, the complexity of the system makes thoroughly isolating one area difficult. We hypothesized there were four parts of this system where we could improve AEA's killing activity in cancer cells that have both COX-2 and FAAH activity. These four areas of improvement are 1) inherent cytotoxicity of the final J-series prostamide metabolites, 2) rate of COX-2 metabolism, 3) rate of and bias for PGDS metabolism, and 4) resistance to degradation by FAAH (Scheme 1.6). The approach outlined below, which employs 10 AEA derivatives, allows us to make informed judgments as to which of the four areas leads to the greatest enhancement of anti-cancer activity and what structural features are required to achieve this.

**Scheme 1.6: Contributing Factors in AEA's Cytotoxic Pathway**

1. Cytotoxicity of 15d-$PMJ_2$
2. COX-2 metabolism
3. PGDS metabolism
4. FAAH degradation




**Specific Aim 1: Synthesize and screen initial AEA derivatives for cytotoxic activity in JWF2 and HCA-7 cell lines and select candidates with equal or greater activity than AEA.**

It was demonstrated in previous studies that linking ethanolamine to AA via an amide bond increases cytotoxicity, thus only modifications at the ethanolamine end of AEA were explored (Kuc et al. 2012). Modifications at this location were also predicted to have minimal or no impact on its ability to be metabolized by COX-2 into prostaglandin analogs, specifically cytotoxic J-series prostamides, because metabolism takes place on the long carbon chain backbone of AEA (Scheme 1.1). AEA derivatives were synthesized from AA and an appropriate amine to yield the desired modification on the ethanolamide arm when coupled using the methodology employed for the synthesis of AEA.

A first generation of seven AEA analogs was designed to create a broad overview of modifications that induce positive or negative effects on anti-cancer activity. The modifications were made based on aspects of AEA's metabolic pathways and their role in cytotoxicity. Six of the seven AEA analogs, R1-methanandamide, arvanil, NAGly, arachidonoyl diethanolamine (ADA), arachidonoyl serinol (AS), and arachidonoyl propanolamine (A-Pro) were included in the first generation because of their demonstrated or hypothesized resistance to FAAH degradation. All seven of the AEA analogs were included in the first generation because of their demonstrated or hypothesized metabolism by COX-2 and PGDS. The cytotoxicity of the first generation AEA analogs was determined in COX-2 overexpressing cancer cells and was classified as hits if they demonstrated equal or better cytotoxicity than AEA when applied to cancer cells. A second generation of AEA analogs was then created for the most potent first generation molecule(s) to further refine our understanding of the structural modifications associated with cytotoxicity.

**Specific Aim 2: Determine the role of the endocannabinoid system on the cytotoxic AEA derivatives identified in the SAR studies.**

The AEA derivatives from the SAR study that produce cytotoxicity that is equal or greater than AEA will be biochemically characterized. The endocannabinoid system is composed of cannabinoid receptors as well as COX-2 and FAAH, which regulate the activity of AEA. Therefore, we examined the role of the most important receptors and enzymes of the endocannabinoid system in the anti-cancer activity of the AEA derivatives. These studies were also utilized to determine the importance of the added functional groups and their effect on the cytotoxicity of their J-series metabolite

# CHAPTER TWO: MATERIALS AND METHODS

## 2.1: Synthesis Reagents

Arachidonic acid was purchased from NuChek Prep (Elysian, MN). AEA, R1-methanandamide, AM404, arachidonoyl glycine, arvanil, arachidonoyl 2'-chloroethylamide, arachidonoyl serinol, AM251, and AMG9810 were purchased from Cayman Chemical Company (Ann Arbor, MI). 4-hydroxybenzylamine, 3,4-dimethoxybenzylamine, propanolamine, and diethanolamine were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile, O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU), N,N-diisopropylethylamine (DIEA), and 1-hydroxybenotriazole (HOBt) were purchased from Sigma-Aldrich (St. Louis, MO).

## 2.2: Synthesis, Purification, and Characterization of AEA and All AEA Derivatives

This is the general synthetic procedure for producing AEA and the related AEA derivatives. 20 mg of AA was first added to 20 mL of acetonitrile followed by 1:1 molar equivalents of DIEA and TBTU. This solution was allowed to mix for 5 minutes to ensure the AA was primed for reaction. Four molar equivalents of ethanolamine or appropriate amine reagent (to produce the target derivative) was then mixed into the solution and stirred for 1 hour at room temperature. Once stirred and reacted sufficiently, the mixture was added to a separation funnel followed by 20 mL of distilled water and 20mL of diethyl ether and the funnel was swirled gently for 30 seconds. The water was drained from the separation funnel and the previous step was repeated two more times to remove the polar reaction products and all impurities. The ether layer containing the water-insoluble product was dried on a Rotovap at 40$^{o}$C. All synthesized and purified molecules were characterized via $^{1}$H-NMR on a Bruker 400 MHz NMR

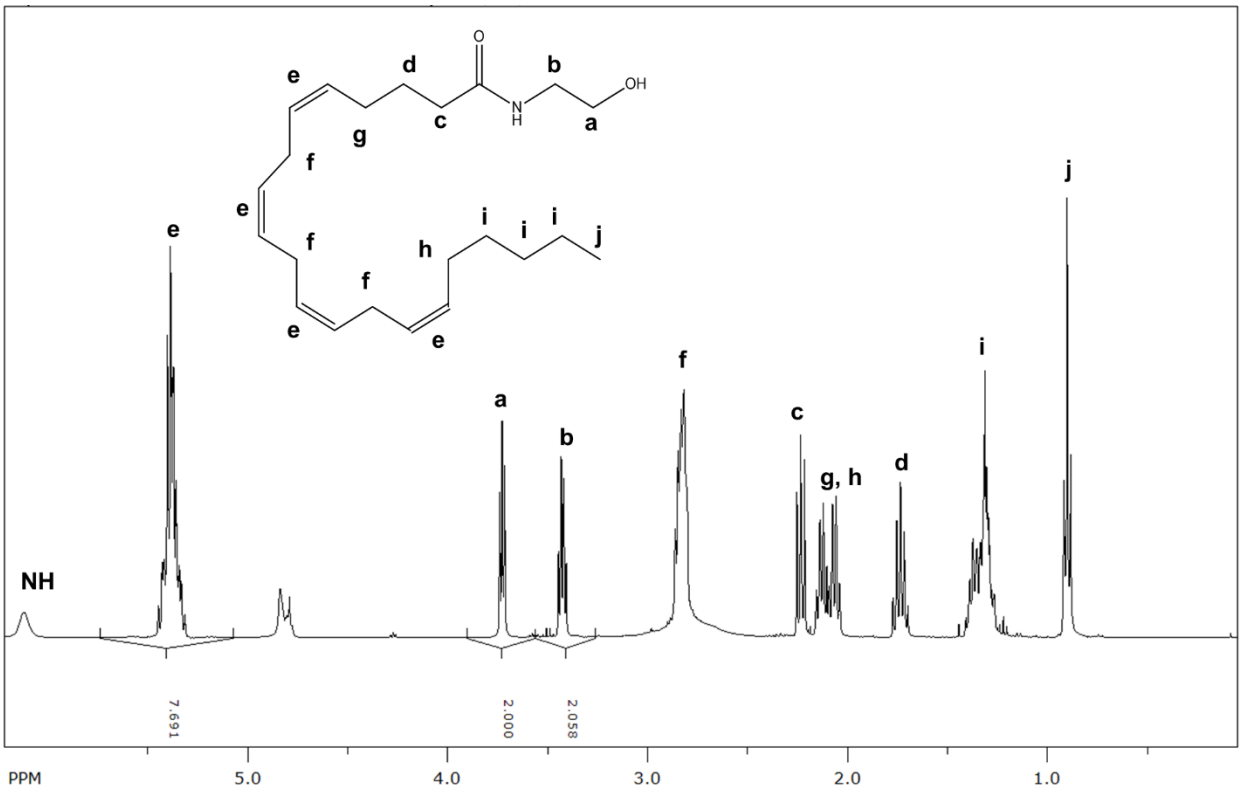



**Figure 2.1: NMR Characterization of Synthesized AEA**

## 2.3: Cell Culture

The JWF2 murine squamous carcinoma cell line (MD Anderson Cancer Center, Smithville, TX) was cultured in Eagle's minimal essential media (US Biological) containing 5% heat-inactivated fetal bovine serum (FBS), 100 mg/ml penicillin, 100 mg/ml streptomycin, nonessential amino acids, and glutamine. HCA-7 human colorectal cancer cell line (Sigma-Aldrich, St. Louis, MO) were cultured in Dulbecco's minimal essential media (Invitrogen) containing 10% heat-inactivated FBS, 100 mg/mL penicillin, 100 mg/mL streptomycin, and 1mM sodium pyruvate.

## 2.4: MTS Cell Viability Assays

Cells were plated in 96-well plates and cultured for 48 hours until 80% confluency was achieved. Serum-free media containing varying concentrations of appropriate molecules were

added to the cells for 24 hours. A 20 µL aliquot of MTS reagent (Promega, Madison, WI) was added to each well as described by manufacturer and the absorbance was measured at 495 nm.

### 2.5: ELISA Assay

JWF2 and HCA-7 cells were treated with vehicle, AEA, NAGly, and arvanil for 8 hours. Media was obtained after 8 hours and D-series prostaglandins, prostamides, and prostaglandin analogs were measured with ELISA kits as described by the manufacturer's instructions.

### 2.6: Caspase-3/7 Activity Assay

HCA-7 cells were plated in 96-well plates and cultured for 48 hours until 80% confluency was achieved. Serum-free media containing 20 µM of vehicle, AEA, NAGly, and arvanil alone, and each agent was also pre-treated and co-treated with $SeCl_4$. A 100 µL aliquot of Caspase-Glo 3/7 reagent (Promega, Madison, WI) was added to each well as described by manufacturer and luminescence was measure using a Tecan luminometer.

### 2.7: Liquid Chromatography-Mass Spectrometry (LC-MS)

HCA-7 cells were treated with arvanil for 8 hours and cell media was removed. Lipids were extracted from acidified media using solid phase extraction (SPE). 1 mL Strata X reverse phase cartridges were equilibrated then applied with 1 mL of acidified media. The sample was washed with ultrapure deionized water followed by 15% acetonitrile. Lipids were eluted with 100% hexane followed by 100% acetonitrile and then allowed to dry overnight. Samples were then reconstituted in 100 µL of 100% acetonitrile with 0.1% formic acid. LC-MS was conducted on a SCIEX Eskigent/5600+ Triple TOF mass spectrometer in negative ionization mode with an

electrospray ionization source. LC was equipped with a Luna $NH_2$ normal phase column (150 mm x 0.3 mm, 3 µm particle size) and a gradient with A: Water with 0.1% formic acid and B: Acetonitrile with 0.1% formic acid.

### 2.8: Statistical Analysis

Presented data is shown as mean ± standard error of mean. One-way analysis of variance (ANOVA) succeeded by Tukey's post-hoc analysis has been carried out using Graphpad Prism statistical software

# CHAPTER THREE: SYNTHESIS AND CYTOTOXICITY OF AEA DERIVATIVES

## 3.1: Design of Experimental Approach

AA, the non-ethanolamine conjugated molecule, and AEA are metabolized by COX-2 to series of prostaglandins and prostamides, respectively. Previously, we demonstrated that AEA was more cytotoxic than AA, suggesting that the ethanolamine moiety enhanced its activity (Kuc et. al 2012). Understanding the importance of the ethanolamine end, we hypothesized that this portion of the molecule could be modified to create a more cytotoxic chemotherapeutic. After we determined that AEA's anti-cancer activity was due to its metabolism by COX-2 to 15d-$PMJ_2$, we also hypothesized that modifications of the ethanolamide region of the molecule will increase anti-cancer activity without interfering with COX-2 metabolism (Soliman et al. 2016a). Our data showed that the pharmacophore of 15d-$PMJ_2$ was the double bond located in the cyclopentonone ring (Ladin et al. 2017). As such, modifying the ethanolamide region of AEA should not interfere with the COX-2 metabolism of the AEA analogs or the pharmacophore responsible for the anti-cancer activity of 15d-$PMJ_2$.

The intent of this investigation is to determine how structural modification of the ethanolamine moiety of AEA affects anti-cancer activity with the aim of creating improved therapeutics. Using a series of AEA derivatives designed to alter the action of key enzymes in the metabolism of the prodrug, including COX-2, PGDS, and FAAH, allows us to postulate whether the final J-series prostamide analog metabolite will be cytotoxic.

The target of 15d-$PMJ_2$ which induces apoptotic cancer cell death is currently unknown, however we hypothesize that the physiochemical features of the ethanolamide group enhances recognition or binding to this target compared to 15d-$PGJ_2$ or that the ethanolamide group stabilizes the molecule. Using the ethanolamide as a template, 10 AEA derivatives were designed and are shown in Table 3.1. These molecules will allow us to explore the influence of 1) branching along the chain, 2) linkage via a tertiary amide, 3) presence of an aromatic ring, and 4) polarity of the terminus on the derivative's cytotoxicity.

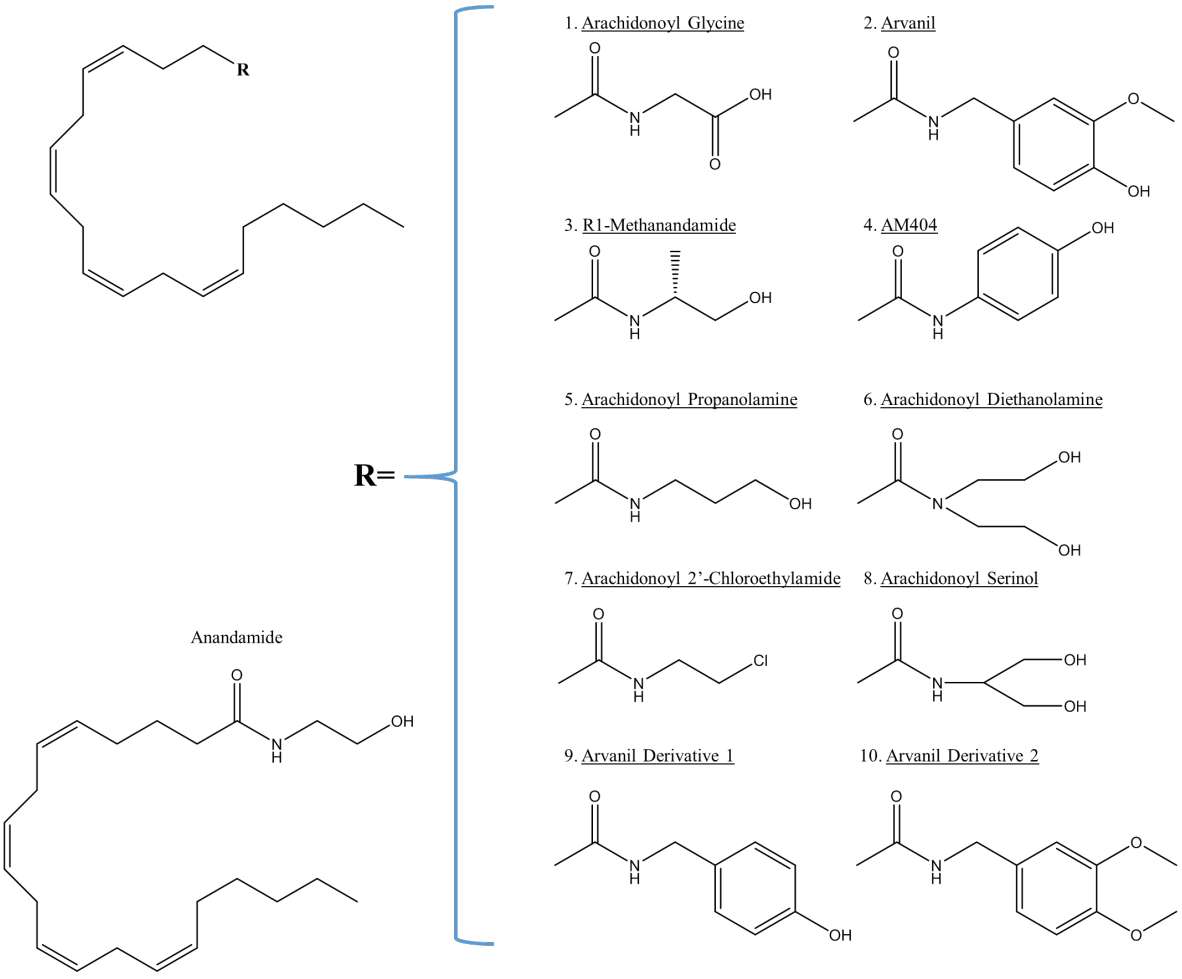
With the goal of this study to improve the chemotherapeutic AEA, the activity demonstrated by AEA was utilized as a baseline for minimum anti-cancer activity for this SAR study. AEA contains a 20 carbon-chain backbone with a secondary amide connection to an ethanolamine end. The 20 carbon-chain dominates the overall polarity of the molecule leading to a lipohilic molecule, but the ethanolamine slightly contributes to the overall polarity of AEA which is slightly different than the polarity of AA and its carboxylic acid end. As discussed earlier, the ethanolamine greatly increases the anti-cancer activity of the molecule. When considering what chemical changes are made to the molecule that could increase its activity, the slight change in polarity may contribute to differences in the ability to bind to the catalytic pocket of COX-2 and the subsequent metabolism. With this in mind, modifications to AEA investigated in this SAR should explore the hypothesis that minor changes in polarity of the molecule could enhance or disrupt the metabolism and cytotoxic activity of AEA analogs.

The difference in molecular size or length of AA and AEA are not huge, but are noticeable when considering the difference in anti-cancer activity. When compared to AA, the size of AEA is slightly larger. AEA still contains the 20 carbon-chain backbone of AA, but extends the molecule by two more carbons and a hydroxyl group. Just as the polarity change of the molecule is slight

but could be vitally important to the activity of the molecule, this size and length difference is minor but could be important to the anti-cancer activity of AEA. To investigate these changes, modifications made in some molecules in the SAR will be made to determine if molecule size in the ethanolamide region or distance between the amide bond and terminal hydroxyl end in AEA are crucial to the improvement or disruption of anti-cancer activity. These changes in anti-cancer activity could be related to increased or decreased target recognition, rate of COX-2 metabolism, or inhibition of FAAH's hydrolase activity.

A key aspect of AEA's anti-cancer activity is its metabolism by COX-2. Within the SAR study, modifications are limited to the ethanolamine region of AEA due to the hypothesis that altering the 20-carbon chain alters COX-2 metabolism of the molecule. Using this strategy, we can identify the importance of each modification to its chemotherapeutic activity without disrupting metabolism by COX-2, demonstrated vital to the anti-cancer activity of AEA. Identified in Scheme 1.1, the important region of AA responsible for COX-2 metabolism is between C-8 and C-15. With this knowledge, the assumption was made that any changes to this region of the molecule or even any part of the molecule close to this region would hinder COX-2 metabolism of AEA and its analogs to prostaglandins thus erasing the anti-cancer activity of the product, 15d-$PMJ_2$.

**Table 3.1: AEA Analogs Investigated for Anti-Cancer Activity in SAR Study**



### 3.1.1: Expression of COX-2 and FAAH in JWF2 NMSC and HCA-7 Cell Lines

We have shown that AEA is cytotoxic in cancer cell lines that overexpress COX-2 (Van Dross 2009, Soliman et al. 2016a). Also, we understand FAAH's hydrolase activity on AEA is detrimental to its cytotoxicity (Kuc et al. 2012). Therefore, previously we screened epithelial cancer cell lines, including NMSC and colorectal cancer cell lines, to observe COX-2 and FAAH expression. We identified two cell lines, JWF2 NMSC and HCA-7 colorectal cancer, that overexpress COX-2 and have little expression of FAAH. Utilizing these two cell lines, we established a model for our SAR study and any further studies.

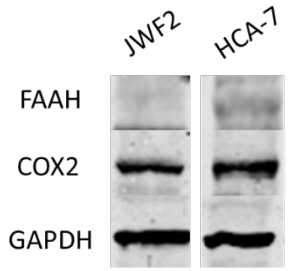



**Figure 3.1: Expression of COX-2 and FAAH in JWF2 NMSC and HCA-7 Cell Lines**
Courtesy of Eman Soliman (Soliman, 2014). Western blot analysis was conducted to compare COX-2 and FAAH expression in HCA-7 colorectal cancer cells and JWF2 tumorigenic keratinocytes. JWF2 and HCA-7 cells overexpress COX-2 and have a low expression of FAAH.

### 3.2: Cytotoxicity of AEA in JWF2 and HCA-7

To establish a benchmark of anti-cancer activity for comparison with all AEA analogs, JWF2 NMSC cells and HCA-7 colorectal cancer cells were treated with AEA at three different concentrations, 10 μM, 20 μM, and 40 μM. The activity demonstrated by AEA was utilized as a baseline for minimum anti-cancer activity for an AEA analog to be considered a "hit" that will be studied in greater detail. In both JWF2 and HCA-7 cell lines, AEA caused a statistically significant reduction in cell viability at 20 μM, but it did not significantly reduce cell viability at 10 μM. Results at 40 μM were not used for comparison in the SAR because 20 μM supplied the lowest concentration of AEA with maximum reduction in cell viability. This creates a concentration dependent comparison in both cell lines for all AEA analogs tested for "hits". Using the established cytotoxicity data for AEA, each AEA analog's % cell viability reduction at concentrations of 10 μM and 20 μM can be compared to AEA. For an AEA analog to be considered a "hit", we set the following % cell viability thresholds:

JWF2

- 80% cell viability (20% reduction in cell viability) at 10 μM, or
- 25% cell viability (75% reduction in cell viability) at 20 μM

HCA-7

- Less than 100% cell viability (Any % reduction in cell viability) at 10 μM, or
- 50% cell viability (50% reduction in cell viability) at 20 μM

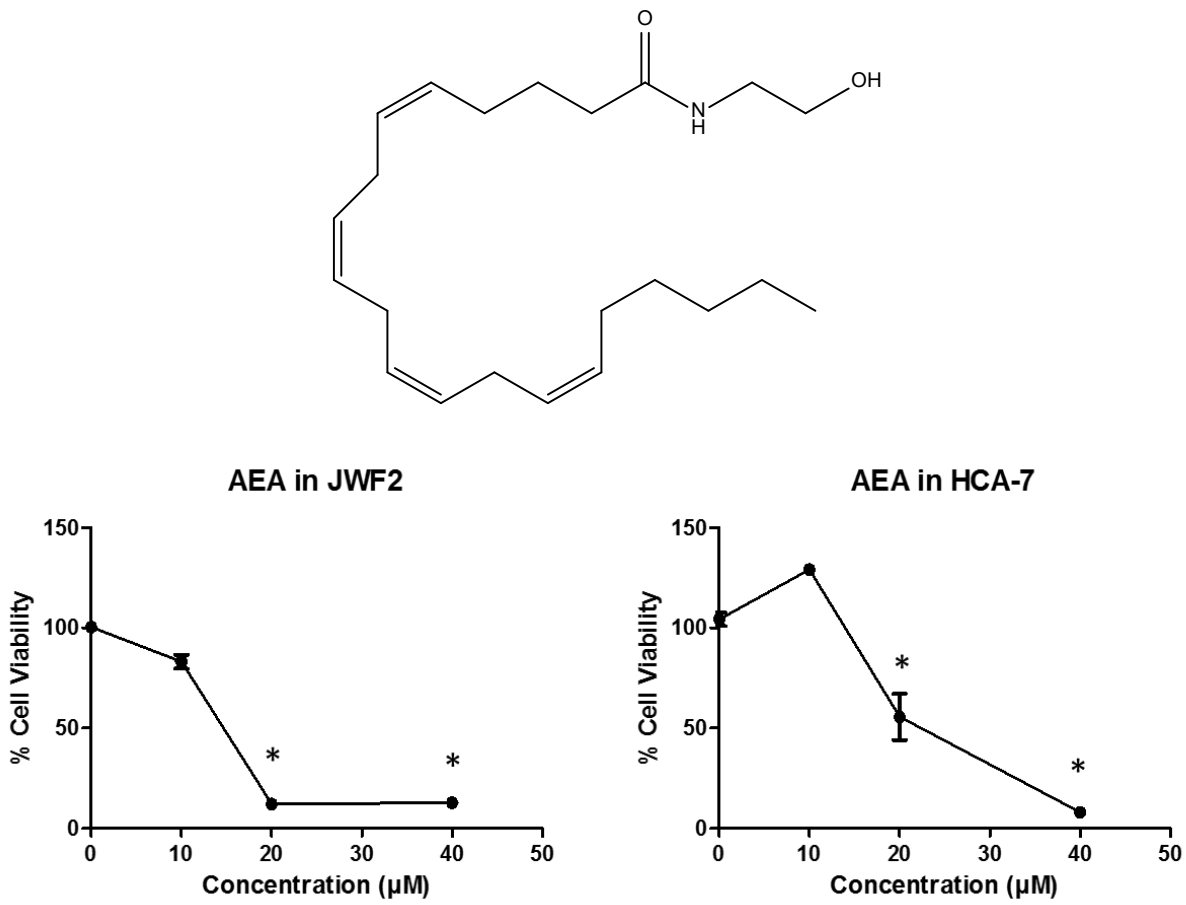



**Figure 3.2: Cytotoxicity of AEA in JWF2 and HCA-7**

JWF2 tumorigenic keratinocytes and HCA-7 colorectal cancer cells were treated with 10 µM, 20 µM, and 40 µM concentrations of AEA. Cell viability was evaluated by MTS assay according to manufacturer's instructions. Data represents mean ± SEM of three independent experiments and are expressed as percent viability compared to untreated cells. Data were analyzed using one-way ANOVA followed by Tukey's analysis ($P < 0.05$ = *) when compared to vehicle treated cells.

### 3.3: Cytotoxicity of R1-Methanandamide in JWF2 and HCA-7

R1-methanandamide's structure is identical to AEA except for the addition of a methyl group on the alpha carbon of the ethanolamide moiety. The methyl group addition makes the molecule a poor substrate for FAAH, in turn making R1-methanandamide a more metabolically stable molecule than AEA (Abadji et al. 1994). As shown in Scheme 1.2, FAAH is responsible for degrading AEA to AA and ethanolamine which inhibits its cytotoxicity (Kuc et al. 2012). With its resistance to FAAH degradation, we expected to observe an increase in cytotoxicity due to an increase in J-series prostamide production.

When JWF2 NMSC cells were treated with R1-methanandamide we observed a 40.5% reduction in cell viability at a concentration of 10 μM and a 78.4% reduction in cell viability at 20 μM. When HCA-7 colorectal cancer cells were treated with R1-methanandamide *in vitro* we did not observe a reduction in cell viability at 20 μM. These data suggest a modification to prevent degradation by FAAH does not correlate with an increase in anti-cancer activity when compared to AEA.

Our hypothesis stated that modifying the molecule with a methyl group on the alpha carbon of ethanolamide would resist degradation by FAAH leading to an increase in cytotoxicity. Although R1-methanandamide meets the % reduction in cell viability in JWF2 cells, it does not in HCA-7 cells. Therefore, it was not examined further in our studies.

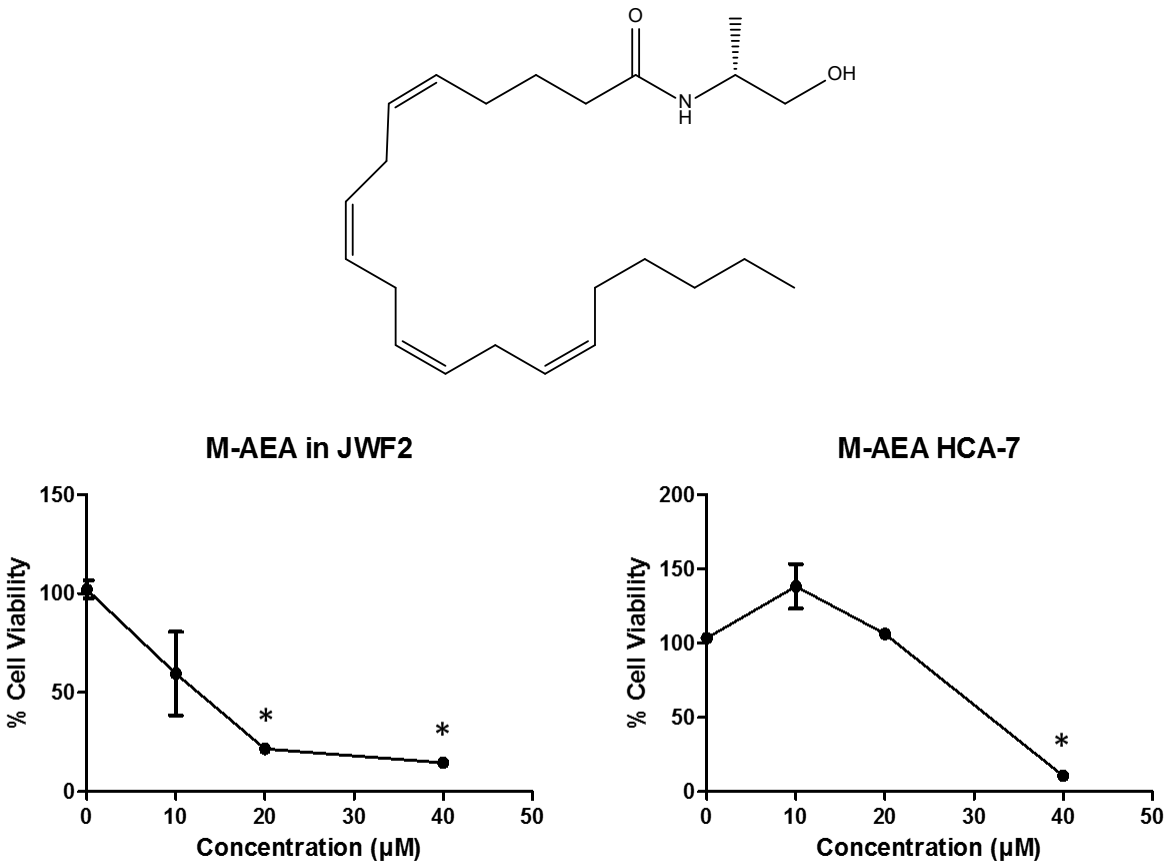



**Figure 3.3: Cytotoxicity of R1-Methanandamide in JWF2 and HCA-7**

JWF2 tumorigenic keratinocytes and HCA-7 colorectal cancer cells were treated with 10 μM, 20 μM, and 40 μM concentrations of M-AEA. Cell viability was evaluated by MTS assay according to manufacturer's instructions. Data represents mean ± SEM of three independent experiments and are expressed as percent viability compared to untreated cells. Data were analyzed using one-way ANOVA followed by Tukey's analysis ($P < 0.05$ = *) when compared to vehicle treated cells.

### 3.4: Cytotoxicity of Arachidonoyl Glycine (NAGly) in JWF2 and HCA-7

NAGly is an endocannabinoid and a carboxylic metabolite of AEA (Burstein et al. 2002, Bradshaw et al. 2009). The molecular structure differs from AEA by substituting a carboxylic acid moiety for the terminal alcohol introducing a more polar molecule. The structure of NAGly is like AA as it contains a terminal carboxylate moiety which we predict will allow for the same binding events to a target that takes place with AA. NAGly is reported to be a selective substrate for COX-2 over COX-1 and has a higher rate of metabolism by COX-2 compared to AEA and other known arachidonoyl derivatives (Prusakiewicz et al. 2002, Prusakiewicz et al. 2007, Kozak et al. 2002).

When JWF2 NMSC cells were treated with NAGly we observed an 88.4% reduction in cell viability at a concentration of 20 μM. When HCA-7 colorectal cancer cells were treated with NAGly we did not observe a reduction in cell viability at 20 μM. NAGly demonstrates a decrease in anti-cancer activity in HCA-7 cells, but we examined it in further studies based on its cytotoxicity in JWF cells.

NAGly has a COX-2 metabolic rate 40% that of AA, which as stated earlier is high compared to other arachidonoyl derivatives. (Prusakiewicz, et al. 2007) Considering our model system is dependent on COX-2 metabolism, our hypothesis was that an AEA analog containing a high rate of COX-2 metabolism would lead to a large concentration of prostamides. With an increase in prostamides, there should be an increase in cancer-killing J-series prostamides which would lead to increased anti-cancer activity. In these results, we do not see that increased anti-cancer activity, but we do still see anti-cancer activity that meets our hit thresholds in JWF2. Considering all of this, we wanted to take NAGly to further studies and explore its production of D- and J-series prostaglandin analogs. These studies can identify if rate of metabolism by COX-

2 is important, if there is bias for other prostaglandin synthases other than PGDS, or if the prostaglandin analogs produced do not have the same chemotherapeutic power as 15d-PMJ$_2$.

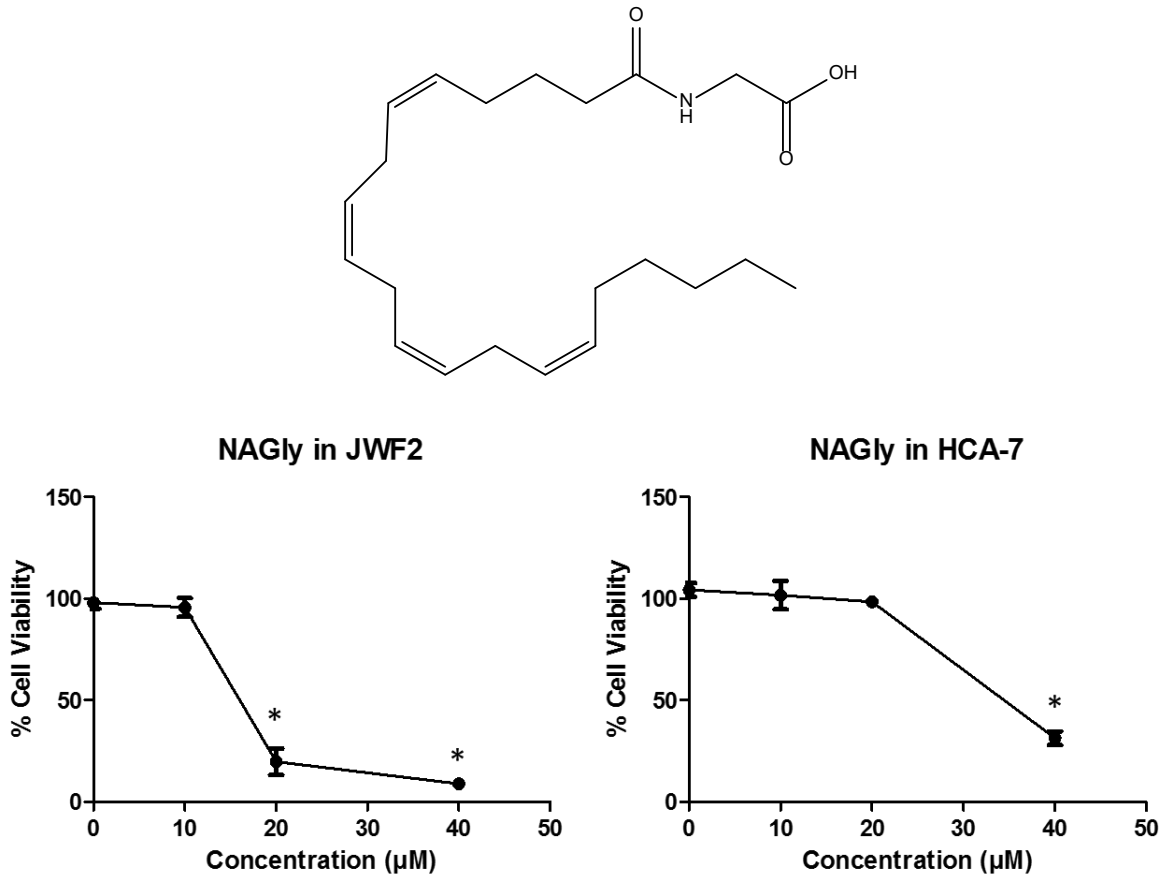



**Figure 3.4: Cytotoxicity of Arachidonoyl Glycine (NAGly) in JWF2 and HCA-7**
JWF2 tumorigenic keratinocytes and HCA-7 colorectal cancer cells were treated with 10 µM, 20 µM, and 40 µM concentrations of NAGly. Cell viability was evaluated by MTS assay according to manufacturer's instructions. Data represents mean ± SEM of three independent experiments and are expressed as percent viability compared to untreated cells. Data were analyzed using one-way ANOVA followed by Tukey's analysis ($P < 0.05$ = *) when compared to vehicle treated cells.

### 3.5: Cytotoxicity of Arvanil in JWF2 and HCA-7

Arvanil is a hybrid endocannabinoid/capsaicin molecule with affinity for the $CB_1$ receptor and the TRPV1 receptor (Di Marzo et al. 2001). It is metabolized by COX-2 with a rate that is 5.6% that of AA (Prusakiewicz et al. 2007). The molecular structure of arvanil contains the biggest difference in structure compared to AEA of any of the derivatives tested. Our goal was to observe how adding steric bulk with the addition of a benzene ring between the amide bond and the terminal moieties, would affect COX-2 metabolism and in turn, anti-cancer activity. The structure still contains a terminal hydroxyl group, but also contains a terminal methyl ether which is a moiety that has not been explored in previous studies. The methoxy group not only adds more steric bulk to the ring, but also increases the electron density. Another critical property of arvanil is that it is resistant to degradation by FAAH.

When JWF2 NMSC cells were treated with arvanil, an 89.5% reduction in cell viability at 10 μM and an 86% reduction in cell viability at 20 μM occurred. When HCA-7 colon cancer cells were treated with arvanil, a 46.3% reduction in cell viability at 10 μM and an 89.4% reduction in cell viability at 20 μM was observed.

The cytotoxicity results from arvanil suggest that steric bulk added to the ethanolamide end via a benzene ring can be tolerated when considering metabolism by COX-2 and PGDS. It is reported that arvanil's rate of metabolism by COX-2 is lower than AEA, but that lower rate of metabolism does not necessarily contribute to a decrease in anti-cancer activity and in this case the anti-cancer activity was increased. This leads to the hypothesis that the concentration of J-series prostamides is not as important as the activity of those J-series prostamides. To investigate this hypothesis further, more studies were completed to identify novel D- and J-series prostaglandin

analogs that were derived from arvanil with increased cytotoxicity when compared to 15d-PMJ$_2$. Based on its cytotoxicity results, arvanil is the best hit of any AEA analog that were tested.

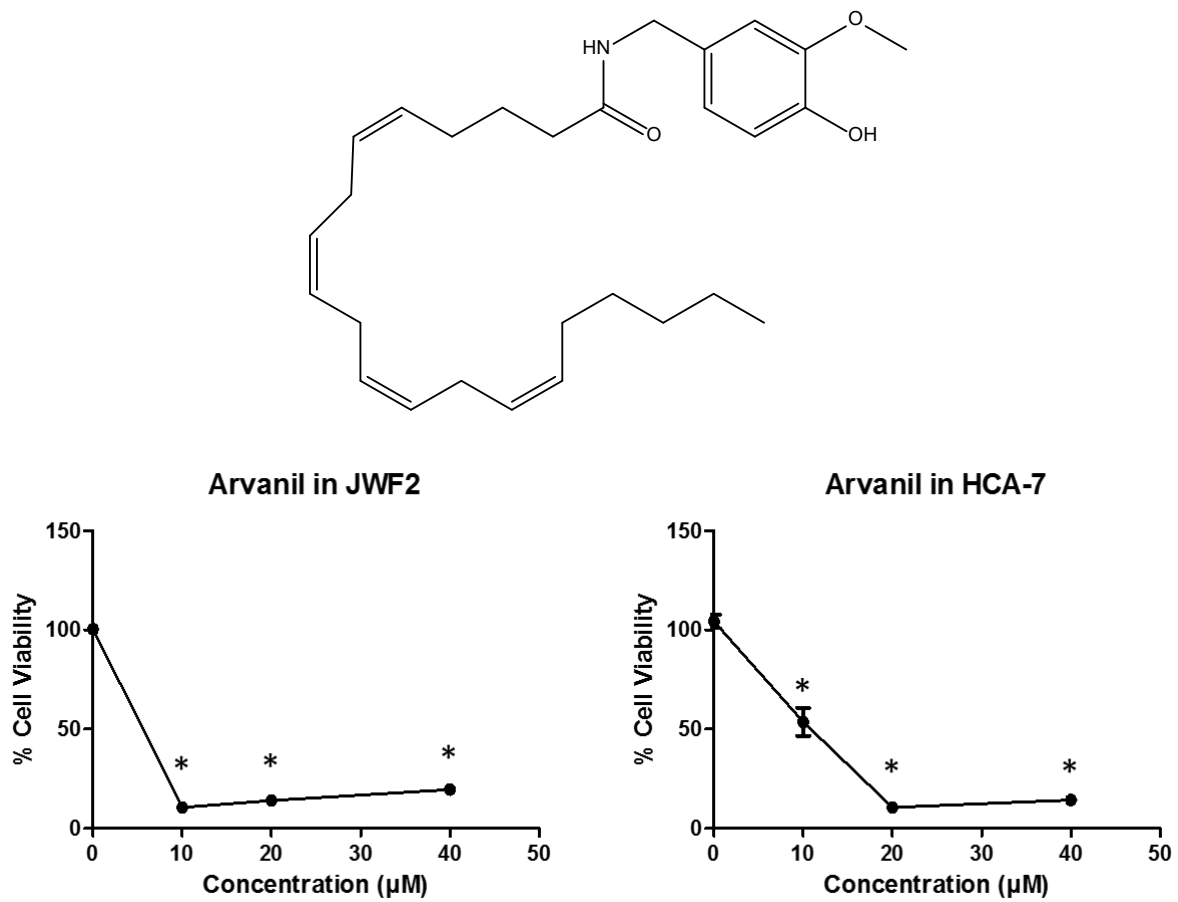



**Figure 3.5: Cytotoxicity of Arvanil in JWF2 and HCA-7**

JWF2 tumorigenic keratinocytes and HCA-7 colorectal cancer cells were treated with 10 μM, 20 μM, and 40 μM concentrations of arvanil. Cell viability was evaluated by MTS assay according to manufacturer's instructions. Data represents mean ± SEM of three independent experiments and are expressed as percent viability compared to untreated cells. Data were analyzed using one-way ANOVA followed by Tukey's analysis ($P < 0.05$ = *) when compared to vehicle treated cells.

### 3.6: Cytotoxicity of Arachidonoyl Diethanolamine (ADA) in JWF2 and HCA-7

Arachidonoyl diethanolamine (ADA) retains similar structure to AEA, but with the addition of a second ethanolamine end. The tertiary amide in ADA removes the hydrogen from the amide bond, thus affecting any binding or signaling event that requires a hydrogen bond associated with a secondary amide. If binding of the hydroxyl group to its target regulates the cytotoxicity of AEA, then adding a second terminal hydroxyl group should increase the probability of this binding event in turn increasing anti-cancer activity.

When JWF2 NMSC cells were treated with ADA we observe a 22.5% reduction in cell viability at a concentration of 10 μM and a 7.2% reduction in cell viability at a concentration of 20 μM. When HCA-7 colorectal cancer cells were treated with ADA we observe no reduction in cell viability at 10 μM or 20 μM.

With these results, our hypothesis that adding a second branch from the amide bond with a terminal hydroxyl did not come to fruition. Adding a second terminal hydroxyl did not increase anti-cancer activity, but instead decreased the anti-cancer activity of AEA. With these results, ADA is not considered a hit for further studies.

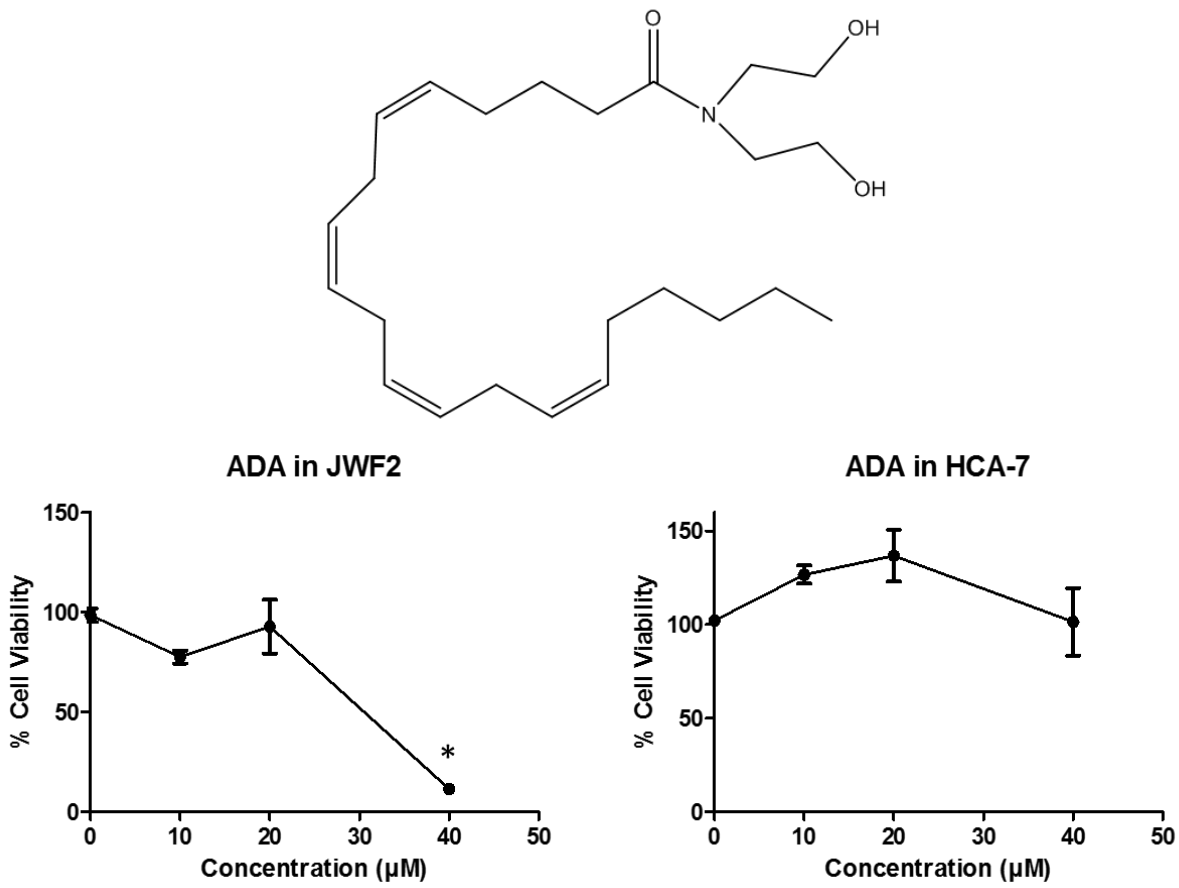



**Figure 3.6: Cytotoxicity of Arachidonoyl Diethanolamine (ADA) in JWF2 and HCA-7**

JWF2 tumorigenic keratinocytes and HCA-7 colorectal cancer cells were treated with 10 μM, 20 μM, and 40 μM concentrations of ADA. Cell viability was evaluated by MTS assay according to manufacturer's instructions. Data represents mean ± SEM of three independent experiments and are expressed as percent viability compared to untreated cells. Data were analyzed using one-way ANOVA followed by Tukey's analysis ($P < 0.05$ = *) when compared to vehicle treated cells.

### 3.7: Cytotoxicity of Arachidonoyl Serinol (AS) in JWF2 and HCA-7

On the other hand, the decreased cytotoxicity of ADA may be due to the change from a secondary amide to a tertiary amide. This amide bond change could have decreased the molecule's ability to bind to its target. To test this theory, the next molecule investigated was arachidonoyl serinol (AS) which contains the same second terminal hydroxyl moiety but retains the secondary amide of AEA. AS is structurally similar to AEA and ADA in terms of polarity and size of the molecule. The modification, a second terminal hydroxyl moiety, which were investigated with ADA is the same being explored by AS. The difference between AS and ADA is, with AS, the secondary amide present in AEA is retained instead of replaced by a tertiary amide.

When JWF2 NMSC cells were treated with AS we observe no reduction in cell viability at a concentration of 10 μM and a 14.3% reduction in cell viability at a concentration of 20 μM. When HCA-7 colorectal cancer cells were treated with AS we observe no reduction in cell viability at 10 μM or 20 μM.

These results confirm our hypothesis established by ADA that adding a branch with a second hydroxyl reduces or abrogates the anti-cancer activity of the molecule. By removing the tertiary amide of ADA and replacing it with the secondary amide of AEA, but keeping the branched ethanolamine end with two hydroxyl moieties, we tested the hypothesis that the tertiary amide is responsible for removal of anti-cancer activity. AS maintained the same decrease in anti-cancer activity, so it was determined the tertiary amide did not play the major role in the reduction of anti-cancer activity. As such, we do not consider AS a hit and further study of this molecule will not be conducted.

This leads to the assumption that either of these structural modifications, a branched ethanolamine end with two terminal hydroxyls or a tertiary amide, lead to a removal of anti-cancer

activity. This could be through removal of binding ability at the target or through obstruction of COX-2 or PGDS metabolism.

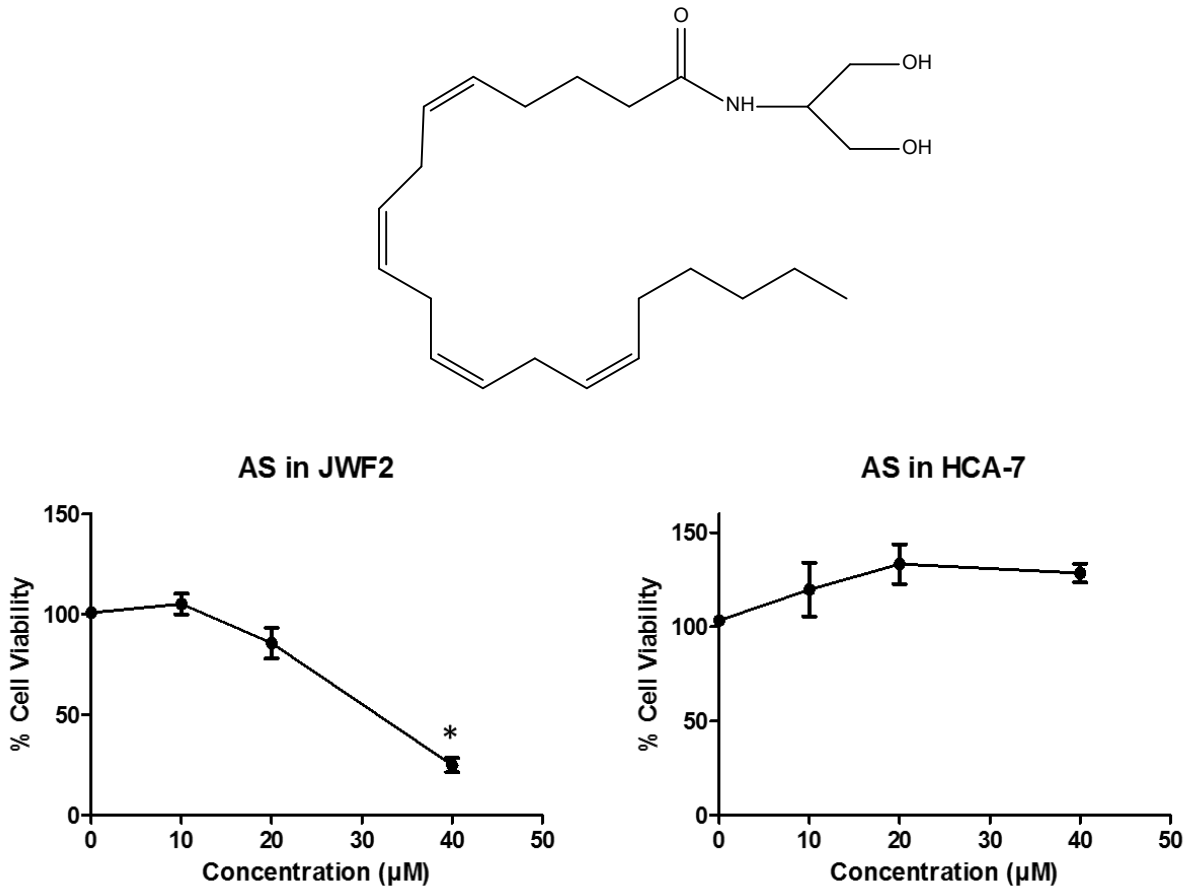



**Figure 3.7: Cytotoxicity of Arachidonoyl Serinol (AS) in JWF2 and HCA-7**
JWF2 tumorigenic keratinocytes and HCA-7 colorectal cancer cells were treated with 10 μM, 20 μM, and 40 μM concentrations of AS. Cell viability was evaluated by MTS assay according to manufacturer's instructions. Data represents mean ± SEM of three independent experiments and are expressed as percent viability compared to untreated cells. Data were analyzed using one-way ANOVA followed by Tukey's analysis ($P < 0.05$ = *) when compared to vehicle treated cells.

### 3.8: Cytotoxicity of Arachidonoyl Propanolamine (A-Pro) in JWF2 and HCA-7

AEA contains a two-carbon chain separating the alcohol from the amide bond in the ethanolamine arm. With the molecule arachidonoyl propanolamine (A-Pro), the theory that a two-carbon chain is the perfect number and length was put to the test. AP contains a three-carbon chain and allows us to investigate whether an odd number carbon chain and a slightly longer chain affects anti-cancer activity. The molecular structure is identical to AEA in every other way, as A-Pro retained the secondary amide and terminal hydroxyl group.

When JWF2 NMSC cells were treated with A-Pro we observed an 11.4% reduction in cell viability at a concentration of 10 μM and a 37.8% reduction in cell viability at a concentration of 20 μM. When HCA-7 colorectal cancer cells were treated with A-Pro we observe no reduction in cell viability at 10 μM or 20 μM.

When compared to AEA, we see a reduction in cytotoxicity with A-Pro. These data demonstrate that the anti-cancer activity is very sensitive to the distance between the amide bond and the terminal hydroxyl moiety. This modification to the structure should not interfere with COX-2 or PGDS metabolism, so it appears the effect is directly related to the inherent cytotoxicity of the 15d-$PMJ_2$ analog. Extending the length between the amide bond and hydroxyl moiety causing reduction in anti-cancer activity contrasts with arvanil. The distance between these two groups in arvanil is five carbons whereas the distance in A-Pro is only three carbons. This suggests that there is not a single fixed length we must maintain, but there are multiple distances that produce anti-cancer activity, and others that remove anti-cancer activity. With these results, A-Pro is not considered a hit.

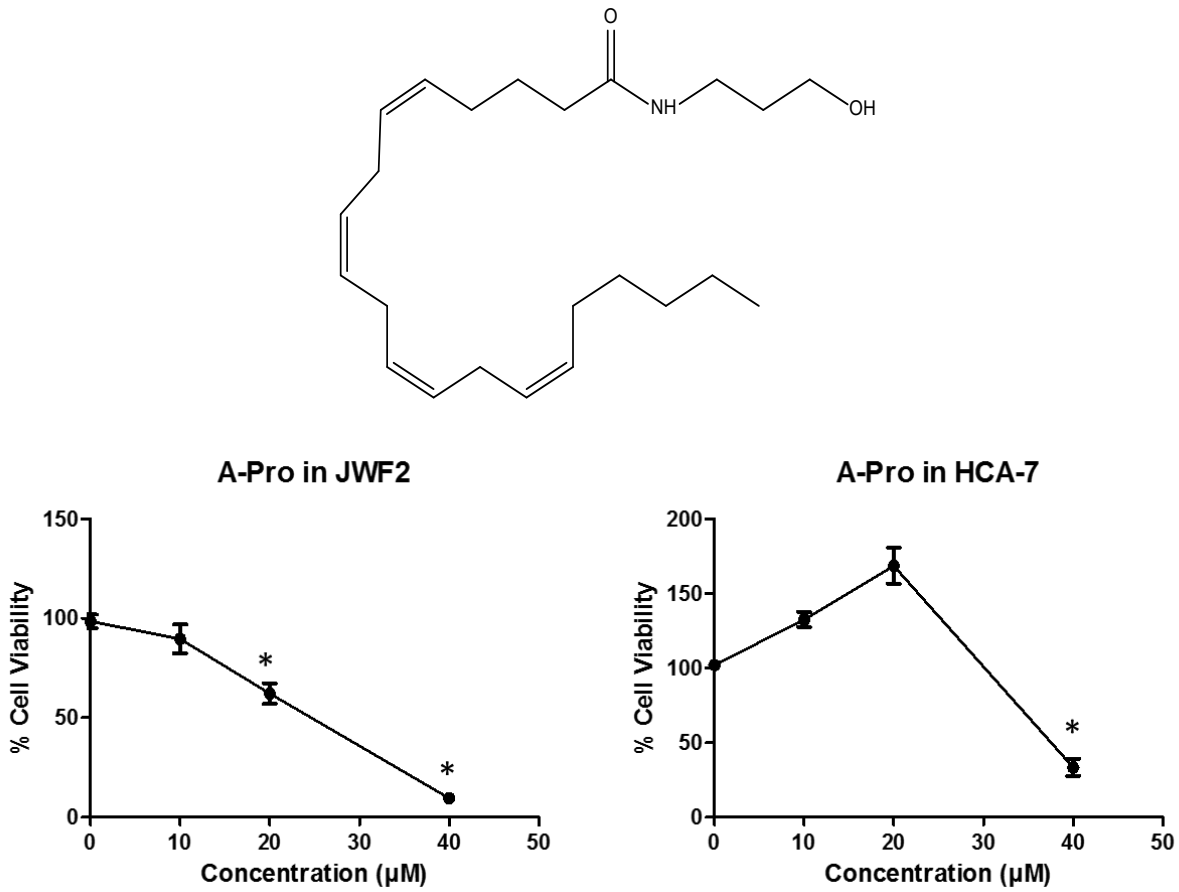



**Figure 3.8: Cytotoxicity of Arachidonoyl Propanolamine in JWF2 and HCA-7**

JWF2 tumorigenic keratinocytes and HCA-7 colorectal cancer cells were treated with 10 μM, 20 μM, and 40 μM concentrations of A-Pro. Cell viability was evaluated by MTS assay according to manufacturer's instructions. Data represents mean ± SEM of three independent experiments and are expressed as percent viability compared to untreated cells. Data were analyzed using one-way ANOVA followed by Tukey's analysis ($P < 0.05$ = *) when compared to vehicle treated cells.

### 3.9: Cytotoxicity of Arachidonoyl 2'-Ethylchloroamide (AC) in JWF2 and HCA-7

In these cytotoxicity experiments we wanted to investigate the importance of the terminal alcohol end of the ethanolamine of AEA for maintaining cytotoxicity. In arachidonoyl 2'-chloroethylamide (AC) the hydroxyl group of AEA was replaced with a chlorine. The approach here was to observe whether the terminal alcohol, and its ability to serve as a hydrogen bond donor, was vital to the cytotoxicity of AEA or if any polar atom or moiety at the end of the ethanolamide arm still elicits anti-cancer activity.

When JWF2 NMSC cells were treated with AC we observed a 17.5% reduction in cell viability at a concentration of 10 μM and a 57.5% reduction in cell viability at a concentration of 20 μM. When HCA-7 colorectal cancer cells were treated with AC we observe no reduction in cell viability at 10 μM or 20 μM.

Hence, AC does have anti-cancer activity in JWF2 cells, but it is less cytotoxic than AEA. This leads us to conclude that polar groups are not interchangeable at the terminal end of AEA. We anticipate that this modification will not affect the metabolism of AC by COX-2 or PGDS. One of the goals of this modification was to identify if hydrogen bond accepting or donating is crucial for the anti-cancer activity of AEA. Examination of the results for NAGly and AC reveals that having a negatively charged group, i.e. a carboxylate, or hydrogen bonding donor is important for preserving cytotoxicity similar to that of AEA. AC is not considered a hit and was not carried on to further studies.

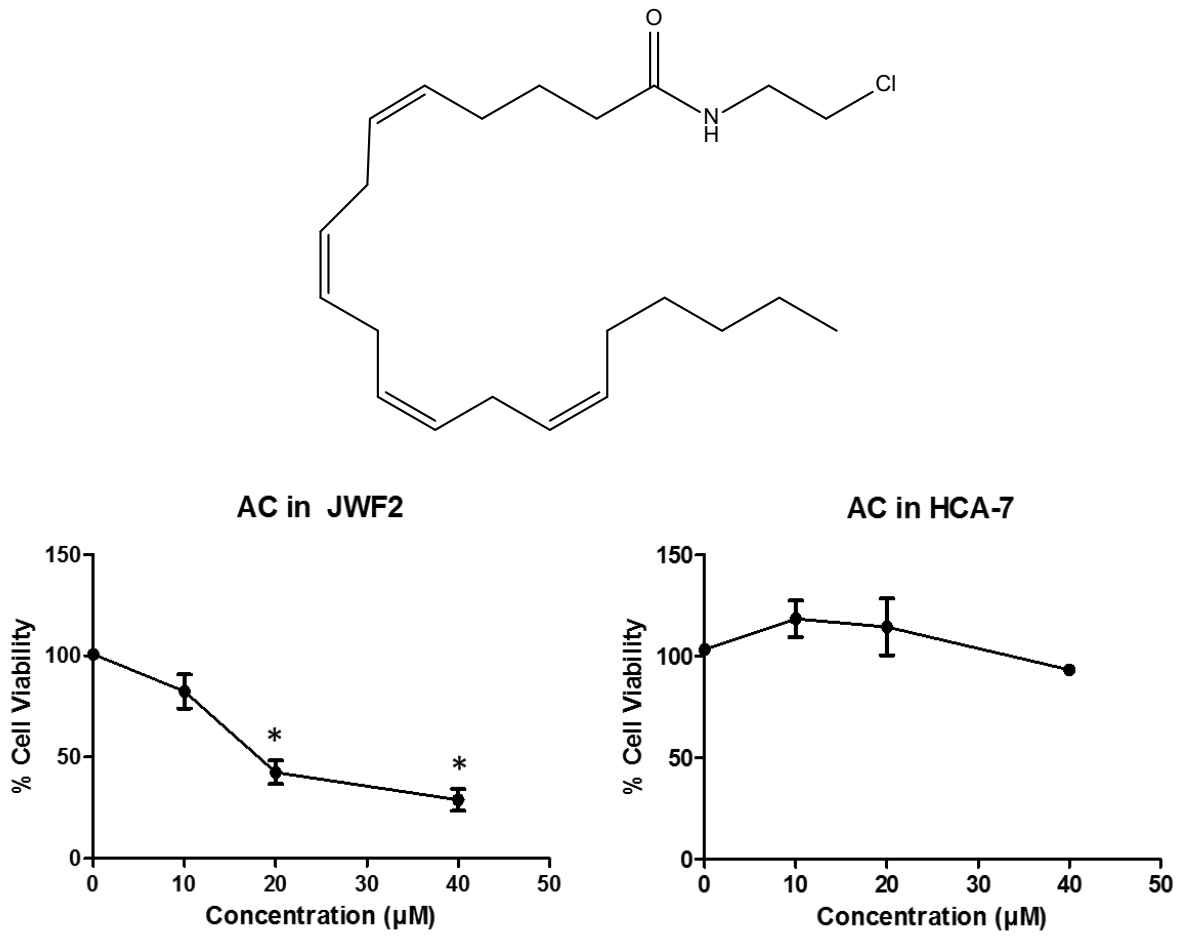



**Figure 3.9: Cytotoxicity of Arachidonoyl 2'-Ethylchloroamide in JWF2 and HCA-7**

JWF2 tumorigenic keratinocytes and HCA-7 colorectal cancer cells were treated with 10 µM, 20 µM, and 40 µM concentrations of AC. Cell viability was evaluated by MTS assay according to manufacturer's instructions. Data represents mean ± SEM of three independent experiments and are expressed as percent viability compared to untreated cells. Data were analyzed using one-way ANOVA followed by Tukey's analysis ($P < 0.05$ = *) when compared to vehicle treated cells.

### 3.10: Second Generation SAR: Arvanil Analogs

Results from the first generation SAR produced three hits: R1-methanandamide, NAGly, and arvanil. Of these three hits, arvanil demonstrated the greatest cytotoxicity. Therefore, three derivatives of arvanil, AM404, arvanil D1, and arvanil D2, were designed and synthesized or purchased. When creating these arvanil analogs, we focused on the substituent pattern of the benzene ring (arvanil D1 and arvanil D2), and the length of the carbon chain separating the benzene ring from the amide bond (AM404) where a carbon was removed between the amide bond and the benzene ring. Moreover, because each of the three molecules are analogs of arvanil, we anticipate that their metabolism by COX-2 and PGDS metabolism will not be compromised. Hence our goals were to: 1) identify the arvanil derivative with the greatest cytotoxicity and compare its cytotoxicity to arvanil, and 2) examine whether arvanil or the arvanil derivatives are metabolized by COX-2 and PGDS to J-series prostaglandins. However, we only chose one, arvanil or arvanil derivative, with the greatest cytotoxicity to pursue further studies investigating COX-2 and PGDS metabolism.

### 3.10.1: Cytotoxicity of AM404 in JWF2 and HCA-7

AM404 is a metabolite of acetaminophen (paracetamol). Acetaminophen undergoes a deacetylation to p-aminophenol in the central nervous system. P-aminophenol is conjugated with arachidonic acid via FAAH to produce AM404, a molecule reported to be responsible for the analgesic action of acetaminophen (Hogestatt et al. 2005, Bisogno et al. 2002, Mallet et al. 2008, Muramatsu et al. 2016). AM404 also inhibits proliferation in C6 glioma cells, an event that occurs due to blockade of AEA uptake (De Lago et al. 2006). This molecule maintains the steric bulk of arvanil via the benzene ring contained between the amide bond and the terminal alcohol, but the chain linking the amide bond to the benzene ring is one carbon shorter.

When JWF2 NMSC cells were treated with AM404 we observe a 20% reduction in cell viability at a concentration of 10 μM and a 68% reduction in cell viability at a concentration of 20 μM. When HCA-7 colorectal cancer cells were treated with AM404 we observe a 16.7% reduction in cell viability at 20 μM.

Based on these results, AM404 contains some anti-cancer activity, but does not meet the cell viability threshold needed to be considered a hit. In contrast to the rest of our AEA analogs, the interest in AM404 came from reported ability to inhibit COX-2 metabolism (Hogestatt et al. 2005). It is an interesting modification because its structure is similar to arvanil and arvanil D1, although the removal of one carbon in the chain length between the amide bond and hydroxyl group inhibits COX-2 metabolism. When compared to the rate of metabolism by arvanil discussed earlier, this slight structure change is crucial for anti-cancer activity when comparing arvanil and AM404. These results suggest that arvanil's ability to be metabolized by COX-2 (AM404 is not metabolized by COX-2) is responsible for an increase in cytotoxicity of the molecule. This supports the hypothesis that COX-2 metabolism is required for increased anti-cancer activity.

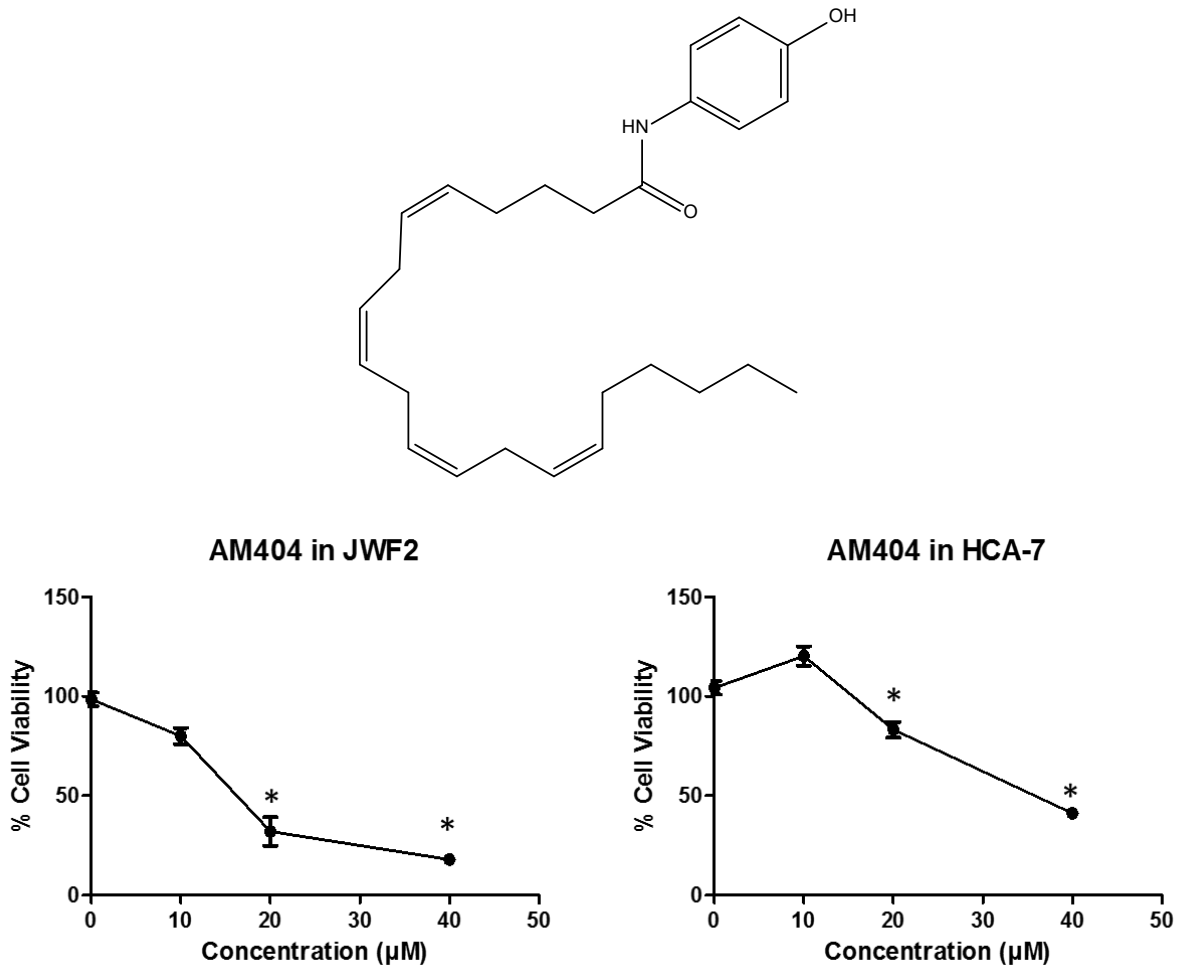



**Figure 3.10: Cytotoxicity of AM404 in JWF2 and HCA-7**

JWF2 tumorigenic keratinocytes and HCA-7 colorectal cancer cells were treated with 10 μM, 20 μM, and 40 μM concentrations of AM404. Cell viability was evaluated by MTS assay according to manufacturer's instructions. Data represents mean ± SEM of three independent experiments and are expressed as percent viability compared to untreated cells. Data were analyzed using one-way ANOVA followed by Tukey's analysis ($P < 0.05$ = *) when compared to vehicle treated cells.

### 3.10.2: Cytotoxicity of Arvanil D1 in JWF2 and HCA-7

When creating arvanil D1, the methoxy substituent was completely removed from the benzene ring. This was to explore the importance of the methoxy group for arvanil's anti-cancer activity. The terminal hydroxyl group of ethanolamide in 15d-$PMJ_2$ is necessary for its anti-cancer activity. Therefore, arvanil D1 investigates whether a hydroxyl substituent leads to an increase in anti-cancer activity when compared to arvanil. Removal of the methoxy group not only decreases the steric bulk of the ring, but it also effects the charge density on the ring which, in turn, lowers the pKa of the hydroxyl group.

When JWF2 NMSC cells were treated with arvanil D1 we observe an 80.3% reduction in cell viability at a concentration of 10 μM and a 97.8% reduction in cell viability at 20 μM. When HCA-7 colorectal cancer cells were treated with arvanil D1 we observe no reduction in cell viability at 10 μM and a 64.5% reduction in cell viability at 20 μM.

Based on these results, it has been demonstrated that the methoxy substituent is not required for the anti-cancer activity of arvanil. The cytotoxicity results of arvanil and arvanil D1 compared with the cytotoxicity results of AM404 suggests that the removal of the methoxy substituent does not interrupt the COX-2 metabolism of the molecule. These results also continue to confirm the hypothesis that hydrogen bonding of a terminal hydroxyl moiety is required for the cytotoxicity of 15d-$PMJ_2$ analogs. To continue testing this hypothesis we synthesized another arvanil analog, arvanil D2, which removes the terminal hydroxyl moiety to further investigate its importance.

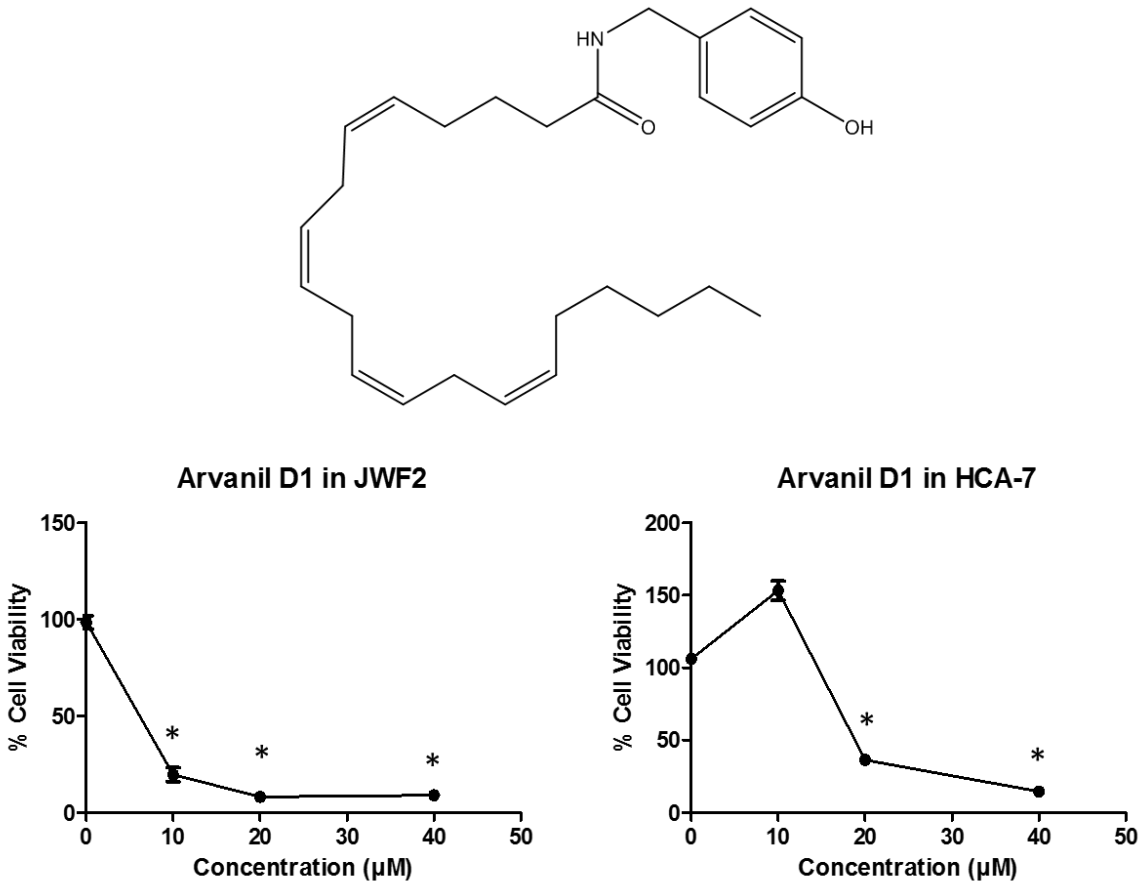



**Figure 3.11: Cytotoxicity of Arvanil D1 in JWF2 and HCA-7**

JWF2 tumorigenic keratinocytes and HCA-7 colorectal cancer cells were treated with 10 µM, 20 µM, and 40 µM concentrations of arvanil D1. Cell viability was evaluated by MTS assay according to manufacturer's instructions. Data represents mean ± SEM of three independent experiments and are expressed as percent viability compared to untreated cells. Data were analyzed using one-way ANOVA followed by Tukey's analysis ($P < 0.05$ = *) when compared to vehicle treated cells.

### 3.10.3: Cytotoxicity of Arvanil D2 in JWF2 and HCA-7

When designing arvanil D2, instead of removing the methoxy substituent, like was done with arvanil D1, the hydroxyl substituent was replaced with a methoxy substituent. This was to explore the importance of a hydroxyl group on the aromatic ring to the cytotoxicity of the molecule. This modification changes the polarity of the molecule slightly and removes the hydrogen bond donating capability of the molecule.

When JWF2 NMSC cells were treated with arvanil D2 we observed a 26.1% reduction in cell viability at a concentration of 10 μM and a 48.7% reduction in cell viability at 20 μM. When HCA-7 colorectal cancer cells were treated with arvanil D2 we did not observe a reduction in cell viability at 10 μM and an 18.4% reduction in cell viability at 20 μM.

Based on these results, it was determined the hydroxyl substituent of arvanil is crucial to its anti-cancer activity. The ongoing hypothesis of all AEA analogs is that hydroxyl moieties are required for maintaining a high level of cytotoxicity, whether for hydrogen bonding or other interactions with the binding site of 15d-$PMJ_2$. This hypothesis is further supported by the cytotoxicity results of the second generation arvanil analogs. The reduction in cytotoxicity of arvanil D2, compared to arvanil D1 and arvanil, may be attributed to reduced COX-2 metabolism because of the increased steric bulk of a second methoxy substituent. If reduced COX-2 metabolism is the reason for the reduced cytotoxicity of arvanil D2, then substituents on the benzene ring aren't as important as the steric bulk those substituents add.

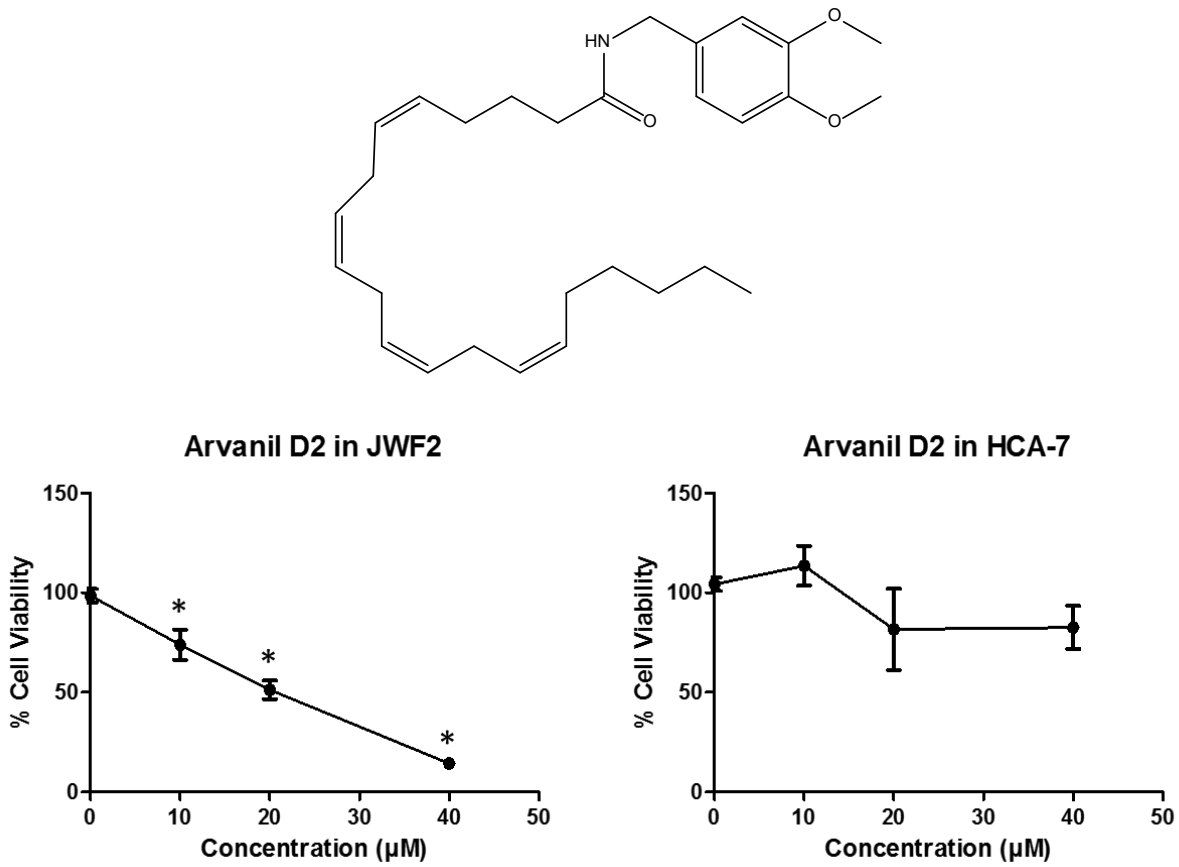



**Figure 3.12: Cytotoxicity of Arvanil D2 in JWF2 and HCA-7**

JWF2 tumorigenic keratinocytes and HCA-7 colorectal cancer cells were treated with 10 μM, 20 μM, and 40 μM concentrations of arvanil D2. Cell viability was evaluated by MTS assay according to manufacturer's instructions. Data represents mean ± SEM of three independent experiments and are expressed as percent viability compared to untreated cells. Data were analyzed using one-way ANOVA followed by Tukey's analysis ($P < 0.05$ = *) when compared to vehicle treated cells.

### 3.11: Summary and Conclusions of SAR Study

In this study, we wanted to expand on our previous findings that demonstrate the chemotherapeutic activity of AEA. Through an expansive structure-activity relationship study, the goal was to identify modifications that can be made to AEA to increase its overall anti-cancer activity (Table 3.2). Each of the following parameters were taken into consideration when designing the analogs of AEA; (1) cytotoxicity of the prostamide analog, (2) its ability to be metabolized by COX-2, (3) its ability to be metabolized by PGDS, and (4) its resistance to FAAH degradation. When designing AEA analogs, modifications were made to the ethanolamide portion of the structure to specifically identify characteristics that are important or can improve AEA's anti-cancer activity. These modifications can be grouped into one or multiple of the following categories; branching along the carbon chain, alteration of the hydrogen-bonding ability at the chain terminus, and the addition of an aryl ring. Many of these modifications are known to affect rate of metabolism by one or more of the aforementioned enzymes. In this SAR, the analogs were screened for their cytotoxicity and those with comparable cytotoxicity to AEA were selected for further screening to determine if the J-series prostaglandin analogs were produced and necessary for cytotoxicity. With the knowledge of how certain modifications affect an analog's metabolism combined with that analog's cytotoxicity profile, we can better assess how a structural modification impacts the J-series prostamide analog's cytotoxic potential.

The results of cytotoxicity studies with AEA in JWF2 NMSC cells and HCA-7 colorectal cancer cells gave us a minimum activity level to use as a benchmark for all of the AEA analogs investigated in this study. The cell viability at concentrations of 10 μM and 20 μM in both cell lines was compared to AEA's cell viability at the same concentrations to generate AEA analog hits. The AEA analogs meeting the hit threshold for the first-generation SAR were R1-

methanandamide, NAGly, and arvanil, and the only hit from the second-generation SAR was arvanil D1. Of these AEA analogs, NAGly and arvanil were examined further to determine if these molecules are metabolized COX-2/PGDS to J-series prostaglandins. R1-methanandamide, even though its cytotoxicity was within the hit threshold, was not carried on to further studies. The methyl group modification of R1-methanandamide, responsible for resisting degradation by FAAH, was hypothesized to increase cytotoxicity compared to AEA. Without seeing an increase in its cytotoxicity in JWF2 and a loss of cytotoxicity in HCA-7 at 20 μM, we deemed it to possess weak potential as an anti-cancer candidate. Arvanil D1 was a part of the SAR second generation of arvanil analogs, which we stated were not going to be carried on to further studies, unless a statistically significant increase in cytotoxicity was demonstrated compared to arvanil. This compound did not meet this threshold and thus was not studied further.

Degradation by FAAH is an important aspect in the cytotoxic potential of AEA, and it was previously demonstrated that blockade by a FAAH inhibitor enhanced cytotoxicity of AEA (Kuc et al. 2012). First generation AEA analogs that are hypothesized or have demonstrated an inherent resistance to FAAH degradation were R1-methanandamide, NAGly, arvanil, AS, and ADA (Abadji et al. 1994, Burstein et al. 2002, Di Marzo et al. 1998). These four AEA analogs demonstrated dramatically different anti-cancer activity compared to AEA at 10 μM and 20 μM, no cytotoxicity from AS and ADA, similar or decreased cytotoxicity depending on the cell line from R1-methanandamide and NAGly, increased cytotoxicity from arvanil. These results demonstrated resistance to FAAH degradation isn't as important as the inherent cytotoxicity of the J-series prostaglandin analogs assumed to be produced by the COX-2 metabolism of the AEA analogs.

The COX-2/PGDS metabolic pathway was demonstrated to be vital to the anti-cancer activity of AEA in our system (Soliman et al. 2016a). There are two characteristics important to COX-2 metabolism, overall ability to be metabolized by COX-2 and rate of metabolism. All seven AEA analogs in the first-generation SAR study were hypothesized or have demonstrated COX-2 metabolism, but the rates of COX-2 metabolism ranged from 40% that of AA with NAGly, to 5.6% that of AA with arvanil, to unknown rates of metabolism (Prusakiewicz et al. 2007). AEA is known to have a rate of metabolism 27% that of AA (Kozak et al. 2002). We hypothesized that rates of metabolism, and in turn, concentrations of prostamide-$D_2$ and prostamide-$J_2$ played a role in the anti-cancer activity of AEA analogs. This hypothesis was disproved by comparing cytotoxicity of AEA, NAGly, and arvanil. Arvanil demonstrates the lowest rate of metabolism of the three, but demonstrates the greatest cytotoxicity. This lends to the hypothesis that if the AEA analog can be metabolized by COX-2, then the most important aspect of the AEA analog is the inherent cytotoxicity of its J-series prostaglandin analogs which is determined by the modifications made to the ethanolamide region of AEA.

When considering inherent cytotoxicity of the COX-2/PGDS metabolized J-series prostaglandin analogs, we identified modifications that both enhance, retain, or decrease anti-cancer activity. Modifications to the terminal end group determined hydrogen bond accepting or donating capability is crucial, because AEA analogs in both the first and second generation that removed the terminal hydroxyl moiety or did not replace it with a carboxylate, AC and arvanil D2, lost all or most of their cytotoxicity. Modifications that created a branched structure in the ethanolamide region, AS and ADA, dramatically reduced cytotoxicity. Modifications that added little to dramatic steric bulk demonstrated mixed cytotoxic results, so we determined size of this region, as long as it does not interfere with COX-2 metabolism, has no significant role in anti-

cancer activity. Modifications that changed the distance between the amide bond and terminal group, A-Pro, arvanil, arvanil D1, arvanil D2, and AM404, produced mixed cytotoxicity results, demonstrating different chain lengths are permitted but there was no longer or shorter length trend apparent.

These findings suggest a real opportunity to increase the anti-cancer activity and develop novel AEA and arvanil analog chemotherapeutics. To aid in future studies and investigate the hypotheses and assumptions formed in this SAR study, two of the hit molecules, arvanil and NAGly, were carried on to further studies to determine their COX-2/PGDS metabolic profiles and cytotoxic pathways.

**Table 3.2: Cell Viability Results of Cytotoxicity SAR Study**

| Molecule | JWF2 | | HCA-7 | |
|---|---|---|---|---|
| | 10 µM | 20 µM | 10 µM | 20 µM |
| AEA | 83.2 ± 3.4 % | 12.2 ± 2.3 % | 129.2 ± 1.9 % | 55.7 ± 11.5 % |
| R1-methanandamide | 59.5 ± 21.1 % | 21.6 ± 2.8 % | 138.2 ± 14.9 % | 106.3 ± 2.5 % |
| NAGly | 95.7 ± 4.6 % | 19.8 ± 6.5 % | 101.7 ± 6.9 % | 98.4 ± 1.9 % |
| Arvanil | 10.5 ± 2.9 % | 14.0 ± 2.1 % | 53.7 ± 6.9 % | 10.6 ± 1.4 % |
| ADA | 77.5 ± 3.2 % | 92.8 ± 13.5 % | 126.8 ± 4.9 % | 136.9 ± 13.8 % |
| AS | 105.1 ± 5.2 % | 85.7 ± 7.6 % | 119.8 ± 14.3 % | 133.4 ± 10.6 % |
| AC | 82.5 ± 8.4 % | 42.5 ± 5.8 % | 118.4 ± 8.9 % | 114.5 ± 14.0 % |
| A-Pro | 89.6 ± 7.2 % | 62.2 ± 5.1 % | 132.7 ± 5.1 % | 168.6 ± 12.1 % |
| Arvanil D1 | 19.7 ± 3.7 % | 8.2 ± 0.7 % | 153.2 ± 6.6 % | 36.5 ± 3.2 % |
| Arvanil D2 | 73.9 ± 7.6 % | 51.3 ± 4.6 % | 113.6 ± 9.9 % | 81.6 ± 20.4 % |
| AM404 | 80.1 ± 4.1 % | 32.1 ± 7.3 % | 120.3 ± 2.6 % | 83.3 ± 3.9 % |

Green highlighted values represent the hit threshold set by AEA's cytotoxicity. Yellow highlighted values indicate that the cytotoxicity meets the hit threshold set forth by AEA's cytotoxicity.

# CHAPTER FOUR: ESTABLISHING CYTOTOXIC AND COX-2 METABOLIC PROFILES OF NAGLY AND ARVANIL

## 4.1: Design of Experiment Approach

The AEA analog SAR study resulted in two hits, NAGly and arvanil, which were carried on to further studies. This was done to explore their cytotoxic and metabolic profiles through the role of cannabinoid receptors, vanilloid receptors, COX-2, PGDS, and prostaglandin analog production. Previous studies in our group demonstrated the role of COX-2 metabolism in the mechanism of AEA's anti-cancer activity (Kuc et al. 2012, Soliman et al. 2016a). By using a similar study, we can better identify how NAGly and arvanil are inducing cancer cell death and investigate the production of any novel D- and J-series prostaglandins.

COX-2 metabolizes AEA to D- and J-series prostamides, which we have demonstrated is required for its anti-cancer activity in JWF2 cells. Therefore, our goal was to determine the mechanism by which NAGly and arvanil were cytotoxic towards cancer cells. Studies in this chapter will examine the role of the endocannabinoid system in the cytotoxicity of both molecules.

Apoptosis, or programmed cell death, is vitally important for cellular processes including normal cell turnover and immune system development. It is also the preferred method of cell death induced by chemotherapeutic agents. Previous studies in our group determined that the cytotoxicity of both AEA and 15d-$PMJ_2$ occurred via the apoptotic pathway (Soliman et al. 2016a, Soliman and Van Dross 2016b, Ladin et al. 2017). Since this controlled cell death via apoptosis is preferred, our goal in Figure 4.4 was to investigate whether NAGly and arvanil induced COX-2-dependent apoptotic cell death to establish their potential and their COX-2 metabolized J-series prostaglandin analogs as chemotherapeutics.

### 4.2: Concentration-Response Relationship Curve of AEA, NAGly, and Arvanil

The first step in establishing the cytotoxic profile of NAGly and arvanil is to compare their $LC_{50}$'s to AEA. To establish these $LC_{50}$'s, we used the JWF2 NMSC cells because each molecule demonstrated maximum cytotoxicity at 20 μM in JWF2, but NAGly does not reach maximum cytotoxicity in HCA-7 cells. Cells were treated with increasing concentration of AEA, NAGly, and arvanil on a logarithmic scale (Figure 4.1). The $LC_{50}$ data demonstrates AEA and NAGly have similar cytotoxicity in JWF2 cells, but arvanil has greater cytotoxicity and a correspondingly lower $LC_{50}$. This begins to establish a cytotoxic profile for arvanil that demonstrates it has the potential to be a more potent chemotherapeutic and produces more potent J-series prostaglandin analogs. The beginning of the cytotoxic profile for NAGly demonstrates an $LC_{50}$ similar to AEA in JWF2 cells. These results lead to a need for further studies to establish if NAGly or the J-series prostaglandin analogs produced by COX-2 metabolism of NAGly are more potent than that of AEA.

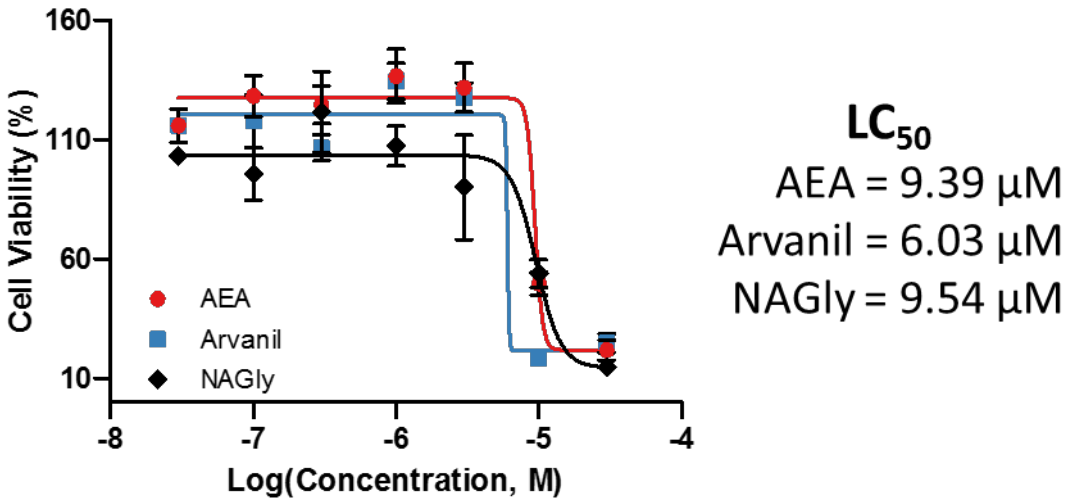



**Figure 4.1: Concentration-Response Relationship Curve of AEA, NAGly, and Arvanil**

JWF2 NMSC cells were treated with varying concentrations of AEA, arvanil, and NAGly to determine a logarithmic dose response curve. Cell viability was evaluated by MTS assay according to manufacturer's instructions. Data represents mean ± SEM of three independent experiments and are expressed as percent viability compared to untreated cells.

### 4.3: NAGly and Arvanil's Cytotoxicity is $CB_1$ and TRPV1 Receptor-independent

AEA has demonstrated anti-cancer activity in $CB_1$ and TRPV1 receptor-dependent mechanisms, yet other groups have demonstrated AEA's anti-cancer activity is due to receptor-independent mechanisms (Contassot et al. 2004, Melck et. al 2000, Melck et al. 1999, De Petrocellis et al. 1998, Kuc et al. 2012, Soliman and Van Dross 2016b). Before investigating the COX-2 metabolism and metabolic products of arvanil and NAGly, we first wanted to examine if $CB_1$ or TRPV1 receptors play a role in the anti-cancer activity of both molecules. To determine the role of the $CB_1$-receptor and TRPV1-receptor in the anti-cancer activity of the AEA analogs, NAGly and arvanil, receptor antagonists, AM251 ($CB_1$) and AMG9810 (TRPV1), were used to inhibit receptor-mediated activity.

It was demonstrated that when JWF2 cells were treated with AEA analog and either AM251 or AMG9810, that the cells were not rescued from death, suggesting that the anti-cancer activity of arvanil and NAGly is $CB_1$- and TRPV1 receptor-independent. These data suggests that these pathways are not responsible for the anti-cancer activity observed with these three molecules in this cell line.

An interesting phenomenon was observed in HCA-7 colorectal cancer cells. Arvanil's anti-cancer activity is suggested to be $CB_1$- and TRPV1 receptor-independent. In HCA-7 cells that were treated with NAGly and AM251, an increase in cytotoxicity was observed in comparison to cells that were treated with NAGly alone. Similarly, an increase in cytotoxicity occurred in cells treated with NAGly and AMG9810. This suggests that inhibiting $CB_1$ and TRPV1 receptor binding by NAGly, we can produce a stronger cytotoxic effect. This could be attributed to the portion of the molecule that interacts with this receptor instead diverting to the COX-2 metabolic

pathway in the presence of the antagonist, producing a higher concentration of cytotoxic 15d-$PMJ_2$ analogs.

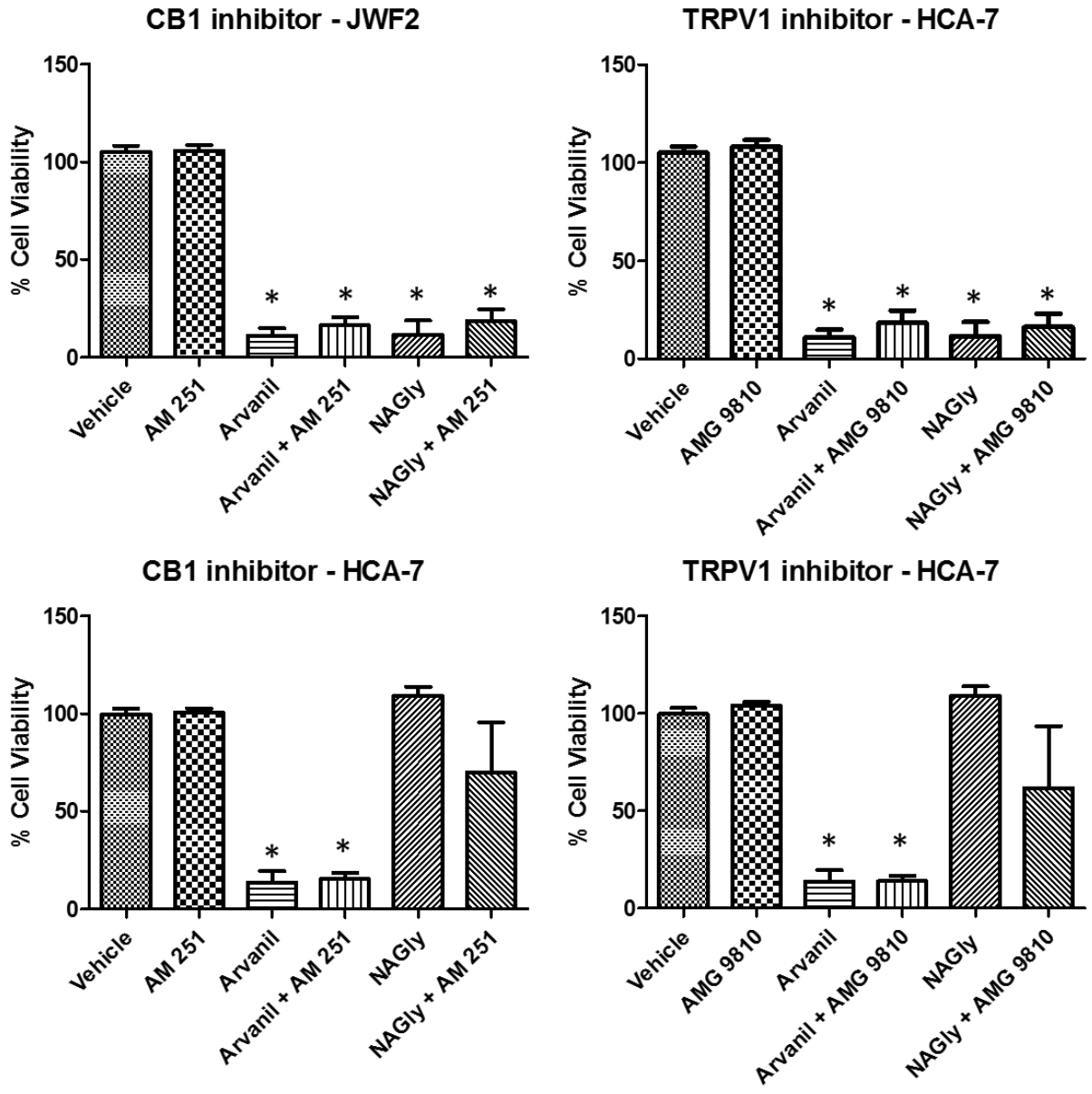



**Figure 4.2: NAGly and Arvanil's Cytotoxicity is $CB_1$ and TRPV1 Receptor-independent**
JWF2 NMSC cells and HCA-7 colorectal cancer cells were treated with 20 μM of arvanil, and NAGly with and without 1 μM of $CB_1$ receptor antagonist, AM251, and with and without 1 μM of TRPV1 receptor antagonist, AMG 9810. Cell viability was evaluated by MTS assay according to manufacturer's instructions. Data represents mean ± SEM of three independent experiments and are expressed as percent viability compared to untreated cells. Data were analyzed using one-way ANOVA followed by Tukey's analysis ($P < 0.05$ = *) when compared to vehicle treated cells.

### 4.4: AEA, NAGly, and Arvanil Produce D-series Prostaglandins and Prostamides

To begin investigation of the COX-2 and PGDS metabolism of arvanil and NAGly, using AEA as a positive control, we conducted an $PGD_2$ ELISA assay with agent-treated media removed from JWF2 and HCA-7. These results demonstrate, like AEA, NAGly and Arvanil are metabolized by COX-2 followed by PGDS producing D-series prostaglandin analogs, $PGD_2$-NAGly and $PGD_2$-arvanil. These findings are crucial in linking the anti-cancer activity produced by NAGly and arvanil to the COX-2 metabolic pathway. Neither NAGly nor arvanil produced as high of a concentration of prostamide-$D_2$ or prostamide-$D_2$ analogs as AEA at 8 hours post treatment (Figure 4.3). As mentioned earlier, the rate of metabolism of arvanil by COX-2 is reported to be lower than that of AEA. This is important to note because arvanil's anti-cancer activity is more powerful than AEA's even though the concentration of $PGD_2$-arvanil is lower than AEA. This suggests $PGD_2$-arvanil, once dehydrated to 15d-$PGJ_2$-arvanil, produces stronger anti-cancer activity than 15d-$PMJ_2$, the cancer-killing metabolite of AEA.

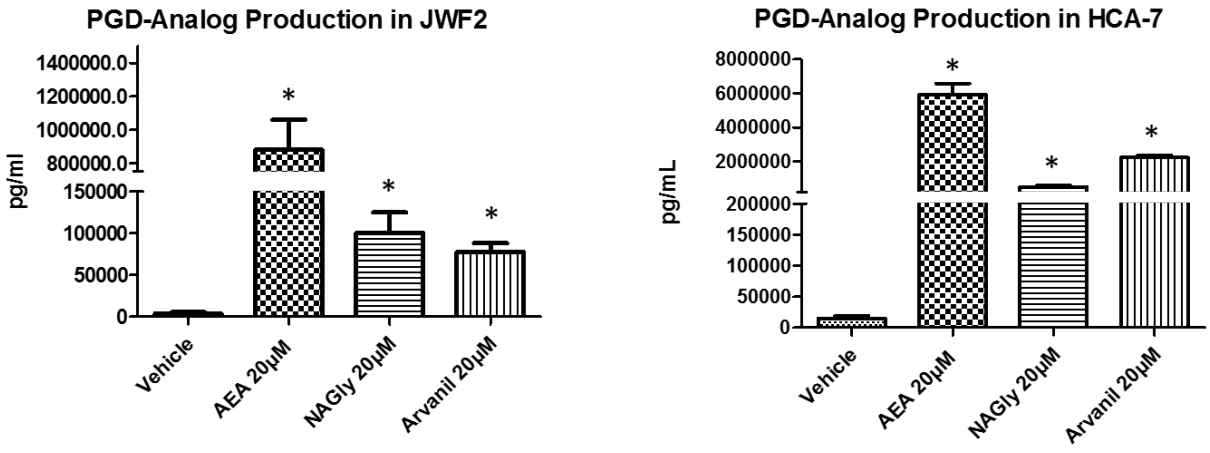



**Figure 4.3: AEA, NAGly, and Arvanil Produce D-series Prostamides and Prostaglandin analogs**
JWF2 NMSC cells and HCA-7 colorectal cancer cells were treated with 20 μM of AEA, arvanil, and NAGly for 8 hours. $PGD_2$, $PMD_2$, and $PMD_2$ analogs accumulated in the media were analyzed via ELISA analysis conducted via manufacturer's instructions to measure D-series prostaglandins, prostamides, and prostaglandin analogs. Data are represented by mean ± SEM and were analyzed using one-way ANOVA followed by Tukey's analysis ($P < 0.05$ = *) when compared to vehicle treated cells.

### 4.5: Metabolism by COX-2 and PGDS is Required for Arvanil-induced Apoptotic Activity

We previously demonstrated AEA-induced apoptotic cell death is initiated via COX-2 metabolism to D-series prostamides and ultimately, 15d-$PMJ_2$ (Kuc et al. 2012, Soliman et al. 2016a). Caspase-3 and caspase-7 are known to be universally activated during apoptosis no matter the specific apoptotic cell death pathway (Luthi and Martin 2007, Timmer and Salvesen 2007). Using this information, we were able to use caspase-3/7 activity as a distinct marker for COX-2 dependent apoptotic activity.

In Figure 4.3, we demonstrated that arvanil and NAGly are metabolized by COX-2 and PGDS, producing D-series prostaglandin analogs. In this experiment, we sought to determine if these D-series prostaglandin analogs were dehydrated to 15d-$PMJ_2$-analogs which would suggest that these metabolites were responsible for the anti-cancer activity of arvanil and NAGly. To accomplish this, we used $SeCl_4$, a PGDS inhibitor, to eliminate the production of D-series prostaglandin analogs and determine how this affected the anti-cancer activity of arvanil and NAGly. Using AEA as a positive control, we were able to demonstrate that arvanil's apoptotic anti-cancer activity is dependent upon the activity of PGDS (Figure 4.4). These data suggest that arvanil is metabolized by COX-2 and PGDS to D-series prostaglandin analogs that are spontaneously dehydrated to J-series prostaglandin analogs. Hence these molecules may mediate the cytotoxicity of arvanil. Mass spectrometry experiments, which are described in Figure 4.5, can confirm if these novel J-series prostaglandin analogs are actually present.

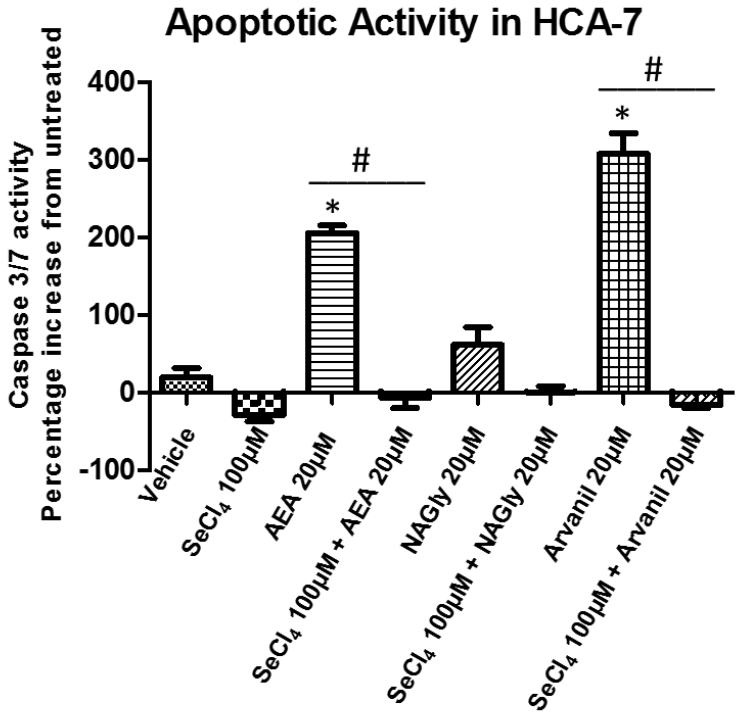



**Figure 4.4: Metabolism by COX-2 and PGDS is Required for AEA and Arvanil-induced Apoptotic Activity**
HCA-7 colorectal cancer cells were treated with 100 µM of $SeCl_4$, 20 µM of AEA, arvanil, NAGly and a combination of 100 µM $SeCl_4$ and the three drugs. Caspase 3/7 activity was analyzed via Caspase Glo according to manufacturer's instructions. Data represents mean ± SEM of three independent experiments and are expressed as percent viability compared to untreated cells. Data were analyzed using one-way ANOVA followed by Tukey's analysis ($P < 0.05$ = *) when compared to vehicle treated cells. # represents statistically significant difference between the samples treated with a combination of 100 µM of $SeCl_4$ and drug compared to cells treated with drug alone.

### 4.6: Mass Spectrometry Confirmation of 15d-PMJ$_2$-arvanil Analog

Throughout the studies exploring the mechanism of cell death, we determined that NAGly and arvanil follow the same metabolic pathway as AEA. Because arvanil is more potent (Figure 4.1) than both AEA and NAGly, and NAGly is not as efficacious in HCA-7 cells as AEA and arvanil (Figure 3.4), we decided to use LC-MS to investigate the presence of only 15d-PMJ$_2$-arvanil given the time- and resource-consuming nature of the experiments.

LC-MS was conducted with lipid-extracted cell supernatant and we identified two peaks that contained m/z 450.278, the target m/z predicted for 15dPMJ$_2$-arvanil. As a result of in-source fragmentation, these peaks also contained m/z 270.965 and m/z 243.165, the m/z associated with common fragment ions associated with 15d-PMJ$_2$, which are the same fragments that would be associated with 15d-PMJ$_2$-arvanil. It was interesting to identify two chromatographic peaks associated with the m/z of 15d-PMJ$_2$-arvanil. We theorize that a second peak could be associated with the COX-2/PGES metabolized 15d-PMA$_2$-arvanil. These two molecules would produce identical m/z, identical m/z fragments, but could differ slightly in retention time due to slightly differing polarities. In prior studies, 15d-PMA$_2$ was not identified and therefore its anti-cancer activity has not been characterized.

These results confirmed the identification of a novel J-series prostamide, 15d-PMJ$_2$-arvanil, the end product of the COX-2, PGDS, and dehydration pathway. In collaboration with the production of prostamide-D$_2$ in arvanil-treated media established in figure 4.3 and the apoptotic activity data for arvanil established in Figure 4.4, we can suggest that arvanil's anti-cancer activity is due to the production of this novel J-series prostamide-analog. These data provide a novel molecule to synthesize and carry out a cytotoxic profile study to determine its anti-cancer activity and mechanism of cell death.

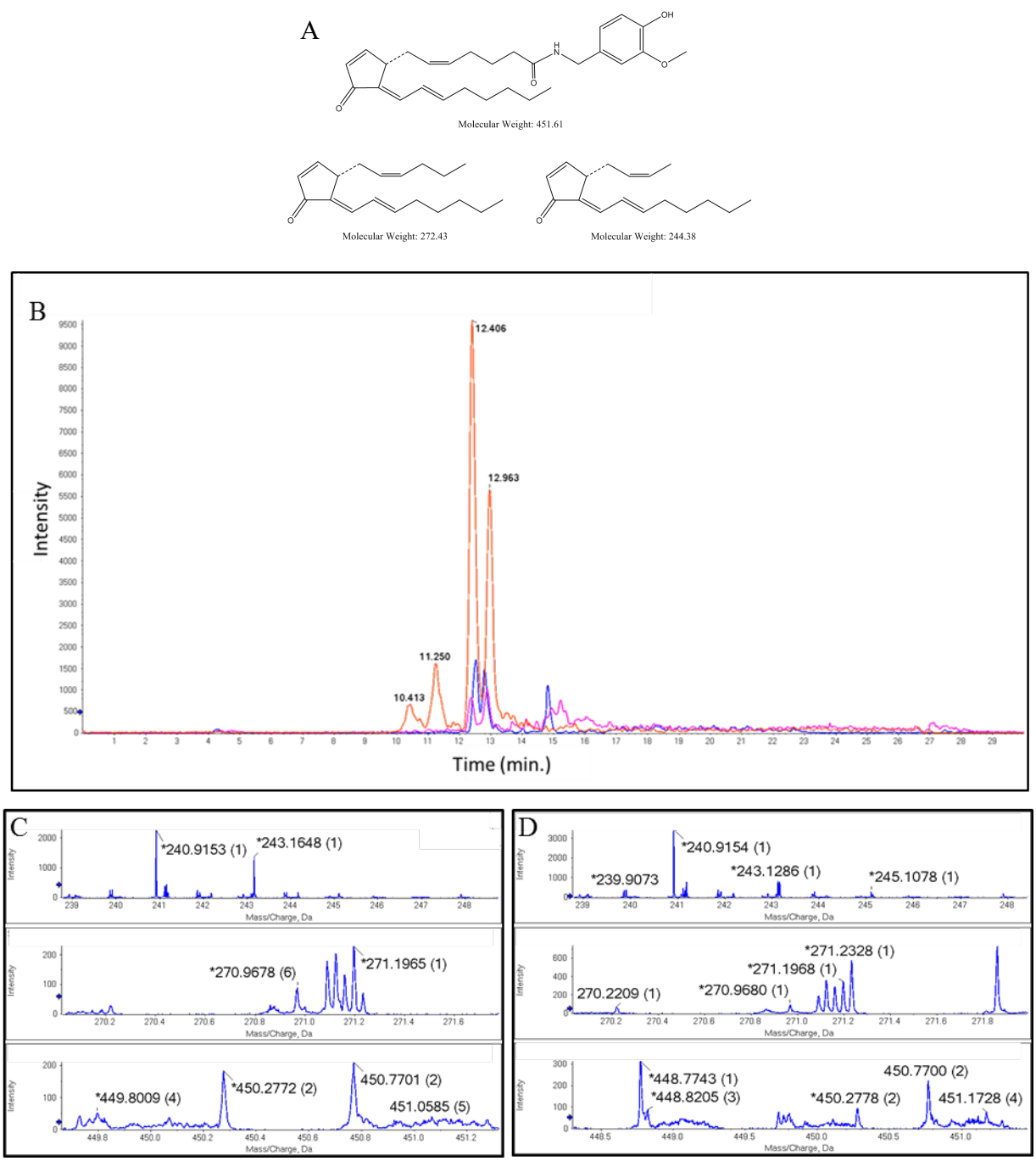



**Figure 4.5: Mass Spectrometry Confirmation of 15d-PMJ$_2$-arvanil Analog**

**A.** 15d-PMJ$_2$-arvanil and common prostaglandin fragments. **B,C,D.** LC-MS analysis was conducted on arvanil-treated HCA-7 colorectal cancer cell media to determine the production of 15d-PMJ$_2$-arvanil. Media was removed after 8 hours of treatment and a solid phase lipid extraction was conducted. LC-MS analysis was run on a SCIEX 425 Eskigent/5600+ Triple TOF mass spectrometer analyzed using negative ionization mode. Reverse phase LC was conducted using a Luna $NH_2$ column with a gradient of A: water with 0.1% formic acid and B: Acetonitrile with 0.1% formic acid

### 4.7: Conclusions of Cytotoxic and COX-2 Metabolic Profiles of Arvanil and NAGly

In this part of the study, the goal was to characterize the cytotoxic mechanism of arvanil and NAGly by determining the role of COX-2, PGDS, $CB_1$ receptor, TRPV1 receptor, and the production of novel prostaglandin analogs in its activity. In previous studies by our group, it was demonstrated that AEA's anti-cancer activity in COX-2 overexpressing JWF2 was attributed to its metabolism by COX-2 and PGDS to 15d-$PMJ_2$ (Van Dross 2009, Kuc et al. 2012). Using these same study guidelines, we conducted a study to investigate the mechanism of arvanil and NAGly's cytotoxicity with a particular focus on its dependence of COX-2 and PGDS.

To compare the activity of AEA with arvanil and NAGly, a concentration-response experiment was conducted. The results demonstrated that arvanil's was more potent ($LC_{50}$ = 6.03 μM) than both AEA ($LC_{50}$ = 9.39 μM) and NAGly ($LC_{50}$ = 9.54 μM) (Figure 4.1). Next, we determined if the activity of arvanil and NAGly were dependent on cannabinoid receptors. The data suggested that the anti-cancer activity of both arvanil and NAGly were independent of the $CB_1$ and TRPV1 receptors in JWF2 and HCA-7 cells (Figure 4.2). This indicates that these receptors have no role in their anti-cancer activity.

To determine the role of COX-2 and PGDS metabolism, studies were completed to examine the production of $PMD_2$ and J-series prostaglandin analogs. The results demonstrated both, arvanil and NAGly, are metabolized by PGDS to $PMD_2$-arvanil and $PMD_2$-Gly (Figure 4.3). It has been demonstrated that AEA is metabolized by COX-2 and PGDS to $PMD_2$ and spontaneously dehydrated to 15d-$PMJ_2$, which is mainly responsible for its anti-cancer activity. In addition, both arvanil and NAGly J-series prostaglandin-analogs were produced. Furthermore, these results demonstrated that blockade of PGDS, which prevents production of $PMD_2$-arvanil,

$PMJ_2$-arvanil, $PMD_2$-Gly, and $PMJ_2$-Gly, inhibits the apoptosis mediated by arvanil and NAGly (Figure 4.4).

Since arvanil demonstrated it is the most cytotoxic hit throughout the SAR study, it is metabolized by COX-2 and PGDS, and its apoptotic anti-cancer activity was COX-2 and PGDS metabolism-dependent, we identified it has the greatest potential to produce novel J-series prostamide-analogs. To investigate the production of these novel J-series prostamide-analogs, we conducted LC-MS analysis with arvanil-treated media. The results of this analysis demonstrated the production of the novel J-series prostamide-analog, 15d-$PMJ_2$-arvanil

# CHAPTER FIVE: REVIEW OF MAJOR FINDINGS AND DISCUSSION

Ten structurally modified AEA analogs were designed with the intent to decipher how each structural modification affected cytotoxicity. We identified four parameters of our model system that could affect the anti-cancer activity of AEA analogs, (1) inherent cytotoxicity of J-series prostaglandin analogs, (2) COX-2 metabolism, (3) PGDS metabolism, and (4) FAAH degradation resistance. Modifications made to the molecule to explore these parameters were part of one of the following groups; branching along the carbon chain, alteration of the hydrogen-bonding ability at the chain terminus, and addition of an aryl ring (Table 3.1). Results and analysis from the SAR revealed the inherent cytotoxicity of the J-series prostaglandin analogs has the biggest role in the anti-cancer activity of AEA analogs (Table 3.2). Two hits, arvanil (Figure 3.5) and NAGly (Figure 3.3), were carried on to more in-depth analysis of their COX-2/PGDS metabolism and the role it has in their respective anti-cancer activity as well as the identification of novel 15d-$PMJ_2$-arvanil by COX-2 metabolism of arvanil, the greatest cytotoxic AEA analog.

The first parameter tested for its importance to cytotoxic activity was the hydrolase activity of FAAH, specifically the reduction in cytotoxicity of AEA due to rapid degradation by FAAH. It has been previously reported that blockade of FAAH's hydrolase activity increases cytotoxicity of AEA (Kuc et al. 2012). R1-methanandamide has demonstrated activity *in vivo* for long periods of time lending to its metabolic stability (Romero et al. 1996). To test the role of FAAH in our model system the FAAH-resistant AEA analog, R1-methanandamide, was the first AEA analog investigated for its cytotoxicity compared to AEA. R1-methanandamide demonstrates a slight increase at 10 μM concentrations in JWF2 compared to AEA, but we don't see increased cytotoxicity in HCA-7 (Figure 3.3). R1-methanandamide adds steric bulk at the α-carbon of the ethanolamine, which is adjacent to the hydrolyzed amide bond, and this dramatically reduces the

hydrolase activity of FAAH, so we believed that adding any steric bulk at this carbon or even to the amide bond itself with the AEA analogs ADA and AS would also block FAAH's hydrolase activity (Abadji et al. 1994). Without specifically investigating the FAAH metabolism of ADA and AS, we relied on the cytotoxicity results to suggest if they were resisting degradation. Since these two molecules weren't cytotoxic, we couldn't decipher if they resist FAAH degradation or if their COX-2 metabolized J-series prostaglandin analogs aren't as cytotoxic. It has been reported amides of propanolamine's three-carbon chain or higher were hydrolyzed by FAAH at reduced rates compared to ethanolamine (Schmid et al. 1985). We assumed any AEA analog with three carbons or more between the amide bond and the terminal end moiety, A-Pro, arvanil, AM404, arvanil D1, arvanil D2 would resist hydrolase activity of FAAH or dramatically lower the rate of degradation. Through all these modifications to resist FAAH degradation, we believed we could decipher if FAAH was playing the most important role in our model system. Of the molecules believed to resist FAAH's hydrolase activity, R1-methanandamide (Figure 3.3), A-Pro (Figure 3.7), ADA (Figure 3.6), AS (Figure 3.8), AM404 (Figure 3.10), and arvanil D2 (Figure 3.12) have no anti-cancer activity or reduced cytotoxicity compared to that of AEA, but arvanil (Figure 3.5) and arvanil D1 (Figure 3.11) have increased cytotoxicity. These results suggested FAAH, specifically resistance to FAAH's hydrolase activity, doesn't have the most important role in the anti-cancer activity of AEA analogs.

Another focus of our original hypotheses was the metabolic activity of COX-2, including the rate of metabolism of AEA analogs. All AEA analogs, except for AM404, were designed or known to retain the COX-2 metabolism of AEA, by not making modification on the 20-carbon chain backbone of AEA, but their rates of metabolism differed (Prusakiewicz et al. 2007, Dietz et al. 1988, Hamberg and Samuelsson 1967, Hogestatt et al. 2005). We predicted molecules that have

a higher rate of COX-2 metabolism, and in turn produce a higher concentration of J-series prostaglandin analogs, would have increased cytotoxicity compared to that of AEA. This theory was disproved by a comparison of NAGly and arvanil. NAGly has a COX-2 rate of metabolism 40% that of AA and arvanil exhibits the lowest rate of COX-2 metabolism, 5.6% that of AA, yet arvanil demonstrated the greatest cytotoxicity (Figure 3.5) due to its COX-2/PGDS metabolism (Figure 4.4) and subsequent production of D-series prostaglandin analogs (Figure 4.3) and 15d-$PMJ_2$-arvanil (Figure 4.5) (Prusakiewicz et al. 2007). Further investigation of the correlation between COX-2 rates of metabolism and anti-cancer activity should have produced a relationship based on production of prostamide-$D_2$ and prostamide-$D_2$ analogs. NAGly has the highest COX-2 metabolism rate of any AEA analog but didn't lead to a dramatically greater concentration of $PGD_2$ analogs compared to AEA, which has a COX-2 metabolism rate 27% that of AA, or arvanil, which has a COX-2 metabolism rate 5.6% that of AA (Prusakiewicz et al. 2007, Kozak et al. 2002) (Figure 4.3). These results indicate there is no relationship between rate of COX-2 metabolism and cytotoxicity of AEA analogs and continues to demonstrate the inherent cytotoxicity of the J-series prostaglandin analogs play the most important role in the overall anti-cancer activity.

Previously, it has been reported that arvanil demonstrates anti-invasive activity towards small cell lung cancers mediated by the AMPK pathway and induces apoptosis in Jurkat cells through an FADD/caspase-8-dependent pathways (Hurley et al. 2016, Sancho et al. 2003). In our studies, we suggest arvanil's anti-cancer activity is attributed to its COX-2/PGDS metabolism and subsequent dehydrations to 15d-$PMJ_2$-arvanil. The requirement of this activity is the COX-2 overexpression in our model cell lines, but in future studies 15d-$PMJ_2$-arvanil will be synthesized and investigated for its COX-2 independent cytotoxicity in a variety of cancers.

The cytotoxicity results from the SAR study (Table 3.2), in comparison with the $PGD_2$ production (Figure 4.3), lead us to believe this system is more complex than we originally thought. When developing a pro-drug all receptors and enzymes that have interactions with the molecule can play a role in its intended activity. As shown in Scheme 1.1, after AA, AEA, and other AEA analogs are metabolized by COX-2 there are multiple prostaglandin synthases that can metabolize $PGH_2$, $PMH_2$, and $PMH_2$ analogs to different series of prostaglandins, prostamides, and prostaglandin analogs. We have demonstrated previously and in this study the COX-2/PGDS metabolism is responsible for the anti-cancer activity of AEA and AEA analogs (Soliman et al. 2016a). On the other hand, COX-2/PGES metabolism of AA to E-series prostaglandins has been shown to be responsible for tumor growth in skin cancer and colon cancer (Ansari et al. 2008, Pai et al. 2002). Metabolism preferences by PGES and PGDS compared to AEA were not explored. If these differences lead to a higher concentration of $PME_2$ analogs compared to AEA, then not only could this be responsible for less than expected concentrations of $PMD_2$, but it could be responsible for the decreased apoptotic activity and cytotoxicity in HCA-7 cells demonstrated by NAGly for example. Without knowing the concentrations of $PME_2$-NAGly compared to $PMD_2$-NAGly we can't suggest bias for specific prostaglandin synthases is responsible for the reduced production of D-series prostaglandin analogs, but future studies can investigate this theory.

We set forth to improve on the chemotherapeutic activity of the endocannabinoid AEA and its COX-2/PGDS metabolized 15d-$PMJ_2$. To accomplish this a SAR study and follow up studies to identify cytotoxic and COX-2 metabolic profiles of the two most promising hits were conducted. With the results from two generations of a SAR study, we were able to identify two hits with increased or similar cytotoxicity in COX-2 overexpressing cancer cells, NAGly and arvanil.

Through investigation of its COX-2 metabolic profile and its apoptotic anti-cancer activity, NAGly didn't demonstrate potential as an improved chemotherapeutic.

$PGA_2$ has shown anti-proliferation effects in esophageal cancer cells and an increase in total tyrosine kinase activity (Joubert et al. 1999). In RKO rectal carcinoma cells, $PGA_2$ induces cell death correlated with a lack of cyclin-dependent kinase inhibitor p21 (Gorospe and Holbrook 1996). In Figure 4.5, we identified two distinct peaks correlated with the target m/z of 15d-$PMJ_2$-arvanil and associated fragments. This phenomenon hasn't occurred in our previous studies identifying 15d-$PMJ_2$, leading us to believe these peaks are associated with two different molecules, 15d-$PMA_2$-arvanil and 15d-$PMJ_2$-arvanil. We theorize both of these molecules are present in arvanil-treated media and the production of 15d-PMA2-arvanil could be playing a role in the increased cytotoxicity of arvanil, although Figure 4.4 suggested 15d-$PMJ_2$-arvanil is responsible for the entirety of the apoptotic cell death associated with arvanil. Future studies to confirm the production of 15d-$PMA_2$-arvanil and its role in cytotoxicity will be pursued.

We were the first to identify arvanil's J-series prostamide, 15d-$PMJ_2$-arvanil, and suggest its contribution to its apoptotic anti-cancer activity. To further demonstrate 15d-$PMJ_2$-arvanil's anti-cancer activity, it will be synthesized using the synthetic techniques set forth previously by our group (Ladin et al. 2017). Once synthesized, 15d-$PMJ_2$-arvanil's cytotoxic profile will be investigated to determine its potential as a novel chemotherapeutic.

# FUTURE DIRECTIONS

In this study we provided evidence of a novel COX-2 metabolized J-series prostaglandin analog, 15d-$PMJ_2$-arvanil, identified via LC-MS. This metabolite was a product of the COX-2/PGDS metabolism of arvanil, which demonstrated increased COX-2 dependent apoptotic anti-cancer activity compared to that of AEA. In previous studies, our group has characterized the anti-cancer activity of 15d-$PMJ_2$, the novel J-series prostamide metabolite of AEA, and demonstrated its powerful selective toxicity in a variety of cancer cell lines as well as *in vivo*. These studies have laid out an experimental roadmap for future studies of novel J-series prostamide chemotherapeutics, such as 15d-$PMJ_2$-arvanil. Using an identical synthetic route to produce various AEA analogs in this study as well as 15d-$PMJ_2$ in previous studies by Ladin et al., we will synthesize and characterize 15d-$PMJ_2$-arvanil (Figure 5.1) (Ladin et al. 2017). Once synthesized, 15d-$PMJ_2$-arvanil's selective chemotherapeutic potential will be investigated via cytotoxicity experiments in various cancer cell lines including NMSC, colorectal cancer, melanoma, and corresponding nontumorigenic cell lines. If its selective toxicity is established and its chemotherapeutic potential is similar or greater than 15d-$PMJ_2$, we will perform studies to investigate its mechanism of cell death, including its induction of apoptosis and whether it is inducing ER-stress mediated cell death which is the cell death pathway identified for 15d-$PMJ_2$.

As demonstrated in Figure 4.5, we identified a potential second metabolite with identical m/z and fragmentation pattern as 15d-$PMJ_2$-arvanil. We hypothesize this peak and its associated ion fragments are produced by the A-series metabolite, 15d-$PMA_2$-arvanil. To test this hypothesis, we will conduct a PGDS inhibition experiment identical to the Figure 4.4 and perform LC-MS on the lipid extracted media. If we observe a disappearance of both peaks it would disprove our theory of A-series metabolite production, and if we observe a disappearance

of only one peak it would suggest our theory is correct. If the latter comes to fruition, we would then synthesize this A-series metabolite, 15d-PMA$_2$-arvanil and establish if it is cytotoxic on its own and thus contributing to the increased cytotoxic power of arvanil compared to that of AEA.

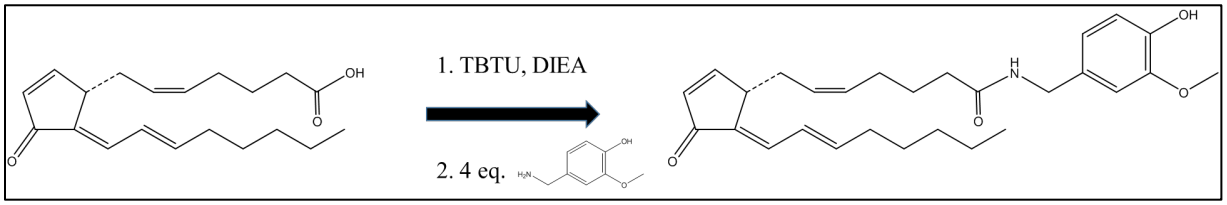
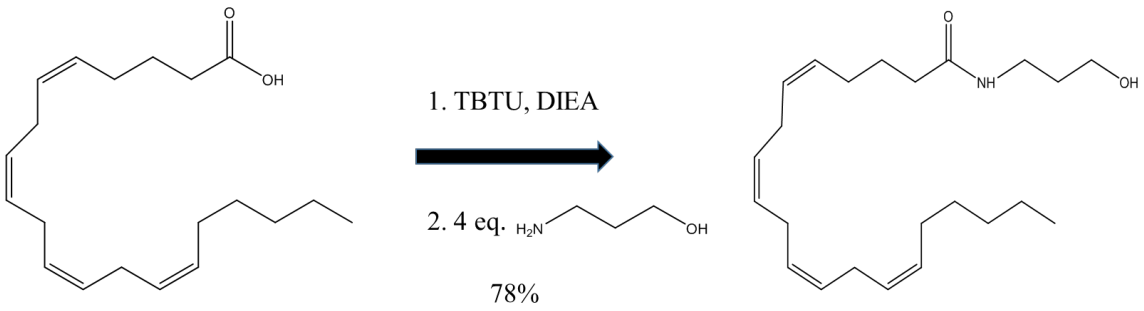
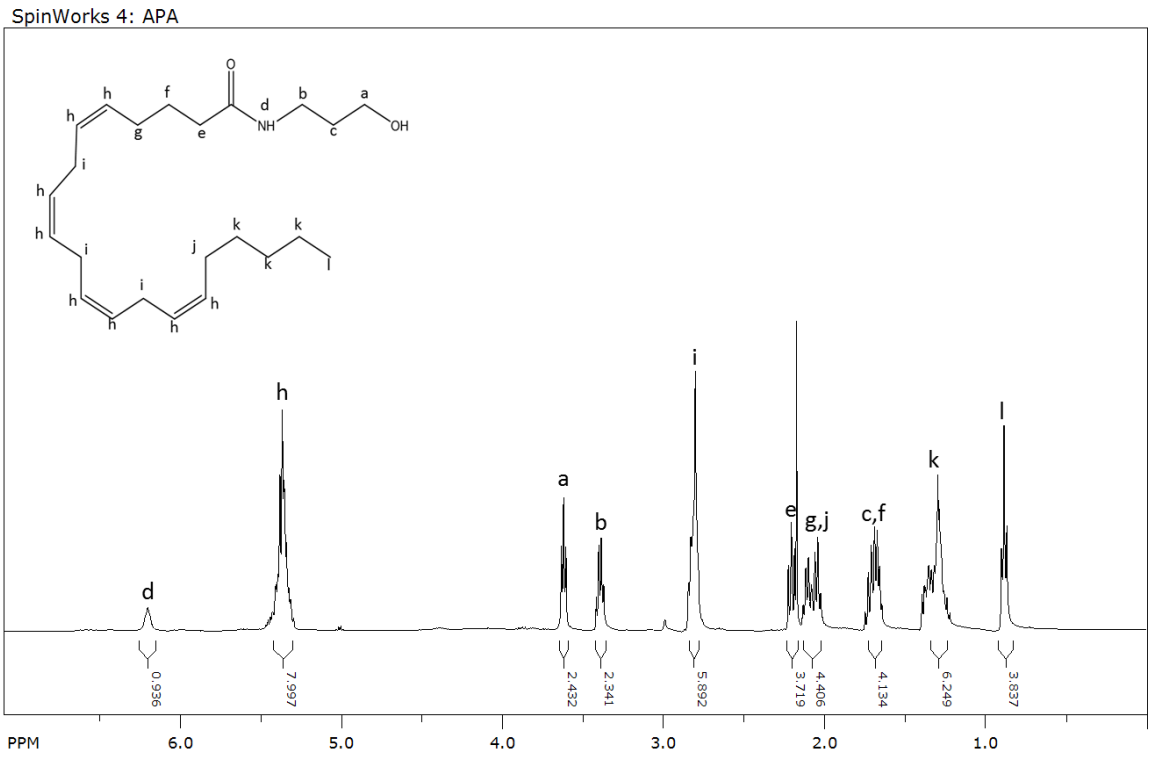



**Figure 5.1: Proposed Synthesis of 15d-PMJ2-arvanil from 15d-PGJ2**

# SUPPLEMENTARY FIGURES A: SYNTHESIS AND CHARACTERIZATION OF AEA ANALOGS

**Figure A.1: Synthesis and Characterization of Arachidonoyl Propanolamine (A-Pro)**

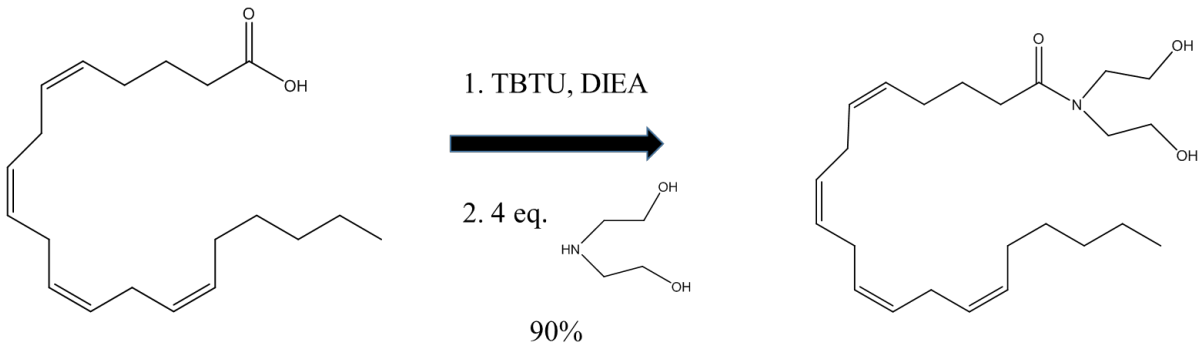


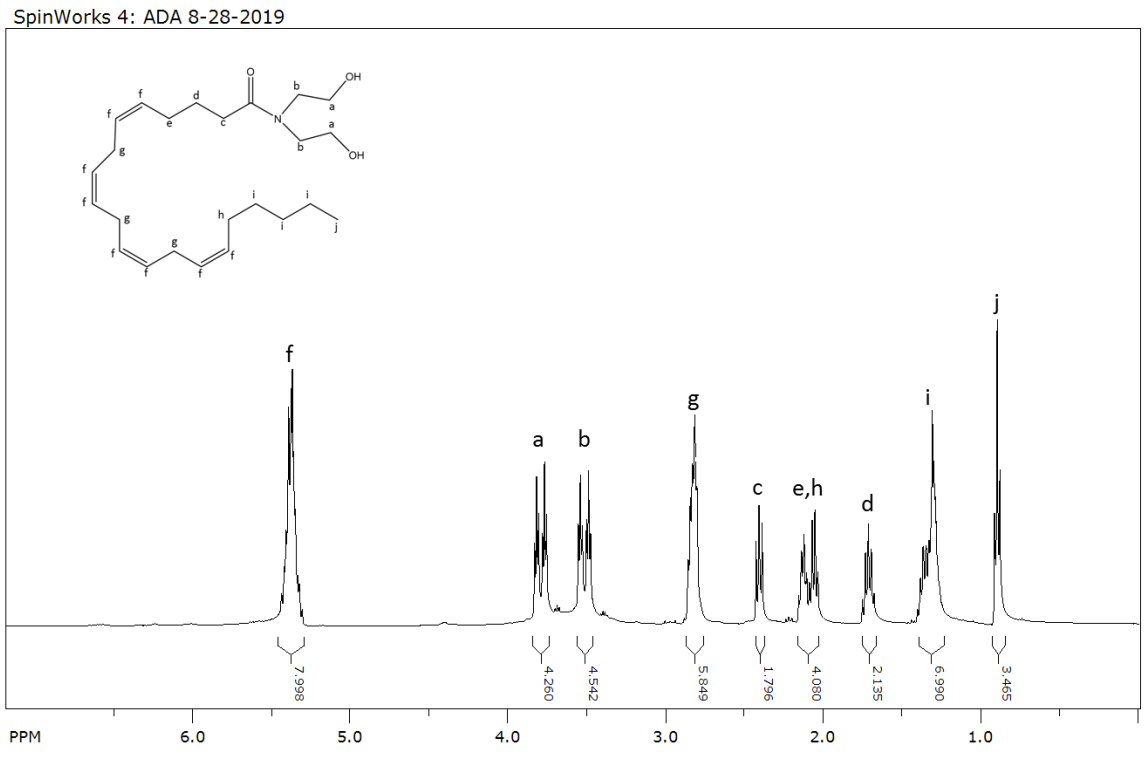




file: ...:\Arachidonoyl Propanolamine\1\fid expt: <zg30>
transmitter freq.: 400.132471 MHz
time domain size: 65536 points
width: 8012.82 Hz = 20.0254 ppm = 0.122266 Hz/pt
number of scans: 16

freq. of 0 ppm: 400.130000 MHz
processed size: 65536 complex points
LB: 0.300 GF: 0.0000

**Figure A.2: Synthesis and Characterization of Arachidonoyl Diethanolamine (ADA)**

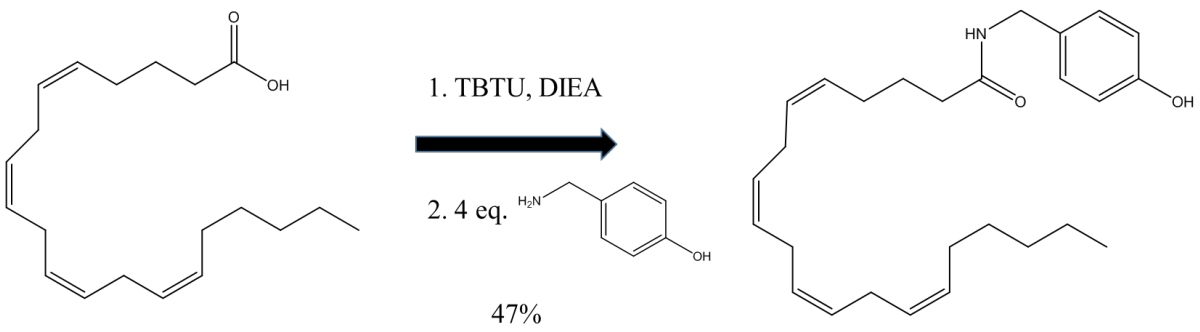



file: J:\ABM ADA 8-28-2019\1\fid expt: <zg30>
transmitter freq.: 400.132471 MHz
time domain size: 65536 points
width: 8012.82 Hz = 20.0254 ppm = 0.122266 Hz/pt
number of scans: 16

freq. of 0 ppm: 400.130000 MHz
processed size: 65536 complex points
LB: 0.300 GF: 0.0000

**Figure A.3: Synthesis and Characterization of Arvanil D1**



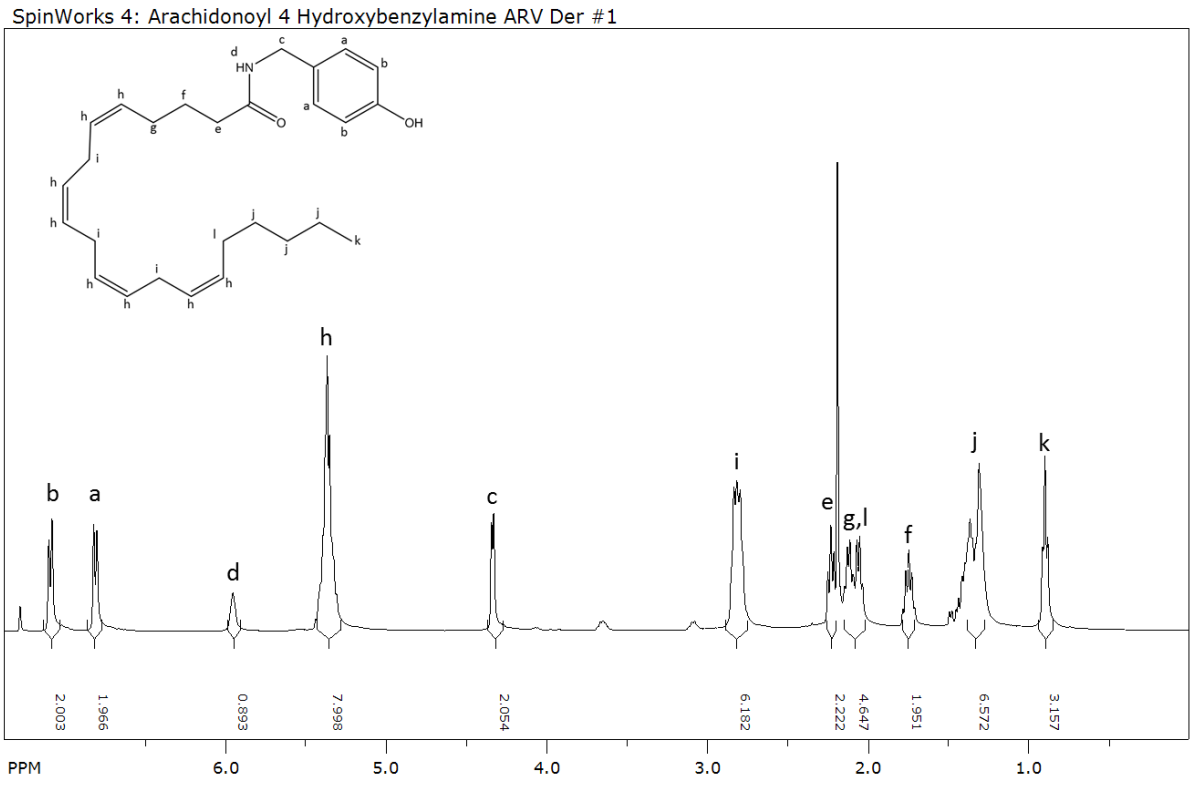
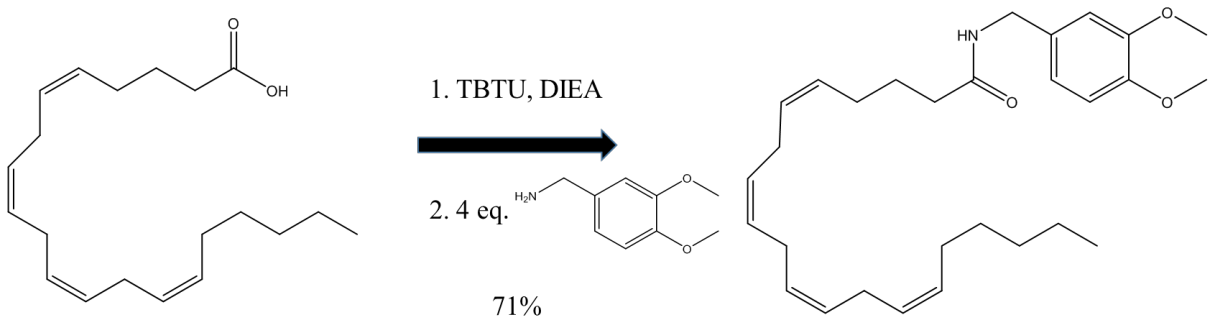
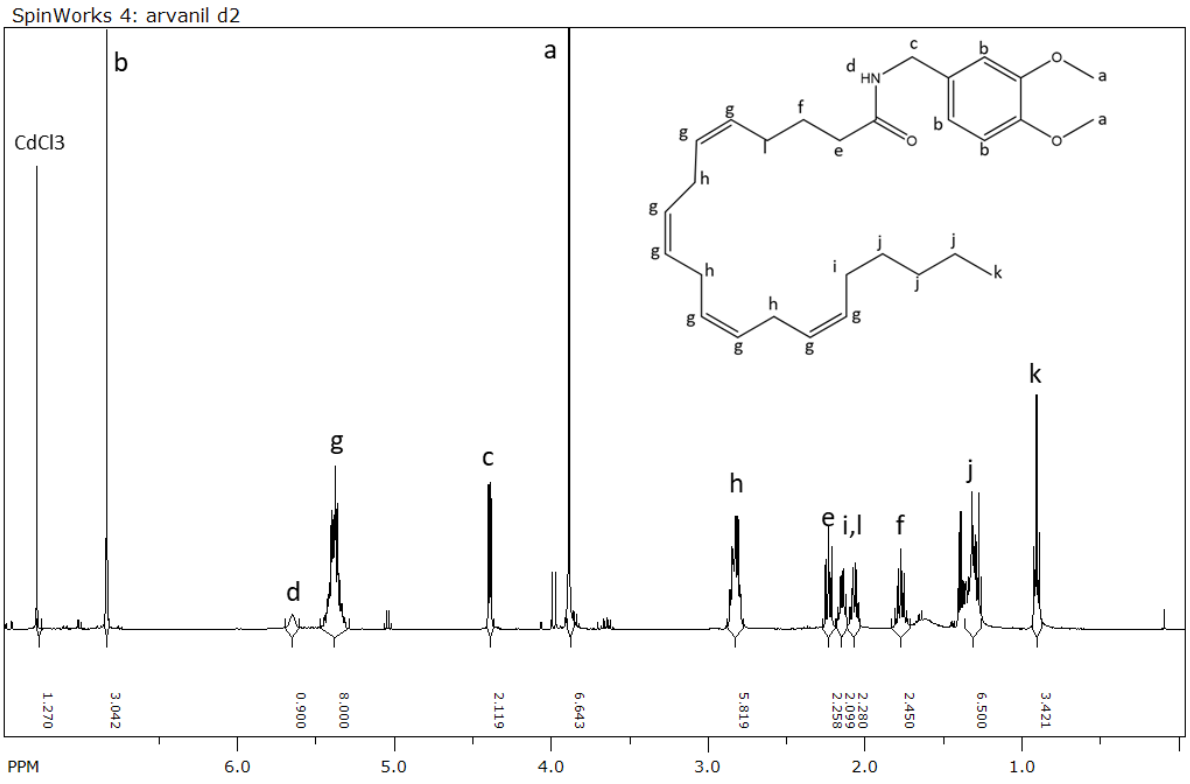



**Figure A.4: Synthesis and Characterization of Arvanil D2**




file: J:\arvanil d2 abm2\1\fid expt: <zg30>
transmitter freq.: 400.132471 MHz
time domain size: 65536 points
width: 8012.82 Hz = 20.0254 ppm = 0.122266 Hz/pt
number of scans: 16

freq. of 0 ppm: 400.130000 MHz
processed size: 65536 complex points
LB: 0.300 GF: 0.0000

# REFERENCES


Abadji V, Lin S, Taha G, Griffin G, Stevenson LA, Pertwee RG, Makriyannis A. 1994. (R)-Methanandamide: A Chiral Novel Anandamide Possessing Higher Potency and Metabolic Stability. J Med Chem. 37:1889-1893.

Alpini G, DeMorrow S. 2009. Changes in the endocannabinoid system may give insight into new and effective treatments for cancer. Vitam Horm. 81:469-485.

Ansari KM, Rundhaug JE, Fischer SM. 2008. Multiple signaling pathways are responsible for prostaglandin E2-induced murine keratinocyte proliferation. Mol Cancer Res. 6(6):1003-1016.

Armstrong BK, Kricker A. 2001. The epidemiology of UV induced skin cancer. J Photochem Photobiol. B. 63(1-3):8-18.

American Cancer Society Facts and Figures. http://www.cancer.org/research/cancerfactsstatistics/index (accessed September 2, 2016).

Bifulco M, Laezza C, Valenti M, Ligresti A, Portella G, Di Marzo V. 2004. A new strategy to block tumor growth by inhibiting endocannabinoid inactivation. FASEB J. 18(13):1606-1608.

Bisogno T, Maurelli S, Melck D, De Petrocellis L, Di Marzo V. 1997. Biosynthesis, uptake, and degradation of anandamide and palmitoylethanolamide in leukocytes. J Biol Chem. 272(6):3315-3323.

Bisogno T, De Petrocellis L, Di Marzo V. 2002. Fatty acid amide hydrolase, an enzyme with many bioactive substrates. Possible therapeutic implications. Curr Pharm Des. 8(7):533-547.

Bisogno T, Ligresti A, Di Marzo V. 2005. The endocannabinoid signaling system: biochemical aspects. Pharmacol Biochem Behav. 81(2):224-238.

Boukamp P. 2005. Non-melanoma skin cancer: what drives tumor development and progression. Carcinogenesis. 26(10):1657-1667.

Bradshaw HB, Rimmerman N, Hu SS, Benton VM, Stuart JM, Masuda K, Cravatt BF, O'Dell DK, Walker JM. 2009. The endocannabinoid anandamide is a precursor for the signaling lipid N-arachidonoyl glycine by two distinct pathways. 10:14.

Brown I, Cascio MG, Wahle KW, Smoum R, Mechoulam R, Ross RA, Pertwee RG, Heys SD. 2010. Cannabinoid receptor-dependent and –independent anti-proliferative effects of omega-3 ethanolamides in androgen receptor-positive and –negative prostate cancer cell lines: Carcinogenesis. 31(9):1584-1591.

Buckman SY, Gresham A, Hale P, Hruza G, Anast J, Masferrer J, Pentland AP. 1998. COX-2 expression is induced by UVB exposure in human skin: implications for the development of skin cancer. Carcinogenesis. 19(5):723-729.

Burstein SH, Huang SM, Petro TJ, Rossetti RG, Walker JM, Zurier RB. 2002. Regulation of anandamide tissue levels by N-arachidonoylglycine. Biochem Pharmacol. 64(7):1147-1150.

Clay CE, Namen AM, Atsumi G, Willingham MC, High KP, Kute TE, Trimboli AJ, Fonteh AN, Dawson PA, Chilton FH. 1999. Influence of J series prostaglandins on apoptosis and tumorigenesis of breast cancer cells. Carcinogenesis. 20(10):1905-1911.

Contassot E, Wilmotte R, Tenan M, Belkouch MC, Schnuriger V, de Tribolet N, Burkhardt K, Dietrich PY. 2004. Arachidonoylethanolamide induces apoptosis of human glioma cells through vanilloid receptor-1. J Neuropathol Exp Neurol. 63(9):956-963.

Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB. 1996. Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature. 384(6604)83-87.

De Lago E, Gustafsson SB, Fernandez-Ruiz J, Nilsson J, Jacobsson SO, Fowler CJ. 2006. Acyl based anandamide uptake inhibitors cause rapid toxicity to C6 glioma cells at pharmacologically relevant concentrations. J Neurochem. 99(2):677-688.

De Petrocellis L, Melck D, Palmisano A, Bisogno T, Laezza C, Bifulco M, Di Marzo V. 1998. The endogenous cannabinoid anandamide inhibits human breast cancer cell proliferation. Proc Natl Acad Sci USA. 95(14):8375-8380.

Deutsch DG, Chin SA. 1993. Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. Biochem Pharmacol. 46(5):791-796.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R. 1992. Isolation and structure of a brain constituent that bind to the cannabinoid receptor. Science. 258(5090):1946-1949.

Diffey BL, Langtry JA. 2005. Skin cancer incidence and the ageing population. BR J Dermatol. 153(3):679-680.

Dietz R, Nastainczyk W, Ruf HH. 1988. Higher oxidation states of prostaglandin H synthase. Rapid electronic spectroscopy detected two spectral intermediates during the peroxidase reaction with prostaglandin G2. Eur J Biochem. 171(1-2):321-328.

Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz JC, Piomelli D. 1994. Formation and inactivation of endogenous cannabinoid anandamide in central neurons. Nature. 372(6507):686-691.

Di Marzo V, Bisogno T, Melck D, Ross R, Brockie H, Stevenson L, Pertwee R, De Petrocellis L. 1998. Interactions between synthetic vanilloids and the endogenous cannabinoid system. FEBS Lett. 436(3):449-454.

Di Marzo V, Melck D. De Petrocellis L, Bisogno T. 2000. Cannabimetic fatty acid derivatives in cancer and inflammation. Prostaglandins Other Lipid Mediat. 61(1-2):43-61.

Di Marzo V, Bisogno T, De Petrocellis L, Brandi I, Jefferson RG, Winckler RL, Davis JB, Dasse O, Mahadevan A, Razdan RK, Martin BR. 2001. Highly selective CB1 cannabinoid receptor ligands and novel CB(1)/VR(1) vanilloid receptor "hybrid" ligands. Biochem Biophys Res Commun. 281(2):444-451.

Di Marzo V, Bifulco M, De Petrocellis L. 2004. The endocannabinoid system and its therapeutic exploitation. Nat Rev Drug Discov. 3(9):771-784.

Di Marzo V. 2009. The endocannabinoid system: its general strategy of action, tools for its pharmacological manipulation and potential therapeutic exploitation. Pharmacol Res. 60(2):77-84.

Fischer SM, Hawk ET, Lubet RA. 2011. Coxibs and other nonsteroidal anti-inflammatory drugs in animal models of cancer chemoprevention. Cancer Prev Res (Phila). 4(11):1728-1735.

Fowler CJ, Jonsson KO, Andersson A, Juntunen J, Jarvinen T, Vandevoorde S, Lambert DM, Jerman JC, Smart D. 2003. Inhibition of C6 glioma cell proliferation by anandamide, 1-arachidonoylglycerol, and by a water soluble phosphate ester of anandamide: variability in response and involvement of arachidonic acid. Biochem Pharmacol. 66(5):757-767.

Funk CD. 2001. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294(5548):1871-1875.

Glaser ST, Abumrad NA, Fatade F, Kaczocha M, Studholme KM, Deutsch DG. 2003. Evidence against the presence of an anandamide transporter. Proc Natl Acad Sci USA. 100(7):4269-4274.

GLOBOCAN 2018. Global Cancer Observatory. https://gco.iarc.fr/ (Accessed November 21, 2019).

Gorospe M, Holbrook NJ. 1996. Role of p21 in Prostaglandin $A_2$-mediated Cellular Arrest and Death. Cancer Res. 56(3):475-479.

Grimaldi C, Pisanti S, Laezza C, Malfitano AM, Santoro A, Vitale M, Caruso MG, Notarnicola M, Iacuzzo I, Portella G, Di Marzo V, Bifulco M. 2006. Anandamide inhibits adhesion and migration of breast cancer cells. Exp Cell Res. 312(4):363-373.

Gustafsson SB, Lindgren T. Jonsson M, Jacobsson SO. 2009. Cannabinoid receptor-independent cytotoxic effects of cannabinoids in human colorectal carcinoma cells: synergism with 5-fluorouracil. Cancer Chemother Pharmacol. 63(4):691-701.

Guy GP, Ekwueme DU. 2011. Years of potential life lost and indirect costs of melanoma and non-melanoma skin cancer: a systematic review of the literature. Pharmacoeconomics. 29(10):863-874.

Hamberg M, Samuelsson B. 1967. On the Mechanism of the Biosynthesis of Prostaglandins $E_1$ and $F_{1\alpha}$. J Biol Chem. 242(20):5536-5343.

Hogestatt ED, Jonsson BA, Ermund A, Andersson DA, Bjork H, Alexander JP, Cravatt BF, Basbaum AI, Zygmunt PM. 2005. Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. J Biol Chem. 280(36):31405-31412.

Huang SM, Bisogno T, Petros TJ, Chang SY, Zavitsanos PA, Zipkin RE, Sivakumar, Coop A, Maeda DY, De Petrocellis L, Burstein S, Di Marzo V, Walker JM. 2001. Identification of a new class of molecules, the arachidonoyl amino acids, and characterization of one member that inhibits pain. J Biol Chem. 276(46):42639-42644.

Hurley JD, Akers AT, Friedman JR, Nolan NA, Brown KC, Dasgupta P. 2017. Non-pungent long chain capsaicin-analogs arvanil and olvanil display better anti-invasive activity than capsaicin in human small cell lung cancers. Cell Adh Migr. 11(1):80-97.

Joubert AM, Panzer A, Joubert F, Lottering ML, Bianchi PC, Seegers JC. 1999. Comparative study of the effects of polyunsaturated fatty acids and their metabolites on cell growth and tyrosine kinase activity in oesophageal carcinoma cells. Prostaglandins Leukot Essent Fatty Acids. 61(3):171-182.

Kiraly AJ, Soliman E, Jenkins A, Van Dross RT. 2016. Apigenin inhibits COX-2, PGE2, and EP1 and also initiates terminal differentiation in the epidermis of tumor bearing mice. Prostaglandins Leukot Essent Fatty Acids. 104:44-53.

Kozak KR, Crews BC, Ray JL, Tai HH, Morrow JK, Marnett LJ. 2001. Metabolism of prostaglandin glycerol esters and prostaglandin ethanolamide in vitro and in vivo. J Biol Chem. 276(40):36993-46998.

Kozak KR, Crews BC, Morrow JD, Wang LH, Ma YH, Weinander R, Jakobsson PJ, Marnett LJ. 2002. Metabolism of the endocannabinoids, 2-arachidonoylglycerol and anandamide, into prostaglandin, thromboxane, and prostacyclin glycerol esters and ethanolamides. J Biol Chem. 277(47):44877-44885.

Kricker A, Armstrong Bk, English DR, Heenan PJ. 1995. A dose-response curve for sun exposure and basal cell carcinoma. Int J Cancer. 60(4):482-488.

Kuc C, Jenkins A, Van Dross RT. 2012. Arachidonoyl ethanolamide (AEA)-induced apoptosis is mediated by J-series prostaglandins and is enhanced by fatty acid amide hydrolase (FAAH) blockade. Mol Carcinog. 51(2):139-149.

Ladin DA, Soliman E, Escobedo R, Fitzgerald TL, Yang LV, Burns C, Van Dross RT. 2017. Synthesis and Evaluation of the Novel Prostamide, 15-Deoxy, Δ12,14-Prostamide J2, as a Selective Antitumor Therapeutic. Mol Cancer Ther. 16(5):838-849.

Linsalata M, Notarnicola M, Tutino V, Bifulco M, Santoro A, Laezza C, Messa C, Orlando A, Caruso MG. Effects of anandamide on polyamine levels and cell growth in human colon cancer cells. Anticancer Res 30(7):2583-2589.

Luthi AU, Martin SJ. 2007. The CASBAH: a searchable database of caspase substrates. Cell Death Differ. 14(4):641-650.

Mallet C, Daulhac L, Bonnefont J, Ledent C, Etienne M, Chapuy E, Libert F, Eschalier A. 2008. Endocannabinoid and serotonergic systems are needed for acetaminophen-induced analgesia. Pain. 139(1):190-200.

Matias I, Chen J, De Petrocellis L, Bisogno T, Ligresti A, Fezza F, Krauss AH, Shi L, Protzman CE, Li C, Liang Y, Nieves AL, Kedzie KM, Burk RM, Di Marzo V, Woodward DF. 2004. Prostaglandin ethanolamides (prostamides): in vitro pharmacology and metabolism. J Pharmacol Exp Ther. 309(2):745-747.

Melck D, Rueda D, Galve-Roperh I, De Petrocellis L, Guzman M, Di Marzo V. 1999a. Involvement of the cAMP/protein kinase A pathway and of mitogen-activated protein kinase in the antiproliferative effects of anandamide in human breast cancer cells. FEBS Lett. 463(3):235-240.

Melck D, Bisogno T, De Petrocellis L, Chuang H, Julius D, Bifulco M, Di Marzo V. 1999b. Unsaturated long-chain N-acyl-vanillyl-amides (N-AVAMs): vanilloid receptor ligands that inhibit anandamide-facilitated transport and bind to CB1 cannabinoid receptors. Biochem Biophys Res Commun. 262(1):275-284.

Melck D, De Petrocellis L, Orlando P, Bisogno T, Laezza C, Bifulco M, Di Marzo V. 2000. Suppression of nerve growth factor Trk receptors and prolactin receptors by endocannabinoids leads to inhibition of human breast and prostate cancer cell proliferation. Endocrinology. 141(1):118-126.

Metterle L, Nelson C, Patel N. 2016. Intralesional 5-fluorouracil (FU) as a treatment for nonmelanome skin cancer (NMSC): A review. 74(3):552-557.

Muramatsu S, Shiraishi S, Miyano K, Sudo Y, Toda A, Mogi M, Hara M, Yokoyama A, Kawasaki Y, Taniguchi M, Uezono Y. 2016. Metabolism of AM404 From Acetaminiophen at Human Therapeutic Dosages in the Rat Brain. 6(1):e32873.

NIH: National Cancer Institute, *What is Cancer?* https://www.cancer.gov/about-cancer/understanding/what-is-cancer (accessed November 21, 2019).

Pai R, Soreghan B, Szabo IL, Pavelka M, Baatar D, Tarnawski AS. 2002. Prostaglandin E2 transativates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. Nat Med. 8(3):289-293.

Patsos HA, Greehough A, Hicks DJ, Al Kharusi M, Collard TJ, Lane JD, Paraskeva C, Williams AC. 2010. The endogenous cannabinoid, anandamide, induces COX-2-dependent cell death in apoptosis-resistant colon cancer cells. Int J Oncol. 37(1):187-193.

Prusakiewicz JJ, Kingsley PJ, Kozak KR, Marnett LJ. 2002. Selective oxygenation of N-arachidonoylglycine by cyclooxygenase-2. Biochem Bioophys Res Commun. 296(3):612-617.

Prusakiewicz JJ, Turman MV, Vila A, Bell HL, Al-Mestarihi AH, Di Marzo V, Marnett LJ. 2007. Oxidative Metabolism of Lipoamino Acids and Vanilloids by Lipoxygenases and Cyclooxygenases. Arch Biochem Biophys. 464(2):260-268.

Romero J, Garcia-Palomero E, Lin SY, Ramos JA, Makriyannis A, Fernandez-Ruiz JJ. 1996. Extrapyramidal effects of methanandamide, an analog of anandamide, the endogenous CB1 receptor ligand. Life Sci. 58(15):1249-1257.

Sancho R, de la Vega L, Appendino G, Di Marzo V, Macho A, Munoz E. 2003. The CB1/VR1 agonist arvanil induces apoptosis through an FADD/caspase-8-dependent pathway. Br J Pharmacol. 140(6):1035-1044.

Schmid PC, Zuzarte-Augustin ML, Schmid HH. 1985. Properties of rat liver N-acylethanolamine amidohydrolase. J Biol Chem. 260(26):14145-14149.

Schrag D. 2004. The Price Tag on Progress – Chemotherapy for Colorectal Cancer. N Engl J Med. 351:317-319.

Sheng H, Shao J, Kirkland SC, Isakson P, Coffey RJ, Morrow J, Beauchamp RD, DuBois RN. 1997. Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. J Clin Invest. 99(9):2254-2259.

Siegmund SV, Seki E, Osawa Y, Uchinami H, Cravatt BF, Schwabe RF. 2006. Fatty acid amide hydrolase determines anandamide-induced cell death in the liver. J Biol Chem. 281(15):10431-10438.

Soliman E. 2014. Molecular mechanism of anandamide-induced apoptosis in non-melanoma skin cancer. (Doctoral Dissertation): Retrieved from Proquest Database.

Soliman E, Henderson KL, Danell AS, Van Dross RT. 2016a. Arachidonoyl-ethanolamide activates endoplasmic reticulum stress-apoptosis in tumorigenic keratinocytes: Role of cyclooxygenase-2 and novel J-series prostamides. Mol Carcinog 55(2):117-130.

Soliman E, Van Dross RT. 2016b. Anandamide-induced endoplasmic reticulum stress and apoptosis are mediated by oxidative stress in non-melanoma skin cancer: Receptor-independent endocannabinoid signaling. Mol Carcin. 55(11):1807-1821.

Timmer JC, Salvesen GS. 2006. Caspase substrates. Cell Death Differ. 14:66-72.

US Department of Health and Human Services, Office of the Surgeon General. (2014). The Surgeon General's call to action to prevent skin cancer. http://surgeongeneral.gov.

Van Dross RT. 2009. Metabolism of anandamide by COX-2 is necessary for endocannabinoid-induced cell death in tumorigenic keratinocytes. Mol Carcin. 48(8):724-732.

Van Dross RT, Soliman E, Jha S, Johnson T, Mukhopadhyay S. 2013. Receptor-dependent and Receptor-independent Endocannabinoid Signaling: A Therapeutic Target for Regulation of Cancer Growth. Life Sci. 92(0):463-466.

Winawer SJ, Zauber AG, Ho MN, O'Brien MJ, Gottlieb LS, Sternberg SS, Waye JD, Schapiro M, Bond JH, Panish JF, Ackroyd F, Shike M, Kurtz RC, Hornsby-Lewis L, Gerdes H, Stewart ET, and the National Polyp Study Workgroup. 1993. Prevention of Colorectal Cancer by Colonoscopic Polypectomy. N Engl J Med. 329:1977-1981