INHIBITION OF FATTY ACID AMIDE HYDROLASE INCREASES RATES OF APOPTOSIS IN CELL LINE MODELS OF DLBCL AND BREAST CANCER

A Thesis

Presented to

The Faculty of the Department of Biomedical Engineering

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in Biomedical Engineering

by

Jared Henderson

August 2016

INHIBITION OF FATTY ACID AMIDE HYDROLASE INCREASES RATES OF APOPTOSIS IN CELL LINE MODELS OF DLBCL AND BREAST CANCER

Jared Henderson

Approved:

Chair of the Committee
Dr. Chandra Mohan, Hugh Roy and Lillie Cranz Cullen Endowed Professor, Department of Biomedical Engineering

Committee Members:

Dr. Ahmet Omurtag, Associate Professor, Department of Biomedical Engineering

Dr. Daniel Frigo, Assistant Professor, Center for Nuclear Receptors and Cell Signaling, Biology and Biochemistry Department

Dr. Suresh K. Khator, Associate Dean, Cullen College of Engineering

Dr. Metin Akay, Founding Chair, John S. Dunn Cullen Endowed Chair Professor, Department of Biomedical Engineering

Acknowledgements

I would like to thank my advisor, Dr. Chandra Mohan, for welcoming me into his lab and providing me with the opportunity to work on such a fascinating project. It has been humbling and inspiring to work with such a knowledgeable, supporting, and interested mentor. I would also like to thank Dr. Simanta Pathak for the invaluable training and advice he gave me as I began my stint in the lab and for constantly being available as to bounce ideas off of when my work hit rough spots. I credit him for no small part of the skills and knowledge developed during my tenure.

I would also like to thank Sanam Soomro and Samantha Stanley for their friendship and shared laughter as well as for selflessly lending a helping hand each time one was needed. I also owe Dr. Huihua Ding a debt of gratitude for her patient and sober thoughts on everything from how best to lay out a graph to where someone put my timer. I am also appreciative of Dr. Yong Du for lending his expertise in animal studies to smooth my way through protocol writing.

Lastly, I would like to thank my family, who has been so kind and supportive of me through the last year. You've helped me make it through the rough patches and helped me stay focused on the important things and I can never say thank you enough.

INHIBITION OF FATTY ACID AMIDE HYDROLASE INCREASES RATES OF APOPTOSIS IN CELL LINE MODELS OF DLBCL AND BREAST CANCER

An Abstract

of a

Thesis

Presented to

The Faculty of the Department of Biomedical Engineering

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in Biomedical Engineering

by

Jared Henderson

August 2016

Abstract

Despite recent advances in cancer treatment, cancers with high heterogeneity such as DLBCL and breast cancer remain difficult to treat, with many patients having few treatment options. The endocannabinoid system has been identified as a potential wellspring of therapeutic agents that can potentially increase cell death and prevent metastasis. Since FAAH is a hydrolytic enzyme that degrades potentially therapeutic endocannabinoids, FAAH inhibitors were tested as apoptosis inducing agents based on their ability to prevent endocannabinoid degradation. In this study, multiple human DLBCL and breast cancer cell lines were treated with FAAH inhibitors, then tested for apoptosis via flow cytometry or with MTT viability testing. DLBCL lines treated with FAAH inhibitors induced apoptosis at dramatically higher rates than vehicle control, and breast cancer cell lines show potential for use of FAAH inhibitors to dramatically decrease cell viability. FAAH inhibition may prove useful as a combination therapeutic for DLBCL or breast cancer.

Table of Contents

Table of Figures

List of Tables

Abbreviations and Symbols

AA	Arachidonic Acid
AEA	Anandamide (*N*-arachidonoylethanolamine)
AM251	CB1 Antagonist
AM630	CB2 Antagonist
CB1	Cannabinoid Receptor 1 (Referred to as CB1R in Figure 2)
CB2	Cannabinoid Receptor 2 (Referred to as CB2R in Figure 2)
CBR	Cannabinoid Receptor
Celecoxib	COX-2 Inhibitor (Commercial name Celebrex®)
COX-2	Cyclooxygenase-2 (Prostaglandin-Endoperoxide Synthase 2)
CXCR4	Chemokine Receptor 4
DLBCL	Diffuse Large B-Cell Lymphoma
eCB	Endocannabinoid
EGFR	Epidermal Growth Factor Receptor
FAAH	Fatty Acid Amide Hydrolase – Enzyme
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid – Cell culture media buffering reagent
IFN-γ	Interferon γ
IL-2	Interleukin-2
IL-6	Interleukin-6
IVIS	*In-Vitro* Imaging System
NHL	Non-Hodgkin Lymphoma
PEA	(*N*-Palmitoylethanolamine)
PF-750	FAAH Inhibitor
PKA	Protein Kinase A

PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
ROS	Reactive Oxygen Species
SB366971	TRPV1 Antagonist
SR1664	PPARγ Inhibitor
TNF-α	Tumor Necrosis Factor α
TRPV1	Transient Receptor Potential Cation Channel Subfamily V Member 1 (Vanilloid Receptor 1)
URB597	FAAH Inhibitor

Chapter 1: Background of FAAH and the Endocannabinoid System

1.1 Introduction to Fatty Acid Amide Hydrolase

Human Fatty Acid Amide Hydrolase (FAAH) is a transmembrane protein primarily found in the endomembrane system with varied distributions and concentrations in body tissues. It is the primary enzyme responsible for regulating levels of a family of lipid signaling molecules called the endocannabinoids (eCBs) by hydrolyzing them into inactive fatty acids and ethanolamine[1]. **By hydrolyzing the eCBs, FAAH reduces the availability of the endocannabinoid ligands**, and by extension for the manipulation of many cellular processes by extension[2]. Because of its role in altering both intra and extracellular eCB concentrations, FAAH has been examined as a potential therapeutic target for a slew of medical purposes including nociception[3], anti-inflammatory response[4], drug addiction, anxiety[5], and even cancers such as melanoma[6], and lung and prostate cancers[7]. This work explores the potential to induce apoptosis in cancer cells by inhibiting FAAH function.

1.2 Introduction to the Endocannabinoid System

The rationale for inhibiting FAAH rests mainly upon the cellular signaling ability of the compounds hydrolyzed by FAAH, the endocannabinoids. Discussed more fully below, the eCBs serve as neurotransmitters, inflammatory regulators, immune modulators, and cellular differentiation, proliferation and homeostatic mechanisms, but most relevant here is that the **eCBs have been shown to induce apoptosis in cancer cells through multiple pathways, notably through cannabinoid receptors 1 and 2**.The eCBs are high affinity ligands for the cannabinoid receptors, cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2), from which they derive their namesake. In addition to direct high affinity activation of the CB1 and CB2 receptors, the eCBs exert a broad influence over a number of other receptors and pathways[8]. Table 1 highlights the key members of the eCB family discussed below.

Table 1: Survey of the Endocannabinoids and Their Affecters

Description	Species	Functional Role
Endocannabinoid	AEA & 2-AG	Primary ligands for CB1 & CB2 activation
Endocannabinoid-like	PEA & OEA	Exert bystander effect – do not directly bind CB1/2 but can potentiate eCB activity
Hydrolytic Enzyme	FAAH	Degrades eCB and eCB-like molecules in a diffusion limited manner
Target Receptors	CB1 & CB2	Primary target of eCBs
	PPARγ	Target of eCBs with lower affinity than CB1/2
	TRPV1	Target of AEA
Catalytic Enzyme	COX-2	Can degrade both AEA and AA into prostaglandins and thereby ROS

The main eCBs themselves include Anandamide (AEA) and 2-Arachidonylglycerol (2-AG), with eCB-like compounds *N*-Palmitoylethanolamine (PEA) and *N*-Oleoylethanolamine (OEA). The chemical structures of these molecules are shown in Figure 1[9]. AEA and 2-AG are well known to activate CB1 and CB2 with high affinity, while PEA and OEA act upon the endocannabinoid system through the bystander effect[8], by not directly activating CB1 and CB2, but rather by serving as a diffusion cushion and reluctant activators of other eCB receptors such as the PPAR family.

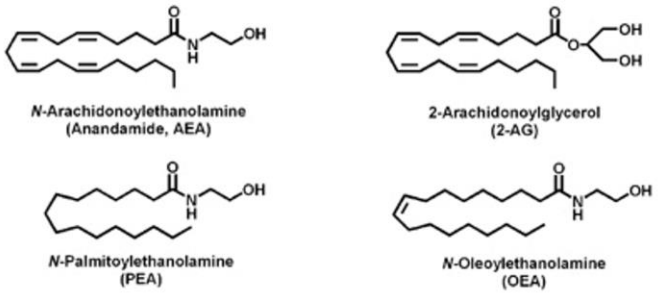

Figure 1: Chemical Structures of the Major Endocannabinoids [9]

The eCBs perform the majority of their physiological roles though the activation of a set of four receptors: CB1, CB2, peroxisome proliferator-activated receptors (PPAR), and transient receptor potential cation channel subfamily V member 1 (TRPV1). CB1 and CB2 have been shown to trigger multiple responses in B-cells including repression of IL-2, stimulation of IL-6,

inhibiting TNF-α and IFN-γ, stimulate serotonin release, and regulate inflammatory responses[10], [11]. Of interest here is the potential for CB1 and CB2 receptors to mediate apoptotic response, which occurs through several intermediaries shown and described in Figure 2. TRPV1, referred to as vanilloid receptor 1, demonstrates a roughly equivalent affinity for AEA as it does for its most well researched ligand, capsaicin, but is rarely activated by AEA due to relatively low availability of AEA caused by FAAH dependent degradation[12]. PPARγ is activated by AEA and 2-AG and has been shown to reduce inflammation and neutrophil invasion in response to elevated levels of endocannabinoids[13].

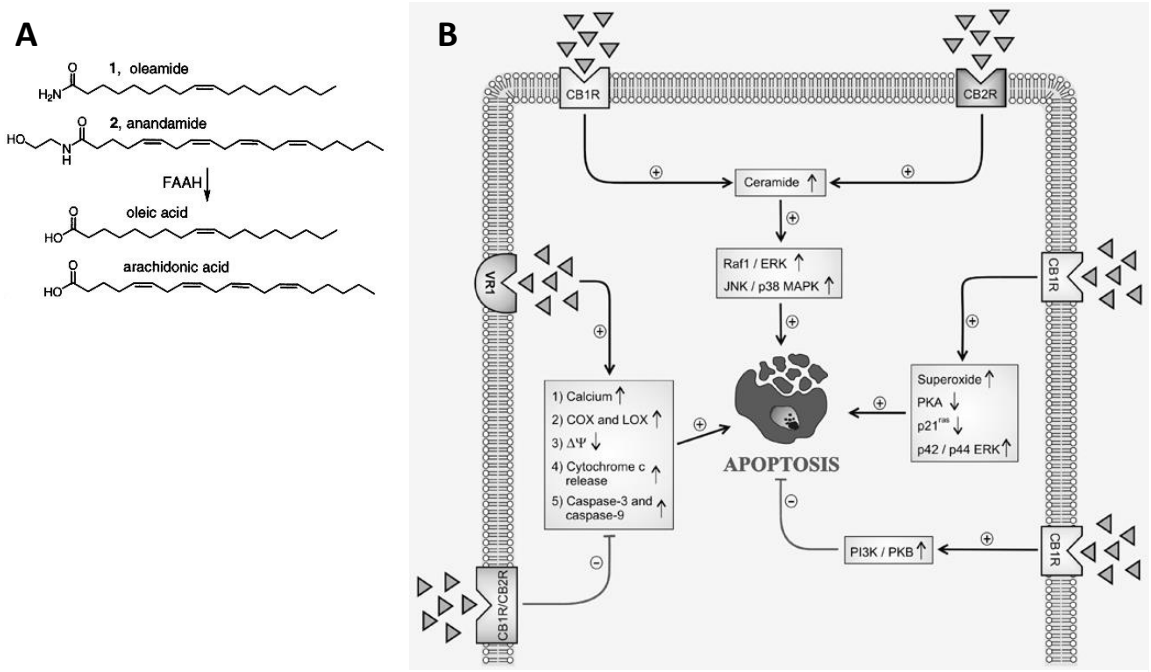

Figure 2: The Role of Endocannabinoids in Promoting Apoptosis [11]
Reprinted by permission from Macmillan Publishers Ltd: *Cell Death Differ.*, vol. 10, no. 9, pp. 946–955, 2003, copyright 2003.

In Figure 2, part A, FAAH is a hydrolytic enzyme that degrades the endocannabinoid ligands AEA, 2-AG, OEA, and PEA into their constituent fatty acids and ethanolamine. In Figure 2, Part B, AEA, cannabinoid receptors, vanilloid receptors cause apoptosis. Binding of extracellular anandamide (triangles) to type 1 or 2 cannabinoid receptors (CB1R or CB2R) triggers different signal transduction pathways, depending on the cell type. Activation of either CB1R or CB2R

increases intracellular levels of ceramide, which activates Raf1/ERK cascade, thus engaging JNK/p38 MAPK along the pathway leading to apoptosis. In addition, binding of anandamide to CB1R can trigger superoxide ion production, inhibition of protein kinase A (PKA) and of the K-*ras* oncogene product $p21^{ras}$, and activation of p42/p44 ERK, all leading to apoptosis. Alternatively, anandamide can activate VR1 by binding to an intracellular site, thus triggering a proapoptotic series of events including elevation of intracellular calcium, activation of the arachidonate cascade through the COX and the LOX pathways, drop in mitochondrial potential ($\Delta\Psi$), increased release of cytochrome c and activation of caspase-3 and caspase-9. These effects of AEA at VR1 are prevented by simultaneous activation of CB1R (in neuronal cells) or CB2R (in immune cells). In astrocytes, CB1R activation by anandamide can also activate the PI3K/PKB pathway, resulting in protection against apoptosis[11].

Cyclooxygenase-2 (COX-2) is the final member of the endocannabinoid family discussed here despite that it is but a grudging adoptee. COX-2 has no true affinity for the endocannabinoids themselves, but rather typically uses a byproduct of AEA degradation, arachidonic acid (AA), to produce prostaglandin G2, which is then converted into prostaglandin H2. The functional relevance in the context of FAAH inhibition is that COX-2 also has the ability to degrade excess AEA, albeit more slowly than FAAH, into members of the prostaglandin family, which has important consequences for cellular maintenance and apoptosis. The primary substrate for COX-2 is AA, which is ubiquitous in cells as a component of cell membranes. Because of the rapid rate at which AA is taken up by the membrane and thereby rendered unavailable to COX-2, the COX-2 enzyme cannot remain constituently active. When excess AEA is present in the cytosolic space, however, COX-2 activity levels increase dramatically[14].

1.3 The Role of FAAH and the Endocannabinoid System in Apoptosis

FAAH inhibition plays a role in inducing apoptosis in mammalian cells by allowing for the buildup of eCBs and eCB-like compounds in the cellular and extracellular environments. Especially in cell types that have relatively high concentrations of either FAAH or endocannabinoid receptors, the inhibition of FAAH activity has the potential to skew cell biology in a clinically relevant direction. In particular, FAAH inhibition, through the effects of the endocannabinoid system, has a great potential to cause apoptosis in cells that, for example, are already under oxidative stress due to poor angiogenesis.

In response to excess AEA in the extracellular environment, TRPV1 initiates apoptosis in cells by triggering a calcium influx that then activates cyclooxygenases and lipoxygenases. These enzymes, which include COX-2, then lower the mitochondrial membrane potential, and releasing cytochrome c that subsequently triggers the caspase cascade as shown in Figure 2[15]. CB1 and CB2 have numerous mechanisms triggering apoptosis cataloged in Figure 2, and activation of PPARγ has been shown to have potential as an anti-cancer treatment due to its induction of apoptosis in tumors[16].

Overall, the endocannabinoids AEA, 2-AG, PEA, and OEA can promote apoptosis though activation of the cannabinoid receptors or though several other pathways. FAAH is responsible for the hydrolysis of these signaling molecules, thus rendering an important regulatory mechanism impotent. By pharmacological inhibition, this work seeks to increase the availability of eCBs, which can then trigger apoptosis in cancer cells.

Chapter 2: FAAH Inhibition in DLBCL Cell Lines

2.1 Background

Diffuse Large B-Cell Lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma (NHL) in the United States, accounting for 30-40% of all cases of NHL[17]. [18], [19]. Patients diagnosed with DLBCL have poor prognosis, generally marked by a 50% five year survival rate, due to the dearth of effective treatments for multiple variants of the disease and the fact that DLBCL manifests primarily in older adults that are more difficult to treat aggressively. There is a need to further investigate the molecular root of DLBCL pathogenesis and evaluate potential targets for both stand alone and combination therapies.

FAAH levels have previously been shown to be heavily increased in DLBCL tissues, with FAAH mRNA levels loosely tied to patient outcomes[20]. Though a broad transcriptomic screen by Shipp et al. did not show a direct correlation between directional regulation of FAAH and patient outcome, the efforts to establish relationships between molecular subtypes and patient outcomes have been hampered by the heterogeneous nature of the disease itself and poorly differentiated classification of DLBCL.

However, more researched than FAAH itself has been the effect of the eCBs on B-cell populations, with several studies associating increased levels of the eCBs AEA, 2-AG, PEA, and OEA regulating a variety of cellular functions such as apoptosis, hematopoiesis, immunocompetent cell migration, and platelet aggregation, due primarily to the influence of relatively high expression of CB2 in the cells of the peripheral immune system[8]. A recent study by Zhang et al. examining DLBCL serum levels of eCBs demonstrates a correlation between increased disease progression and levels of serum eCBs[21]. Furthermore, multiple studies have shown that the eCBs AEA and 2-AG have been highly effective at inducing apoptosis in B-cells[14], [15], [22]–[25]. However, the extremely poor half-life of these compounds, mainly due

to degradation by FAAH[26], has been shown to be less than five minutes precluding them from direct therapeutic use in the treatment of DLBCL. As such, this project seeks to establish that FAAH inhibition can induce apoptosis in DLBCL tissues by increasing levels of circulating eCBs. The following experiments establish FAAH inhibition does indeed result in significant cellular apoptosis in DLBCL cells.

2.2 Materials and Methods

Cell Lines: The human DLBCL cell lines DB, HBL-1, Ly-7, Ly-8, Ly-18, and U-2932 were a generous gift from Dr. Richard E. Davis at The University of Texas MD Anderson Cancer Center. Cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 25 mmol HEPES. Cells were maintained at 37°C at 5% CO_2.

Drugs: AEA and PEA were purchased from Cayman Chemical Company. The inhibitors URB597, PF-750, Celecoxib, and SB366791 as well as the antagonists AM251, AM630, and SR1664 were purchased from Tocris Bioscience. All drugs were prepared as stock solutions in DMSO according to manufacturer recommendations and diluted to experimental concentrations in cell culture media just prior to use with cell lines. Therefore DMSO was used as vehicle control in all cell line experiments with the final concentration of DMSO kept under 0.2%.

Western Blot: Cell lysates were immobilized on PVDF membrane and probed first with FAAH monoclonal antibody purchased from Abcam (ab54615), then stripped using MilliporeSigma's ReBlot Plus Strong Antibody Stripping Solution and probed again for α-Tubulin as loading control. Quantification was performed by Bio-Rad Image Lab™ software.

Apoptosis Assays: Cells were plated in 24-well plates at a concentration of 2.5×10^5 cells/well in 2.0 mL total media and allowed to acclimatize for one to two hours. Cells were then treated and incubated 24 hours. Apoptosis was measured via flow cytometry using PE Annexin V

from BD Biosciences (556421) and/or CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit from Thermo Scientific, both according to manufacturer specifications. Flow cytometry was performed on both a BD Accuri C6 and BD FACSAria II. Flow cytometer data was analyzed in FlowJo™ analysis software.

RT-PCR: Total RNA was extracted from cultured cells using Ambion TRIzol® Reagent according to manufacturer's instructions. To measure mRNA expression, reverse transcription was performed using Bio-Rad SYBR® Green Supermix, and the generated cDNA was amplified by PCR using primers selected through the PrimerBank platform[27]. Primers were chosen for the FAAH, CB1, CB2, α-Tubulin, and β-Actin genes as described in Table 2.

Table 2: PCR Primers

Gene Name	PrimerBank ID	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')
FAAH	166795286c3	GGGGACCTGGTCTCAATTCTG	CAATCACGGTTTTGCGGTACA
CB1	237681172c1	TTACAACAAGTCTCTCTCGTCCT	GGCTGCCGATGAAGTGGTA
CB2	206725541c1	GGGTGACAGAGATAGCCAATGG	TGAACAGGTATGAGGGCTTCC
α-Tubulin	17986283a1	TCGATATTGAGCGTCCAACCT	CAAAGGCACGTTTGGCATACA
β-Actin	4501885a1	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT

2.3 FAAH Inhibition Results in Apoptosis in DLBCL Lines

To evaluate the potential of FAAH inhibition as a therapeutic option for inducing apoptosis in DLBCL, the human cell lines DB, HBL-1, Ly-7, Ly-8, Ly-18, and U-2932 were first evaluated for expression of FAAH by western blot as shown in Figure 3. Following verification that the selected DLBCL lines expressed FAAH, each cell line was treated with the selective, irreversible FAAH inhibitors URB597 and PF-750 at various concentrations to establish if FAAH inhibition resulted in increased apoptotic rates. Cells were originally tested at concentrations from 10 to 70 μM for each inhibitor and incubated for 24, 48, and 72 hours to establish the experimental parameters (data not shown). Through this process it was determined that optimal treatment was between 10 and 50 μM for a 24 hour period. All cell lines showed dose dependent response to both FAAH

inhibitors as determined by Annexin V and Caspase 3/7 staining and flow cytometry with the corresponding data shown in Figure 4.

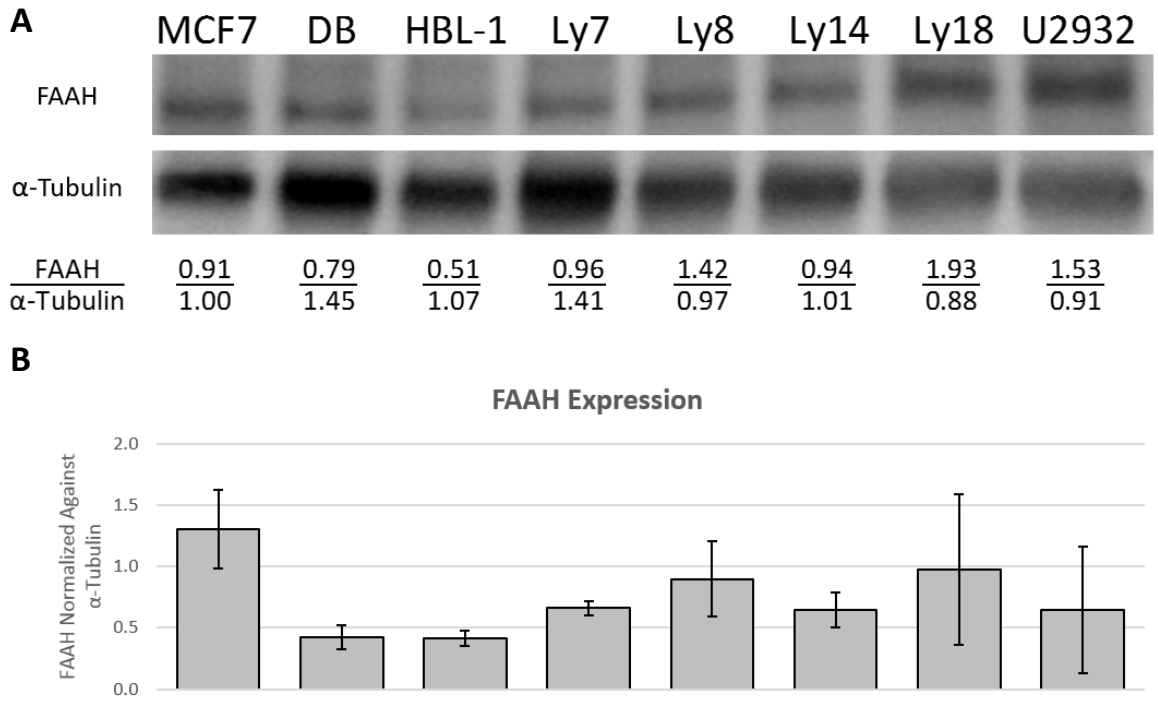

Figure 3: Expression of FAAH in Human DLBCL Cell Lines

In Figure 3, part A, All cell lines express Fatty Acid Amide Hydrolase (FAAH) as demonstrated by western blot, using α-Tubulin as house-keeping control. DB, HBL-1, Ly-7, Ly-8, Ly-14, and Ly-18 are human DLBCL cell lines, and MCF7 was used as positive control. Ratio is shown for visible blots only. In Figure 3, Part B, FAAH is shown normalized against α-Tubulin. Quantification was performed by image analysis using Image Lab™ software. Error bars represent SE, and *n*=3.

In Figure 4, parts A and B, FACS Analysis of Ly7 cells treated with Vehicle (DMSO) and 50µM URB597, stained with Annexin V and SYTOX AADvanced shown in part A, and Caspase 3/7 Detection Reagent and SYTOX AADvanced shown in part B, representative of twelve trials. In part C, apoptosis rates of cell lines Ly7, Ly18, Ly8, HBL-1, and U-2932 caused by FAAH inhibition by URB597 and PF-750 relative to vehicle control is shown as determined by Annexin V positive staining via flow cytometry. In part D, apoptosis rates are shown relative to vehicle

control as determined by Caspase 3/7 positive staining via flow cytometry. Data are expressed as fold increase in apoptosis over vehicle control with $n \geq 3$. Statistical significance is relative to vehicle control and is assigned as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

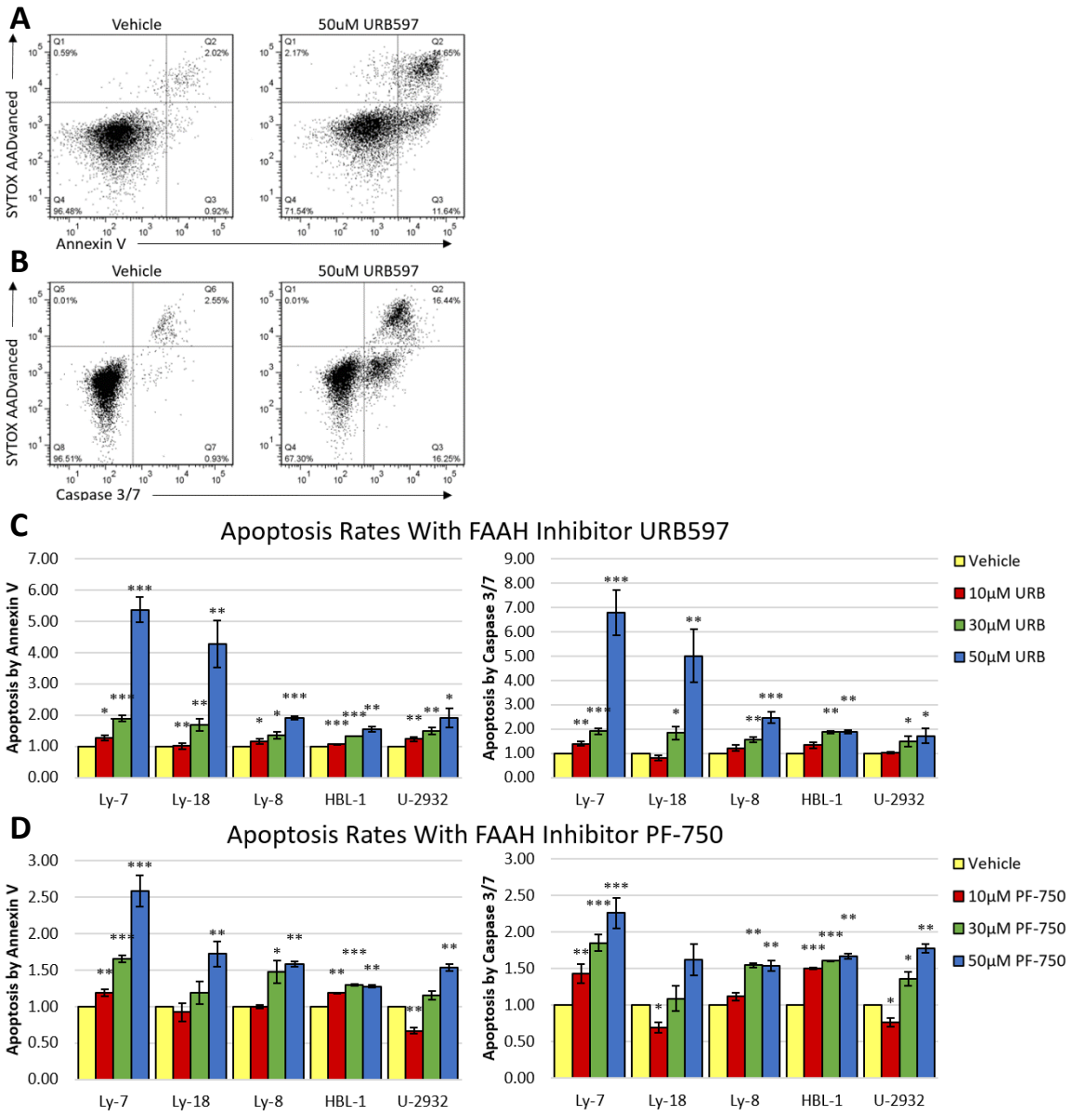

Figure 4: Relative Rates of Apoptosis Dependent Upon FAAH Inhibition

To confirm the hypothesis that FAAH inhibition initiates apoptosis indirectly by allowing the buildup of eCBs which in turn activate the apoptotic machinery, cells were directly incubated with both the direct eCB AEA and the bystander eCB PEA alone and in the presence of URB597. AEA alone induced significant levels of apoptosis over vehicle control in a dose dependent manner, while the bystander eCB PEA did not produce meaningful changes in apoptotic rates over vehicle control shown in Figure 5. This data demonstrates that direct eCB signaling is responsible for induced apoptosis when FAAH is inhibited.

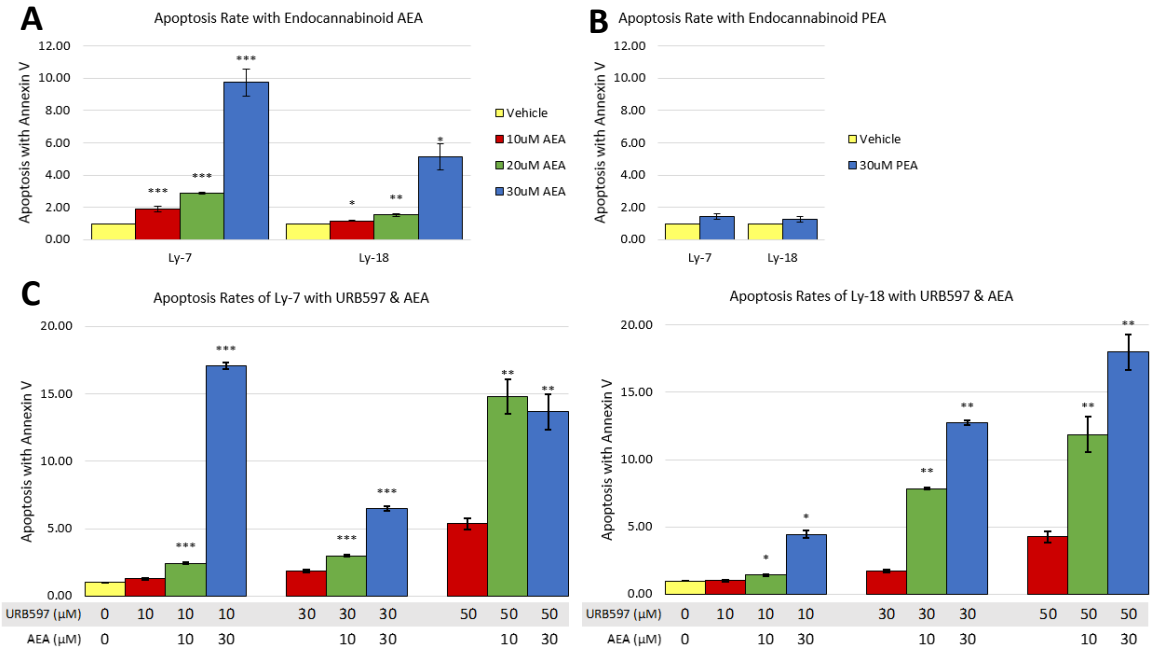

Figure 5: Apoptosis Is Induced by the Endocannabinoid AEA in a Supra-Additive Manner, but Not PEA

In Figure 5, part A, relative fold change of apoptosis rates of Ly7 and Ly18 treated with AEA relative to vehicle control is shown. In Figure 5, part B, relative fold change of apoptosis rates of Ly7 and Ly18 treated with PEA relative to vehicle control is shown. In Figure 5, part C, apoptosis rates relative to vehicle control. Cells were plated according to materials and methods, then pre-incubated with URB597 for 30 minutes before the addition of AEA, then incubated for 24 hours. Data are expressed as fold increase in apoptosis over vehicle control via Annexin V stain and measured though flow cytometry with *n*=3. Statistical significance is

relative to cells treated with equivalent doses of URB597 alone (relative to bar shown in red) with p-values as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

When combined with URB597, AEA increased cell death dramatically in a supra-additive manner in both Ly-7 and Ly-18 cell lines while PEA combined with URB597 did not produce enhanced effect over URB597 alone (PEA data not shown). Clearly, the increased apoptotic rate is an effect of direct endocannabinoid signaling and cannot simply be attributed to the accumulation of eCB-like molecules such as PEA, which only exert their influence through the bystander effect on the eCB receptors. To establish a link between rates of apoptosis and eCB receptor levels, mRNA was isolated from each cell line and reverse transcription and qPCR were performed using the primers indicated in Table 2 to determine the relative expression levels of CB1 and CB2, which are displayed in Figure 6.

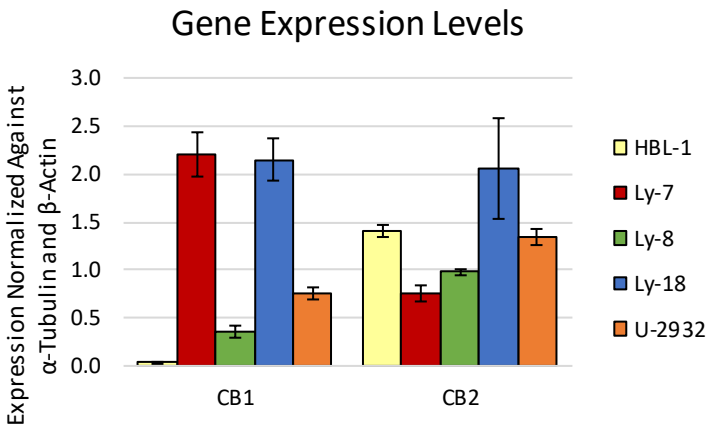

Figure 6: CB1 and CB2 mRNA Expression Levels
Represents mRNA expression normalized against β-actin and α-tubulin. Experiment performed in duplicate and error bars indicate SE.

The cell lines Ly-7 and Ly-18, which had the highest rates of apoptosis due to FAAH inhibition shown in Figure 4, clearly overexpressed CB1 relative to the other three cell lines, suggesting a correlation between the rate of apoptotic response and CB1 levels, noting that the eCB AEA preferentially binds to CB1. In light of this, further experiments were designed to

elucidate the potential cellular mechanisms that trigger apoptosis, focusing on pathways in which the eCB AEA is active.

2.4 Apoptotic Mechanisms Mediated by Cannabinoids and FAAH

As discussed earlier in Chapter 1, AEA is a metabolically promiscuous lipid, playing significant roles not only in the eCB pathway, but also causing activity in several other significant areas, all of which have been shown to potentiate apoptosis. The receptors which hold most promise for apoptotic trigger in DLBCL are CB1, CB2, PPARγ, COX-2, and TRPV1[14], [15], [24], [28]. In order to determine which, if any, of these pathways is responsible for the apoptosis demonstrated in Figure 4 and Figure 5, inhibitors were purchased for each potential target, then used to determine if blocking the activity of any of these proposed receptors would reduce or silence the pro-apoptotic effects of FAAH inhibition. The names and types of the drugs used are listed in Table 3.

Table 3: Inhibitors Used in Pathway Identification

Target Receptor/Enzyme	Drug Name	Drug Type
CB1	AM251	Irreversible Antagonist
CB2	AM630	Irreversible Antagonist
PPARγ	SR1664	Irreversible Inhibitor
COX-2	Celecoxib	Irreversible Inhibitor
TRPV1	SB366971	Irreversible Antagonist

Before testing the combined effect of these inhibitors plus FAAH inhibitors, a baseline apoptosis test was performed for each molecule at various concentrations to ensure that the effect of adding these drugs to the DLBCL lines would not cause change in baseline apoptosis levels in the absence of FAAH inhibition. The levels of apoptosis were again determined by flow cytometry using Annexin V and Caspase 3/7, with data shown in Figure 7. Concentrations that did not significantly affect baseline apoptosis were then selected for the next experiment. The inhibitors in Table 3 were added in conjunction with URB597 to determine if blockade of one of

the five pathways could reverse the apoptosis demonstrated by URB597. Table 4 shows the selected concentrations of each inhibitor used. The cell lines Ly-7 and Ly-18 were then treated with AEA and URB597 in conjunction with additional inhibitors shown in Figure 8 and Figure 9.

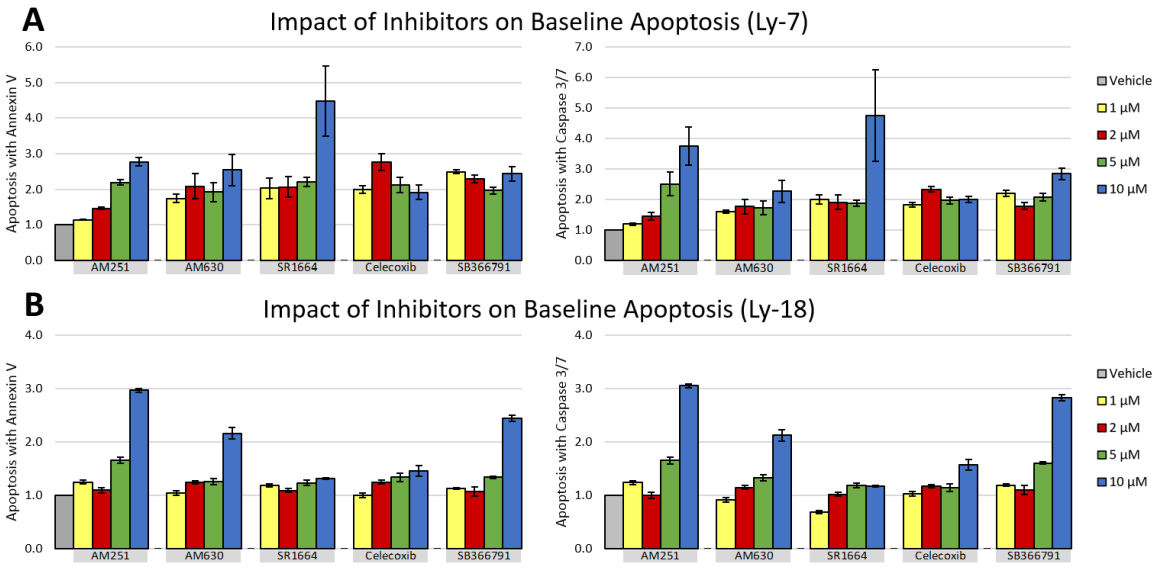

Figure 7: Baseline Apoptosis Induction for Inhibitors AM251, AM630, SR1664, Celecoxib, and SB366791

In Figure 7, baseline apoptosis levels in Ly-7 and Ly-18 cell lines cultured with various concentrations of AM251 (CB1 Inhibitor), AM630 (CB2 Inhibitor), SR1664 (PPARγ Inhibitor), Celecoxib (COX-2 Inhibitor), and SB366971 (TRPV1 Inhibitor) were established. Cells were plated according to materials and methods, then cultured for 24 hours. Data are expressed as fold increase in apoptosis over vehicle control via Annexin V or Caspase 3/7 stain and measured though flow cytometry with *n*=3. Error bars represent SE. In part A, Ly-7 cells show low to moderate apoptotic response in the presence of tested inhibitors. In part B, Ly-18 cells show relatively low response to the presence of tested inhibitors.

Table 4: Selected Inhibitor Concentrations

Target Receptor / Enzyme	Drug Name	Selected Concentration (µM)
CB1	AM251	1
CB2	AM630	2
PPARγ	SR1664	2
COX-2	Celecoxib	10
TRPV1	SB366971	2

In Figure 8, High rates of FAAH dependent apoptosis are prevented in Ly-7 cells by co-incubation with AM630 (CB2 Inhibitor), Celecoxib (COX-2 Inhibitor), and SB366971 (TRPV1

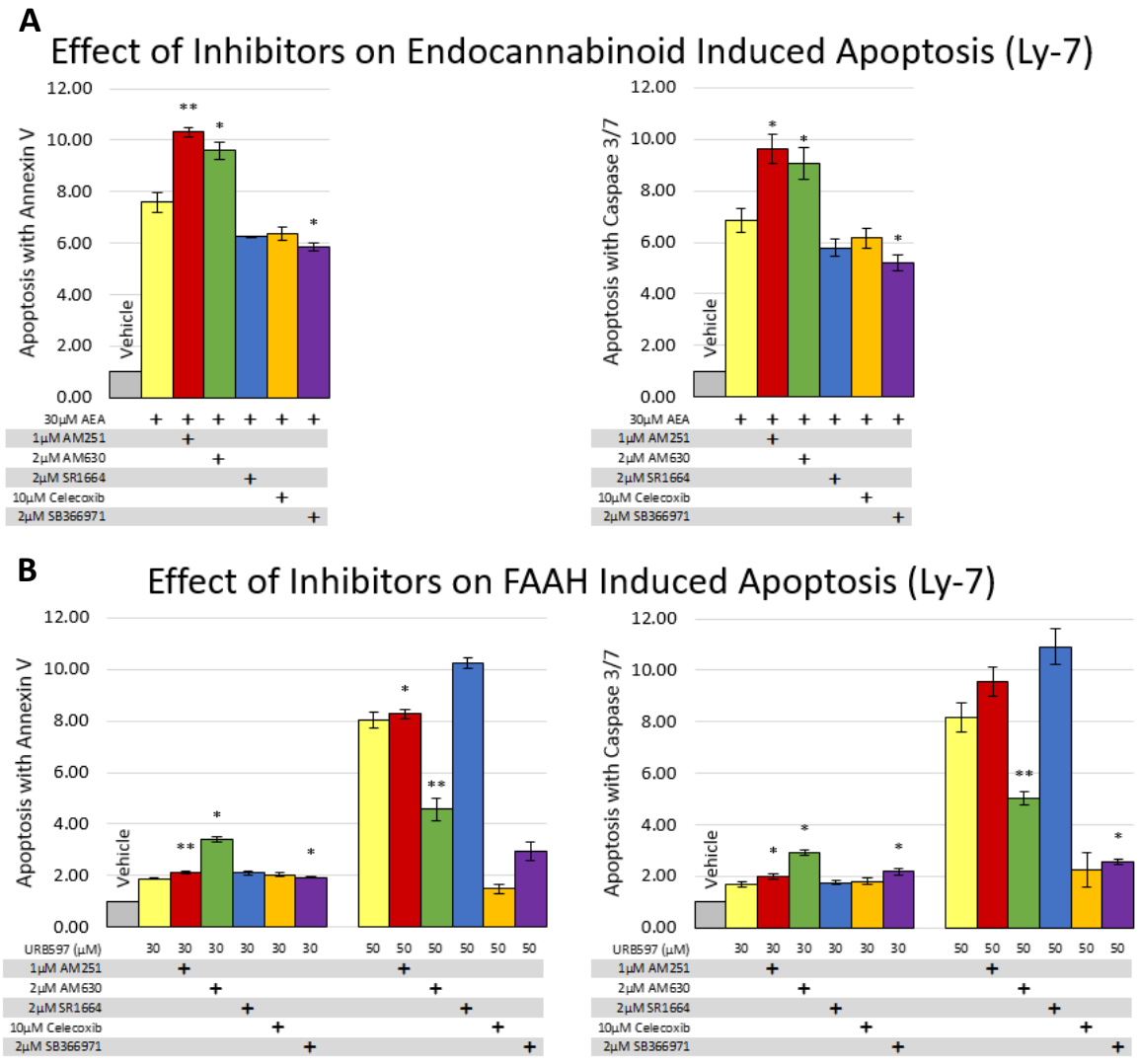

Figure 8: Effect of Secondary Inhibitors on FAAH Pathway Induced Apoptosis (Ly-7)

Inhibitor). In part A, Ly-7 cells were treated with the endocannabinoid AEA with and without additional downstream inhibitors as shown. Apoptosis was determined by Annexin V and Caspase 3/7 stains respectively. In part B, Ly-7 cells were treated with the FAAH inhibitor URB597 in the doses shown with and without additional downstream inhibitors. Apoptosis was determined by Annexin V and Caspase 3/7 stains respectively. Apoptosis rates shown are relative to vehicle control. Cells were plated according to materials and methods, then pre-incubated with either the endocannabinoid AEA or FAAH inhibitor URB597 for 30 minutes before the addition of secondary inhibitors AM251, AM630, SR1664, Celecoxib, and SB366791, then incubated for 24 hours. Data are expressed as fold increase in apoptosis over vehicle control via Annexin V or Caspase 3/7 stain and measured though flow cytometry with n=3. Statistical significance is assigned relative to cells treated with equivalent doses of URB597 alone (relative to bar shown in yellow) with p-values as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

In Figure 9, apoptotic rates were not conclusively decreased by inhibition of pathways directly affected by the endocannabinoid AEA or FAAH inhibition. In parts A and B, Ly-18 cells were treated with the endocannabinoid AEA with and without additional downstream inhibitors as shown. Apoptosis was determined by Annexin V and Caspase 3/7 stains respectively. In parts C and D, Ly-18 cells were treated with the FAAH inhibitor URB597 in the doses shown with and without additional downstream inhibitors as shown. Apoptosis was determined by Annexin V and Caspase 3/7 stains respectively. Apoptosis rates shown are relative to vehicle control. Cells were plated according to materials and methods, then pre-incubated with either the endocannabinoid AEA or FAAH inhibitor URB597 for 30 minutes before the addition of secondary inhibitors AM251, AM630, SR1664, Celecoxib, and SB366791, then incubated for 24 hours. Data are expressed as fold increase in apoptosis over vehicle control via Annexin V or Caspase 3/7 stain and measured though flow cytometry with *n*=3. Statistical significance is

assigned relative to cells treated with equivalent doses of URB597 alone (relative to bar shown in yellow) with p-values as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

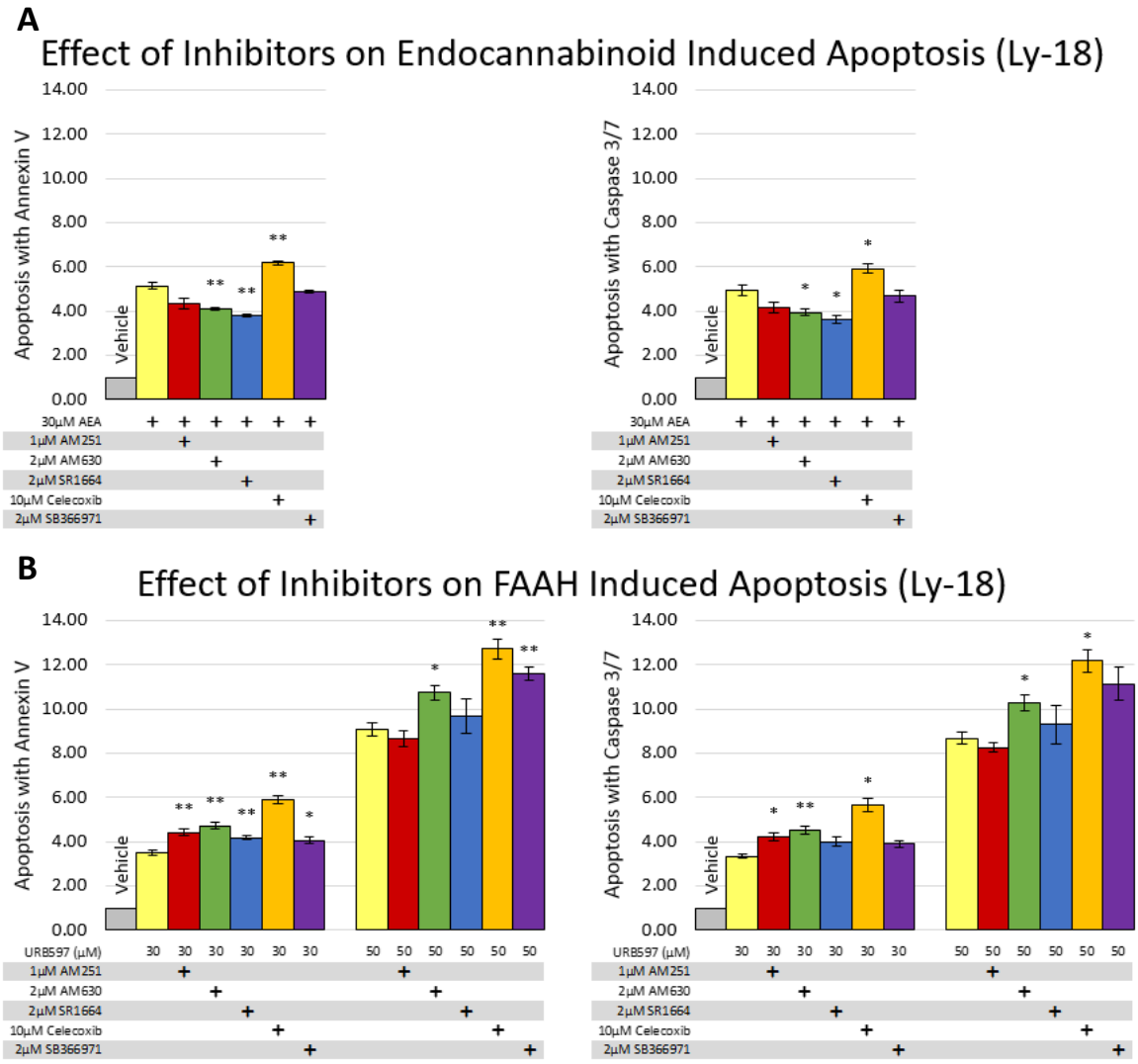

Figure 9: Effect of Secondary Inhibitors on FAAH Pathway Induced Apoptosis (Ly-18)

Data collected in Figure 8 part (A) for Ly-7 reveals several clues as to the mechanism of apoptosis. When treated with AEA, blockade of the cannabinoid receptors CB1 and CB2 using antagonists AM231 and AM630 resulted in marked increase in apoptosis, while blockade of PPARγ, COX-2, and TRPV1 resulted in lower rates of apoptosis relative to treatment with AEA alone. The implication is that CB1 and CB2 must confer some protective or proliferative role in Ly-7 cells[8]. By eliminating cannabinoid activation, apoptosis rates ticked up due to increased

eCB availability to serve as a ligand for other binding partners. By the same token, there was a slight down tick in apoptosis when the non-cannabinoid binding partners were inhibited, perhaps allowing greater bioavailability for AEA to perform a proliferative role in conjunction with the cannabinoid receptors. Results for Ly-18 shown in Figure 9 part (A) did not yield definitive answers about what pathway induced the previously demonstrated eCB and FAAH inhibitor dependent apoptosis.

The data in Figure 8, part B paints a slightly less coherent picture, but nonetheless indicates an increase in cell survival when blocking CB2, COX-2, and TRPV1, while CB1 and PPARγ blockade did not significantly affect the apoptotic rate. The differences in response between FAAH inhibition and direct AEA incubation could certainly be accounted for by the fact that FAAH inhibition allows the buildup of a number of additional molecules not tested here, with many of them certainly at play to generate the outcomes of this experiment.

However, based on the above data, inhibition of FAAH does result in moderate to heavy apoptosis in DLBCL. Though more work is needed to fully flesh out the complete mechanism and pathway activated, it is clear that FAAH inhibition mediates dose dependent apoptosis in keeping with the original hypothesis.

2.5 Proposed and Ongoing Studies

The next rational step in establishing FAAH inhibition as a viable treatment for DLBCL is an *in vivo* study. To that aim, plans are underway to perform a mouse xenograft study using fluorescently labeled Ly-7 and Ly-18 cells. This mouse study will provide much greater insight into the effectiveness of FAAH blockade for induction of apoptosis in DLBCL tumors by answering questions that *in vitro* culturing is simply ill equipped to answer.

The most glaring deficiency in the existing data is that while B-cells seem to respond in various ways to eCBs, they synthesize very little themselves, relying on other tissues to

synthesize most of the eCBs and eCB-like compounds[29]. Therefore, *in vitro* testing of FAAH inhibition is self-limiting due to the lack of available eCBs in a homogenous plastic test environment. In mice, however, physiological levels of eCBs can approach levels 15 times greater than normal when FAAH is inhibited[30]. This will allow a much more complete picture of how FAAH inhibition will actually affect the cancer cells and the tumor itself.

After labeling the cells with the florescent tag luciferase, mice will be injected in the left flank with 1.0×10^6 cells. The tumor will then be allowed to grow to a critical mass of 45-70 mm^3, at which time mice will undergo a treatment of 5 mg/kg URB597 daily. The tumor will be imaged via an In Vitro Imaging System (IVIS) every 3 days to monitor tumor progression with the hypothesis that FAAH inhibition will inhibit tumor growth by inducing apoptosis in the tumor cells and thereby reduce the tumor burden overall.

Chapter 3: FAAH Inhibition in Breast Cancer Cell Lines

3.1 Background

The endocannabinoids, as described in earlier chapters, have been implicated in a variety of important biological pathways, regulating cell proliferation, differentiation, and apoptosis though both cannabinoid receptor activation and other pathways. They have also been explored as an avenue to cancer treatment, with several studies confirming the role of the endocannabinoids as anti-proliferative and pro-apoptotic in a variety of cancers[31]. However, there have also been reports of eCB signaling resulting in cancer cell proliferation, which should sound a cautionary alarm about approaching the endocannabinoids as a panacea for cancer[32].

Breast cancer in particular has been targeted for treatment with eCBs, both endogenous and exogenous, with a swath of data collected and categorized by Guindon and Hohmann showing that a preponderance of breast cancer models have responded in a clinically beneficial way to treatment with eCBs. From AEA to tetrahydrocannabinol (THC), a host of experiments representing both *in vitro* and *in vivo* studies have confirmed that endocannabinoid treatment of breast cancers holds potential[33]. Based on the evidence that artificially increased eCB levels have a tendency to perform well as anti-cancer agents (as measured by decrease in tumor burden) in breast cancer, the question asked here is whether or not the inhibition of FAAH can be used to treat breast cancer by decreasing the degradation rate of endogenously produced eCBs.

FAAH regulates levels of eCBs by hydrolyzing eCB ligand molecules into inactive membrane component fatty acids and ethanolamine, which are then incapable of performing the myriad signaling tasks attributed to the eCBs. Breast cancer tissues and cell lines in particular have been demonstrated to possess high levels of FAAH relative to other body tissues[34]. Because of the high expression of FAAH, previous studies have reported vanishingly small eCB concentrations

with many cell lines so efficient at hydrolyzing eCBs that the levels are undetectable[35]–[37]. Such high rates of eCB hydrolysis by FAAH make elucidating the role of eCBs in various breast cancer models challenging since even after administering exogenous eCB , the rate of degradation is so rapid that the ligand is potentially eliminated before rendering its full meaningful clinical effect[38]. However, the inhibition of FAAH with selective, irreversible drugs results in dramatic increases in eCB levels even in cell lines with FAAH concentrations high enough to render eCB levels undetectable before inhibition[37].

Even so, no study to date has examined the potential for treatment of breast cancer solely with FAAH inhibitors. Thus, the inhibition of FAAH could prove to be an untapped resource in the quest to utilize eCB signaling for breast cancer treatment. The goal of this study will be to assess the potential for FAAH inhibitors to reduce the viability of and induce apoptosis in human breast cancer cell lines, with the hypothesis that FAAH inhibition will directly contribute to apoptosis in these lines, likely through an eCB mediated pathway.

3.2 Materials and Methods

Cell Lines: The human breast cancer cell lines MCF7, MDA-MB-231, and T-47D were donated by Dr. Chin-Yo Lin from the Center for Nuclear Receptors & Cell Signaling in the Biology and Biochemistry Department at the University of Houston. Cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 25 mmol HEPES. Cells were maintained at 37°C at 5% CO_2.

Drugs: AEA and PEA were purchased from Cayman Chemical Company. The inhibitors URB597 and PF-750 were purchased from Tocris Bioscience. All drugs were prepared as stock solutions in DMSO according to manufacturer recommendations and diluted to experimental concentrations in cell culture media just prior to use with cell lines. Therefore DMSO was used

as vehicle control in all cell line experiments with the final concentration of DMSO kept under 0.2%.

Western Blot: Cell lysates were immobilized on PVDF membrane and probed first with FAAH monoclonal antibody purchased from Abcam (ab54615), then stripped using MilliporeSigma's ReBlot Plus Strong Antibody Stripping Solution and probed again for α-Tubulin as loading control. Quantification was performed by Bio-Rad Image Lab™ software.

Viability Assays: Cells were plated in 96-well plates at a concentrations of 5.0 x 10^4 cells/well for MCF7 and 2.0 x 10^4 cells/well in 200 µL total media and allowed to acclimatize and attach overnight. Cells were then treated and incubated 24 hours. Cell viability was measured using the MTT Cell Proliferation Assay (30-1010K) from ATCC® according to manufacturer's directions. Optical Density readings were performed at 500 and 690 nm using a BioTek® ELx808 plate reader and analyzed with Gen5 software.

RT-PCR: Total RNA was extracted from cultured cells using Ambion TRIzol® Reagent according to manufacturer's instructions. To measure mRNA expression, reverse transcription was performed using Bio-Rad SYBR® Green Supermix, and the generated cDNA was amplified by PCR using primers selected through the PrimerBank platform[27]. Primers were chosen for the FAAH, CB1, CB2, α-Tubulin, and β-Actin genes as described in Table 2. qPCR was performed on a Bio-Rad CFX96 Touch™ in an 96-well plate in duplicates with analysis performed in Bio-Rad CFX Manager software. A-Tubulin and β-Actin were used as housekeeping control genes.

3.3 FAAH Inhibition Results in Apoptosis in Breast Cancer Lines

To assess the potential for FAAH inhibition to induce apoptosis in breast cancer, three model human breast cancer lines were chosen to represent a variety of well characterized cancer subsets. MCF7, and T-47D were chosen for their ubiquity in breast cancer literature and relative similarity as Lumina A, ER^+, $PR^{+/-}$, $HER2^-$ lines for use in direct comparison between

similarly classified cells, and MDA-MB-231 due to its triple negative and chemotherapy resistant nature. [39]–[41]. Following selection and initial culture, each cell line was probed by western blot for expression of FAAH, as shown in Figure 10.

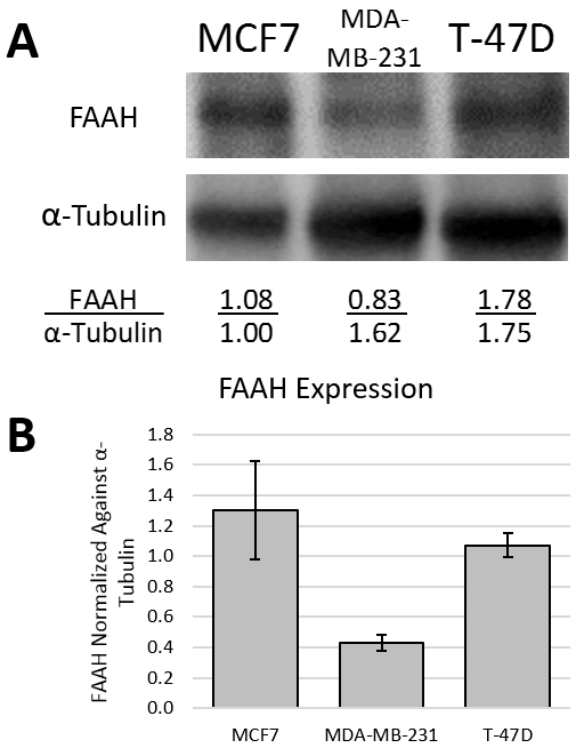

Figure 10: Expression of FAAH in Human Breast Cancer Cell Lines

In Figure 11, part A, cell lines express Fatty Acid Amide Hydrolase (FAAH) as demonstrated by Western blot, using α-Tubulin as housekeeping control. MCF7, MDA-MB-231, and T-47D are human breast cancer cell lines. MCF7 is used as positive control. Ratio is shown for visible blots only. In part B, FAAH is shown normalized against α-Tubulin. Quantification was performed by image analysis using Image Lab™ software. Error bars represent SE, and *n*=2.

Following verification of FAAH expression, MCF7 and MDA-MB-231 cell lines were incubated with the selective, irreversible FAAH inhibitors URB597 and PF-750 for 24 hours, then assayed for cell viability, as described in materials and methods, to establish the effect of FAAH inhibition *in vitro*. As shown in Figure 11, MDA-MB-231 shows a marked decrease in cell viability when

cultured with either URB597 or PF-750 in a dose dependent manner. This establishes that the hypothesis that FAAH inhibition can produce a decrease in cell viability and induce apoptosis is correct. However, MCF7 displays inconsistent dose response with a negligible decrease in viability triggered at low doses but cellular proliferation triggered at higher dosing. In either case, FAAH inhibition generated a directional viability response, demonstrating that a biological effect can be produced in breast cancer lines by FAAH inhibition alone.

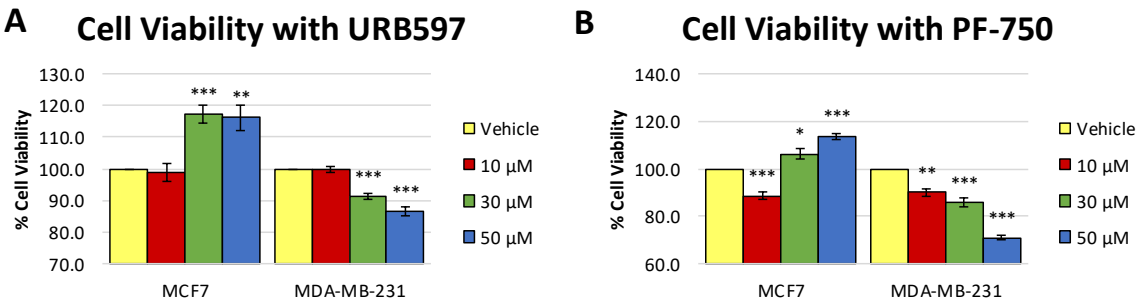

Figure 11: FAAH Inhibition Causes Changes in Cell Viability

In Figure 11, part A, MCF7 and MDA-MB-231 cells were treated with shown concentrations of the selective, irreversible FAAH inhibitor, URB597. In part B, MCF7 and MDA-MB-231 cells were treated with shown concentrations of the selective, irreversible FAAH inhibitor, PF-750. Data are expressed as percentage change in viability as measured by MTT assay over vehicle control and are representative of data collected in two tests performed in quadruplicate. Statistical significance is relative to vehicle control and is assigned as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

In order to determine a potential effector for the change in viability demonstrated in Figure 11, both cell lines were then tested with the eCBs AEA and PEA directly, and again assayed for viability after 24 hours, with results shown in Figure 12. This data demonstrates that on their own, the eCBs AEA and PEA have only marginal effects on MCF7 and MDA-MB-231.

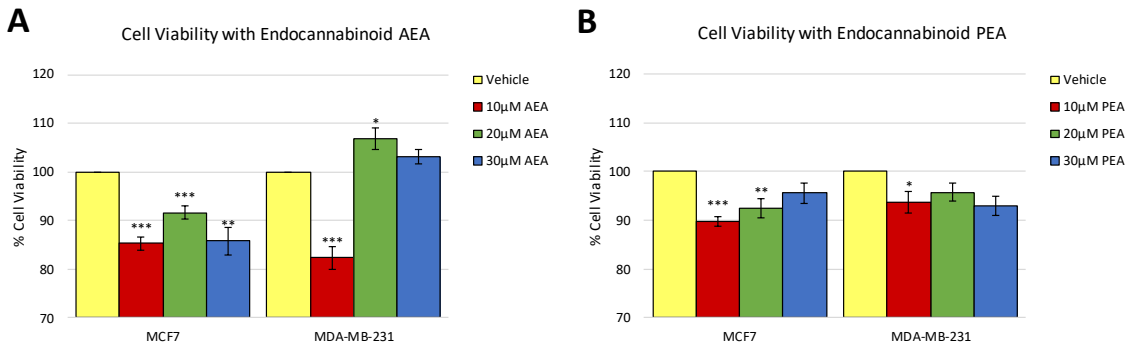

Figure 12: Breast Cancer Cell Viability is Marginally Impacted by Endocannabinoids

In Figure 12, MCF7 and MDA-MB-231 cells treated with shown concentrations of the endocannabinoids AEA in part A and PEA in part B. Data are expressed as percentage change in viability over vehicle control as measured by MTT assay and are representative of data collected in two tests performed in quadruplicate. Statistical significance is relative to vehicle control and is assigned as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

AEA was able to reduce the viability of MCF7 cells marginally, but not in a dose dependent manner. This phenomenon could be due to the rapid degradation of the eCBs following initial dosing of the ligand, which would have been able to activate cell surface receptors CB1, CB2, and TRPV1 upon deposition into the culture vessel, but the supply of AEA would have been degraded by the high concentration of FAAH expressed in MCF7 even when higher concentrations of AEA were introduced. The addition of PEA caused a slight, but uniform drop in viability of both cell lines. These results are in line with previously published work[42].

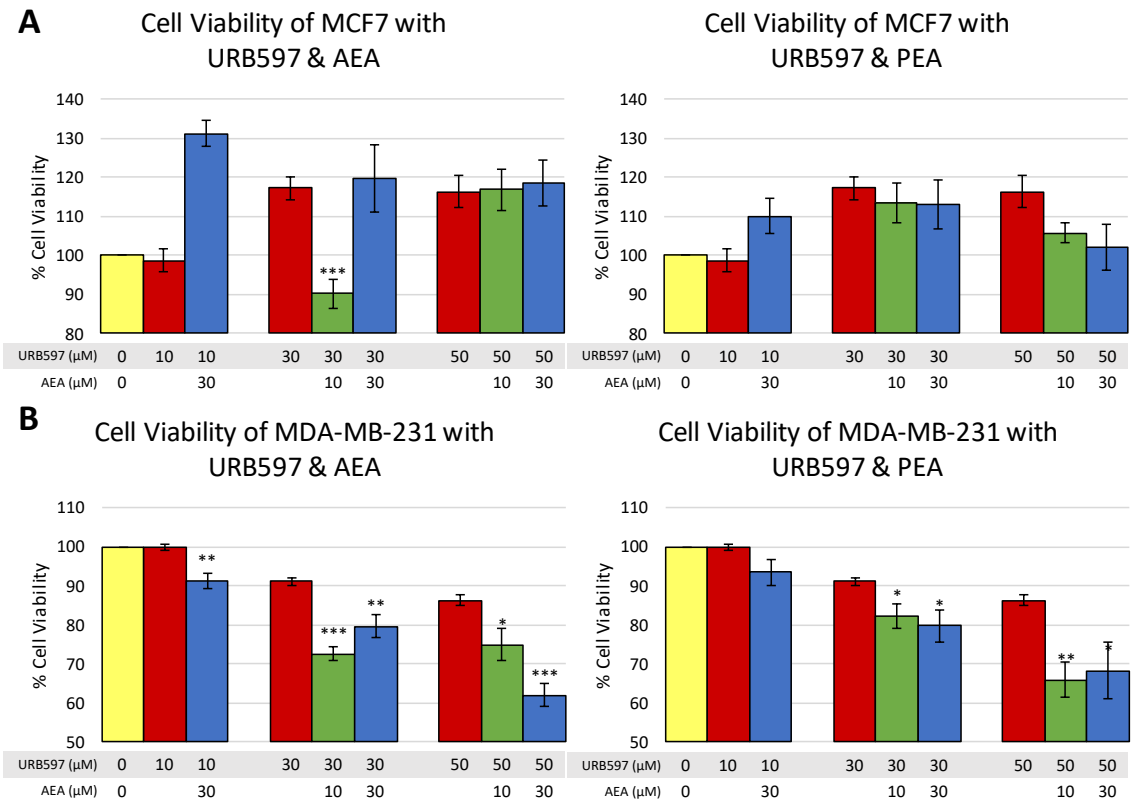

Figure 13: FAAH Inhibited Breast Cancer Lines Respond to Increased Endocannabinoid Loading in a Variable Manner

In Figure 13, the percent viability of MCF7 (A) and MDA-MB-231 (B) cells is shown being treated with concentrations of URB597 with and without the endocannabinoids AEA and PEA. Cells were plated according to materials and methods, then pre-incubated with URB597 for 30 minutes before the addition of AEA & PEA respectively, then incubated for 24 hours. Data are expressed as percentage change in viability over vehicle control as measured by MTT assay and are representative of data collected in two tests performed in quadruplicate. Statistical significance is assigned relative to cells treated with equivalent doses of URB597 alone (relative to bar shown in red) with P-values as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

To further establish the potential effect of system wide FAAH inhibition on a tumor site, each cell line was co-treated with URB597 and eCBs, serving to simulate eCB buildup in cancer tissues. This step was performed because as noted above, MCF7 and MDA-MB-231 produce

noticeably low levels of eCBs on their own in culture[37]. The data, shown in Figure 13, follows the initial dose responses originally shown in Figure 12. MDA-MB-231 clearly demonstrates a decrease in cell viability relative both to vehicle control and the wells containing similar dosing of URB597 alone. Additionally, both AEA and PEA generated roughly the same drop in viability when co-incubated relative to URB597 incubation alone, suggesting a generic effect of endocannabinoid increase that correlates to decreased cell viability. MCF7 results were again somewhat sporadic, but with a clear trend toward increased proliferation after treatment. Of final interest is that FAAH inhibition appears to generate the same pattern of response as co-incubation with eCBs, lending further credence to the hypothesis that FAAH inhibition can produce results similar to previous studies independently of direct addition of eCBs.

Because MCF7 and MDA-MB-231 have been shown to express both CB1 and CB2, both of which have also been shown to potentiate changes in cellular invasion and proliferation, mRNA was isolated from MCF7, MDA-MB-231, and T-47D and reverse transcription and qPCR was performed using primers cataloged in Table 2 to determine expression levels of CB1 and CB2, the results of which are shown in Figure 14.

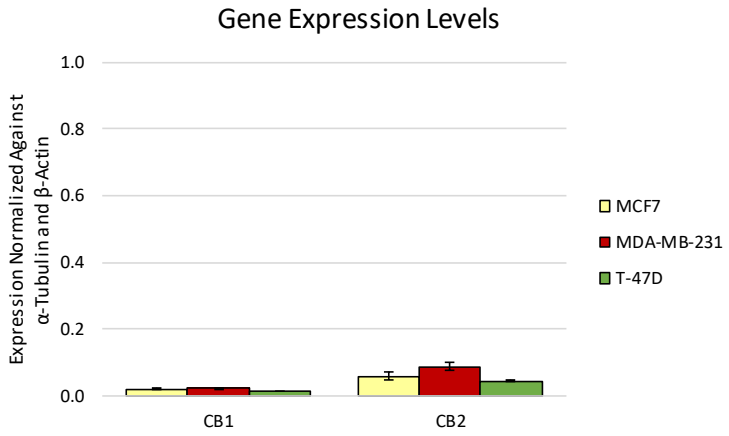

Figure 14: CB1 and CB2 mRNA Expression Levels
Represents mRNA expression normalized against β-actin and α-tubulin. Experiment performed in duplicate and error bars indicate SE.

While trace amounts of CB1 and CB2 mRNA were detected, there was no significant difference between the expression levels of any of the lines to account for the apparent differences in eCB activities. It stands to reason that one of several other members of the eCB pathway must be responsible for the differences in response between these two archetypal cell lines.

While results varied among the breast cancer models tested, the data above show a clear indication that FAAH inhibition can be used to reduce cellular proliferation and induce apoptosis in a similar manner to what has been described elsewhere using the eCB system for at least some breast cancer models. FAAH inhibition is able to reduce cell potential for proliferation and increase pro-apoptotic responses.

3.4 Proposed and Ongoing Studies

In light of the results cataloged above, much work must be done both to validate the above findings and parse them out to their logical conclusion. First, the T-47D cell line must also be tested to determine response rates to FAAH inhibition, while at the same time, more replicates must be tested for each of the viability studies above. While two trials is sufficient to determine an initial course of action, each trial must will be repeated an additional two times to provide reasonable assurances that the data are not artifacts. Further, while cell viability indexes are a commonly used method to ascertain metabolic activity of a culture that can then be extrapolated to assume death or distress in that culture, there is no way to determine with certainty what percentage of cells are actually undergoing apoptosis. Therefore, selected assays will be repeated and tested using flow cytometry paired with viability staining to determine the true percentage of cells undergoing apoptosis.

Moving forward, if FAAH inhibition is indeed a viable option to push cells toward apoptosis, the mechanism through which the reduction in viability acts will need to be better understood.

Several players constitute valid targets, firstly CB1 and CB2, but additionally TRPV1, PPAR family, and cyclooxygenase for reasons described in previous chapters, but also potentially the epidermal growth factor receptor (EGFR) and chemokine receptor 4 (CXCR4). The additional EGFR and CXCR4 pathways have been shown to be activated in breast cancer when eCBs are introduced and could be responsible for the uptick in cell proliferation or for greater cell migration in some cell lines[43], [44].

Planned studies also include a mouse xenograft model to determine the role of FAAH inhibition on tumor growth and metastasis. As in section 2.5 with DLBCL, mice will be implanted with a breast cancer cell line tagged with the florescent marker luciferase to monitoring tumor growth during treatment with the FAAH inhibitor URB597. This study will shine a light on how the complex physiological environment may alter the results obtained *in vitro*.

The data represented here confirms the hypothesis that FAAH inhibition can be used to elicit responses previously attributed solely to eCB dependent pathways, including anti-proliferative and pro-apoptotic responses. With additional research, FAAH inhibition could possibly lead to effective breast cancer treatments.

Chapter 4: Concluding Thoughts

This study represents an opportunity to fill a gap in current treatment options for patients with DLBCL and breast cancer, two cancers that have fairly standard treatment courses that, while generally considered effective, leave gaps for patients that have tumors that are resistant to the standard of care. Due to the incredible heterogeneity presented in each of these cancers and their subsets, no treatment can possibly be effective for every patient, but by investigating FAAH inhibition as an alternate path of attack, it is possible that in the future, we will be able to close some of the gaps by illuminating previously shrouded oncogenic pathways.

While this study represents evidence that FAAH inhibition has the potential to be used as a tool to induce apoptosis in DLBCL and breast cancer cells, at the present time that potential comes with many caveats that must be addressed. First, while the effect of FAAH inhibition on overall levels of eCB substrates in various body tissues has been fairly well documented thanks largely to studies involving FAAH knockout mice[7], [45], the overall impact of the various eCBs are certainly not. Significantly more study on the role of each of the substrates of FAAH is needed. Without greater knowledge related to the functions of each molecular player, this course of study will be largely a shot in the dark. Even here, several lines responded well to treatment with FAAH, resulting in what would be a marked improvement for patients, but due to the dizzying amount of heterogeneous DLBCL and breast cancer subtypes, there will be many tumors that will respond to FAAH inhibition by proliferating instead of dying depending on the particular molecular drivers of that tumor. To advance the potential for use of FAAH inhibitors in cancer treatment, a concerted effort must be made to fully characterize what causes once cell to proliferate while another terminates. Along this line, it will also be important to determine just which apoptotic pathways are activated in each cell line. It has been clear that while CB1 and CB2 can generate apoptotic responses, the vast majority of tissues simply do not possess

CB1 and CB2 but have nonetheless been shown to undergo apoptosis in the presence of FAAH inhibitors or eCBs[46].

Perhaps most exciting about the potential of using FAAH inhibiting drugs as a cancer treatment is in the idea that FAAH inhibition seems to have relatively few side effects other than mild and even potentially desirable analgesic, anti-inflammation, and anti-depressant qualities[47]–[50]. In this capacity, drugs inhibiting FAAH activity make a strong case for combination therapy with existing treatments or for palliative care. Also, inhibition of FAAH has been proposed and explored as a promising therapeutic in several other cancers, with more testing needed, especially in lung and prostate cancers[7], [51]–[54].

References

[1] B. Q. Wei, T. S. Mikkelsen, M. K. McKinney, E. S. Lander, and B. F. Cravatt, “A Second Fatty Acid Amide Hydrolase with Variable Distribution Among Placental Mammals,” *J. Biol. Chem.*, vol. 281, no. 48, pp. 36569–36578, 2006.

[2] F. R. de Fonseca, I. del Arco, F. J. Bermudez-Silva, A. Bilbao, A. Cippitelli, and M. Navarro, “The Endocannabinoid System: Physiology and Pharmacology,” *Alcohol Alcohol.*, vol. 40, no. 1, pp. 2–14, 2005.

[3] M. Sałaga, M. Sobczak, and J. Fichna, “Inhibition of Proteases as a Novel Therapeutic Strategy in the Treatment of Metabolic, Inflammatory and Functional Diseases of the Gastrointestinal Tract,” *Drug Discov. Today*, vol. 18, no. 15–16, pp. 708–715, 2013.

[4] J. E. Schlosburg, S. G. Kinsey, and A. H. Lichtman, “Targeting Fatty Acid Amide Hydrolase (FAAH) to Treat Pain and Inflammation.,” *AAPS J.*, vol. 11, no. 1, pp. 39–44, 2009.

[5] A. S. Bhagwat and C. R. Vakoc, “Targeting Transcription Factors in Cancer,” *Trends in Cancer*, vol. 1, no. 1, pp. 53–65, 2015.

[6] L. Hamtiaux, J. Masquelier, G. G. Muccioli, C. Bouzin, O. Feron, B. Gallez, and D. M. Lambert, “The Association Of N-Palmitoylethanolamine With the FAAH Inhibitor URB597 Impairs Melanoma Growth Through a Supra-Additive Action.,” *BMC Cancer*, vol. 12, no. 1, p. 92, 2012.

[7] M. P. Endsley, R. Thill, I. Choudhry, C. L. Williams, A. Kajdacsy-, W. B. Campbell, and K. Nithipatikom, “Expression and Function of Fatty Acid Amide,” vol. 123, no. 6, pp. 1318–1326, 2008.

[8] V. Gasperi, D. Evangelista, I. Savini, D. Del Principe, L. Avigliano, M. Maccarrone, and M. V. Catani, “Downstream Effects of Endocannabinoid On Blood Cells: Implications for Health And Disease,” *Cell. Mol. Life Sci.*, vol. 72, no. 17, pp. 3235–3252, 2015.

[9] N. Battista, M. Di Tommaso, M. Bari, and M. Maccarrone, “The Endocannabinoid System: An Overview,” *Front Behav Neurosci*, vol. 6, no. March, p. 9, 2012.

[10] M. L. Wolfson, D. O. Muzzio, J. Ehrhardt, A. M. Franchi, M. Zygmunt, and F. Jensen, “Expression Analysis of Cannabinoid Receptors 1 And 2 in B Cells During Pregnancy and Their Role on Cytokine Production,” *J. Reprod. Immunol.*, vol. 116, pp. 23–27, 2016.

[11] M. Maccarrone and A. Finazzi-Agró, “The Endocannabinoid System, Anandamide and the Regulation of Mammalian Cell Apoptosis,” *Cell Death Differ.*, vol. 10, no. 9, pp. 946–955, 2003.

[12] R. Ross, “Anandamide and Vanilloid TRPV1 Receptors.,” *Br. J. Pharmacol.*, vol. 140, no. 5, pp. 790–801, 2003.

[13] S. E. O’Sullivan, “Cannabinoids Go Nuclear: Evidence for Activation of Peroxisome Proliferator-Activated Receptors.,” *Br. J. Pharmacol.*, vol. 152, no. 5, pp. 576–82, 2007.

[14] C. Kuc, A. Jenkins, R. T. Van Dross, and N. Carolina, “Arachidonoyl Ethanolamide (AEA) - Induced Apoptosis is Mediated by J-Series Prostaglandins and is Enhanced by Fatty Acid Amide Hydrolase (FAAH) Blockade,” vol. 149, no. July 2010, pp. 139–149, 2012.

[15] M. Maccarrone, T. Lorenzon, M. Bari, G. Melino, and A. Finazzi-Agro, “Anandamide Induces Apoptosis in Human Cells Via Vanilloid Receptors. Evidence for A Protective Role Of Cannabinoid Receptors,” *J. Biol. Chem.*, vol. 275, no. 41, pp. 31938–31945, 2000.

[16] H. A. Elrod and S. Y. Sun, “PPARγ and Apoptosis in Cancer,” *PPAR Res.*, vol. 2008, 2008.

[17] C. H. Lawrie, S. Gal, H. M. Dunlop, B. Pushkaran, A. P. Liggins, K. Pulford, A. H. Banham, F. Pezzella, J. Boultwood, J. S. Wainscoat, C. S. R. Hatton, and A. L. Harris, “Detection of Elevated Levels of Tumour-Associated Microrna in Serum of Patients With Diffuse Large B-Cell Lymphoma.,” *Br. J. Haematol.*, vol. 141, no. 5, pp. 672–5, 2008.

[18] K. Dybkær, M. Bøgsted, S. Falgreen, J. S. Bødker, M. K. Kjeldsen, A. Schmitz, A. E. Bilgrau, Z. Y. Xu-Monette, L. Li, K. S. Bergkvist, M. B. Laursen, M. Rodrigo-Domingo, S. C. Marques, S. B. Rasmussen, M. Nyegaard, M. Gaihede, M. B. Møller, R. J. Samworth, R. D. Shah, P. Johansen, T. C. El-Galaly, K. H. Young, and H. E. Johnsen, “Diffuse Large B-Cell Lymphoma Classification System That Associates Normal B-Cell Subset Phenotypes With Prognosis,” *J. Clin. Oncol.*, vol. 33, no. 12, pp. 1379–1388, 2015.

[19] F. Jardin, “Classification of Diffuse Large B-Cell Lymphoma by Immunohistochemistry Demonstrates That Elderly Patients Are More Common in the Non-GC Subgroup and Younger Patients in the GC Subgroup (Reply),” *Haematologica*, vol. 97, no. 2, p. 6769, 2012.

[20] M. a Shipp, K. N. Ross, P. Tamayo, A. P. Weng, J. L. Kutok, R. C. T. Aguiar, M. Gaasenbeek, M. Angelo, M. Reich, G. S. Pinkus, T. S. Ray, M. a Koval, K. W. Last, A. Norton, T. A. Lister, J. Mesirov, D. S. Neuberg, E. S. Lander, J. C. Aster, and T. R. Golub, “Diffuse Large B-Cell Lymphoma Outcome Prediction by Gene-Expression Profiling and Supervised Machine Learning.,” *Nat. Med.*, vol. 8, no. 1, pp. 68–74, 2002.

[21] J. Zhang, D. Medina-cleghorn, L. Bernal-mizrachi, P. M. Bracci, A. Hubbard, L. Conde, J. Riby, D. K. Nomura, and C. F. Skibola, “The Potential Relevance of the Endocannabinoid, 2-Arachidonoylglycerol , In Diffuse Large B-Cell Lymphoma,” *Oncoscience*, vol. 3, no. 1, 2016.

[22] T. Sugiura, S. Kondo, T. Miyashita, T. Kodaka, Y. Suhara, H. Takayama, and K. Waku, “CARBOHYDRATES , LIPIDS , AND OTHER NATURAL PRODUCTS: Evidence That 2-Arachidonoylglycerol but Not N -Palmitoylethanolamine or Anandamide Is the Physiological Ligand for the Cannabinoid CB2 Receptor: COMPARISON OF THE AGONISTIC ACTIVITIES OF VARIOUS CANN,” *J. Biol. Chem.*, vol. 275, no. 1, pp. 605–12, 2000.

[23] B. Herrera, A. Carracedo, M. Diez-Zaera, M. Guzman, and G. Velasco, “p38 MAPK is Involved in CB2 Receptor-Induced Apoptosis of Human Leukaemia Cells,” *FEBS Lett.*, vol. 579, no. 22, pp. 5084–5088, 2005.

[24] R. J. Mckallip, C. Lombard, M. Fisher, B. R. Martin, S. Ryu, S. Grant, P. S. Nagarkatti, M. Nagarkatti, W. Dc, R. J. Mckallip, C. Lombard, M. Fisher, B. R. Martin, S. Ryu, S. Grant, P. S. Nagarkatti, and M. Nagarkatti, “Targeting CB2 cannabinoid receptors as a Novel Therapy To Treat Malignant Lymphoblastic Disease,” vol. 100, no. 2, pp. 627–634, 2013.

[25] S. Jiang, Y. Fu, and H. K. Avraham, “Regulation of Hematopoietic Stem Cell Trafficking and Mobilization by the Endocannabinoid System,” *Transfusion*, vol. 51, no. SUPPL. 4, pp. 65–71, 2011.

[26] M. K. McKinney and B. F. Cravatt, “Structure and Function of Fatty Acid Amide Hydrolase,” *Annu. Rev. Biochem.*, vol. 74, no. 1, pp. 411–432, 2005.

[27] A. Spandidos, X. Wang, H. Wang, and B. Seed, “PrimerBank: A Resource of Human and Mouse PCR Primer Pairs for Gene Expression Detection and Quantification,” *Nucleic Acids Res.*, vol. 38, no. SUPPL.1, pp. 792–799, 2009.

[28] A. H. Benz, C. Renne, E. Maronde, M. Koch, U. Grabiec, S. Kallendrusch, B. Rengstl, S. Newrzela, S. Hartmann, M. L. Hansmann, and F. Dehghani, “Expression and Functional Relevance of Cannabinoid Receptor 1 in Hodgkin Lymphoma,” *PLoS One*, vol. 8, no. 12, 2013.

[29] J. M. Sido, P. S. Nagarkatti, and M. Nagarkatti, “Production of Endocannabinoids by Activated T Cells and B Cells Modulates Inflammation Associated With Delayed Type Hypersensitivity.,” *Eur. J. Immunol.*, pp. 1472–1479, 2016.

[30] B. F. Cravatt, K. Demarest, M. P. Patricelli, M. H. Bracey, D. K. Giang, B. R. Martin, and A. H. Lichtman, “Supersensitivity to anandamide and Enhanced Endogenous Cannabinoid Signaling In Mice Lacking Fatty Acid Amide Hydrolase.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 98, no. 16, pp. 9371–6, 2001.

[31] S. Oesch and J. Gertsch, “Cannabinoid Receptor Ligands As Potential Anticancer Agents — High Hopes For New Therapies?,” *J. Pharm. Pharmacol.*, vol. 61, pp. 839–853, 2009.

[32] S. Hart, O. M. Fischer, and A. Ullrich, “Cannabinoids Induce Cancer Cell Proliferation Via Tumor Necrosis Factor Alpha-Converting Enzyme (TACE/ADAM17)-Mediated Transactivation Of The Epidermal Growth Factor Receptor,” *Cancer Res.*, vol. 64, no. 6, pp. 1943–1950, 2004.

[33] J. Guindon and A. G. Hohmann, “The Endocannabinoid System And Cancer: Therapeutic Implication,” *Br. J. Pharmacol.*, vol. 163, no. 7, pp. 1447–1463, 2011.

[34] M. Uhlén, L. Fagerberg, B. M. Hallström, C. Lindskog, P. Oksvold, A. Mardinoglu, Å. Sivertsson, C. Kampf, E. Sjöstedt, A. Asplund, I. Olsson, K. Edlund, E. Lundberg, S. Navani, C. A.-K. Szigyarto, J. Odeberg, D. Djureinovic, J. O. Takanen, S. Hober, T. Alm, P.-H. Edqvist, H. Berling, H. Tegel, J. Mulder, J. Rockberg, P. Nilsson, J. M. Schwenk, M. Hamsten, K. von Feilitzen, M. Forsberg, L. Persson, F. Johansson, M. Zwahlen, G. von Heijne, J. Nielsen, and F. Pontén, "Tissue-Based Map of the Human Proteome," *Science (80-.).*, vol. 347, no. 6220, Jan. 2015.

[35] D. Melck, D. Rueda, I. Galve-Roperh, L. De Petrocellis, M. Guzmán, and V. Di Marzo, "Involvement of the cAMP/Protein Kinase A Pathway and of Mitogen-Activated Protein kinase in the Anti-Proliferative Effects of Anandamide in Human Breast Cancer Cells.," *FEBS Lett.*, vol. 463, no. 3, pp. 235–240, 1999.

[36] V. Di Marzo, C. S. Breivogel, Q. Tao, D. T. Bridgen, R. K. Razdan, A. M. Zimmer, A. Zimmer, and B. R. Martin, "Levels, Metabolism, and Pharmacological Activity of Anandamide in CB1 Cannabinoid Receptor Knockout Mice: Evidence For Non-CB1, Non-CB2 Receptor-Mediated Actions of anandamide in Mouse Brain," *J. Neurochem.*, vol. 75, no. 6, pp. 2434–2444, 2000.

[37] H. Li, J. T. Wood, K. M. Whitten, S. K. Vadivel, S. Seng, A. Makriyannis, and H. K. Avraham, "Inhibition of fatty Acid Amide Hydrolase Activates Nrf2 Signalling and induces Heme Oxygenase 1 Transcription in Breast Cancer Cells," *Br. J. Pharmacol.*, vol. 170, no. 3, pp. 489–505, 2013.

[38] G. Alpini and S. DeMorrow, “Changes in the Endocannabinoid System May Give Insight into new and Effective Treatments for Cancer,” *Vitam. Horm.*, vol. 81, no. C, pp. 469–485, 2009.

[39] J. Kao, K. Salari, M. Bocanegra, Y. La Choi, L. Girard, J. Gandhi, K. A. Kwei, T. Hernandez-Boussard, P. Wang, A. F. Gazdar, J. D. Minna, and J. R. Pollack, “Molecular Profiling of Breast Cancer Cell Lines Defines Relevant Tumor Models and Provides A Resource for Cancer Gene Discovery,” *PLoS One*, vol. 4, no. 7, 2009.

[40] J. C. Kathryn, G. Sireesha V, and L. Stanley, “Triple Negative Breast Cancer Cell Lines: One Tool in the Search for Better Treatment of Triple Negative Breast Cancer,” *Breast Dis*, vol. 32, pp. 35–48, 2012.

[41] D. L. Holliday and V. Speirs, “Choosing the Right Cell Line for Breast Cancer Research,” *Breast Cancer Res.*, vol. 13, no. 4, pp. 1–7, 2011.

[42] V. Di Marzo, D. Melck, P. Orlando, T. Bisogno, O. Zagory, M. Bifulco, Z. Vogel, and L. de Petrocellis, “Palmitoylethanolamide Inhibits the Expression of Fatty Acid Amide Hydrolase and Enhances the Anti-Proliferative Effect of Anandamide in Human Breast Cancer Cells,” *Biochem. J.*, vol. 358, no. 1, pp. 249–255, 2001.

[43] M. W. Nasser, Z. Qamri, Y. S. Deol, D. Smith, K. Shilo, X. Zou, and R. K. Ganju, “Crosstalk Between Chemokine Receptor CXCR4 and Cannabinoid Receptor CB 2 in Modulating Breast Cancer Growth and Invasion,” *PLoS One*, vol. 6, no. 9, pp. 1–9, 2011.

[44] Z. Qamri, A. Preet, M. W. Nasser, C. E. Bass, G. Leone, S. H. Barsky, and R. K. Ganju, “Synthetic Cannabinoid Receptor Agonists Inhibit Tumor Growth and Metastasis of Breast Cancer.,” *Mol. Cancer Ther.*, vol. 8, no. 11, pp. 3117–29, 2009.

[45] S. Ortega-Gutierrez, E. G. Hawkins, A. Viso, M. L. Lopez-Rodriguez, and B. F. Cravatt, "Comparison of Anandamide Transport in FAAH Wild-Type and Knockout Neurons: Evidence for Contributions by Both FAAH and the CB1 Receptor to Anandamide Uptake," *Biochemistry*, vol. 43, no. 25, pp. 8184–8190, 2004.

[46] I. Svíženská, P. Dubový, and A. Šulcová, "Cannabinoid Receptors 1 and 2 (CB1 and CB2), Their Distribution, Ligands and Functional Involvement in Nervous System Structures — A Short Review," *Pharmacol. Biochem. Behav.*, vol. 90, no. 4, pp. 501–511, 2008.

[47] J. R. Clapper, G. Moreno-Sanz, R. Russo, A. Guijarro, F. Vacondio, A. Duranti, A. Tontini, S. Sanchini, N. R. Sciolino, J. M. Spradley, A. G. Hohmann, A. Calignano, M. Mor, G. Tarzia, and D. Piomelli, "Anandamide Suppresses Pain Initiation Through a Peripheral Endocannabinoid Mechanism.," *Nat. Neurosci.*, vol. 13, no. 10, pp. 1265–1270, 2010.

[48] O. Sasso, R. Bertorelli, T. Bandiera, R. Scarpelli, G. Colombano, A. Armirotti, G. Moreno-Sanz, A. Reggiani, and D. Piomelli, "Peripheral FAAH Inhibition Causes Profound Antinociception and Protects Against Indomethacin-Induced Gastric Lesions," *Pharmacol. Res.*, vol. 65, no. 5, pp. 553–563, 2012.

[49] M. Solinas, G. Tanda, Z. Justinova, C. E. Wertheim, S. Yasar, D. Piomelli, S. K. Vadivel, A. Makriyannis, and S. R. Goldberg, "The Endogenous Cannabinoid Anandamide Produces delta-9-tetrahydrocannabinol-like Discriminative and Neurochemical Effects that are Enhanced by Inhibition of Fatty Acid Amide Hydrolase but Not by Inhibition of Anandamide Transport.," *J. Pharmacol. Exp. Ther.*, vol. 321, no. 1, pp. 370–380, 2007.

[50] S. Pisanti, A. M. Malfitano, C. Grimaldi, A. Santoro, P. Gazzerro, C. Laezza, and M. Bifulco, "Use of Cannabinoid Receptor Agonists in Cancer Therapy as Palliative and Curative

Agents," *Best Pract. Res. Clin. Endocrinol. Metab.*, vol. 23, no. 1, pp. 117–131, 2009.

[51] L. Thors, A. Bergh, E. Persson, P. Hammarsten, P. Stattin, L. Egevad, T. Granfors, and C. J. Fowler, "Fatty acid amide hydrolase in prostate cancer: Association With Disease Severity and Outcome, CB1 Receptor Expression and Regulation by IL-4," *PLoS One*, vol. 5, no. 8, 2010.

[52] J. Ravi, A. Sneh, K. Shilo, M. W. Nasser, and R. K. Ganju, "FAAH Inhibition Enhances Anandamide Mediated Anti-Tumorigenic Effects in Non-Small Cell Lung Cancer by Downregulating The EGF/EGFR Pathway," *Oncotarget*, vol. 5, no. 9, pp. 2475–2486, 2014.

[53] K. Winkler, R. Ramer, S. Dithmer, I. Ivanov, J. Merkord, and B. Hinz, "Fatty Acid Amide Hydrolase Inhibitors Confer Anti-Invasive and Antimetastatic Effects On Lung Cancer Cells," *Oncotarget*, vol. 7, no. 12, pp. 15047–15064, 2016.

[54] L. Hamtiaux, J. Masquelier, G. G. Muccioli, C. Bouzin, O. Feron, B. Gallez, and D. M. Lambert, "The Association of N-palmitoylethanolamine With the FAAH Inhibitor URB597 impairs Melanoma Growth Through a Supra-Additive Action.," *BMC Cancer*, vol. 12, no. 1, p. 92, 2012.