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METABOLISM OF OMEGA-3 ENDOCANNABINOIDS BY CYTOCHROME P450s AND
THE ROLE OF THEIR METABOLITES IN NEURODEGENERATIVE DISEASES

BY

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DISSERTATION

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ABSTRACT

There has been growing interest in unveiling the mechanisms by which nutritional status influences the immune response, especially related to the consumption of omega-3 fatty acids. Cytochrome P450 (CYP) epoxygenases are versatile heme-containing enzymes capable of metabolizing xenobiotics, sterols, fatty acids, eicosanoids, and vitamins. The omega-3 and omega-6 fatty acid metabolizing CYPs produce anti-inflammatory and immunomodulatory lipid mediators. Furthermore, lipids also serve as a key structural component for biological function. Therefore, the research in this dissertation focuses on three major areas: 1) unveiling the biological properties of omega-3 endocannabinoid lipid mediators with respect to T-cell activity in a multiple sclerosis animal model, 2) identifying how CYP-mediated metabolism can be influenced by xenobiotics, and 3) unraveling the potential role of structural lipids in ensuring homeostatic function in neurodegenerative conditions induced by influenza virus.

Chapter 2 is the investigation of the immunomodulatory capability endocannabinoids possess in inflammatory and neurodegenerative diseases such as multiple sclerosis. While epidemiological studies correlated dietary intake of omega-3 fatty acids such as docosahexaenoic acid (DHA) with improved quality of life in multiple sclerosis (MS), the precise mechanism is not well understood. As DHA can be endogenously metabolized to form endocannabinoid lipid mediators, the goal of this study was to identify how endocannabinoids can modulate neuroinflammatory responses in a preclinical model of MS. In this study, we discovered that docosapentaenoyl ethanolamine (DHEA) and epoxydocosapentaenoyl ethanolamine reduces Th1 and Th17 polarization, both of which contribute to the pathogenesis of MS. In a preclinical animal model of MS, we discover that DHEA delays the disease onset, time to relapse, and the overall percentage of infiltrating CD4⁺ T-cells in the central nervous system. Collectively, we

identify the relationship of endocannabinoids and CD4⁺ T-cells in a neuroinflammatory disease model.

In Chapter 3, we explore how Cytochrome P450 2J2 (CYP2J2) mediated metabolism of arachidonic acid to form cardioprotective endocannabinoid epoxyeicosatrienoic acids can be influenced by xenobiotics. Specifically, anthracycline chemotherapeutics are highly effective, but their clinical usefulness is hindered by adverse side effects such as cardiotoxicity. As CYP2J2 is expressed in the heart and can convert arachidonic acid to cardioprotective epoxyeicosatrienoic acid (EET) regioisomers, we performed biochemical studies to understand the interaction of anthracycline derivatives with CYP2J2. Through biochemical assays and iterative ensemble docking, we unveil the interactions of anthracycline derivatives with CYP2J2. Collectively, these studies will help identify alternative mechanisms of how anthracycline cardiotoxicity may be mediated through the inhibition of cardiac P450, and broadly provides insight into how endocannabinoid production can be influenced by xenobiotics.

In Chapter 4, we explore the role of lipids as structural components in the myelin. The notion that myelin remains static during adulthood has been challenged in recent years, and is crucial for proper cognitive function, behavior, and can be influenced by external factors. As peripheral infection and subsequent systemic inflammation can exacerbate neurological diseases, we explore how an upper respiratory infection can alter the myelin lipidome in an animal model. In this study, we infect C57BL/6 mice with mouse-adapted human influenza A virus and isolate the myelin from the brain. Using a shotgun lipidomic approach, we determine that a majority of lipid classes with the exception of sphingosine and triglycerides increase as a result of infection. Importantly, we identify those lipids that are differentially expressed belong to the glycerophospholipid and sphingolipid signaling pathways. As these lipids play a critical role in

the structural integrity of myelin, we identify how upper respiratory infection can cause changes to the biochemical and anatomical characteristics of myelin.

In summary, our work presented in this dissertation advances our understanding of the role of structural lipids and lipid mediators in the larger context of neurodegenerative and autoimmune disease states.

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CHAPTER 1: INTRODUCTION

1.1 THE ENDOCANNABINOID SYSTEM

Cannabis sativa historically has been used for both medical and recreational purposes and has been gaining popularity recently for its beneficial health effects^{1, 2}. In the early 1950s, Δ^9 -tetrahydrocannabinol (Δ^9 -THC), the primary psychoactive molecule, was isolated from *Cannabis sativa*³. This led to a strong interest in the synthesis of cannabinoid (CB) analogs to understand the structure-activity relationship with putative CB receptors⁴⁻⁸. Eventually, two G-protein coupled receptors (GPCRs), cannabinoid receptor 1 and 2 (CB₁ and CB₂), were discovered^{9, 10}. While CB₁ is highly expressed in the central nervous system (CNS)¹¹, CB₂ is present primarily in immune cells and others outside of the CNS¹². Since Δ^9 -THC and other CBs that bind to CB₁ and CB₂ are lipophilic, there was a search for endogenous lipids that interacted with these receptors.

The first endogenous cannabinoids, or endocannabinoids (eCBs), that were shown to activate CB₁ were anandamide (AEA) and 2-arachidonoylglycerol (2-AG)¹³⁻¹⁵. AEA and 2-AG are synthesized endogenously from omega-6 polyunsaturated fatty acid (PUFA), arachidonic acid (AA). Similarly, there are endocannabinoids derived from omega-3 fatty acids, such as docosahexaenylethanolamine (DHEA). While these endocannabinoids can activate cannabinoid receptors, they are also susceptible to enzymatic degradation in the body. For instance, ethanolamide derivatives (AEA or DHEA) can be hydrolyzed by fatty acid amide hydrolase (FAAH), whereas glycerol derivatives such as 2-AG can be degraded by monoacylglycerol lipases (MAGL). In addition, these endocannabinoids can be further metabolized by eicosanoid synthesizing enzymes such as lipoxygenases (LOX), cyclooxygenase 2 (COX-2) and cytochrome P450 epoxygenases (CYP450) to form new bioactive molecules^{16, 17}. Together, the

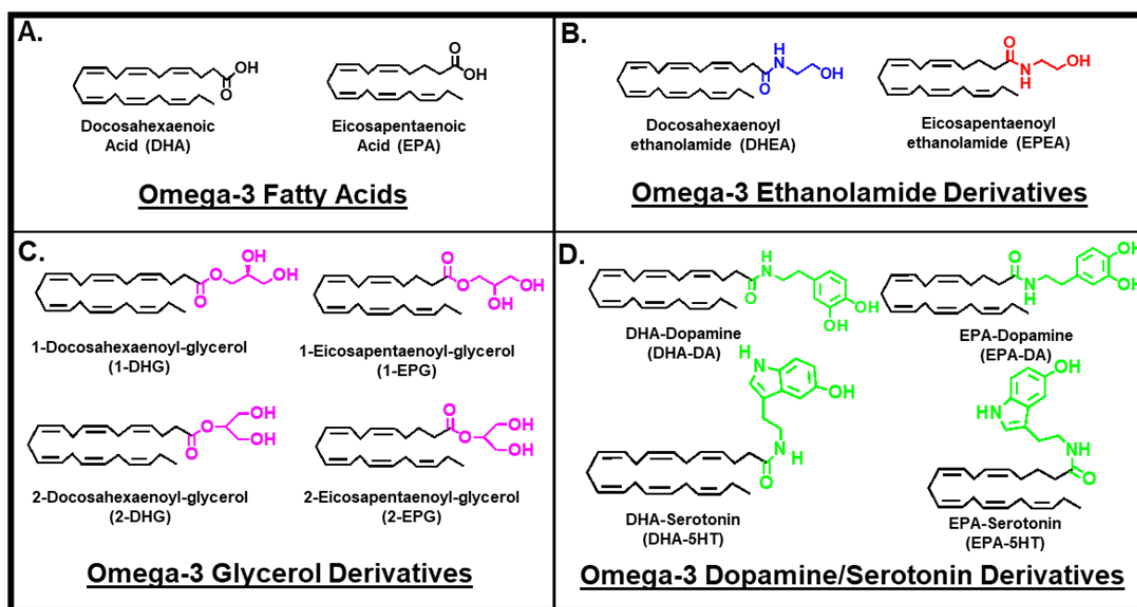
interactions between the CB receptors, eCB ligands, as well as their biosynthetic and degradative enzymes, constitute the eCB system (ECS) and are involved in maintaining homeostasis in the biological system ¹⁸.

Broadly, the eCBs are members of a large group of structurally related fatty acid derived signaling molecules. It is important to note that eCBs are traditionally defined as ligands with significant affinity and agonism to CB₁ and CB₂ according to the Nomenclature and Standards Committee of the International Union of Basic and Clinical Pharmacology (NC-IUPHAR) committee ¹⁹. This definition resulted in the discovery and synthesis of several other eCB and “eCB-like” analogues ²⁰⁻²³. Thus, many of these fatty acid derived molecules are eCB-like ligands that have weak affinity to CB receptors and cannot be considered as classical eCBs. Additionally, as the definition of the ECS system is expanding, it is currently known that CBs and other eCB-like compounds, such as N-arachidonylglycine (NA-Gly), can target more receptors such as transient receptor potential cation channel subfamily V member 1 (TRPV1) or G-protein-coupled receptors (GPR) GPR18, GPR119 and GPR55 ²⁴⁻²⁸.

With the increasing focus towards greater consumption of omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), there has been growing interest in the discovery of omega-3 fatty acid derived eCB, or eCB-like molecules with novel bioactivity²⁹. Currently, there exist many endogenously discovered endocannabinoids such as glycerol derivatives, or more recently discovered dopamine and serotonin derivatives³⁰. The most well studied omega-3 endocannabinoids are the DHA-derived ethanolamide derivative, where they have been shown to increase synaptic activity, promote neurite growth and axon development, and increase cortical neuron growth^{26, 31}. In the periphery the DHA-derived

ethanolamide derivative, or docosahexaenoyl ethanolamide (DHEA), reduced inflammation macrophages, and reduced headache severity^{32, 33}.

More recently, a new endocannabinoid derived from DHEA was discovered³⁴. DHEA metabolized by cytochrome P450 epoxygenases form corresponding epoxides at the olefin position to form any one of six epoxydocosahexaenoyl ethanolamide (DHEA-Epoxy) endocannabinoids. Specifically, the DHEA-Epoxy with a corresponding epoxide at the 19-20 carbon position reduced pro-inflammatory nitric oxide (NO) and IL-6, while increasing anti-inflammatory IL-10 production in microglia cultures. Notably, results from cannabinoid activation assays performed in this study showed that DHEA-Epoxyes were more potent and a stronger activator of cannabinoid receptors than other endocannabinoids³⁴. Collectively, these data suggest that omega-3 fatty acids may get converted into DHEA-Epoxyes within the body to elicit the beneficial health properties observed with high omega-3 consumption.



Watson, Kim, and Das. *POLM* 2019.

Figure 1. Structures of emerging class of omega-3 metabolites. (A) Docosahexaenoic acid and eicosapentaenoic acid precursors to **(B)** ethanolamide derivatives, **(C)** glycerol derivatives, and **(D)** dopamine and serotonin derivatives.

1.2 MULTIPLE SCLEROSIS

History of Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic inflammatory and neurodegenerative disease of the central nervous system (CNS). The first evidence of MS dates back to the 14th century, but it was not until the 19th century upon which the disease was described³⁵. While Robert Carswell and Jean Cruveilhier are among the first to describe neuronal lesions similar to that of MS during years 1835-1838, Jean Martin Charcot is credited with the description of the disease in 1868³⁶. Charcot, also known as the Father of Neurology, was the first to correlate clinical manifestations of MS and pathological changes post-mortem³⁵.

According to the most recent global assessment representing 87% of the world's population, there has been an increase in the prevalence of this disease, with a current total of 2.8 million people living with MS³⁷. This equates to an incidence of nearly 1 in 3,000 people worldwide with MS, and as high as 1 in 300 people in countries with the highest prevalence³⁷. Interestingly, early studies indicate an increase of prevalence at higher latitudes³⁸⁻⁴⁰. Specifically within the United States, there exists a higher prevalence in northern states compared to the southern states³⁹. Authors of a later review observed that for every 10 degree increase of latitude away from the equator, there was a corresponding incidence of 30% in women, and 50% in men⁴¹. These associations of latitude gradients and prevalence suggest that environmental factors play a role in the etiology of MS. Such environmental factors include low vitamin D and lack of sun exposure⁴².

Genetic Influence

There is overwhelming evidence of genetic involvement with MS. For instance, it is well established that there exists a propensity for females rather than males, where MS is three times more common in women than in men⁴³⁻⁴⁶. While prevalence is higher in females, males for unknown reasons have a greater progression of disability^{47, 48}. In addition to sex, there are familial genetic factors that contribute to MS that was first postulated by Eichhorst in 1896^{49, 50}. Family recurrence rates of MS are approximately 15%⁵¹. While first degree relatives have increased risk^{52, 53}, there is an associated decrease with genetic distance⁵⁴. Studies show higher concordance rates for monozygotic twins (24-25%) than dizygotic twins (3-5%)^{55, 56}.

Natural History of Multiple Sclerosis

The natural history of multiple sclerosis is heterogeneous across patients⁵⁷⁻⁵⁹. The relapse-remitting (RRMS) course of MS occurs in approximately 85% of patients⁶⁰, where there are periods of flare-up of symptoms (relapse) followed by periods of remission. Initial symptoms at onset may include optic neuritis, as well as brainstem and spinal cord syndrome leading to ataxia, vertigo, or involuntary movement⁶¹. During relapse, lesions can be visualized using magnetic resonance imaging (MRI). Specifically, gadolinium-enhancing T1-MRI imaged lesions at relapse indicates a breakdown of the blood brain barrier (BBB), and T-2MRI imaged lesions indicate subpial demyelination and atrophy⁶².

Over time, a large proportion of patients with RRMS will develop secondary progressive multiple sclerosis (SPMS). While there is a decrease of occurrence of new T2 lesions as patients transition from RRMS to SPMS, there is a worsening of symptoms. It is difficult to identify when a patient has transitioned from RRMS to SPMS as there can be new lesions during the

SPMS phase of the disease⁶³. Clinically however, it is characterized as a progression of symptoms with few or no relapses⁴⁷. The last form of MS is primary progressive multiple sclerosis (MS), and is present in approximately 10% of patients⁶³. PPMS is characterized by gradual worsening of neurological function from the onset of symptoms without relapses or remission⁶³.

Animal Models of Multiple Sclerosis

Multiple sclerosis is a complex disease that is influenced by both genetic and environmental factors. Furthermore, the etiology is not fully understood. While it is difficult to fully replicate the human disease in animal models due to the complexity of the disease, there are several models that mimic pathological outcomes of multiple sclerosis.

Experimental autoimmune encephalomyelitis (EAE) is the most extensively utilized *in vivo* model to study multiple sclerosis. EAE is a model by which there is immunization with self-antigens derived from myelin-specific proteins. Immunization of SJL/J mice with proteolipid protein 139-151 (PLP₁₃₉₋₁₅₁) possess a translational value of relapse-remitting MS⁶⁴⁻⁶⁶, whereas immunization of C57BL/6J mice with myelin oligodendrocyte glycoprotein 35-55 (MOG₃₅₋₅₅) is reflective of a progressive form of MS⁶⁷⁻⁶⁹. Considering approximately 85% of MS patients have the RRMS form⁶⁰, EAE in SJL/J mice resemble the closest model due to its similar relapsing remitting disease course. In rodents, clinical disability is measured as previously described⁷⁰. On a 5-point scale, mice are scored from having no overt signs of disease to death. The gradual progression however includes tail weakness, followed by tail paralysis, limb weakness, and eventual limb paralysis⁷⁰. Key pathological features of EAE pathogenesis include infiltration of Th1 and Th17 T-cells into the CNS, like that of MS in humans⁷¹. Furthermore, concentrations of

inflammatory immune cells around lesions, and the lesions present in the white matter closely resemble that in humans⁷¹. As a result, EAE is a strong model for studying how neuroinflammation leads to demyelination of the CNS.

Viral models such as Theiler's murine encephalomyelitis virus (TMEV) are also used to study how viruses can promote inflammatory demyelination⁷²⁻⁷⁴. TMEV is a picornavirus that results in a biphasic response of early acute encephalomyelitis, followed by chronic immune-mediated demyelination^{73, 75}. Importantly, there are inflammatory infiltrates at lesions similar to human MS patients, and thus is a strong model for studying inflammatory demyelination⁷³. In contrast, other models such as the cuprizone model are better suited for studying the remyelination process. While cuprizone-fed mice have previously been used to study demyelination, the response is a result of an artificial copper chelator that reproducibly induces toxic demyelination of specific anatomical regions⁷⁶. After withdrawal of the toxicant from the food, remyelination ensues⁷⁷. There is resulting oligodendrocyte apoptosis, however the precise mechanism by which cuprizone-induced demyelination occurs is unknown^{78, 79}. As a result, cuprizone-induced models are not strong for studying demyelination, but rather is suitable for studying remyelination.

1.3 STRUCTURAL AND FUNCTIONAL LIPIDS

Classification of Lipids

Lipids are essential to maintaining homeostatic function of the body. Lipids can be defined as an organic biological molecule soluble in nonpolar solvents⁸⁰. Common types of lipids include triglycerides, phospholipids, waxes, and sterols⁸¹. These various lipids possess different

roles within our body acting as either functional lipids or structural lipids. Here, the various roles lipids have in our body are briefly discussed.

Structural Lipids

Lipids play a critical role as structural components in our body. For instance, eukaryotic membrane bilayers are composed of amphipathic structural lipids such as glycerophospholipids. Glycerophospholipids contain a glycerol backbone and hydrophilic fatty acid tails esterified to various head groups. Importantly, these amphipathic lipids allow for the formation of a barrier for cells, and compartmentalize organelles within cells to allow for discrete functional units in the cell⁸². There is a wide diversity in these types of structural lipids including their backbone, chain length, and degree of saturation. In addition to phospholipids, other classifications of lipids that play a major role in membrane structure include sphingolipids and sterol lipids.

Structural lipids play an important role in maintaining healthy function in our central nervous system. For instance, myelin is an insulating layer surrounding axons that increases neuronal conduction and provides trophic support for axons⁸³. Myelin is approximately 70% lipids and 30% proteins that work in conjunction to insulate axons⁸⁴. In this respect, lipids play a critical role by spanning and keeping intact multi-lamellar layers, while simultaneously providing a hydrophobic side on the outer lamellar layer. For instance, 2-hydroxylated sphingolipids play a critical role in maintaining a hydrophobic outer lamellar layer that aids in the compaction of myelin⁸⁵. Studies demonstrate that the absence of 2-hydroxylated sphingolipids result in myelin degeneration. Additionally, other transgenic mice lacking the ability to form galactosylceramide lipids develop tremors, uncoordinated gait, and eventual paralysis⁸⁶, all of which are indications of dysmyelination.

Functional Lipids

Functional lipids are broadly referenced as molecules that possess bioactive properties. For instance, lipids in the diet are predominantly in the form of triglycerides that provide energy when consumed. Within the human body however, triglycerides can serve as an energy source, or an energy reserve. Broadly, when excess calories are consumed, triglycerides are synthesized and stored as an energy reserve. In scenarios of acute and prolonged starvation, triglycerides are broken down to serve as an energy source for the body.

Lipids can serve as functional lipids by acting on signaling pathways as secondary messengers. Secondary messenger lipids can be released from lipid stores or be produced as an end product. One pathway in which this occurs is following the activation of the phosphoinositide signaling pathway by which diacylglycerol and inositol 1,4,5-triphosphate are produced. Diacylglycerol can activate protein kinase C⁸⁷, while inositol 1,4,5-triphosphate can bind to receptors on the endoplasmic reticulum to release calcium⁸⁸. The regulation of these secondary messenger lipids and their action have direct implications to maintaining homeostasis⁸⁷.

Alternatively, lipids can act as mediators of the immune system through the formation of eicosanoids. For instance, omega-6 arachidonic acid can be metabolized by the cyclooxygenase (COX), lipoxygenase (LOX), and epoxygenase (EPOX) pathway. Prostaglandins formed through the COX pathway are lipid autacoids that can mediate inflammatory processes, platelet aggregation, and regulate the vasculature⁸⁹. The LOX pathway can produce leukotrienes that also mediate inflammatory processes and influence constriction in the smooth muscle⁹⁰. Lastly, the EPOX pathway can generate epoxide molecules such as epoxygenic acids or

epoxydocosapentaenoyl ethanolamine that are widely implicated in mediating inflammation and other physiological effects^{91, 92}.

CHAPTER 2: ROLE OF OMEGA-3 ENDOCANNABINOIDS IN THE MODULATION OF T-CELL ACTIVITY IN A MULTIPLE SCLEROSIS EAE MODEL

2.1 ABSTRACT

Epidemiological studies show that omega-3 fatty acid consumption is associated with improved conditions in neurodegenerative diseases such as multiple sclerosis (MS). However, the mechanism of this association is not well understood. Emerging evidence suggests that parent molecules such as docosahexaenoic acid (DHA) are converted into downstream metabolites that are capable of directly modulating immune responses. *In vitro*, we found that docosahexaenoyl ethanolamide (DHEA), another dietary component and its epoxide metabolite reduced the polarization of naïve T-cells toward Th1 and Th17 phenotypes. Furthermore, we identified that DHEA and related endocannabinoids are changing during the disease progression in mice undergoing RR-EAE. Additionally, daily administration of DHEA to mice delayed the onset of disease, the rate of relapse, and the severity of clinical scores at relapse in relapse-remitting EAE (RR-EAE), an animal model of multiple sclerosis. Collectively, these data indicate that DHEA and their metabolites reduce the disease severity in the RR-EAE model of MS and can be potential dietary adjuvant to existing MS therapeutics.

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2.2 INTRODUCTION

Lipid mediators contribute to inflammation resolution following an inflammatory challenge⁹³.⁹⁴. There has been growing interest in unveiling the mechanisms by which nutritional status influences the immune response, especially related to the consumption of omega-3 fatty acids. Epidemiological studies correlated dietary intake of omega-3 fatty acids such as docosahexaenoic acid (DHA) with improved disease outcomes including asthma, diabetes, and neurodegenerative diseases such as multiple sclerosis^{95, 96}. Consequently, there is strong interest in unveiling the changes in the DHA metabolome in the body following an enzymatic transformation of DHA into bioactive DHA derivatives. Previously, it was shown that DHA is converted into docosahexaenoyl ethanolamide (DHEA). Docosahexaenoyl ethanolamide, an analogue of omega-6 derived endocannabinoid anandamide (AEA)^{14, 29, 97-99}, is also known as synaptamide which plays an important role in promoting synaptogenesis and is found endogenously in the brain and retina^{31, 33, 99-101}. Previous studies have demonstrated the ability of DHEA to attenuate lipopolysaccharide-induced activation of microglia^{102, 103}. Notably, a study focusing on bacterial and viral meningitis demonstrated that DHEA was reduced in the cerebrospinal fluid (CSF) samples obtained from patients with neuroinflammation¹⁰⁴. Within the central nervous system (CNS), DHEA prevented neuropathic pain after spinal cord injury by suppressing spinal microgliosis¹⁰⁵. DHEA also influenced inflammatory markers and attenuated macrophage activation in adipocytes¹⁰⁶⁻¹⁰⁸, indicating its potential influence on the peripheral immune system.

DHEA is a polyunsaturated lipid that can be metabolized by eicosanoid synthesizing enzymes such as the cyclooxygenase (COX), lipoxygenase (LOX), or Cytochrome P450 (CYP450) epoxygenase enzymes to form oxidized metabolites. It was previously reported that DHEA is converted by LOX to form 10,17-dihydroxydocosahexaenoyl ethanolamide (10,17-

DiHDHA-EA) and 15-hydroxy-16(17)-epoxy-docosapentaenoyl ethanolamide (15-HEDP-EA) that reduced the migration of polymorphonuclear leukocyte chemotaxis²². In 2017, we demonstrated that CYP450 enzymes can convert DHEA into DHEA-Epoxydes (epoxydocosahexaenoyl-ethanolamide, or EDP-EA)³⁴. One of the regioisomers 19,20-EDP-EA significantly reduced pro-inflammatory markers IL-6 and nitric oxide production, while promoting anti-inflammatory marker IL-10 in microglia³⁴. Overall, DHEA is further metabolized to downstream oxidized metabolites that are immunomodulatory and may be beneficial as a potential nutrition-based intervention in diseases with aberrant inflammatory responses such as multiple sclerosis (MS).

MS is a chronic inflammatory, demyelinating, and debilitating disease of the CNS in which 85% of patients manifested a relapsing-remitting (RR-MS) form where they experience episodic neurological dysfunction (relapses) followed by periods of partial recovery (remission)^{60, 109}. While the natural history of MS is heterogeneous across patients⁵⁷⁻⁵⁹, disease onset and exacerbation likely stems from an aberrant inflammatory response brought on by autoreactive T- and B- cells in the CNS parenchyma¹¹⁰⁻¹¹². Additionally, several studies suggest that the inflammatory response and the subsequent axonal damage is exacerbated by responses including the production of pro-inflammatory molecules by activated microglia and infiltrating macrophages¹¹³⁻¹¹⁵. Epidemiological studies suggest that omega-3 rich diets are associated with lower rates of MS¹¹⁶, and possess beneficial anti-inflammatory^{95, 117, 118}, and neurological¹¹⁹⁻¹²¹ health properties. A double-blind controlled trial in patients with RRMS indicated a trend favoring those consuming an omega-3 supplementation over placebo pills¹²². Another study showed that individuals consuming high omega-3 fatty acids through their diet reduced the risk of experiencing a clinical isolated syndrome¹²³. While there exists a correlation of improved quality of life in MS

patients from increased omega-3 fatty acid intake, the precise mechanism is not well understood. Separately, studies utilizing phytocannabinoids Δ^9 -tetrahydrocannabinol (Δ^9 THC) has been shown to reduce disease severity in experimental autoimmune encephalomyelitis (EAE) and Theiler's murine encephalomyelitis virus (TMEV) models of MS^{11, 124-127}. Furthermore, cannabinoid treatment of MS patients decreased spasticity^{128, 129}, muscle stiffness^{128, 130}, and neuropathic pain¹³⁰. Endocannabinoids are derived from fatty acids and previous studies that showed that omega-6 endocannabinoids such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG) decrease inflammation through their interactions with cannabinoid receptors¹³¹. As endocannabinoids and cannabinoids interact with the same receptors⁹², it is possible that DHEA and DHEA-Epoxydes that are DHA based endocannabinoids will be efficacious in ameliorating aberrant inflammatory diseases such as MS.

The goal of the current study is to show that DHEA reduces inflammatory responses while simultaneously inhibiting relapses and disease severity in a preclinical animal model of MS. The rationale of this study is based on the following observations: (A) endocannabinoids modulate neuroinflammatory responses in MS, (B) phytocannabinoids from *Cannabis Sativa*, and cannabinoid receptors play important role in MS and (C) there is correlation of omega-3 fatty acid consumption with better quality of life in patients with MS. Overall, this demonstrates that DHEA, a dietary component also found endogenously in the human body, is dysregulated in experimental autoimmune encephalomyelitis (EAE) and influences the disease progression of relapse-remitting EAE (RR-EAE) animal model of multiple sclerosis. The fundamental understanding about endocannabinoid interactions with T-cells will help in the design of natural therapeutics in autoimmune diseases and will provide insight into understanding the mechanisms behind omega-3 consumption and conferred health benefits.

2.3 MATERIALS AND METHODS

Experimental autoimmune encephalomyelitis (EAE) experiments in animals

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Illinois Urbana-Champaign and were performed in accordance with the NIH Guidelines for Care and Use of Laboratory Animals. Six- to eight-week old female SJL/JCrHsd mice (Envigo No. 052) were obtained from Envigo (Indianapolis, IN). All animals were housed under a reverse 12-hour light/dark cycle with food (Teklad 2918) and water ad libitum. One week prior to induction of RR-EAE, mice were stratified by weight and housed four per cage. Mice were treated daily with vehicle or DHEA (0.5 mg/kg/day) beginning on the first day of immunization. Treatments were administered via intraperitoneal injection on alternating lateral sides. C57BL/6 mice (age 9-12 weeks) were utilized for T-cell polarization experiments, and C57BL/6-Tg(Tcra2D2, Tcrb2D2)1Kuch/J mice were utilized for primary splenocyte cultures.

Induction of Relapsing- Remitting EAE

RR-EAE was induced by subcutaneous injection of Complete Freund's Adjuvant and PLP₁₃₉₋₁₅₁ emulsion on 9-week old mice. Complete Freund's Adjuvant (CFA) was prepared by mixing Incomplete Freund's Adjuvant (Sigma Aldrich #F5506) and inactivated mycobacterium tuberculosis H37 RA (Difco # 231141) at a concentration of 4 mg/mL. PLP₁₃₉₋₁₅₁ (AS-63912, Anaspec Inc, Fremont, California) was solubilized in phosphate-buffered saline (pH 7.4) at a concentration of 1.5 mg/mL. Equivalent volumes of PLP₁₃₉₋₁₅₁ and CFA were mixed in a dropwise manner while vortexing between each drop. The PLP₁₃₉₋₁₅₁ and CFA emulsion was then vortexed for 1 hour. Mice were anesthetized with 4% isoflurane and then subcutaneously injected with 100

μ L PLP₁₃₁₋₁₅₁ and CFA emulsion distributed equally over two spots across the flank. Each mouse received a total of 200 μ g mycobacterium tuberculosis and 75 μ g PLP₁₃₉₋₁₅₁. Mice were weighed and monitored daily for clinical disability in a blinded manner.

Clinical Scoring of RR-EAE

Active RR-EAE was scored as previously described¹³² with slight modification. Clinical score severity was assessed on a scale of 0 to 5 utilizing the following scoring system: 0, normal mouse with no signs of disease; 0.5, tail weakness or hindlimb weakness; 1, tail paralysis, or tail weakness and hindlimb weakness; 1.5, tail paralysis and hindlimb weakness; 2, tail paralysis, loss of righting reflex, and hindlimb weakness; 2.5, partial hindlimb paresis; 3, one complete limb paralysis; 3.5, one complete limb paralysis and weakness in the other limb; 4, two limb paralysis; 4.5, two limb paralysis and weakness in forelimb; 5, moribund state/death by EAE. All atypical symptoms were also monitored. To be considered a relapse, a corresponding 5 % body weight loss and decreased clinical score of 0.5 was required.

Tissue Harvesting

Mice were asphyxiated with CO₂, right atrium cut and blood collected. Mice were then perfused with 30 mL of sterile 1x PBS at a rate of 1 mL/ minute. Blood was processed for serum and stored for later analysis. Brain, spinal cord, and spleens were collected under sterile conditions in RPMI 1640 and stored on ice for culturing. Tissues collected for endocannabinoid extraction were collected in a pre-weighed cryovial and flash frozen in liquid nitrogen for analysis.

Endocannabinoid Extractions

All extractions were completed on ice using solvent extraction systems with volumes relative to tissue mass. Briefly, 9:1 Ethyl Acetate:Hexane supplemented with TPPU (10 μ M, Cayman Chemical #11120) and phenylmethanesulfonyl fluoride (1 mM, Gold Bio #P-470) were utilized in a 1:20 tissue mass (g): volume (mL) ratio. Tissues were homogenized with a biohomogenizer for 30 seconds on ice, and 9:1 Ethyl Acetate: Hexane and distilled water containing TPPU and PMSF were added. The contents were then vortexed, and subsequently centrifuged at 800xg for 5 minutes. The upper organic layer was collected, dried under continuous stream of nitrogen gas, and endocannabinoid quantification conducted.

Quantification of Endocannabinoids

Samples were analyzed with the 5500 QTRAP LC/MS/MS system (Sciex, Framingham, MA) in Metabolomics Lab of Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. Software Analyst 1.7.1 was used for data acquisition and analysis. The 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) includes a degasser, an autosampler, and a binary pump. The LC separation was performed on an Agilent Eclipse XDB-C18 (4.6 x 150mm, 5 μ m) with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). The flow rate was 0.4 mL/min. The linear gradient was as follows: 0-2min, 90%A; 10min, 5%A; 23-30min, 0%A; 30.1-37min, 90%A. The autosampler was set at 10°C. The injection volume was 10 μ L. Separation chromatograms can be viewed in Supplementary Figure 3. Mass spectra were acquired under both positive (ion spray voltage was +5500 V) and negative (ion spray voltage was -4500 V) electrospray ionization (ESI). The source temperature was 450 °C. The curtain gas, ion source gas 1, and ion source gas 2 were 32, 50, and 55, respectively.

Multiple reaction monitoring (MRM) was used for quantitation: in the positive mode, AEA m/z 348.3 $\rightarrow$ m/z 62.1; 2-AG m/z 379.2 $\rightarrow$ m/z 79.0; EPEA m/z 346.3 $\rightarrow$ m/z 62.1; DHEA m/z 372.4 $\rightarrow$ m/z 62.1; DHG m/z 403.1 $\rightarrow$ m/z 119.0; 19/20-EDP-EA m/z 388.1 $\rightarrow$ m/z 62.1; 19/20-DiHDPA-EA m/z 406.3 $\rightarrow$ m/z 388.3. In the negative mode, 19/20-EDP m/z 343.2 $\rightarrow$ m/z 281.2; 19/20-DiHDPA m/z 361.1 $\rightarrow$ m/z 273.1. Internal standard d4-AEA was monitored at m/z 352.3 $\rightarrow$ m/z 287.2 (positive ESI) and d5-DHA was monitored at m/z 332.2 $\rightarrow$ m/z 288.2 (negative ESI).

Liver Toxicity

Female SJL/JCrHsd (n=6 per group) were treated with 100 mg/kg/day DHEA via intraperitoneal injection for 33 days. Mice were euthanized and tissues were collected. The blood was collected without anticoagulants, and centrifuged at 1000 $\times g$ for 5 minutes at 4 °C. The serum was isolated and immediately frozen. Samples (200 μ L each) were pooled (n=3) and submitted to the Veterinary Diagnostic Laboratory at the University of Illinois at Urbana-Champaign for liver chemistry profile analysis of ALT, ALP, GGT, Total Bili, BUN, Glucose, Albumin and Cholesterol.

Primary Cell Isolation

Tissues were placed on a 70 μ m cell strainer and mashed with a sterile plunger. Red blood cells were lysed using 1x Red Blood Cell Lysis Buffer and subsequently inhibited with equal volume of RPMI 1640. Following centrifugation at 400 $\times g$ for 5 minutes and removal of supernatant, single cell suspensions were created in RPMI 1640 containing 10% FBS, 1x Glutamax, 1 mM Sodium Pyruvate, and 100 U/mL Penicillin/Streptomycin. 5×10^{10} cells/well were

plated in 96-well plate prior to treatments and stimulation. For analysis of infiltrating cells in the CNS, brains and spinal cords were mashed, cells dissociated with Accutase (ThermoFisher #A1110501) for 30 minutes at 37 ° C, and filtered through a 70 µm cell strainer. Cells were resuspended in 30 % Percoll (ThermoFisher #45-001-747) and an underlayer of 70 % Percoll was added. Cells were spun at 20,000 $\times g$ for 1 hour and the middle interface was collected.

Naïve T-cell Isolation and Polarization

Naïve CD4⁺ T-cells (ThermoFisher #8804-6824-74) were isolated and then polarized into Th1 cells (R&D Systems #CDK018). Th17 cells were obtained using the same Naïve CD4⁺ T-cell isolation kit but polarized to Th17 (R&D Systems #CDK017). Medium used was X-VIVO 15 hematopoietic cell media (Lonza, #BE02-060Q) supplemented with 10% FBS and 100 U/mL Penicillin/Streptomycin. Following polarization to Th1, cells were pre-treated for 30 minutes with endocannabinoids prior to stimulation. Cells were stimulated with phorbol 12-myristate 13-acetate and ionomycin (0.081 µM and 1.34 µM, respectively), and Brefeldin A added. Following a 4 hour incubation in 37° C, cells were harvested and processed for flow cytometry as described below.

Flow Cytometry and Antibodies

Following treatment, cells were resuspended in flow buffer (2% FBS in 1x PBS) and cell surface marker antibodies added. Following a 15 minute incubation at 4° C in the dark, cells were washed twice and prepared for intracellular cytokine staining. Cells were fixed in IC Fixation Buffer (eBioscience #00-8222-49) for 20 minutes at room temperature, followed by 10 minutes incubation in permeabilization buffer (eBiosciences #00-8333-56). Cells were then resuspended

in permeabilization buffer and incubated with intracellular antibodies for 20 minutes in the dark at room temperature. Cells were washed twice and analyzed on the Invitrogen Attune NxT flow cytometer. Compensations were conducted using UltraComp eBeads (Invitrogen, #01-2222). Antibodies used were as follows; IFN γ (rat anti-mouse, PE-Cyanine7, Clone XMG1.2, eBioscience, FisherSci #50-156-14), FOXP3 (rat anti-mouse, FITC, Clone FJK-16s, eBioscience, FisherSci #50-112-9051), IL-10 (rat anti-mouse, APC, Clone JES5-16E3, eBioscience, FisherSci #50-122-3185), IL-17A (rat anti-mouse, FITC, Clone eBio17B7, eBioscience, FisherSci #50-112-9414), IL-4 (rat anti-mouse, APC, Clone 11B11, eBioscience, FisherSci #5015301), CD3 (rat anti-mouse, Alexa Fluor 700, Clone 17A2, eBioscience FisherSci #50-168-42), and CD4 (rat anti-mouse, APC-Cy7, Clone RM4-5, Biolegend 100526).

Chemical Synthesis of Docosahexaenoyl Ethanolamide

For primary cultures and EAE experiments, Docosahexaenoyl ethanolamide (Cayman Chemical #10007534) was utilized. Molecules were checked via ESI mass spectrometry in positive ion mode as seen in Supplementary Figure 4. For DHEA treatments to non-disease mice, chemical syntheses were performed, and analysis conducted as follows. Unless otherwise noted, all reactions were carried out under an ambient atmosphere. All chemicals were purchased from commercial suppliers and used as received. Dry dichloromethane (CH₂Cl₂) was obtained by passing commercially available anhydrous, oxygen-free HPLC-grade solvents through activated alumina columns. Analytical thin-layer chromatography was performed on Merck silica gel 60 F254 glass plates. Visualization was accomplished with UV light and/or potassium permanganate (KMnO₄). Retention factor (R_f) values reported were measured using a 5 $\times$ 2 cm TLC plate in a developing chamber containing the solvent system described. Flash column chromatography was performed

using Silicycle SiliaFlash® P60 (C18-functionalized SiO₂, 40-63 µm particle size, 230-400 mesh). ¹H and ¹³C NMR spectra were recorded on Bruker 500 (500 MHz, ¹H; 126 MHz, ¹³C) or Varian Unity Inova 500 (500 MHz, ¹H) spectrometers. Spectra are referenced to residual chloroform (δ = 7.26 ppm, ¹H; 77.16 ppm, ¹³C). Chemical shifts are reported in parts per million (ppm). Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). Coupling constants J are reported in Hertz (Hz). Mass spectrometry (MS) was performed by the University of Illinois Mass Spectrometry Laboratory. Electrospray ionization (ESI+) spectra were performed using a time-of-flight (TOF) mass analyzer. Data are reported in the form of m/z (intensity relative to the base peak = 100). Infrared spectra were measured neat on a Perkin-Elmer spectrum BX FT-IR spectrometer. Peaks are reported in cm⁻¹ with indicated relative intensities: s (strong, 0–33% T); m (medium, 34–66% T), w (weak, 67–100% T), and br (broad).

To a mixture of EDC (233 mg, 1.22 mmol, 2 equiv.), N-hydroxyphthalamide (109 mg, 670 µmol, 1.1 equiv.) and DMAP (7.45 mg, 60.8 µmol, 0.1 equiv.) in DCM (6.0 mL) was added docosahexaenoic acid (200 mg, 608 µmol, 1 equiv.). The mixture stirred for 3 h and the solvents were removed in vacuo. Hexanes (20 mL) was then added, and the resulting suspension was filtered through a pad of silica. The silica was then washed with another 40 mL hexanes to make sure all of the product was washed off the silica. The filtrate was then concentrated, and THF (12 mL) was then added. To this solution was added K₂CO₃ (168 mg, 1.22 mmol, 2 equiv.), H₂O (12 mL), and Ethanolamide (74.5 mg, 1.22 mmol, 2 equiv.). The resulting mixture was stirred for 3 h, then the reaction was diluted with 1M HCl (12 mL) and CH₂Cl₂ (30 mL). The layers were separated, and the aqueous phase was extracted with CH₂Cl₂ (2 × 30 mL), and the combined organic extracts were washed with brine (50 mL), dried over MgSO₄, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (C18-functionalized

SiO₂, H₂O:MeCN = 3:1 → 1:3) to yield a yellow oil [211 mg, 510 μmol, 84%]. These molecules were verified through ¹H NMR and ¹³C NMR as seen in Supplementary Figure 7.

R_f = 0.30 (SiO₂, CH₂Cl₂:MeOH = 16:1)

¹H NMR (500 MHz, CDCl₃) δ 5.93 (s, 1H), 5.48 – 5.25 (m, 12H), 3.72 (t, J = 5.3 Hz, 2H), 3.42 (q, J = 5.3 Hz, 2H), 2.93 – 2.76 (m, 10H), 2.62 (s, 1H), 2.46 – 2.38 (m, 2H), 2.27 (t, J = 8.0 Hz, 2H), 2.07 (p, J = 7.5 Hz, 2H), 0.97 (t, J = 7.5 Hz, 3H).

¹³C NMR (126 MHz, CDCl₃) δ 173.8, 132.2, 129.7, 128.7, 128.5, 128.44, 128.42, 128.23, 128.21, 128.20, 128.1, 128.0, 127.2, 62.7, 42.6, 36.5, 25.80 (2 overlapping peaks), 25.78, 25.76, 25.69, 23.5, 20.7, 14.4.

HRMS (ESI-TOF, m/z) calcd. For C₂₄H₃₈NO₂ [M+H]⁺ calc.: 372.2903; Found: 372.2900.

IR (ATR, neat, cm⁻¹): 3299 (br), 3012 (m), 2962 (m), 2931 (m), 2873 (m), 1644 (s), 1584 (m), 1433 (m), 1067 (m), 704 (m).

Chemical Synthesis of 19,20-EDP and 19,20-EDP-EA

Chemical synthesis of 19,20-EDP and 19,20-EDP-EA were performed using a two-step procedure. 0.1435 mmol DHA (Nu-Chek Prep, Inc #U84A) was added to 2 mL of dichloromethane in an 18 x 150 mm borosilicate test tube. *meta*-Chloroperoxybenzoic acid (0.2897 mmol) was added and pulse vortexed until dissolved. The resulting mixture was stirred and incubated for 1 h at 25 °C shielded from light. The non-specific DHA-epoxidation was then added to a separatory funnel containing 15 mL DCM. 20 mL H₂O was added and mixture inverted, followed by addition 10 mL 10% NaHCO₃ (w/v) and inversion. The layers were separated and organic phase collected. The extraction in the separatory funnel was repeated twice more with the addition of 20 mL DCM, and the collected organic phase was dried for separation on HPLC. The subsequent product was

resuspended in EtOH and purified under reverse phase HPLC as described below. 19,20-EDP-EA was next synthesized using a 1:40:40:40 molar ratio of 19,20-EDP : 1-Ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride : N-hydroxysuccinimide : ethanolamide on a rocker at room temperature for 18 hours. Synthesis schematic can be viewed on Supplementary Figure 1.

Separation of Epoxide Regioisomers by HPLC

Extracted products of non-specific DHA-epoxidation were separated under reverse phase HPLC using the Waters Alliance e2695 HPLC system with the 2998 PDA detector. Purification was achieved using the SunFire C₁₈ OBD 100 Å, 5 µm, 19 mm x 50 mm preparatory column (SunFire #186002566). Solvents used were solvent A (95% H₂O, 4.9 % ACN, 0.1 % AcOH) and solvent B (95% ACN, 4.9 % H₂O, 0.1 % AcOH). The flow rate was at 3 mL/min. The gradient is as follows: 0 min (50% A); 25 min, (25% A); 40 min, (25% A); 50 min, (0% A); 60 min, (50% A). The resulting peak at 16 minutes was collected and verified by ESI TOF Mass Spectrometry in positive ion mode prior to use.

Synthesis of 19,20-DiHDPA and 19,20-DiHDPA-EA standards for LC-MS/MS analysis

Terminal epoxides of DHA and DHEA were synthesized and purified as described above. The conversion of 19,20-EDP and 19,20-EDP-EA to their corresponding diol was accomplished as follows. Purified epoxides were solubilized in 2 mL acetonitrile, and 2 mL of 1:1 glacial acetic acid:water was added. The reaction was incubated at 45 °C for 1 hour (Supplementary Figure 1). The resulting diols were purified by reverse phase HPLC (Supplementary Figure 2). Successful synthesis of the standards was confirmed by high resolution mass spectrometry. Peak at elution

time 12 minutes was confirmed to be 19,20-DiHDPA-EA, and elution time at 23 minutes was 19,20-DiHDPA.

2.4 RESULTS

Endocannabinoids inhibit T-cell polarization

Helper T-cells (Th) play an important role in MS pathogenesis and are essential for induction of EAE¹³³. Therefore, we measured the capacity of DHEA or DHEA-Epoxyde (endocannabinoid) and/or DHA-Epoxyde (non-endocannabinoid)) to inhibit T-cell responses *in vitro* (Figure 2A). Splenocytes were isolated from 2D2 mice, a T-cell receptor transgenic mouse line in which 90% of helper T cells bear a receptor specific for the myelin peptide antigen myelin oligodendrocyte glycoprotein (MOG)₃₅₋₅₅, pre-incubated with DHEA or DHEA-Epoxyde, and stimulated with antigen (Figure 2B). Antigenic stimulation of 2D2 T-cells in this fashion promotes IFN γ production (Bettelli et al., 2003). Treatment with DHA-epoxyde (19,20-) did not inhibit IFN γ production from antigen stimulated cultures. However, both DHEA and DHEA-epoxyde (19,20-) significantly reduced IFN γ production at high dose. Importantly, 10 μ M DHEA-epoxyde completely inhibited IFN γ production without inducing cell death as measured by lactate dehydrogenase (LDH) production (Figure 2B). It was previously shown that O-1966, a selective CB₂R agonist, can suppress T-cell proliferation and proinflammatory cytokine production¹³⁴ after stimulation with CD3 and CD28 crosslinking antibodies. To determine if DHEA can influence the polarization of naïve T-cells into proinflammatory Th1 or Th17 subsets, purified naïve T-cells were cultured under Th1 or Th17 polarizing conditions in the presence of DHEA or DHEA-epoxyde (19,20-) (10 μ M). After 48 hours, the percentage of IFN γ and IL17A producing cells was determined by flow cytometry. Both DHEA and DHEA-Epoxyde treatment during the polarization

phase reduced the percentage Th1 and Th17 cells without altering viability (Figure 2C-E). Importantly, DHEA-Epoxyde treatment was more efficient at reducing Th17 polarization (Figure 2E).

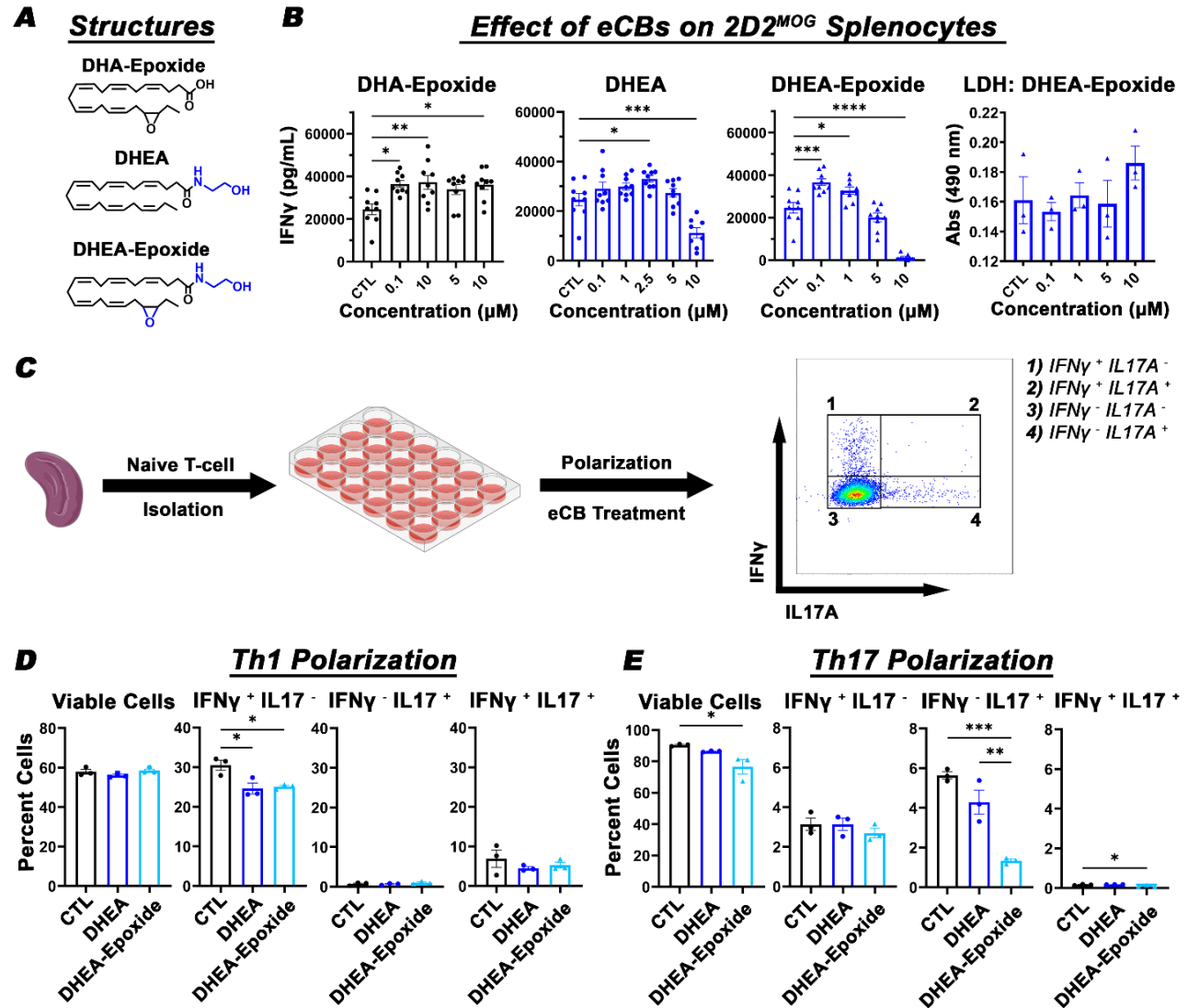


Figure 2. Endocannabinoids influence T-cells. (A) Structures of non-endocannabinoid epoxydocosahexaenoic acid (19,20-DHA-Epoxyde) and endocannabinoids docosahexaenoyl ethanolamide (DHEA) and epoxydocosahexaenoyl ethanolamide (19,20-DHEA-Epoxyde). (B) Endocannabinoids DHEA and DHEA-Epoxyde, but not non-endocannabinoid DHA-Epoxyde (19,20-) inhibits IFN γ production in TCR^{MOG} splenocytes without toxicity as measured by LDH. (C) Schematic of DHEA or DHEA-Epoxyde treatment on T-cell polarization to Th1 and Th17 and gating strategy. (D) Effects of endocannabinoid treatment during naïve T-cell to Th1 or (E) Th17 polarization. One-Way ANOVA and Tukey's Multiple Comparisons test performed.

Central and peripheral levels of endocannabinoid levels in mice undergoing RR-EAE

Endocannabinoid mediated suppression of T-cell responses *in vitro* indicates that treatment may prove efficacious in the suppression of inflammatory diseases. Anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) have been shown to change during neuroinflammation¹³⁵, and in a progressive model of EAE. We measured the levels of AEA, 2-AG and DHEA endocannabinoids at each stage of disease progression in relapse-remitting model of EAE (RR-EAE). Throughout the course of the experiment, the associated weight change and clinical disability score of SJL/JC mice (N=24, n=6 per group) with EAE were measured (Figure 3A-B). Mice were sacrificed on day of immunization (day 0 p.i.), peak (day 14 p.i.), remission (day 19 p.i.) and at relapse (day 34 p.i.) time points of disease (Figure 3B). Targeted LC-MS/MS analysis was used to analyze the levels of DHEA, AEA and 2-AG to identify whether their levels change during disease progression. We report the levels of these three endocannabinoids in the CNS (brain and spinal cord), and in the periphery (spleen).

In the spinal cord, DHEA was reduced at both peak and relapse time points compared to the baseline levels in RR-EAE mice. Additionally, levels of omega-6 derived ethanolamide derivative AEA were reduced throughout the progression of disease (peak, remission, or relapse) compared to the baseline. In the brain, DHEA and AEA levels did not change during RR-EAE (Figure 3C). However, 2-AG increased at peak and relapse compared to baseline. During remission, 2-AG recovered to similar levels as the baseline. As endocannabinoids influenced T-cells isolated from the spleen of the periphery (Figure 2B-E), we next investigated whether endocannabinoids were changing in the spleen in the RR-EAE studies. DHEA levels were increased at remission and relapse compared to baseline. Similarly, AEA levels increased at remission compared to baseline and peak. While 2-AG levels increased at peak, there was recovery

at remission and relapse (Figure 3D). Interestingly, the changes in splenic levels of 2-AG were comparable to the brain. Overall, during the progression of the disease, the levels of the ethanolamine-modified fatty acids DHEA and AEA change significantly in the spinal cord and spleen, but not in the brain.

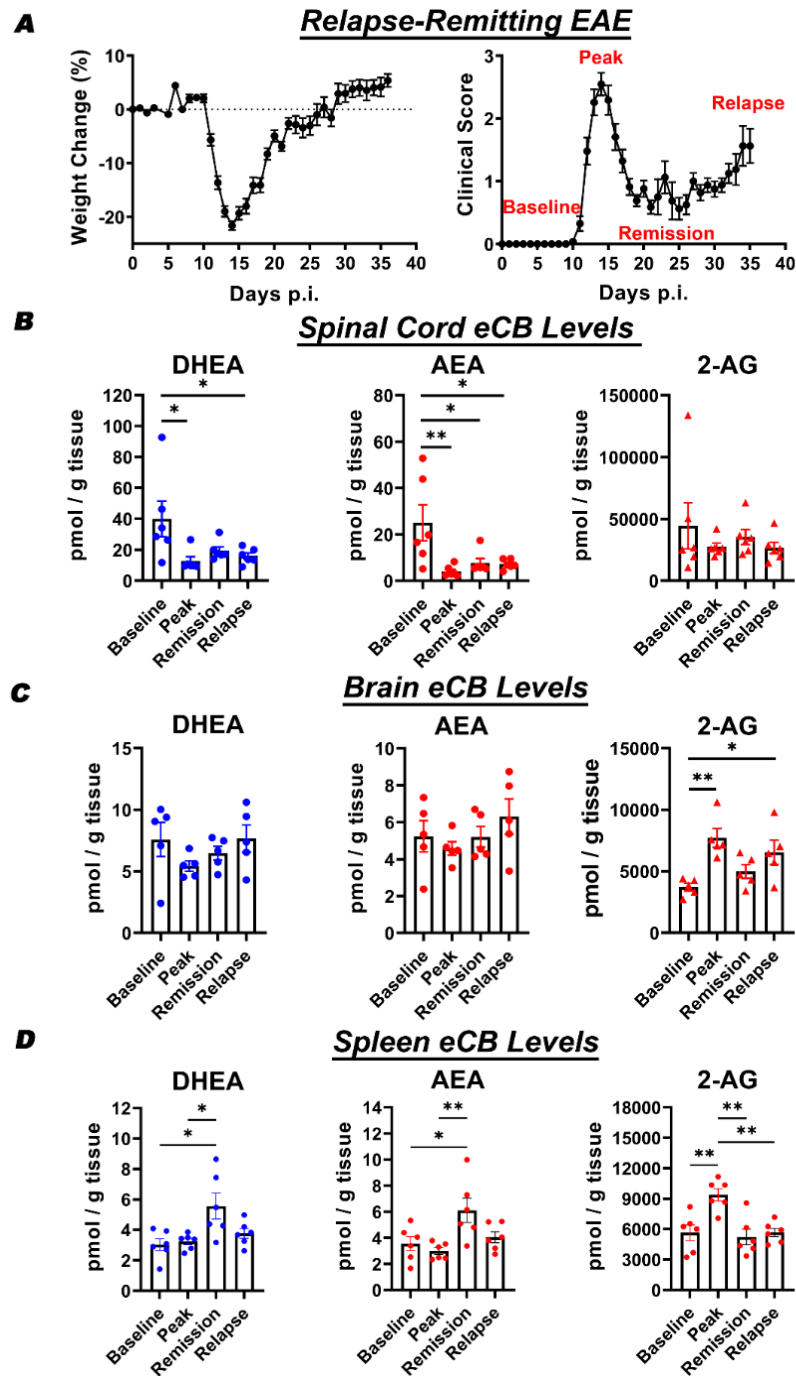


Figure 3. Endocannabinoid System is dysregulated in EAE. (A) EAE disease progression shown as clinical score and weight change (%) on immunized SJL/J mice (N=40). Dysregulation of endocannabinoids in the (B) spinal cord, (C), brain, or (D) spleen. Endocannabinoids docosahexaenoyl ethanolamide (DHEA), anandamide (AEA), and 2-arachidonoyl glycerol (2-AG) were detected and expressed as pmol / g tissue. Tissues were harvested and analyzed as follows: Baseline - day 0 p.i., Peak - day 14 p.i. Remission - day 21 p.i., Relapse - day 35. n=6 SJL/J mice per group were analyzed for statistical differences via One-Way ANOVA, and Tukey's Multiple Comparisons Test performed.

DHEA treatment suppresses RR-EAE

To verify that 100 mg/kg/d DHEA maintains the baseline immune profile and is not toxic, we administered vehicle (saline containing 10% PEG 400 and 0.1% Tween 80) and DHEA daily to SJL/J mice in a non-disease state (Supplementary Figure 5). There were no differences of weight loss, behavioral, or clinical side effects in the DHEA treatment group compared to the vehicle control group. Since endocannabinoids possess immunomodulatory properties, we next questioned whether DHEA treatment in healthy mice would alter the immune profile of the spleen. Treatment did not affect the overall percentages of CD4⁺ T-cells in the spleen. Furthermore, treatment neither altered the percentage of IFN γ producing Th1, nor IL17A producing Th17 T-cells in this organ (Supplementary Figure 5). Lastly, there were no differences in endocannabinoid levels in the brain or spinal cord in a non-disease state (Supplementary Figure 5D-E), suggesting that DHEA treatment in RR-EAE mice likely influences the peripheral immune cells involved in EAE disease pathogenesis. Lastly, fatty acid administration could lead to liver toxicity, but there were no effects of liver toxicity measured through serum analysis of liver metabolites (Supplementary Figure 6).

To determine if DHEA treatment could suppress EAE, we induced RR-EAE in SJL/J mice and treated the mice with either vehicle or a low dosage of DHEA (5 mg/kg/d). At this dose we did not observe an effect of treatment on disease progression as determined by clinical score and weight change (Supplementary Figure 9, Supplementary Table 5). Thus, we next investigated the effects of DHEA at a higher and more relevant dosage of 100 mg / kg using the same RR-EAE model.

In this single-blinded experiment, there were three treatment groups (n=12 per group) receiving vehicle (“Vehicle” - black, saline containing 10% PEG 400, 0.1% Tween 80 beginning day 0), DHEA from day of immunization (“DHEA” – blue, beginning day 0), or DHEA after the

peak disease (“DHEA Post-Peak” – orange, beginning day 19). Analysis of clinical scores indicated a delay of disease onset between the vehicle and DHEA (from day of immunization, blue) groups (Figure 4A-C, Figure 4H). These data suggest that the DHEA treatment decreased the severity of the disease as compared to the vehicle group. Within the DHEA treated group, there were responders and non-responders to DHEA. We observe comparable disease progression of non-responders to the vehicle control group, albeit with a slight delay of time to relapse by 3 days (Supplementary Figure 8). In the responders, we observed a delay in the time to disease onset but a greater disease severity at peak. The responders also exhibited a longer time to relapse from peak by approximately 18 days compared to 12 days in the vehicle group. To better visualize our observations, we plotted the data as percent of mice that were disease free (Figure 4G). We observed that 40% of the mice in the DHEA group (blue, beginning day 0) develop disease at day 22 post immunization compared to vehicle or the group receiving DHEA treatment after peak disease (orange, beginning day 19). Furthermore, these data revealed that the DHEA treatment group had a delay in the time to relapse compared to those in the vehicle control group (Figure 4H). There was also a delay in time to relapse when DHEA was administered after the initial peak disease (Post-Peak; Figure 3I). While there was no effect of time to relapse in mice administered DHEA from day of immunization, it is likely because DHEA initially delayed the day of onset in EAE mice (Figure 3I). The summary of day of onset, maximum score, and cumulative scores between all three treatment groups are provided in Supplementary Table 6.

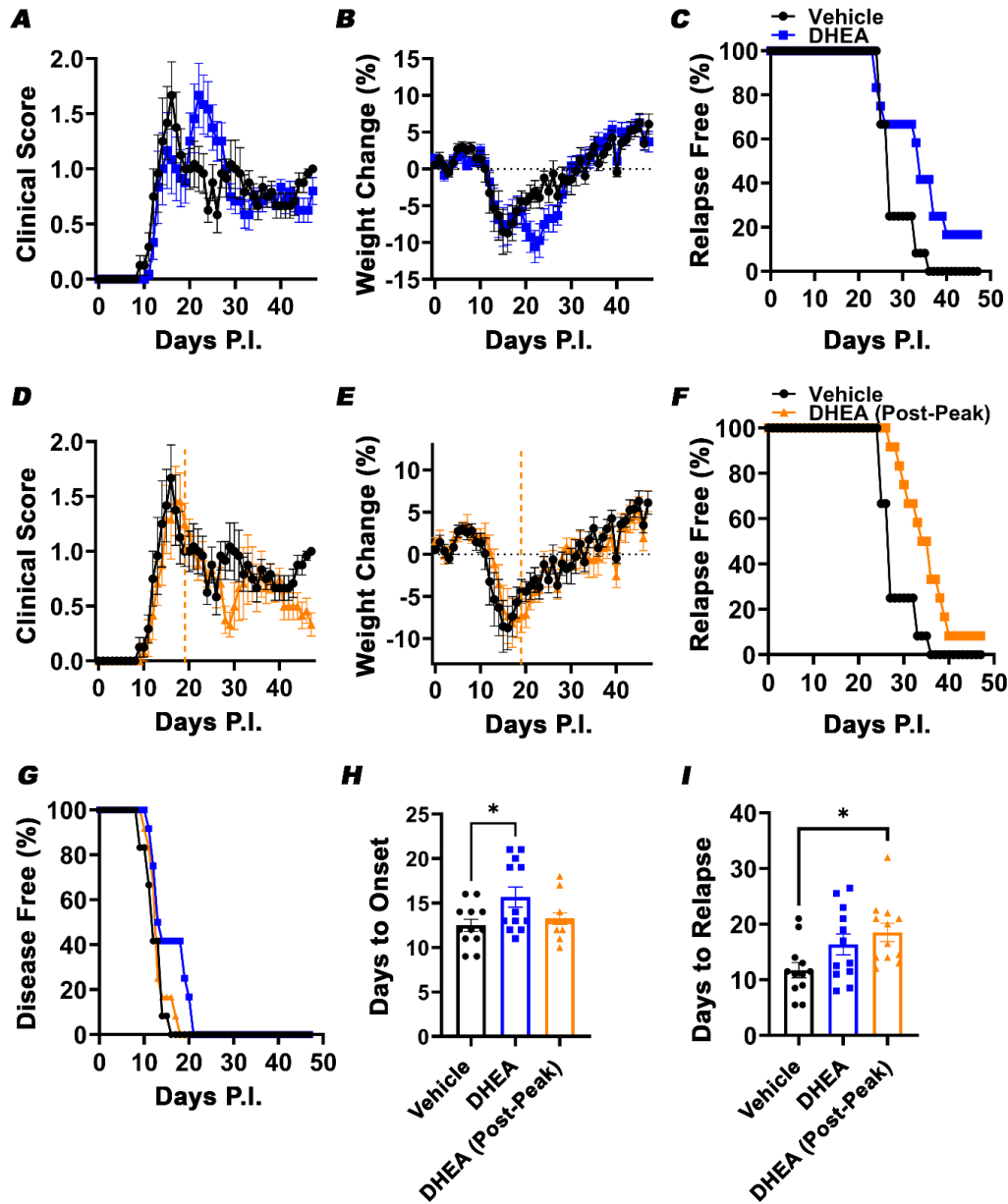


Figure 4. DHEA (100 mg / kg) treatment to EAE mice. SJL/J mice immunized with Complete Freund's Adjuvant containing PLP₁₃₁₋₁₅₉ (n=12 per treatment group). **(A)** Clinical disability scores, **(B)** Weight change (%) of immunized mice treated with vehicle (black, saline containing 0.1% Tween 80, 10% PEG 400), or DHEA beginning day of immunization (blue, 100 mg / kg body weight), and **(C)** Percent relapse free. **(D)** Clinical disability scores, **(E)** Weight change (%) and **(F)** percent relapse free of vehicle, or DHEA administered post peak beginning on day 19 (orange, 100 mg / kg body weight). **(G)** Percent disease free from day of immunization, **(H)** average day of onset, and **(I)** time to relapse from day of peak disease for DHEA and DHEA post-peak compared to vehicle group. Analysis conducted by One-Way ANOVA with Holm-Šidák Multiple Comparisons Test.

Infiltrating CD4⁺ cells in the CNS in RR-EAE mice treated with DHEA at 100 mg / kg body weight

Because CD4⁺ T-cells play an imperative role in the pathogenesis of EAE, we questioned whether treatment affected their number or effector function within the CNS (Figure 5). CD3 in the spinal cord was not detected. Therefore, cytokine productions on CD45^{hi} CD19⁻ populations were examined. At days 48 and 49 post immunization, there was a lower percentage of IFN γ ⁺IL17A⁻ cells in the spinal cord from DHEA treatment beginning post-peak (Figure 5E). While there were lower percentage and number of CD45^{hi}CD19⁻ cells from treatment compared to vehicle, there was no effect. Considering the clinical disability scores of mice are observable after a change in immune response, it is likely that mice in the vehicle and DHEA group were undergoing remission, and mice the DHEA post-peak group were relapsing. Therefore, to get a better sense of how treatment affected CNS infiltrating immune cells we repeated the experiment and sacrificed mice just as the control group were beginning to relapse. We observed similar disease disability scores (Figure 6A) and weight change (%) (Figure 6B) in mice treated with either vehicle or DHEA (Post-Peak) as in our previous experiment (Figure 5). DHEA (Post-Peak) treatment decreased the percentage of infiltrating IFN γ ⁺IL17A⁺ cells in the brain (Figure 6D). In the spinal cord, DHEA (Post-Peak) treatment reduced the percentage of IFN γ ⁺IL17A⁻ producing cells (Figure 6F).

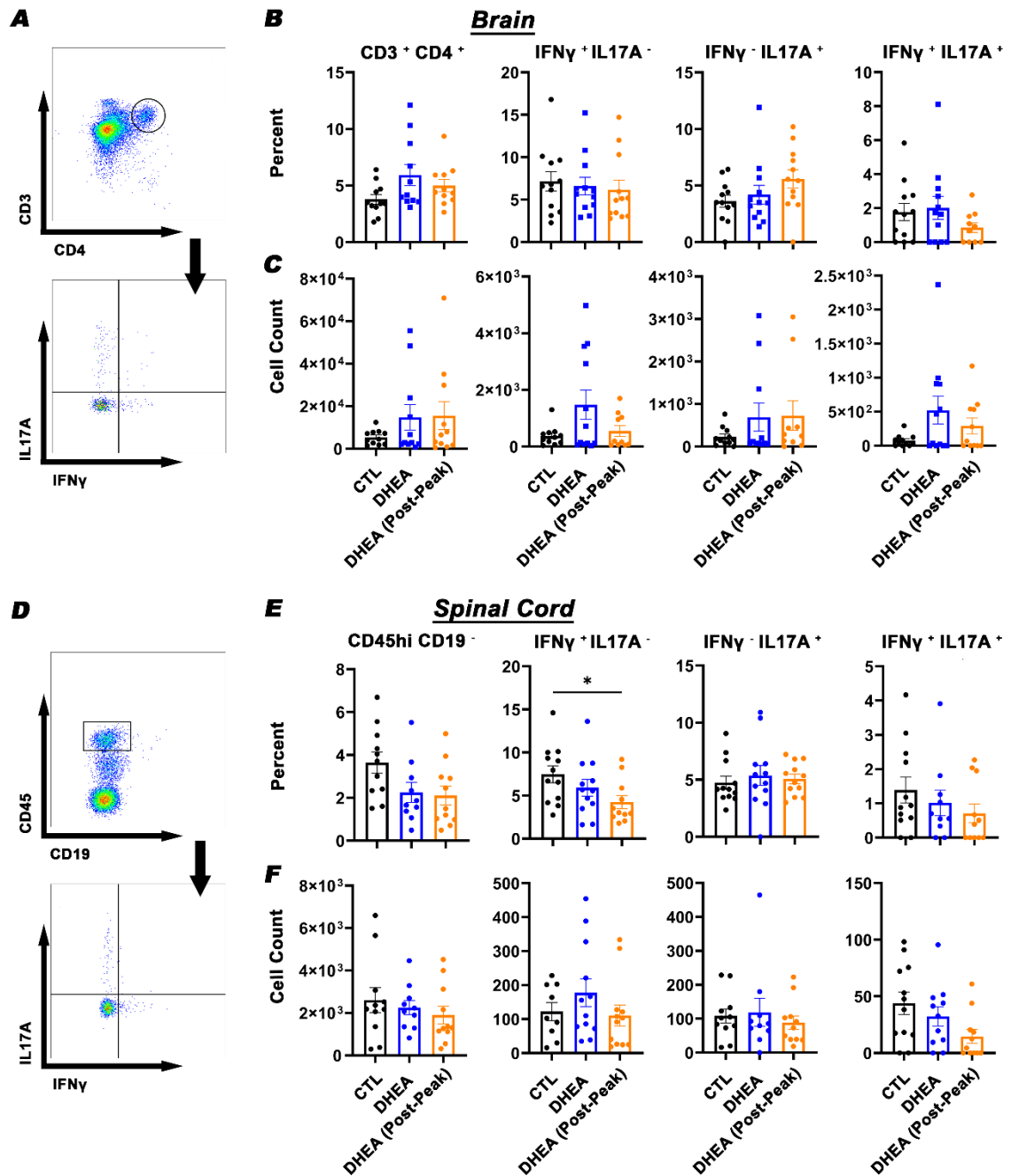


Figure 5. Infiltrating CD4⁺ T-cells in CNS of DHEA-treated RR-EAE SJL/J Mice. SJL/J mice immunized with Complete Freund's Adjuvant containing PLP₁₃₁₋₁₅₉ (n=12 per treatment group). **(A)** Gating strategy for **(B)** Percent and **(C)** Number of CD3⁺CD4⁺, IFN γ ⁺IL17A⁻, IFN γ ⁻IL17A⁺, or IFN γ ⁺IL17A⁺ T-cells in the brain. **(D)** Gating strategy for **(E)** Percent and **(F)** Number of Infiltrating CD45^{hi}CD19⁻, IFN γ ⁺IL17A⁻, IFN γ ⁻IL17A⁺, or IFN γ ⁺IL17A⁺ T-cells in the spinal cord. Analysis conducted by One-Way ANOVA with Holm-Šidák Multiple Comparisons Test.

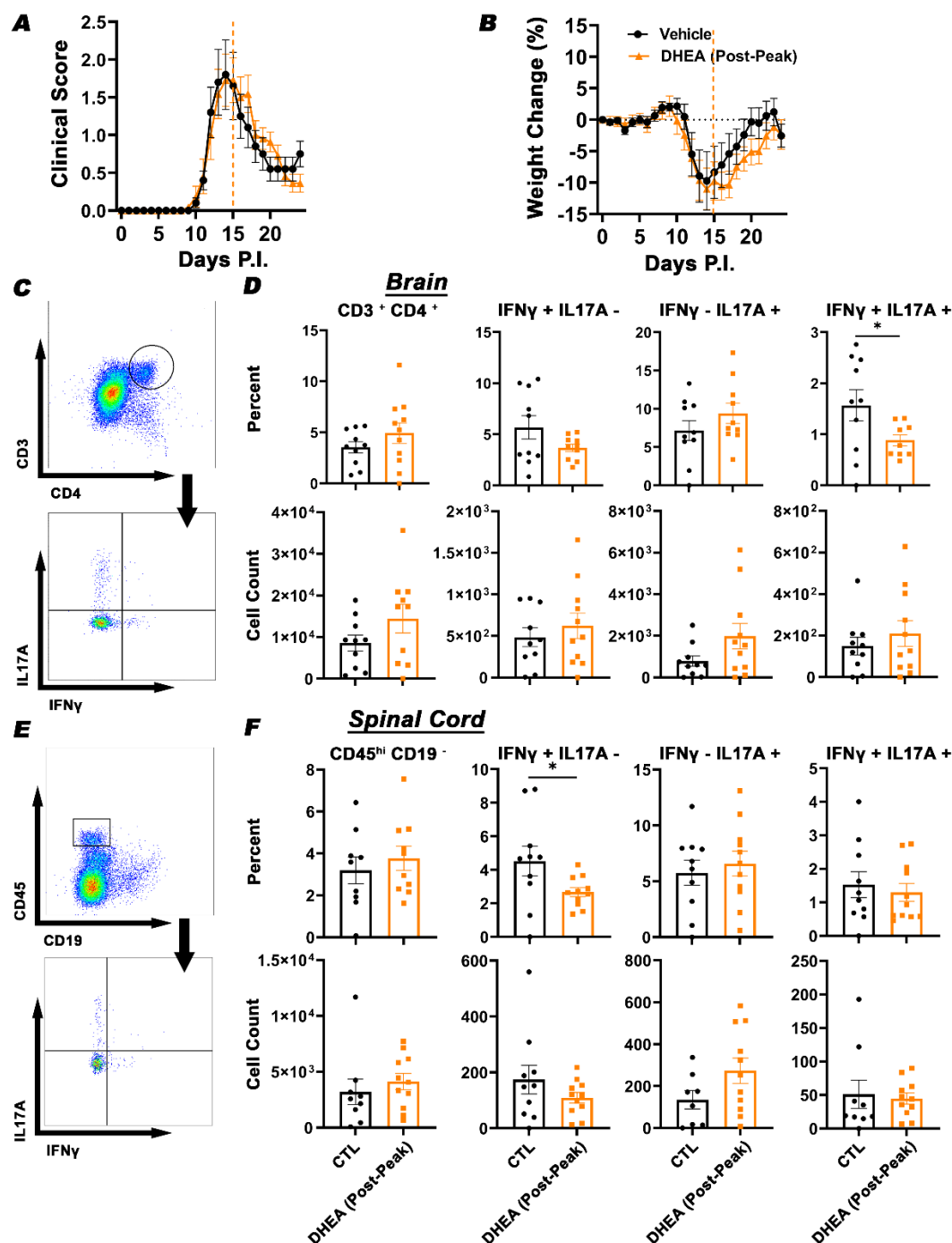


Figure 6. DHEA (Post-Peak) treated RR-EAE mice beginning day 15. Tissues from SJL/J mice immunized with Complete Freund's Adjuvant containing PLP₁₃₁₋₁₅₉ (n=10 for saline, n=11 for DHEA) were collected at day 24 to identify infiltrating cells in the CNS. **(A)** Clinical disability score and **(B)** Weight change (%) of RR-EAE mice treated with saline or DHEA (Post-Peak). **(C)** Gating strategy for **(D)** Infiltrating CD3⁺CD4⁺, IFNγ⁺IL17A⁻, IFNγ⁻IL17A⁺, or IFNγ⁺IL17A⁺ T-cells in the brain. **(E)** Gating strategy for **(F)** Infiltrating CD45^{hi}CD19⁻, IFNγ⁺IL17A⁻, IFNγ⁻IL17A⁺, or IFNγ⁺IL17A⁺ T-cells in the brain. Analysis conducted by unpaired Student's t-test.

2.5 DISCUSSION

Omega-3 fatty acids are associated with anti-inflammatory^{95, 117, 118} and neurological¹¹⁹⁻¹²¹ health properties. Herein, we demonstrated that DHEA, derived from DHA, influences the polarization of Th1 and Th17 cells *in vitro*. As both Th1 and Th17 cells are suspected to have a pivotal role in disease progression of autoimmune diseases such as MS, we utilized RR-EAE to model the effects of DHEA on MS. We found that eCBs are dysregulated in the CNS during various disease stages, in a manner that was tissue dependent. Finally, we show that systemic DHEA treatment increased the time to relapse in RR-EAE mice which was correlated to a reduction in CNS infiltrating IFN γ producing Th1 cells, suggesting a promising natural diet-based therapeutic to help manage symptoms in MS patients.

Previously, it was shown that DHA-treated dendritic cells were poor stimulators of antigen-specific Th1 cell proliferation and differentiation¹³⁶, a process that would result in reduced IFN γ production by Th1 cells. Herein we show that in isolated splenocytes from TCR^{MOG} mice, DHEA significantly reduced levels of IFN γ production and DHEA-Epoxy abolished production of IFN γ . In contrast, DHA-Epoxy did not decrease the IFN γ production, likely because it lacks the ethanolamide moiety and does not interact with cannabinoid receptors³⁴. As *Cnr2* has been shown to be expressed on T-cells¹²⁷ and cannabinoids can modulate T-cell responses¹³⁷, the ability of DHEA-derived molecules to act as cannabinoid receptor agonists is likely important for their immunomodulatory capacity. In addition to influencing TCR^{MOG} splenocyte response to its cognate self-antigen (MOG₃₅₋₅₅), we found that DHEA and DHEA-epoxide reduced T-cell polarization of both Th1 and Th17 cells. Lastly, previous studies demonstrated that DHA and DHEA increase levels of IL-10^{34, 138} which can have a suppressive function on T-cells¹³⁸. While our study focuses on TCR^{MOG} splenocytes or isolated T-cells, it can be surmised that eCBs also

influence other immune cells in the body leading to a synergistic immunomodulatory capacity. Indeed, we observed greater suppression by DHEA and DHEA-epoxides when we used splenocyte cultures containing antigen presenting cells including B-cells, macrophages, and dendritic cells than when we treated purified T-cell cultures.

Previously it was shown that endocannabinoids maintain homeostasis and their dysregulation occurs in many chronic inflammatory diseases¹³⁹⁻¹⁴¹. Considering the epidemiological evidence that support the beneficial effects of omega-3 fatty acids in persons with MS, we sought to determine whether endocannabinoids (AEA, DHEA and 2-AG) were dysregulated in an animal model of this disease. In mice undergoing RR-EAE, we observed a dysregulation of omega-3 derived DHEA and omega-6 derived AEA as disease progressed. This is contrary to another report where both AEA and 2-AG increased in striatum in a progressive model of MS¹³⁵. As omega-3 fatty acids are located throughout the brain and demyelination of white matter occurs in various regions, we investigated the whole tissue for an encompassing representation of endocannabinoid dysregulation. Another notable difference between our current study and those published previously is the type of disease being modeled. Progressive EAE models typically exhibit more neurodegeneration than relapsing remitting models which may affect endocannabinoid levels.

We further showed that the administration of DHEA to non-disease mice does not alter the endocannabinoid levels in the CNS (Supplementary Figure 5). Thus, the positive effects of DHEA treatment, including delay of disease onset and delay of relapse in our EAE studies, are likely a result of the effect on the peripheral immune system. While the mechanisms surrounding omega-3 fatty acid transport into the brain are still being studied, there are emerging data suggesting that fatty acid transporters including fatty acid binding protein 5 (FABP5)^{142, 143} or major facilitator

superfamily domain-containing protein 2 (MFSD2A)^{144, 145} at the blood-brain barrier play a critical role in this process. For instance, MFSD2A deficiencies resulted in hypomyelination¹⁴⁶, and MFSD2A knockout mice had reduced levels of DHA in the brain¹⁴³. Considering that MFSD2A is required for both fatty acid transport and myelination in the brain, it is likely that EAE also affects MFSD2A on endothelial cells at the blood-brain-barrier, leading to reduced intake of fatty acids during a diseased state. Collectively, these data further strengthen the likelihood that DHEA treatment affects the peripheral immune system during RR-EAE. In addition, we show that DHEA administration to non-disease mice do not alter percentages of CD4⁺ T-cells in the spleen. These data may indicate that overt activation is required to see an effect of DHEA treatment to splenic responses. Additionally, it is plausible that DHEA treatment influences peripheral immune response and their infiltration into the CNS in a disease state, but not in a healthy state. Alternatively, DHEA may affect their effector function rather than being cytotoxic, which is supported by the data presented herein. Hence, an increase of omega-3 consumption may be beneficial to patients diagnosed with MS and may alleviate symptoms of other autoimmune diseases.

In our RR-EAE mice study, we administered DHEA at either 5 mg / kg body weight or 100 mg / kg body weight. Importantly, treatments were administered via intraperitoneal injection on alternating lateral sides, a safe method for administering treatment¹⁴⁷. Furthermore, we selected to administer DHEA via intraperitoneal injection to first establish the systemic effects of endocannabinoid treatment as lipids can be altered through digestive processes within the gastrointestinal tract. When mice were treated with DHEA at a dose of 5 mg / kg body weight, we observed some positive trends (S9). Specifically, while there were no effects of DHEA treatment compared to vehicle, it appeared that DHEA reduced disease severity and time to relapse. One

study describes that FDA-recommended dosage of omega-3 fatty acids are 3 g / day in chronic cardiovascular diseases¹⁴⁸. In previous double-blind omega-3 clinical trials, MS patients were administered 10 grams omega-3 fatty acids a day¹²². According to the Meeh-Rubner formula, a 75 kg human receiving only 40 mg / kg body weight omega-3 equates to approximately 160 mg/ kg/ day omega-3 in a 25 gram mouse¹⁴⁹. Therefore, despite the considerably low administration of DHEA at 5 mg / kg body weight, our observations were promising and served as motivation for administering a higher dose. Indeed, we observed a delay of disease onset of mice treated with a higher dose (100 mg/ kg body weight) of DHEA from day of immunization suggesting that DHEA is acting as an immunosuppressive molecule¹⁵⁰. In addition, DHEA was administered after peak (DHEA Post-Peak) to observe whether there was a delay in relapse, or reduction of the relapse severity. We observed that in comparison to vehicle, DHEA administered post-peak delays the time to relapse and severity of the relapse. These data collectively suggest that DHEA is acting to suppress the immune response. Considering that RR-EAE is a CD4⁺ T-cell mediated process^{64, 65}, these *in vivo* data correspond with our findings that DHEA and DHEA-Epoide endocannabinoids influence T-cell polarization, Th17 effector function, and antigen-specific activation in TCR^{MOG} splenocytes. Flow cytometry analysis did not indicate any differences in the T-cells in the brain of our first study RR-EAE study (Figure 5), which may be due to the stage of the disease progression when mice were sacrificed. At the time of sacrifice, the vehicle and DHEA groups were at their peak of relapse while the DHEA post-peak group was at remission. While clinical scores are indicative of disease progression, symptoms present itself after the immune response occurs within the body. As a result, the lack of differences in infiltrating CD4⁺ T-cells in the brain from treatment may not be representative of the effects of DHEA. In the spinal cord, we observed a decrease of IFN γ ⁺IL17A⁻ cells. While these cells may be indicative of overall cytokine production by cells

present in the spinal cord, the precise cell types cannot be determined. It is plausible that in the spinal cord, there was a loss of CD3 and CD4 expression due to the activation of T-cells. Upon repeating DHEA (Post-Peak) treatment to RR-EAE mice, there was an observed decrease of percent of IFN γ +IL17A+ cells in the brain. This is likely a result of reduction of IFN γ ⁺ production, and not IL17A production. In the spinal cord of CD45^{hi}CD19⁻ cells, there was a decrease in IFN γ ⁺IL17A⁻ producing cells. Nevertheless, these data align with our TCR^{MOG} and Th1 polarization data that DHEA and DHEA-Epoxyde treatment influence Th1 cells.

Modulation of the endocannabinoid system has been shown to affect the pathogenesis of EAE. For instance, CB₂ knockout mice had greater disease score in Ac1-11 peptide-induced immunization¹⁵¹. In the same study, treatment with 25 mg / kg body weight THC suppressed EAE in ABH Biozzi mice suggesting that cannabinoid receptor activation may ameliorate EAE¹⁵¹. Another study showed that in C57BL/6 mice immunized with MOG₃₅₋₅₅, highly selective CB₂ agonist Gp1a administration reduced incidence of EAE by 60% and reduced the maximum score and total cumulative score over the course of 36 days¹⁵². In our study, DHEA-Epoxyde (19,20-) was consistently more potent than DHEA at 10 μ M *in vitro*. In cannabinoid receptor activation studies, DHEA had an EC₅₀ value of 1044 nM for CB₁ compared to DHEA-Epoxyde (19,20-) of 108nM³⁴. Similarly, DHEA-Epoxyde (19,20-) had greater affinity for CB₂ than DHEA, where EC₅₀ values were 280 nM and 305 nM, respectively³⁴. Considering that endocannabinoids possess greater affinities for cannabinoid receptors, it is likely that the immunosuppressive capacity of DHEA-Epoxyde is a direct result of its ability to activate the cannabinoids receptors¹⁵³⁻¹⁵⁵.

In conclusion, this is the first study to demonstrate that downstream molecules of omega-3 fatty acid DHA can influence EAE progression by modulating the immune profiles. Importantly, this further illustrates the hypotheses that conversion of DHA into DHEA and further to DHEA-

Epoxides leads to the formation of potent molecules that are immunosuppressive. Previously similar molecules such as resolvin D1, resolvin D2, and maresin 1 are non-endocannabinoid metabolites that inhibited secretion of IFN γ and IL17 in human CD4⁺ T-cells¹⁵⁶, processes that are involved in MS progression. Overall, DHEA and other lipid metabolites can influence T-cell polarization, and influence T-cell effector function thereby showing a positive outcome in autoimmune diseases such as MS.

2.6 ACKNOWLEDGEMENTS

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CHAPTER 3: ANTHRACYCLINE DERIVATIVES INHIBIT CARDIAC CYP2J2

3.1 ABSTRACT

Anthracycline chemotherapeutics are highly effective, but their clinical usefulness is hampered by adverse side effects such as cardiotoxicity. Cytochrome P450 2J2 (CYP2J2) is a cytochrome P450 epoxygenase in human cardiomyocytes that converts arachidonic acid (AA) to cardioprotective epoxyeicosatrienoic acid (EET) regioisomers. Herein, we performed biochemical studies to understand the interaction of anthracycline derivatives (daunorubicin, doxorubicin, epirubicin, idarubicin, 5-iminodaunorubicin, zorubicin, valrubicin, and aclarubicin) with CYP2J2. We utilized fluorescence polarization (FP) to assess whether anthracyclines bind to CYP2J2. We found that aclarubicin bound the strongest to CYP2J2 despite it having large bulky groups. We determined that ebastine competitively inhibits anthracycline binding, suggesting that ebastine and anthracyclines may share the same binding site. Molecular dynamics and ensemble docking revealed electrostatic interactions between the anthracyclines and CYP2J2, contributing to binding stability. In particular, the glycosamine groups in anthracyclines are stabilized by binding to glutamate and aspartate residues in CYP2J2 forming salt bridge interactions. Furthermore, we used iterative ensemble docking schemes to gauge anthracycline influence on EET regioisomer production and anthracycline inhibition on AA metabolism. This was followed by experimental validation of CYP2J2-mediated metabolism of anthracycline derivatives using liquid chromatography tandem mass spectrometry fragmentation analysis and inhibition of CYP2J2-mediated AA metabolism by these derivatives. Taken together, we use both experimental and theoretical methodologies to unveil the interactions of anthracycline derivatives with CYP2J2. These studies will help identify alternative mechanisms of how anthracycline cardiotoxicity may

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be mediated through the inhibition of cardiac P450, which will aid in the design of new anthracycline derivatives with lower toxicity.

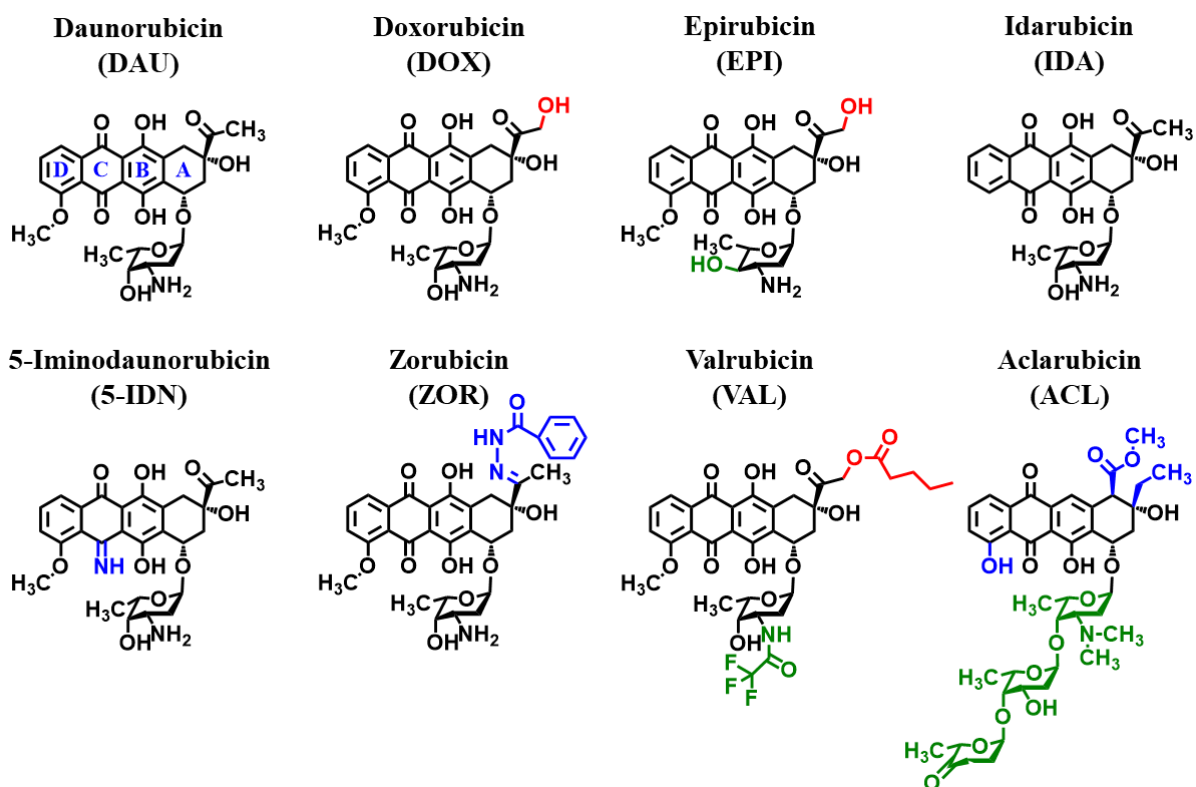


Figure 7. Chemical structure of anthracycline derivatives. Daunorubicin contains ring label identifiers from A-D where the A-ring represents the ring closest to the glycosylation moiety.

3.2 INTRODUCTION

Anthracyclines are among the major classes of common anti-cancer chemotherapeutics developed¹⁵⁷⁻¹⁵⁹. They elicit their antitumorigenic effect by site-specific DNA damage¹⁶⁰, DNA intercalation^{161, 162}, and topoisomerase II α inhibition^{163, 164}. Notably, it has been observed that anthracyclines may also be toxic to cancer cells by formation of reactive oxygen species (ROS)^{160, 165}. The first anthracycline discovered was daunorubicin, a naturally occurring molecule by the microorganism *Streptomyces peucetius*¹⁶⁶. Shortly after, doxorubicin, a 14-hydroxy derivative was discovered and shown to be a more effective anticancer agent^{159, 167, 168}. While anthracycline chemotherapeutics are highly effective in suppressing tumor growth, its clinical usefulness is hampered by adverse side effects such as dose-dependent cardiotoxicity^{160, 169-173}. Although mitochondrial damage and free radical production have been suggested to induce cardiotoxicity, unknown mechanisms and preventative treatments for anthracycline-induced cardiotoxicity remain^{174, 175}. Due to the different degrees of chemotherapeutic activity and associated toxicity, there have since been extensive studies to further discover analogues with reduced toxicity³. Having a better understanding of the biochemical mechanisms underlying anthracycline cardiotoxicity will aid in the development of efficacious and nontoxic anthracycline derivatives that can be used for chemotherapy. Herein, we chose to study the interaction of select anthracycline derivatives with human cardiac cytochrome P450 2J2 (CYP2J2) epoxide hydrolase.

The anthracyclines selected for this study include daunorubicin (DAU), doxorubicin (DOX), epirubicin (EPI), idarubicin (IDA), 5-iminodaunorubicin (5-IDN), zorubicin (ZOR), valrubicin (VAL), and aclarubicin (ACL) (Figure 7). While DAU was the first anthracycline discovered, the 14-hydroxy derivative known as DOX was of high interest due to it being more effective as an anticancer agent. DOX, though highly effective as an antineoplastic agent,

influences cardiac function that is attributed to the formation of ROS. For instance, a one-electron-reduction on the C-ring of DOX (Figure 7) can form a semiquinone free radical, upon which the unpaired electron donated to O₂ can form superoxide free radicals¹⁷⁶⁻¹⁷⁹. These radicals have been attributed to the cardiotoxic effects in the myocardium as measured in adult rats and cultured neonatal myocytes¹⁸⁰. Considering that the C-ring of DOX or DAU can form a quinone free radical, other derivatives were synthesized in efforts to circumvent this process. For instance, 5-IDN is a derivative of DAU for which an imine group is substituted in place of the quinone at the C-ring position¹⁸¹⁻¹⁸³. The imine substitution does not increase superoxide levels over control levels in bovine heart submitochondrial particles, compared to that of DAU or DOX¹⁸¹. The quinone modification is demonstrated to reduce cardiotoxicity, however there are still adverse side effects unrelated to ROS production. For instance, one study utilized rat electrocardiogram models to compare the toxicity levels between 5-IDN and DOX¹⁸⁴. The less cardiotoxic, 5-IDN widened the QRS complex and caused electrophysiological changes.

Other analogues with high structural similarity to DOX include IDA and EPI. IDA is a 4-desmethoxy analogue of DAU with reduced toxicity observed in Phase II Clinical trials and isolated rat heart models¹⁸⁵. Conversely, EPI is a 4'-epimer of DOX, with an epimerization of the hydroxyl group on the 4' position of the amino-sugar moiety¹⁸⁶. Importantly, EPI has similar or greater antitumor properties than DOX in some cancer models, while simultaneously being less cardiotoxic^{186, 187}. These derivatives are quinone-producing and thus it is not surprising that EPI and DOX generate similarly high levels of ROS¹⁸⁸. However, these studies further suggest that alternative mechanisms of cardiotoxicity exist as EPI when compared to DOX is similarly effective, produces similar levels of ROS, but is less cardiotoxic. Other anthracycline derivatives with lower cardiotoxic potential despite its ability to produce quinones include ZOR, VAL, and

ACL. ZOR is another derivative of DAU containing a benzoyl-hydrazone modification that is used as an antineoplastic agent in breast cancer. VAL is a derivative of DOX that contains an N-trifluoroacetyl modification (Figure 7). ACL is isolated from *Streptomyces galilaeus* and is antineoplastic through similar mechanisms as DOX and DAU. Importantly however, ACL is considered to be less cardiotoxic than DOX and DAU¹⁸⁹. Overall, DAU derivatives have been designed or isolated to improve efficacy in cancer treatment while simultaneously decreasing their cardiotoxicity. Therefore, unveiling novel mechanisms of the potential cardiotoxicity of the anthracycline derivatives can aid in the development of further analogues with reduced cardiac interactions.

Cytochrome P450s (CYPs) are involved in the metabolism of sterols, fatty acids, vitamins, and xenobiotics¹⁹⁰⁻¹⁹⁵. While some P450s found in endothelial cells such as CYP2C8 and CYP2C9 are known to participate in drug metabolism, other CYPs such as CYP2J2 have been investigated to produce lipid metabolites¹⁹⁶⁻¹⁹⁹. Highly expressed in human cardiomyocytes^{200, 201}, CYP2J2 is responsible for the metabolism of AA to produce four epoxyeicosatrienoic acid (EET) regioisomers^{21, 200, 202-204}, and two hydroxyeicosatetraenoic acids (HETEs)²⁰³⁻²⁰⁶. These EET metabolites are known to possess beneficial vasoactive properties such as anti-inflammation and vasodilation^{197, 206}. *In vitro*, P450-derived EETs have been shown to protect cardiomyocyte function²⁰⁷⁻²¹⁰. Additionally, CYP2J2 specifically has been shown to play a critical role in improving conditions in various disease states²¹¹⁻²¹⁶. Notably, overexpression of CYP2J2 in transgenic mice protected against DOX-induced cardiac dysfunction¹⁹⁹. This was attributed to an increase in EET production, as EET supplementation to *Rattus norvegicus* H9c2 cardiac cells limited DOX injury. It is recognized that many P450s are upregulated in heart failure, including that of the CYP2J subfamily²¹⁷. In transgenic mice overexpressing human CYP2J2, there was an

observed decrease of anthracycline-induced cardiotoxicity through the production of bioactive EETs by CYP2J2¹⁹⁹. The molecular interactions of how anthracyclines interact with CYP2J2 to modulate the bioactive EETs was unknown until recently. The first direct measurement of AA metabolism inhibition by anthracyclines was studied using three DAU analogues, DOX, ZOR, and 5-IDN²¹⁸. Notably, DOX was found to inhibit AA metabolism by CYP2J2, resulting in reduction of total EETs available to exert its cardioprotective functions.

Very few studies have looked into anthracycline interactions with cytochrome P450s. Specifically, the impact of molecular dynamics (MD) on drug design: applications for the characterization of ligand-macromolecule complexes^{219 220}. With respect to modeling studies with anthracyclines, a recent study with CYP3A4 (the major drug metabolizing CYP found in human liver) has shown that the binding of anthracyclines via hydrophobic interactions is stabilized by electrostatic interactions with arginine residues, in particular R212, within the binding pocket²²⁰. In the case of DOX binding to CYP3A4, the predicted productive binding modes depict the tetracyclic rings, in particular the A-ring of DOX coordinating with the heme. While this may be the case for CYP3A4, these productive poses have their net dipole facing antiparallel from the heme and cannot address the questions of cardiotoxicity, thus warranting further anthracycline binding studies, particularly with CYP2J2, the less studied and highly expressed in cardiac tissue cytochrome P450. In addition, the reported A-ring coordination from DOX in CYP3A4 lends credibility of an alternative mechanism to the C-ring quinone induced free radical formation.

Using both experimental models and MD, we investigate how a screen of anthracycline derivatives (Figure 7) affect human cardiac CYP2J2 in metabolizing AA into bioactive lipid metabolites. We first determine the binding parameters of the anthracyclines to CYP2J2 using fluorescence polarization spectroscopy. Ensemble molecular docking of the select anthracyclines

onto CYP2J2 resulted in several prominent clusters, which allowed for gauging potential effects on regioisomer production and inhibition of AA metabolism via an iterative AA docking protocol. This was followed by experimental validation of CYP2J2 mediated metabolism of anthracycline derivatives using LC-MS/MS fragmentation analysis and inhibition of CYP2J2 mediated AA metabolism by these derivatives. Taken together, we use both experimental and theoretical methodologies to unveil the interactions of anthracyclines with CYP2J2. These studies help in illuminating alternative mechanisms of anthracycline cardiotoxicity, establishing a framework for the design of new anthracycline derivatives with lower cardiotoxicity.

3.3 MATERIAL AND METHODS

Materials

Doxorubicin was obtained from Santa Cruz Biotechnology. ZOR was obtained from the National Cancer Institute. ACL was obtained from Gold Biotechnology (Olivette, MO). All other anthracycline drugs were obtained from Cayman Chemical (Ann Arbor, MI). δ -aminolevulinic acid was obtained from Frontier Scientific. Ampicillin, Chloramphenicol, arabinose, isopropyl β -D-1-thiogalactopyranoside (IPTG), and Ni-NTA resin were purchased from Gold Biotechnology. 1-palmitoyl-2oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-L serine (POPS) were purchased from Avanti Polar Lipids, Inc. Human CYP2J2 (hCYP2J2) cDNA was obtained from OriGene (Cat. No. SC321730). UniProt Accession IDs of CYP2J2 is UniProtKB - P51589 (CYP2J2_HUMAN)

Recombinant Expression of CYP2J2 in Escherichia coli

CYP2J2 containing a His-tag and 34-residue N-terminal truncation was expressed and purified as previously described^{221, 222}.

Recombinant Expression of Cytochrome P450 Reductase

Expression of cytochrome P450 reductase (CPR) was expressed and purified as previously described²²¹

Arachidonic Acid Metabolism Kinetics

The metabolism of arachidonic acid (AA) by CYP2J2 in the presence and absence of anthracyclines were performed as follows. 0.5 mL reaction mixtures containing Arachidonic Acid (100 μ M), CYP2J2 (0.6 μ M), CPR (0.6 μ M), POPS:POPC lipid reconstituted system (50 μ M), 0.1M Potassium Phosphate Buffer (pH 7.4), ROS scavengers (10 U/mL of superoxide dismutase and 10 U/mL catalase, final concentration), and respective anthracyclines (5 and 50 μ M) were pre-incubated at 37 ° C for 10 minutes. Reactions were initiated with 6 mM NADPH and terminated after 5 minutes with 100 μ L acetic acid. The metabolites were extracted three times with 0.5 mL ethyl acetate, centrifuged at 3,000 rpm for 5 minutes, and dried under N₂ gas. Samples were then submitted for LC-MS/MS analysis as described below.

Liquid chromatography – tandem mass spectrometry for quantification of EETs

Analyses were performed at the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. EETs were quantified using a 5500 QTRAP LC-MS/MS system (SciEx,

Framingham, MA). 10 μ L of samples were injected with autosampler set to 4° C. The liquid chromatography and mass spectrometry conditions were set as previously described²⁰⁴.

Fluorescence Polarization Binding

Steady-state fluorescence polarization measurements were obtained with an ISS K2 multi-frequency phase and modulation fluorometer (ISS, Urbana, IL). Fluorescence polarization spectra were recorded using Vinci 3 Software (Urbana, IL). Measurements were taken with a 1 nm slit width filter and 594 nm cutoff filter. Excitation and emission parameters were similar to reported literature values. Excitation and emission spectra chromatograms are in Supplementary Figure 10, and the summary of excitation and emission parameters used for fluorescence polarization binding in Supplementary Table 7.

The polarization spectra were obtained using optimal emission absorbance determined experimentally. Specifically, an excitation wavelength of 460nm was used for ACL, and excitation wavelength of 550nm was used for 5-IDN. An excitation wavelength of 480nm was used for all other derivatives. 0-50 μ M CYP2J2 solubilized in 0.1% cholate were titrated into a mixture containing 0.1M Potassium Phosphate Buffer (pH 7.4), 0.1% cholate, and 1 μ M derivative. Each titration was incubated for 2 minutes at 37°C using a circulating water bath prior to measurement. The polarization values were plotted and fitted to the one-site binding equation (Eq 1) in Origin (version 2018b, Northampton, MA), where r = fluorescence polarization value, S = substrate concentration (CYP2J2), r_0 = intrinsic polarization value, r_1 = maximum polarization value, and K = dissociation constant (K_d). All data points were unweighted to minimize bias during the fit. In this fitting model, the intrinsic polarization values were unique to each drug, and there was not a limiting maximum polarization value utilized for any drug.

$$\text{Equation 1 (One-Site Binding Equation): } r = r_o + \frac{r_1 \frac{[S]}{K}}{1 + \frac{[S]}{K}}$$

Competitive one-site binding parameters seen in Supplementary Figure 11 and Supplementary Table 8 were fit to the following equation:

$$\text{Equation S1 (Competitive One-Site Binding Equation): } r = r_o + \frac{r_1 * [S]}{K \left(1 + \frac{I}{K_i} \right) + [S]}$$

where r = fluorescence polarization value, S = substrate concentration (CYP2J2), r_o = intrinsic polarization value, r_1 = maximum polarization value, I = inhibitor concentration (60 μ M ebastine), and K = K_d obtained from one-site binding. The calculated K_i using this methodology can be seen in in Supplementary Table 8

LC-MS/MS Identification of Anthracycline Metabolites

Samples were analyzed using the Q-Exactive MS system (Thermo. Bremen, Germany) in the Metabolomics Laboratory of Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. XCalibur 3.0.63 software was used for data acquisition and analysis. The liquid-chromatography separation was performed using the Phenomenex Prodigy 5 μ m ODS-2 column, and the Dionex Ultimate 3000 series HPLC system was used. With the autosampler set to 10 ° C, 10 μ L was injected. Mass spectra were acquired under positive electrospray ionization (sheath gas flow rate, 49; aux gas flow rate: 12; sweep gas flow rate, 2; spray voltage, 3.5 kV; capillary temp, 259 ° C; aux gas heater temp, 419 ° C). Mass scan range is 70 – 1,000. For full scan, the resolution was set to 70,000 , and the automatic gain control (AGC) target was 1E6 with a maximum injection time of 50 ms. For MS/MS scan, the resolution was set to 17,500 and the

AGC target was 5E4 with a maximum injection time of 50 ms. Normalized collision energy was 25 and 30.

Fold Change Calculations

To calculate the fold change of regioisomers, the following calculation was conducted:

$$\text{Fold Change} = \frac{\text{Average EET produced in presence of anthracycline}}{\text{Average EET produced with 0 } \mu\text{M anthracycline}}$$
 A fold change of 1 indicates that there is no difference in levels of EETs in the presence of anthracyclines compared to the arachidonic acid control.

Molecular Docking and Molecular Dynamics Simulations

To characterize binding of the studied anthracyclines to CYP2J2, ensemble molecular docking was done using Autodock Vina²²³. The structure of CYP2J2 was obtained from a previous study in which CYP3A4 was used as a homology template and then bound to a POPC membrane using an accelerated membrane binding mimetic²¹⁸. Once the constructed CYP2J2 system was bound to the membrane mimetic, an equilibrium simulation was done with full tail lipids. The resulting MD simulation was used to extensively sample the dynamics of membrane bound CYP2J2, allowing for a myriad of conformational snapshots to be used for the ensemble molecular docking protocol²²⁴. Specifically, the last 20 ns of the simulation trajectory were used for the docking, where 200 unique protein snapshots were taken every 100 ps. To perform the initial ensemble molecular docking, a 28 Å x 28 Å x 28 Å grid box was used, positioned at the center of the catalytic site of CYP2J2, containing the heme moiety. Every anthracycline was then individually docked into each of the 200 snapshots via Autodock Vina with an exhaustiveness of 10. The top 10 poses were collected for further analysis for each snapshot, yielding a total of 2000

docked poses per drug. The poses were then clustered using a root-mean-square deviation (RMSD) metric²²⁵. The cutoff distance between clusters implemented was 4.5 Å, selecting only the carbons shared by ring A and B, ring B and C, and ring C and D for clustering. These six atoms are shared among all the studied anthracyclines, and are highly rigid relative to the functional groups and glycols of the drugs. For each anthracycline the four largest clusters were selected for further filtering. Poses were filtered again by the output vina scoring function energies, with the lowest poses within the lower inner quartile of the distribution selected for further simulation.

The holo-CYP2J2 simulations containing membrane bound protein-ligand system were minimized for 2000 steps and equilibrated for 1 ns. During the equilibration the protein backbone, the alpha carbons were harmonically restrained ($k=1 \text{ kcal mol}^{-1} \text{ Å}^{-2}$). Following the equilibration, a production run was done without any restraints for an additional 50 ns. The simulation was performed using the molecular dynamics software package, NAMD2²²⁶, using the CHARMM36m force field for the protein²²⁷ and lipids²²⁸. The simulation parameters for each anthracycline were obtained from the CHARMM General Force field²²⁹. In addition, the solvent used a TIP3P model for water²³⁰. The simulation was performed using an NPT ensemble at 310 K with a pressure of at 1.0 atm. Moreover, an integration time step of 2 fs was used. The Nose-Hoover Langevin piston method was used to maintain a constant pressure, with temperature maintain constant via Langevin dynamics with a 0.5 ps^{-1} damping coefficient. A 12 Å cutoff was used for nonbonded interactions with a smoothing function implemented after 10 Å. The bond distances of the hydrogen atoms were constrained using the SHAKE algorithm. For long range electrostatic calculations, the particle mesh Ewald (PME) method was used, with a grid density of less than 1 Å^{-3} . Visualization and analyses of the simulations were done using Visual Molecular Dynamics (VMD)²³¹. Molecular interaction diagrams were prepared using the Molecular Operating Environment (MOE) software.

The final snapshot for each CYP2J2-anthracycline bound simulation was prepared using the QuickPrep feature of MOE, with default parameters and a tethering strength of 10 for both the receptor and the ligand of interest. Following the simulations, an iterative docking was used onto each holo-CYP2J2, using the same docking protocols described, with the AA as the new ligand. An example of aclarubicin docking to CYP2J2 can be visualized in Supplementary Movie 2, along with the visualization of the occupation of the active site by aclarubicin in Supplementary Movie 3.

3.4 RESULTS

Determination of the binding parameters of anthracyclines to CYP2J2 using fluorescence polarization spectroscopy

We first investigated whether anthracyclines could bind to CYP2J2 using fluorescence polarization spectroscopy. While Soret-induced spin state ligand binding studies are commonly conducted to investigate CYP-ligand interactions, anthracyclines possess fluorescent and absorbance properties that interfere with the readout on the UV-spectrum. Therefore, utilizing the fluorescent properties of the anthracyclines (Supplementary Figure 10), we measured the increase in anisotropy to assess the binding of the anthracycline analogues to CYP2J2 (Figure 8A). For all studies, the G factor was approximately 1.0. The G factor is an indication of the polarization bias of the detection system of the instrument's sensitivity towards vertically and horizontally polarized light. A G factor of 1.0 indicates there is no polarization bias between detecting vertical or horizontal polarization.

DOX was previously reported to tightly bind to CYP2J2 with a $K_d = 5.33 \pm 0.89 \mu\text{M}^{218}$ fit to the One-Site Binding Equation (Equation 1). DAU had comparable binding affinity to CYP2J2

with $K_d = 9.56 \pm 1.01 \mu\text{M}$. Other analogues with the greatest structural similarities include IDA and EPI. IDA demonstrated strong binding to CYP2J2 with $K_d = 4.99 \pm 0.66 \mu\text{M}$ (Figure 8D, Table 1), comparable to that of DOX. Unlike IDA however, EPI displayed the weakest binding to CYP2J2 with $K_d = 14.70 \pm 1.99 \mu\text{M}$ (Figure 8C, Table 1). While they were structurally the most similar and reported to have among the highest toxicities due to their structural similarities, in our studies with respect to interaction with CYP2J2, EPI interacts weaker as compared to IDA. Overall, the binding interaction of all the derivatives with CYP2J2 are moderately different from each other.

The specific anthracyclines commonly reported to have lower toxicities than that of DOX include 5-IDN, ZOR, VAL, and ACL. ACL is reported to possess low cardiotoxic effects while having a higher therapeutic index compared to that of DOX or DAU⁵². We measured comparable binding affinity of 5-IDN and ZOR to CYP2J2 with $K_d = 6.08 \pm 0.58 \mu\text{M}$ and $10.13 \pm 1.11 \mu\text{M}$, respectively. In comparison to DOX, there was an observed stronger binding affinity to CYP2J2 with both VAL and ACL with $K_d = 2.51 \pm 0.14 \mu\text{M}$ and $K_d = 2.44 \pm 0.24 \mu\text{M}$, respectively. Overall, we show that all the anthracyclines tested, including those that are reported to have high therapeutic indices and low cardiotoxicity such as ACL, interact with CYP2J2, a cardiovascular CYP, and exhibit tight binding.

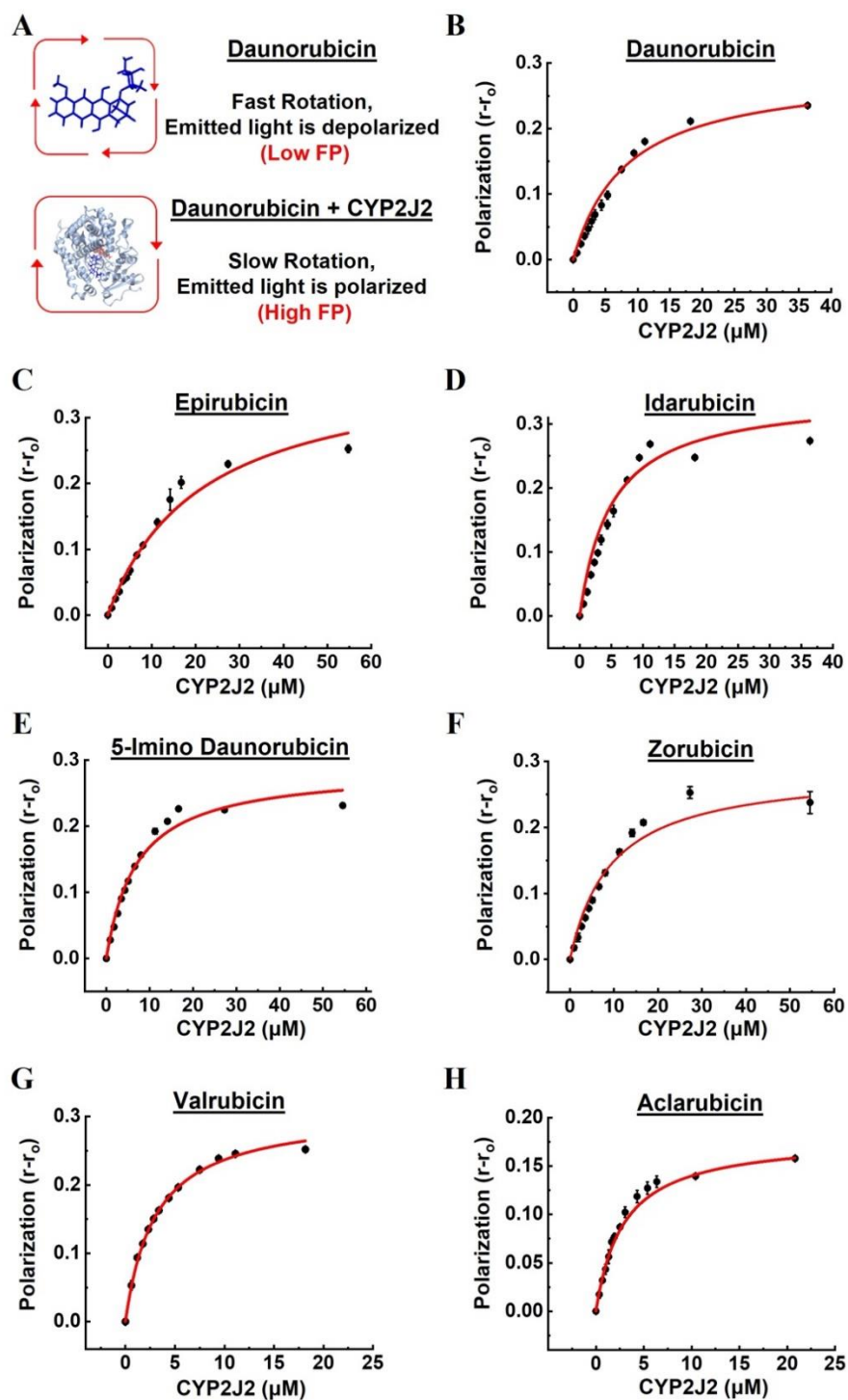


Figure 8. Fluorescence Polarization. (A) Schematic of steady state fluorescence polarization dependent on binding of anthracycline (daunorubicin) to CYP2J2 epoxigenase. Steady-state fluorescence measurements of anthracyclines (1 μM) to increasing concentrations of CYP2J2 (0-60 μM) (B) Daunorubicin, (C) Epirubicin, (D) Idarubicin, (E) 5-Iminodaunorubicin, (F) Zorubicin, (G) Valrubicin and (H) Aclarubicin. Values are expressed as mean and standard error of three replicates.

Table 1: Summary of Binding Parameters			
	r_o	r_I	K_D (μ M)
DOX*	0.083 ± 0.007	0.219 ± 0.010	5.326 ± 0.890
DAU	0.92 ± 0.00	0.33 ± 0.01	9.56 ± 1.01
EPI	0.09 ± 0.01	0.36 ± 0.02	14.70 ± 1.99
IDA	0.45 ± 0.01	0.36 ± 0.14	4.99 ± 0.66
5-IDN	0.15 ± 0.01	0.29 ± 0.01	6.08 ± 0.58
ZOR	0.09 ± 0.01	0.33 ± 0.01	10.13 ± 1.11
VAL	0.14 ± 0.00	0.29 ± 0.01	2.51 ± 0.14
ACL	0.21 ± 0.00	0.19 ± 0.01	2.44 ± 0.24

Table 1. Summary of binding parameters of anthracyclines in comparison to doxorubicin as previously published²¹⁸. Fluorescence polarization values were fit to the One-Site Binding Equation (Equation 1). * indicates data was obtained from Arnold et al., 2017²¹⁸.

Computational Characterization of Anthracycline Binding

To probe potential binding modes of the studied anthracyclines, we employed molecular docking and molecular dynamics (MD) techniques. Using the last 20 ns of the simulation trajectory of CYP2J2 done previously²¹⁸, glycosylated states of the anthracyclines were docked into the catalytic site of CYP2J2. Snapshots from the 20 ns simulation trajectory were taken every 100 ps, amounting to 200 unique protein conformations for ensemble molecular docking of the anthracycline set. Resultant normalized cumulative Autodock Vina scores show comparable affinity of almost every anthracycline to the docked cavity, with the lowest affinity observed for ZOR (Figure 9A). Ligand interaction analysis shows a consistent tendency of the docked anthracyclines to orient with the D-ring of the tetracycline group facing the heme iron in CYP2J2 (Figure 9C and 9D).

Following the ensemble docking of the anthracyclines onto CYP2J2, each set of compounds was clustered using a root mean squared clustering algorithm²²⁵, with four resulting clusters per anthracycline. The binding energy scores of the resultant clustered poses are seen in

Figure 9A, with the minimum valued poses, within the lower inner quartile of the score distributions, selected for an additional 50 ns production run. The lower inner quartile represents values within 2.698σ of the distribution of these data, and beyond that distribution represents outlier values.

Figure 9B depicts the occupancy density over the 50 ns simulation trajectory of the docked ACL. This occupancy describes the space accessible for the largest studied glycosylated anthracycline, in which all the other anthracyclines can also occupy. Figure 9A computationally predicts the binding affinity of anthracyclines to CYP2J2. Here, zorubicin is predicted to bind with the lowest affinity than other anthracyclines, which may be due to the rigidity of the benzoylhydrazone moiety. As opposed to the other anthracycline functional groups, the benzoylhydrazone moiety on zorubicin receives minimal electrostatic stabilization (Supplementary Figure 12.H). The bulky and rigid aromatic ring is lacking favorable aromatic interactions, and is instead in proximity of C237, N231, H244, L247, and Y230, which are overall entropically unfavored for benzoylhydrazone affinity. This agrees with our experimental data showing that the binding of zorubicin is weaker compared to aclarubicin. Moreover, Figure 9C, Figure 9D, and Supplementary Figure 12 shows the molecular interactions between CYP2J2 and the respective bound anthracycline. The poses shown in the figure represent the 50 ns mark of the equilibrium simulations of the docked anthracyclines, showing interactions with heme, nearby residues, and solvent exposure. The anthracycline interaction diagrams are shown in Figure 9C, Figure 9D and Supplementary Figure 12. The key interactions noted throughout all the anthracyclines included arginine coordination with carbonyl and carboxylate groups, mainly through R117, and glycosamine stabilization via E314 and E222. In addition, we observe hydrophobic stabilization of the tetracyclic rings and a predominant binding mode with the D-ring

facing the heme throughout the anthracycline set. In addition, each anthracycline induced a suppression of residue fluctuations throughout each 50 ns simulation. This unique residue fluctuation fingerprint was quantified via a root mean squared fluctuation analysis (RMSF) as seen in Supplementary Figure 13.

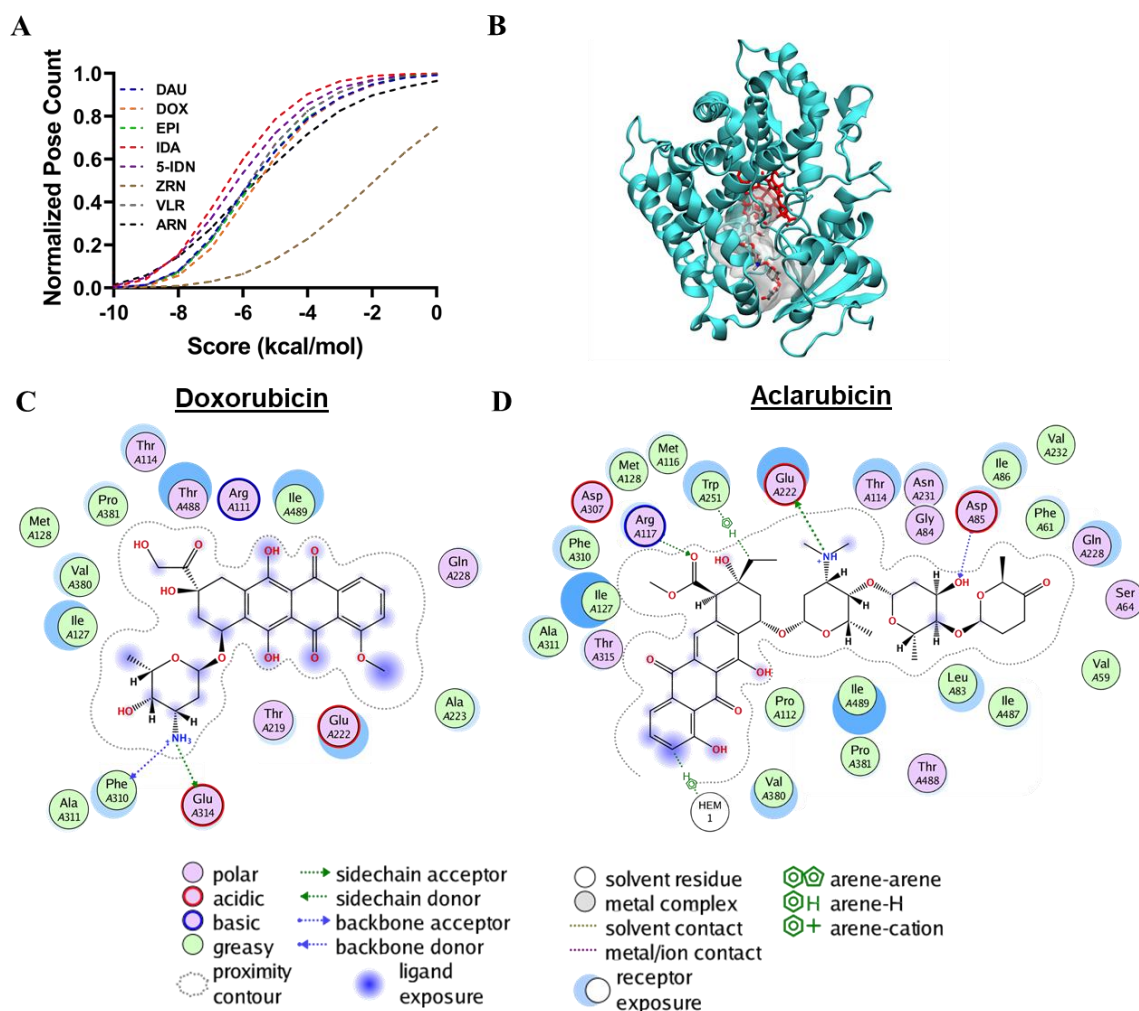


Figure 9. (A) Normalized pose count versus binding affinity score for each computationally treated anthracycline. (B) Volume of aclarubicin accessible during the first round of molecular docking in relation to the heme (red). (C) Doxorubicin and (D) Aclarubicin. Other interactions diagrams for every cluster are in the supplemental information Supplementary Figure 12.

Direct metabolism of anthracyclines by CYP2J2 measured using LC-MS/MS fragmentation analysis

Anthracyclines can undergo reductive aglycosylation by cytochrome P450 Reductase (CPR), the redox partner of CYPs to yield aglycone products⁵⁸⁻⁶⁰. It has been previously suggested that major toxic effects of anthracyclines may in fact be caused by accumulation of aglycosylated products that are more hydrophobic rather than the anthracycline parent molecule itself. While there exists aglycosylated products, there can also exist 7-deoxyaglycone products of anthracyclines²¹⁸. Previously, it has been shown that 7-deoxy-aglycosylated DOX can concurrently bind to CYP2J2 and alter AA metabolism²¹⁸. Using the same LC-MS/MS fragmentation analysis, we identified the 7-deoxy-aglycosylated product of DAU, EPI, and IDA with m/z of 383.11, 399.107, and 353.102, respectively. Previously, we have showed that the aglycosylated product of DOX played an important role in altering the metabolism of AA in the active site of CYP2J2. Interestingly, we did not detect the formation of the aglycones of the other anthracycline tested. It has been reported in literature that other anthracyclines form similar aglycosylated products²¹⁸.

Inhibition of CYP2J2 mediated arachidonic acid metabolism to form EETs by anthracyclines

As CYP2J2 is known to metabolize AA into cardioprotective EETs (Figure 10A), we were interested in determining if the binding of the anthracycline analogues to CYP2J2 affected its function as an AA metabolizing enzyme. Using AA concentrations near previously determined K_m values, we measured total EET production in the presence and absence of anthracyclines. Total EET production in the absence of anthracyclines yielded $84.50 \pm 1.21 \text{ pmol}_{\text{EETs}} (\text{min})^{-1}$

(nmol_{CYP2J2})⁻¹. Inhibition of EET production by DOX, ZOR, and 5-IDN were consistent with previously reported values⁵².

DAU, DOX, EPI, ZOR, and ACL inhibited total EET production at two different concentrations (Figure 10B). There was substantial inhibition of total EET production at the lowest concentration (5 μ M) of IDA and 5-IDN. This suggests that these drugs may interact with CYP2J2 as an inhibitor more strongly at lower concentrations compared to other anthracyclines. Additionally, some drugs did not appear to inhibit total EET production at all. For instance, VAL did not significantly inhibit the production of total EETs by CYP2J2-mediated AA metabolism. However, there were changes in regioisomer production indicating that drugs can influence CYP2J2-mediated metabolism of AA through mechanisms other than direct inhibition (Figure 10C).

As previously mentioned, examining the changes in regioisomer production in addition to total EET inhibition may yield extra information as to how the anthracyclines are interacting with cardioprotective CYP2J2 (Figure 10C, 10D, 10E). For instance, ACL is a comparatively less toxic anthracycline compared to DOX, however accumulated cardiotoxicity still occurs through unknown mechanisms. Upon looking at regioisomer changes of EETs generated, ACL resembles similar properties of DOX influencing CYP2J2-mediated AA metabolism. ACL increased 5,6-EET production by 1.471 fold, similar to DOX's 1.474 fold increase. Additionally, ACL reduced 14,15-EET production by 0.750 fold, similar to 0.753 fold by DOX. These data of 5,6-EET production increase and 14,15-EET decrease, in conjunction with a decrease of total EET production (Figure 10B) further strengthens one mechanism of anthracycline-induced cardiotoxicity through interactions with CYP2J2-mediated metabolism of AA.

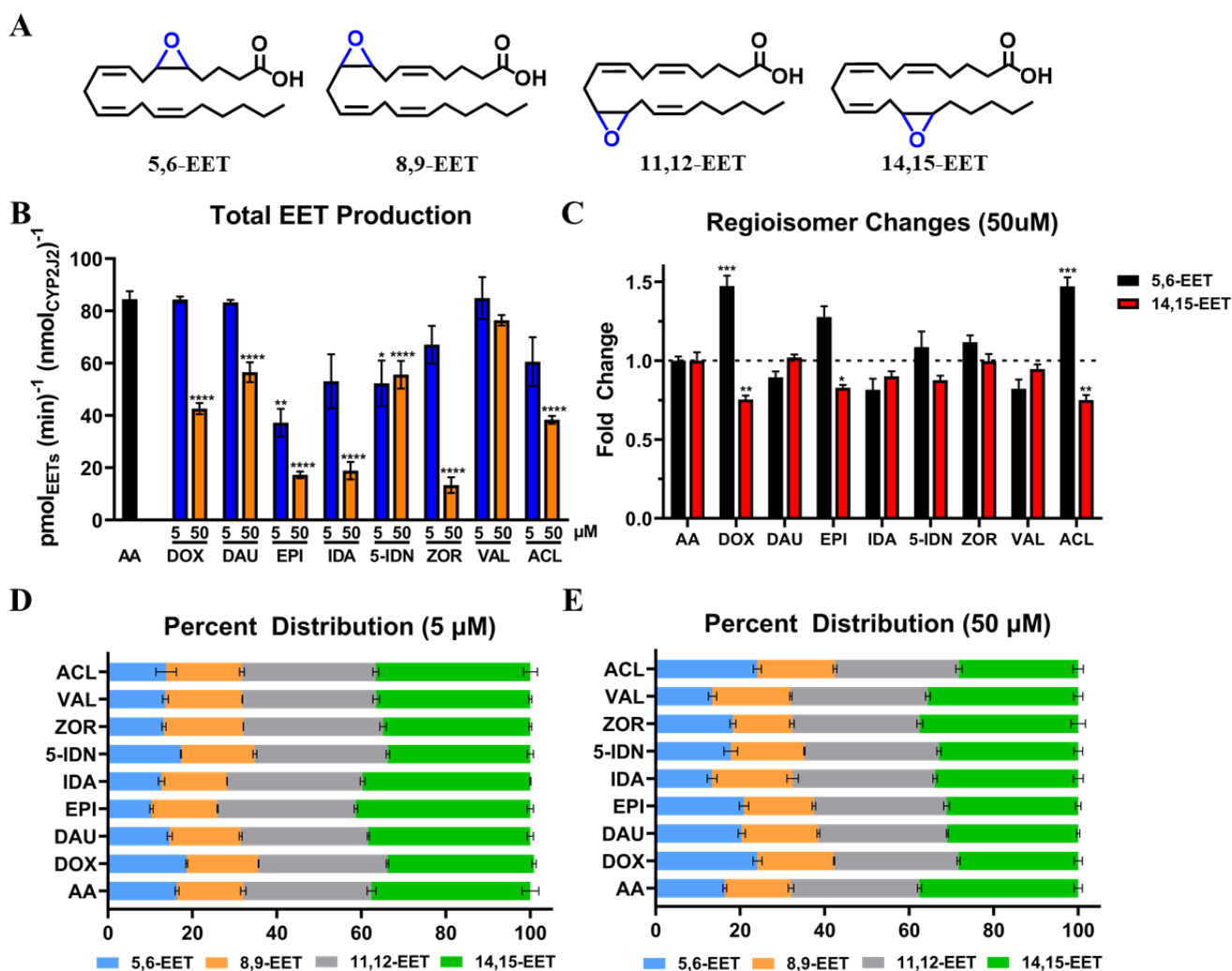


Figure 10. EET Production/Metabolism. (A) Structures of omega-6 derived epoxides 5,6-EET (5,6-epoxyeicosatrienoic acid), 8,9-EET (8,9-epoxyeicosatrienoic acid), 11,12-EET (11,12-epoxyeicosatrienoic acid), and 14,15-EET (14,15-epoxyeicosatrienoic acid). (B) Inhibition of total EET production at 5 and 50 μ M anthracyclines. (C) Regioisomer changes under inhibition by 50 μ M anthracyclines of 5,6-EET and 14,15-EET regioisomers, and (D) Percent distribution of all regioisomers by 5 μ M and (E) 50 μ M anthracyclines. Mean and standard error values are represented from $n=3$ with statistical outliers removed. One-way ANOVA with Tukey's post-hoc analysis was conducted.

Computational Characterization of Anthracycline Binding to CYP2J2 altering the metabolism of AA

To predict the effects of DAU derivatives on the metabolism of AA, we further implemented an iterative ensemble molecular docking scheme. In this scheme, the anthracyclines were docked onto an apo-CYP2J2, no anthracycline but still heme bound, MD trajectory, clustered, filtered based on energy score, then solvated, and simulated for 50 ns. The resultant holo-CYP2J2 trajectories were then used to dock and simulate AA. This iterative scheme allowed for us to observe the effects of the anthracycline set on AA positions in CYP2J2, amounting to 1.6 μ s of anthracycline bound simulation used for AA ensemble docking. Analysis of the unsaturated double bonds on AA docked to holo-CYP2J2, primarily their distance to the heme iron, was used as the metric for prediction of AA metabolism, EETs regioisomer production or inhibition (Figure 11 and Supplementary Table 9).

The productive poses observed for the various anthracyclines, show a preferential binding mode in with the D-ring of the anthracyclines facing the heme in CYP2J2. In the case of ACL, as seen in Figure 11A, the tetracyclic rings can be seen clearly facing the heme iron, creating an excluded volume effect for the iteratively docked AA. Our data shows that this excluded volume limits the available AA poses, indicative a mode of action for regioselective metabolism. Analysis of double bond distances (Figure 11B and Supplementary Table 9) shows the distribution of likely sites of metabolism given each studied anthracycline. Furthermore, upon analysis of the contact probability (Figure 11C) for the carboxylate group of AA, we observe the highest coordination with the PUFA binding residue R321.²¹⁸

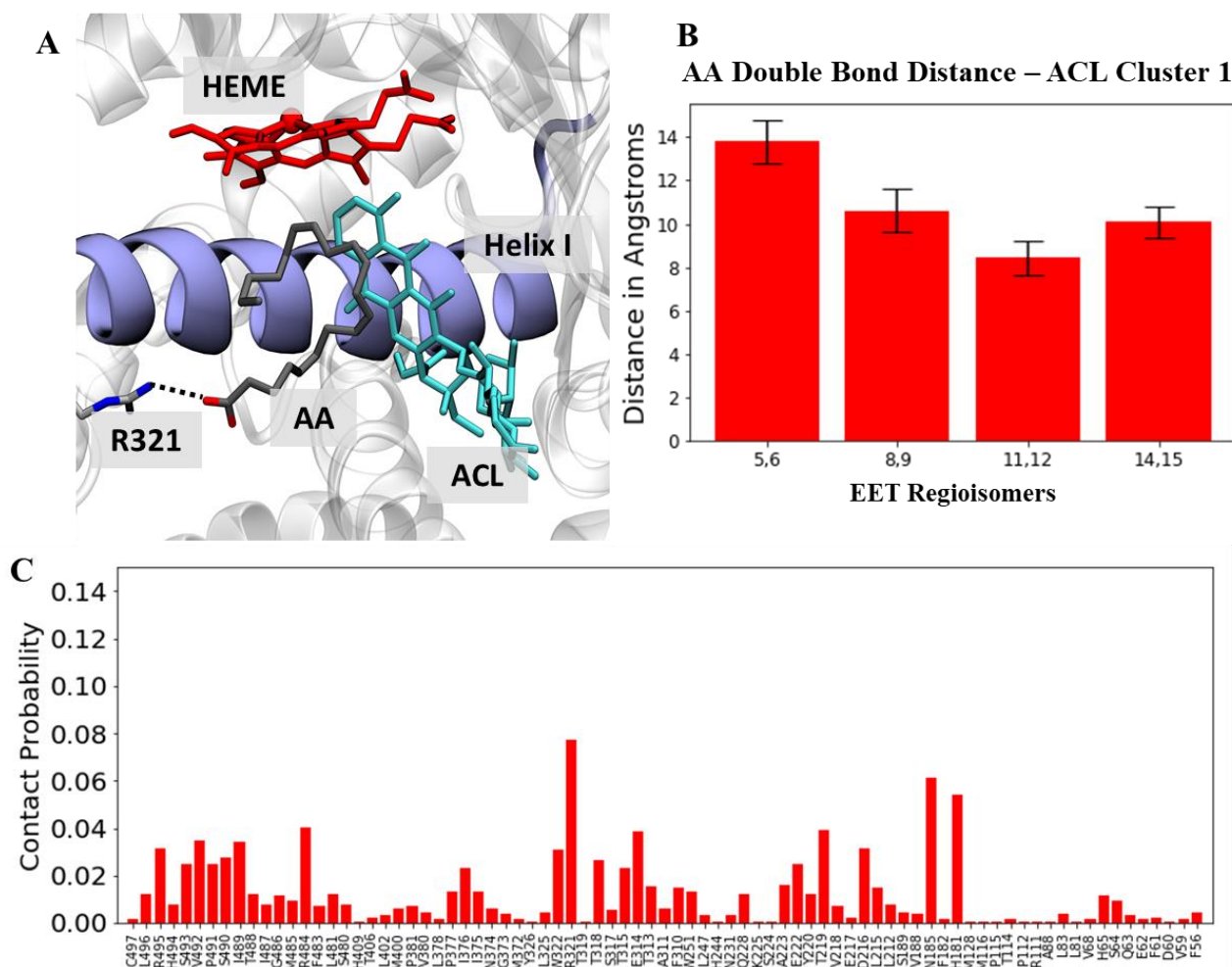


Figure 11. A) Arachidonic acid bound to an equilibrated aclarubicin-CYP2J2 complex, showing stabilization via the canonical PUFA binding residue R321. B) Distribution of arachidonic acid double bonds relative to the central heme iron in CYP2J2, lower values correspond to increased likelihood of epoxy formation. C) Contact probability in Figure 11C is defined as residues within 3 angstroms of the ligand of interest. The total number of residue hits were counted and divided by the frame total for every residue.

3.5 DISCUSSION

CYP2J2 is highly expressed in human cardiomyocytes and is known to metabolize polyunsaturated fatty acids including arachidonic acid to form cardioprotective epoxides such as epoxyeicosatrienoic acids (EETs). As previous studies have investigated the cardioprotective potential of EETs, we sought to investigate whether other commonly used anthracyclines similarly inhibited CYP2J2 function. As these anthracycline analogues were developed with the objective of reducing toxic side effects and cardiotoxicity in patients, we hypothesized that it will be of importance to evaluate their interaction with cardiac CYP2J2 to help in the design of better future analogues with little to no adverse cardiovascular effects. We observed that these anthracycline derivatives bind strongly to CYP2J2 and inhibit CYP2J2-mediated formation of EETs. This aids in hypothesizing that while the cardiotoxicity of these anthracyclines is not evident in smaller time scales of the cardiotoxicity study, their inhibition of cardiac CYP2J2 may manifest in long-term cardiotoxic effects that are yet to be studied in a pre-clinical model. While these data presented here does not present a clear model correlating anthracycline effects on CYP2J2 with degree of cardiotoxicity found in literature, we do find some interesting observations that are summarized below.

By utilizing the fluorescent properties of the anthracyclines, we first measured the binding affinity of the anthracycline derivatives to CYP2J2. For instance, DOX bound tightly to CYP2J2. DOX and DAU have comparable binding affinity to CYP2J2 due to their structural similarities, which differs by a single hydroxyl group. ACL on the other hand bound with even greater affinity to CYP2J2 with $K_d = 2.44 \pm 0.24 \mu\text{M}$. This is an interesting observation since compared to DOX, ACL has a large steric bulk, lowest cardiotoxicity and greater therapeutic index¹⁸⁹. While both drugs intercalate into DNA and interact with topoisomerase complexes to inhibit DNA replication,

it was thought that there must exist other mechanisms of anthracycline-induced cardiotoxicity. An important distinction is that while ACL has a high therapeutic index compared to DOX, it is not entirely non-cardiotoxic¹⁸⁹. Hence, it is possible that one of the potential mechanisms of ACL's cardiotoxicity is due to the inhibition of EET production by CYP2J2. Due to its large sugar moiety which may cause steric hindrance to enter the active site of CYP2J2, we hypothesized ACL would poorly interact with CYP2J2. However, the strong binding of ACL to CYP2J2 and altered EET production further provides evidence for the need to study alternative mechanisms of toxicity. Notably, others are further modifying anthracyclines to reduce its side-effects²³². Similar modifications to also reduce the inhibition of CYPs by anthracyclines may additionally contribute to increasing therapeutic indices.

To obtain molecular understanding of the nature of the binding of the anthracycline derivatives to CYP2J2, we use ensemble docking and molecular dynamics. We show that the dynamic and flexible nature of CYP metabolic cavities leads to promiscuity in protein-ligand interactions. This dynamical nature is advantageous for the metabolism and breakdown of xenobiotics but can lead to toxicity when the CYP is involved in the metabolism of endogenous lipids and xenobiotics. When modeling CYP-ligand interactions it is vital to incorporate protein dynamics to capture any cryptic or transient sites that may aid in binding²³³. In the case of CYP2J2 we used a 50 ns simulation trajectory with snapshots taken every 100 ps to increase our sampling. Utilizing the snapshots of the simulation trajectory we performed ensemble molecular docking on CYP2J2 with the anthracycline derivative set. The resultant binding scores showed ACL had the highest overall average binding energy scores. This matches nicely with the experimental finding in Table 1. Both the experimental and molecular dynamics demonstrated that ACL, the bulkiest anthracycline derivative in our set, interacts with CYP2J2 most tightly. Close inspection of

interacting residues showed that the glutamic acids in the cavity is coordinating with the glycosamine group of the studied anthracyclines. Furthermore, the carbonyl and carboxylate groups showed interactions with nearby cavity arginine residues, in a similar fashion as found in previous CYP3A4-anthracycline studies ²²⁰. Moreover, an overall trend of the D-ring of anthracyclines being in proximity to the heme iron (Supplementary Figure 12) lends credibility to a C-ring quinone free radical contribution to cardiotoxicity.

In addition, there may be several factors of the ligand structures that contribute to the experimentally and computationally obtained binding affinities. Overall, we see agreement for aclarubicin, whose larger sugar moiety allows for increased contact with cavity residues, ultimately increasing the binding affinity. Furthermore, due to the bulky nature of aclarubicin, it likely will remain bound once in the protein cavity. However, in the case of epirubicin compared to doxorubicin, idarubicin, 5-iminodaunorubicin, and daunorubicin, the difference in binding is likely due to the change of hydroxyl position from the equatorial to axial on the sugar moiety (Figure 7). Any noticeable binding affinity difference due to the change in stereochemistry was only apparent in the fluorescence polarization experiments, where epirubicin had a binding affinity of an order of magnitude higher (Table 1). This subtle difference in stereochemistry leading to a large binding affinity difference was not captured in the computational binding affinities (Figure 9A), likely due to limitations of the empirical scoring function of AutoDock Vina²³⁴. In the case of zorubicin, the docking model reveals two binding modes: one in which the D-ring is proximal to the heme, and one where the benzoylhydrazone moiety is proximal to the heme. The increase in structural rigidity provided by the benzoylhydrazone moiety likely decreases the accessible conformational modes of zorubicin, leading to a subsequent entropically unfavored binding and decrease in binding affinity. Lastly, valrubicin contains a trifluoroacetyl modification on the sugar

moiety that likely contributes to the strong binding affinity. In particular, fluoride molecules are highly electronegative and may contribute to increased binding affinity²³⁵.

To predict the effects of DAU derivatives on the metabolism of AA, we implemented an iterative ensemble molecular docking scheme. In this scheme, the DAU derivatives were docked onto an apo-CYP2J2 MD trajectory, clustered, filtered based on energy score, re-solvated and simulated, and the resultant holo-CYP2J2 trajectory used to dock AA. This iterative scheme allowed for us to observe the effects of the DAU derivatives on AA positioning in CYP2J2. Analysis of unsaturated double bonds on AA docked to holo-CYP2J2 and their distance the heme iron was used as the metric for prediction of AA metabolism to EETs.

We further utilized fluorescence polarization (FP) measurements to assess whether anthracyclines are competitively inhibiting CYP2J2's binding to ebastine (Supplementary Figure 11, Supplementary Table 8), a known CYP2J2 substrate. Previously, it has been shown that DOX can competitively bind to CYP2J2 in the presence of ebastine with $K_i = 13.80 \pm 1.60 \mu\text{M}$. While other anthracyclines were not as strong an inhibitor of CYP2J2, ACL was comparable with $K_i = 35.60 \pm 2.56 \mu\text{M}$. Again, this is surprising considering ACL has bulky groups and is among one of the anthracyclines with greater therapeutic index than DOX. This further strengthens the notion that the unknown mechanism of ACL-mediated cardiotoxicity may be attributed to the inhibition of CYP2J2, thus resulting to a reduced production of bioactive and cardioprotective EETs. These binding data indicate that ACL was comparable to DOX as a competitive inhibitor. However, it is important to acknowledge that there may be alternative fitting models including the non-competitive inhibition model. While this is a possibility, the experimental design is such that CYP2J2 is embedded and solubilized into a lipid-reconstituted system. The partition coefficient of anthracyclines (LogP) suggests that to interact with CYP2J2, the anthracyclines must enter through

the lipids in which the protein (and subsequent active site) is anchored to. Furthermore, it is worth acknowledging that the experimental design is such that the protein is titrated into the experimental reaction mixture containing the fluorescent anthracycline, with or without ebastine. However, to most accurately assess whether anthracyclines are competitively inhibiting the binding of ebastine to CYP2J2, anthracyclines must be titrated into the mixture containing the protein and ebastine. Due to the fluorescent properties of anthracyclines, there is an experimental limitation in thoroughly and accurately determining the IC_{50} and subsequent K_i . Thus, while informative, binding studies must be followed up by direct metabolism studies to assess whether anthracyclines truly influence CYP2J2-mediated AA metabolism.

To investigate whether anthracyclines are directly metabolized by CYP2J2, we measured their direct metabolism using LC-MS/MS. In absence of authentic standards of the aglycosylated products, we were able to show that the aglycosylated, or 7-deoxy aglycosylated products for some of the anthracycline derivatives (DAU, EPI and IDA) were formed (Supplementary Figure 14). Furthermore, we showed that the anthracyclines differentially inhibited CYP2J2-mediated metabolism of AA to EETs. In comparison to the control, VAL did not inhibit EET production. Conversely, the other anthracyclines all decreased total EET production at either 5 or 50 μM concentrations. Interestingly, drugs with greater therapeutic indices than DOX had comparable or greater inhibitory effects on EET production. For instance, ZOR is an anthracycline with lower cardiotoxic effects than DOX as measured by nucleotide leakage²³⁶. While the precise mechanism of toxicity is not fully understood, ZOR was among the top inhibitors of EET production. Similarly, ACL had comparable inhibition of 14,15-EET while increasing 5,6-EET production to DOX at 50 μM . Of note, there was inhibition of total EET production by EPI and 5-IDN at 5 μM compared to the other anthracycline derivatives. These data suggests that these drugs may interact

with CYP2J2 as an inhibitor more strongly at lower concentrations compared to other anthracyclines. Although there are key differences in the ligand structures that may contribute to the binding affinities determined by fluorescence polarization and computational docking, experimental measurements are conducted in the presence of arachidonic acid. It is likely that these findings are different than the measured binding affinities due to the presence and availability of CYP2J2 substrates or ligands. This further illustrates the need for a multi-approach assessment of the toxicity of anthracyclines.

Of note, P450 enzymes possess multiple properties in addition to the formation of epoxides. Specifically, P450 enzymes are widely known to be abundantly expressed in the liver where they metabolize xenobiotics²³⁷. Several P450s including CYP1A2, CYP2C9, CYP2D6, CYP3A4, and CYP3A5 metabolize a wide majority of drugs^{238, 239}. Furthermore, polymorphisms contribute to a patient's ability to metabolize drugs²⁴⁰. It has been shown that anthracyclines can alter the expression of P450s in both the liver²⁴¹ and heart²⁴². In the liver where xenobiotic metabolism primarily occurs, chemotherapeutic agents were found to induce expression and activity of CYP3A4²⁴³. The role of xenobiotic metabolizing P450s is particularly important as it will process and clear the anthracyclines. With respect to the heart, P450 genes were induced by DOX in cardiac H9c2 cells²⁴⁴. Since epoxidizing P450s are primarily expressed in cardiac tissue²⁴⁵, it is imperative that both xenobiotic metabolizing and epoxidizing P450s are investigated for their relationship to anthracycline derivatives. While the specificity of CYP2J2 compared to other P450s can be predicted computationally, there exists compounding bias due to iterative docking. As such, the direct binding and alteration of P450-mediated epoxidation must be measured experimentally.

In conclusion, the effects of anthracyclines on AA metabolism may not be the primary mechanism of cardiotoxicity. Rather, the effects undermine the ability to combat the toxicity and

weaken the heart against toxic events overall. Therefore, the remaining toxicity of less-toxic analogues, like ACL, may be explained by their effects on EET production. However, more studies that directly address the effect on EET production *in vivo* will have to be conducted to clarify this hypothesis. While ROS production is an important mechanism linked to cardiotoxicity, it is also known that P450s can be uncoupled to generate ROS. As uncoupling requires P450 reductase, which can also interact with the C-ring of anthracyclines to produce quinones and subsequent ROS, this was not measured experimentally. As such, future studies examining the uncoupling of P450s by anthracyclines is warranted.

3.6 ACKNOWLEDGEMENTS

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CHAPTER 4: UTILIZING LIPIDOMIC TECHNIQUES TO EXAMINE MYELIN LIPIDOME UNDER UPPER RESPIRATORY INFECTION

4.1 INTRODUCTION

Systemic Inflammation

Systemic inflammation induces behavior modifications including cognitive impairment, anxiety, lethargy and depression²⁴⁶ and can accelerate the progression of many neurological disorders and diseases including schizophrenia, Alzheimer's disease²⁴⁷, Parkinson's disease²⁴⁸, and multiple sclerosis^{249, 250}. Exacerbation of these diseases by systemic infection is strongly associated with inflammatory sequelae occurring within the central nervous system. It is well-known that microglia become reactive in response to systemic inflammation²⁵¹ or viral infection²⁵²⁻²⁵⁴ and that this phenomenon is linked with the development of sickness behaviors. Microglial reactivity is also a prominent feature of most white matter diseases²⁵⁵ and the proinflammatory cytokines that are secreted by microglia during systemic inflammation influence oligodendrocyte (OL) homeostasis²⁵⁵. Whether systemic inflammation brought on by an infectious pathogen alters myelin in adults has not yet been investigated. However, recent data suggest that chronic lung inflammation resulting from asthma predicts a concurrent decline in both myelination and cognitive function²⁵⁶. Additionally, we have found that lung inflammation caused by influenza infection profoundly decreases OL-specific gene expression and likely affects myelin²⁵⁷. Since influenza infection causes microglial reactivity and cognitive impairment^{252, 254} it is possible that systemic inflammation stimulates microglia to produce factors that affect myelin plasticity and that these alterations underlie changes to behavior.

In submission to Nature Communications.

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Myelin, the multilamellar substance of brain white matter, forms compact insulating sheaths that surround axons. In humans, white matter constitutes approximately 40% of the brain, by which myelin is the primary component (approximately 50% dry weight of white matter)²⁵⁸. Proper myelin formation increases neuronal conduction velocity by up to 100 fold²⁵⁹, promotes energy-efficient action potentials²⁶⁰, and provides trophic support for axons⁸³. Myelin is imperative for normal motor function and higher order cognition and behavior. Additionally, proper myelination during development corresponds to cognitive functions including vocabulary and decision making^{261, 262}. In contrast, prenatal white matter disease periventricular leukomalacia is the leading cause of cerebral palsy, and causes permanent motor, cognitive, and behavioral deficits²⁶³. In addition, demyelination is a key pathological feature in numerous neurological diseases. Specifically, white matter abnormalities are observed in schizophrenia²⁶⁴⁻²⁶⁶, cognitive decline due to aging, Alzheimer's disease²⁶⁷, multiple sclerosis²⁶⁸ and depression²⁶⁹.

In the central nervous system (CNS), oligodendrocytes (OL) are responsible for the formation of myelin. As one oligodendrocyte can create an estimated myelin surface area of approximately $20 \times 10^5 \mu\text{m}^2$ ²⁷⁰, it is clear that OLs are among the most imperative membrane producing cells in the body. Interestingly, oligodendrocytes have the capacity to completely renew myelin membranes up to three times in one day²⁷¹, a finding corroborated by recent studies in zebrafish demonstrating the capacity for oligodendrocytes to make new myelin sheaths in five hours²⁷². It has been known for some time that alterations in OL homeostasis affect cognition and behavior. However, the notion that myelin remains static during adulthood has recently been challenged. For instance, while neurogenesis in rodents is promoted by exercise and environmental enrichment^{273, 274}, it can be negated by social isolation^{275, 276}. Furthermore, it is evident that not all axons in white matter are myelinated, and the degree of myelin on axons can differ^{277, 278}. White

matter changes also occur when learning complex motor skills such as juggling²⁷⁹ and playing the piano²⁸⁰. Yeung et al., leveraged atmospheric ¹⁴C to date oligodendrocytes sorted from post-mortem tissues obtained during various stages of life to explore the turnover of OL. In doing so, they provided definitive evidence that OL cell turnover is slow, but the myelin membrane itself is highly dynamic²⁸¹.

The myelin sheath is a multilamellar structure characterized by a high proportion of lipids to proteins, which differs from most biological membranes. The lamellar layers of myelin can be visualized by electron microscopy by which there are apposition of the cytoplasmic leaflets (major dense line) and the apposition of the extracellular leaflets (intraperiod line)⁸⁴. Myelin is comprised of approximately 70% lipids and 30% proteins, which collectively contributes to the stability and integrity of the myelin²⁸². The most abundant lipids in myelin are cholesterol, galactosylceramides, and plasmalogens. Lipids such as cholesterol are a prerequisite for the synthesis of myelin. Other lipids such as galactosylceramides and plasmalogens play a critical role in the compaction and stability of myelin. For instance, galactosylceramides are highly hydrophobic by which long hydrocarbon fatty acid chains intercalate into the inner membrane leaflet and galactose head group presents itself at the intraperiod line⁸⁴. The hydrophobic interactions of the hydrocarbon chains attract myelin membranes together and repel extracellular fluids^{283, 284}. In addition, galactose head groups in the intraperiod line have attractive properties that contribute to the compaction of the lamellar layers in myelin^{285, 286}. In addition to lipids, proteins play a critical role for the formation of myelin. For instance, proteolipid protein (PLP)²⁸⁷ and myelin basic protein (MBP)²⁸⁸ are the two most abundant proteins in CNS myelin. These hydrophobic membrane proteins form covalent, or electrostatic interactions with negatively charged lipids that promote the proper structural

formation of myelin. Collectively, the interactions of both lipids and proteins within the myelin contribute to the overall compaction and subsequent structural integrity of myelin.

Assessment of Myelination and Demyelination

The degree of myelination and demyelination is often measured to assess degree of development, neurological decline, and neurological recovery²⁸⁹⁻²⁹¹. Myelination is often assessed in several ways including examining myelin proteins by staining, or measuring the thickness of myelin through electron microscopy²⁹²⁻²⁹⁴. While it is true that myelin proteins such as myelin basic protein are necessary for the proper formation of myelin, these do not take into consideration lipid composition. This is more evident by the findings that the half-life of myelin proteins is estimated to be 6 months²⁹⁵. Furthermore, PLP and MBP turns over at a slower rate than cyclic nucleotide phosphodiesterase (CNP)²⁹⁶, a marker of non-compact myelin. Given the slow turnover of myelin proteins, it is necessary to evaluate both proteins and lipids to obtain a comprehensive understanding of the degree of myelination.

It is imperative to note that while myelin proteins are unique to myelin, lipids are present in other regions of the brain. Thus, isolation of purified myelin as previously described²⁹⁷ is a requisite for the proper assessment of lipid changes in the myelin. Following confirmation of myelin isolation by techniques such as western blot for myelin proteins or non-myelin proteins such as glial fibrillary protein, lipidome profiling can be conducted through mass spectrometry

4.2 MATERIALS AND METHODS

Animals and Infection

C57BL/6J male and female mice were obtained from Jackson Laboratories (No. 000664). All animals were housed 4-5 per cage under constant 12-h light/dark cycles (10am-10pm) and constant temperature and fed with a standard rodent diet (Envigo 2918) ad libitum. Mice were anesthetized with 4% isoflurane and then inoculated with either sterile PBS or mouse-adapted human influenza A virus (IAV) (strain A/Puerto Rico/8/1934 H1N1; PR8) diluted in sterile PBS in a total volume of 30 μ L at 8- to 11 weeks of age. Animals were sacrificed at day 4, 8, and 16 post-infection for follow-up experiments. All animal care protocols were in accordance with National Institutes of Health Guidelines for Care and Use of Laboratory Animals and were approved by the University of Illinois Laboratory Animal Care and Use Committee.

Myelin Extraction

Animals were euthanized via CO₂ asphyxiation and perfused with phosphate buffer saline (pH 7.4). Whole brains were harvested and flash frozen under liquid nitrogen within 2 minutes of euthanasia. Myelin was extracted from brains under sucrose density centrifugation and osmotic shock as previously described²⁹⁷. All solvents were prepared fresh at 4 ° C, and extractions conducted under ice. Additionally, all extractions were conducted using the same solvents on the same day. Briefly, brains were homogenized with a dounce homogenizer in 0.3M sucrose containing 0.7M Tris-HCl (pH 7.4), 10 μ g/mL Leupeptin (Sigma Aldrich, No. 103476-89-7), 10 μ g/mL Antipain (Sigma Aldrich, No. 37691-11-5), and 100 μ M phenylmethylsulfonyl fluoride (GoldBio, No. P-470). Homogenized brain samples were layered on 0.83 M sucrose containing Tris-HCL, leupeptin, antipain and phenylmethylsulfonyl fluoride (PMSF). The dounce

homogenizer was washed twice with 0.3 M sucrose and additionally layered. Samples were centrifuged for 30 minutes at 75,000 xg , 4 ° C, and without brakes to prevent disruption of density gradient layers. Following centrifugation, the crude myelin present in the interface between 0.83 M sucrose and 0.3 M sucrose was collected and transferred to a new centrifuge tube containing MQ H₂O for centrifugation at 75,000 xg , 4 ° C for 30 minutes. Osmotic shocks were then conducted twice by resuspending myelin in 5 mL MQ H₂O and homogenized thoroughly. Following a 10 minute incubation on ice, samples were centrifuged for 15 minutes at 12,000 xg , 4 ° C and without brakes. After two osmotic shocks, pelleted crude myelin was resuspended in 2 mL 0.83 M sucrose and layered on 16 mL 0.83 M sucrose, followed by a 0.3 M sucrose. The samples were centrifuged for 15 minutes at 75,000 xg , 4 ° C and without brakes. The interface was then transferred to a new tube and resuspended in MQ H₂O for final centrifugation at 75,000 xg , 4 ° C and without brakes. The supernatants were carefully discarded to obtain the purified myelin. The purified myelin was then resuspended in 100 μ L 1M Tris-HCl and stored in -80 ° C.

Lipid Extraction

Purified myelin was extracted for total lipids using the Bligh & Dyer method as previously described²⁹⁸. Briefly, extracted myelin sheath samples were added to 750 μ L 1:2 CHCl₃:MeOH and vortexed for 15 minutes at 25°C (room temperature). Following a 5 minutes incubation on ice, 250 μ L CHCl₃ and 250 μ L MQ H₂O were added to the sample. Samples were then vortexed for 5 minutes at 25 ° C, and centrifuged for 5 minutes at 800 xg at 4°C. The organic layer was collected and dried under steady flow of N₂ gas and resuspended in 90% ethanol for analysis via LC-MSMS.

Instrument Analysis

The samples were spiked with 5 μ L of 50 μ g/mL internal standard mixture (Ceramide 18:1/12:0; Phosphatidylcholine 12:0/12:0; Phosphatidylethanolamine 14:0/14:0; phosphatidylglycerol 14:0/14:0; phosphatidylserine 14:0/14:0) before instrument injection. The samples were analyzed at the Metabolomics Laboratory of the Roy J. Carver Biotechnology Center of the University of Illinois at Urbana-Champaign²⁹⁹. Samples were injected into the Dionex Ultimate 3000 series HPLC system (Thermo, Germering, Germany) which included an autosampler, degasser, and a binary pump. Liquid chromatography separation was conducted on a Thermo Accucore C18 column (2.1 mm x 150 mm, 2.6 μ m) with a flow rate of 0.4 mL/min. Mobile phase A (60% acetonitrile: 40% H₂O containing 10 mM ammonium formate and 0.1% formic acid) and mobile phase B (90% isopropanol: 10% acetonitrile containing 10mM ammonium formate and 0.1% formic acid) was utilized. The detailed method was similar to previously published paper from the laboratory²⁹⁹. The following linear gradient was used: 0 min, 70% A; 4min, 55% A; 12 min, 35% A; 18 min: 15% A, 20-25 min: 0% A, 26-33 min: 70% A. The injection volume was 10 μ L, autosampler was set to 15°C, and column was kept at 45°C. Thermo Q-Exactive MS system (Bremen, Germany) was used for obtaining tandem mass spectrometry under both positive (sheath gas flow rate, 50; aux gas flow rate, 13; sweep gas flow rate, 3; spray voltage, 3.5 kV; capillary temp, 263 ° C; aux gas heater temp, 425 ° C) and negative electrospray ionization (sheath gas flow rate, 50; aux gas flow rate, 13; sweep gas flow rate, 3; spray voltage, -2.5 kV; capillary temp, 263 ° C; aux gas heater temp, 425 ° C). The full scan mass-spectrum resolution was set to 70,000 resolution at m/z 200 with scan range of m/z 230-1,600. The automatic gain control target was 1,000,000 using the maximum injection time of 200 ms. For MSMS, the mass spectrum resolution was set to 17,500. The automatic gain

control (AGC) target was 50,000 with a maximum injection time of 50 ms. Loop count was 10. Isolation window was m/z with normalized collision energy (NCE) of 25 and 30 eV.

Data analysis

Lipids were identified with Thermo Fisher software LipidSearch (Version 4.1.30) as previously described^{300, 301}. The lipid signals were normalized to whole brain mass and the corresponding internal standard signal responses. For lipid classes without corresponding internal standard, positive lipid ion signals were normalized to signal of Ceramide 18:1/12:0 and negative lipid ion species normalized to signal of Phosphatidylglycerol 14:0/14:0. We only focused on monoisotopic species. LipidSearch software was used to predict the possible fragment ions for lipid species within the precursor ion tolerance using the accurate m/z values of the precursor ion. The LipidSearch database are built using the known fragment ions for lipid classes and the intensity pattern based on measured spectra. Identification was done by using the parent ion that was based on the accurate mass of precursor ion with the mass shift tolerance of 5 ppm. Identification of the product ion was based on the accurate mass of precursor ion with mass shift tolerance of 8 ppm and MSMS spectral pattern.

Statistical Testing

Differential expression analysis between PBS and IAV was carried using the two-sided non-parametric Wilcoxon ranksum statistic. We used the Python SciPy package for calculating the ranksum statistic for each lipid species. We also computed expression fold-change of IAV versus PBS as a second measure for evaluating differential expression. We required p -value < 0.05 jointly with fold-change > 2 or fold-change < 0.5 for significant differential expression. In

total, only 108 lipid species belonging to 13 distinct lipid families passed these thresholds. Since only a small number of species characterized as differentially expressed lipids, the chance of having lipids wrongly identified as significant (Type 1 error) is small. Therefore, we did not use any p-value correction method and worked with p-values directly obtained from statistical testing.

4.3 RESULTS

Lipid extractions of both the mPFC and from the myelin of the whole brain was analyzed. To isolate crude myelin, we homogenized the brain and isolated through sucrose gradients. To obtain pure myelin from crude myelin, we conducted osmotic shocks on the samples, prior to analysis on LC-MSMS. During every stage of the myelin isolation, the debris was collected, and western blot performed in comparison to pure myelin. Our data indicate that proteolipid protein, a protein found in myelin, was indeed found in the myelin fraction and not the brain fraction (Figure 12A). In addition, glial fibrillary acidic protein (GFAP), a protein found in astrocytes, was found in the brain fraction but not myelin fraction, indicating that our isolation was pure. Having established purity, we questioned whether infection changed the lipidomic profile of myelin. Figure 12B and Figure 12C illustrate differential expression analysis between PBS and IAV groups using the two-sided non-parametric Wilcoxon rank sum test. Expression fold-change of IAV vs. PBS was computed as a second measure for evaluating differential expression. Since only a small number of species were characterized as differentially expressed lipids, the Type 1 error is small and therefore we did not use any P-value correction method, and worked with P-values directly obtained from statistical testing.

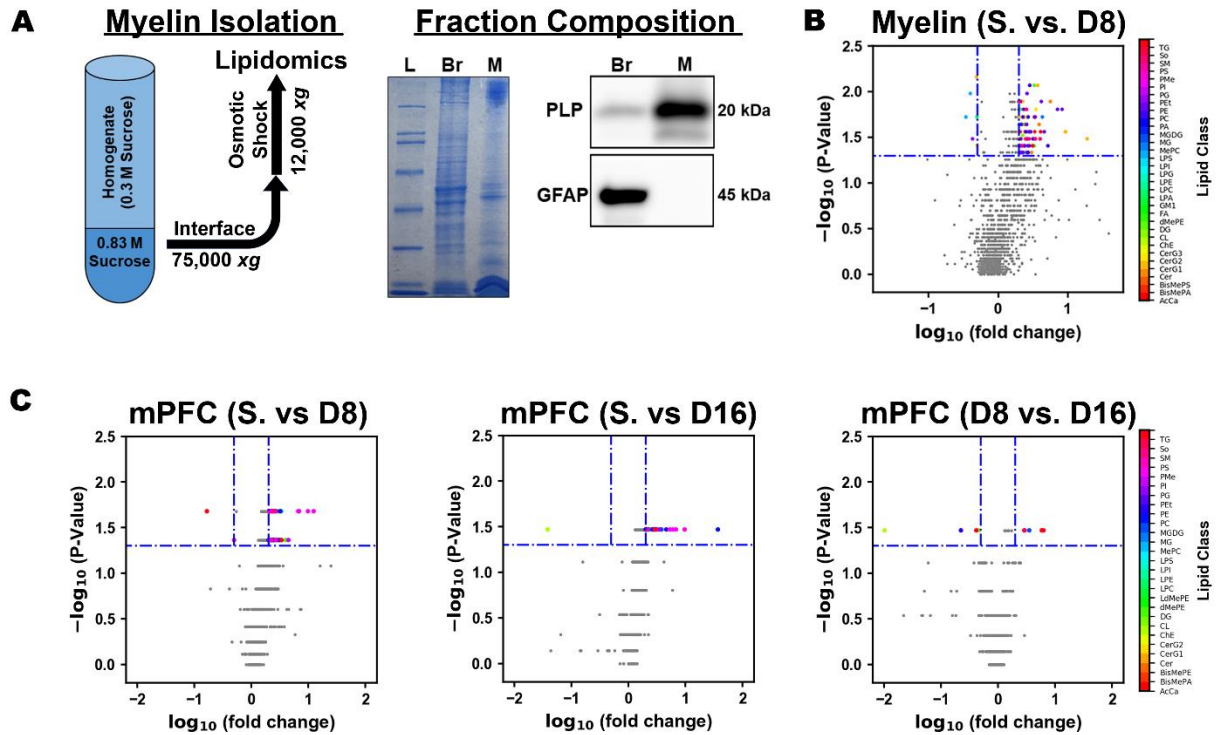


Figure 12. Lipidomics experimental design and statistical analysis. (A) Experimental design for conducting lipidomics on myelin from mice at day 8 p.i. Purified myelin isolated via fractionation by ultracentrifugation and osmotic shock was confirmed by western blot (top) and analyzed for lipid species (bottom). (B) Two-sided non-parametric Wilcoxon Ranksum Analysis plotted against fold change (IAV, n=9 vs. PBS n=12) with p-value significance $p < 0.05$, or (C) in mPFC (IAV, n=4 vs PBS n=4).

The prefrontal cortex is responsible for complex cognitive processes, and white matter in this region is compromised in several neurological disorders including schizophrenia^{302, 303}, autism spectrum disorder³⁰⁴, and major depressive disorder³⁰⁵. Therefore, we first sought to determine if there were any lipidome changes in the medial prefrontal cortex (mPFC) due to influenza infection. We isolated the mPFC from PBS or IAV mice at day 8 and day 16 p.i. In total, 1091 lipid species belonging to 28 major lipid classes were identified in the mPFC. Three pair-wise comparison analyses were conducted in the following manner: A) PBS vs. Day 8, B) PBS vs. Day 16, and C) Day 8 vs Day 16. In the mPFC at day 8 compared to PBS, there were a

total of 215 lipid species that were differentially expressed (Figure 13A). While a majority of the major lipid classes increased in IAV Day 8 of the mPFC, three lipid species belonging to sphingosine and triacylglycerol classes were decreased. At day 16 post IAV infection, 338 lipid species belonging to 25 major lipid classes were differentially expressed when compared to PBS controls (Figure 13B). Interestingly, the only major lipid class that was not increased in IAV Day 16 of the mPFC was cholesterol. When comparing IAV Day 8 to IAV Day 16, there were minimal differences (Figure 13C). Specifically, there were only 16 lipid species that were differentially expressed. These data indicate that modulation of lipids by infection did not recover to baseline levels by day 16, despite recovery of gene expression and body condition score by Day 16 post-infection (data not shown).

To further assess the effects of respiratory viral infection on the myelin, we isolated purified myelin from the whole brain for lipidomic analysis. Myelin was purified from whole mouse brain by density gradient fractionation and osmotic shock. Myelin enrichment was verified by SDS-PAGE and western blot, which confirmed abundant PLP and negligible levels of the astrocyte protein glial fibrillary acidic protein (GFAP) in extraction myelin fractions (Figure 12A). A total of 176 lipid species were differentially expressed out of 1356 total lipid species identified (Figure 13D). Apart from lysophosphatidic acid, lysophosphatidyl serine, and phosphatidylethanolamine which decreased by nearly 2-fold, most of the affected lipid classes were increased in IAV-inoculated mice compared to PBS controls at day 8 post-infection. These data corroborate the lipidomic findings in the mPFC, where lipid species are greatly increased as a result of upper respiratory infection.

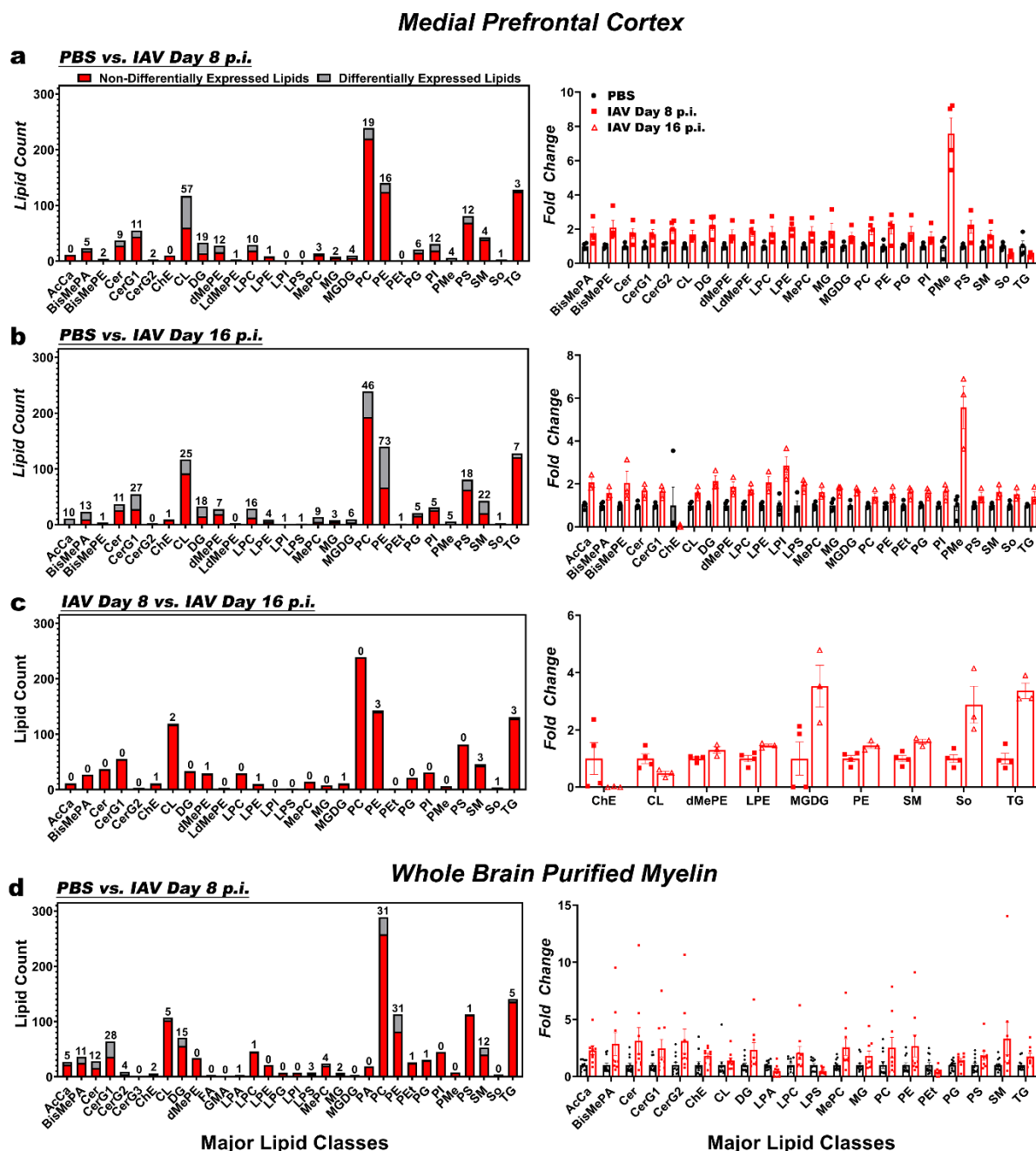


Figure 13. Lipidomics analyses reveals lipid changes in purified myelin. (A-C) Left; Differential expression of lipid species between PBS (n=4), IAV day 8 (n=4), and IAV day 16 (n=3) in the medial prefrontal cortex. 2-3 animals were pooled for each PFC sample. Values above histogram represent number of individual lipid species unique to its respective major lipid class. Right; Differentially expressed lipids ($p < 0.05$) of overall major lipid classes plotted as fold change of PBS (black circle), IAV day 8 (red square), and IAV day 16 (red triangle). (D) Differential expression of lipid species between PBS (n=12), and IAV day 8 (n=12) in purified myelin isolated from whole brain. Differentially expressed lipids ($p < 0.05$) of overall major lipid classes plotted as fold change of PBS (black circle) and IAV day 8 (red square).

In addition to the increase of major lipid classes that are differentially expressed in IAV-infected mice, there are other underlying similarities. For instance, lipids that are known to be involved in the structural integrity of myelin including Ceramides, Glucosylceramides, Galactosylceramides, Phosphatidylethanolamines, Phosphatidylcholine, Sphingomyelin and sphingosines are all differentially expressed in both mPFC and myelin isolated from the whole brain. Therefore, we conducted bioinformatic analysis using LIPEA. Following Bonferroni correction, we discovered that glycerophospholipid metabolism and sphingolipid signaling pathways were the most highly enriched pathways in both the mPFC and myelin (Figure 14C). Collectively, these data demonstrate that influenza infection results in alterations of the myelin lipidome.

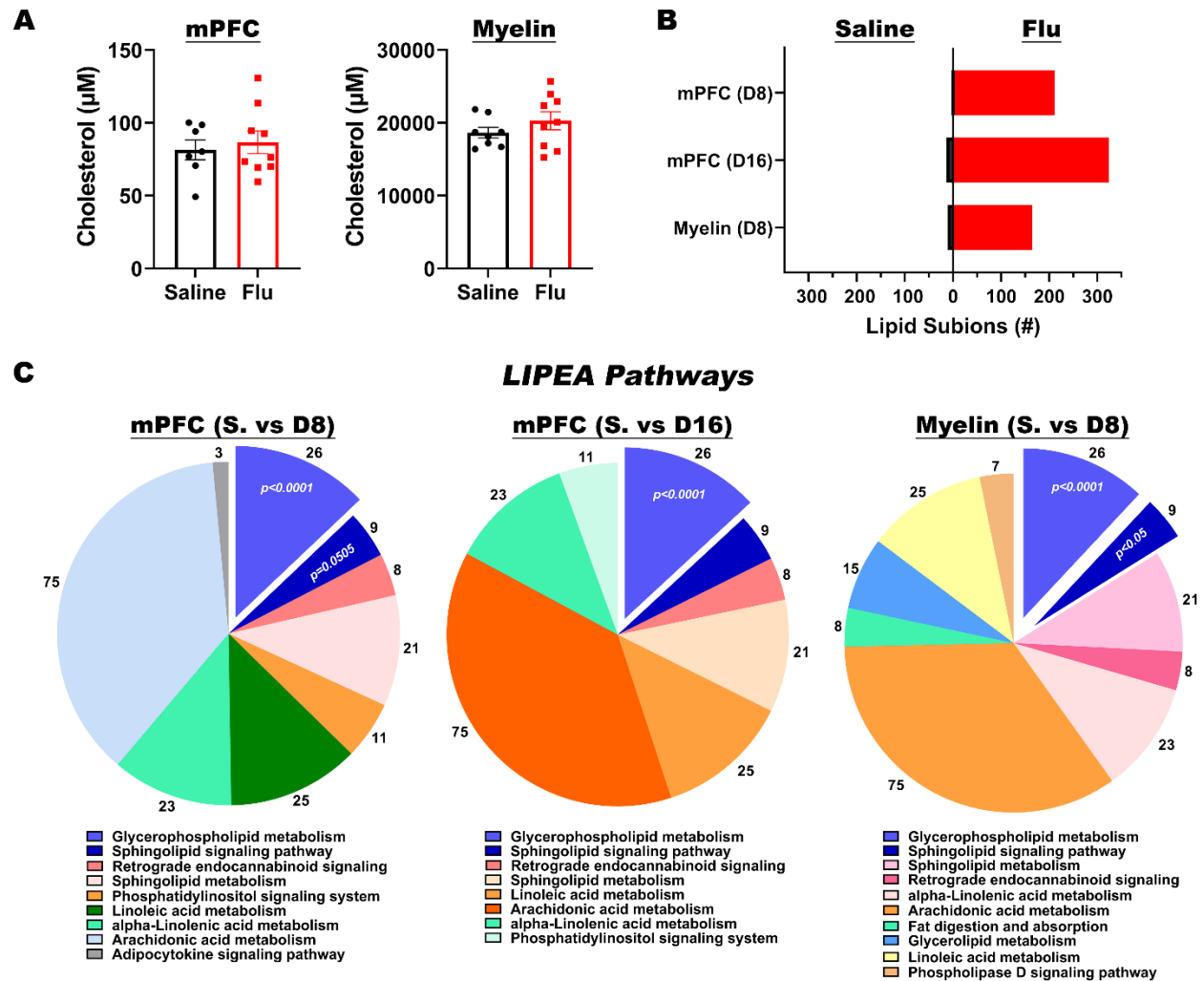


Figure 14. LIPEA Bioinformatics reveal changes in signaling pathways. (a) Cholesterol levels in mPFC and isolated myelin from whole brain in PBS or IAV day 8 p.i. mice. (b) Number of lipid subions greater in PBS or IAV treated mice in mPFC day 8 p.i., mPFC day 16 p.i., or myelin day 8 p.i. mice. (c) Differentially expressed lipids with biological pathways associated with lipid synthesis and degradation analyzed by LIPEA with Bonferroni correction. Values represent number of lipid species involved in each pathway.

As cholesterol is a requisite for myelin and a core limiting factor in tandem mass spectrometry methods include the detection of esterified cholesterol, we performed an Amplex Red Cholesterol Assay on purified myelin or the mPFC from C57/BL6 mice. This methodology

measures both esterified and free cholesterol species. Interestingly, we did not observe differences in the levels of cholesterol between the PBS or IAV inoculated mice in either the myelin or the mPFC at day 8 post-infection (Figure 14A).

4.4 DISCUSSION

In recent years, it has become clear that myelin is dynamic and subject to remodeling even in adulthood. Myelin can be remodeled by environmental factors such as learning a new task, social isolation and reintegration, and by social defeat stress. Stress is considered a potent risk factor for the onset of depression and mood disorders. In socially-isolated or stressed mice, OL and myelin transcripts were decreased in the prefrontal cortex, a region that is compromised in depression and other disorders associated with white matter abnormalities. We previously reported that influenza infection altered myelination genes²⁵⁷. Therefore, we investigated the effect of infection on lipid biochemistry of the mPFC, and the myelin isolated from the whole brain. Previous studies found that infection invoked widespread downregulation of transcripts involved in myelination in the adult mouse CNS²⁵⁷. We found that infection caused changes to the myelin lipidome. These changes were predominantly characterized by upregulation of SM, Cer, and CerG lipid species, but also entailed downregulation of the LPA, LPS, and PEt lipid classes. To our knowledge, this is the first study to demonstrate that respiratory viral infection can facilitate lipidomic changes to the myelin.

By lipidomic analysis of the myelin isolated from the whole brain, we found that 13% (176 out of 1356) of total lipid species identified were affected by non-neurotropic influenza infection occurring in the periphery, the majority of which were upregulated. Although RNA analyses revealed the transcription of genes involved in lipid biosynthetic pathways was

downregulated, it is important to recognize that lipids are often short-lived and precursors are continuously recycled. Thus, downregulation of an enzyme involved in a lipid biosynthetic pathway may in fact cause a bottle-neck accumulation of certain lipid substrates. Furthermore, although the downstream product may be the same in a given pathway, the substrate preference of different lipid synthases varies. For instance, CerS5 and CerS6 favors Acyl-CoA lengths of C14:0 to C18:0, while CerS2 prefers Acyl-CoA lengths of C20:0 to C26:0, but these ceramide products containing different R-chain lengths are all substrates to UDP-galactosyltransferase, for which transcription was suppressed. As such, suppression of *Ugt8a* might promote accumulation of ceramide and sphingomyelin species.

While influenza infection increased structural lipids in the myelin, there are three major lipid classes that decreased due to infection. Phosphatidylethanol and lysophosphatidylserines have not been documented within the myelin. Lysophosphatidylserine however has been studied as a signaling molecule through its interaction with Toll-Like-Receptor 2 (TLR2)³⁰⁶, as well as GPR174 present on T cells³⁰⁷. Lysophosphatidic acid has been previously studied in regards to myelination. Lysophosphatidic acid (LPA) within the central nervous system is present in the embryonic brain, neural tube, choroid plexus, meninges, blood vessels, spinal cord, and cerebrospinal fluid. LPA lipids interact through LPA Receptors (LPAR)³⁰⁸. Most studied is LPAR1, a receptor first discovered in the brain³⁰⁸ is necessary for proliferation, differentiation and survival of neuronal precursors. One study found that LPAR1 expression in postnatal mature oligodendrocytes was associated with the onset of myelination in the brain. Additionally, LPAR1-null mice possessed a higher percentage of unmyelinated fibers compared to control mice³⁰⁹. In support of these findings, Anliker et al. reported that LPA release by DRG neurons was necessary to promote Schwann cell migration. In LPAR1-null mice, there was an observed

decrease myelin thickness³¹⁰. In our study, we observed a decrease in LPA lipids in day 8 p.i. mice, as well as a correlated decrease in *LPAR1* expression (FC = -1.3178 with FDR $p < 0.001$) in the cerebellum at day 8 p.i. Since LPA and LPAR are necessary for myelination and their subsequent decrease will lead to reduced myelination, it is possible that an increase in glycerophospholipids and sphingolipids at day 8 p.i. is to aid in the maintenance of the existing myelin structure.

In the mPFC, the majority of lipids were upregulated due to influenza infection. We observed changes to phosphatidylcholines, which is like other neurological diseases such as schizophrenia. Patients with schizophrenia have dysregulated levels of phosphatidylcholine in gray matter. Furthermore, it has been shown that phosphatidylcholine and phosphatidylethanolamine plasmalogens were increased in the frontal cortex in Alzheimer's patients. Despite these trends, there are some conflicting data in the field. In contrast to previous studies, others have shown a decrease in phosphatidylcholine and phosphatidylethanolamine lipids in the prefrontal cortex of patients with cognitive disorders. While changes of the lipids in the mPFC may be indicative of behavioral changes and cognitive function that, it is not a measurement of myelin. Since we previously observed changes to the OL transcriptome, we next measured the changes in the myelin lipid biochemistry.

The overarching trend in the mPFC and the myelin was an increase of lipids as a result of influenza infection. While seemingly counterintuitive, the increase of lipids does not guarantee that the myelination process is occurring, or that the myelin structure is stable. In the myelin sheath specifically, we found only ~13% (176 out of 1356 total) lipid species to be differentially expressed. Within the differentially expressed lipids, bioinformatics analysis identified these lipids to be involved in the glycerophospholipid and sphingolipid pathways. Lipids directly

involved in maintaining the structure of myelin include cholesterol, ceramide, glucosyl- or galactosyl-ceramides, and phosphatidylethanolamine. Given that compaction, structure, and function of myelin is dependent on both structural proteins and lipids, it is possible that the observed increase of lipids with known compaction properties is a natural means to counteract the decreased transcriptional expression of structural proteins MBP and PLP. Previous studies support this hypothesis where absence of 2-hydroxylated sphingolipids resulted in myelin degeneration⁸⁵. Another study showed that transgenic mice lacking UDP-galactose:ceramide galactosyltransferase had a deficiency in galactosylceramides. Subsequent to this deficiency, the mice developed tremors, uncoordinated gait, and eventual paralysis⁸⁶, all of which are indications of dysmyelination.

While shotgun lipidomics have advantages of high throughput, it is difficult to delineate isobaric species³¹¹. Additionally, several other critical lipids such as cholesterol that are often stored in their esterified form in the membranes cannot be detected without de-esterification process that might cause other changes in the lipids extracted from the myelin of mice. Specifically, cholesterol is composed of 3 major components: A) tetracyclic carbon ring, B) polar hydroxyl group on Ring A, and C) non-polar carbon chain on Ring D³¹². Within the body however, cholesterol species are often found in the esterified form. While mass spectrometric methods including GC-MS and LC-MS/MS can detect these molecules, a de-esterification process in the sample preparation is often required to maximize detection. Thus, other methodologies such as the Amplex Red Cholesterol Assay may be needed in conjunction with high throughput lipidomic techniques. Importantly, our results align with previously published work, indicating that myelin extraction from 8-10 week old mice analyzed via LC-MSMS does not yield high levels of cholesterol³¹³. Additionally, while we observe changes to the lipid

profile, we do not know the structural changes to the myelin. To confirm this, further studies measuring the myelin diameter (thickness) through serial-block electron microscopy, and degree of compaction through stimulated Raman scattering microscopy³¹⁴ are needed.

In conclusion, our results demonstrate that peripheral infection with influenza A alter the myelin lipidome in the adult mouse CNS. Myelination is often measured through levels of structural proteins present within the myelin. However, given that myelin protein turnover varies and myelin composition is predominantly lipids, it is clear that a direct investigation of the lipid species is necessary to measure demyelination.

4.5 ACKNOWLEDGEMENTS

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APPENDIX A: REVIEW – EMERGING CLASS OF OMEGA-3 FATTY ACID ENDOCANNABINOIDS & THEIR DERIVATIVES

A.1 Abstract

Cannabinoid receptor activation is involved in homeostatic regulation of the body. These receptors are activated by cannabinoids, that include the active constituents of *Cannabis sativa* as well as endocannabinoids (eCBs). The eCBs are endogenously synthesized from the omega-6 and omega-3 polyunsaturated fatty acids (PUFAs). The consumption of omega-3 fatty acids shifts the balance towards a higher proportion of omega-3 eCBs, whose physiological functions warrants further investigation. Herein, we review the discovery of omega-3 fatty acid derived eCBs that are generated from long chain omega-3 PUFAs - docosahexaenoyl ethanolamide (DHA-EA or synaptamide), docosahexanoyl-glycerol (DHG), eicosapentaenoyl ethanolamide (EPA-EA), eicosapentanoylglycerol (EPG). Furthermore, we outline the lesser known omega-3 eCB-like molecules that arise from the conjugation of the omega-3 fatty acids with neurotransmitters serotonin and dopamine - DHA-serotonin (DHA-5HT), EPA-serotonin (EPA-5HT), DHA-dopamine (DHA-DA) and EPA-dopamine (EPA-DA). Additionally, we describe the role of these omega-3 eCBs and their derivatives in different disease states such as pain, inflammation and cancer. Moreover, we detail the formation and potential physiological roles of the oxidative metabolites that arise from the metabolism of omega-3 eCBs by eicosanoid synthesizing enzymes - cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 epoxygenase (CYP450). In summary, we outline the novel findings regarding a growing class of signaling molecules, omega-3 eCBs, that can control the physiological and pathophysiological processes in the body.

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A.2 INTRODUCTION

Cannabis sativa is used for both medical and recreational use ^{1, 2}. In the early 1950s, Δ^9 -tetrahydrocannabinol (Δ^9 -THC), the primary psychoactive molecule, was isolated from *Cannabis sativa* ³. This led to a strong interest in the synthesis of cannabinoid (CB) analogs to understand the structure-activity relationship with putative CB receptors ⁴⁻⁸. Eventually, two G-protein coupled receptors (GPCRs), cannabinoid receptor 1 and 2 (CB₁ and CB₂), were discovered ^{9, 10}. While CB₁ is highly expressed in the central nervous system (CNS), CB₂ is present primarily in immune and peripheral cells. Since Δ^9 -THC and other CBs that bind to CB₁ and CB₂ are lipophilic, there was a search for endogenous lipids that interacted with these receptors.

In the 1990s, the first endogenous cannabinoids (eCBs) that were shown to activate CB₁ were anandamide (AEA) and 2-arachidonoylglycerol (2-AG) ¹³⁻¹⁵. AEA and 2-AG are synthesized from omega-6 polyunsaturated fatty acid (PUFA), arachidonic acid (AA), and are hydrolyzed by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively. Later studies identified other eCB-metabolizing enzymes, such as FAAH 1 and 2 for AEA; MAGL, α , β -hydrolase domain-containing 6 and 12 (ABDH6 and ABDH12) for 2-AG. Both AEA and 2-AG are also metabolized by eicosanoid synthesizing enzymes such as lipoxygenases (LOX), cyclooxygenase 2 (COX-2) and cytochrome P450 epoxygenases (CYP450) to form new bioactive molecules ^{16, 17}. Together, the interactions between the CB receptors, eCB ligands, as well as their biosynthetic and degradative enzymes, constitute the eCB system (ECS) and are involved in maintaining homeostasis in the biological system ¹⁸.

Broadly, the eCBs are members of a large group of structurally related fatty acid derived signaling molecules. It is important to note that eCBs are traditionally defined as ligands with

significant affinity and agonism to CB₁ and CB₂ according to the NC-IUPHAR committee ¹⁹. This definition resulted in the discovery and synthesis of several other eCB and “eCB-like” analogues ²⁰⁻²³. Thus, many of these fatty acid derived molecules are eCB-like ligands that have weak affinity to CB receptors and cannot be considered as classical eCBs. Additionally, as the definition of the ECS system is expanding, it is currently known that CBs and other eCB-like compounds, such as N-arachidonylglycine (NA-Gly), can target more receptors, such as TRPV1, GPR18, GPR119 and GPR55 ²⁴⁻²⁸.

With the increasing focus towards the consumption of omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), there has been a growing interest in the discovery of omega-3 fatty acid derived eCB or eCB-like molecules with novel bioactivity ²⁹. Herein, we review the discovery and physiological role of omega-3 eCBs that are derived from DHA and EPA (Figure 15A). We illustrate that the derivatization of carboxylic acid end of the omega-3 fatty acid with ethanolamide, glycerol or other groups, such as neurotransmitters, leads to substantial changes in their biological activity and receptor interactions. We also review the discovery, mechanism and activity of the following omega-3 eCBs- DHA-ethanolamide (DHA-EA), 2-docosahexaenoyl-glycerol (2-DHG) and eCB-like- DHA-serotonin (DHA-5HT), DHA-dopamine (DHA-DA) and the EPA analogs. Additionally, we also evaluate the formation and potential physiological roles of their oxidative metabolites, which arise from the metabolism of omega-3 eCBs by eicosanoid synthesizing enzymes.

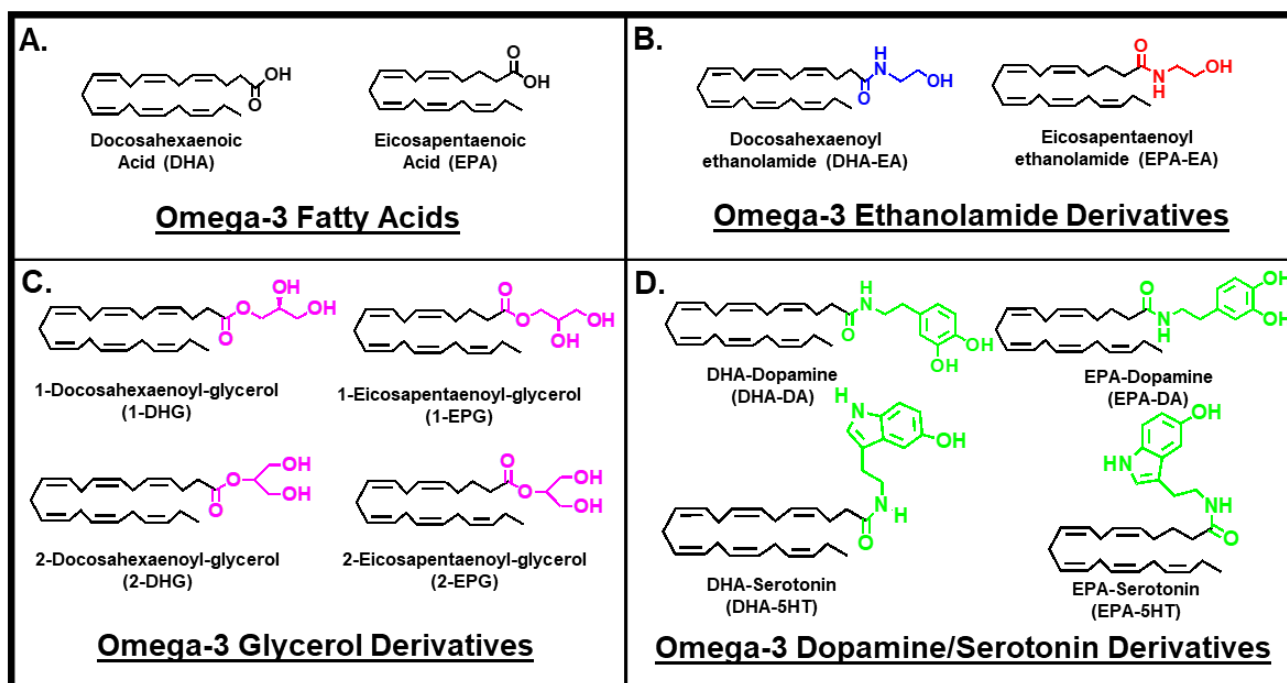


Figure 15. Omega-3 endocannabinoids and derivatives. Chemical Structures of **(A)** Omega-3 fatty acids, Docosahexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA) and N-acyl amide derivatives that have been identified in literature. **(B)** Docosahexaenoyl ethanolamide (DHA-EA), Eicosapentaenoyl ethanolamide (EPA-EA), **(C)** 1-Docosahexaenoyl-glycerol (1-DHG), 1-Eicosapentaenoyl-glycerol (1-EPG), **(D)** DHA-Dopamine (DHA-DA), EPA-Dopamine (EPA-DA), DHA-Serotonin (DHA-5HT) and EPA-Serotonin (EPA-5HT).

A.3 ENDOCANNABINOID AND THEIR DERIVATIVES

Omega-3 fatty acid DHA ethanolamide (DHA-EA or DHEA)

To study the structure-activity relationship of eCB-like analogues to CB₁, an omega-3 derivative of DHA, N-docosahexaenylethanolamide (DHA-EA or DHEA), was synthesized. In comparison to AEA, DHA-EA was shown to be 8- to 22.5-fold less potent to CB₁ receptor (Figure 15B & Table 2)^{14, 315}. Later, DHA-EA was found endogenously in brain and retina^{31, 100}. When piglets were supplemented with a DHA diet, a several fold increase in DHA-EA levels were measured (Supplementary Table 1)³¹. Additionally, dietary consumption of fish oil led to higher levels of DHA-EA in rodent brain, jejunum, liver and adipose tissue (Supplementary Table 1)^{32, 316-318}. DHA-EA was also measured under inflammatory conditions in rat

cerebrospinal fluid and the spinal cord tissue (Supplementary Table 1) ³¹⁹. Most recently, DHA-EA has been detected in human milk (Supplementary Table 1) ³²⁰. As DHA-EA is identified in the body, further investigation of its physiological and pharmacological roles are necessitated.

Role of DHA-EA in the Central Nervous System (CNS)

Since DHA-EA is detected in the brain, its role in synaptogenesis (formation of synapses between neurons) were investigated ³²¹. Previously, DHA-EA production in neurons correlated with enhanced glutamatergic synaptic activity ^{33, 101}. This activity is required for the synaptogenesis process; hence, DHA-EA was named “synaptamide” ⁹⁹. In dose-dependent studies, DHA-EA significantly induced synaptic activity at 0.1 μ M and 1 μ M. Additionally, DHA-EA treatment increased hippocampal neurite growth 10-fold more than that of DHA following three days of supplementation. Similar to AEA, DHA-EA is hydrolyzed to DHA by FAAH. Interestingly, in the presence of a FAAH inhibitor, the synaptogenic properties of DHA were potentiated and DHA-EA levels increased ^{33, 99}. Collectively, results from these studies helped in establishing the dual significance of the combined amide moiety and omega-3 scaffold in promoting synaptogenesis (Table 3) ³²².

Given that eCBs elicit their activities through receptor activation, it was hypothesized that DHA-EA promoted synaptogenesis by acting as an endogenous ligand of GPR110. In the study, use of GPR110 KO mice abolished DHA-EA bioactivity by significantly reducing synaptic protein expression and synapse number in developing brains. DHA-EA also induced neurogenic differentiation and neurite outgrowth, which were also dependent upon GPR110 activation. Hence, the DHA-EA/GPR110 signaling is hypothesized to be an essential mechanism

for active neurogenesis and synaptogenesis, proving vital for the development of brain function³²³.

The role of DHA-EA in neural development may further be supported by the detection of DHA-EA in murine embryonic stem cells and neural stem cells (NSCs)^{324, 325}. In NSCs, DHA-EA treatment induced neuronal differentiation for seven days. The maximum effect for DHA-EA was measured at 10 nM alone and at 5 nM in the presence of a FAAH inhibition. At nanomolar concentrations, DHA-EA was 50 to 100-fold more effective, than DHA, at increasing expression of neuron-specific markers, microtubule-associated protein 2 (MAP2) and class III beta-tubulin (Tuj-1). Interestingly, DHA-EA also increased phosphorylation of protein kinase A (PKA) substrates, which have been demonstrated to mediate the CREB signaling pathway³²⁵. For instance, ethanol reduced levels of cAMP and hindered the ability of NSCs to differentiate, yet, DHA-EA treatment reversed these ethanol-induced effects. Additionally, at concentrations between 1 and 10 nM, DHA-EA significantly increased the cellular cAMP production and upregulated expression of adenylyl cyclase's, AC7 and AC8, which are important regulators of cAMP levels. Notably, in the presence of 25 mM ethanol, 1 to 5 nM of DHA-EA completely reversed cAMP reduction³²⁶.

Furthermore, DHA-EA has shown to promote axon development by negatively regulating sonic hedgehog (Shh) signaling in cortical neurons³²⁷. Shh signaling is activated when Shh binds to patched-1 receptor that leads to transactivation of a smoothened GPCR (SMO). In the presence of SMO, 1 μ M DHA increased levels of DHA-EA and caused a 28% increase in cortical neuron growth. Additionally, at 100 nM, DHA-EA prevented SMO-dependent transcription of GLI family zinc finger and blocked signaling induced by Shh. Intriguingly, when SMO was overexpressed in cortical cells, these cells failed to significantly decrease axonal

growth caused by DHA-EA. Yet, in the presence of adenylyl cyclase inhibition, DHA-EA axonal growth was decreased, highlighting the demand for cAMP to drive this process. Collectively, these studies showed that DHA-EA enhances synapto- and neuro-genesis several folds more than DHA ³²⁵⁻³²⁷.

Role of DHA-EA in Inflammation

DHA-EA has been shown to be anti-inflammatory in several different inflammation models. For instance, the production and anti-inflammatory properties of DHA-EA were first demonstrated in 3T3-L1 adipocytes (Table 3). After 24 hours, 10 to 50 μ M of DHA supplementation increased the conversion of DHA to DHA-EA by 2- to 7-fold higher compared to controls. To investigate the properties of DHA-EA in adipocytes, a pro-inflammatory response was evoked using lipopolysaccharide (LPS), a component from gram-negative bacteria commonly used to study inflammatory processes. DHA-EA supplementation at 1 nM to 10 μ M reduced production of pro-inflammatory cytokines, such as IL-6 and monocyte chemotactic protein-1 (MCP-1), in differentiated adipocytes. Notably, both markers were reduced by 50% in the presence of 10 nM DHA-EA, compared to DHA, which had no effect at this concentration ¹⁰⁶. Furthermore, receptor inhibition studies demonstrated that the reduction of pro-inflammatory markers were partially mediated by PPAR γ and CB₂. These studies confirmed that DHA-EA in adipocytes exhibit anti-inflammatory properties that are mediated through both CB- and non-CB receptors mediated pathways ¹⁰⁶.

The effects of DHA-EA were further investigated in macrophages. At 10 μ M, DHA-EA significantly inhibited pro-inflammatory nitric oxide (NO) and MCP-1 production in LPS-stimulated RAW264.7 and peritoneal macrophages (Table 3) ¹⁰⁷. To elucidate the potential role

of toll-like receptor (TLR) signaling in the anti-inflammatory effects caused by DHA-EA, the involvement of the TLR3/TLR4 myeloid differentiation primary response 88 pathway was examined. Interestingly, DHA-EA treatment in LPS and poly-IC induced macrophages led to significant reduction in the level of NO. However, DHA-EA did not inhibit NF- κ B or IFN- β activation that is associated with this TLR3/TLR4 pathway. Additionally, pharmacological inhibition studies determined that CB₁, CB₂, and PPAR γ minimally contributed to NO reduction by DHA-EA. Further suggesting that reduction in NO production is mediated through an unknown or alternate pathway. Currently, it is hypothesized that DHA-EA may indirectly reduce the pro-inflammatory response by reducing AA-derived COX eicosanoids or it is converted further by eicosanoid synthesizing enzymes to form anti-inflammatory lipid metabolites ¹⁰⁸.

Role of DHA-EA in Cancer

The bioactivity of omega-3 eCBs have also been demonstrated in cancer models. In this study, DHA-EA significantly induced the cell death of prostate cancer cells sensitive to androgen (LNCaP) and insensitive to androgen (PC3). Moreover, expression of CB₁ and CB₂ in PC3 and LNCaP were determined and radioligand binding studies confirmed DHA-EA as an agonist of both the CB receptors. Additionally, degradation of DHA-EA by FAAH was measured and the inhibition of this enzyme in LNCaP cells led to a potent increase in its anti-proliferative properties ²⁰. In other cancer cell types, such as breast cancer cells, DHA-EA mediated anti-proliferation effects through the PPAR γ activation pathway ³²⁸.

Role of DHA-EA in Exercise and Pain

In recent years, eCBs have been evaluated for their potential role in exercise and pain. Exercise-induced hypoalgesia (EIH) is characterized by elevations in pain tolerance which has been attributed to the involvement of opioid and non-opioid systems. Previously, activation of CBs receptors and EIH have shown to lower nociceptor activity, enhance exercise-induced analgesia and release endogenous opioid-like molecules to modulate and reduce pain ³²⁹⁻³³¹. Thus, it has been suggested that EIH may be reduced via elevated levels of eCBs after exercising to reduce pain sensitivity. In fact, DHA-EA levels become significantly increased in human serum after exercise ³³². Additionally, exogenous treatment of DHA-EA to muscle tissue increased glucose-uptake and expression of CB₁, CB₂, and NAPE-PLD, a key enzyme involved in the synthesis of AEA (Table 3) ³³³.

DHA-EA bioactivity was also demonstrated in humans undergoing a high omega-3 and low omega-6 fatty acid diet intervention. This diet was shown to reduce headache frequency, headache severity, and psychological distress. Adults undergoing a high omega-3 and low omega-6 fatty acid diet intervention had significantly higher levels of DHA-EA in circulation, but lower levels of 2-AG in blood plasma (Supplementary Table 1). Notably, DHA-EA also caused an increase of anti-nociceptive omega-3 metabolites, such as 18-HEPE and 17-HDHA. Individuals on a high omega-3 diet reduced medication use for pain by 37%, while these results were not paralleled in a high omega-6 diet. Collectively, this evidence demonstrates that omega-3 dietary interventions increase the production of DHA-EA. Likewise, when DHA-EA increases in circulation, a downstream increase in other eCBs and DHA metabolites that may aid in reducing pain activities ³³⁴.

Biosynthesis of DHA-EA

DHA-EA was first isolated from bovine retina. A potential precursor, N-docosahexanoyl-phosphatidylethanolamine (NDHPE), was identified¹⁰⁰. There are three hypothesized models for DHA-EA endogenous biosynthesis. Similar to AEA synthesis, one mechanism involves a phospholipase D2 enzyme that cleaves NDHPE from the phospholipid backbone to form free DHA-EA. Secondly, it is possible that α/β -hydrolase 4 can be used to create an intermediate susceptible to hydrolysis of the NDHPE such that free DHA-EA is released. Thirdly, direct condensation of DHA and ethanolamine to form DHA-EA can occur by FAAH, though this reversed hydrolysis would likely occur at significantly higher concentrations. Collectively, more studies are needed to elucidate the synthesis of DHA-EA and confirm which mode(s) of synthesis occurs predominantly *in vivo*^{100, 335}.

Metabolism of DHA-EA and Role of Metabolites

The eCBs are further metabolized by three eicosanoid synthesizing enzymes – COX, LOX and CYP450 – to form eCB metabolites. Studies have extensively investigated the generation of AEA and 2-AG oxy metabolites that have been produced through these enzymes¹⁷. In recent years, DHA-EA was proposed to be a substrate for the LOX and CYP450 enzymes (Figure 16). The first reported metabolism of DHA-EA was through the LOX pathway. The primary DHA-EA metabolites were identified in mouse brain, human hemoglobin and polymorphonuclear (PMN) leukocytes (Figure 16A). During inflammation, PMN leukocytes migrate to the originating sites of inflammation, a process known as chemotaxis. In contrast, chemotaxis can be reduced in the presence of anti-inflammatory molecules, such as resolvins³³⁶. Among the LOX products formed, 10,17-diHDHA-EA and 15-HEDPEA reduced the migration

of PMN chemotaxis up to 10 nM and decreased platelet-leukocyte aggregation formation by ~30-40% in human whole blood at 10 and 100 pM (Table 3). In addition, 10,17-diDHA-EA and 15-HEDPEA demonstrated CB₂ receptor agonist activity with EC₅₀ values at 3.9 nM and 1 nM, respectively (Table 2) ²².

Recently, our group demonstrated that the CYP450 pathway plays a role in the metabolism of DHA-EA (Figure 16B). We have developed a targeted liquid chromatography tandem mass spectroscopy (LC-MS/MS) method to quantitatively measure and detect DHA-EA epoxide metabolites in rat tissues. Additionally, when rat brain homogenates were incubated with DHA-EA, six DHA-EA epoxide regioisomers called epoxydocosapentaenoic-ethanolamides (EDP-EAs) were produced. As proof of concept, one brain CYP450 isozyme, CYP2J2, was shown to be able to metabolize DHA-EA to form the corresponding epoxides. Furthermore, one regioisomer, 19,20-EDP-EA, was predominately produced by CYP450s in rat brain. This metabolite significantly reduced pro-inflammatory markers, IL-6 and NO, while inducing the expression of anti-inflammatory marker, IL-10, in microglial cells. Additionally, 19,20-EDP-EA mediated platelet aggregation, vasodilation, angiogenesis, while activating CB₁ and CB₂ receptors with EC₅₀ values of 108 nM and 280 nM, respectively (Table 2 and 3) ²¹. These studies suggest that omega-3 eCB metabolites may serve as potential therapeutic targets in neuroinflammation and cerebrovascular diseases.

Omega-3 fatty acid EPA ethanolamide (EPA-EA or EPEA)

When AEA was discovered to bind and activate CB₁, researchers synthesized AEA analogs to investigate which structural requirements were essential for binding to CB₁. One of the compounds synthesized was eicosapentanoyl ethanolamide (EPA-EA or EPEA) (Figure

15B) ³³⁷. Competitive binding experiments showed that EPA-EA had a 4- to 16.5-fold weaker binding to CB₁ compared to AEA (Table 2) ^{315, 337, 338}.

Years later, EPA-EA was isolated from animal tissues, compared to AEA and DHA-EA, and levels were significantly lower or undetectable at basal levels ³²⁴. Additionally, when piglets were fed a fish oil diet, phospholipids containing esterified EPA significantly increased, while EPA-EA levels were undetectable in the brain, jejunum and liver ³¹⁶. Yet, in adipose tissue, mice fed with omega-3 oil, levels of EPA-EA increased ³¹⁷. Lastly, EPA-EA has also been detected in human milk, similar to DHA-EA ³²⁰ (Supplementary Table 1). Together, these findings further support that endogenous levels can be altered through dietary interventions and the levels of EPA-EA vary by tissue type.

Role of EPA-EA in Inflammation

Since EPA has shown to possess anti-inflammatory properties, the anti-inflammatory effects of EPA-EA was studied in adipocytes and myoblasts. In 3T3-L1 adipocytes, 10 to 50 μ M of EPA supplementation led to a 3.5- to 10.9-fold increase of EPA-EA. When LPS-stimulated adipocytes were treated with EPA-EA, levels of pro-inflammatory cytokines, IL-6 and MCP-1, were reduced by 50% at 100 nM and 10 nM, respectively (Table 4). Moreover, LPS-stimulation increased EPA-EA levels several fold higher than DHA-EA, possibly due to the higher endogenous levels of esterified EPA than DHA ^{106, 339}.

In C₂C₁₂ myoblasts treated with EPA-EA, the expression of CB receptors and glucose-uptake receptors were increased. Additionally, EPA-EA-induced CB receptor expression was investigated for regulating insulin sensitivity via activation of the mitogen-activated protein kinase (MAPK) pathway. Interestingly, when C₂C₁₂ myoblasts were supplemented with EPA-

EA, p38 MAPK phosphorylation was altered. Additionally, N-acyl phosphatidylethanolamine phospholipase mRNA levels increased with EPA-EA supplementation (Table 4) ³³³.

Collectively, these findings propose that EPA-EA may be indirectly mediating its anti-inflammatory effects through increased levels of other fatty acid-derived eCBs. Ultimately, they may aid in the reduction of pro-inflammatory cytokines and increase insulin sensitivity through the MAPK pathway.

Role of EPA-EA in Cancer

EPA-EA has been shown to inhibit prostate cancer cell growth and induce cell death of LNCaP and PC3 cell lines. In these cells, EPA-EA was determined to be an agonist to CB₁ and CB₂ (Table 2). Similar to DHA-EA, the anti-proliferative effects of EPA-EA were significantly reduced in the presence of FAAH inhibitors ²⁰. Additionally, in breast cancer cells (MCF-7), EPA-EA inhibited proliferation in a dose-dependent manner with an IC₅₀ value of 1.5 μ M ³²⁸. EPA-EA also demonstrated to activate PPAR γ , which plays a key role in the autophagy process (Table 4). When PPAR γ is activated, an autophagy process occurs in which activation of beclin-I is observed. When the autophagy process becomes inhibited, beclin-I interacts with Bcl-2. Remarkably, EPA-EA reduced the beclin-I and Bcl-2 interaction and ultimately inhibited the AKT-mTOR signaling pathway, which plays a key role in the autophagy and apoptotic processes of the cell cycle ³²⁸.

Metabolism of EPA-EA and Role of Metabolites

EPA-EA is metabolized by CYP450 to form corresponding epoxides that are found endogenously in rat tissues (Figure 16B). Specifically, CYP2J2, metabolized EPA-EA into five regioisomers called epoxyeicosatetraenoic acid-ethanolamides (EEQ-EAs). Of the five regioisomers produced, 17,18-EEQ-EA was the predominant metabolite formed (Table 4). Similar to 19,20-EDP-EA, 17,18 EEQ-EA significantly reduced pro-inflammatory NO and IL-6, whereas anti-inflammatory IL-10 production significantly increased in microglia cultures. Additionally, 17,18-EEQ-EA was also determined to be three- and six-fold more susceptible to FAAH and soluble epoxide hydrolase (sEH) hydrolysis, respectively, when compared to 19,20-EDP-EA. EPA-derived metabolites are more susceptible to breakdown compared to DHA-derived metabolites, which could be due to their structural differences. Interestingly, 17,18 EEQ-EA also inhibited platelet aggregation regulated vasodilation and angiogenesis, as well as activated CB₁ and CB₂ with an EC₅₀ value of 18.5 nM and 1.4 nM, respectively (Table 2) ²¹.

Summary of DHA-EA and EPA-EA

In conclusion, endogenous DHA and EPA are non-oxidatively converted to DHA-EA and EPA-EA, respectively. These omega-3 eCBs are precursors to more potent bioactive molecules that interact through CB-independent and -dependent-pathways. For instance, DHA-EA and EPA-EA can undergo further downstream modifications through the canonical oxygenase pathway involving COX, LOX, and CYP450. The mechanism of action of these newly formed metabolites remains to be investigated. Thus far, DHA-EA and EPA-EA possess anti-proliferative as well as anti-inflammatory properties and may be key modulators of the inflammatory cascade. Additionally, DHA-EA and EPA-EA possess some affinity to CB and

non-CB receptors (Table 12)^{21, 22, 340}. Although we focus on the nutritionally relevant omega-3 derived eCBs, from DHA and EPA, it is important to highlight reports that have demonstrated that similar analogues, such as α -linolenoyl ethanolamine (ALA-EA), possess pharmacological significance through activation of transient receptor potential vanilloid 1 (TRPV1) and not CB₁^{315, 341}. Therefore, there is a large class of omega-3 fatty acid derived eCB-like molecules that can modulate cell physiology through other non-CB receptors.

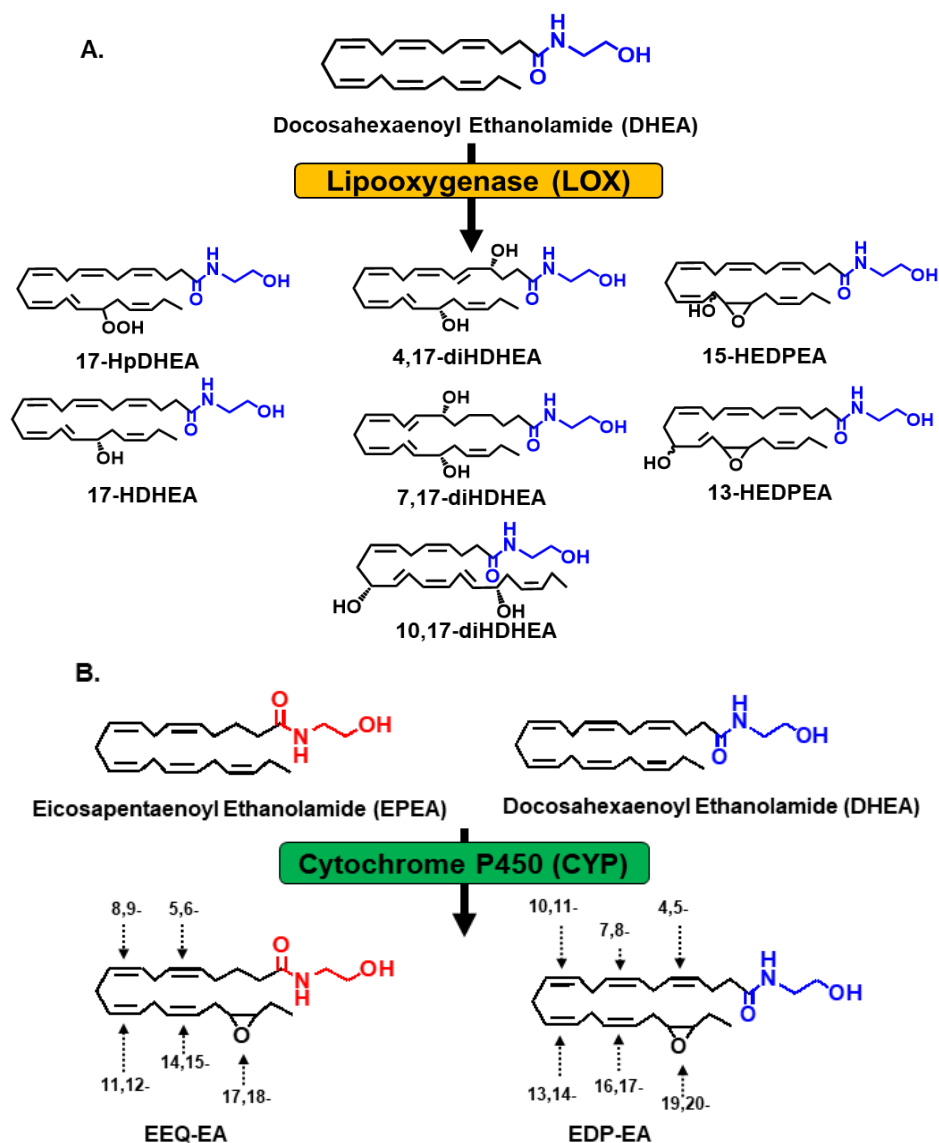


Figure 16. Oxidation of Omega-3 endocannabinoids (DHA-EA and EPA-EA) by the LOX and CYP450 pathway. (A) For the LOX pathway, oxidation can occur once and metabolites formed can undergo additionally oxidation or reduction to form seven DHA-EA-LOX metabolites; 17-hydroperoxydocosahexaenoyl ethanolamide (17-HpDHA-EA), 17-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoyl ethanolamide (17-HDHA-EA), 10,17-dihydroxydocosahexaenoyl ethanolamide (10,17-diHDHA-EA), 7,17-dihydroxydocosahexaenoyl ethanolamide (7,17-diHDHA-EA), 4,17-dihydroxydocosahexaenoyl ethanolamide (4,17-diHDHA-EA), 13-hydroxy-16(17)-epoxy-docosapentaenoyl ethanolamide (13-HEDPEA) and 15-hydroxy-16(17)-epoxy-docosapentaenoyl ethanolamide (15-HEDPEA). **(B)** The CYP450 pathway oxidizes DHA-EA and EPA-EA into six epoxydocosapentaenoic acid-ethanolamide (EDP-EA) and five epoxyeicosatetraenoic acid-ethanolamide (EEQ-EA) regioisomers, respectively.

Omega-3 fatty acid DHA and EPA glycerol (DHG and EPG)

Another class of potential omega-3 eCBs with structural similarities to 2-AG have also been discovered. DHA-derived, docosahexaenoyl glycerol (DHG) and EPA-derived, 2-5,8,11,14,17-eicosapentaenoyl glycerol (EPG), have a glycerol ester linkage (Figure 15C). Both 2-DHG and 2-EPG were first reported in a study investigating 2-AG analogues. Since 2-AG induced a rapid, increase in intracellular free calcium levels, the effects of structural analogues were also assessed. When compared, 2-AG and 2-EPG both induced cAMP levels, that are partially mediated by CB₁, with similar agonist activities. However, 2-DHG showed weak agonist activity at the CB₁²³. These initial studies set the precedence for investigating the endogenous production of 2-DHG and 2-EPG as well as their bioactive roles.

It is important to note that 2-AG isomerizes to 1-AG and is also hydrolyzed by active MAGL to form AA. Similarly, 2-DHG and 2-EPG are not thermodynamically stable and can isomerize to 1-DHG and 1-EPG. Furthermore, they are susceptible to become hydrolyzed to form DHA and EPA. Therefore, the effects observed with 2-DHG or 2-EPG might be partially be due to the unesterified DHA and EPA that is formed after the corresponding monoacyl-glycerol cleavage.

Endogenous Levels of 2-DHG and 2-EPG

Both 2-EPG and 2-DHG were found in mouse embryonic stem cells (mES) and embryoid bodies. In mES cells, there were comparable concentrations of 2-DHG and 2-EPG³²⁴. Additionally, a high omega-3 and low omega-6 diet in humans produced a significant increase in 2-DHG levels, whereas 2-AG levels decreased. Furthermore, EPG and DHG levels have shown to increase in human milk upon maturation (Supplementary Table 1)³²⁰. Strikingly, an increase

in 2-DHG was correlated with a decrease in pain (Table 4 & Supplementary Table 1) ³³⁴.

Despite the detection of 2-DHG and 2-EPG, the biological and physiological significance were not evident.

Bioactivity of 1-DHG

2-AG is one of the most well studied eCB. However, it isomerizes spontaneously into 1-AG under physiological conditions and is considered a potential mechanism of its deactivation ³⁴²⁻³⁴⁴. In fact, it has been reported that commercially available 2-AG contains approximately 10% 1-AG, thus the isomerization process is inevitable in biochemical research assays ³⁴⁴. Hence, testing of the potential effects of 2-AG is undermined due to the rate of isomerization and the chemical instability of these molecules ^{343, 344}. 1-DHG is structurally similar to 2-DHG, containing a DHA attached to the sn-1 instead of the sn-2 position of the glycerol molecule via an ester bond. 1-DHG is also referred to as monoacylglyceride docosahexaenoic acid (MAG-DHA) ³⁴⁵. Its effects in pulmonary arterial hypertension (PAH) and cystic fibrosis have recently been investigated (Table 3).

PAH is a disease characterized by a progressive increase in pulmonary pressure and vascular resistance ³⁴⁶. It has been previously demonstrated that CYP450s in the lungs, including CYP2J2, CYP2C8 and CYP2C9, can metabolize AA, EPA and DHA into oxygenated eicosanoids, which have been reported to modulate vascular and airway smooth muscle tone ³⁴⁶⁻³⁴⁸. Thus, the ability for 1-DHG to inhibit vasoconstriction was first investigated using human pulmonary cells. In these studies, 1-DHG was found to reduce vasoconstriction in a dose-dependent manner, with an EC₅₀ of 1.25 μ M. In addition, 1-DHG was able to reduce tension in human pulmonary arteries by 50% at 3 μ M. Interestingly, this tension was further reduced to

95% in the presence of a Rho-kinase inhibitor, H-1152³⁴⁵. Since 1-DHG reduced vasoconstriction modulated by Rho-kinase, further studies showed that 30 μ M of 1-DHG was able to inhibit RhoA activity, a known activator of Rho-kinase³⁴⁶. Furthermore, it was revealed that 1-DHG treatment reduced phosphorylation of downstream members of the Rho-kinase pathway (Table 3)^{345, 346}. Thus 1-DHG may represent a new pharmacological alternative in modulating vasoconstriction in patients with pulmonary hypertension.

Homeostatic response caused by omega-3 eCBs and their metabolites are not limited to cancer, inflammation or pain, as these molecules can act broadly in different diseases states as inflammation is common etiology in many diseases. For instance, cystic fibrosis is the most common autosomal, recessive, inherited disease with fatal outcome in Caucasians, and is characterized by persistent lung infections and losing the ability to breathe over time³⁴⁹. Previously, cystic fibrosis patients have reported a fatty acid deficiency with an imbalance between omega-3 and omega-6 levels in their blood lipids^{350, 351}. Since omega-3 fatty acid supplements were found to reduce inflammation in cystic fibrosis patients, the effects of 1-DHG were investigated utilizing lung adenocarcinoma Calu-3 cells. At 3 μ M, 1-DHG treatment was found to increase the mean DHA/AA ratio with at least a two-fold increase in DHA/AA ratio from control. Moreover, 1-DHG treatment also led to a reduction of pro-inflammatory markers, such as IL-6 and IL-8, induced by LPS. Notably, docosaepentaenoic glycerol (DPG) and EPG showed a more effective reduction (Table 3)³⁵². This study was the first to demonstrate the anti-inflammatory properties of 1-DHG and its potential in the management of cystic fibrosis. While there are some studies reported for 1-DHG, there are no current reports on 2-DHG.

Bioactivity of 1-EPG

Similar to DHA, EPA also has a monoacylglyceride derivative known as MAG-EPA or 1-EPG (Figure 15C). Previously, 1-EPG has shown to be anti-inflammatory through reduction of pro-inflammatory NF- κ B and COX-2. Use of an asthma model has demonstrated that 1-EPG treatment reduced the reactivity of airway smooth muscles with an EC₅₀ value of 0.77 nM. Interestingly, 1-EPG significantly reduced calcium sensitivity and decreased the omega-6/omega-3 ratio in the asthma model (Table 4)³⁵³. These findings directly support the previous findings that 1-EPG caused these effects, as omega-6 fatty acid levels are positively correlated with asthmatic symptoms. In related studies, the effects of 1-EPG on airway hyper-responsiveness were investigated. In guinea pig models, 1-EPG reduced tracheal ring tension with an IC₅₀ = 1.03 μ M. It was further reported that an EPA metabolite, 17,18-epoxyeicosatetraenoic acid (17,18-EEQ) had a greater effectiveness compared to its precursor 1-EPG³⁵⁴. The findings of this study suggest that 1-EPG may have potential in the treatment of respiratory-related diseases, possibly through its metabolism to the highly bioactive 17,18-EEQ metabolite³⁵⁴.

Since omega-3 supplementation causes a decrease in the production of inflammatory cytokines involved in ulcerative colitis pathogenesis, the effects of 1-EPG in ulcerative colitis were also investigated. In a colitis rat model, orally administered 1-EPG was found to reduce the severity of colitis, further suggesting that 1-EPG may prevent the acceleration of ulcerative colitis³⁵⁵. To investigate the potential mechanism of 1-EPG in colitis, pro-inflammatory cytokines were measured. In colon tissue, an 81% reduction of NF- κ B was observed with 1-EPG treatment. As in previous studies, TNF- α , IL-1 β , IL-6 and COX-2 expression levels were also decreased when treated with 1-EPG, suggesting that 1-EPG may act on a transcriptional level.

Collectively, these studies suggest that 1-EPG is anti-inflammatory in several models, including asthma, airway hyper-responsiveness, and ulcerative colitis.

It is known that DHA and EPA have beneficial effects in cancer models *in vitro* and *in vivo* ³⁵⁶. Compared to 1-DHG, 1-EPG decreased cell growth of human colorectal carcinoma cells, where apoptosis was even induced in a dose-dependent manner (0.3 μ M to 10 μ M) by 1-EPG ³⁵⁷. *In vivo* mouse model studies demonstrated that 1-EPG reduced tumor sizes by 3-fold after daily administration. Moreover, 1-EPG treatments reduced VEGF and HIF-1 α expression levels in tumors, both of which are commonly overexpressed in many cancers. 1-EPG downregulated the gene products of NF- κ B, VEGFR, and AKT within the colorectal carcinoma study, all of which are associated with angiogenesis, metastasis and proliferation of tumors (Table 4) ³⁵⁷. While these effects were observed in mice, a study is currently in progress to investigate whether the 1-EPG in cell cultures translates in humans. The effects of 1-EPG on prostate cancer on randomized, double-blind, placebo-controlled trial is currently being conducted as well, highlighting the potential far-reaching implications of eCB-based therapeutics ³⁵⁸.

N-acyl neurotransmitter derivatives of DHA and EPA: Dopamine and Serotonin

Neurotransmitters enable neurotransmission in the brain and are synthesized from amino acids precursors. Biogenic amines such as monoamine neurotransmitters and catecholamine neurotransmitters are crucial for transmitting signals across a chemical synapse from one neuron to the target cells. Recently, DHA and EPA derived conjugates of monoamine neurotransmitters have become of interest due to the potential dual physiological effects of omega-3 fatty acids

and neurotransmitters. Herein, we discuss omega-3 derived N-acyl conjugates of neurotransmitters and their potential roles in immunomodulation and neurodegeneration.

Expression and biological effects of DHA-Dopamine and EPA-Dopamine (DHA-DA or EPA-DA)

N-arachidonoyl dopamine (NADA) was first reported as a novel CB₁ ligand³⁵⁹ (Figure 15D). Similar to NADA, DHA-dopamine (DHA-DA) is of interest due to its potential physiological properties that are linked to brain synapses and retinal neuroprotection³⁶⁰. The synthesis and biological effects of omega-3 derived neurotransmitters, such as DHA-DA has been shown to possess analgesic and antioxidative effects (Table 3)^{361, 362}. In addition, DHA-DA decelerates the development of Parkinson's disease symptoms and recently has shown to be anti-inflammatory³⁶³. In rodent macrophages and microglia cells, DHA-DA dose-dependently, at 10 nM to 2.5 μ M, suppressed the production of four pro-inflammatory markers: NO, MCP-1, macrophage-inflammatory protein 3 α and IL-6. Other immunomodulatory effects by DHA-DA is suppressing levels of prostaglandin E₂ (PGE₂), a COX-2 product that promotes inflammation, pain and fever. Notably, PGE₂ was suppressed dose dependently by DHA-DA up to 75% at 1 μ M. Similarly, COX-2 transcript levels were also dose-dependently decreased by DHA-DA³⁶⁴. However, DHA-DA did not affect NF- κ B, a transcriptional regulator of COX-2. Notably, similar findings of DHA-DA in macrophages were consistent in BV-2 microglia cells, however COX-2 gene expression in LPS-activated microglial cells were unaffected³⁶⁴. Collectively, this evidence suggests that DHA, the most abundant fatty acid in the brain, and dopamine, an important neurotransmitter in the brain, together form a lipid mediator conjugate that can modulate neuroinflammation.

In human breast cancer cell lines, DHA-DA and EPA-DA has been reported to induce cell death through its interaction with PPAR γ . Both DHA-DA and EPA-DA dose-dependently reduced cell viability in MCF-7, SKBR3 and MDA-MB-231 cell lines. In MCF-7, SKBR3 and MDA-MB-231, DHA-DA dose- and time-dependently reduced cell viability in all cell lines with IC₅₀ values of 25, 20 and 6 μ M, respectively. Furthermore, DHA-DA did not appear to influence proliferation of non-tumorigenic breast MCF-10A cells. Notably, this data combined with the IC₅₀ values of tumorigenic breast cancer cell lines indicate DHA-DA has greater effects on more aggressive cell types than others. Increased expression of PPAR γ was measured and further studies demonstrated that activation of PPAR γ by DHA-DA and EPA-DA mediate the antiproliferative effects in cancer cells. Notably, DHA-DA and EPA-DA also upregulated autophagic protein, Beclin-1, expression in these cells ³⁶⁵. Together, this suggests that DHA-DA and EPA-DA activation of PPAR γ may have potential in adjuvant therapeutic interventions in breast cancer.

Expression and Biological effects of DHA-Serotonin and EPA-Serotonin (DHA-5HT or EPA-5HT)

It has been demonstrated that the endogenous production of N-acyl amides and serotonin levels are increased in intestinal tissues with omega-3 supplementation ³⁶⁶. In the first study, the serotonin conjugate of DHA, DHA-serotonin (DHA-5HT), significantly inhibited pro-inflammatory mediators, IL-6 and PGE₂, at 2.5 μ M in LPS-stimulated murine macrophages (Table 3). Similar to DHA-DA, gene expression of COX-2, IL-6 and NO synthase 2 were all suppressed by DHA-5HT at the transcriptional level. Furthermore, DHA-5HT treatment led to downregulation of co-stimulatory molecules needed for T cell activation, demonstrating that

DHA-5HT reduces gene expression of mediators involved in inflammatory T cell responses. Moreover, microarray analysis found DHA-5-HT to reduce the gene expression of metalloproteinases, chemokines and pro-inflammatory interleukins such as IL-12b, IL-23a, IL-6, and IL-1 β . Notably, these observations were consistent in LPS-stimulated RAW264.7 cells in which DHA-5HT treatments inhibited migration of macrophages by 52% and 77%, at 1 μ M and 2 μ M, respectively (Table 3) ³⁶⁷. DHA-5HT also reduced the production of MCP-1 and CCL-20, a chemokine released by pathogenic pro-inflammatory T helper 17 (Th17) cells, to that of DHA-DA ³⁶⁴. Collectively, this evidence suggests that DHA-5HT may have therapeutic potential for patients with intestinal immune-dysregulation of inflammatory T cells.

Recently the presence and effects of DHA-5HT in human tissues was demonstrated. LC-MS/MS analysis, found that both DHA-5HT and EPA-5HT were detected in addition to other N-acyl-5HT. Notably, out of the six N-acyl serotoninins detected, DHA-5HT was most effective at suppressing pro-inflammatory cytokine IL-17 release in peripheral blood mononuclear cells (PBMCs) in a dose-dependent manner. DHA-5HT treatments decreased IL-17 production by PBMCs by 80% and 39% at 10 μ M and 1 μ M, respectively. Interestingly, DHA and serotonin separately did not influence IL-17 release, suggesting that the N-acyl serotonin moiety is vital for this physiological effect. Measuring CCL-20 production helped probe the immunomodulatory effects of DHA-5HT. Similar to IL-17, CCL-20 production was dose-dependently and significantly attenuated, by 39.5% and 45.95%, at 5 and 10 μ M treatments, respectively ³⁶⁸. Hence, DHA-5HT regulates the production of two important Th17 mediators involved in pathogenesis of intestinal diseases and is a class of immunomodulatory omega-3 derived lipid mediators.

The mechanism of action of DHA-DA and DHA-5HT still remains unknown. However, similar conjugates such as, ALA-DA and ALA-5HT, can activate CB and TRPV1 receptors and inhibit FAAH degradation of AEA ^{362, 369}. Thus, the potential mechanism of action of DHA-DA, DHA-5HT and the EPA analogues may follow similar receptor interactions. As eCBs also act through TRPV1 ³⁷⁰⁻³⁷², they are termed as “endovanilloids”. In addition to mediating the pain associated from stimuli, TRPV1 exhibits pro- and anti-inflammatory effects ³⁷³⁻³⁷⁶. Recently, it has been postulated that there is a crosstalk between CB₁ and TRPV1 receptors, which are co-localized in dorsal root ganglion. Therefore, the ECS and TRPV1 axis provides a promising target to develop pain and inflammation therapeutics.

A.4 CONCLUDING REMARKS

In the past few decades, a significant amount of work was performed to understand the role of omega-3 and omega-6 derived eCBs and their derivatives the overall physiology of the body. Despite structural similarities, common pathways for formation and enzymatic degradation, eCBs are intriguing lipid signaling molecules with varied interactions with several different types of receptors leading to varied physiological roles. Though previous studies focus on cannabinoid receptor activation, it is important to acknowledge that omega-3 and omega-6 eCBs could possibly activate other receptors as they are multi-functional molecules. Therefore, systems-based approaches are required to fully characterize these molecules with respect to receptor interactions and downstream signaling. Their on-demand signaling capabilities could suggest that they act at multiple receptors in tandem until they resolve the homeostatic challenge or are converted to new molecules with altered bioactivity through different pathways including eicosanoid synthesizing enzymes. All of these create possibilities for multiple modes of action of

eCBs, create new areas of cross-talk to investigate. As shown, omega-3 and omega-6 fatty acid eCBs have demonstrated metabolism by LOX-, COX- and CYP450-mediated pathways, yet only LOX and CYP450 metabolites have been reported for DHEA and only CYP450 metabolites for EPEA (Figure 16). Thus, the physiological function of the LOX and CYP450 generated metabolites remains to be elucidated further to understand how these lipids modulate cell signaling under diseased and non-diseased states.

We must continue to focus on the influence of naturally produced omega-3 eCB derivatives to better understand how they display such ubiquitous biological roles. Referencing the molecular origins and fates of these molecules while discussing their physiological roles is critical in driving future research surrounding omega-3-derived eCBs. Many of the studies mentioned in this review limit our understanding of these molecules, as this basic characterization is biased towards measuring just CB receptor interaction and direct inflammatory responses. Hence, more research is needed to describe these molecules across multiple disease models.

Table 2. Cannabinoid Receptor Binding and Activation of DHA-EA, EPA-EA and oxygenated metabolites

Compound	CB₁ EC₅₀ values (nM)	CB₂ EC₅₀ values (nM)	References
AEA	0.7 ^a	1352 ^a	21
	39.2 ± 5.7 ^b	ND ^b	315
	543 ± 83 ^b 160 ± 13 ^c	ND ^b ND ^c	14
	89 ± 10 ^b (with PMSF) 5400 ± 1600 ^b	ND ^b ND ^b	338
DHA-EA	1044 ^a	305 ^a	21
	9.8 ^a	ND ^a	22
	12200 ± 520 ^b 6000 ± 800 ^c	ND ^b	14
	324.1 ± 9.2 ^b	ND ^b	315
19,20 EDP-EA	108 ^a	280 ^a	21
10,17-diHDHA-EA	ND ^a	3.9 ^a	22
15-HEDPEA	ND ^a	1 ^a	22
EPA-EA	0.1 ^a	2.1 ^a	21
	1470 ± 500 ^b (with PMSF)	ND ^b	338
	162.3 ± 13.6 ^b	ND ^b	315
17,18 EEQ-EA	18.5 ^a	1.4 ^a	21

Abbreviations: N-docosahexaenoyl ethanolamine (DHA-EA), eicosapentaenoyl ethanolamide (EPA-EA), epoxydocosapentaenoic acid-ethanolamide (EDP-EA), epoxyeicosatetraenoic acid-ethanolamide (EEQ-EA), dihydroxydocosahexaenoyl ethanolamide (diHDHA-EA), hydroxy-epoxy-docosapentaenoyl ethanolamide (HEDPEA) and not determined (ND).

^a Experiments were performed with cell cultures transfected with CB receptors

^b Experiments were performed with displacement of CB receptor agonist

^c Experiments were measured by inhibition of cAMP

Table 3. Biological Effects of DHA-derived eCBs and Derivatives

<i>Effects of DHEA in Brain</i>		References
	9.5-fold increase in piglet brain from DHA supplementation	31
	Synaptic activity induced in hippocampal neurons	33
	Neurite growth increased 10-fold greater than DHA	33
	50 to 100-fold more effective than DHA in differentiating NSCs	325
	Phosphorylation of PKA substrates increased	325
	Ethanol impairment reversed	326
	Cellular cAMP production increased	326
	GPCR activation increased	326
	Reversed cAMP reduction caused by ethanol (25mM)	326
	Promoted axon development	327
	Negatively regulated sonic hedgehog signaling in cortical neurons	327
	Increased cortical neuron growth	327
	Inhibited sonic hedgehog induced signaling	327
<i>Effects of DHEA in adipose, peritoneum and pain</i>		
	Reduced IL-6 and MCP-1 levels in LPS-stimulated adipocytes	106
	Causes negative correlation in size of adipocytes	317
	Reduced nitric oxide production and MCP-1 levels in peritoneal macrophages and RAW264.7 cells	107
	Increases glucose up-take, CB ₁ , CB ₂ and NAPE-PLD expression in C ₂ C ₁₂ myoblasts	333
	Reduced headache severity by High ω -3/Low ω -6 diet	334
<i>Effects of DHEA in cancer</i>		
	Induced death of LNCaP and PC3 prostate cancer cells to androgen	20
	Inhibits proliferation of MCF-7 breast cancer cells	328
	Yield reduction of AKT-mTOR pathway	328
<i>Effects of DHEA metabolized by lipoxygenase (LOX)</i>		
	Reduced migration of PMN chemotaxis by IL-8	22
	Activates CB ₂ receptors (10,17-diDHEA & 15-HEDPEA)	22
	Decreased platelet-leukocyte aggregation by 30%-40%	22
<i>Effects of Docosahexaenoyl Ethanolamide Epoxides (EDP-EA)</i>		
	Significantly reduced pro-inflammatory nitric oxide and IL-6	21
	Induced anti-inflammatory cytokine production in primary microglial cultures	21
<i>Effects of Monoacylglyceride Docosahexaenoic Acid (1-DHG)</i>		
	Reduced vasoconstriction from human pulmonary tissues	346
	Reduced arterial tension comparably to H-1152 by 50%	346

Table 3 (cont.)

	Reduced RhoA activity by lowering coupling to GTP	346
	Reduced phosphorylation of Rho-kinase, MYPT-1 and MLC	345, 377
	Reverse hypertension in human pulmonary arteries	345, 377
	Reduced expression of VEGF	345, 377
	Reduced inflammatory markers in cystic fibrosis induced by LPS	352
<i>Effects of Docosahexaenoic Acid Dopamine (DHA-DA)</i>		
	Decelerated development of Parkinson's disease symptoms	363
	Elicits antioxidative properties under oxidative stress conditions	363
	Suppressed nitric oxide production	363
	Reduced pro-inflammatory markers, MCP-1, CCL-20 and IL-6	363
	Suppressed PGE ₂ by 75%	363
	Reduced breast cancer cell viability	365
	Decreases COX-2 transcript levels	355
<i>Effects of Docosahexaenoic Acid Serotonin (DHA-5HT)</i>		
	Inhibited IL-6 by 75% in LPS-stimulated RAW262.7 cells	367
	Inhibited PGE ₂ in LPS-stimulated RAW262.7 cells	367
	Suppressed pro-inflammatory mediator transcript levels	367
	Downregulated pro-inflammatory IL's and co-stimulatory T cell molecules	367
	Inhibited migration of RAW264.7 cells	367
	Suppressed IL-17 in PBMC's by 80%	368

Table 4. Biological effects of EPA-derived eCBs and Derivatives

<i>Effects of Eicosapentaenoyl Ethanolamide (EPEA)</i>		<i>References</i>
	Reduced IL-6 levels in LPS-stimulated 3T3-L1 adipocytes	106
	Reduced MCP-1 production by 50% in LPS-stimulated 3T3-L1 cells	106
	Inhibited MCF-7 cancer cell proliferation	328
<i>Effects of Eicosapentaenoyl Ethanolamide Epoxides (EEQ-EA)</i>		
	Reduced pro-inflammatory nitric oxide and IL-6	21
	Induced production of anti-inflammatory IL-10 in microglia	21
<i>Effects of Monoacylglyceride Eicosapentaenoic Acid (1-EPG)</i>		
	Dose administration reduced reactivity of airway smooth muscles	353
	Reduced expression NFκβ and COX-2	353
	Reduced tracheal ring tension induced by U-46619	354
	Reduced severity of DSS-induced colitis	355
	Reduced pro-inflammatory cytokines TNFα, IL-1β, and IL-6	355

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A.6 SUPPLEMENTARY INFORMATION

Supplementary Table 1. Detection of AA, DHA and EPA N-acyl ethanolamide derivatives and metabolites in tissue

Tissue	Compound	Detection in Tissue			Reference		
Piglet Brain (pmol/g of wet weight)		Control Diet	AA and DHA Diet	Sow's milk	31		
	Brainstem	AEA	1.106 ± 0.09	1.606 ± 0.23		2.506 ± 0.35	
		DHA-EA	0.706 ± 0.06	4.906 ± 1.79		9.906 ± 1.39	
	Auditory cortex	AEA	3.006 ± 0.23	4.006 ± 0.69		7.306 ± 1.33	
		DHA-EA	0.806 ± 0.23	4.406 ± 0.87		8.006 ± 1.62	
	Visual cortex	AEA	3.506 ± 0.35	ND		4.006 ± 0.58	
		DHA-EA	3.306 ± 0.17	ND		2.106 ± 0.35	
	Cerebellum	AEA	0.506 ± 0.06	ND		0.806 ± 0.06	
		DHA-EA	0.506 ± 0.17	ND		0.406 ± 0.06	
	Striatum	AEA	1.806 ± 0.02	1.506 ± 0.06		1.106 ± 0.17	
		DHA-EA	1.006 ± 0.12	2.006 ± 0.29		3.206 ± 0.17	
	Hippocampus	AEA	2.206 ± 0.46	2.206 ± 0.17		2.006 ± 0.17	
DHA-EA		1.206 ± 0.12	0.906 ± 0.06	1.406 ± 0.17			
Human Plasma (ng/mL)	2-DHG DHA-EA 2-AG AEA	Basal 166 ± 74.7 0.47 ± 0.22 793 ± 324.1 0.49 ± 0.18			334		
Mice Brain (ng/g)	DHA-EA EPA-EA AEA DHG EPG AG	Control Diet 3.77 ± 0.66 4.59 ± 1.04 1.88 ± 0.46 2,500 ± 1,470 54.1 ± 20.3 5700 ± 2100		DHA Diet 4.62 ± 0.74 4.37 ± 1 1.47 ± 0.42 2,970 ± 1,640 205 ± 77 5480 ± 1950	32		
Mice Plasma (ng/mL)	DHA-EA EPA-EA AEA DHG EPG AG	Control Diet 1.25 ± 0.28 0.3 ± 0.26 0.55 ± 0.38 2,150 ± 990 204 ± 113 1570 ± 750		DHA Diet 2.34 ± 0.58 0.21 ± 0.21 0.38 ± 0.26 2,550 ± 1,030 485 ± 30 876 ± 442			
	Rat Brain (pmol/g of wet weight)	AEA DHA-EA EPA-EA	Control Diet 11.3 ± 0.7 30.6 ± 2.3 ND	AA-Diet 17.7 ± 0.8 28.3 ± 2.1 ND		FO-Diet 14.2 ± 0.7 29.9 ± 1.8 ND	316

Supplementary Table 1 (cont.)

Rat Small intestine (pmol/g of wet weight)	AEA DHA-EA EPA-EA	Control Diet 19.2 ± 2.2 6.8 ± 0.9 ND	AA-Diet 35.9 ± 4.4 5.3 ± 0.8 ND	FO-Diet 9.2 ± 0.6 26.6 ± 2.0 21.6 ± 2.4	
Rat Liver (pmol/g of wet weight)	AEA DHA-EA EPA-EA	Control Diet 4.2 ± 0.9 2.0 ± 0.2 ND	AA-Diet 7.4 ± 1.4 1.2 ± 0.2 ND	FO-Diet 1.9 ± 0.2 5.2 ± 0.5 ND	
Mice Plasma (ng/g)	AEA DHA-EA EPA-EA	Control Diet 0.48 ± 0.02 0.81 ± 0.01 ND	ω3-TG Diet 0.13 ± 0.01 2.55 ± 0.04 0.23 ± 0.06	ω3-PL Diet 0.09 ± 0.01 5.15 ± 0.05 0.49 ± 0.09	317
Mice Adipose (ng/g)	AEA DHA-EA EPA-EA	Control Diet 2.68 ± 0.12 2.40 ± 0.15 ND	ω3-TG Diet 1.08 ± 0.07 14.92 ± 1.63 3.96 ± 0.55	ω3-PL Diet 1.08 ± 0.07 31.37 ± 7.26 6.33 ± 1.00	
Rat Spinal Cord (pg/mg)^a		Carrageenan injection at Hour 0		Carrageenan injection at Peak Hour	319
Ipsilateral	AEA DHA-EA EPA-EA	30.6 ± 3.36 38.1 ± 2.31 ND		50 ± 7.58 47.8 ± 1.13 0.17 ± 0.13	
Contralateral	AEA DHA-EA EPA-EA	33.9 ± 2.78 41.6 ± 1.16 ND		52.1 ± 3.36 47.5 ± 2.33 0.258 ± 0.15	
Human Milk (ng/mL)	AEA DHA-EA EPA-EA DHG EPG	Transitional Milk 0.15 ± 0.05 0.11 ± 0.01 0.07 ± 0.03 673.50 ± 198.01 24.70 ± 7.70		Mature Milk 0.08 ± 0.01 0.07 ± 0.01 0.11 ± 0.04 866.3 ± 383.60 61.99 ± 28.85	320

Supplementary Table 1. Detection of AA, DHA and EPA N-acyl ethanolamide derivatives and metabolites in tissue. ^aNote: Following carrageenan injection, lipid levels were measured at hour 0, 0.5, 1, 2, 4, 8, 12, 18 and 24. The levels reported in ST1 are the highest measured.

APPENDIX B: REVIEW – CYP2J2 MOLECULAR RECOGNITION: A NEW AXIS FOR THERAPEUTIC DESIGN

B.1 ABSTRACT

Cytochrome P450 (CYP) epoxygenases are a special subset of heme-containing CYP enzymes capable of performing the epoxidation of polyunsaturated fatty acid (PUFA) substrates in addition to metabolizing xenobiotics. This dual functionality positions epoxygenases along a metabolic crossroads, making study of their structure-function critical towards understanding drug-PUFA interactions. In order to design direct therapeutics that target epoxygenases, there is a need for improved understanding of epoxygenase structure-function. Among the known epoxygenases, there is a rising interest in CYP2J2 due to its important role in the human cardiovascular system. In this review, the early research on the discovery and activity of epoxygenases is contextualized to more recent advances in CYP epoxygenase enzymology with respect to PUFA and drug metabolism. Additionally, the review spotlights CYP2J2 epoxygenase by covering its interactions with PUFAs and drugs, its tissue-specific physiological roles during diseased states, as well as its structural features that enable epoxygenase function. Additionally, the enumeration of research on CYP2J2 identifies the future needs for the molecular characterization of CYP2J2 to enable a new axis of therapeutic design.

Keywords: cytochrome P450 enzymes, epoxygenase, polyunsaturated fatty acids, bioactive lipid mediators, structure-function, drug-drug interactions

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Abbreviations

2-AG, 2-arachidonyl glycerol; 7-deoxydoxorubicin aglycone, 7-de-aDOX; Å³, cubic ångström; AA, arachidonic acid; ADX., adrenodoxin; ADXR, adrenodoxin reductase; AEA, anandamide; CB1, cannabinoids receptor 1; CB2, cannabinoids receptor 2; CBC, cannabichromene; CBD, cannabidiol; CBG, cannabigerol; CBN, cannabinol; COX, cyclooxygenase; CPR, cytochrome P450 reductase; CYP, cytochrome P450; CYP2J2fl, Full length/native N-terminus CYP2J2; CYP2J2tr, N-terminus truncated CYP2J2; DHA, docosahexanoic acid; DOX, doxorubicin; DPA, docosapentaenoic acid; EBS, ebastine; eCB, endocannabinoid; EDP, epoxydocosapentaenoic acid; EET, epoxyeicosatrienoic acid; EET-EA, epoxyeicosatrienoic ethanolamide (synonymous with AEA-epoxide); EPA, eicosapentaenoic acid; EPOX, epoxygenase; ER, endoplasmic reticulum; FP, fluorescence polarization; HMMM, highly mobile membrane-mimetic; LA, linoleic acid; LOX, lipoxygenase; MD, molecular dynamics; MDMA, 3,4-methylenedioxymethamphetamine; MOE, Molecular Operating Environment; NSAID, non-steroidal anti-inflammatory drug; O-AEA, virodhamine; pCB, phytocannabinoids; PDB, protein data bank; PGH₂, prostaglandin; PGI₂, prostaglandin I₂; POPC, 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine; POPS, – 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine; PUFA, polyunsaturated fatty acid; she, soluble epoxide hydrolase; sEH_i, soluble epoxide hydrolase inhibitors; SM, sphingomyelin; SNP, single nucleotide polymorphism; TRP, non-selective transient receptor potential cation channel; TRPV1, TRP vanilloid 1; Δ⁸-THC, Δ⁸-tetrahydrocannabinol; Δ⁹-THC, Δ⁹-tetrahydrocannabinol

B.2 INTRODUCTION

Epidemiological evidence suggests that a diet rich in polyunsaturated fatty acids (PUFAs) is beneficial for various health conditions ^{378, 379}. The biochemical mechanisms facilitating these beneficial effects are being gradually unveiled ^{380, 381}. Previous studies demonstrated that PUFAs are converted to bioactive lipid metabolites through both oxidative and non-oxidative routes of metabolism. The oxidative metabolism of PUFAs are broadly initiated by three classes of enzymes: cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 epoxygenase (EPOX) (Figure 17) ³⁸²⁻³⁸⁴. While the COX and LOX pathways are best known for converting arachidonic acid (AA) into pro-inflammatory mediators, termed “prostaglandins” and “leukotrienes” ³⁸³, the EPOX pathway converts AA to produce anti-inflammatory epoxide mediators “epoxyeicosatrienoic acid (EETs)” ^{91, 384, 385}. Mechanistic studies of both the COX and LOX pathway enzymes have led to the development of several drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) ³⁸⁶, anti-allergic drugs ³⁸⁷. Additionally, it lead to an improved understanding of pro-resolving mediators such as resolvins ³⁸⁸. In contrast, there are limited therapeutics from the EPOX pathway. This is likely due to an incomplete understanding of the total biosynthetic machinery used to generate bioactive epoxides, structure-function of the enzymes involved, as well as the overall physiology of the bioactive epoxides produced during various disease states. Recent studies indicate that the ω -6 PUFA epoxides such as EETs have beneficial anti-inflammatory and cardioprotective effects ³⁸⁹. Likewise, the ω -3 PUFA epoxides protect against cardiac arrhythmias, reduce inflammatory and neuropathic pain, and prevent tumor metastasis ^{384, 390}. Recently, Schunck and coworkers summarized the recent advances on omega-3 PUFA epoxides, their generation by EPOX enzymes, their metabolic fate, known biological activities, and roads to clinical applications ³⁸¹.

The PUFA-epoxides are rapidly degraded via soluble epoxide hydrolase (**I**) to make diols. Advances in studying the physiological role of the PUFA-epoxides have come from the use of potent selective **I** inhibitors (sEHi) that indirectly increase the levels of the PUFA-epoxides ³⁹¹, ³⁹². Recently, bioisosteric analogues of PUFA-epoxides were reported in which the epoxide group was replaced by urea and oxamide groups, which were then used to target cardiovascular diseases and pain ³⁹³. However, there exist no current drugs that directly regulate the EPOX enzymes. In order to design direct therapeutics and inhibitors that target the EPOX enzymes, there is a need for improved understanding of the structure-function of these enzymes. Herein, the recent structure-function studies of selected EPOX enzymes will be reviewed with respect to PUFA-lipid metabolism that will aid in designing therapeutics targeting this pathway.

Many of the cytochrome P450 (CYP) epoxygenase enzymes characterized as lipid metabolizers belong to the CYP2 and CYP4 class. Although “lipid” is a broad term, for this review, we refer to lipids as either polyunsaturated fatty acids (PUFAs) or endocannabinoid signaling molecules (eCBs). And while lipid-metabolizing CYPs may be involved with a great array of catalytic function on lipid substrates, we want to specifically focus here on the ability for these enzymes to perform monooxygenation in the form of epoxide generation from PUFAs or eCBs. These CYP2 class enzymes are involved in the metabolism of PUFA alkenes into PUFA-epoxides, which are primarily anti-inflammatory in nature ²⁰². There are also a set of PUFA-metabolizing CYPs within the CYP4 class that convert PUFAs into hydroxy-PUFAs, which are pro-inflammatory in nature ³⁹⁴. Certain selected CYPs have been linked with PUFA epoxidation, primarily because of research findings which associated a physiological role with their discovered epoxygenase function ^{384, 395}.

CYPs, in general, are expressed as many isoforms in humans, with each isoform exhibiting several polymorphisms. CYPs also exhibit high degrees of substrate permissiveness. Many of these CYP isoforms have the potential to perform PUFA hydroxylation. However, in this review, we are focusing on their ability to form PUFA-epoxides (Table 5).

In order to help increase the effectiveness and drug development for the EPOX pathway, a thorough understanding of the tissue-specific expression, structure-function relationships, biochemical mechanism, and physiological roles of both EPOX bioactive metabolites and biosynthetic machinery is required. In this review, studies on one specific human CYP epoxidase, CYP2J2, are used as a model for better understanding the biochemical and physiological functions; the kinetics of PUFA metabolism; and the structural details, of CYP epoxidases.

Functional classification of cytochrome P450s and their interactions with redox partners.

CYPs comprise a superfamily of membrane-bound hemoproteins that are located in either the endoplasmic reticulum (ER) or the mitochondria ³⁹⁶. CYPs are named for the characteristic absorbance at 450 nm when CO is bound to the reduced CYP ³⁹⁷. In humans, 57 CYPs have been identified ¹⁹⁰ although there are discrepancies in the number of enzymes considered “functional” due to differences in genetic processing and stability, thus preventing all CYPs from being studied proportionately. CYPs have universal ability to insert an oxygen atom at C-H, C=C, or N- bonds, and sometimes perform degradative functions such as N- or O-dealkylation ^{398 399}. CYPs can catalyze a wide variety of chemistry, ranging from hydroxylation, heteroatom release and oxygenation, functional group migration, aromatization, and epoxidation ⁴⁰⁰⁻⁴⁰⁶. In addition, many other types of “atypical” CYP reactions are also possible ³⁹⁹. For

example, CYP5A1 and CYP8A1 are atypical CYPs insofar as they are isomerases as opposed to oxidases^{407 408}.

Most human CYPs require redox partners to perform their catalysis excluding isomerases (Figure 17A). In particular, cytochrome P450 reductase (CPR) is the primary redox partner of various microsomal CYPs⁴⁰⁹. The other reductases such as adrenodoxin (ADX) and adrenodoxin reductase (ADXR) can perform the redox transfer for mitochondrial CYPs⁴¹⁰. Additionally, cytochrome b5 can act as an electron mediator between CPR and CYPs and subsequently modulate CYP substrate metabolism, as seen with CYP17A1⁴¹¹⁻⁴¹⁴. Additionally, the membrane itself has been shown to be an important component in controlling the putative activity of CYPs⁴¹⁵.

CYPs are broadly classified into two categories based on their function: (1) biosynthetic CYPs are involved in synthesizing endogenous oxy-lipids or steroids and (2) xenobiotic metabolizing CYPs that are involved in drug metabolism. While these two classes of CYPs utilize the same overall catalytic mechanism, their ability to uniquely position substrates for metabolism enables different physiological functions.

There are several CYPs involved in steroidogenesis. These include CYP11A1 (converting cholesterol to pregnenolone)⁴¹⁰, CYP17A1 (hydroxylating or lyasing pregnenolone and progesterone)⁴¹⁶, CYP19A1 (converting testosterone to estrogen)⁴¹⁷, and CYP11B1 (converting 11-deoxycortisol and 11-deoxycorticosterone to cortisol and corticosterone, respectively)⁴¹⁸. Additionally, CYP2R1 hydroxylates vitamin D to calcifediol⁴¹⁹ and CYP27B1 hydroxylates calcifediol to calcitriol⁴²⁰. Importantly, these biosynthetic steroidogenic CYPs are very selective for their substrate recognition, and generally do not metabolize extraneous substrates^{417, 421, 422 423}.

The xenobiotic metabolizing CYPs are involved in phase 1 drug metabolism^{399, 424, 425}. Contrary to steroid synthesizing CYPs, the xenobiotic CYPs, such as CYP3A4 can effectively metabolize any foreign substance that enters the body. CYPs in the 2C family, as well as 3A4, can leverage their promiscuity through cooperative binding mechanisms, or use their larger active sites, to accommodate more, as well as bulky, molecules. Hence, the “*one enzyme one substrate*” hypothesis breaks down for several xenobiotic CYPs such as CYP3A4, as they exhibit complex substrate-substrate interactions due to the concurrent, or cooperative, binding of multiple drugs at allosteric sites or even at the active site^{191, 426, 427}. For example, the modulation of midazolam binding by carbamazepine in CYP3A4 leads to a shift in the site of metabolism from 1'-hydroxylation of midazolam to 4-hydroxylation⁴²⁸⁻⁴³⁰.

Poly unsaturated fatty acid (PUFA) metabolizing CYPs are at the crossroads of bioactive oxy-lipid synthesis and drug metabolism. Hence, it becomes imperative to study these CYPs, as they can show complex drug-PUFA interactions. PUFA metabolizing-CYPs can function as either omega-hydroxylases or -epoxygenases, where differences in product distribution bear physiological significance. Certain CYPs in the CYP4 class insert oxygen to form a hydroxyl group along the omega-terminal carbon of AA, leading to mid-chain hydroxylation akin to lipoxygenases. Epoxygenases function by conversion of the alkene bonds in PUFAs to epoxides^{91, 380, 382, 384, 431}. Most of the PUFA-metabolizing CYPs are also involved in drug metabolism. Hence, it is apparent that drugs binding to PUFA-metabolizing CYPs will affect PUFA metabolism and vice versa through some form of cooperative interactions. For instance, 6-hydroxyflavone was shown to noncompetitively inhibit CYP2C9 and it was predicted to bind to a site just outside the heme pocket and preventing substrate ingress^{432, 433}. The impact of lipid-drug interactions on the metabolism of PUFAs by these CYPs is an area of active interest

and highlights the importance of studying CYP epoxygenases if these enzymes are to be targeted or modulated by therapeutics.

Discovery and functional elucidation of CYP epoxygenase activity

CYP-dependent AA metabolism was demonstrated in 1981^{434, 435}. The first instance of AA epoxidation using rat liver microsomes (LM₂) was shown in 1982^{436, 437}. It was confirmed that CYP activity was responsible for PUFA mono-oxygenation based on the ratio of the rate of NADPH oxidation and oxygen consumption during PUFA monooxygenation.

Research capacities to validate proper product identification were subsequently improved following developments in mass spectrometry, where authentic oxy-lipid standards were synthesized to elucidate the structure of the products⁴³⁸. Although it was recognized that liver microsomal activity was due to multiple CYP enzymes, the specific CYP isoform(s) responsible for epoxygenase function was unknown. As the field emerged, identifying the regioselectivity of endogenous epoxidation was critical as it was later used as a metric to differentiate between epoxygenase activity of CYP isoforms⁴³⁹. The comparison of epoxide regio- and enantio-selectivity formed by microsomal epoxygenases with that of purified CYP isoforms, as well as the use of antibody-based inhibition, finally indicated the CYP2 class to be involved in epoxidation (Figure 17B)⁴⁴⁰⁻⁴⁴².

The most studied epoxidation reaction is the metabolism of AA to all four *cis*-EET (5,6-, 8,9-, 11,12-, and 14,15-EET) regioisomers by CYPs. However, there are differences in the ratio of these regioisomers depending on the CYP isoform^{205, 382, 443, 444}. For instance, most CYP epoxygenases generate a higher ratio of the terminal epoxide, 14,15-EET, compared to the others. This is likely due to the facilitated entry of the fatty acid tail towards the heme active site

as opposed to the carboxylic acid end of the molecule, as CYP access channels must be able to direct lipophilic molecules into the deeply buried active site cavity⁴⁴⁵⁻⁴⁴⁹. In mammals, the epoxidation of PUFAs to non-allylic, *cis*-epoxides is unique to the P450 system, as other epoxides that are formed from hydroperoxide-dependent CYP oxidation, or from lipoxygenase oxidation of AA, can form both *cis*- and *trans*- EETs^{450, 451}. While the EETs generated from CYPs are mostly anti-inflammatory and vasodilatory³⁸⁵, the hydroxylated products (19- and 20-HETE) are vasodilatory⁴⁵² and vasoconstrictive^{453 454-457}.

CYPs generate the EETs as either (*R,S*) or (*S,R*) stereoisomers. The ratio of the two enantiomers varies between different CYPs and between different regioisomers formed by single CYPs^{458, 459}. The exact biological effects elicited by individual EET enantiomers and regioisomers is limited as most biological studies are done with racemic EET mixtures^{91, 460-466}. The known enantioselectivity preferences of individual CYP epoxygenases with respect to AA metabolism have been summarized in Table 5.

Interestingly, the ratio of the regioisomers produced *in vivo* depends on which tissues the EETs were extracted from, indicating differential CYP expression in different tissues. For example, the ratio of 14,15-EET and 11,12-EET in the heart was revealed to be approximately 34% and 37% of all total EETs, respectively. On the other hand, it has been shown that 5,6-EET is the predominant EET found within the lung (45.4%) (El-sherbeni et al. 2013). A major factor underscoring these differences in EET regioisomer bioavailability is the presence of specific CYP epoxygenases expressing their signature regioselective product distributions across different tissues in which they are present. Therefore, knowledge of the tissue-distribution of EET regioisomers may aid in the identification of CYPs, which can then be targeted or modulated for treating pathological conditions where AA metabolism is altered.

Although the majority of characterized CYP epoxygenases belong to the CYP2J or CYP2C families, other families of cytochrome P450s have been shown to generate PUFA-epoxides at detectable levels, albeit to a lesser extent. These include the CYP2D and CYP4F classes (Guengerich et al. 2005). The CYP4 family, including CYP4A and CYP4F, utilizes AA as a substrate. The CYP4A and CYP4F classes were originally characterized for their roles as prostaglandin and leukotriene hydroxylases. For example, CYP4F was shown to be the major LTB₄ degradative pathway (Figure 17C) and CYP4A as a key player in prostaglandin hydroxylation (Kalsotra and Strobel 2006). However, it was shown that CYP4F12 and CYP4F8 metabolized DHA in a NADPH-dependent manner to the four epoxides 19,20-epoxydocosapentaenoic acid (19,20-EDP), 16,17-EDP, 13,14-EDP, and 10,11-EDP. CYP4F8 also hydroxylates AA to 18-HETE, and CYP4F12 has metabolized DPA to four epoxides, 19,20-epoxydocosatetraenoic acid (19,20-EDPA), 16,17-EDPA, 13,14-EDPA, and to a lesser extent 10,11-EDPA (Stark et al. 2005). Additionally, CYP4F2 can convert AA, EPA, DHA, and docosapentaenoic acid (DPA) to epoxy and hydroxyl metabolites (Arnold et al. 2010). CYP4F2 functioned almost exclusively as a hydroxylase, indicating that the formation of PUFA epoxides is a minor product in comparison. CYP4F2 converted DHA into 22-HdoHE, 21-HdoHE, and 19,20-EDP.

The identification of epoxygenase function in the CYP4 family raises an important point in that the earlier declarations of “primary epoxygenases” was biased from the extent of screening certain CYPs in certain tissues from certain organisms. However, one should note that the epoxygenase activity of CYP4Fs is a minor reaction and is also selective for certain PUFAs.

With the advent of recombinant expression of membrane-bound human CYPs, researchers became interested in *in vitro* AA metabolism by human enzymes. Earlier research had

established the CYP 2C family as the primary epoxxygenases in rat and rabbit livers, which prompted the purification of CYPs 2C8 and 2C9 from human livers for further studies ⁴⁶⁷. Both CYP2C8 and CYP2C9 favor the production of 14,15-EET compared to the non-terminal epoxides (Chang and Waxman 1995). Human liver microsomal CYP2C8 was found to generate only 14,15- and 11,12-EETs in a ratio of 5:4, while CYP2C9 made 14,15-, 11,12-, and 8,9-EETs in ratios of 2.3:1.0:0.5 ⁴⁶⁷. Zeldin and coworkers heterologously expressed and purified human CYP 2C8 to determine the absolute configuration of its resulting EET metabolites ⁴⁶⁸. The metabolic trends previously found for CYP2C8 liver microsomes were in agreement with Zeldin group's data, though it was found that the (*R,S*) enantiomer for both 11,12- and 14,15-EETs were preferred. CYP2C8 also selectively generates only the (*R,S*) enantiomers of 14,15-EET and 11,12-EET. Similarly, CYP2C9 exhibits a catalytic preference towards the formation of the 14*I*,15(*S*)-EET enantiomer and it produces a higher ratio of 11(*S*),12*I*-EET compared to the 11*I*,12(*S*)-EET regioisomer (Kalsotra and Strobel 2006).

CYP2J2 is another major EET-producing CYP2 enzyme. It generates all four EET regioisomers. It displays unique enantiomeric selectivity with the terminal 14,15-EET and 11,12-EET. CYP2J2 can only produce the 14*I*,15(*S*)-EET enantiomer, but it can produce an approximately equal ratio of the 11,12-EET enantiomers ^{200, 469}.

Overall, as the lipid metabolizing CYPs from CYP2 and CYP4 class are also involved in drug metabolism, it is difficult to identify structural features for strictly defining CYP epoxxygenases. While CYPs 2 are mainly classified as primary epoxxygenases, they are still mainly characterized by their ability to perform monooxygenation on a large variety of xenobiotics ^{470, 471}. Importantly, the definition of a CYP epoxxygenase is not limited to any specific substrate class, *per se*, but by the tissue-specific expression of CYP epoxxygenases, along

with the disease-specific physiological roles associated with PUFA epoxide metabolites. This refined definition of a CYP epoxygenase will aid in studying the pharmacology of these epoxides throughout different regions of the body (i.e. the cardiovascular system, brain, intestines, etc.). Moving forward, this review will contextualize PUFA-epoxides with respect to the current body of work on the expression, physiological role, biochemical function, and structures associated with modeling CYP2J2.

B.3 EXPRESSION OF HUMAN CYP2J2 EPOXYGENASE, KNOWN POLYMORPHISMS, AND PHYSIOLOGICAL FUNCTION

Expression of CYP2J2 in extrahepatic tissues

The 2C and 2J families contain enzymes which have been characterized as primary CYP epoxygenases that produce EETs, PUFA epoxides, and epoxy-endocannabinoids^{34, 385, 472}. In humans, the most well-studied of these enzymes include CYP2J2 (the only 2J enzyme in humans), CYP2C8, CYP2C9, and to a lesser extent CYP2C19³⁸⁵. The 2C enzymes, much like their other xenobiotic counterparts, have the highest levels of expression in the liver^{425, 473}.

CYP2J2 was first discovered in the liver. However, it is considered an extrahepatic P450 with predominant expression in the heart^{200, 201, 443, 474}. Specifically, CYP2J2 is highly expressed in the aortic endothelium and cardiomyocytes and in particular in the right ventricle of the heart⁴⁷⁵. CYP2J2 is also expressed in the brain⁴⁷⁶, and the gastrointestinal (GI) tract⁴⁷⁷⁻⁴⁸⁰.

Corroborating these reports is genomic analysis by the GTEx database (Supplementary Table 2). Primary expression of CYP2J2 was 23.9 RPKM in heart muscle (n=412), followed by the liver (19.19 RPKM, n=119), small intestine (18.5 RPKM, n=88), and brain⁴⁸¹ (Supplementary Table 2). Importantly, the GTEx database sampled from a large number of patient samples.

Other databases, such as the FANTOM5 project database, suggested primary CYP2J2 expression in the small intestine (275.5 tags/million, n=1), followed by liver (223.6 tags/million, n=1), brain, and then the heart (48.4 tags/million, n=1) ⁴⁸² (Supplementary Table 3).

Collectively, human genome analysis coupled with expression analysis by various research groups worldwide indicate CYP2J2 is primarily expressed in the heart, resulting in cardiovascular implications due to its ability to generate PUFA-epoxides.

CYP2J2 mRNA levels were also found to be almost 20,000-fold higher than CYP2C8 or CYP2C9 in the myocardium and also has high levels of expression in the vascular endothelium ^{198, 475, 483}. Importantly, it was found that the EET profile observed in the heart matches the EET profile produced by CYP2J2, thus demonstrating that CYP2J2 as the primary epoxygenase in the heart ²⁰⁰. This type of analysis – coupling specific CYP expression and EET regioisomer profiles – can be extended to the study of any CYP epoxygenase to identify the primary metabolizers within any given tissue. It is important to consider that regardless of the extent of expression in different tissues, a CYP epoxygenase will contribute to the localized production of EETs, and therefore the resulting physiology.

CYP2J2 is one of the most expressed CYPs found in many regions of the brain including the hippocampus, cerebellum, and astrocytes ⁴⁸⁴⁻⁴⁸⁶. Human CYP2J2 activity is orthologously compared to the activity of a variety of CYP2J enzymes in rodents, as rats and mice have many different CYP2Js whereas humans only have one. In rodents, these CYP2J enzymes have been shown to also be expressed in various rat brain-specific regions and cell types ⁴⁸⁷⁻⁴⁸⁹. Similar to the heart, CYP2J in the brain has been shown to provide a protective role against various brain diseases, such as Parkinson's ⁴⁹⁰. Despite its dominance in the heart, the biochemical functions

of human CYP2J2 persist in other tissues in which it is expressed, providing new justifications for study on the physiological relevance of CYP2J2 function in other tissues.

CYP2J2 homologs in other vertebrates and transgenic models

To assess the physiological function of CYP2J2, it is important to also understand the cross-species differences between phylogenetically-related CYP2J enzymes. Vertebrates with the closest homology sequence to human CYP2J2 include rhesus macaques or chimpanzees ⁴⁹¹⁻⁴⁹⁴. CYP2J2 isoforms between humans and monkeys are reportedly 95% similar ⁴⁹⁵, an observation supported by genome sequencing databases ⁴⁹²⁻⁴⁹⁴.

There exist several CYP2J2 homologs in mice and rat rodent models for facilitating the study of the physiological role of PUFA-epoxides. For instance, there are seven genes within the mouse CYP2J locus, including CYP2J5, CYP2J6, CYP2J8, CYP2J9, CYP2J11, CYP2J12, and CYP2J13 ⁴⁸⁹. CYP2J6 is the closest homolog to CYP2J2 with 84% homology at the primary amino acid sequence level ⁴⁹¹. The Rat Genome Database indicates that rat homologs to human CYP2J2 include CYP2J3, CYP2J4, CYP2J10, CYP2J13, and CYP2J16 ⁴⁹⁶, where CYP2J4 is closest in homology sequence at 76% similarity ⁴⁹¹. To better understand the rodent genome and cell-specific expression of genes, a recent study conducted single cell transcriptomics on 20 tissues from eight C57BL/6 mice ¹²⁷. Exploring the cell-specific expression dataset may provide insight into cell-specific expression of human cytochrome P450s. In C57BL/6 mice, CYP2J6 was highly expressed in glial cells, such as oligodendrocyte progenitor cells, oligodendrocytes, and astrocytes. As the cross-talk of glia plays an important role in neuroinflammatory and neurodegenerative diseases ⁴⁹⁷, this data suggests that PUFA epoxides in astrocytes and

oligodendrocytes may act to encourage microglial polarization towards an anti-neuroinflammatory microglial phenotype.

In addition to single-cell transcriptomic data, there exist CYP2J2 transgenic models (Supplementary Table 4). For example, there exist a few studies demonstrating that mice expressing CYP2J2 are more resistant to experimentally-induced diseases^{213, 214, 460, 498-502}. The Zeldin lab was the first to generate a transgenic CYP2J2 (CYP2J2-Tr) animal model^{211, 503}. To do this, CYP2J2 cDNA was cloned into pBS- α MHC-hGH, a vector containing the α MHC promotor for cardiomyocyte-specific expression of the transgene. In addition, the human growth hormone (hGH)/polyA was incorporated into the vector to enhance the transgene mRNA stability. This transgene was then injected into the pronuclei of C57BL/6 mouse embryo that was implanted into pseudo-pregnant mice^{211, 503}. The resulting transgenic mice expressing CYP2J2 are a powerful tool that has been utilized since its inception. Studies using these transgenic mice were the first to verify the role of epoxygenases *in vivo*. For example, CYP2J2-Tr mice had improved recovery of ventricular developed pressure compared to wild type mice after ischemia reperfusion²¹¹. This confirmed previous studies that CYP2J2 is essential in generating EETs, eicosanoids involved in improving cardiac health. Another study provided evidence towards the effects of EETs on cardiomyocyte L-type Ca^{2+} channels. For instance, one study reported that EETs increase I_{Ca} in rat cardiomyocytes⁵⁰⁴, while another reported that EETs have inhibitory properties on porcine cardiac L-type Ca^{2+} channels⁵⁰⁵. Utilizing CYP2J2-Tr mice, it was demonstrated that CYP2J2-mediated generation of EET metabolites had a stimulatory effect on I_{Ca} in cardiomyocytes, and thus in modulating basal cardiac L-type Ca^{2+} channel activity⁵⁰³. Collectively, CYP2J2-Tr mice proved to be a powerful resource in understanding the roles of P450 epoxygenases *in vivo*.

Physiological role of CYP2J2

Given its expression in the heart, CYP2J2 plays important role in cardiovascular diseases. For instance, cardiac remodeling is a key pathological process in cardiovascular diseases, resulting in numerous complications including cardiac dysfunction and arrhythmia⁴⁹⁸. In transgenic mice overexpressing human CYP2J2 specific to cardiomyocytes, arrhythmia inducibility was reduced compared to WT mice²¹⁴. An additional study demonstrated that CYP2J2 was able to inhibit cardiac remodeling altogether, a process that inevitably leads to arrhythmia⁴⁹⁹. Other studies demonstrated that overexpression of CYP2J2 alleviated cardiac injury induced by TNF α ⁵⁰⁰, a common cytokine produced and involved in systemic inflammation⁵⁰⁶. The involvement of CYP2J2 in cardiovascular diseases has been reviewed extensively before^{507 491}. Overexpression of CYP2J2 has also been shown to reduce cardiotoxicity induced by chemotherapeutics, such as doxorubicin (DOX). As EETs prevent the myocardium from ischemia and reperfusion injury, transgenic mice overexpressing CYP2J2 specific to cardiomyocytes were generated to test whether CYP2J2 was able to reduce DOX-induced cardiotoxicity¹⁹⁹. Convincingly, transgenic mice overexpressing CYP2J2 exhibited less cardiomyocyte apoptosis and improved echocardiography readings compared to that of wild type mice hearts¹⁹⁹.

CYP2J2 is also the second most expressed CYP in the brain, implicating potential roles in mediating neurodegenerative and cerebrovascular diseases that require deeper examination. Neurodegenerative diseases such as Alzheimer's diseases are associated with the CYP2J2 polymorphisms in the Chinese Han population⁵⁰⁸. *In vivo*, CYP2J enzymes in rodents protected against Parkinson's diseases models⁵⁰⁹. However, studies of brain diseases utilizing CYP2J2 transgenic mice with overexpression specific to the brain are currently unavailable.

Nevertheless, endothelial overexpression of CYP2J2 protected against experimental cerebral ischemia in mice ¹⁹⁶. Coupling these works with the importance of tissue-specific expression will hopefully usher in new work elucidating the role of CYP2J2 epoxygenase function in combatting brain diseases.

In addition to cardiovascular and cerebrovascular properties ^{510, 511}, CYP2J2 overexpression improves cardiopulmonary function and protects against ischemia ^{460, 501}. And while PUFA epoxides are effective against diabetic diseases, there are only a few studies directly examining the effects of CYP2J2 in diabetes ²¹³, or in high-fat diet-induced NAFLD ⁵⁰². Given the role of CYP epoxygenases and their EET products in maintaining homeostasis, it is likely that epoxygenases like CYP2J2 participate in the physiological responses to disease in other tissues where the basal expression is low.

Despite tissue-specific expression differences, CYP2J2 and other epoxygenases are upregulated in cancer, promoting angiogenesis and tumorigenesis and can therefore be considered as cancer-promoting. In particular, CYP2J2 expression was elevated as measured by mRNA and protein levels of human-derived cancer cell lines and human cancer tissues as compared to healthy human cell lines or tissues ⁵¹². Following studies demonstrated that CYP2J2 overexpression promoted tumor metastasis in four different human cancer cell lines via production of EETs ¹². Interestingly, let-7, a common miRNA dysregulated in human cancers ^{513, 514}, increased the expression of CYP2J2 in human cancers ⁵¹⁵. Although used to manage cancer tumorigenesis ⁵¹⁶, let-7 miRNA therapy similarly reduced cancer phenotypes as well as CYP2J2 expression ⁵¹⁵, suggesting greater complexity in the regulatory networks involving CYP2J2 and its activity in cancerous state. As CYP epoxygenases produce multiple EET regioisomers, it is likely that the regioisomers display different physiological behaviors based upon their

subsequent anabolic and catabolic pathways. Rand et al. found that 8,9-, 5,6-, and 11,12-EET were substrates for both COX-1 and COX-2, in decreasing order, resulting in products that can be further metabolized by I, ultimately forming an angiogenic lipid species known as *ct*-8,9-E-11-HET⁵¹⁷. While a single downstream omega-6 metabolite was found to be angiogenic following CYP bioactivation, the formation of EDP omega-3 epoxides has shown to display anti-cancer properties^{518, 519}.

In summary, CYP2J2 and PUFA epoxide-generating epoxygenases may be detrimental in cancer due to their angiogenic tendencies⁵²⁰, overwhelming evidence suggests that PUFA epoxides are important to modulating cardiovascular, and broadly, inflammatory diseases^{389, 507, 521, 522}. However, it is important to note here that the functional interactions between a single epoxygenase and a substrate class do not necessarily follow clear-cut rules leading to a specified physiological implication. This is perhaps one of the greatest challenges faced within CYP enzymology, which is confounded even further when considering how CYP epoxygenase structure-function results in the accommodation of many different substrates to derive similar looking molecules with sometimes contrasting bioactivity. Because of their lipophilic nature, endogenous CYP-epoxygenase products then interact with receptors given their CYP-derived form or after further enzymatic transformations as downstream metabolites (i.e. interactions within the endocannabinoid system). Thus, while the presence and activity of a specific CYP epoxygenase is important to measure how epoxygenase function contributes to or detracts from a given physiological state, these metrics must also be contextualized with that of other enzymatic and receptor machineries which contribute to the assumed bioactivity of CYP epoxygenase activity. This rationale also applies to functions specified by different epoxygenase polymorphisms.

Known polymorphisms of CYP2J2

Although CYP2J2 is implicated in diabetes⁵²³⁻⁵²⁵ and inflammatory gastrointestinal diseases^{480, 524}, most studies have focused on its cardioprotective role, provided its predominant expression in heart tissues. Accordingly, most of the known CYP2J2 polymorphisms are associated with cardiovascular diseases⁵²⁶⁻⁵²⁹. An example is the CYP2J2*7 polymorphism found in Chinese populations, which, albeit has function associated with cerebrovascular physiology through ischemic stroke, is inherently cardiovascular in nature⁵³⁰. The CYP2J2*7 polymorphism has a “T” substituted with “G” at the 50th position in the promoter region²¹⁰. Additionally, the differences in allele frequencies are observed across different populations worldwide⁵³¹. This single nucleotide polymorphism (SNP) is associated with a wide array of other cardiovascular diseases including coronary artery disease^{210, 532-534}, hypertension⁵³⁵, and myocardial infarction⁵³⁴⁻⁵³⁶. In addition, stroke patients were observed to have a higher frequency of the CYP2J2*7 allele compared to a healthy human control group⁵³⁶. As it pertains to CYP2J2*7, evidence points to an imbalance of PUFA epoxides generation for its physiological malfunction. While only the CYP2J2*7 allele has been shown to be associated with diseases, other variations also exist and demonstrated impaired metabolic capabilities. For example, CYP2J2 *2, *3, *4 and *6 variations all exhibit reduced arachidonic acid metabolism^{210, 531}. Additionally, *8 and *10 had complete loss⁵³⁷, or reduced protein function⁵³⁸. Broadly, the reduced protein function or ability to metabolize arachidonic acid suggests that the body would have lower circulating PUFA epoxides, an important class of anti-inflammatory and immunomodulatory lipid mediators which are cardioprotective in nature. Given the importance of substrate turnover to form these metabolites, much of the availability and bioactivity of PUFA epoxides are defined by epoxxygenase kinetics.

Kinetics of metabolism of PUFAs and their endocannabinoid derivatives by CYP2J2 epoxygenase

CYP2J2 was discovered in 1996²⁰⁰ and much of its initial characterization was based on exploring its role in human physiology. CYP2J2 was first recombinantly expressed using the baculovirus system²⁰⁰. The expression of recombinant protein under these conditions was 100-150 nmol/liter of infected Sf9 cells. Following protein purification however, the final yields were approximately 10% of that from cell harvesting²⁰⁰. In 2002, five variants of CYP2J2, including R158C, I192N, D342N, N404Y, and T143A were identified that were recombinantly expressed with the Sf9/baculovirus system. The yields were low, with P450 expression levels ranging from 3 to 10 nmol/liter of Sf9 cells²¹⁰. Due to the need for large amounts of protein for structure-function studies, CYP2J2 was expressed in bacterial expression systems. Several constructs of N terminus-deleted mutants of CYP2J2 with silent mutations were expressed in bacterial expression systems²²¹. All constructs were co-transformed into DH5 α *E. coli* cells with the pTGro7 plasmid encoding for the GroEL-GroES chaperonin system. The protein yields of the N-terminally modified constructs ranged up to 200 nmol/liter protein, a yield that would allow for detailed structure-function studies. Using the CYP2J2-CPR-Nanodisc system, a model lipid bilayer that mimics the endogenous membrane environment of the protein, rates of ebastine (EBS) metabolism were confirmed that were similar to that of previously reported rates. Collectively, this was the first report of high yielding expression of functional full-length CYP2J2 protein²²¹. The large-scale expression of CYP2J2 subsequently facilitated several kinetic studies on direct metabolism of PUFAs and drugs by CYP2J2, which are outlined below.

CYP2J2-mediated metabolism of PUFA and omega-6 endocannabinoids –asymmetric binding and metabolism

CYP2J2 metabolizes arachidonic acid (AA) to form all EET regioisomers, with the preferred product being the terminal epoxide 14,15-EET. On the contrary, 2-arachidonyl glycerol (2-AG) (an omega-6 endocannabinoid) is epoxxygenated only at the 14,15- and 11,12- positions, as it has a larger head group at the carboxy terminus of the arachidonic acid. CYP2J2 metabolizes anandamide (AEA) with a $V_{\max}$ of 53.3 ± 2.9 pmol product $\cdot$ min $^{-1}\cdot$ nmol $^{-1}$ P450⁵³⁹, AA with a $V_{\max}$ of 189 ± 9 pmol product $\cdot$ min $^{-1}\cdot$ nmol $^{-1}$ P450, linoleic acid (LA) with a $V_{\max}$ of 146 ± 17 pmol product $\cdot$ min $^{-1}\cdot$ nmol $^{-1}$ P450, EPA with a $V_{\max}$ of 259 ± 77 pmol product $\cdot$ min $^{-1}\cdot$ nmol $^{-1}$ P450, and DHA with a $V_{\max}$ of 254 ± 11 pmol product $\cdot$ min $^{-1}\cdot$ nmol $^{-1}$ P450²⁰⁴. These values are consistent with other reported rates of metabolism of PUFAs by CYPs, which typically range between 100 to 1000 pmol product $\cdot$ min $^{-1}\cdot$ nmol $^{-1}$ P450. Previously, a similar rate of 200 pmol product $\cdot$ min $^{-1}\cdot$ nmol $^{-1}$ P450 for eicosapentaenoic acid (EPA) metabolism was reported using rat CYP2J3, which is 70% similar in amino acid sequence to CYP2J2⁵⁴⁰. Although these fatty acid substrates can assume multiple conformations in the active site due to flexibility in the alkyl chain, changes in the structure of the lipid head group can influence overall substrate orientation and atomic distance from the heme, thereby influencing the different product distributions seen for AA, DHA, EPA, 2-AG and AEA. Molecular Operating Environment (MOE) modelling done between CYP2J2 and 2-AG had revealed that greater polar interactions between the glycerol moiety and residues within CYP2J2 restricted orientations to favor epoxidation at selected positions⁵³⁹. Meanwhile, AA and AEA interactions with a single polar residue, Q228, enabled more freedom for other sites of epoxidation. With an increase in the number of studies that explored the metabolism of different fatty acids and their endocannabinoid derivatives, it was

revealed that changing head groups of the carboxylic acid changes the rate of metabolism of the lipids^{204, 218}. Previous data suggested that a three-carbon spacer length between the carboxylic head group and the neighboring alkene moiety caused a relatively fixed orientation to favor quicker terminal epoxidation due to polar contacts, ultimately influencing regioselectivity of the epoxidation. Specifically, a triad of the amino acids T318, R321, and S493 directly contacted and trapped the PUFA carboxylic end to a certain extent of motions during the simulations involving CYP2J2. Regioselectivity is also determined by the steric constraints imposed by the presence of multiple methylene bridges along a PUFA alkyl chain. From these molecular dynamics (MD) studies, some conformational freedom provided to the lipid substrate enables the generation of multiple epoxides, while enough rigidity within the protein structure biases regioselectivity.

We additionally showed that CYP2J2 was able to differentiate reactive moieties within the active site (i.e. different olefinic bonds and their methylene bridges) so that individual oxygenations can be selected. The rates of metabolism of PUFAs and regioisomers formed by human CYP epoxygenases are reported in. Similar data for non-human CYP epoxygenases can be found in Supplemental Information.

CYP2J2-mediated metabolism of omega-6 and omega-3 endocannabinoids

The functions of PUFAs are partially mediated by their non-oxidative conversion to endocannabinoids (eCBs) that act on the same cannabinoid receptors as the principle components of *Cannabis*. The functionalization of the PUFA headgroups leads to dramatic changes in biological activities, rate of metabolism, and receptor selectivity^{97, 98, 541, 542}. Anandamide (AEA) consists of the AA scaffold conjugated to an *N*-linked ethanolamine,

whereas 2-AG is AA conjugated to glycerol at the second position. Ethanolamide and glycerol derivatives of other PUFAs such as EPA and DHA have been discovered^{97, 98, 541 31, 106}. Further eCB-like modifications include amide-linked derivations with amino acids and taurine^{542, 543}. Together, eCBs mediate several divergent physiological responses primarily through cannabinoid receptors 1 and 2 (CB1 and CB2). The non-selective transient receptor potential cation (TRP) channels have also been shown to be activated by eCBs, notably TRP vanilloid 1 (TRPV1)⁵⁴⁴⁻⁵⁴⁸. CYP2J2 along with other important CYP isoforms in brain tissue, such as CYP2D6 and CYP4X1, are capable of metabolizing endocannabinoids to form endocannabinoid epoxides, which exhibit anti-inflammatory and vasodilatory properties partly through interactions with cannabinoid receptors, as well as with an unknown PUFA epoxide receptor^{549 34, 550, 551}. Furthermore, 5,6-EET-EA (AEA-epoxide) and many of the ω -3 eCB epoxides show a greater selectivity for CB2 binding, suggesting that the epoxidation of eCBs switches CB1/CB2 preference^{34, 550, 552}. Thus, endocannabinoids and endocannabinoid-like molecules can be metabolized by several CYPs to play an important role in maintaining homeostasis during inflammation-based pathologies. More specifically, the bioactivities of epoxide metabolites can be altered from those of the parent molecule substrate metabolized by CYP epoxygenases. Combining these relative pools of unreacted and bioactivated parent compound scaffolds in specific tissues can impose variable consequences on the handling of different inflammatory states. This systems-level metabolic equilibrium becomes further complicated when inhibitors are introduced that modulate epoxygenase function through singular or cooperative interactions.

Inhibition of CYP2J2 by drugs and endogenous lipids

Given that CYPs are generally capable of binding multiple substrates due to large active sites, and that lipid conformational flexibility enables active CYP conformations leading to beneficial bioactive products, it becomes important to understand the molecular mechanisms of lipid-drug interactions through inhibition studies. CYPs can be inhibited through the formation of reversible, pseudo-irreversible, or irreversible complexes with an inhibitor. Reversible inhibitors interfere with CYP function prior to electron transfer through non-covalent interactions with certain residues and regions of the protein, usually by binding into the active site. Pseudo-irreversible inhibitors form a coordinate bond to the heme, the extent of inhibition being dependent upon the affinity of the lone pair of electrons on a heteroatom in the inhibitor scaffold. Alternatively, an inhibitor could covalently modify active site residues within the CYP active site to induce a catalytically inactive protein (irreversible inhibition). In some cases, the inhibitors can prevent the oxy-heme intermediates from proceeding productively to product generation; they essentially “trap” the enzyme in an unproductive state upon reaction⁵⁵³⁻⁵⁶³. Such mechanism-based inhibitors are difficult to design but can be very specific and therefore can facilitate physiological studies related to the enzyme. One such mechanism-based inhibitor is reported for CYP19A1⁵⁶⁴⁻⁵⁶⁶. Other forms of inhibition result from binding to lipophilic regions of the protein, which impairs the CYP’s ability to sample conformations necessary for catalysis. Regardless of the overall mode of inhibition, inhibitors can also inhibit the enzyme by competing against CYP substrates for access to the heme. Overall CYP inhibition is reviewed in the book chapter by Correia and Hollenberg⁵⁶⁷.

Specific and mechanism-based inhibitors of CYP2J2

CYP epoxygenases metabolize both lipids and drugs and are able to recognize a wide variety of ligands, including those that are either polycyclic, aliphatic, or both ^{471, 567-569}. To this point, it has been postulated that the drug substrates of CYP2J2 interfere with its epoxygenase activity and contribute to cardiotoxicity ^{570 571}. Therefore, it is of pharmacological interest to determine which drugs' off-target effects involve CYP2J2 inhibition, which may contribute to cardiotoxicity. Additionally, CYP2J2 expression is upregulated in many cancers and EETs are implicated in tumor progression, and so the inhibition of CYP2J2 may be important in combating cancer ⁵⁷². This subsection provides examples of various drugs that target CYP2J2 specifically or with unique implications. Figure 18 shows the structures of some of these drugs and how they inhibit CYP2J2. The rest of Section 6 will outline specific interactions of drugs regarding CYP2J2's endogenous metabolisms.

Commercially available xenobiotic substrates were determined as CYP2J2 substrates using either human liver microsomes or screens against CYP2J2 activity. Preliminary work with CYP2J2 identified several inhibitors/substrates, many of which are antihypertensive, antiarrhythmic, or cardiotoxic drugs. Many of these cardiotoxic drugs are Class II antihistamines. Some of these inhibitors/substrates include albendazole, amiodarone ^{573, 574}, apixaban ⁵⁷⁵, astemizole ^{478, 574}, cyclosporine ⁵⁷⁴, danazol ^{574, 576}, ebastine (EBS) ^{201, 524, 577}, fenbendazole ⁵⁷⁸, mesoridazine ⁵⁷⁴, nabumetone ⁵⁷⁴, ritonavir ⁵⁷⁹, terfenadine ^{201, 574, 580-583}, tamoxifen ⁵⁷⁴, thioridazine ⁵⁷⁴, and vorapaxar ⁵⁸⁴.

In 2010, Totah's group led a large 139 compound screen to identify novel substrates of CYP2J2, where specific inhibitors for predominant hepatic CYPs – 1A2, 2C8, 2C9, 2C19, 2D6, and 3A4, all of which also exhibit epoxygenase function – were implemented to identify 2J2-

specific activity within supersomes ⁵⁷⁴. The ten compounds identified from the 139 compounds screened were not specific to CYP2J2. The catalytic turnover of these ten molecules was less than that for previously identified CYP2J2 substrates terfenadine and astemizole. Furthermore, besides astemizole and danazol forming five and six metabolites, respectively, the remaining eight selected substrates were metabolized to form primarily one or two products when metabolized by CYP2J2. This initial screen provided the postulate that CYP2J2 possesses a large active site to accommodate larger, bulkier substrates.

In another study, terfenadine hydroxylation and astemizole *O*-demethylation, both of which are primarily performed by CYP2J2, were used as probe reactions to screen for CYP2J2 inhibition ⁵⁷³. In the screen, 42 compounds inhibited terfenadine hydroxylation by 50% or more, whereas eight compounds reduced CYP2J2 activity by 90% or more. Of the compounds that inhibited terfenadine hydroxylation by 50% or more, 24 of them overlapped with those that inhibited astemizole *O*-demethylation by 50% or more. The candidate compounds inhibiting astemizole metabolism by greater than or equal to 90% were danazol, ketoconazole, loratadine, miconazole, and nicardipine. To further elucidate the specificity of the observed inhibition, the top 20 inhibitors from the previous screen were further screened using human liver microsomes metabolizing terfenadine and astemizole. Danazol demonstrated the greatest selectivity against CYP2J2-mediated metabolism. Later, it was shown that Danazol could be a potential index inhibitor of CYP2J2, as it inhibited several CYP2J2 based reactions with sub-micromolar IC₅₀ values, making it a substrate-independent inhibitor ⁵⁷⁶. While this work was critical for developing tools to study CYP2J2, for clinical work, it underscores the difficulty in extending CYP epoxide research beyond specific case studies as danazol also inhibited CYP2C9 and CYP2D6, which also have epoxide function ^{573 585}.

A more recent study suggested some structure-activity relationships among antihypertensive drugs to further characterize the requirements for inhibition of CYP2J2⁵⁸⁶. In a luciferin-2J2/4F12 *O*-dealkylase activity assay, Ikemura et al. evaluated the inhibitory capacities of drugs amlodipine, azelnidipine, barnidipine, benidipine, cilnidipine, efonidipine, felodipine, manidipine, nicardipine, nifedipine, nilvadipine, nisoldipine, nitrendipine, telmisartan, and delapril.

While the rationale for screening these drugs against CYP2J2 was to suggest that CYP2J2-mediated interactions with antihypertensive drugs could explain some of the side effects and bioavailability of these therapeutics, the study's choice of substrates recapitulated the major findings of the CYP2J2 substrate/inhibitor screenings from 2010. Each of the molecules were polycyclic, containing two to six rings. Those molecules which acted as competitive inhibitors all possessed bulky and largely asymmetric scaffolds, allowing them to take up more space within CYP2J2's larger active site. With largely asymmetric structures containing a long linker to separate a bulky substitution from their tetrasubstituted dihydropyridine core, these competitive inhibitors share a trend of having dihydropyridine moieties with complex substitutions. Meanwhile, the drugs considered to be mixed inhibitors shared a trend of having mostly 1,4- and 1,8-annulated planar cores, *para* substituted benzenes containing electron-withdrawing groups, and more or less symmetric non-bulky *meta* substitutions. With less bulk and more freely accessible lone pairs, these mixed inhibitors have more opportunities to bind to various parts of CYP2J2 to induce mixed inhibition.

While structure-activity trends from previous studies suggest an importance of bulk and relative symmetry on how a ligand may interact with CYP2J2, they do not necessarily provide strict guidelines beyond examples of strongly CYP2J2-specific drugs, nor do they suggest exact

physiological consequences which may translate following CYP2J2 inhibition.

Terfenadine/tefenadone and derivatives thereof have shown remarkable specificity in inhibiting CYP2J2^{587, 588 582, 589}. Importantly, terfenadine is a Class II antihistamine that is cardiotoxic, but another Class II congener, EBS, is not. Many of these terfenadone derivatives show greater inhibition of CYP2J2 when even compared to CYP2J2's closest epoxigenase relatives, CYP2C8 and CYP2C9. These derivatives also show many modes of reversible and irreversible inhibition. The ketone moiety of terfenadone is key to binding to CYP2J2 and positioning the drug near the heme^{588 590} while the tail is important for determining reversible or mechanism-based inhibition⁵⁹¹. Another CYP2J2-specific inhibitor, LKY-047, was developed as a competitive and an uncompetitive inhibitor of CYP2J2. It inhibited CYP2J2-mediated metabolisms with IC₅₀ values less than 3 μ M, whereas the IC₅₀ of all other tested CYPs was greater than 50 μ M⁵⁹². More CYP2J2-preferring inhibitors appear to demonstrate multiple modes of inhibition when maintaining specificity.

Some mechanism-based inhibitors of CYP2J2 have also been found. The importance of these types of inhibitors is that they inactivate CYP2J2 irreversibly and so would have long-lasting implications. These contain a highly reactive functional group, such as an alkynyl or quinone moiety. 17 α -Ethinylestradiol, a component of oral contraceptives, was found to inactivate CYP2J2 by forming two reactive intermediates. During the oxidation of the ethynyl group, mass spec analysis suggested that the oxidation of the terminal ethynyl carbon forms adducts with the CYP2J2 protein and oxidation of the penultimate carbon forms adducts with the heme⁵⁹³. Dronedarone was developed as an antiarrhythmic that was discontinued due to an increased risk of heart failure. Like its congener, amiodarone, dronedarone is metabolized by CYP2J2. Amiodarone showed noncompetitive inhibition and dronedarone showed mixed inhibition.

Both amiodarone and dronedarone were also mechanism-based inhibitors, with dronedarone showing the greatest potency. This was thought to have arisen from the formation of a reactive quinone-oxime (Figure 18)^{594, 595}. Lastly, Ikemura et al. discovered azelnidipine to be a CYP2J2 mechanism-based inactivator, which possesses a mixture of structural characteristics shared by the competitive and mixed inhibitors found from the study, being largely asymmetric with one side being considerably bulkier than the other⁵⁸⁶.

Typically, a probe substrate is used for screening inhibitors. The use of probe substrates to determine inhibition of CYP epoxygenases can confound efforts towards inhibitor discovery because an epoxygenase may assume different conformations in response to one class of molecules (e.g. polycyclic xenobiotics) that may not account for a different substrate type (e.g. PUFAs or lipids). For example, both AA and ebastine (EBS) are structurally dissimilar substrates of CYP2J2. Doxorubicin (DOX), a cardiotoxic chemotherapeutic, is converted to 7-deoxydoxorubicin aglycone (7-de-aDOX) in the body. While DOX inhibits EBS and AA metabolism and demonstrates competitive inhibition, 7-de-aDOX only inhibits AA, but not EBS, metabolism²¹⁸. From this example, it can be noted that multiple molecules can concurrently bind to CYP2J2, as supported by kinetic and fluorescence polarization (FP) measurements, but the conformation with which these molecules occupy the active site is highly dependent on the structures of the molecules in the study (Figure 19). Taken together, interactions between a specific CYP and a specific ligand are unique with respect to that pairing, and this observation needs to be taken into consideration when extrapolating from studies using probe substrates. Inhibition studies must then highlight the value of determining the effects of inhibitors or drugs directly on the metabolism of endogenous substrates on CYP epoxygenases, such as AA for CYP2J2, instead of relying solely on probe substrate reactions. This might also indicate why

screening of inhibitors using probe substrate reactions does not always translate into *in vivo* studies. Overall, it is important to screen for inhibitors that are the most specific for certain CYPs using both exogenous and endogenous substrates as probe substrates.

To date, there is not a lot of structure-function data to accurately predict how a drug will inhibit CYP2J2. Indeed, Figure 18 shows that CYP2J2 binds a variety of differently structured ligands. In general, drugs that are more linear in shape tend to be competitive inhibitors. However, the shape of the drug cannot always predict actions. Some of these drugs have similar structures, like EBS and terfenadine, and inhibit CYP2J2 in similar ways. Others, like amiodarone and dronedarone, inhibit CYP2J2 in unique ways. Mechanism-based inhibition cannot always be predicted as well. Both danazol and 17 α -ethynylestradiol contain an ethynyl moiety, but only the latter has been shown to inactivate CYP2J2. Meanwhile, a drug like azelnidipine, which has a biphenyl terminus like those found in EBS and terfenadine, acts as a mechanism-based inhibitor. These nontransitive actions further exemplify the need to investigate ligand-ligand interaction on a per-need basis. Furthermore, Figure 18 also demonstrates that inhibiting CYP2J2 does not always lead to an observed cardiotoxic event. Additionally, not all cardioactive drugs that modulate CYP2J2 are cardiotoxic. For example, the antihypertensive drug telmisartan is a mixed inhibitor of CYP2J2 and has shown no adverse cardiotoxicity⁵⁸⁵. Instead, we conjecture that cardiotoxic drugs may precipitate a toxic event and the inhibition of CYP2J2 exacerbates the toxicity by preventing the recovery⁵⁷¹. Despite the unpredictable nature regarding CYP2J2 inhibition and cardiotoxicity, the preponderance of cardiotoxic drugs modulating CYP2J2 exemplifies the need to include CYP2J2 in toxicity studies.

B.4 CYP2J2-LIGAND INTERACTIONS

Molecular Basis of lipid-lipid interactions in CYP2J2

The inhibitor screen of CYP2J2 provided evidence that the CYP2J2 active site is large and can therefore bind structurally divergent xenobiotics. This implies that the CYP2J2 active site will be large enough to accommodate lipids and drug simultaneously as seen for CYP3A4⁵⁹⁶,⁵⁹⁷. Therefore, these drug-lipid interactions would impose noticeable effects on PUFA metabolism. Early instances of drug-lipid interactions within CYP2J2 were shown when AA was found to inhibit EBS hydroxylation⁵²⁴. The rate of EBS hydroxylation by CYP2J2 was significantly greater than other microsomal CYPs, allowing for EBS to act as a probe substrate of CYP2J2. Use of 50 μ M AA reduced EBS hydroxylation by ~90% in human intestine microsomes, whereas 100 μ M AA nearly shut down the CYP2J2-mediated EBS hydroxylation⁵²⁴. In a separate study, astemizole *O*-demethylation was inhibited by 33% relative to control in the presence of 100 μ M AA⁵⁷⁴. Taken together, these studies were the first examples of lipid-based inhibition of CYP2J2 activity, but without any mechanistic detail. These studies highlighted that endogenous lipids are inhibitors of drug metabolism by CYP2J2, and that endogenous ligands can prove to be strong inhibitors due to their physiological and evolutionary relevance to epoxygenase function.

We recently studied the metabolism of endogenous PUFAs and their endocannabinoid derivatives by CYP2J2 and how exogenous drugs modulate these lipids' metabolism. We provided molecular details which contextualize epoxygenase action when presented with both endogenous lipids and exogenous drugs. PUFA metabolism by CYP2J2 was shown to exhibit mixed inhibition²⁰⁴. Mixed inhibition experiments were conducted such that the PUFAs docosahexanoic acid (DHA), eicosapentanoic acid (EPA) and linoleic acid (LA), were

individually co-incubated with AA. In these experiments, AA acted as either an inhibitor or a substrate. For AA metabolism experiments, concentrations of 15 μ M and 50 μ M for each PUFA were used for inhibition studies. It was found that DHA inhibited AA metabolism the strongest with a $K_i^{\text{DHA} \rightarrow \text{AA}}$ of $16.5 \pm 2.2 \mu\text{M}$, whereas the presence of AA inhibited DHA metabolism by four-fold less potency with a $K_i^{\text{AA} \rightarrow \text{DHA}}$ of $65.2 \pm 15.8 \mu\text{M}$. EPA and AA did not noticeably inhibit each other's metabolism, which we speculated to be because of their similarities in lipid chain length and only varying in degree of unsaturation²⁰⁴. Competitive titrations against probe substrate EBS were then performed to evaluate this asymmetric trend in inhibition amongst PUFAs and AA. Here, DHA bound twice as tightly as AA to CYP2J2. Following this mode of analysis, LA and EPA therefore bound more loosely to CYP2J2 than AA and DHA, which accurately reflected the trends seen from the inhibition of AA studies. Using MD simulations, few critical residues in CYP2J2 were identified as interacting with DHA, LA, EPA and AA. Specifically, a CYP2J2-T318V single mutant and a CYP2J2-T318V/S493A double mutant were made and the inhibitory binding constants for DHA and AA were found to be weaker for both the single and double mutants during the EBS competitive binding assay. However, the K_i for DHA in the single mutant was ~50% that of AA, but approaches ~80% that of AA when tested on the double mutant. Trends in weaker binding also translated to weaker inhibition of AA metabolism. As mutation of T318 and S493 resulted in DHA binding CYP2J2 with affinities similar to AA, it can be implied that these residues are involved in maintaining CYP2J2 selectivity between different fatty acid substrates. By focusing on these evolved interactions between a class of preferred lipid substrates and CYP2J2, we were able to uncover a "PUFA binding pocket", the existence of which was able to justify an emerging pathway of DOX-induced cardiotoxicity mediated through CYP2J2⁵⁷¹.

Molecular basis of doxorubicin-lipid interactions in CYP2J2 and potential alternate mechanism of doxorubicin-induced cardiotoxicity

Given CYP2J2 expression in the heart, we postulated that cardiotoxic drugs such as DOX modulated AA metabolism to incite cardiotoxicity²¹⁸. This hypothesis was primarily supported by the study that dose-dependent DOX toxicity is reduced either through CYP2J2 overexpression or EET exposure^{199, 385}. This study suggested that CYP2J2 produced EETs or metabolized DOX directly. In a recent study it was shown that DOX gets metabolized by cytochrome P450 reductase (CPR) to form 7-de-aDOX, which can then inhibit AA metabolism with an IC_{50} and K_i of 10.2 ± 0.1 and 5.78 ± 0.03 μ M, respectively. Using competitive fluorescence polarization experiments, 7-de-aDOX was found to inhibit AA binding to CYP2J2 with half the efficacy of DOX. Given that the 7-de-aDOX did not completely inhibit AA metabolism raised the possibility that that there existed an alternative CYP2J2 binding site for 7-de-aDOX. Taken together, the inhibitory data suggested DOX to be a competitive inhibitor of AA whereas the concurrent generation of 7-de-aDOX during the reaction resulted in an incomplete inhibition which allowed AA metabolism that was bound to the PUFA binding site. In comparison, DOX was a competitive inhibitor of EBS metabolism by CYP2J2 whereas 7-de-aDOX exhibited no inhibitory potential on the turnover, suggesting that 7-de-aDOX does not bind to the same site as EBS. MD simulations were used to further elucidate potential binding modes for DOX and 7-de-aDOX to CYP2J2, and to contextualize the effects of DOX and 7-de-aDOX on AA metabolism regioselectivity. The simulations were used to examine how these anthracyclines interacted with the previously identified PUFA-binding pocket. 7-de-aDOX was shown to occupy a large amount of space near the heme and alter the positioning of AA to skew regioselectivity during metabolism. In the case of co-occupation within the CYP2J2 active site,

7-de-aDOX can outcompete AA for the PUFA-binding pocket to prevent terminal epoxidation to favor 5,6- or 8,9-EET generation, which are less capable in serving cardioprotective roles as 14,15- or 11,12-EETs (Figure 19) ²¹⁸. The simulations also depicted EBS as sampling space opposite the PUFA-binding pocket and peripheral to the heme, therefore not interacting with the site of 7-de-aDOX binding. All simulations performed were in agreement with experimental data, as increasing DOX exposure did result in a significant decrease in terminal 14,15-EET formation (Figure 19). Lastly, the generation of 7-de-aDOX illustrated potential complexity of CYP-based inhibitions, as this inhibitor was formed through interactions with CPR to then inhibit CYP2J2 epoxygenation of AA. Taken together, the inhibitory capacities of DOX and 7-de-aDOX were shown to be specific to AA metabolism because it was found that 7-de-aDOX occupied an evolved binding site for fatty acid and lipid substrates. Thus, multiple events occurring within the CYP2J2 active site during DOX metabolism ultimately led to skewed regioselectivity to favor EETs with altered bioactive properties from those otherwise produced during basal conditions.

Molecular basis of endogenous lipid virodhamine interactions with CYP2J2

While it is of importance to find exogenous inhibitors, the body has its own repertoire of endogenous inhibitors that are not well characterized. Recently it was shown that virodhamine (O-AEA), an endogenous lipid derivative, is an endogenous inhibitor of CYP2J2 ²²⁴. The overall catalytic turnover of O-AEA into epoxy-O-AEAs was very low ($V_{\max} = \sim 5 \text{ pmol product} \cdot \text{min}^{-1} \cdot \text{nmol}^{-1} \text{ P450}$). When co-incubated with AEA for inhibition studies, O-AEA was found to be a potent inhibitor and inhibited AEA metabolism (K_i of $8 \pm 2 \text{ } \mu\text{M}$), while the $V_{\max}$ for AEA turnover remained the same. The kinetic data suggested competitive inhibition of AEA by O-

AEA. Competitive titrations against EBS revealed that O-AEA produced a type II bathochromic, or “red”, shift in the CYP2J2 UV-Vis spectra. Type II shifts are typically indicative of direct binding to the heme prosthetic group. Direct binding to the heme also explains the high inhibitory potential of O-EAE on ebastine binding to CYP2J2, as its K_i was $13 \pm 4.7 \mu\text{M}$. MD studies showed that while protonated O-AEA interacted with E314, F310, and T219 aid in directing O-AEA to assume a catalytically competent conformation, some snapshots of neutral O-AEA positioned the amine group as being able to directly bind heme. Thus, the competitive basis of EET metabolism is caused by steric restriction of AA when obstructing access to the heme as a result of hydrophobic contacts with the residues within the CYP2J2 I helix or through direct heme ligation ²²⁴. Furthermore, a dose-dependent wound healing experiment revealed O-AEA dose-dependently inhibits wound healing in hCYP2J2-transfected HUVEC cells with an IC50 value of $0.61 \mu\text{M}$. Taken together, the molecular characterizations of the interactions between O-AEA and CYP2J2 are just one instance of an endogenous interaction between a PUFA and a CYP epoxigenase bearing physiological relevance. It is likely that there are other endogenous molecules which can regulate epoxigenase function through selective inhibition, which might have variable physiological consequences depending upon the specific CYP and tissue localization involved in the inhibition event.

Molecular basis of phytocannabinoids metabolism by CYP2J2

With the previous studies mentioned focused on metabolism of endogenous substrates, it cannot be forgotten that CYP epoxigenases also have xenobiotic metabolizing functions. Phytocannabinoids (pCBs) are the chemical constituents of *Cannabis* and have known cardiovascular effects ⁵⁹⁸⁻⁶⁰³. Extent of cyclization of the pCB rings can become a useful handle

in separating the different types of pCBs from one another (Figure 18). Polycyclic with varying extents of flexibility, different pCBs have altered degrees of unsaturation within their rings. If opened, these rings provide additional aliphatic alkyl chains aside from the side chain off the resorcinol moiety, introducing increasingly lipophilic character ⁶⁰⁴.

Although pCBs have been known to inhibit CYP microsomal drug metabolism ⁶⁰⁵, there was no biochemical study of CYP2J2-mediated metabolism of pCBs. It was shown that CYP2J2 can metabolize six of the most naturally predominant pCBs – Δ 9-tetrahydrocannabinol (Δ 9-THC), Δ 8-tetrahydrocannabinol (Δ 8-THC), cannabinol (CBN), cannabidiol (CBD), cannabichromene (CBC), and cannabigerol (CBG) ⁶⁰⁶. With demonstrated one-site binding through Soret titrations or competitive binding against EBS, all pCBs tested were suggested to be substrates of CYP2J2. End-point metabolism resulted in the discovery that each pCB was monohydroxylated to form a primary metabolite, although there were other deoxygenated side products formed as well. From mass spec analyses, it was suggested that despite the structural differences between each of the pCBs tested, the primary metabolites derived from each species were likely hydroxylated along the pentyl side chain to form 1'-OH products when compared with well-established fragmentation patterns published for phytocannabinoids ⁶⁰⁷.

In the presence of AEA, all pCBs except for CBG demonstrated noncompetitive inhibition against the generation of EET-Eas from anandamide. CBG competitively inhibited AEA metabolism with a K_i of $10.8 \pm 1.4 \mu\text{M}$. The only pCB with a tighter inhibition constant was Δ 9-THC at $9.86 \pm 1.4 \mu\text{M}$. The remaining pCBs inhibited AEA metabolism less efficiently than Δ 9-THC. In decreasing potency, AEA metabolism was inhibited by CBD, followed by CBN, Δ 8-THC, and CBC. Trends in catalytic efficiency mirrored those seen for inhibition constants across the pCBs, except the order of greatest catalytic efficiency between Δ 8-THC and

CBD were opposite that seen within the inhibition data. Across all the binding and metabolism studies, the pCBs bound with lower binding constants and were turned over more efficiently than AEA, apart from CBG, which seemed appropriate given its more lipid-like structure and competitive mode of inhibition. Taken together, pCBs effectively shut down AEA metabolism by CYP2J2 and are instead preferentially metabolized by CYP2J2⁶⁰⁶.

These pCBs provide a noncanonical example of inhibitors, as their mode of inhibition is noncompetitive with respect to AEA metabolism, although the pCBs can be simultaneously metabolized themselves. In the case of CBG, perhaps its more linearized structure gives it more flexibility, making it a more lipid-like molecule and thus a competitive inhibitor. Although the pCBs tested were not epoxidated by CYP2J2, the generation of a single, general metabolite suggests that despite their structural differences, all pCBs tested were able to position their alkyl chains for C-H hydroxylation by CYP2J2 in a similar, if not identical, manner.

B.5 DELINEATING THE ROLE OF THE MEMBRANE ENVIRONMENT AND MACROMOLECULAR CROWDING IN CYP-CPR INTERACTIONS

All human cytochrome P450s are membrane-associated proteins, where the surrounding membrane and macromolecular environments have been shown to affect membrane and soluble protein functions^{415, 608}. Mammalian CYPs interact with their redox partners – including CPR, ADX-ADR – in the membranes. Measuring interaction between the CYP and the membrane bilayer is an active area of both experimental and computational research. Notably, P450 interactions with membranes have been shown to regulate many important aspects of molecular recognition of CYPs including, but not limited to, ligand binding, membrane depth of insertion, redox potentials, enzyme stability, structure, and overall enzyme orientation⁶⁰⁹. Each of these

variables has a direct effect on the function of CYPs, and, in the case of CYP epoxxygenases, the generation of bioactive epoxides. Additionally, advanced computational approaches have revealed the dynamic nature of CYPs towards interactions with the membrane bilayer⁶¹⁰⁻⁶¹⁵. Previous studies have revealed that CYPs are inserted within the membrane, with a partially imbedded active site and deeply immersed N-terminus and F-G loop^{611, 616, 617}. Therefore, including the membrane as a factor in influencing the CYP activity is imperative. Although there are several studies indicating that anionic lipids in the membrane influence CYP-CPR association and activity⁶¹⁸⁻⁶²³, the role of the membrane in these interactions is not totally resolved.

CYP2J2 is an integral membrane protein which is anchored into the membrane bilayer via its N-terminus hydrophobic residues and F-G loop. Recently, the membrane binding and insertion of CYP2J2 was captured using a highly mobile membrane-mimetic (HMMM) model to run molecular dynamics (MD) simulations⁶²⁴. The MD simulations revealed three key residues—W235, I236, and F239—in the F-G loop, as well as W48 in the N-terminus that are responsible for association with the membranes. Noting I236 and F239 are hydrophobic, both were mutated to hydrophilic residues – aspartic acid (D) and histidine (H) which can potentially form a stable salt-bridge between them. MD simulations revealed that I236D causes W235 to begin detaching from the membrane while F239H has less of an effect. The double mutant containing both I236D and F239H has a similar effect to I236D⁶²⁴. These CYP2J2 mutants were then expressed in *E. coli* and characterized spectroscopically. The mutations did not interrupt the P450-fold or the heme-thiolate binding motif to a large extent and were found to be stable. AA metabolism was used to assess the functionality of each mutant where it was discovered that I236D mutant had greater catalytic efficiency compared to the base CYP2J2

construct while F239H dramatically exhibited decreased activity despite the membrane insertion predictions from the MD simulations. The double mutant had a similar catalytic efficiency to the CYP2J2 base construct and none of the mutants changed the ratio of EET regioisomers, indicating little change at the heme active site. Overall, using fluorescence spectroscopy and MD simulations, high resolution interactions between the membrane and CYP2J2 F-G loop were provided. Overall, the results suggested that the introduction of hydrophilic residues in the F-G loop may help in engineering a soluble CYP2J2 without major alteration of the site for substrate recognition or product egress ⁶²⁴.

Likewise, the N-terminus hydrophobic amino acid residues of CYP2J2 are important for CYP-CPR interactions and catalytic efficiency. A previous investigation using stopped-flow spectroscopy studied the role of the N-terminal domain in electron transfer from CPR to CYP, as well as the effects of substrate type and oligomeric state on both full-length and truncated CYP2J2 (denoted by fl and tr, respectively) ⁶²⁵. CO reduction kinetics from 0.2-1000 seconds revealed that the association-dependent rate k_1 is ~200-fold faster for CYP2J2fl than for the truncated protein, indicating the essential role of the N-terminus in CYP-CPR association. This remains true when AA is used as a substrate. Kinetics were also similar between the detergent reconstituted system and CYP2J2 in nanodiscs, showing that oligomerization state is not a key factor ⁶²⁵. The authors hypothesized that the truncated N-terminus caused the CYP-CPR association step to be rate-determining, rather than fast, as it was for CYP2J2fl, implying that CYP2J2 and CPR may still associate rapidly *in vivo*. They also noted that CYP2J2tr may have adopted a less than optimal conformation with CPR, which would signify the importance of the N-terminus in docking. This is supported by both CYP2J2fl and CYP2J2tr having a K_s along the same order of magnitude. Overall, these studies highlight the functional importance of CYP N-

terminus integrity, as the existence of hydrophobic residues along this region of the protein allow for efficient association with the CYP obligate redox partner and the membrane bilayer.

Aside from the structural elements which allow for CYP2J2 and CPR association, it is essential to take into consideration the role of the membrane bilayer composition in overall CYP function. In a recent study, it was shown that lipid membrane composition alters the rate of metabolism of EBS by CYP2J2, as well as CYP2J2s coupling efficiency with CPR ⁶²⁶. Nanodiscs containing 20% and 40% anionic lipids (POPS) had the highest rates of NADPH oxidation and EBS hydroxylation, respectively. Above 40% anionic lipids, there was a noticeable drop in enzymatic activity, resulting in speculation that electrostatic repulsion between anionic phospholipids can destabilize the nanodisc assembly. Nanodiscs containing cholesterol produced slightly less product, and sphingomyelin (SM)-containing nanodiscs produced less than half the primary EBS metabolite, hydroxyebastine, compared to 20% POPS discs. Notably, the secondary metabolite of EBS, carebastine, was only quantifiably produced by nanodiscs containing SM ⁶²⁶. It was postulated that a lipid environment including SM promotes processive metabolism or that SM serves as a potential allosteric regulator. Extending these thoughts, it is likely that the ability for individual CYPs to sample catalytically competent conformations is heavily reliant on membrane composition, as this environment commands how a CYP folds around substrates and associates with CPR. And as CYPs are expected to be in lipid rafts associated with high concentration of SM, these results are relevant to *in vivo* CYP functionality. Membrane insertion then becomes an important factor as well to overall CYP function, holding equally valuable potential for translating to *in vivo* systems.

Taking into consideration the influence of the crowded cellular milieu beyond just membrane content, the same study also examined the effects of macromolecular crowding on

CYP2J2 and CPR through basic characterization experiments in the presence of microviscogens. Microviscogens are viscous reagents which alter the relative solution volume available to proteins with which they share space⁶²⁷, essentially simulating the effects of molecular crowding by changing effective concentrations (a phenomenon sometimes referred to as the excluded volume effect) in an *in vitro* setting⁶²⁸⁻⁶³⁰. Experiments in a lipid-solubilized-system (LSS) revealed an increased K_m for all crowding agents tested⁶²⁶. However, studies were also performed in nanodiscs to further mimic a membrane-like environment. Ficoll 400 increased the K_m of EBS binding to 13.3 μM compared to 9.44 μM for uncrowded nanodiscs. Both Dextran 75 and Dextran 500 slowed the V_{max} , and Dextran 75 significantly decreased the K_m (1.40 nmol product $\cdot\text{min}^{-1}\cdot\text{nmol}^{-1}$ P450 compared to 4.05 nmol product $\cdot\text{min}^{-1}\cdot\text{nmol}^{-1}$ P450 for uncrowded). This difference in effect was attributed to two factors—volume and compressibility. Though similar in molecular weight, Ficoll is known to be more compressible than Dextran and thus takes up less volume overall⁶³¹. An expansion of this study examined whether macromolecular crowders would affect the stability of CYP2J2 and CPR through ΔG unfolding experiments. Data revealed that both Ficoll 70 and sucrose had a destabilizing effect on CYP2J2, whereas CPR was stabilized by sucrose⁶²⁶.

Ultimately, the study concluded that the surrounding environment of integral membrane proteins such as CYP2J2 is critical to protein function and should be taken into account when examining other aspects of the protein. True for all CYPs, the composition of the membrane environment is a critical determinant for how the protein can sample different conformations, associate with CPR, and therefore how it perceives different substrates. Subtle differences between CYP epoxide function, product preference, and regio- and enantio-selectivity must then all be influenced, and perhaps partially explained, by native lipid environments and extent

of membrane insertion. It is therefore likely that the association with human membranes was a critical determinant for the evolved structural architectures of human CYP epoxigenases.

B.6 STRUCTURAL ANALYSIS OF CYP EPOXYGENASES AND HOMOLOGY

MODELS OF CYP2J2

Comparison of crystal structures of CYP2 epoxigenases and CYP3A4

Given how malleable CYPs are in their ability to perform a variety of reactions, it becomes critical to obtain accurate crystal structures so that specific functions can be related to structural features, namely domain orientations and folds. A crystal structure from the CYP2J family is currently unavailable. However, many other CYP2J2 homologous to CYP2J2 have been crystallized, including the other human epoxigenases, CYP2C8 and CYP2C9. These structures have provided the basis for CYP2J2 *in silico* homology models. In this section, the key aspects of the known crystal structures of CYPs used to develop CYP2J2 homology models will be discussed. These crystal structures are of the following CYPs: 2C5, 2C8, 2C9, 2C19, 2A6, 2B4, 2D6, 2R1. Crystal structures of CYP3A4 is also discussed in order to illustrate the importance of experimentally determined active site volumes for explaining observed substrate permissiveness. The factors governing ligand binding to CYPs cannot be generalized, per se, as CYP will have a different interaction with each ligand. However, a special emphasis will be given to structures that show common interaction features, multiple ligand binding, and unique ligand recognition. These features are especially important in evaluating homology models of CYP2J2 obtained from these structures.

All CYPs contain a general conserved CYP-fold⁶³². Although the sequence identity among CYPs can be as low as 20%, they possess a high degree of structural similarity, particularly

around the heme region with regards to the precise positioning of key helices. CYPs generally contain 4 β -sheets and 13 α helices. The conserved core is comprised of a 4-helix bundle (designated as the D, E, I, and L-Helices), the J and K helices, two sets of β -sheets, and a coil. The heme binding loop is comprised of a Phe-X-X-Gly-X-Arg-X-Cys-X-Gly sequence, in which the Cys is crucial for heme ligation. The Glu-X-X-Arg patch on the K-Helix is also conserved that is involved in stabilization of the protein core. The variable regions consist of the 6 substrate recognition sequences (SRS) and the membrane anchoring (N-terminal) region. Further membrane anchoring is facilitated by the F-G loop region. More importantly, the helices that directly contact heme are conserved – especially the I-and L-helices. The B' helix is the least conserved across different CYPs, as this helix controls substrate specificity.

For most of the mammalian CYPs that have been crystallized, the hydrophobic N-terminus is truncated and is replaced by a soluble hydrophilic sequence to increase expression in *E. coli* and *in vitro* solubility following purification²²². These modifications generally do not alter CYP activity and allow them to still maintain association with membranes through other membrane binding regions^{221, 633}.

Rabbit CYP2C5 was the first mammalian CYP to have its crystal structure resolved⁶³⁴. The modified CYP2C5 construct, CYP2C5/3LVdH showed remarkable spatial conservation at the heme binding site to bacterial CYP102A1, given that bacterial CYPs are water soluble^{635, 636}. This is remarkable as there have been millions of years of divergence between bacteria and rabbits and bacterial CYPs are soluble, while mammalian CYPs are membrane-bound. As CPR has a negatively charged surface, a positively charged surface near membrane-bound CYP heme was designated as the CYP-CPR binding interface based also on previous mutational studies demonstrating reduced CPR binding⁶³⁴. Thereafter, structures of CYP2C5/3LVdH were solved

with different drugs such as DMZ ⁶³⁷ and diclofenac ⁶³⁸. The carboxylate moiety of diclofenac formed an extensive H-bond network with water among residues N204, K241, S289, and D290. Such H-bond interactions and charge interactions are key to carboxylate binding with many CYPs. ⁶³⁸.

CYP2C8 and CYP2C9 are two of the main epoxigenases in humans. The closest in homology to CYP2J2 is CYP2C8 followed by CYP2C9. Eric Johnson and coworkers elucidated the structure of substrate free CYP2C8 in 2004 ⁶³⁹. CYP2C8dH crystallized as a dimer with extensive interactions between helices F to G of each monomer. Importantly, two palmitic acid molecules were bound at the interface of these two dimers which may or may not represent a functional fatty acid binding site. Later, they determined the structures of the CYP2C8dH construct bound to different drugs such as montelukast, troglitazone, felodipine, and 9-*cis*-retinoic acid ⁶⁴⁰. However, two molecules of 9-*cis*-retinoic acid were bound concurrently to CYP2C8 in the active site as this ligand is smaller than the other ligands that were tested (Figure 21). Experimental data demonstrated two binding sites for 9-*cis*-retinoic acid in CYP2C8 ⁶⁴⁰. These data are key for establishing the ability for epoxigenases binding multiple ligands.

The structure of CYP2C9 was originally resolved with and without warfarin bound ⁶⁴¹. An important feature of this structure was that S-warfarin was bound in a pocket that was too far away from the heme to be properly metabolized but could allosterically modulate the binding of other substrates. *In silico* modelling confirmed that another warfarin molecule or fluconazole could bind near the heme with warfarin bound in this pocket ⁶⁴¹. A separate study later found that 6-hydroxyflavone noncompetitively inhibited CYP2C9 by binding near this warfarin allosteric site and preventing diclofenac access to the heme ⁴³². These data helped to establish that ligand binding near the heme can allosterically modulate the binding of another. Later,

another CYP2C9 structure was obtained with an anionic substrate, flurbiprofen bound to the active site⁶⁴². The original structure obtained by Williams et al. could not explain the anionic selectivity of CYP2C9 due to the lack of basic residues in the active site⁶⁴¹. However, the new structure by Wester, et al. showed that flurbiprofen can induce conformational changes that allow residues such as R108, D293, and N289 to swivel into position (Figure 21)⁶⁴². Another structure obtained by Liu et al. shows that R108 also interacts with the thiophene group of TCA007, facilitating substrate binding⁶⁴³. Therefore, these structures demonstrated that substrates can induce different interactions with active site residues.

CYP2C19 is 91% homologous to CYP2C9. The structure of CYP2C19 bound to 0XV was determined in 2012⁶⁴⁴. CYP2C19 contains an antechamber that resembles the warfarin site in CYP2C9; however, in CYP2C8 smaller residues merge these two chambers into one large active site. CYP2C8, therefore, has the largest active site, followed by CYP2C19 and then CYP2C9.

Like CYP2C5, rabbit CYP2B4 represents another pinnacle CYP that has become a model CYP for biophysical and structural studies. Particularly, the structures of CYP2B4 have been used to model open and closed conformations of mammalian CYPs. CYP2B4 was crystallized as a dimer in the open (substrate-free) conformation in 2003, due to the presence of a cleft in the F-G region⁶⁴⁵ and its structure was further resolved in the closed conformation in 2004 by binding to 4-CPI⁶⁴⁶. The putative closed conformation was crystallized with 4-CPI bound to the protein and achieved by mutating a histidine residue in CYP2B4dH (CYP2B4dH-H266Y)⁶⁴⁶. The cleft seen in the open conformation moved in towards 4-CPI to isolate it and the active site from the bulk solution. This “closed” structure more closely resembles the structures of CYP2C5, CYP2C8, and CYP2C9 obtained earlier. Later, a substrate-free structure in the closed conformation was obtained⁶⁴⁷. Interestingly, CYMAL-5, a common detergent used to solubilize

CYP2B4, was found not only to bind a peripheral site near the F-G region of WT and mutant (F202W or F224W) CYP2B4 but also at the active site ⁶⁴⁷⁻⁶⁵¹. This demonstrates that detergents can bind to CYPs as well. Finally, several mutational studies provided further insight into substrate recognition by CYP2B4.

CYP2D6 is not considered a primary epoxidase; however, it metabolizes PUFA-derived endocannabinoids. CYP2D6 is known to produce all four regioisomer epoxides of anandamide (EET-Eas), as well as hydroxylated products of anandamide ^{652, 653}. CYP2D6 is a primary CYP of the brain and known to selectively metabolize basic and N-containing substrates that includes psychoactive and psychotherapeutic drugs such as 3,4-methylenedioxymethamphetamine (MDMA) ⁶⁵⁴, tricyclic antidepressants ⁶⁵⁵, and cannabidiol ⁶⁵⁶. The structure of CYP2D6 (allelic variant V347M) was solved in 2006 ⁶⁵⁷. The CYP2D6 architecture was closest to that of CYP2C9, but with key differences. The most prominent difference is in the F-G region. Importantly, negatively charged residues were identified in the active site and were proposed to attract basic ligands to the active site ⁶⁵⁷. Thereafter, several structures of ligand-bound CYP2D6 were reported ^{658, 659}. The first study demonstrated prinomastat bound to CYP2D6 in a closed conformation ⁶⁵⁹. Later, structures of thioridazine demonstrated open and closed structures, with two molecules of thioridazine bound in the open structure ⁶⁵⁸.

CYP2J2 has a high amount of sequence homology to CYP2R1, despite these enzymes having distinct functions. CYP2R1 is responsible for hydroxylating the vitamin D series, notably producing calcifediol ⁴¹⁹. CYP2J2 likewise metabolizes Vitamin D₂, D₃, and 1 α -OH-D₃, with a higher preference for the exogenous D₂ over the endogenous D₃ ⁶⁶⁰. There are 3 PDB entries for the structure of CYP2R1 ⁶⁶¹. The substrate access channel in crystals with vitamin D₃ bound formed an “extended” active site. The conformation of Vitamin D₃ was in a non-steroidal

conformation, consistent with the inability of CYP2R1 to metabolize cholesterol or 7-dehydrocholesterol ⁶⁶¹. A unique structural aspect of the CYP2R1 structure is that the I-Helix is not distorted, whereas I-helix distortion in other mammalian and bacterial CYPs is proposed to help facilitate proton delivery to O₂ during the catalytic cycle ⁶⁶².

CYP3A4 is a highly promiscuous enzyme and the concurrent binding of several substrates has been proposed to be the basis for some drug-drug interactions ^{191, 663, 664}. Although CYP3A4 is not highly homologous to CYP2J2 and has not been used to generate a CYP2J2 homology model, it is one of the most studied human CYPs with respect to drug metabolism. Furthermore, it has a large active site similar to that proposed for CYP2J2. Despite having less than 40% homology to CYP2Cs, CYP3A4 has a structure that is rather similar to CYP2C8 ⁶⁶⁵. Both have large active sites, i.e. 1438 Å³ for CYP2C8 and 1386 Å³ for CYP3A4. However, the key difference is that the active site of CYP3A4 is more open at the vicinity of the heme due in part to shorter F- and G-Helices that do not pass over the active site. This larger volume allows for the accommodation of a large array of substrates and substrate orientations that have been observed experimentally ⁶⁶⁵. Since then, many structures of CYP3A4 with other ligands have been solved with various drugs and endogenous ligands bound, such as progesterone and metyrapone ⁶⁶⁶, ketoconazole and erythromycin ⁶⁶⁷, ritonavir and analogues ⁶⁶⁸⁻⁶⁷¹, bromoergocryptine ⁶⁷², progesterone and citrate ⁶⁷³, midazolam ⁶⁷⁴, and metformin ⁶⁷⁵. Progesterone bound at a peripheral site near the access channel and membrane interface ^{666, 673}. Later, progesterone binding at this site was shown to increase the activity of CYP3A4 and contribute to cooperativity ⁶⁷⁶.

Capturing the different crystal structures of CYP2 enzymes and others has enabled a deeper understanding of important characteristics regarding CYP function. One of the most striking and

defining features of CYPs is their promiscuous substrate selectivity and conformational plasticity. This is perhaps best exemplified by the open, closed, and quasi-closed states of CYP2B4, demonstrating that substrates can induce key structural changes (but not always). Additionally, CYP2C9 demonstrates how a substrate (flurbiprofen) can induce a key interactive residue to swivel into the active site. Complex allosteric binding has also been revealed through these crystal structures. CYP2B4, CYP2C8, and CYP3A4 demonstrate peripheral binding sites for CYMAL-5, palmitic acid, and progesterone, respectively, and warfarin shows allosteric binding to CYP2C9 near the heme. Multiple-ligand binding to CYPs is demonstrated by CYP2C9 (retinoic acid), CYP2D6 (thioridazine), and CYP2B4 (CYMAL-5) providing insight into the complex cooperativity observed with CYPs. Overall, it is this structural malleability between CYPs and their ligands that make generalizations regarding ligand binding difficult. Ligand binding, therefore, must be studied on a per-basis need to gain insight into the unique molecular interactions governing binding. This may especially be true regarding PUFAs, which are highly malleable substrates whose conformations may be highly influenced by interactions with another ligand. More crystal structures of CYPs with PUFAs or PUFA-like ligands may help to provide insight into this conformational pliability. However, the crystal structure of one CYP cannot suffice in explaining or predicting the ligand interactions of another CYP due to these complex, unique interactions. Therefore, no current crystal structure alone can provide insight into how ligands bind to CYP2J2. Other techniques may further our understanding of the dynamic interactions between PUFAs and epoxigenases. Homology and molecular dynamic simulations can provide valuable insight into interactions between ligands and proteins over time, especially if the protein has no known crystal structure. In the next sections, the homology

models and molecular dynamics simulations of CYP2J2 are discussed, which can help bridge the gap in this knowledge.

In silico modeling of the CYP2J2 structure and use of molecular dynamics to perform structure-function studies

Computational approaches can provide insight into the dynamics of molecular interactions, which are hard to ascertain by the static frames of crystallographic data. Several homology models have been developed and have been used in performing molecular docking and molecular dynamic simulations to better describe CYP2J2 structure-function, as this epoxigenase has no available crystal structure. These models rely on the crystal structures of various CYPs as outlined in the previous section. In this section, the development of CYP2J2 homology models and insights provided from these models are described.

One of the earliest models was developed by Lafite et al.⁶⁷⁷. The purpose of the model was to help explain the regioselective hydroxylation of terfenadone derivatives by CYP2J2. Many CYP structures were used to develop the model, which are as follows: CYP2A6 [PDB 1Z11,⁶⁷⁸], CYP2B4 [PDB 2BDM,⁶⁷⁹], CYP2C5 [PDB 1NR6,⁶³⁸], CYP2C8 [PDB 1PQ2,⁶³⁹], and CYP2D6 [PDB 2F9Q,⁶⁵⁷]. The authors used the SWISS-MODEL v3.5 server to build the CYP2J2 model. To test the robustness of the model builder, the authors built a model of CYP2B4 and compared it to its crystal structure. They were able to obtain a model of CYP2B4 that matched within 1.05 Å of the known closed structure of CYP2B4 [PDB 1SUO,⁶⁴⁶] despite the reference structures having only 51%-54% sequence identity to CYP2B4. Molecular docking was then performed on select terfenadone derivatives. One of the most important findings of the study was that R117 is important for forming H-bonds to the ketones of these derivatives. This

helped to position the molecule near the heme for metabolism and would also aid in the understanding of terfenadone-CYP2J2 structure-function for future inhibitor development.

Later, a model was developed by Li, et al. based on the structure of CYP2C9 bound to warfarin [PDB 1OG5, ⁶⁴¹] using Modeller 8v2 software ⁵⁹⁰. They truncated CYP2J2 at the first 43 residues of the N-terminus to reflect the missing residues of CYP2C9 in the crystal structure. MD simulations were then used on the resulting model for 1 ns to test the stability of the structure. Furthermore, molecular docking with terfenadone and related substrates of CYP2J2 was done and, again, the importance of R117 in directing ligand binding was observed. However, it was also observed that R117 formed a salt bridge with E222, which is analogous to other observations for similar CYPs. While the identification of this salt bridge did not occur in the work of Lafite et al., similar results between both efforts ascribe the same amino acid with functional importance in maintaining structural interactions between CYP2J2 and its substrates. Again, homology models may not necessarily replace the accuracy of a crystal structure in a specific conformation. But, as shown from these studies, the ability of models to accurately describe biophysical phenomenon can be validated by cross-referencing results obtained from multiple models, where each model was constructed by different software packages and computational approaches.

It can become difficult for experimentalists to know which homology models to trust when it comes to the best interpretation of a protein-ligand interaction. For example, another CYP2J2 model ⁵⁷⁴ based on the structures of CYP2A6 [PDB 1Z10, ⁶⁷⁸], CYP2B4 [PDB 1PO5, ⁶⁴⁵], and CYP2C8 [PDB 1PQ2, ⁶³⁹] was prepared. The purpose for generating a model in this study was to compare the active site volumes between CYP2J2 to CYP3A4. Referencing active site volumes between these two CYPs served as the authors' basis for comparison of *in vitro* ligand binding,

and so no docking or MD studies were performed and the stability of the model was not robustly tested. Thus, it is always important to remember how and for what purpose a model is prepared for in order to maximize its effectiveness in representing experimental observations or predicting outcomes of specific CYP structure-function studies.

As reiterated throughout this review, the binding of lipid substrates to CYP epoxygenases is distinct from that of more rigid substrates due to differences in conformational flexibility. Accordingly, homology models should be developed to account for intricacies inherent to the desired application. Homology models were later developed to understand AA binding to CYP2J2. The first study investigated the polymorphisms T143A and N404Y⁶⁸⁰. The sequence of CYP2J2 was submitted to PDB to look for sequence homologies and CYP2R1 bound to vitamin D₃ was used for the template based on sequence identity [PDB 3C6G,⁶⁶¹]. The model was then tested for stability with a 1 ns MD simulation. Molecular docking was performed for AA in the protonated form to each polymorphism, and then 10-ns MD simulations were performed. The group found that 5 of the 6 CYP substrate recognition sequences (SRS) were involved in AA binding, which mostly involved hydrophobic interactions. It was suggested that a T143A polymorphism could affect water access and N404Y polymorphism could affect the positioning of residues interacting with the headgroup of AA. The group later developed another model to investigate more CYP2J2 polymorphisms and obtained similar results⁶⁸¹.

Another model was developed later by Proietti et al. to further investigate AA binding to CYP2J2⁶⁸². This model was built based on the structure of CYP2B4 bound to 4-(4-chlorophenyl)imidazole [PDB 1SUO,⁶⁴⁶]. This structure was chosen based on sequence identity to CYP2J2 (39%), completeness of the sequence, and high resolution of the structure. This active site, however, was too small to accommodate a large molecule like arachidonic acid.

Instead, the authors used the active site of CYP2R1 bound to vitamin D₃ [PDB 3C6G, ⁶⁶¹] and likewise used vitamin D₃ as the model ligand to develop the CYP2J2 active site. The study then investigated AA binding to CYP2J2 in the unprotonated form using 30-50-ns MD simulations. It was found that R117 was important for AA binding to CYP2J2, which orients the 11,12-alkene position closest to the heme for metabolism.

A model of CYP2J2 was by See-Hyoung, et al. made based on the structure of CYP2C8 [PDB: 2NNI, ^{640, 683}]. The authors, however, did not provide details into their model validation. The study demonstrated a noncompetitive inhibition of astemizole demethylation in human liver microsomes by a compound found in the gromwell plant, acetylshikonin. As astemizole demethylation is predominantly performed by CYP2J2, the authors then modelled astemizole and acetylshikonin binding to CYP2J2. Acetylshikonin, interestingly, bound in the active near the heme and prevented astemizole from adopting a productive conformation, similar to the noncompetitive inhibition of CYP2C9 by 6-hydroxyflavone ⁴³². As pCBs are noncompetitive inhibitors of AEA metabolism and are also substrates, we suspect that the pCBs similarly inhibit AEA metabolism by binding in a pocket in the active site and preventing AEA metabolism ⁶⁰⁶.

These several homology models vary in their approaches regarding the use of CYP templates and in the software used for modeling. They also vary in the degree of testing, validation, and employment of the models, the latter of which can give valuable insight into the integrity of the model and how they can be used. All the models employed molecular docking or molecular dynamics simulations in their studies to validate them, except for the model proposed by Lee et al. The models developed by Lafite et al., Cong et al., Xia et al., and Proietti et al. have all been used in MD simulations in addition to molecular docking. One of the most striking differences among the models is the range of active site volumes. From largest to smallest, the reported

active site volumes are as follows: 1420 Å³⁵⁷⁴, 945 Å³⁶⁷⁷, 330 Å³⁶⁸¹, 320 Å³⁶⁸⁰, and 234 Å³⁶⁸².

It was suggested that the vast differences in active site volume estimation could be arising from how each program defines the active site volume and implied that including solvent or substrate channel and the heme pocket in determining the active site volume can give larger estimates⁶⁸².

It is important for both experimental and theoretical researchers across the spectrum of basic and translational research to be cognizant of the variability between different homology models when attempting to describe CYP structure-function. Confidence in a model can always be improved through validation measures, which can also include computational sampling techniques, like molecular docking and molecular dynamics simulations for characterizing biophysical interactions.

Molecular docking and molecular dynamics (MD) simulations

Despite the large variations in the models, especially with respect to active site volume estimations, some common features are apparent. Notably, R117 was shown to be an important residue for ligand recognition. This residue was crucial for forming H-bond interactions with the ketone of terfenadone and positioning it to the heme, which was later confirmed by a follow-up study using 200-ps MD simulations^{677, 684}. Similarly, R117 was identified in the binding of terfenadone and several similar ligands⁵⁹⁰. R117 was also shown to form an important salt bridge with E222, and it was demonstrated that *in vitro* mutations of R117 undermined the stability of CYP2J2^{590, 684}. R117 was implicated in directing AA binding to CYP2J2. However, the conformation of AA that interacted with R117 presented the 11,12- position closest to the heme for oxidation at the end of their simulations. The major site of metabolism is at the 14,15- position, which the authors mentioned was not replicated in their simulations⁶⁸². One possibility

is that their model has the smallest active site volume, which may be constraining AA into this position. Without further structural data, the true active site volume of CYP2J2 remains otherwise unknown. The other simulations performed in the protonated state of AA may explain the predominance of hydrophobic interactions in their results^{680, 681}. Still, these models have limited abilities in explaining the exact interactions between CYP2J2 and AA without being accompanied with rigorous thermodynamic and structural experiments.

A difficulty in accurately recapitulating CYP activity *in vitro* is mimicking the membrane environment. The previous simulations were performed in aqueous solutions. However, CYP2J2 exists in a membrane environment, which can influence the architecture of CYP2J2 and its activity. Therefore, Lafite's CYP2J2 model was used to perform MD simulations of CYP2J2 in a highly mobile membrane-mimetic model (HMMM). From the simulations, we identified F-G loop residues that are important for the association of CYP2J2 to the membrane, namely I236 and F239. Building upon the truncated CYP2J2 construct (Δ 34-CYP2J2), a double mutant, Δ 34-CYP2J2-I236D/F239H, was produced that maintained enzymatic activity while also being soluble in aqueous media⁶²⁴.

We further carried out MD simulations to investigate the binding of lipid ligands to CYP2J2, namely AA, EPA, DHA, and O-AEA in their charged states^{204, 224}. These studies led to the determination of a "PUFA binding pocket" comprised of residues T318, R321, and S493, which direct PUFA binding to CYP2J2. The importance of these residues was confirmed by mutational analysis using a T318V and a T318V/S493A double mutant. Meanwhile, other binding clusters were observed where PUFAs bound to sites previously reported terfenadone and EBS binding sites²⁰⁴, and also resembled previously reported orientations assumed by AA in other homology models⁶⁸². However, no significant interactions with R117 were observed in the simulations of

CYP2J2 and the lipid ligands mentioned here. This discrepancy in binding interactions is likely the result of several differences between the previous simulations versus ours. Firstly, our lab's work used the Lafite homology model, which has a much larger reported active site volume than the other models. Secondly, initial docking of each PUFA was done with the predominant position (the ultimate alkene) closest to the heme before running the simulations for 100-200 s. Lastly, the simulations were carried out using a membrane-mimetic model. Controlling these variables allowed for what we believe to be a more representative model: one that arranges substrate geometry in a preferred manner before simulation, and accounts for the membrane environment.

Using these precautions in designing *in silico* experiments allow for more accurate computation that can accompany experimental data. When hypothesizing that DOX-induced cardiotoxicity was mediated by CYP2J2, substrate-bound CYP2J2 MD simulations were performed using different coincubations of PUFAs, DOX and 7-de-aDOX ²¹⁸. It was shown that 7-de-aDOX modulated the site of AA metabolism by CYP2J2 towards greater 5,6-epoxidation. MD simulations demonstrated that 7-de-aDOX can bind into the PUFA binding pocket of CYP2J2, thus displacing AA to place the 5,6- position closest to the heme and bias regioselective metabolism ²¹⁸. Experimentally observed shifts in metabolism validated the simulation results, providing a more solid rationale to explain the known cardiotoxicity of DOX than simulations or modelling would on their own ⁵⁷¹.

Overall, computational analyses give information on the dynamics of ligand binding that are usually corroborated through crystal structure or NMR studies. MD simulation studies increasingly reported no major conformational changes in CYP2J2, which may reflect the rigidity of the CYP2J2 active site. However, caution must be used towards interpretation of the

models as the software used and templates selected can greatly influence the results of the model. Although they may not fully represent the system perfectly, *in silico* modeling can aid experimental data to develop a more comprehensive understanding of physically observed molecular interactions. This is especially true regarding multiple-ligand interactions, such as AA and 7-de-aDOX,²¹⁸ as well as for single-ligand interactions, as was done for determining the primary mode of CYP2J2 inhibition by O-AEA²²⁴. Simulations can help to bridge gaps in comprehending how a ligand engages with conformationally lithe CYPs, but currently these computational approaches are most effective when using a validated crystal structure or homology model; accounting for nuanced information which best mirror the context of the experiment (i.e. membranous versus aqueous environments); and accompanying wet bench experimental data that the simulations are attempting to describe.

B.7 CONCLUSIONS AND FUTURE OUTLOOK

Mammalian CYPs are the “organic chemists of the body” that are equipped with an impressive arsenal of synthetic capabilities, which allows them to metabolize a wide diversity of substrates. The biochemical roles assumed by CYPs are a consequence of their complex structure-function relationships, their large repertoire of substrates, and the membrane environment which encapsulates them. CYP epoxigenases are a specific subclass of CYPs that are worthy of singular attention, as their large active sites and ability to metabolize endogenous lipids, results in the formation of PUFA-epoxides with diverse pharmacological properties.

This review highlights the recent advances in the structure-function studies of CYP epoxigenases, focusing on CYP2J2 due to its prominent role in PUFA epoxidation. Here, we have shown that only a subset of the 57 human CYPs have the ability to metabolize PUFAs into

PUFA-epoxides. The CYP2 class has been designated as the primary epoxygenases based on their widespread expression across different tissue types in murine models⁶⁸⁵, which then translated into further research showing that CYPs 2 demonstrate an important physiological function related to the epoxidation of PUFAs into bioactive lipid mediators.

Despite functional differences, all CYPs utilize the same generalized P450 catalytic cycle to metabolize their substrates. It was noted that CYPs can be broadly classified into two categories based on their function. They can be classified either as biosynthetic CYPs that have evolved to synthesize endogenous steroids, or as xenobiotic-metabolizing CYPs that are involved in drug metabolism. Interestingly, CYP epoxygenases are well-endowed with both functionalities, as they are at the crossroads of bioactive oxy-lipid synthesis and drug metabolism. Most of the lipid-metabolizing CYPs, such as CYP2C8 and CYP2C9, are well known for their important role in drug metabolism. This fluid function of lipid-metabolizing CYPs, on one hand points to our need for understanding what are the determinants for molecular recognition and metabolism of lipids; on the other hand, this dual functionality demonstrates a physiological need for more information on the tissue distribution and activity during different disease states of these CYP epoxygenases. Overall, more studies in nearly every research area – gene expression, *in vivo* animal studies, *in vitro* biochemical assay, biophysical characterizations, crystal and spectroscopic structure determination, improved computational modeling and simulation capacities – are critically needed to understand lipid metabolism by CYP epoxygenases and its physiological relevance in health and disease.

Initial studies in the 1980s focused on identifying the regioselectivity of endogenous epoxidation by the CYPs in lieu of identifying specific CYP isoforms in microsomes that were responsible for the observed epoxidation. This classification of epoxide regioselectivity and

enantioselectivity was later used as a metric to differentiate between key CYP epoxygenase activities in different tissues as well as diverse disease states. Overall, while most P450 research has been “liver centric”, the role of extrahepatic CYPs in localized endogenous lipid metabolism is emerging. Currently there are very few studies that link the expression of CYP epoxygenases in tissues or in disease states *with* the type of epoxide regioisomers or enantiomers respectively formed *to* the outcome of disease progression. For instance, while CYP2J2 has been most widely studied with respect to its cardiovascular role, it is noted here that CYP2J2 expression is high in other extrahepatic tissues, such as the brain and small intestine. It is intriguing that single cell brain expression studies show that CYP2J2 (CYP2J6 in mice) is highly expressed in oligodendrocyte progenitor cells (OPC) as well as endothelial cells, suggesting that CYP2J2, as well as other CYPs and CYP epoxygenases, carry out specific functions within specific cell types⁶⁸⁶. That CYPs are expressed and active in extrahepatic tissues, like the brain, is a stance which researchers need to emphasize further; otherwise, the importance of CYP function across different disease states in different tissues will be stalled. The function of CYP2J2 in the brain and its dynamic expression in brain tissues during neuroinflammation is unknown. As PUFA-epoxides have been detected in brain, it is highly plausible that CYP2J2 produces PUFA-epoxides that act on microglial cells to reduce inflammation and promote pathological resolution. However, the mechanism of CYP2J2 and other epoxygenases to generate PUFA-epoxides in response to an insult or inflammatory event in the brain needs to be studied further. Given differences in regio- and enantioselectivity in epoxidating endogenous substrates, it is very likely that different epoxygenases are differentially involved in the regulation of specific inflammatory responses across all tissues. Hence, there is a need to further elucidate how PUFA-epoxides are locally produced in different tissues to help modulate these responses.

CYP2J2 and other epoxygenases are membrane-bound proteins. Lipids and proteins behave differently in a membrane environment when compared to free fatty acids. Membrane bilayers introduce spatial restrictions to the proteins and thus change the relative diffusion of hydrophobic molecules such as PUFAs. Precisely how lipids or other substrates enter CYPs has not been experimentally proven, although computational studies have been referenced in this review when discussing the influence of the membrane environment on epoxygenase structure-function. Additionally, enzymes that release free fatty acids, those that metabolize these lipids into bioactive molecules, and the receptors which interact with these lipids, are all co-localized to the membrane. Given their close proximity, the effective concentration of PUFAs at the site of action becomes an important variable to consider for *in vitro* and *in vivo* research. A common criticism to *in vitro* structure-function studies on the interactions between bioactive lipid mediators and their protein targets is with respect to experimental design. These experiments are conducted on the micromolar scale, whereas the endogenous production of these molecules is several fold-lower in blood and extracted tissues. However, it is important to take into consideration macromolecular crowding within the membrane environment. Yes, many of the bioactive lipid mediators covered in this review are synthesized on-demand, which results in arguably reduced half-lives and chemical stability. But, the membrane provides a platform to facilitate interactions between these rapidly degrading bioactive lipids and their cognate receptors through dynamic and crowding effects⁶⁸⁷⁻⁶⁸⁹. Note that a single lipid synthesized within 10 nm of its target in the membrane has an effective concentration of ~3.5 mM⁶⁸⁷.

Regardless of its localization, CYP2J2 expression and activity leads to the formation of non-allylic, cis-epoxides of PUFAs. PUFAs are different from other small molecules or biological molecules. There are many unknowns with respect to PUFA biology with respect to

their release from and location within membranes throughout the body. PUFAs are also highly flexible, often adopting various conformations which lead to rattling and dynamically variable motions when associating with membranes and CYP active sites. These motions, which are due to high degrees of conformational flexibility, cooperate with the inherent structural malleability of CYP epoxygenases to influence the formation of various epoxides and hydroxides. Therefore, the PUFAs have less discriminatory molecular handles or reactive moieties aside from their head groups, as any modifications at the carboxylic ends of lipid substrates, such as endocannabinoids, changes the binding interactions and biological properties of PUFAs. This makes their study more challenging because most of the molecular interactions between PUFAs and CYPs become difficult to experimentally modulate. More importantly, PUFAs are hydrophobic substrates with poor solubility in buffer conditions normally used to study biochemistry of protein-lipid interactions, which fundamentally limits the extent of possible biochemical characterizations that can be done on these molecules. Hydrophobicity and flexibility due to unbranched structures with varying degrees of unsaturation makes these molecules highly versatile (they bind several different receptors) and malleable (they can bind into a variety of cavities and can allosteric modulate substrate metabolism). These same features also make it difficult to predict how lipids and PUFAs will interact with a protein. Therefore, there needs to be a greater dataset of lipid-CYP interactions which will help to create better tools by which we may predict how these lipids bind to the epoxygenases, as well as other protein receptors which directly mediate downstream physiological responses and regulatory schemes.

The initial characterization of CYP2J2 was based on exploring its role in human cardiovascular physiology. CYP2J2 was recombinantly expressed using the baculovirus system which was not sufficient for biophysical studies. Later, it was recombinantly expressed in

bacteria to yield enough protein for detailed structure-function studies involved in drug and lipid metabolism. It was shown that CYP2J2 was able to metabolize PUFAs and endocannabinoids. There has been a rigorous search for CYP2J2 inhibitors by several and separate research groups using probe substrate reactions. The inhibitor screens lead to identification of danazol and terfenadine derivatives as the semi-specific inhibitors of CYP2J2. The diversity of identified inhibitors also demonstrated that CYP2J2 has a large active site which can provide greater conformational freedom to small molecules at the active site when compared to other epoxygenases. However, the identification of a specific mechanism-based inhibitor and a crystal structure of CYP2J2 are still ongoing and will be invaluable in delineating the role of this CYP in the presence of other epoxygenases such as CYP2C8 and CYP2C9. The Ikemura et al. study (2019) referenced during the introduction of CYP2J2-specific substrates and inhibitors characterized azelnidipine as a CYP2J2 mechanism-based inhibitor; within that same paper that includes drugs such as ritonavir, dronedarone, amiodarone, 17-ethynylestradiol, 17-octedcynoic acid, *N*-(methylsulfonyl)-6-(2-proparglyoxyphenyl)-hexanamide), as well as some unnamed compounds discovered by Lafite et al.⁵⁹¹, were cited as mechanism-based inhibitors, although with respect to inhibiting specific CYP2J2-ligand interactions. Recently, a near-infrared fluorescent probe was developed for monitoring CYP2J2 activity was developed, showing promise that other molecular design studies can be carried out to develop more specific probes and inhibitors of CYP2J2⁶⁹⁰. It is these types of biochemical advances that will facilitate several physiological studies in other diseases states, such as cancer, where overall expression of all CYPs are high and specific contribution of the different CYP isoforms remains poorly understood.

One of the primary problems in the pharmaceutical industry is drug-drug interactions where concurrent use of two drugs or one drug and one endogenous molecule may mimic, magnify, or oppose the effect of the other drug. Whether intended or unintended targets of small molecules, drug-drug interactions accommodated by CYP active sites could result in impaired homeostatic maintenance due to altered pathways of endogenous metabolism^{691, 692}. CYP2J2 is the primary cardiovascular CYP but is not included regularly in drug toxicity screening as drug metabolism studies are commonly “liver centric”. There is increasing awareness that many current drugs are substrates for CYP2J2 and this enzyme plays a significant part in the metabolism of drugs known to cause cardiotoxicity. Only doxorubicin-CYP2J2 interactions have been studied using both in vivo and in vitro model. Therefore, our understanding of the role of CYP2J2 and EET formation is based largely on the data from this single drug. Fully understanding the role and possible protection by CYP2J2 in the heart against drug toxicity requires extended molecular characterization studies of how CYP2J2 discriminates between xenobiotic and endogenous substrates when presented with either ligand type. The biochemical studies on the doxorubicin-lipid, phytocannabinoids-lipid, and lipid-lipid interactions with CYP2J2 demonstrate that CYP2J2 cooperative recognition of small molecules which should be taken into account, especially with respect to evaluation of cardiotoxicity of chemotherapeutics drugs. These studies showed that multiple molecules can concurrently bind to CYP2J2, but the selected conformation of these molecules occupying the active site is highly dependent on the structures of each molecule studied. Overall the structure-function studies of CYP2J2 have led to a better understanding on how ligands and proteins, specifically CYPs, interact, which can give valuable insight into their functions. More specifically, these experimental findings may lead to

better drug design or therapeutics that exploit these features involved in modes of molecular recognition and conformational sampling by the ligand and protein when in complex.

Considering the variety of health benefits that the epoxides provide to inflammation and the cardio/cerebrovascular systems ⁶⁹³, there is a lot of interest in exploiting this pathway for therapies. Importantly, more research will need to be done regarding the potential effects of drugs. A more provocative design would be to design a drug that can bind and potentiate epoxygenase activity. Positive heterotopic interactions have been observed for other CYPs, mostly the drug-metabolizing CYP3A4. Therefore, eventually developing drugs that can increase endogenous CYP activity may be an effective therapeutic route to increase the levels of PUFA-epoxides, especially during disease conditions such as stroke.

Overall, CYP2J2 is one of the most highly expressed CYPs of the heart and is also highly expressed in the brain compared to other epoxygenases. Therefore, CYP2J2 must be a source of producing in these tissues PUFA epoxides with vasodilatory, anti-inflammatory and other beneficial pharmacologies. An important feature is that CYP2J2 produces all four regioisomers of EETs and can metabolize a variety of different lipids due to its large active site varying from fatty acids to large endocannabinoids, such as 2-AG. Epoxygenases CYP2C8 and CYP2C9 are more limited by their substrates and regioselectivities. Additionally, CYP2J2 has specific enantioselectivity and produces a 74:26 ratio of (*R,S*)- : (*S,R*)- 14,15-EET and an almost racemic mixture of the other regioisomers. Selective product distributions leading to different epoxide regioisomers and enantiomers can influence bioactive lipid binding to downstream receptors, where the result of metabolism often ends with biotransformed molecules exhibiting altered pharmacologies from their parent structures. Although the identity of the EET receptor remains unknown ⁶⁹⁴, it is known to show enantioselective preferences not only in binding but also in

potential downstream signaling. Therefore, the unique ability of CYP2J2 of producing all the different regioisomers and 19-hydroxy product may be important for cardiovascular and anti-inflammatory function. Alternatively, selective regioisomer formation by select CYP epoxygenases in select tissues could be critical in elucidating endogenous mechanisms for responding to the development of different disease pathologies. Biochemically, CYP2J2 is also a very slow enzyme with respect to lipid metabolism as compared to drug metabolism which is physiologically relevant as high PUFA-epoxide production can lead to adverse toxicity. Upon considering the rates and reactive preferences of different CYP epoxygenases, it is imperative to ponder about the extent of crosstalk between all the epoxygenases, their potential lipid substrates, and the resulting downstream interactions with receptors.

There is an overall consensus in the scientific community that acute inflammation needs to be followed by a timely resolution otherwise it leads to chronic inflammation. This inflammation resolution plays a fundamental part in the body's response to inflammation caused by tissue injury, infection and aging. Hence, it can be postulated that there is complex cross-talk and competition between the CYP2J2 and other epoxygenases with cyclooxygenase and lipoxygenase for PUFA substrates during an inflammatory process which needs to be understood. Therefore, as can be seen from this review, there are many unanswered questions with regards to CYP epoxygenases: (a) interactions with PUFA and drugs, (b) tissue specific roles, (c) interactions with membranes, (d) specific mechanism-based inhibitors and inducers and (e) interactions amongst other epoxygenases and with different lipid-metabolizing enzymes especially during disease conditions. The enumeration of the research on CYP2J2 presented here describes the current knowledge on CYP epoxygenases and identifies the future needs for the molecular characterization of CYP2J2 to enable a new axis of therapeutic design.

Figure 17. Overview of fatty acid and endocannabinoid metabolism by cytochrome P450 classes

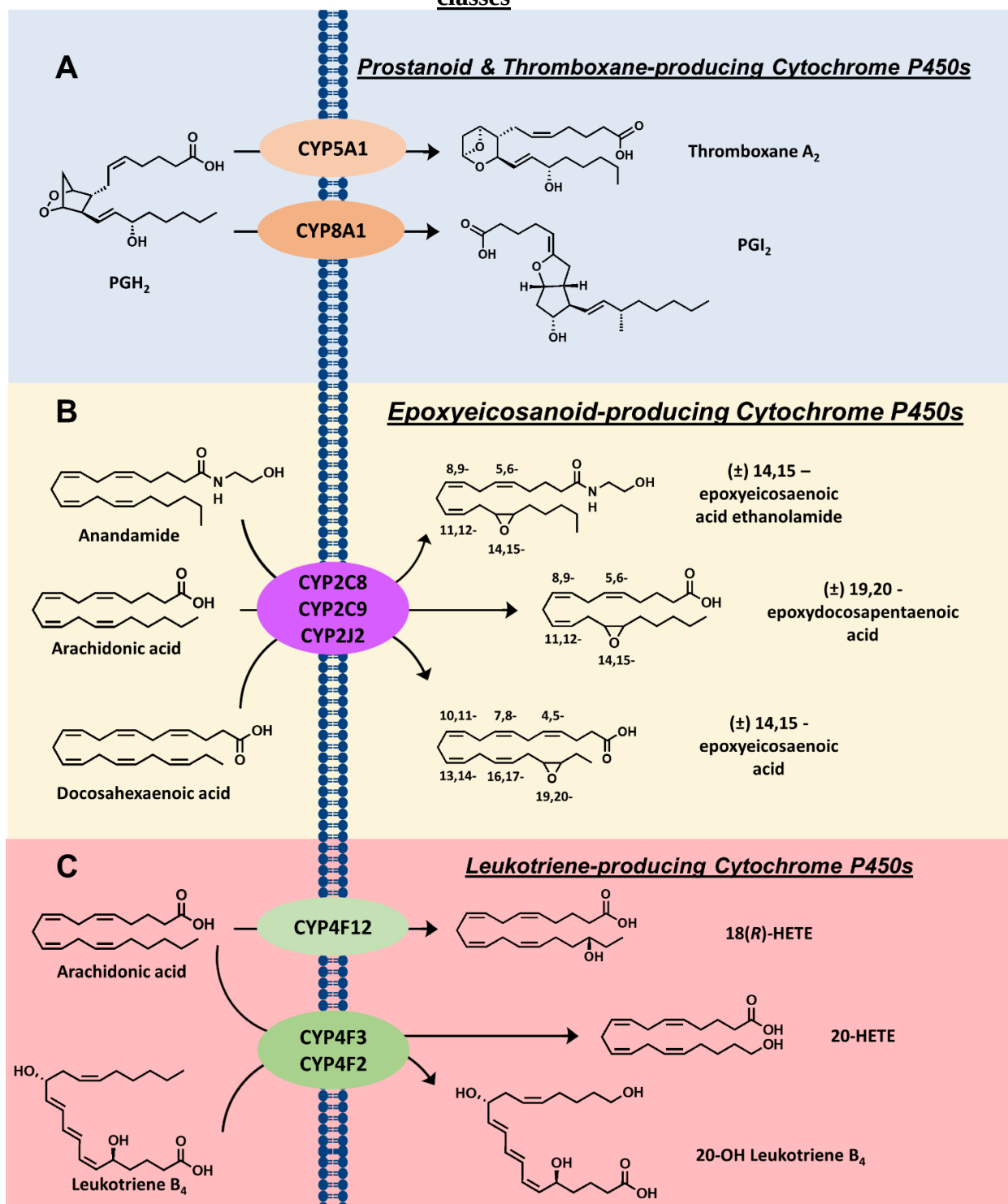


Figure 17. Overview of fatty acid and endocannabinoid metabolism by cytochrome P450 classes. A lipidomic view of prostaglandin H2(PGH2), anandamide (AEA), arachidonic acid (AA), docosahexaenoic acid (DHA) and leukotriene B4 metabolism by functionally-distinct, membrane-bound cytochrome P450s(CYPs). The CYPs are organized into classes based on their primary metabolism pathway and are shown as colored ovals. The substrates of each reaction are shown above each enzyme, while the products are shown below the enzyme. (A) The prostanoid and thromboxane producing cytochrome P450s include CYP5A1 and CYP8A1. While CYP5A1 catalyzes the conversion of PGH2 to Thromboxane A2-a potent vasoconstrictor, CYP8A1 produces PGI2-the opposing vasodilatory metabolite. (B) CYP2C8, CYP2C9, and CYP2J2 display epoxigenase activity and can metabolize AA, DHA, and the endocannabinoid AEA to bioactive metabolites in a regioselective and enantioselective manner. (C) The CYP4F class consisting of CYP4F12, CYP4F2 and CYP4F3 display lipoxygenase activities. In particular, they catalyze hydroxylation reactions to produce the hydroxyeicosatetraenoic acids (HETEs) and hydroxylated leukotriene metabolites, such as leukotriene B4. Although hydroxylation is the primary metabolic pathway for the CYP4F class, they have been shown to metabolize polyunsaturated fatty acids such as AA.

Figure 18. Xenobiotic ligands of CYP2J2

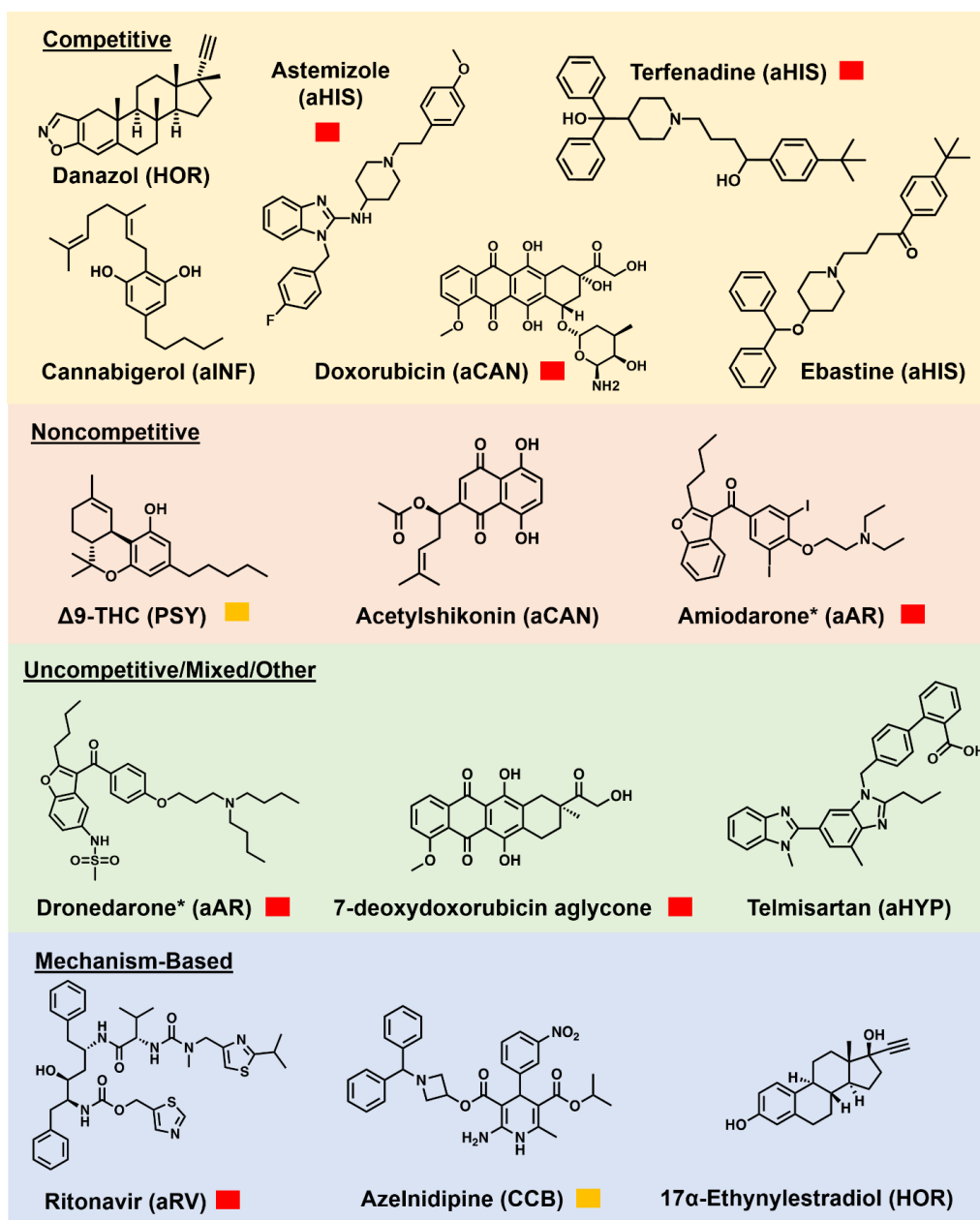


Figure 18. Xenobiotic ligands of CYP2J2. Structures of key ligands mentioned in the text are given. Each is categorized based on their mode of CYP2J2 inhibition (mostly of astemizole metabolism). Xenobiotics that are known to have cardiovascular effects are indicated with an orange square and those that are cardiotoxic are indicated with a red square. Abbreviations for the type of drug are given in parentheses beside each name and are as follows: aAR, antiarrhythmic; 188an, anticancer; aHIS, antihistamine; aHYP, antihypertensive; aINF, anti-inflammatory; aRV, antiretroviral; CCB, calcium channel blocker; HOR, hormonal; PSY, psychoactive. Compounds indicated with asterisks have also been shown to be mechanism-based inhibitors.

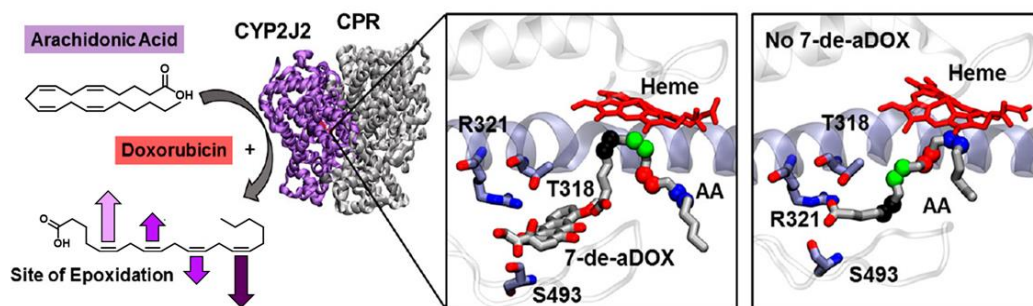


Figure 19. Modulation of arachidonic acid by CPR-mediated generation of 7-deoxydoxorubicin aglycone. Occupation of the PUFA-binding pocket by 7-de-aDOX results in altered regioselectivity in metabolism of arachidonic acid by CYP2J2 (left box). Arachidonic acid metabolism occurring in the absence of 7-de-aDOX assumes native regioselectivity preferences (right box). Image reproduced after slight modifications with permission from the original publisher ²¹⁸.

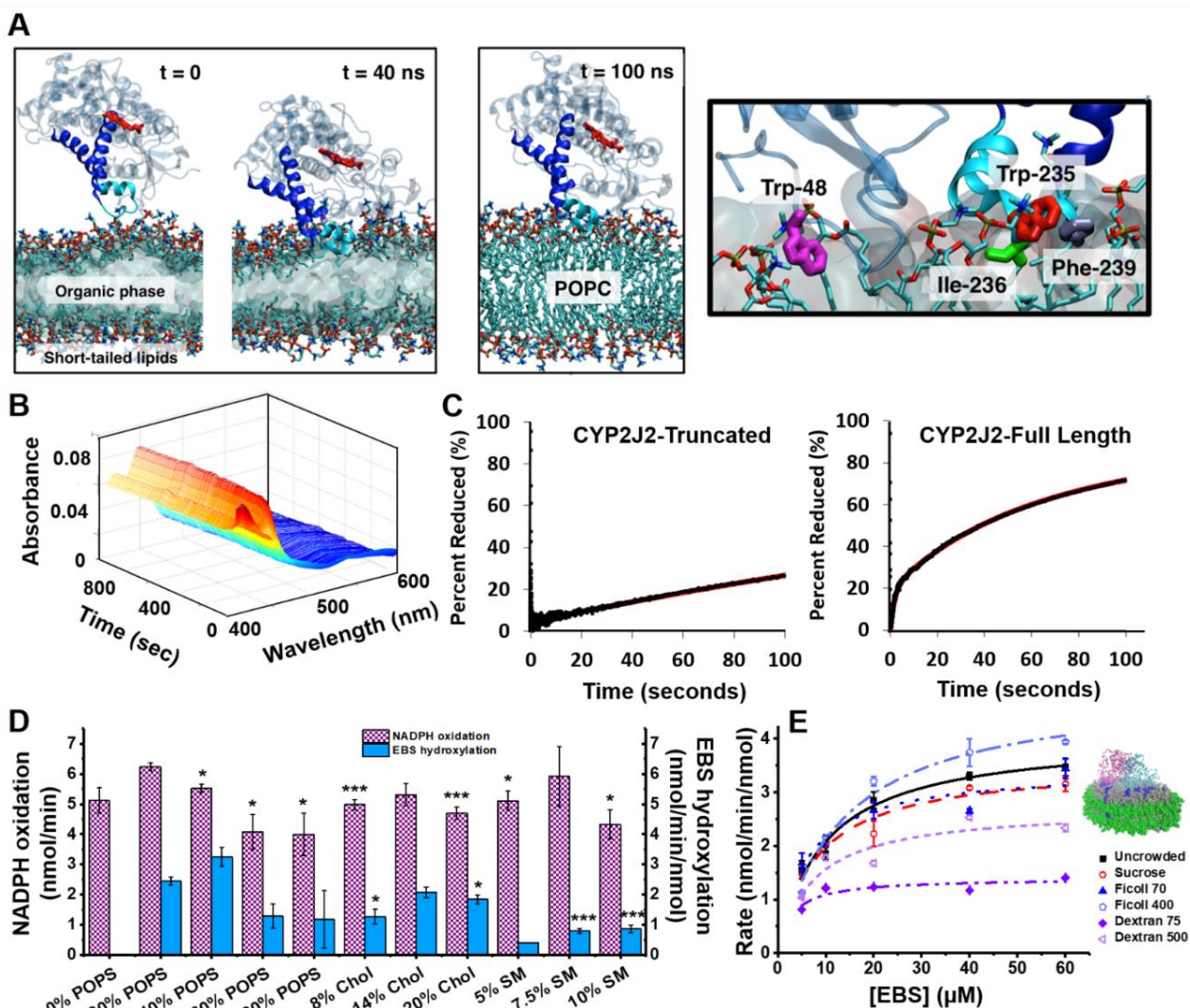


Figure 20. Effect of membrane association on CYP2J2 function. (A) Membrane binding of CYP2J2 captured with the HMMM membrane in 40 ns. The membrane-bound model of CYP2J2 was extended to a POPC membrane and simulated for another 100 ns. Residues Trp-48, Trp-235, Ile-236 and Phe-239, identified to interact with the membrane upon binding, are shown in stick representation⁶²⁴. (B) Three-dimensional plot of representative absorbance spectra from 0 to 1000 s following mixing of NADPH with a CYP2J2–CPR pre-incubated complex⁶²⁵. (C) Association-dependent electron transfer kinetics of the formation of Fe(II)–CO peak at 449 nm over time (100 s)⁶²⁵. (D) Measurement of rate of NADPH oxidation and rate of metabolite formation from ebastine metabolism by CYP2J2–CPR–nanodiscs with varied lipid compositions⁶²⁶. (E) Michaelis-Menten curves for 80%/20% POPS nanodiscs crowded with 100 mg/ml of macromolecular crowding agent⁶²⁶.

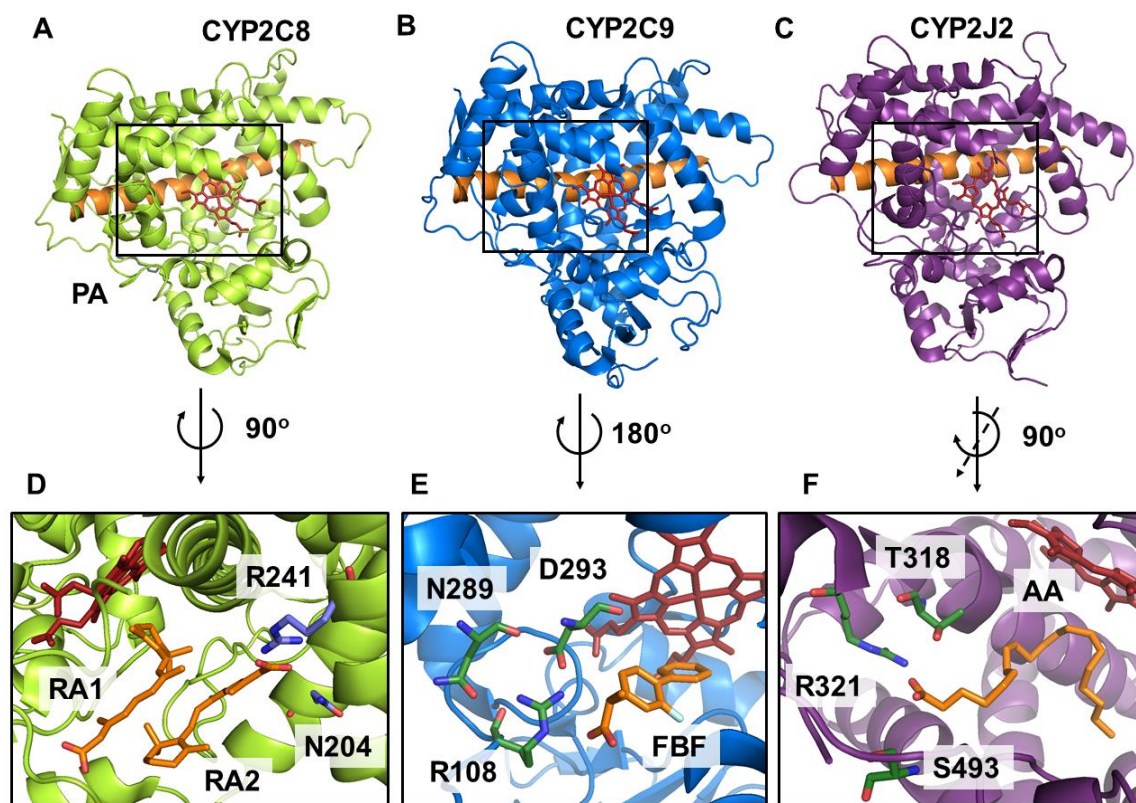


Figure 21. Structures of the most characterized CYP2 human epoxygenases. (A-C) Structures of (A) CYP2C8 (crystal, PDB: 1PQ2), (B) CYP2C9 (crystal, PDB: 1OG2), and CYP2J2 (homology model, ⁶⁷⁷). The hemes are shown as red sticks. The I-Helices are highlighted in orange. The palmitic acid (PA) in the CYP2C8 structure is shown as black sticks. (D-E) Key active site residues for substrate binding as mentioned in the text. Some residues have been omitted for clarity. (D) Residues (blue) involved in 9-*cis*-retinoic acid (RA, orange) binding to CYP2C8 (PDB: 2NNH). Two molecules of RA are bound in the active site and support homotropic cooperativity. (E) Residues (green) involved in flurbiprofen (FBF, orange) binding to CYP2C9 (PDB: 1R9O). These residues are believed to contribute to the selectivity of CYP2C9 for negatively charged substrates. Notably, R108 swings inward by almost 180° to interact with FBF, as compared to the apo structure (PDB: 1OG2). (F) Residues (green) comprising the “PUFA binding pocket” in CYP2J2 as revealed by MD simulations ²⁰⁴. Arachidonic acid (AA, orange) is bound. For each crystal structure, one monomer is shown for clarity.

Table 5. Enantioselectivity of arachidonic acid metabolism by CYP epoxygenases

CYP	Ω -6 epoxide enantioselectivity		ω -9 epoxide enantioselectivity		ω -12 epoxide enantioselectivity		Ref
	(R,S)	(S,R)	(R,S)	(S,R)	(R,S)	(S,R)	
							200
2J2	76	24	49	51	47	53	469
2J3	43	57	62	38	60	40	705
2B1	64	36	14	86	14	86	697
2B2	65	35	16	84	10	90	697
2B12	ND	ND	30	70	61	39	699
2C8	82	18	82	18	ND	ND	441, 469
	86.2	13.8	81.1	18.9	ND	ND	701
2C9	63	37	31	69	ND	ND	469
	62.5	37.5	30.5	69.4	ND	ND	701
2C10	63	37	31	69	34	66	441, 469
2C11	49	51	54	46	42	58	697
	38	62	79	21	54	46	706
2C23	25	75	89	11	94	6	706
2C24	67	33	75	25	39	61	706
2C44	ND	ND	94	6	95	5	707
2C50	63.8	36.2	58	42	57.1	42.9	708
2C54	48.7	51.3	46.5	53.5	46.6	53.4	708
2C55	40.4	59.5	57	43	59	41	708
2D18	71	29	76	24	48	52	709
2N1	49	51	92	8	91	9	710
2N2	32	68	70	30	90	10	710
1A1	85	15	13	87	13	87	697
1A2	63	37	95	5	7	93	697

Table 5. Enantioselectivity of arachidonic acid metabolism by CYP epoxygenases**B.8 ACKNOWLEDGEMENTS**

We would like to thank Dr. Ilia Denisov for the helpful discussions. This research was supported by the American Heart Association Scientist Development Grant 15SDG25760064 (A.D.) and in part by the National Institute of Health Grant R01 GM1155884 (A.D.).

This work was completed in collaboration with Aditi Das, Austin T. Weigle, William R. Arnold, Lauren N. Carnevale, and Hannah C. Huff at the University of Illinois Urbana-Champaign.

B.9 SUPPLEMENTARY INFORMATION

<u>GTE_x</u>		
Tissue	Expression (RPKM)	Sample Size (n)
Heart Muscle	23.9	412
Liver	19.9	119
Small Intestine	18.5	88
Caudate	7.7	117
Hypothalamus	6.5	96
Hippocampus	5.9	94
Cerebral Cortex	4.7	222
Cerebellum	4.2	230
Pituitary Gland	0.6	103

Supplementary Table 2. Human CYP2J2 tissue-specific expression in GTE_x human genome sequencing database. Expression in tissues are ordered in a descending fashion of expression (RPKM).

<u>FANTOM5</u>		
Tissue	Expression (Tags per million)	Sample Size (n)
Small Intestine	275.5	1
Liver	223.6	1
Caudate	73.1	1
Heart Muscle	48.4	2
Cerebellum	48.1	2
Hippocampus	46	2
Brain	31.8	1
Pituitary Gland	9.9	1

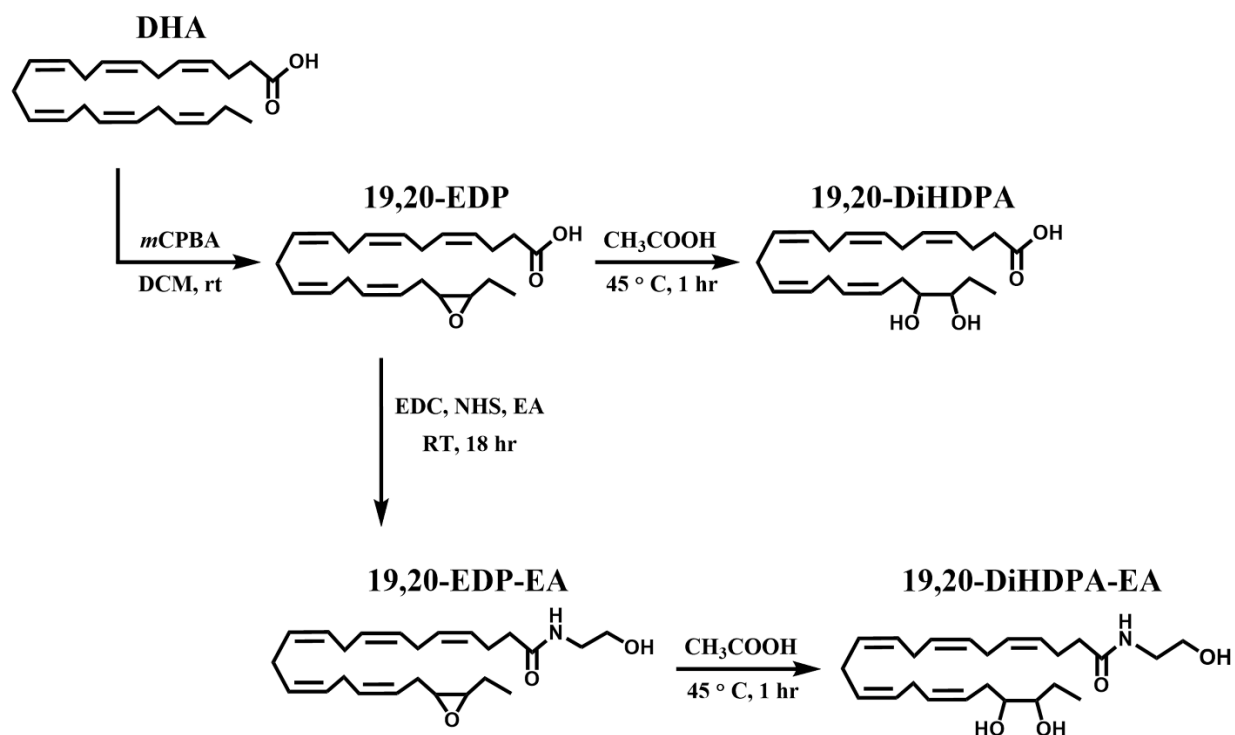
Supplementary Table 3. Human CYP2J2 tissue-specific expression in FANTOM5 Database. Expression in tissues are ordered in a descending fashion of expression (tags per million).

Supplementary Table 4. Expression of Mouse CYP2J6 in single cell transcriptomics.

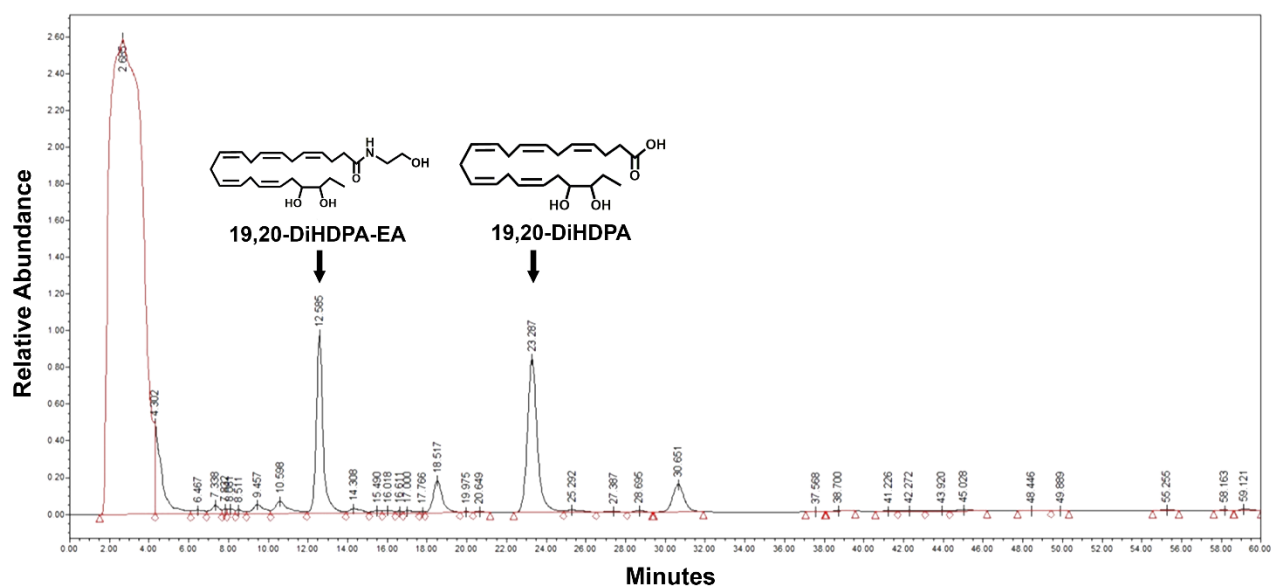
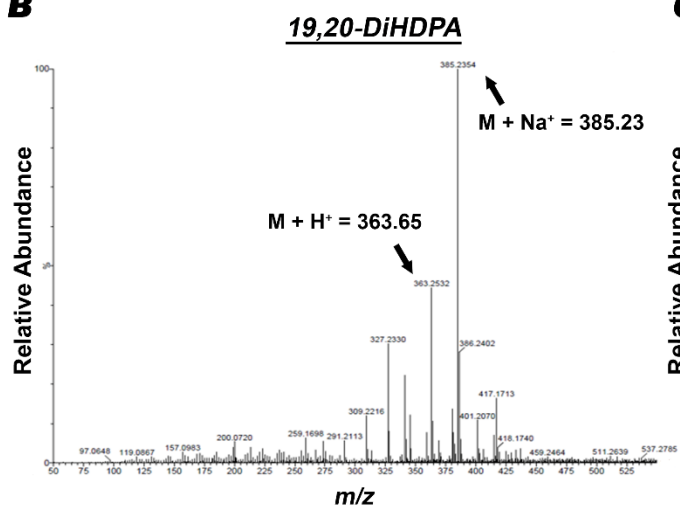
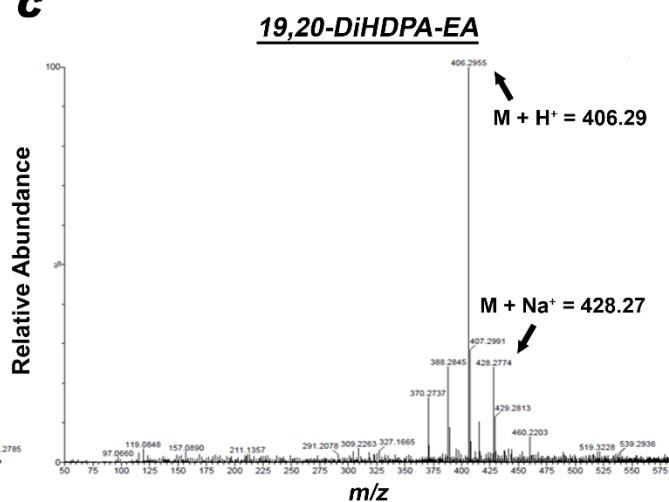
Organ	Tissue	Mean (ln(1+CPM))	# of Cells
Brain	Oligodendrocyte Progenitor Cell	4.31	203
	Astrocyte	1.78	432
	Oligodendrocyte	1.64	1574
	Bergmann glial cell	1.13	40
	Endothelial cell	0.43	715
	Neuron	0.21	281
	Brain pericyte	0.08	156
	Microglial cell	0.1	4394
Heart	Heart fibroblast	1.04	2119
	Endocardial cell	0.76	165
	Cardiac muscle cell	0.58	133
Liver	Hepatocyte	2.12	391
Skin	Epidermal cell	2.9	276
	Stem cell of epidermis	1.6	53
	Basal cell of epidermis	1.59	562
	Keratinocyte stem cell	0.5	1404
Intestines	Large intestine goblet cell	2.3	833
	Epithelial cell of large intestine	1.95	2019
	Enterocyte of epithelium of large intestine	1.68	964
	Brush cell of epithelium proper of large intestine	0.23	63

Supplementary Table 4. Expression of Mouse CYP2J6 in single cell transcriptomics.

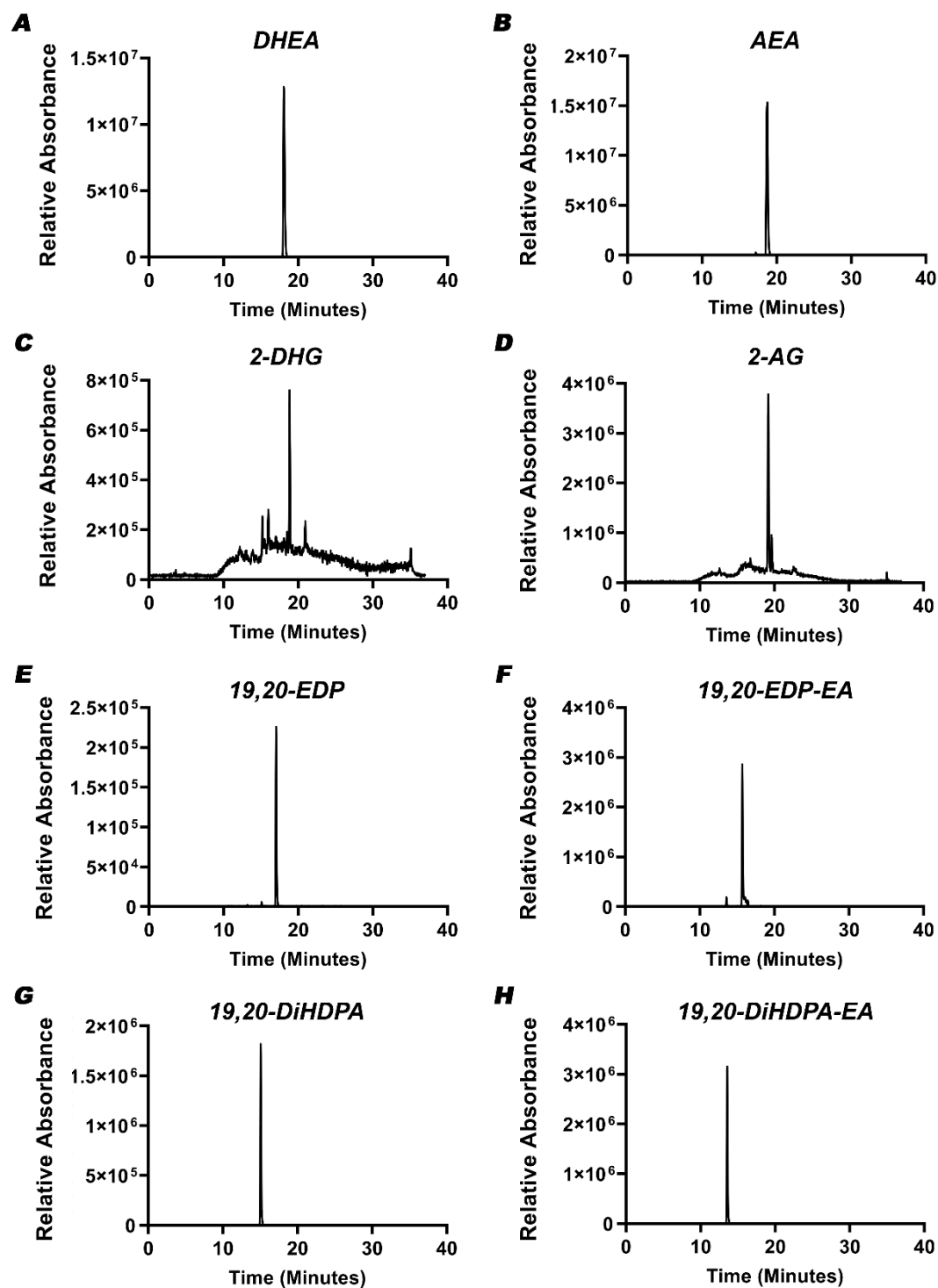
**APPENDIX C: SUPPLEMENTARY INFORMATION - ROLE OF OMEGA-3
ENDOCANNABINOIDS IN THE MODULATION OF T-CELL ACTIVITY IN A
MULTIPLE SCLEROSIS EAE MODEL**



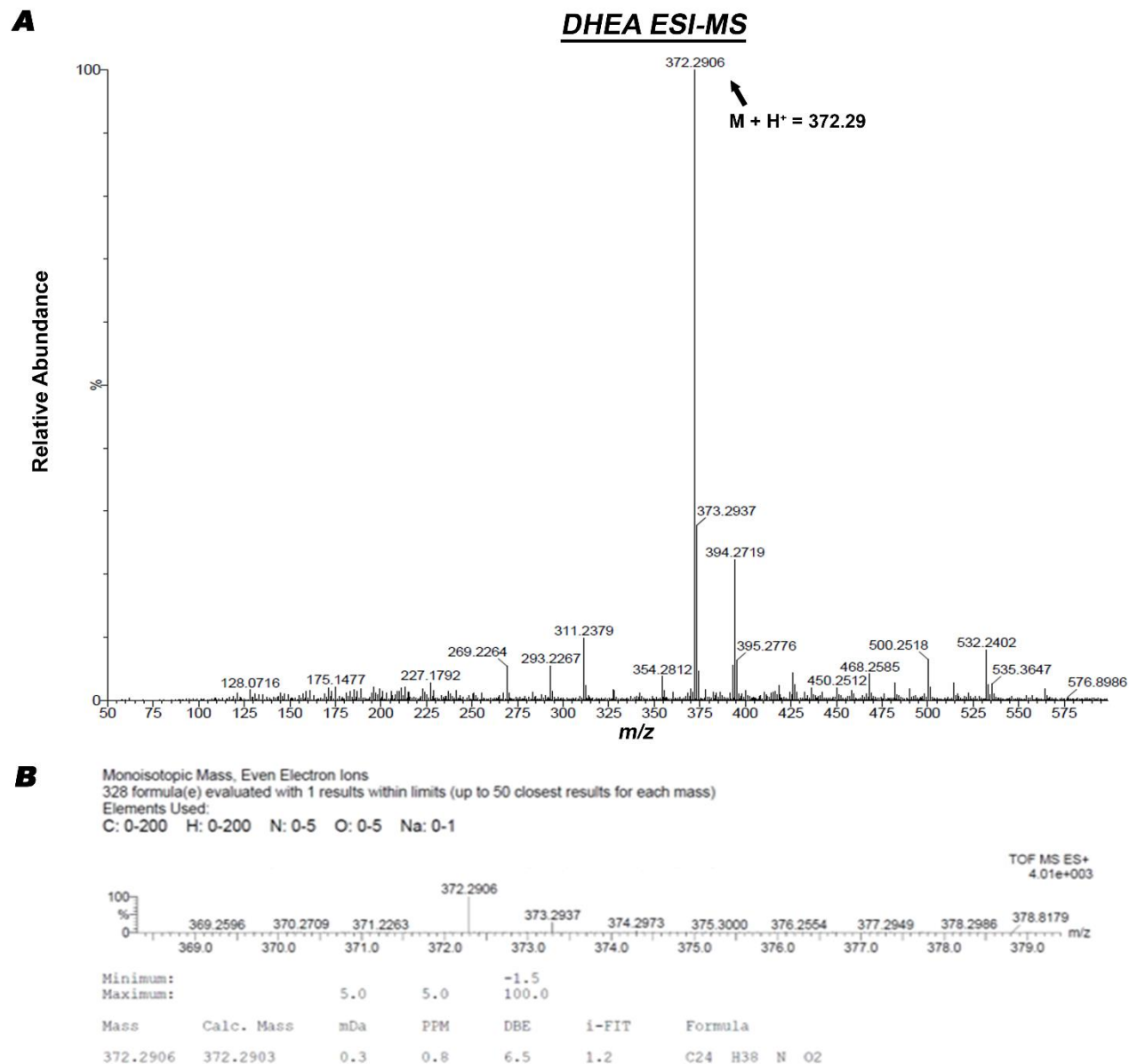
Supplementary Figure 1. Synthesis of Dihydroxy Standards. DHA was synthesized and purified by HPLC to 19,20-EDP, or 19,20-EDP-EA. Pure 19,20-EDP or 19,20-EDP-EA was then resuspended in acetonitrile, and 1:1 Acetic Acid:H₂O added for 1 hr at 45 ° C.

A**B****C**

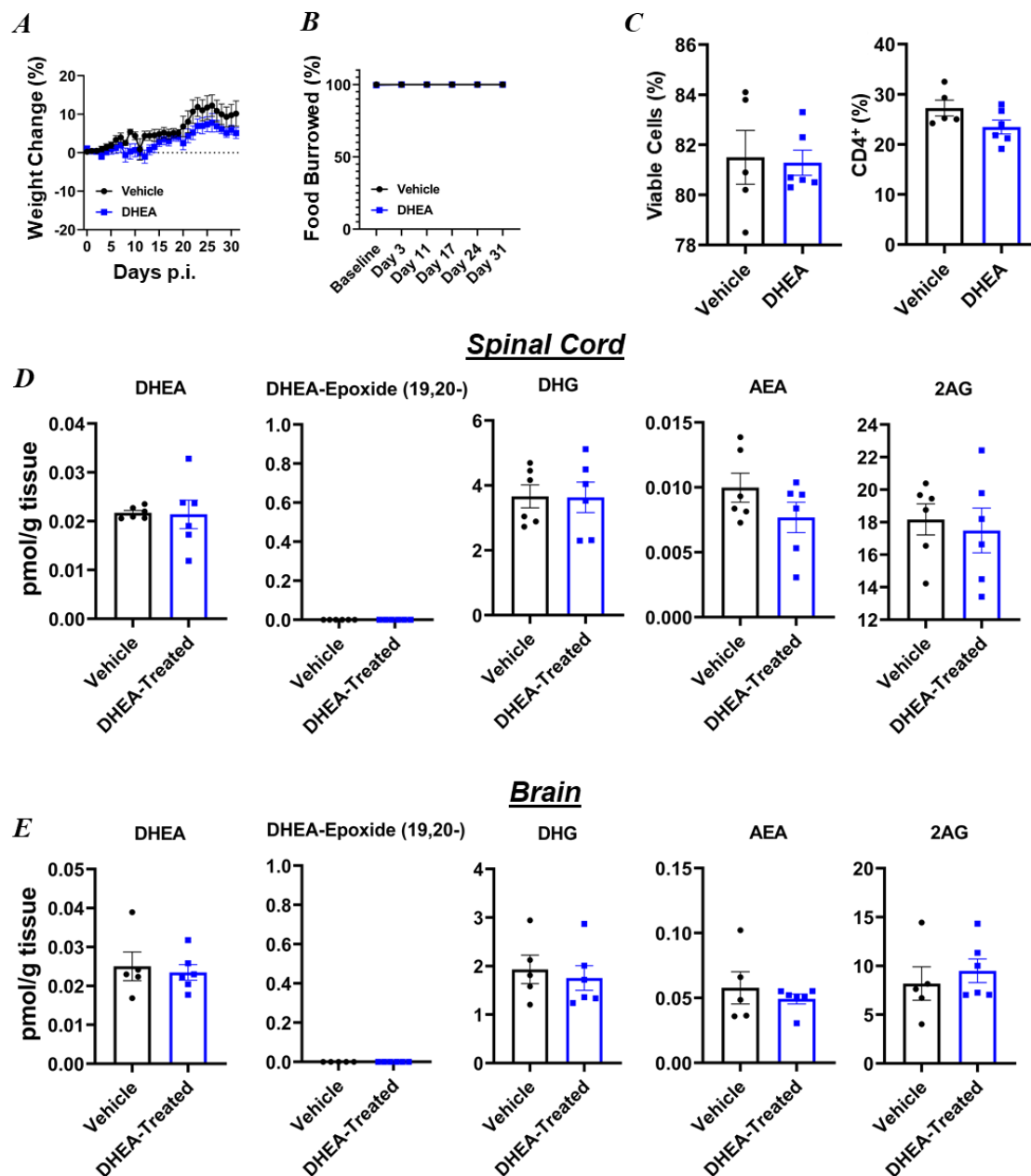
Supplementary Figure 2. Purification of Dihydroxy Standards by HPLC. (A) Purification and isolation of dihydroxy derivatives by reverse-phased high performance liquid chromatography. **(B-C)** Confirmation of synthesized dihydroxy derivatives by ESI Mass Spectrometry in positive ion mode for 19,20-DiHDPA and 19,20-DiHDPA-EA



Supplementary Figure 3. HPLC Chromatograms for LC-MS/MS. Liquid Chromatography separation for (A) DHEA, (B) AEA, (C) 2-DHG, (D) 2-AG, (E) 19,20-EDP, (F) 19,20-EDP-EA, (G) 19,20-DiHDPA, and (H) 19,20-DiHDPA-EA.

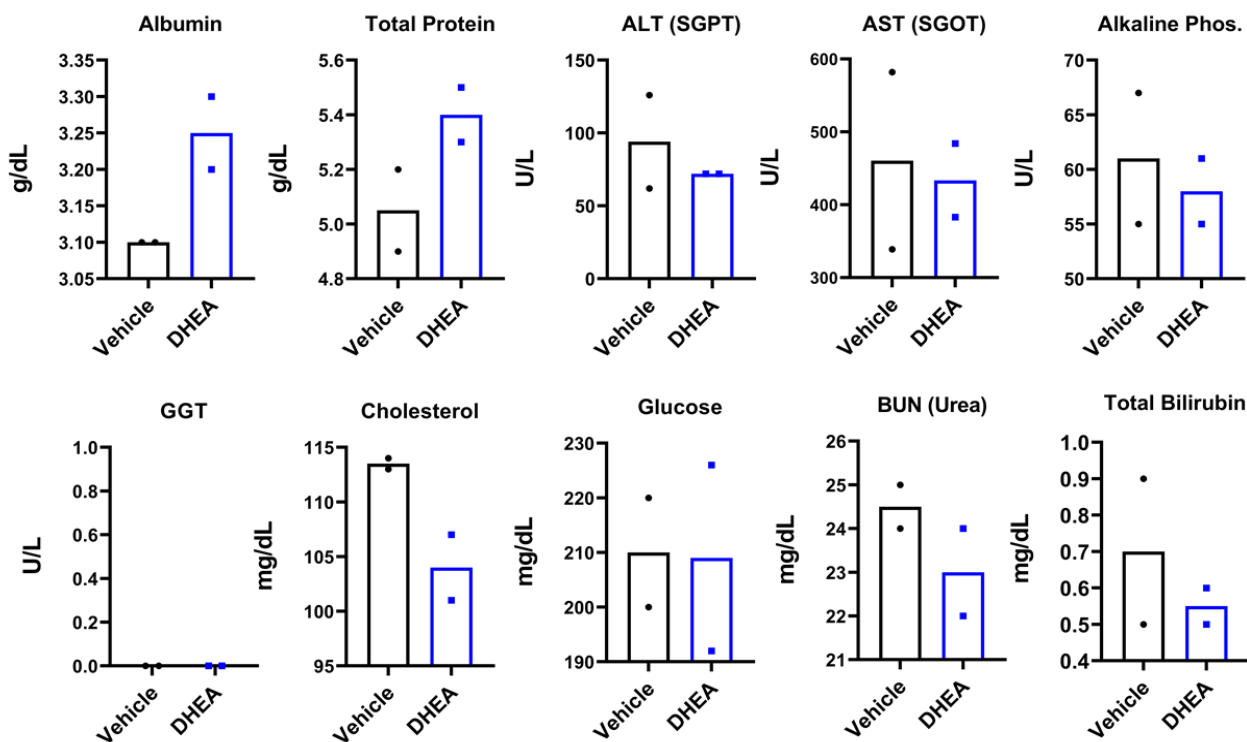


Supplementary Figure 4. Confirmation of DHEA for RR-EAE and Primary Culture Studies. (A) Confirmation of DHEA by ESI mass spectrometry for administration to RR-EAE SJL/JCrHsd mice and primary cultures. (B) Elemental composition report of DHEA.

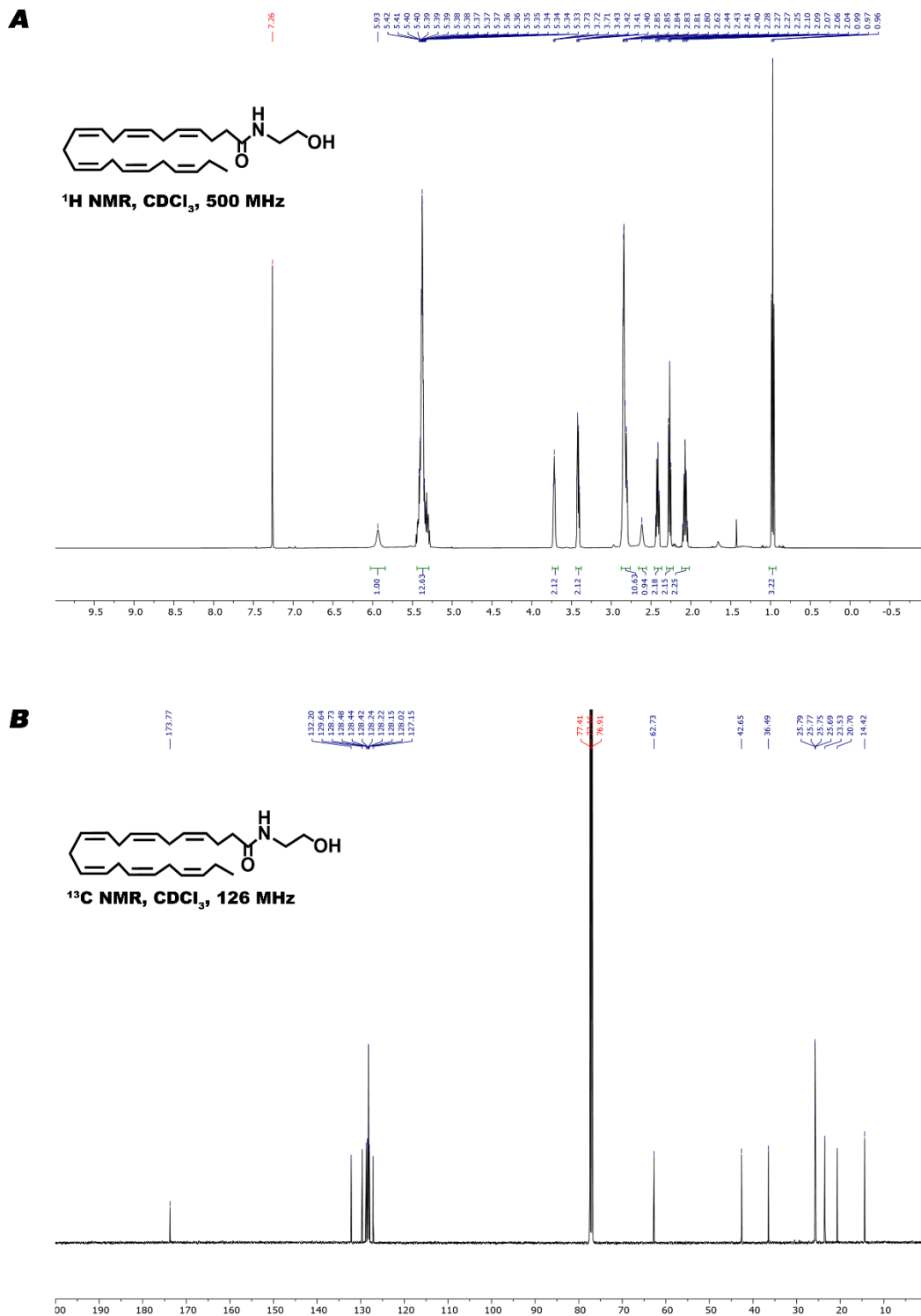


Supplementary Figure 5. Administration of DHEA to non-disease SJL/JCrHsd Mice. (A) Weight change and (B) Percent food burrowed for SJL/JCrHsd mice treated with 100 mg / kg DHEA. (C) Viable cells and CD4⁺ splenic cells are not altered by DHEA treatment. (D) Spinal cord and (E) Brain levels of omega-3 or omega-6 derived endocannabinoids do not change following DHEA treatment.

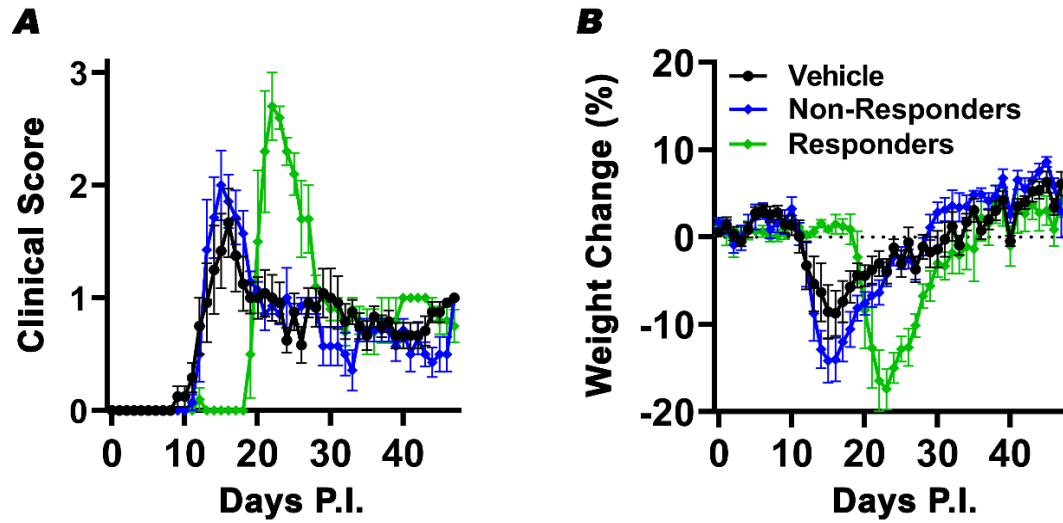
Serum Metabolite Analysis



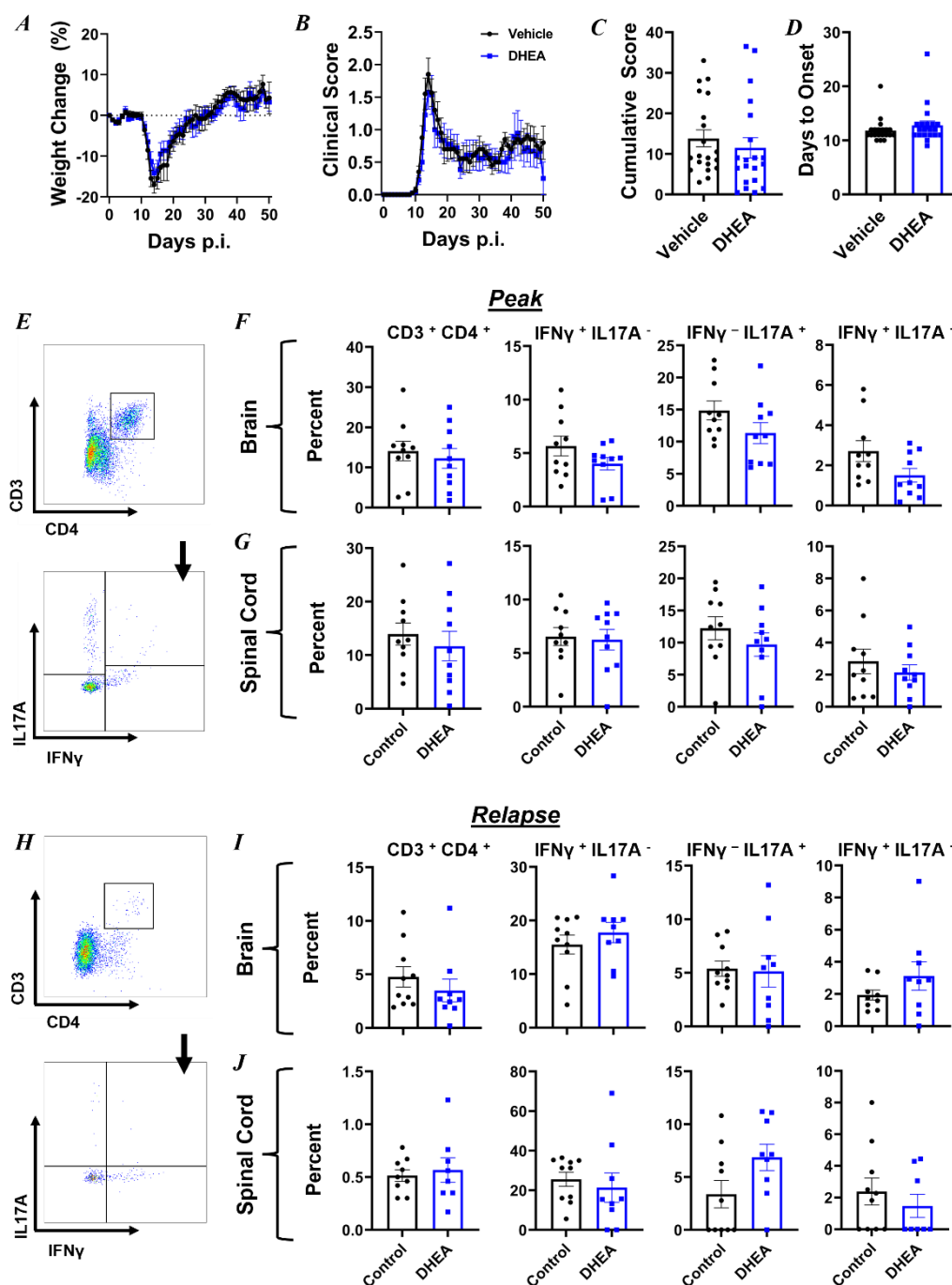
Supplementary Figure 6. Serum Metabolite Analysis of DHEA Treatment to Non-Disease Mice. Signs of liver toxicity by serum metabolite analysis Mice from each group were pooled (3 mice pooled for n=2) for serum metabolite analysis of markers of liver toxicity.



Supplementary Figure 7. ¹H and ¹³C NMR Spectra. Characterization of DHEA synthesis by (A) ¹H NMR and (B) ¹³C NMR.



Supplementary Figure 8. Non-Responder and Responder RR-EAE Mice treated with DHEA. (A) Clinical score and (B) Weight change (%) of mice administered with vehicle (saline containing 0.1% Tween 80, 10% PEG 400), or DHEA (100 mg / kg) beginning on day of immunization. Groups are separated by Non-Responders (Blue) or Responders (Green) to treatment.



Supplementary Figure 9. Infiltrating CD3⁺CD4⁺ Cells into the CNS after DHEA (5 mg/kg) Treatment. (A) Weight change (%) and (B) Clinical score of mice (N=20 per group) administered with vehicle (saline containing 0.1% Tween 80, 10% PEG 400), or DHEA (5 mg / kg) beginning on day of immunization. (C) Cumulative score and (D) Days to disease onset indicate no difference between treatment groups. **I** Gating strategy for infiltrating CD3⁺CD4⁺ T-cells into the (F) Brain and (G) Spinal cord at peak disease (day 15-16). (H) Gating strategy for infiltrating CD3⁺CD4⁺ T-cells into the (I) Brain and (J) Spinal cord at relapse (days 50-51). Unpaired T-Test analysis was performed.

	Vehicle	DHEA
Day of Onset	11.8 ± 0.48	12.8 ± 0.80
Max Score (Median)	2	2
Cumulative Score (Average)	13.8 ± 0.5	11.5 ± 0.8

Supplementary Table 5. DHEA (5 mg / kg) treatment to EAE mice. Mice administered with vehicle (saline containing 0.1% Tween 80, 10% PEG 400), or DHEA (5 mg / kg) beginning on day of immunization. Student's T-test analysis was performed.

	Vehicle	DHEA	DHEA (Post-Peak)
Day of Onset	12.5 ± 3.6	15.7 ± 4.5*	13.3 ± 3.8
Max Score (Median)	2.5	2.5	2.25
Cumulative Score (Average)	33.1 ± 3.1	32.5 ± 2.2	27.5 ± 4.1
Days to Relapse	11.8 ± 1.8	16.4 ± 1.9	18.5 ± 1.7*

Supplementary Table 6. DHEA (100 mg / kg) treatment to EAE mice. Mice administered with vehicle (saline containing 0.1% Tween 80, 10% PEG 400), or DHEA (100 mg / kg) beginning on day of immunization or post-peak disease (day 19). One-Way ANOVA and Holm-Sidak's post-hoc analysis performed. Significance represented in comparison to Vehicle.

APPENDIX D: SUPPLEMENTARY INFORMATION – ANTHRACYCLINE

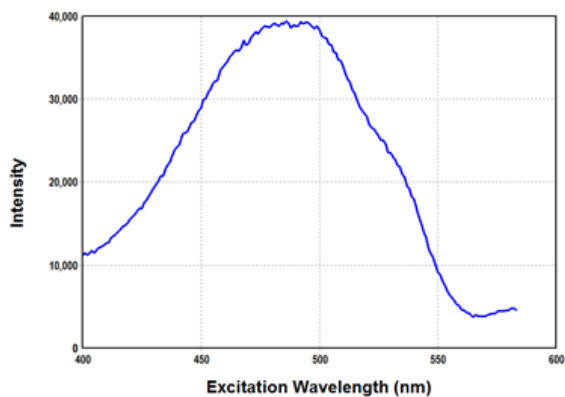
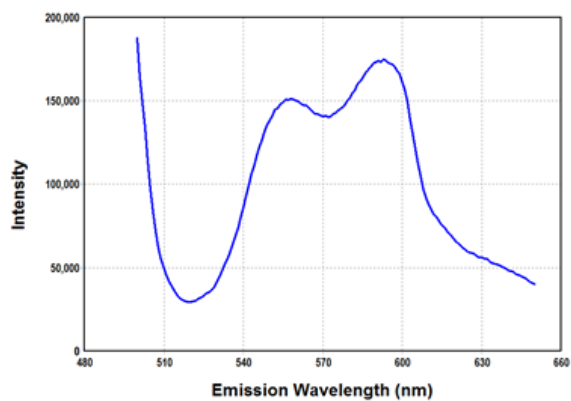
DERIVATIVES INHIBIT CARDIAC CYP2J2

	DOX	DAU	EPI	IDA	5-IDN	ZOR	VAL	ACL
Emission (nm)	450	593	593	580	628	590	593	550
Excitation (nm)	593	480	480	480	550	480	480	450

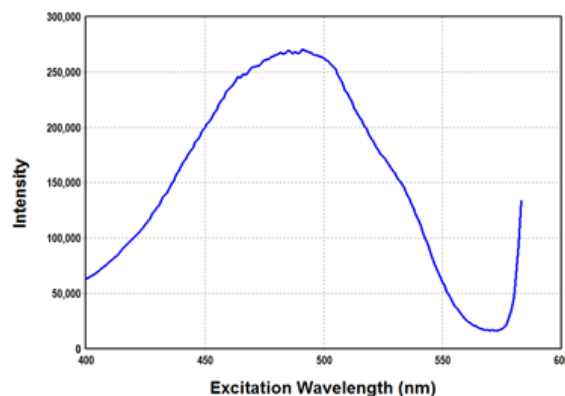
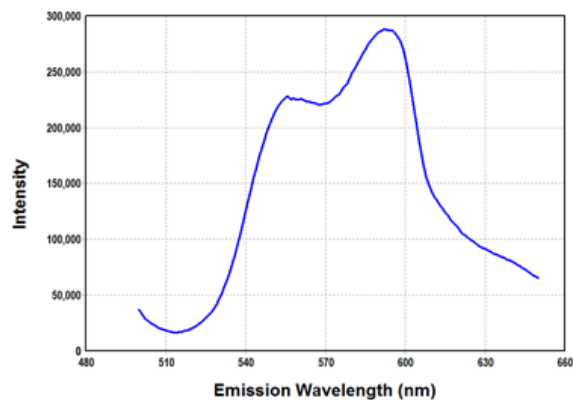
Supplementary Table 7. Summary of Emission and Excitation Parameters. Summary of emission and excitation parameters for fluorescence polarization experiments. All experimentally obtained emission and excitation measurements were taken with a 1 nm slit width filter and 594 nm cutoff filter.

Supplementary Figure 10 (cont.)

Daunorubicin

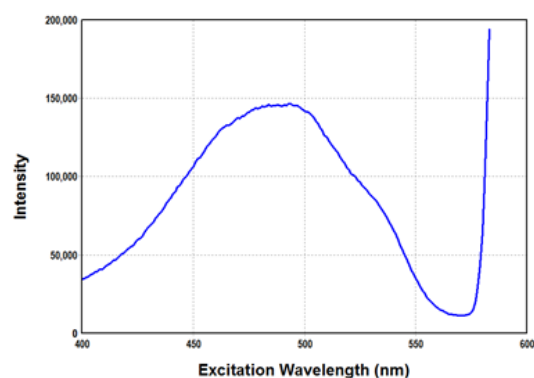
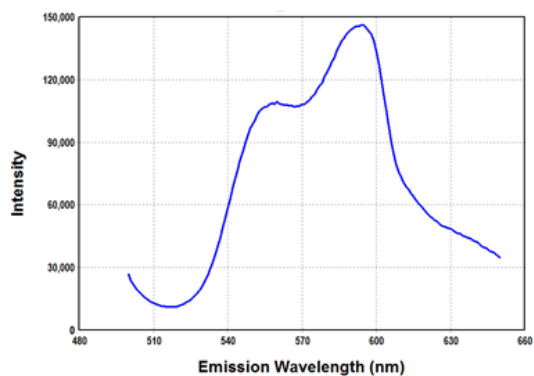


Epirubicin

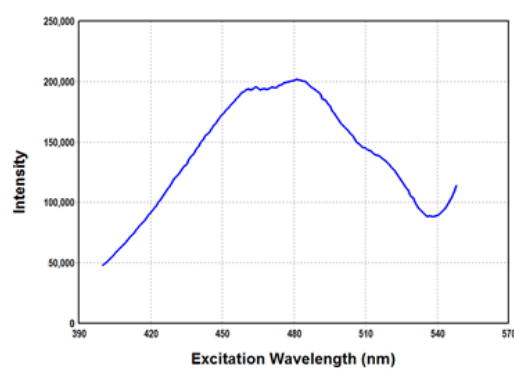
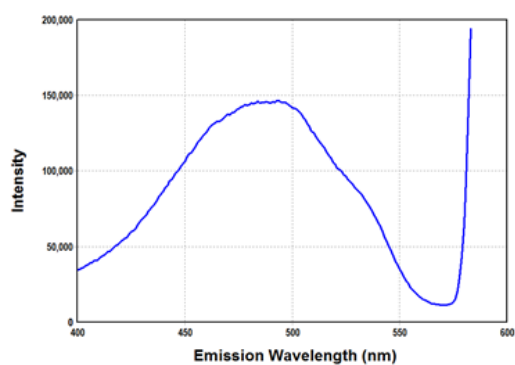


Supplementary Figure 10 (cont.)

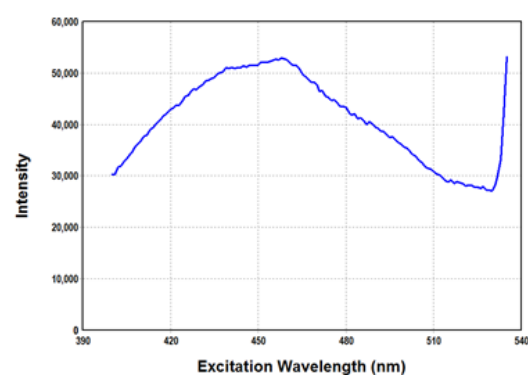
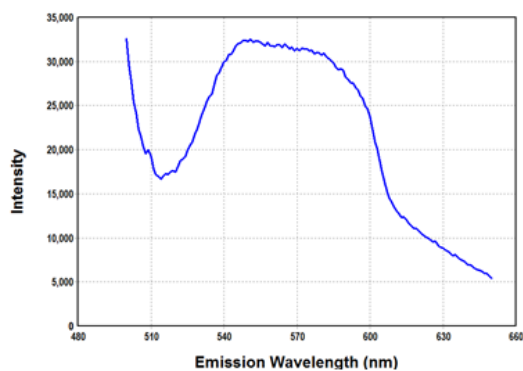
Valrubicin



Idarubicin



Aclarubicin



Supplementary Figure 10. Emission and Excitation Spectra Chromatograms. A full scan of the emission spectra was first conducted to identify the emission wavelength. With the emission wavelength set as a parameter, the excitation spectra was obtained.

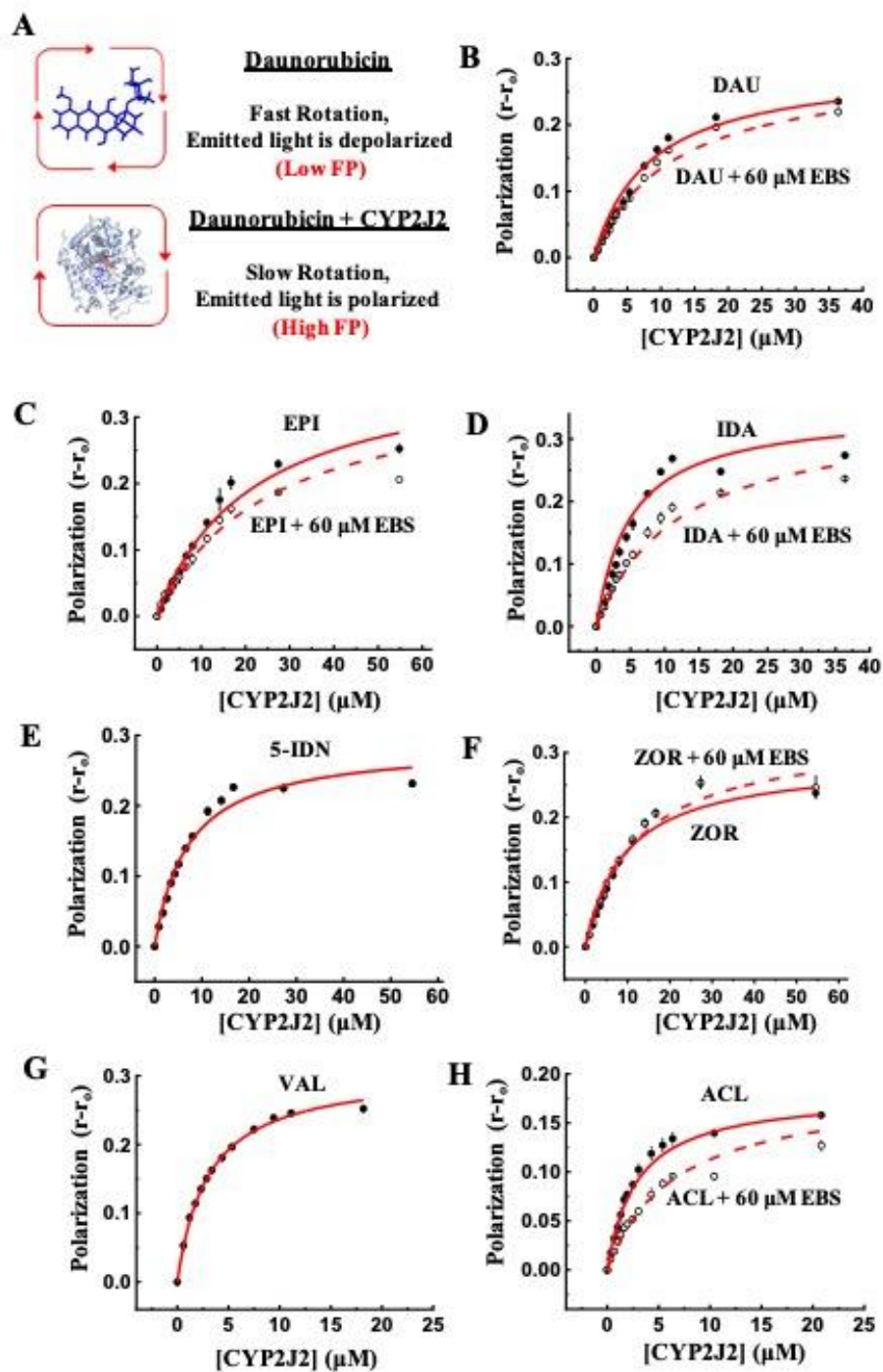
Competitive inhibition of anthracycline binding by ebastine

To obtain insight into how anthracyclines may competitively inhibit ebastine binding to CYP2J2, we conducted the fluorescence polarization experiments in the presence of 60 μM ebastine. Importantly, to accurately calculate the K_i , the IC_{50} must first be calculated. Our experimental design however is such that CYP2J2 is titrated into our experimental solvent (0.1 % cholate, 0.1M Potassium Phosphate Buffer) containing anthracycline (1 μM) and ebastine (60 μM). To test whether anthracyclines inhibit ebastine binding to CYP2J2, the anthracycline must be titrated into the solution. Since anthracyclines have fluorescent properties, titrating anthracyclines or simultaneously titrating anthracyclines and CYP2J2 is not feasible. Thus, the following data is not a direct measurement of K_i and should only be considered as insight into how the different anthracyclines may inhibit CYP2J2. The most reliable data can be seen in the metabolism inhibition studies (Figure 4).

However, to gain insight into how anthracyclines may competitively inhibit ebastine binding to CYP2J2, we conducted the experiment described above, and fit the values to the Competitive One-Site Binding Equation:

$$\textbf{Equation S1 (Competitive One-Site Binding Equation): } r = r_o + \frac{r_1*[S]}{K\left(1+\frac{I}{K_i}\right)+[S]}$$

where r = fluorescence polarization value, S = substrate concentration (CYP2J2), r_o = intrinsic polarization value, r_1 = maximum polarization value, I = inhibitor concentration (60 μM ebastine), and $K = K_d$ obtained from one-site binding. The calculated K_i using this methodology can be seen in in Supplementary Table 2



Supplementary Figure 11. Competitive Inhibition of Anthracycline Binding by Ebastine. Fluorescence Polarization. (A) Schematic of steady state fluorescence polarization dependent on binding of anthracycline (daunorubicin) to CYP2J2 epoxygenase. Steady-state fluorescence measurements of anthracyclines (1 μ M) to increasing concentrations of CYP2J2 (0-60 μ M) (B) Daunorubicin, (C) Epirubicin, (D) Idarubicin, (E) 5-Iminodaunorubicin, (F) Zorubicin, (G) Valrubicin and (H) Aclarubicin. Open circles and dotted line represent competitive binding

inhibition by Ebastine (60 μM). Values are expressed as mean and standard error of three replicates.

Supplementary Table 8. Estimated K_i using Competitive One-Site Binding Equation

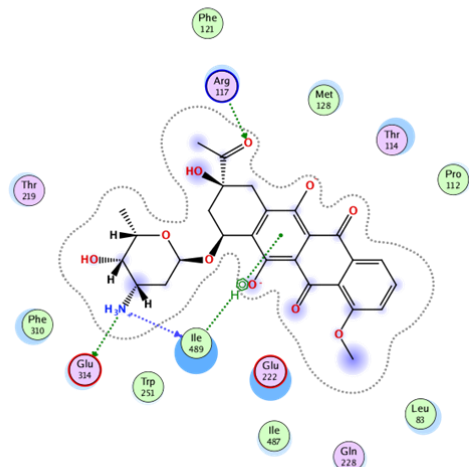
		r_o	r_I	K_D (μM)	K_i (μM)
DOX*	<i>DOX*</i>	0.083 ± 0.007	0.219 ± 0.010	5.326 ± 0.890	---
	<i>DOX + EBS*</i>	0.101 ± 0.003	---	---	13.800 ± 1.600
DAU	<i>DAU</i>	0.92 ± 0.00	0.33 ± 0.01	9.56 ± 1.01	---
	<i>DAU + EBS</i>	0.12 ± 0.00	---	---	243.07 ± 27.69
EPI	<i>EPI</i>	0.09 ± 0.01	0.36 ± 0.02	14.70 ± 1.99	---
	<i>EPI + EBS</i>	0.16 ± 0.01	---	---	100.86 ± 12.95
IDA	<i>IDA</i>	0.45 ± 0.01	0.36 ± 0.14	4.99 ± 0.66	---
	<i>IDA + EBS</i>	0.11 ± 0.00	---	---	55.11 ± 3.31
5-IDN	<i>5-IDN</i>	0.15 ± 0.01	0.29 ± 0.01	6.08 ± 0.58	---
	<i>5-IDN + EBS</i>	---	---	---	---
ZOR	<i>ZOR</i>	0.09 ± 0.01	0.33 ± 0.01	10.13 ± 1.11	---
	<i>ZOR + EBS</i>	---	---	---	---
VAL	<i>VAL</i>	0.14 ± 0.00	0.29 ± 0.01	2.51 ± 0.14	---
	<i>VAL + EBS</i>	---	---	---	---
ACL	<i>ACL</i>	0.21 ± 0.00	0.19 ± 0.01	2.44 ± 0.24	---
	<i>ACL + EBS</i>	0.25 ± 0.00	---	---	35.60 ± 2.56

Supplementary Table 8. Estimated K_i using Competitive One-Site Binding Equation.

Summary of binding parameters of anthracyclines in comparison to doxorubicin as previously published²¹⁸. Fluorescence polarization values were fit to the One-Site Binding Equation (Equation 1) or the Competitive One-Site Binding Equation (Equation S1). * indicates data was obtained from Arnold et al., 2017²¹⁸.

A

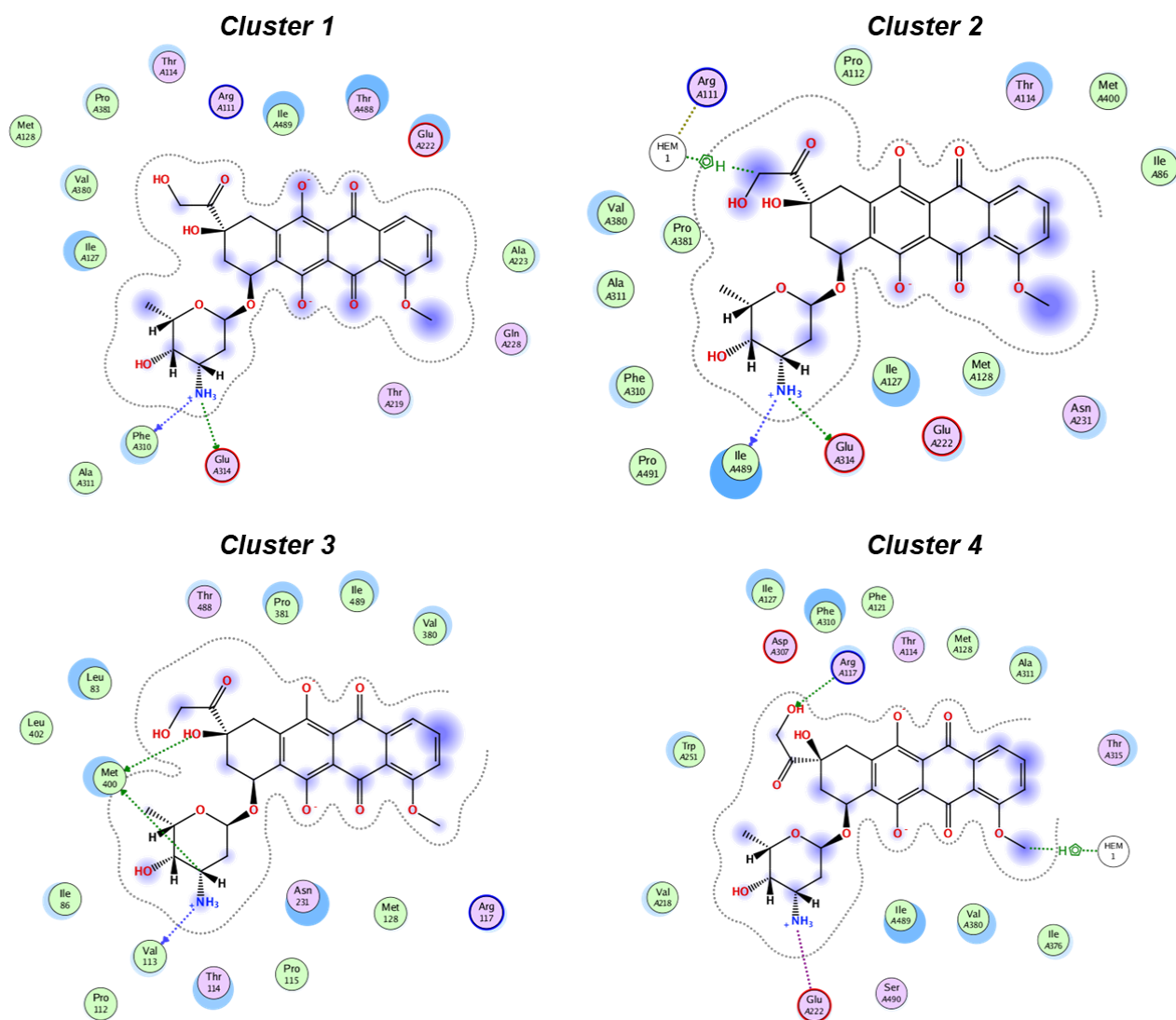
Cluster 1



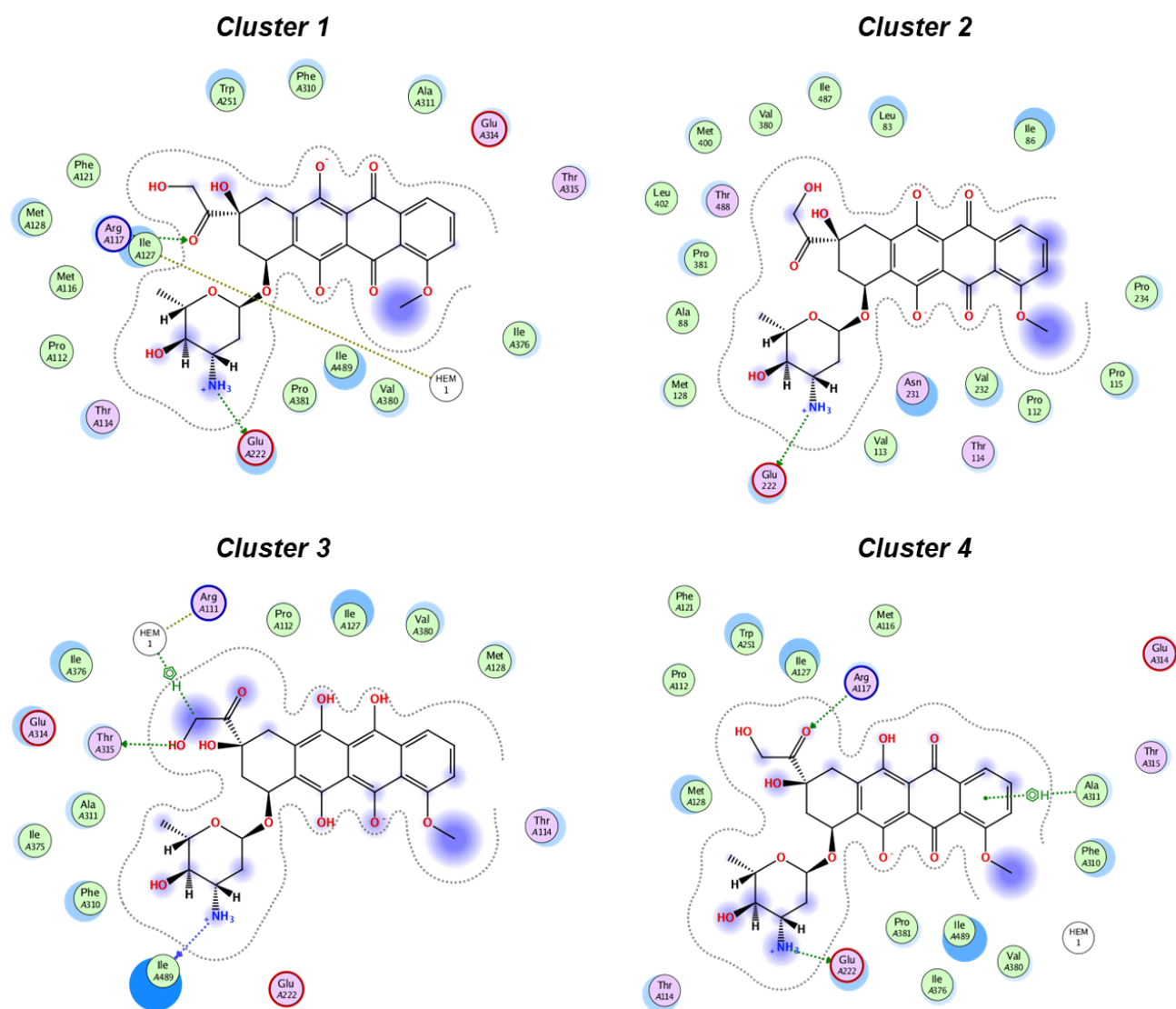
The chemical structure of a flavonoid compound is shown, with several residues highlighted in blue circles: Arg A117, Met A128, Val A189, Thr A314, Thr A468, Glu A222, Ile A489, Glu A314, Ala A311, Thr A315, Pro A491, Asp A107, and Phe A310. The structure features a central chromane core with a hydroxyl group (OH) and a methoxy group (OCH₃). The molecule is surrounded by a dashed line indicating a binding pocket or interaction site.

Supplementary Figure 12 (cont.)
B

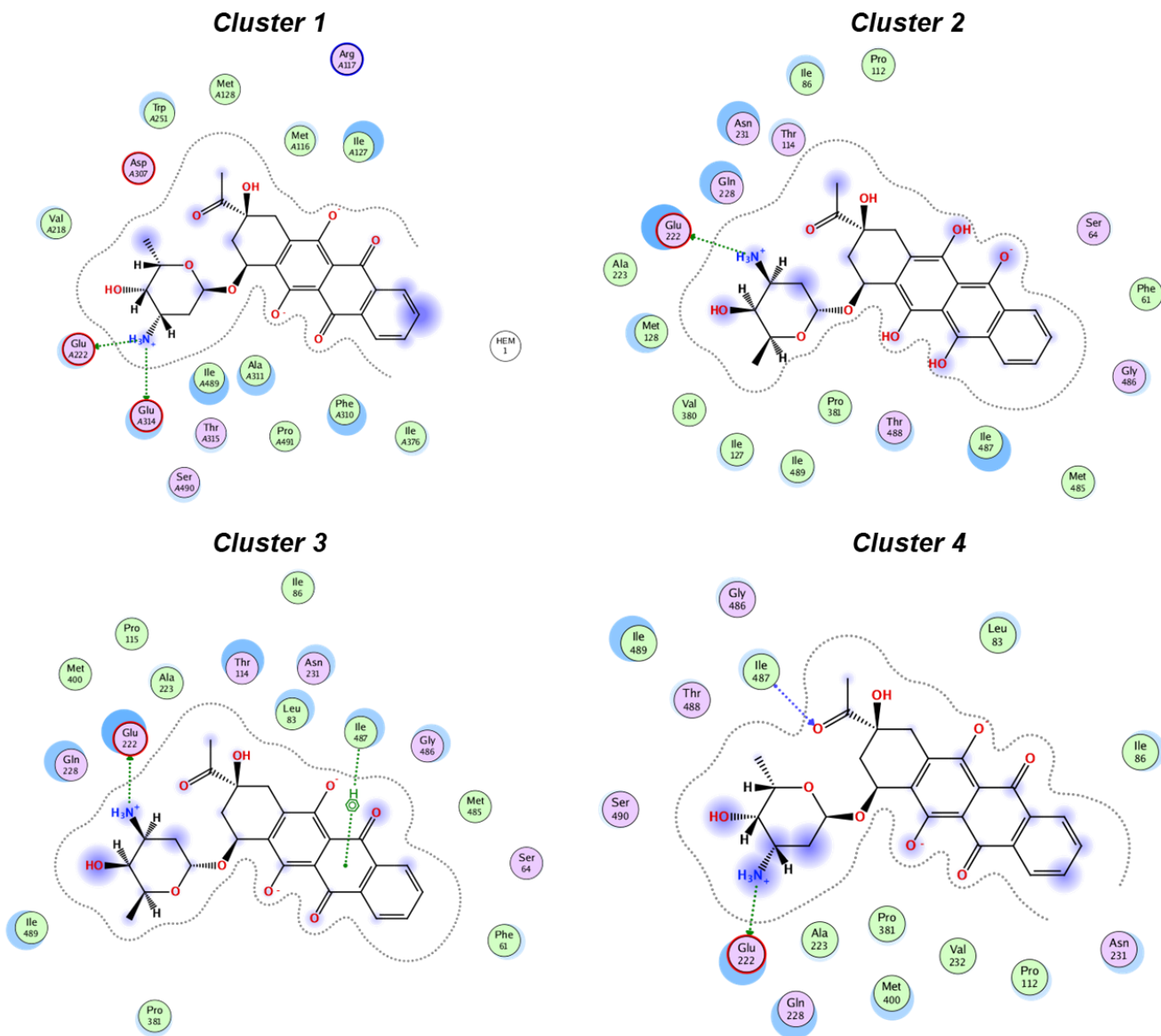
Doxorubicin



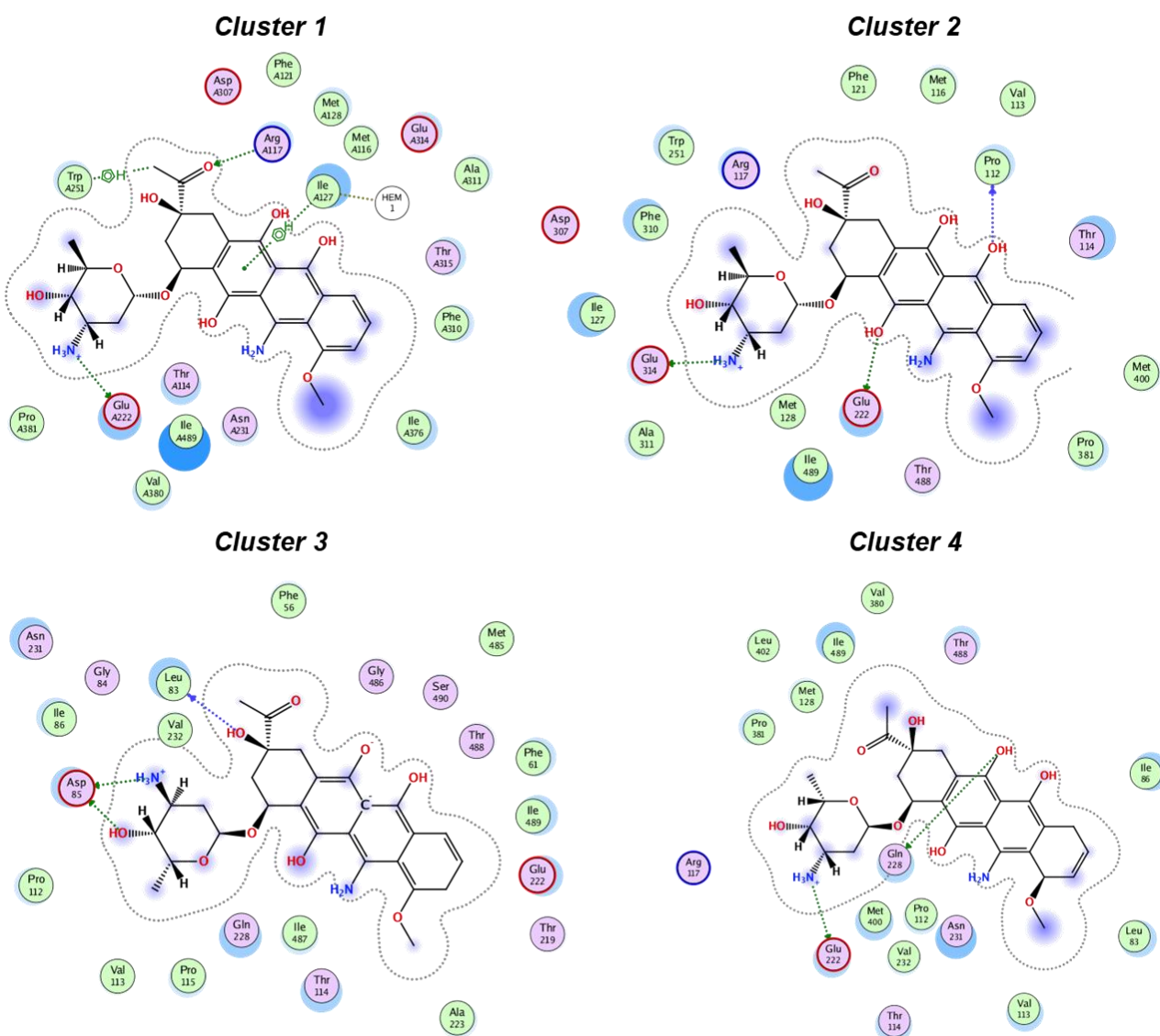
Epirubicin



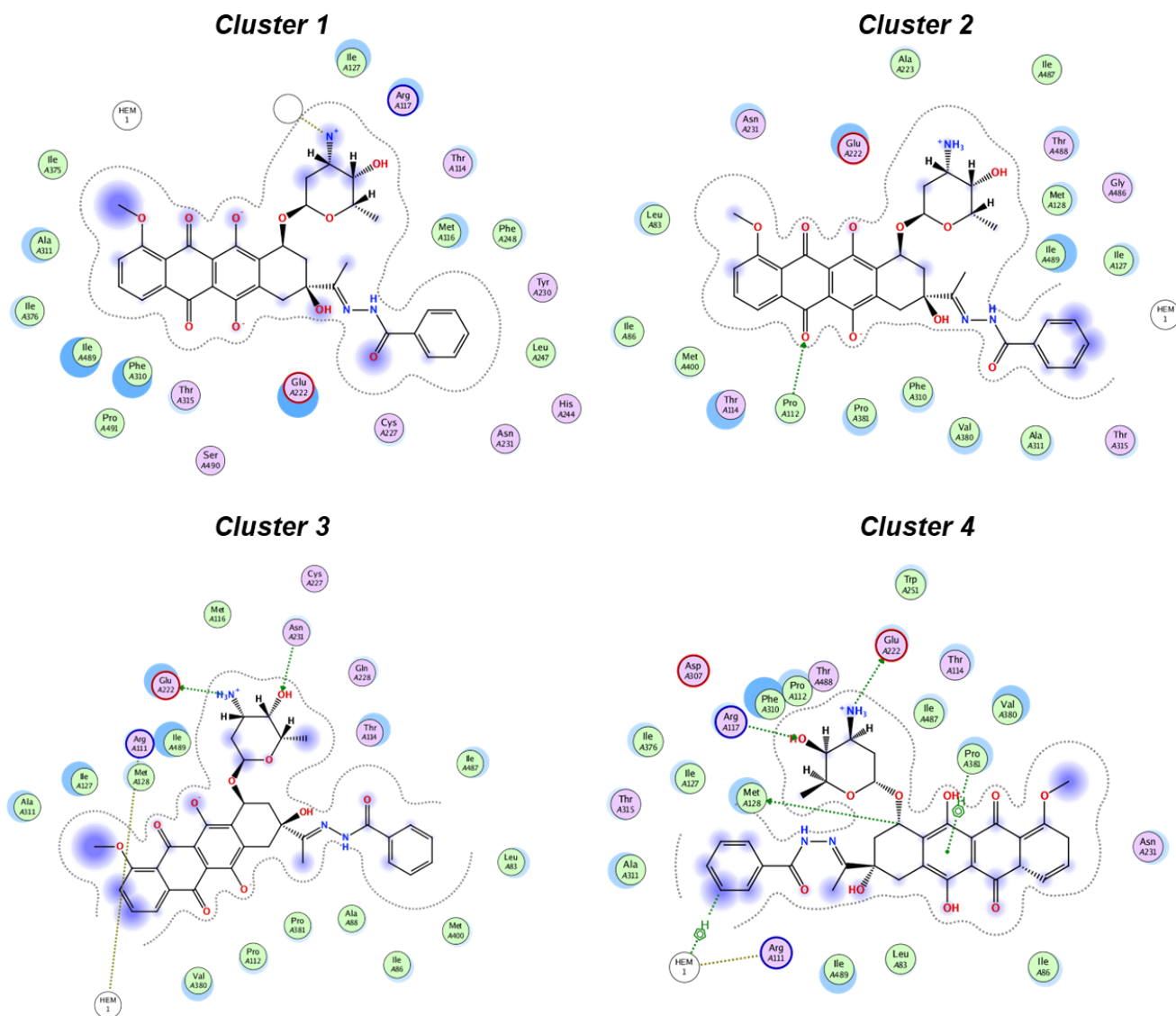
Idarubicin



5-Iminodaunorubicin

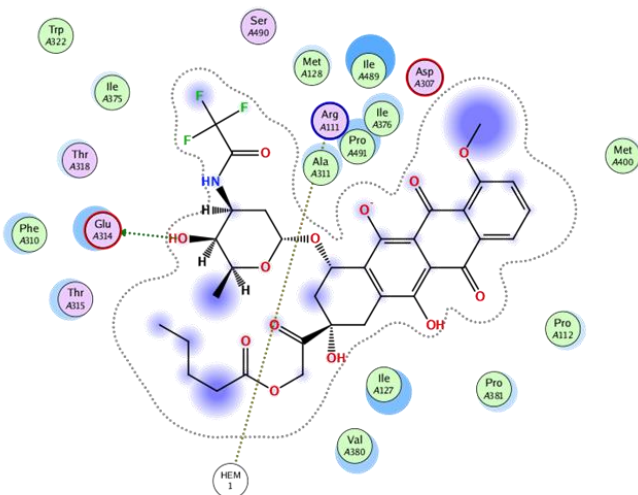


Zorubicin

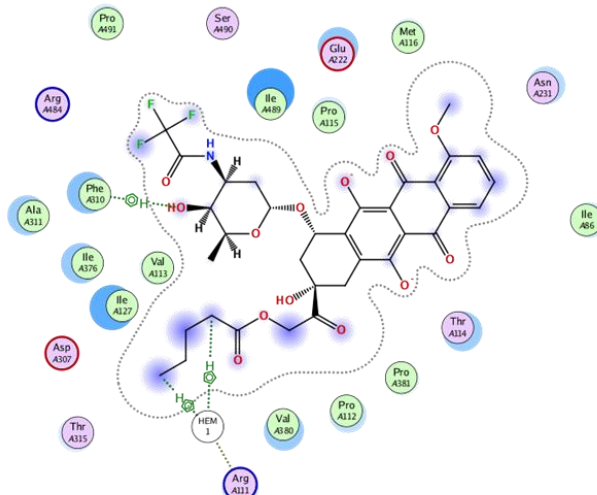


Valrubicin

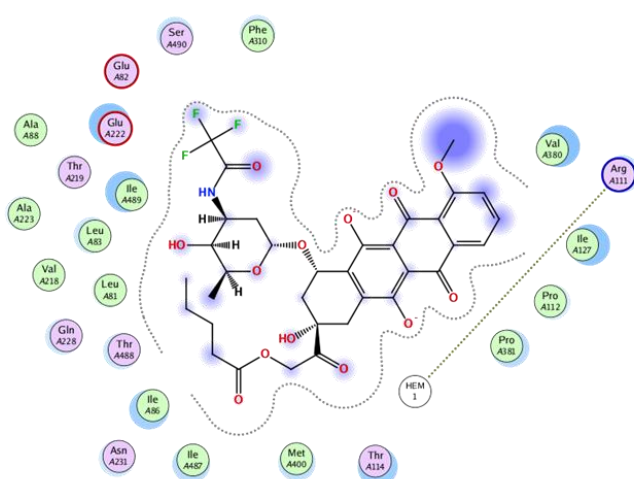
Cluster 1



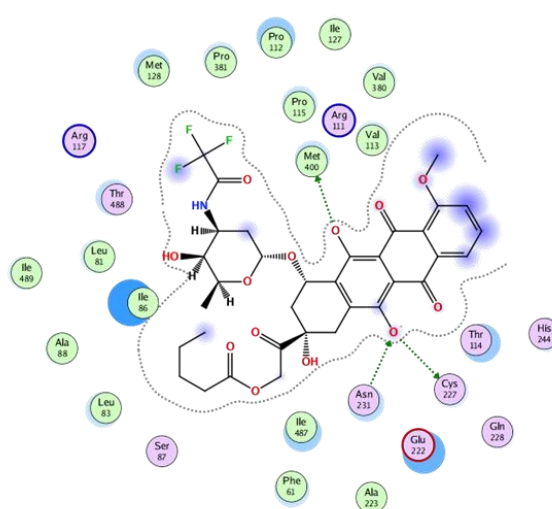
Cluster 2



Cluster 3

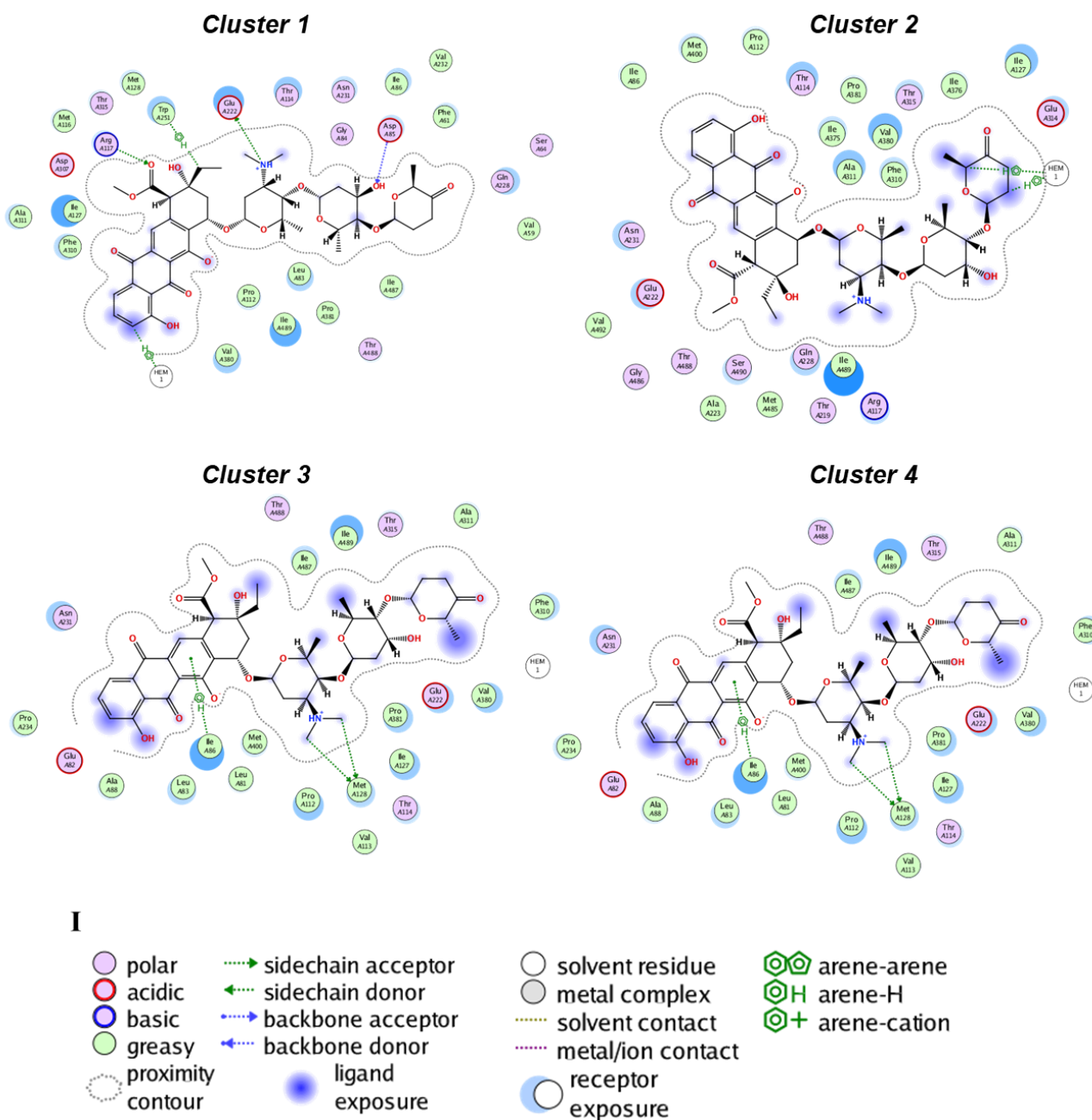


Cluster 4

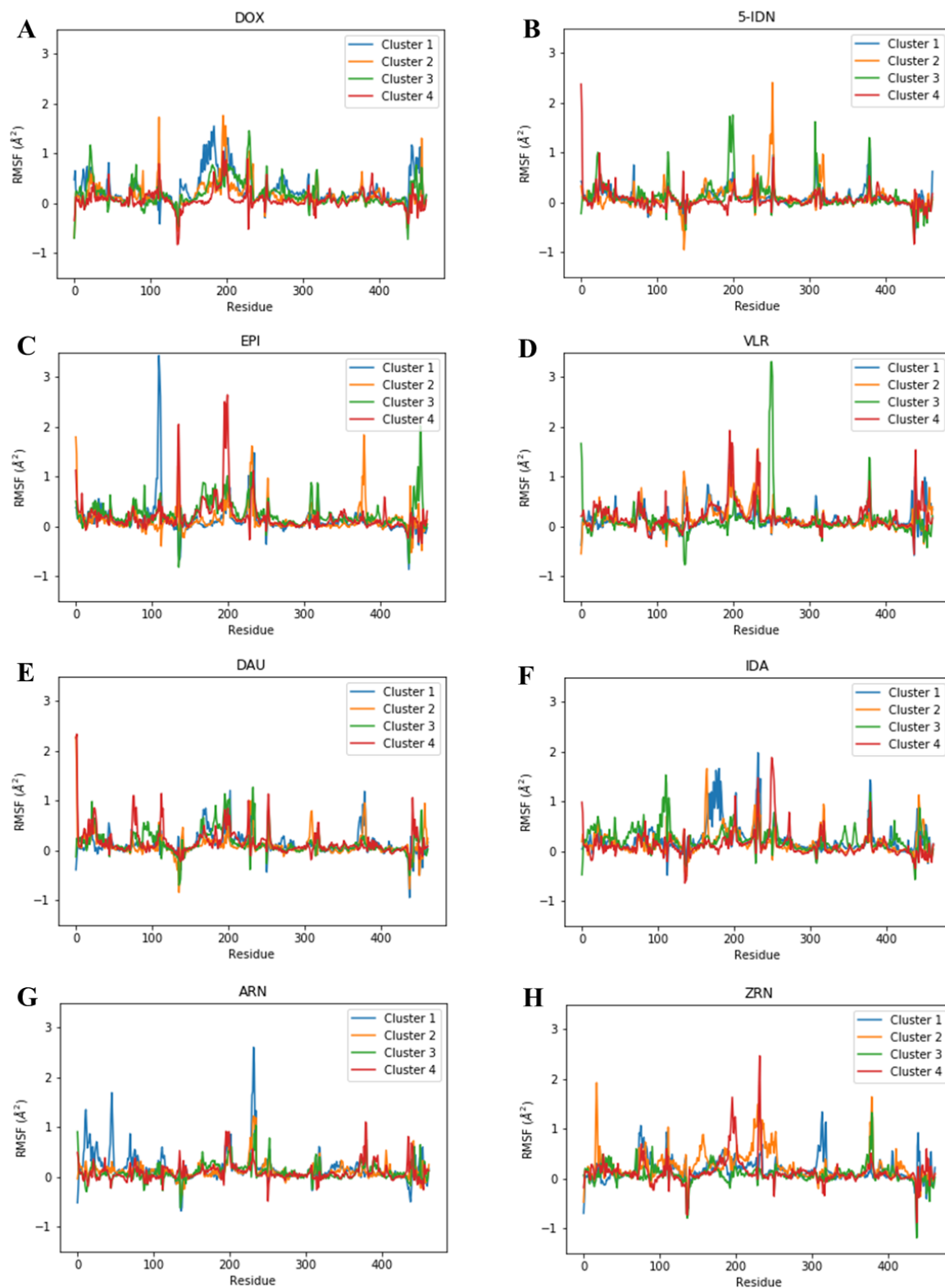


Supplementary Figure 12 (cont.)
H

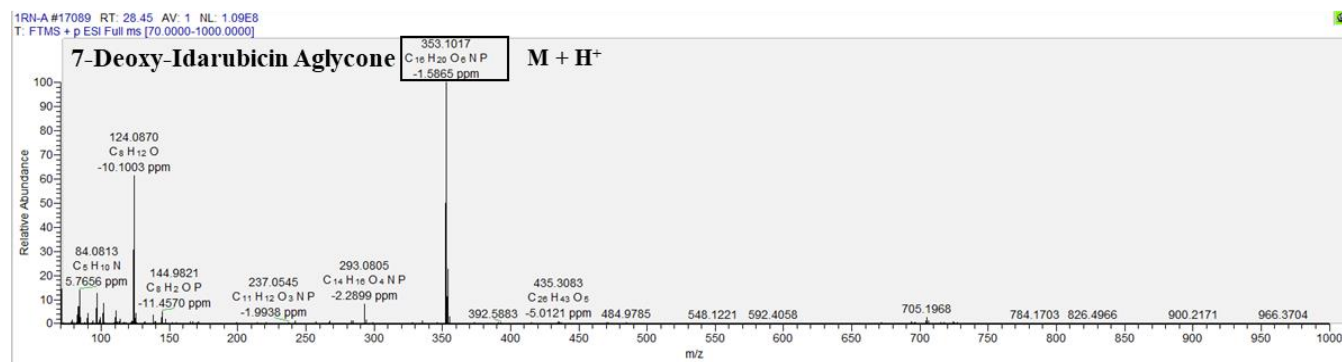
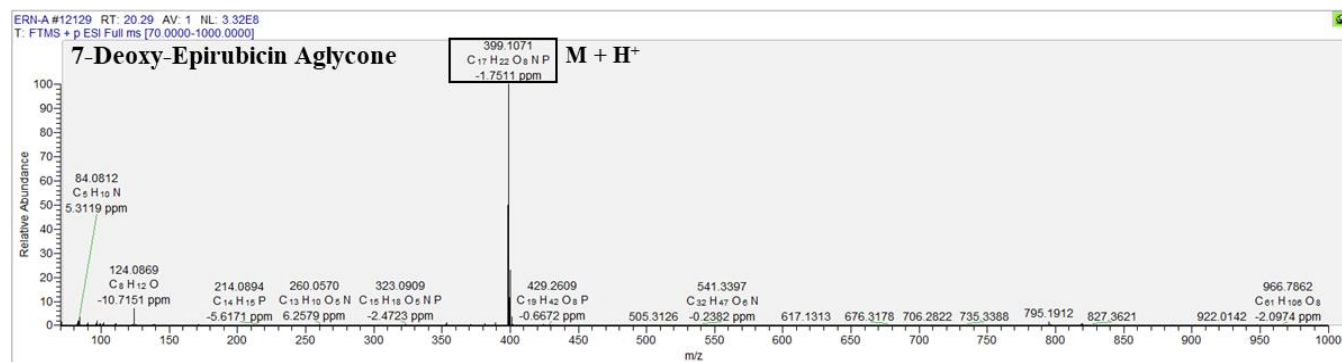
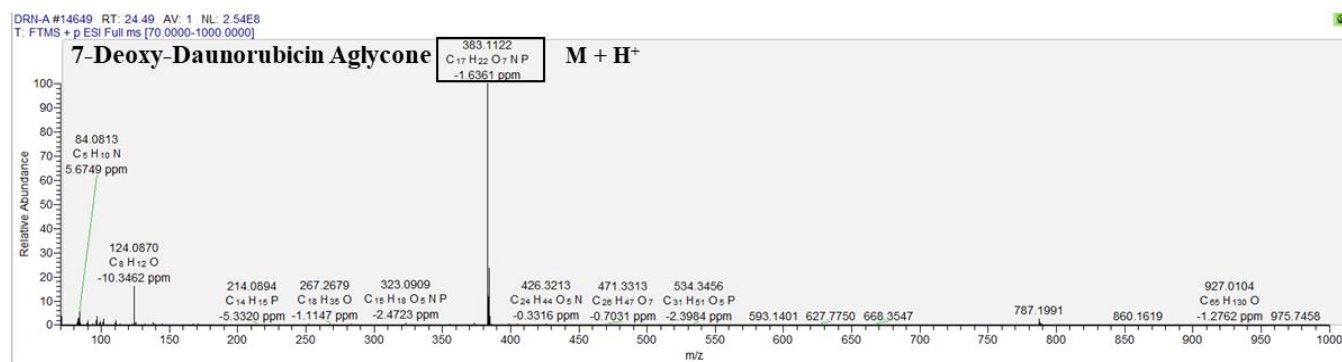
Aclarubicin



Supplementary Figure 12. Molecular Interaction Diagrams. Molecular Interaction diagrams for each cluster of anthracycline at the final frame of each 50 ns equilibrium MD run. Anthracyclines are (A) Doxorubicin, (B) Daunorubicin, (C) Epirubicin, (D) Idarubicin, I 5-Iminodaunorubicin, (F) Zorubicin, (G) Valrubicin, and (H) Aclarubicin. (I) The legend for the Molecular Interaction diagrams.



Supplementary Figure 13. *Root-Mean-Square-Fluctuations of Anthracyclines.* Root mean squared fluctuations from the 50 ns MD simulations of anthracycline bound CYP2J2.



Supplementary Figure 14. LC-MS Analysis of Aglycone Formation. MS Chromatogram of 7-Deoxy Aglycones of Daunorubicin (383.1122 $m/z \pm 5$ ppm), Epirubicin (399.1071 $m/z \pm 5$ ppm), and Idarubicin (353.1017 $m/z \pm 5$ ppm). The MS chromatograms were obtained by conducting mass spectrometry from liquid chromatography timepoints of 24.49 min, 20.29 min, and 28.45 min, respectively.

Supplementary Table 9. Distance of Arachidonic Acid Double Bonds to Heme Iron of CYP2J2

	Cluster #	Mean (14,15-)	Std. Error	Mean (11,12-)	Std. Error	Mean (8,9-)	Std. Error	Mean (5,6-)	Std. Error
ACL – 1	1	10.10	0.72	<u>8.43</u>	0.77	10.62	0.97	13.79	1.01
	2	16.58	0.61	14.37	0.80	11.28	0.78	<u>8.74</u>	0.93
	3	14.20	0.88	11.00	0.88	<u>8.58</u>	0.77	10.02	0.80
	4	18.27	0.95	17.93	1.45	<u>16.65</u>	0.65	18.33	0.80
ACL – 2	1	<u>16.99</u>	0.61	19.40	0.79	21.40	0.79	24.10	0.98
	2	<u>17.39</u>	0.82	20.23	0.80	21.17	0.62	24.22	0.76
	3	17.54	0.84	<u>17.50</u>	0.85	20.22	0.77	21.37	0.88
	4	<u>19.04</u>	0.91	21.49	0.66	24.34	0.84	26.52	0.80
ACL – 3	1	<u>11.34</u>	0.68	14.28	0.70	16.15	0.68	17.78	0.89
	2	17.29	0.98	16.04	0.72	14.10	0.70	<u>11.19</u>	0.72
	3	16.24	0.70	14.57	0.66	11.53	0.73	<u>10.14</u>	0.73
	4	<u>10.23</u>	0.84	12.59	0.95	15.44	0.74	16.49	0.75
ACL – 4	1	<u>11.19</u>	0.70	14.15	0.66	16.12	0.69	17.38	0.90
	2	16.30	0.68	14.60	0.70	11.52	0.81	<u>10.31</u>	0.72
	3	17.46	0.90	16.21	0.72	14.27	0.59	<u>11.30</u>	0.66
	4	<u>10.11</u>	0.65	11.71	0.86	14.72	0.71	16.21	0.70
DAU – 1	1	6.31	0.96	<u>4.50</u>	0.55	6.66	0.85	9.02	0.51
	2	8.45	0.88	6.20	1.10	<u>4.76</u>	0.78	7.25	0.88
	3	6.30	1.05	<u>4.42</u>	0.66	6.36	0.96	8.09	0.63
	4	8.83	0.63	6.27	0.98	<u>4.56</u>	0.69	6.53	0.82
DAU – 2	1	18.92	1.04	19.34	0.88	<u>17.76</u>	1.06	19.33	1.01
	2	19.31	1.09	21.38	0.89	19.60	0.90	<u>17.52</u>	0.73
	3	18.79	0.89	21.08	0.71	20.41	0.76	<u>17.73</u>	0.71
	4	<u>17.80</u>	0.80	20.69	0.70	21.62	1.03	20.00	0.75
DAU – 3	1	<u>10.19</u>	0.80	12.85	0.60	14.23	0.66	16.81	0.65
	2	16.96	0.65	14.39	0.72	13.07	0.64	<u>10.76</u>	0.99
	3	<u>8.58</u>	1.10	11.31	0.89	13.34	0.74	14.98	0.84
	4	<u>11.97</u>	0.88	14.04	0.81	15.23	0.84	17.64	0.82
DAU – 4	1	17.90	1.08	18.28	1.00	<u>16.48</u>	0.54	18.70	0.88
	2	10.23	0.82	<u>9.03</u>	0.82	11.21	0.64	13.73	0.74
	3	11.09	0.55	<u>8.57</u>	0.65	9.49	0.83	12.34	0.93
	4	12.14	0.76	9.83	0.94	<u>8.89</u>	0.92	11.02	0.62
EPI – 1	1	17.14	1.15	17.22	1.68	<u>16.98</u>	0.83	18.15	0.98
	2	<u>17.30</u>	1.45	17.51	1.20	17.49	1.16	19.89	0.98
	3	<u>17.17</u>	0.93	18.42	1.20	17.48	1.38	17.25	<u>0.80</u>
	4	21.19	0.75	18.62	0.93	16.76	0.76	<u>16.16</u>	1.37
EPI – 2	1	8.02	0.62	<u>4.90</u>	0.58	5.93	0.61	9.23	0.71
	2	11.73	0.55	9.57	0.55	6.32	0.62	<u>4.62</u>	0.54
	3	9.53	0.58	6.21	0.57	<u>4.60</u>	0.43	7.60	0.68
	4	11.71	0.71	9.42	0.71	6.20	0.79	<u>4.50</u>	0.62
EPI – 3	1	<u>16.97</u>	0.92	19.73	0.79	20.74	0.66	21.48	0.88
	2	<u>11.27</u>	1.03	13.72	0.67	14.47	0.60	17.30	0.81

Supplementary Table 9 (cont.)

	3	17.50	0.74	<u>17.16</u>	0.66	17.40	0.87	19.67	0.93
	4	18.95	0.97	20.56	0.63	19.20	0.80	<u>16.75</u>	0.88
EPI – 4	1	21.77	0.76	21.76	0.59	19.18	0.90	<u>17.44</u>	0.65
	2	22.17	0.89	22.04	0.85	19.00	0.99	<u>17.23</u>	0.64
	3	<u>17.49</u>	0.95	18.58	1.12	21.34	0.83	21.82	0.87
	4	<u>13.79</u>	0.60	15.14	0.41	17.83	0.73	19.42	0.56
IDA – 1	1	22.32	0.98	22.22	0.71	20.46	0.84	<u>18.20</u>	0.86
	2	18.05	1.19	20.73	0.87	20.18	0.86	<u>17.08</u>	0.96
	3	18.22	0.88	18.38	1.04	<u>16.88</u>	0.97	19.00	0.91
	4	21.61	0.48	18.88	0.56	<u>18.17</u>	0.79	20.52	1.02
IDA – 2	1	<u>15.00</u>	0.77	16.96	1.00	18.14	0.64	16.37	0.65
	2	12.12	0.82	9.28	0.79	6.64	0.99	<u>4.66</u>	0.60
	3	<u>4.75</u>	0.75	6.54	1.11	9.13	0.78	11.92	0.84
	4	18.25	0.76	<u>16.19</u>	0.54	17.65	0.89	16.21	1.03
IDA – 3	1	16.19	0.80	<u>13.91</u>	0.43	14.75	0.87	17.03	1.05
	2	13.56	0.75	10.38	0.84	9.32	0.65	<u>7.14</u>	0.74
	3	<u>16.15</u>	0.99	18.41	0.93	18.75	0.89	17.18	0.94
	4	<u>8.53</u>	0.74	10.19	0.90	12.89	0.81	15.39	0.89
IDA – 4	1	9.46	0.78	6.38	1.06	<u>5.20</u>	0.89	8.21	0.86
	2	9.14	0.83	6.25	1.06	<u>5.52</u>	0.85	8.73	0.83
	3	9.48	0.79	8.62	0.71	5.69	0.70	<u>5.03</u>	0.86
	4	5.62	1.00	<u>5.56</u>	0.66	8.71	0.78	9.76	0.71
VAL – 1	1	<u>18.18</u>	0.94	20.02	1.02	22.43	0.66	21.51	0.84
	2	22.18	0.78	20.08	0.79	<u>17.26</u>	0.83	17.68	1.11
	3	18.16	0.92	<u>17.19</u>	1.10	18.20	0.92	21.15	0.79
	4	21.00	0.90	22.73	0.58	21.03	0.99	<u>18.66</u>	0.78
VAL – 2	1	16.49	1.04	17.53	0.64	<u>16.19</u>	0.57	18.04	0.95
	2	20.53	0.94	21.13	0.85	18.78	0.95	<u>16.33</u>	0.61
	3	16.54	0.71	<u>16.29</u>	0.46	19.16	0.84	20.63	0.83
	4	19.57	0.87	18.27	0.61	16.78	0.91	<u>16.61</u>	0.58
VAL – 3	1	7.00	0.98	<u>5.29</u>	0.63	7.62	0.73	9.96	0.54
	2	9.61	1.04	10.33	0.99	9.84	0.63	<u>7.17</u>	0.75
	3	9.76	0.64	9.14	1.10	9.54	0.94	<u>8.40</u>	0.92
	4	9.94	0.69	8.58	0.84	<u>5.84</u>	0.59	6.42	1.10
VAL – 4	1	18.04	0.81	18.44	1.07	17.53	0.96	<u>16.25</u>	1.16
	2	19.31	0.64	16.97	0.57	16.13	1.35	<u>16.04</u>	0.70
	3	<u>16.22</u>	0.93	18.34	0.88	20.65	0.77	20.61	0.93
	4	16.79	0.93	15.58	1.21	<u>15.46</u>	0.52	18.38	0.66
ZRN – 1	1	18.22	0.76	19.72	0.75	20.12	0.96	<u>17.77</u>	1.00
	2	17.06	1.20	18.59	0.80	19.40	0.78	<u>16.99</u>	1.07
	3	18.36	0.92	19.05	0.88	<u>16.86</u>	1.15	18.12	0.98
	4	19.71	0.75	19.78	0.70	17.69	0.89	<u>14.85</u>	0.75
ZRN – 2	1	<u>16.55</u>	0.78	18.32	0.86	18.65	0.72	18.04	1.01
	2	17.85	0.83	18.71	0.65	<u>17.71</u>	0.95	19.07	0.84
	3	<u>16.46</u>	1.01	16.87	0.92	18.51	1.02	18.71	0.71

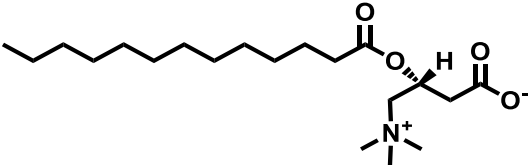
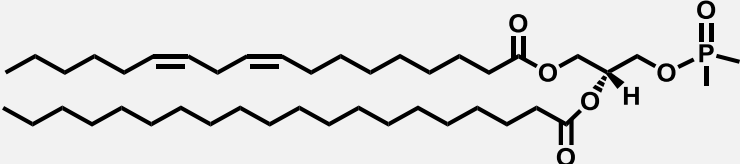
Supplementary Table 9 (cont.)

	4	18.26	0.85	18.64	0.87	18.53	0.83	<u>16.68</u>	0.74
ZRN – 3	1	16.74	1.02	<u>14.32</u>	0.76	15.16	0.70	18.19	1.03
	2	<u>8.51</u>	0.93	10.03	0.80	12.75	0.89	14.85	0.52
	3	<u>17.10</u>	0.60	17.30	0.98	19.98	0.56	21.94	0.53
	4	<u>16.93</u>	0.66	19.69	0.54	22.11	0.68	22.60	0.73
ZRN – 4	1	<u>16.82</u>	0.98	18.86	0.69	19.10	0.68	17.61	0.82
	2	18.34	0.85	19.28	0.70	<u>18.03</u>	1.35	18.97	0.89
	3	<u>16.67</u>	1.10	17.27	0.84	19.18	0.56	19.32	0.64
	4	<u>11.45</u>	0.76	14.26	0.63	15.53	0.53	17.36	0.67
5-IDN – 1	1	<u>5.02</u>	0.73	5.92	0.68	8.98	0.66	10.14	0.96
	2	9.75	0.62	8.59	0.88	5.95	0.84	<u>5.09</u>	0.78
	3	9.24	1.08	10.79	1.05	10.97	0.71	8.56	0.90
	4	5.94	0.76	<u>4.85</u>	0.71	7.90	0.70	10.24	0.83
5-IDN – 2	1	12.24	0.97	9.78	0.67	6.97	0.87	<u>5.38</u>	0.70
	2	7.29	0.85	<u>4.73</u>	0.53	6.84	0.88	9.41	0.82
	3	9.11	0.78	6.49	0.76	<u>4.66</u>	0.70	7.36	1.09
	4	11.02	1.04	9.48	0.60	9.75	0.97	<u>8.14</u>	1.16
5-IDN – 3	1	15.60	0.48	13.83	0.87	11.31	1.40	<u>8.81</u>	1.68
	2	<u>8.46</u>	0.60	10.99	0.72	13.89	0.69	15.65	0.49
	3	14.16	0.74	12.94	0.86	10.47	0.89	<u>8.60</u>	0.73
	4	<u>17.76</u>	0.68	20.48	1.11	23.30	0.68	22.33	0.94
5-IDN – 4	1	6.63	0.84	<u>4.30</u>	0.38	6.51	0.97	9.22	0.80
	2	<u>4.87</u>	0.72	5.40	0.60	8.41	0.59	11.22	0.69
	3	9.34	0.73	6.28	0.79	<u>4.46</u>	0.49	6.83	0.68
	4	<u>4.72</u>	0.70	5.69	0.75	8.55	0.66	11.34	0.69

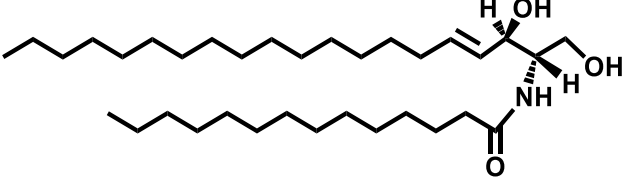
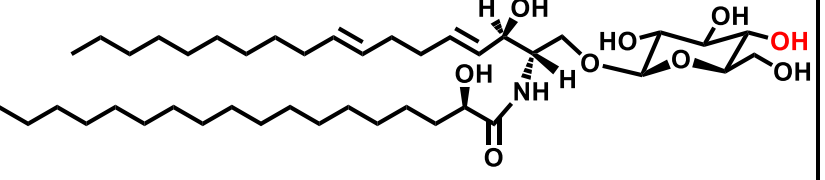
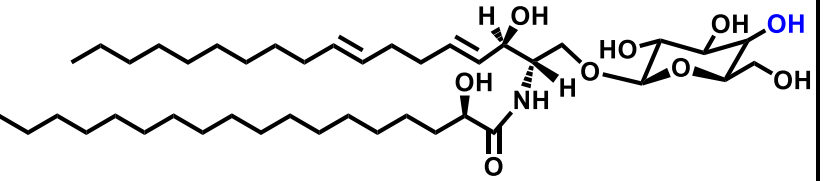
Supplementary Table 9. Distance of Arachidonic Acid Double Bonds to Heme Iron of CYP2J2. Mean distance and standard deviation of EET forming double bonds in AA relative to the heme iron of CYP2J2. The values shown correspond to the second iteration of the ensemble docking, following anthracycline docking. Underlined red values correspond the minimum distance from the heme iron.

APPENDIX E: SUPPLEMENTARY INFORMATION – UTILIZING LIPIDOMIC TECHNIQUES TO EXAMINE MYELIN

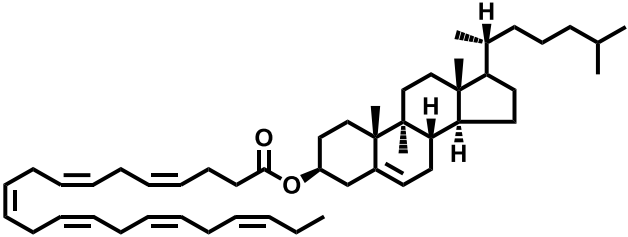
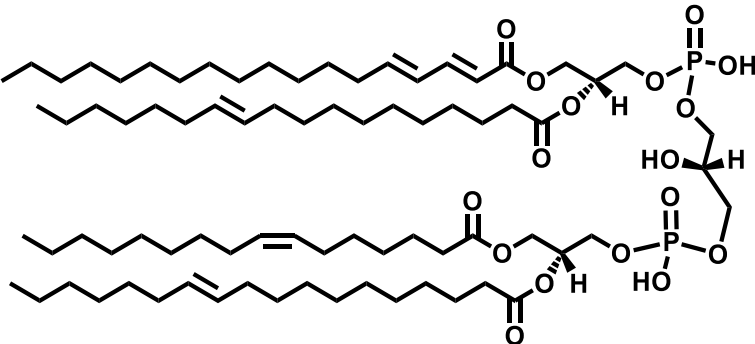
LIPIDOME UNDER UPPER RESPIRATORY INFECTION

Supplementary Table 10: Myelin PBS vs IAV day 8				
<i>Lipid Ion</i>	<i>Lipid Class</i>	<i>Fold Change (IAV/PBS)</i>	<i>p-value</i>	<i>Representative Structure</i>
AcCa(12:0)+H	AcCa	1.70	0.03301	 AcCa (13:0)
AcCa(13:0)+H	AcCa	2.56	0.00065	
AcCa(14:1)+H	AcCa	4.12	0.02759	
AcCa(20:5)+H	AcCa	1.78	0.04660	
AcCa(24:1)+H	AcCa	1.73	0.01052	
BisMePA(18:2p/16:1)+NH4	BisMePA	1.52	0.02759	 BisMePA (18:2/20:0)
BisMePA(16:0/20:4)+NH4	BisMePA	2.08	0.01568	
BisMePA(16:0/20:5)+NH4	BisMePA	2.61	0.03301	
BisMePA(18:2p/19:1)+NH4	BisMePA	2.55	0.04660	
BisMePA(18:2p/20:0)+NH4	BisMePA	2.02	0.03301	
BisMePA(18:0/20:5)+NH4	BisMePA	3.38	0.02759	
BisMePA(18:1/20:5)+NH4	BisMePA	2.60	0.01902	
BisMePA(18:0/22:5)+NH4	BisMePA	2.18	0.01287	
BisMePA(18:2p/22:3)+NH4	BisMePA	2.88	0.00855	
BisMePA(18:2p/22:5)+NH4	BisMePA	3.45	0.01902	
BisMePA(18:2p/22:6)+NH4	BisMePA	2.44	0.02296	
Cer(d18:0/18:0)+H	Cer	3.92	0.02296	
Cer(d18:2/18:0)+H	Cer	5.68	0.01287	
Cer(d18:0/22:0)+H	Cer	1.89	0.03301	
Cer(d18:1/23:1)+H	Cer	2.45	0.01902	
Cer(d18:2/23:0)+H	Cer	2.44	0.02759	

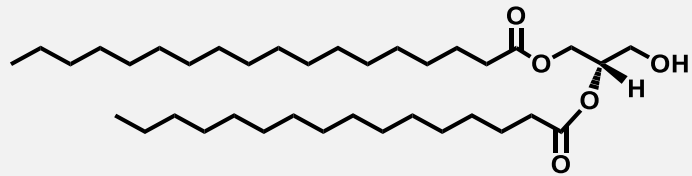
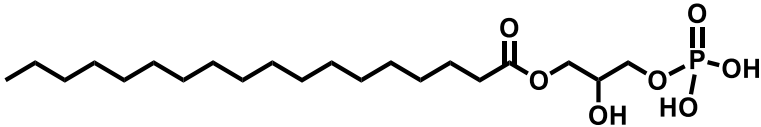
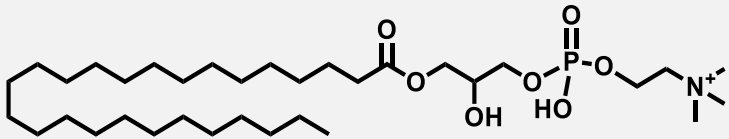
Supplementary Table 10 (cont.)

Cer(d18:0/24:0)+H	Cer	1.43	0.03301	 Cer (18:1/14:0)
Cer(d18:0/24:1)+H	Cer	2.46	0.01568	
Cer(d18:1/24:1)+H	Cer	3.05	0.04660	
Cer(d18:1/25:0)+H	Cer	1.45	0.04660	
Cer(d18:1/25:1)+H	Cer	1.73	0.01287	
Cer(d20:1/24:0)+H	Cer	1.93	0.01568	
Cer(d20:1/24:1)+H	Cer	1.72	0.01568	
CerG1(d18:2/16:0)+H	CerG1	0.57	0.02759	 Glucosylceramide (18:2/18:0)
CerG1(d18:1/18:0)+H	CerG1	2.39	0.03931	
CerG1(d18:1/18:1)+H	CerG1	1.50	0.01287	
CerG1(d18:1/20:0)+H	CerG1	2.04	0.02759	
CerG1(d18:1/21:0)+H	CerG1	2.61	0.02759	
CerG1(d18:1/21:1)+H	CerG1	2.04	0.01902	
CerG1(d18:0/22:0)+H	CerG1	2.01	0.02759	
CerG1(d18:2/22:1)+H	CerG1	2.18	0.03301	
CerG1(d18:1/22:6)+H	CerG1	0.49	0.00692	
CerG1(d18:0/23:1)+H	CerG1	3.73	0.00855	
CerG1(d18:1/23:0)+H	CerG1	2.07	0.03301	
CerG1(d18:2/23:0)+H	CerG1	3.34	0.01902	
CerG1(d18:1/23:2)+H	CerG1	1.91	0.03301	
CerG1(d18:2/23:1)+H	CerG1	1.94	0.01902	
CerG1(d18:0/24:0)+H	CerG1	1.48	0.03301	
CerG1(d18:1/24:0)+H	CerG1	1.93	0.04660	
CerG1(d18:1/24:1)+H	CerG1	3.07	0.03931	
CerG1(d18:2/24:1)+H	CerG1	1.99	0.03301	
CerG1(d18:1/24:2)+H	CerG1	3.16	0.03931	
CerG1(d18:2/24:2)+H	CerG1	1.58	0.02759	 Galactosylceramide (18:2/18:0)
CerG1(d18:1/24:3)+H	CerG1	2.83	0.02759	
CerG1(d18:0/25:1)+H	CerG1	2.28	0.01287	
CerG1(d18:1/25:1)+H	CerG1	1.92	0.03931	

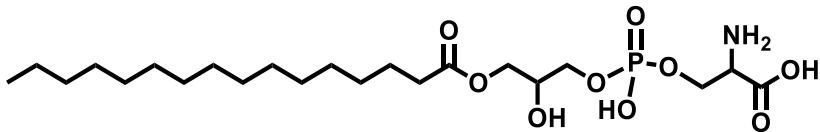
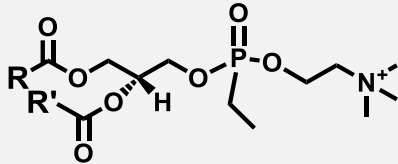
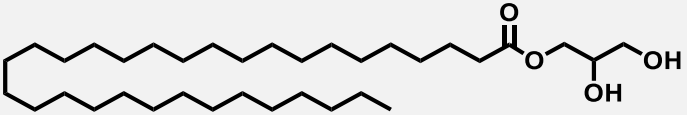
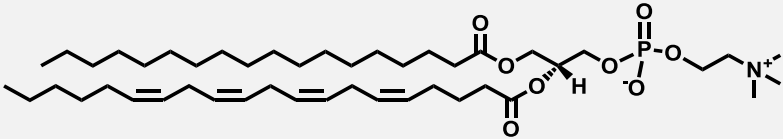
Supplementary Table 10 (cont.)

CerG1(d18:2/25:1)+H	CerG1	1.96	0.01287	
CerG1(d18:1/25:2)+H	CerG1	1.79	0.02759	
CerG1(d18:1/26:1)+H	CerG1	2.60	0.03931	
CerG1(d18:1/26:2)+H	CerG1	9.37	0.02759	
CerG1(d44:4)+H	CerG1	19.23	0.03301	
CerG2(d34:2)+H	CerG2	3.53	0.01568	
CerG2(d18:1/22:6)+H	CerG2	0.50	0.03931	
CerG2(d18:1/24:0)+H	CerG2	2.55	0.01902	
CerG2(d54:4)+H	CerG2	1.90	0.01287	
ChE(22:6)+NH4	ChE	1.83	0.02759	 ChE (22:6)
ChE(24:6)+NH4	ChE	1.64	0.03301	
CL(18:2/18:1/16:1/18:1)-H	CL	1.32	0.02759	 CL (18:2/18:1/16:1/18:1)
CL(20:4/18:1/16:1/18:1)-H	CL	1.21	0.02759	
CL(18:2/18:1/20:4/18:1)-H	CL	1.57	0.01287	
CL(17:1/18:1/20:4/22:6)-H	CL	2.11	0.03931	
CL(23:0/20:4/18:0/20:4)-H	CL	2.28	0.03019	
DG(16:0/16:0)+NH4	DG	3.31	0.03931	

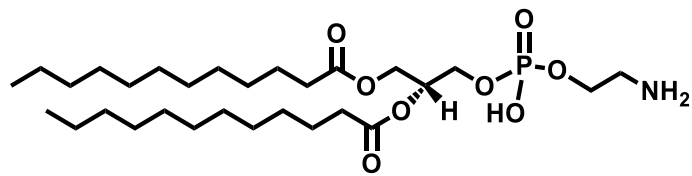
Supplementary Table 10 (cont.)

<i>DG(16:0/16:1)+NH4</i>	<i>DG</i>	3.34	0.00855	 DG (16:0/18:0)
<i>DG(18:0/16:0)+NH4</i>	<i>DG</i>	2.55	0.02296	
<i>DG(18:0/18:2)+NH4</i>	<i>DG</i>	3.24	0.03931	
<i>DG(18:1/18:2)+NH4</i>	<i>DG</i>	5.12	0.00050	
<i>DG(16:1/20:4)+NH4</i>	<i>DG</i>	2.76	0.03931	
<i>DG(20:1/18:2)+NH4</i>	<i>DG</i>	2.05	0.03931	
<i>DG(18:0/20:4)+NH4</i>	<i>DG</i>	2.23	0.02296	
<i>DG(22:1/18:2)+NH4</i>	<i>DG</i>	2.12	0.01568	
<i>DG(18:0/22:3)+NH4</i>	<i>DG</i>	1.86	0.03931	
<i>DG(18:0/22:6)+NH4</i>	<i>DG</i>	2.75	0.03301	
<i>DG(18:2/23:0)+NH4</i>	<i>DG</i>	1.47	0.01052	
<i>DG(18:1/24:2)+NH4</i>	<i>DG</i>	1.58	0.01568	
<i>DG(20:4/23:0)+NH4</i>	<i>DG</i>	1.38	0.02759	
<i>DG(24:1/20:4)+NH4</i>	<i>DG</i>	1.60	0.01287	
<i>LPA(18:0)-H</i>	<i>LPA</i>	0.50	0.01902	 LPA (18:0)
<i>LPC(26:0)+H</i>	<i>LPC</i>	2.09	0.03301	 LPC (26:0)
<i>LPS(16:0)-H</i>	<i>LPS</i>	0.40	0.01052	
<i>LPS(18:1)-H</i>	<i>LPS</i>	0.58	0.01287	

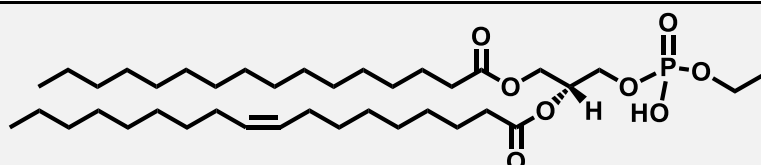
Supplementary Table 10 (cont.)

<i>LPS(20:4)-H</i>	<i>LPS</i>	0.35	0.01902	 LPS (16:0)
<i>MePC(32:2)+NH4</i>	<i>MePC</i>	2.07	0.01287	 MePC
<i>MePC(34:4)+NH4</i>	<i>MePC</i>	2.61	0.04660	
<i>MePC(34:5)+NH4</i>	<i>MePC</i>	1.77	0.01568	
<i>MePC(42:5)+NH4</i>	<i>MePC</i>	4.36	0.01902	
<i>MG(32:0)+H</i>	<i>MG</i>	2.02	0.02296	 MG (32:0)
<i>MG(36:0)+H</i>	<i>MG</i>	1.57	0.02759	
<i>PC(23:3e)+H</i>	<i>PC</i>	0.63	0.04660	 PC (18:0/20:4)
<i>PC(24:4e)+H</i>	<i>PC</i>	0.66	0.03301	
<i>PC(31:0)+H</i>	<i>PC</i>	2.71	0.04660	
<i>PC(31:3)+H</i>	<i>PC</i>	1.09	0.04660	
<i>PC(33:0p)+H</i>	<i>PC</i>	2.27	0.04660	
<i>PC(17:1/16:1)+H</i>	<i>PC</i>	2.09	0.03931	
<i>PC(33:4)+H</i>	<i>PC</i>	1.97	0.02759	
<i>PC(18:0p/16:1)+H</i>	<i>PC</i>	2.04	0.03931	
<i>PC(34:2)+H</i>	<i>PC</i>	2.17	0.02296	
<i>PC(35:5)+H</i>	<i>PC</i>	1.90	0.03301	
<i>PC(18:1/18:1)+H</i>	<i>PC</i>	3.20	0.03301	
<i>PC(36:2e)+H</i>	<i>PC</i>	1.97	0.03931	

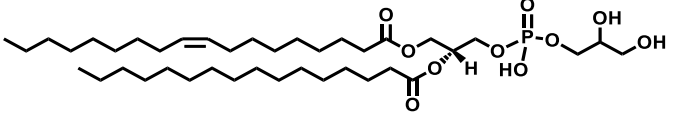
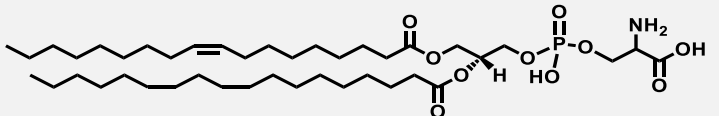
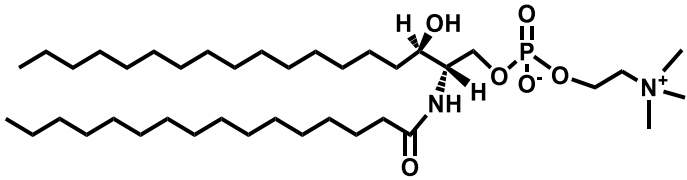
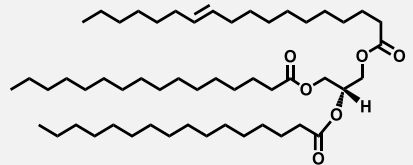
Supplementary Table 10 (cont.)

PC(37:0)+H	PC	1.62	0.04660	
PC(37:5)+H	PC	1.16	0.04660	
PC(38:4)+H	PC	4.05	0.03301	
PC(18:3/20:5)+H	PC	1.97	0.04660	
PC(40:3p)+H	PC	1.80	0.04660	
PC(18:0p/22:6)+H	PC	1.85	0.03931	
PC(40:6p)+H	PC	5.24	0.03931	
PC(18:1/22:6)+H	PC	2.73	0.04660	
PC(18:2/23:1)+H	PC	1.92	0.01902	
PC(42:11)+H	PC	2.87	0.00855	
PC(42:3)+H	PC	2.42	0.03931	
PC(42:5)+H	PC	2.52	0.02759	
PC(43:3)+H	PC	1.95	0.01568	
PC(43:4)+H	PC	1.70	0.02759	
PC(44:5)+H	PC	2.96	0.02759	
PC(45:4)+H	PC	2.29	0.01902	
PC(45:5)+H	PC	1.69	0.03301	
PC(46:5)+H	PC	1.77	0.03931	
PC(60:6)+H	PC	2.72	0.01902	
PE(20:3e)+H	PE	1.65	0.03931	 PE (12:0/12:0)
PE(16:0/16:1)+H	PE	2.62	0.01052	
PE(18:1p/16:1)+H	PE	1.95	0.01902	
PE(16:1/18:2)+H	PE	2.10	0.01287	
PE(16:0p/20:0)+H	PE	2.11	0.04660	
PE(18:0/18:1)+H	PE	2.39	0.03931	
PE(16:0/20:4)+H	PE	2.61	0.03301	
PE(18:0p/19:1)+H	PE	2.06	0.04660	
PE(18:1p/19:1)+H	PE	2.42	0.03931	
PE(18:0p/20:1)+H	PE	1.95	0.04660	
PE(18:0p/20:3)+H	PE	2.14	0.04660	

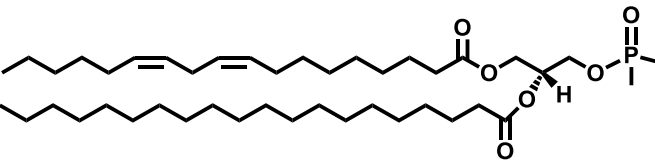
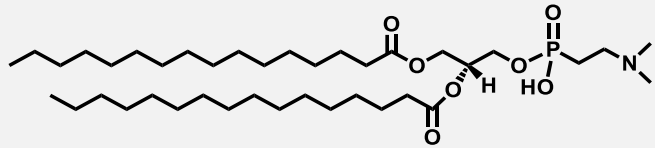
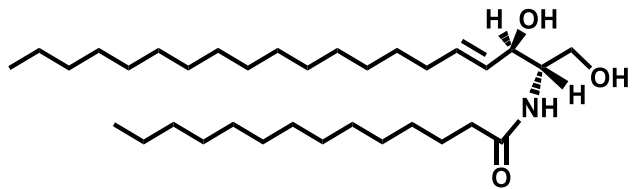
Supplementary Table 10 (cont.)

PE(18:0/20:4)+H	PE	3.34	0.03301	
PE(38:4e)+H	PE	8.39	0.01568	
PE(18:1/20:4)+H	PE	2.59	0.02296	
PE(18:2/20:4)+H	PE	1.18	0.01902	
PE(16:0/22:6)+H	PE	2.29	0.04660	
PE(18:2p/20:4)+H	PE	2.73	0.00224	
PE(16:0p/22:6)+H	PE	2.71	0.03931	
PE(16:1p/22:6)+H	PE	1.30	0.03301	
PE(40:3p)+H	PE	4.64	0.02759	
PE(18:0p/22:4)+H	PE	2.16	0.03931	
PE(18:0/22:6)+H	PE	2.83	0.03931	
PE(18:1p/22:5)+H	PE	2.12	0.03301	
PE(18:0p/22:6)+H	PE	3.36	0.01902	
PE(18:1/22:6)+H	PE	2.79	0.02759	
PE(18:2/22:6)+H	PE	1.43	0.03301	
PE(18:1p/24:2)+H	PE	1.61	0.02759	
PE(20:0p/22:4)+H	PE	2.25	0.03931	
PE(42:4p)+H	PE	7.19	0.01568	
PE(24:0/20:4)+H	PE	2.34	0.01568	
PE(44:4)+H	PE	4.21	0.01287	
PEt(16:0/14:0)-H	PEt	0.43	0.03301	 PEt (16:0/18:1)

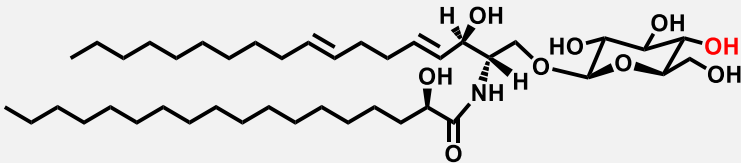
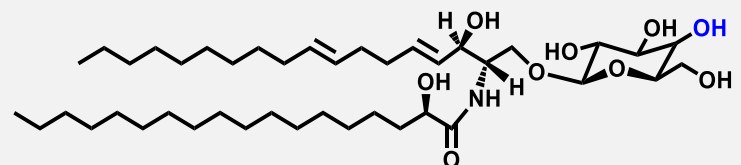
Supplementary Table 10 (cont.)

Table S11 (cont.) PG(18:0/16:0)-H	PG	1.43	0.03931	 PG (18:1/16:0)
PS(18:1/18:2)-H	PS	1.84	0.03301	 PS (18:1/18:2)
SM(d35:1)+H	SM	2.09	0.02759	 SM (d18:0/16:0)
SM(d36:0)+H	SM	3.48	0.03301	
SM(d18:0/18:1)+H	SM	4.03	0.03301	
SM(d18:1/18:1)+H	SM	2.58	0.01568	
SM(d18:2/18:1)+H	SM	0.61	0.04660	
SM(d18:1/20:1)+H	SM	2.82	0.03301	
SM(d18:1/22:0)+H	SM	2.49	0.03301	
SM(d18:1/23:0)+H	SM	2.01	0.01287	
SM(d18:1/25:1)+H	SM	1.78	0.01902	
SM(d18:1/25:2)+H	SM	2.24	0.03931	
SM(d18:1/26:0)+H	SM	1.34	0.04660	
SM(d18:1/26:1)+H	SM	1.84	0.03301	
SM(d18:1/26:4)+H	SM	96.47	0.01287	
TG(40:4e)+NH4	TG	1.93	0.04660	 TG (16:0/16:0/18:1(11E))
TG(42:3e)+NH4	TG	1.63	0.01287	
TG(43:4e)+NH4	TG	1.38	0.02759	
TG(14:0e/10:1/20:4)+NH4	TG	1.60	0.01287	
TG(16:0/16:0/17:0)+NH4	TG	3.17	0.03931	

Supplementary Table 10: Differentially expressed lipids in the myelin of IAV at day 8 vs PBS C57BL/6 mice. Lipid ion, major lipid class, fold change (IAV day 8 / PBS), p-value and representative structure are included.

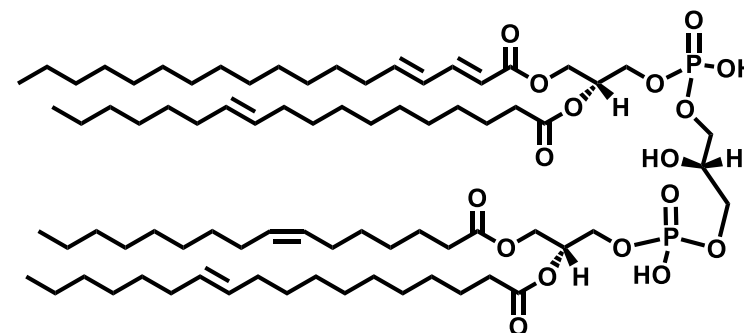
Supplementary Table 11: mPFC (PBS vs. IAV day 8)				
<i>Lipid Ion</i>	<i>Lipid Ion</i>	<i>Fold change (IAV/PBS)</i>	<i>Lipid Ion</i>	<i>Lipid Ion</i>
BisMePA(18:2p/14:0)+NH4	BisMePA	2.52	0.04331	 BisMePA (18:2/20:0)
BisMePA(18:2p/20:1)+NH4	BisMePA	1.73	0.04331	
BisMePA(18:2p/22:6)+NH4	BisMePA	1.71	0.04331	
BisMePA(42:10)+NH4	BisMePA	2.21	0.02092	
BisMePA(42:11)+NH4	BisMePA	2.13	0.02092	
BisMePE(16:0/16:0)+H	BisMePE	1.86	0.02092	 BisMePE (16:0/16:0)
BisMePE(18:0/22:6)+H	BisMePE	2.25	0.02092	
Cer(d18:0/24:1)+H	Cer	2.02	0.04331	 Cer (18:1/14:0)
Cer(d18:1/22:1)+H	Cer	1.61	0.04331	
Cer(d18:1/23:0)+H	Cer	1.55	0.04331	
Cer(d18:1/24:0)+H	Cer	1.88	0.04331	
Cer(d18:1/24:1)+H	Cer	1.71	0.04331	
Cer(d18:2/18:0)+H	Cer	2.70	0.02092	
Cer(d18:2/20:0)+H	Cer	2.43	0.02092	
Cer(d18:2/22:0)+H	Cer	2.22	0.04331	
Cer(d18:2/23:0)+H	Cer	2.26	0.04331	
CerG1(d18:1/20:0)+H	CerG1	1.81	0.04331	
CerG1(d18:1/22:1)+H	CerG1	1.60	0.04331	

Supplementary Table 11 (cont.)

CerG1(d18:1/24:0)+H	CerG1	1.95	0.04331	 Glucosylceramide (18:2/18:0)
CerG1(d18:1/24:1)+H	CerG1	1.59	0.04331	
CerG1(d18:1/24:3)+H	CerG1	1.61	0.04331	
CerG1(d18:2/18:0)+H	CerG1	2.83	0.04331	
CerG1(d18:2/25:0)+H	CerG1	2.25	0.04331	
CerG1(d22:1/24:1)+H	CerG1	2.02	0.04331	
CerG1(d36:2)+H	CerG1	1.78	0.04331	
CerG1(d41:6)+H	CerG1	2.07	0.02092	
CerG1(d42:4)+H	CerG1	1.92	0.04331	
CerG2(d18:1/24:1)+H	CerG2	1.63	0.04331	 Galactosylceramide (18:2/18:0)
CerG2(d54:5)+H	CerG2	2.28	0.04331	
CL(17:1/18:1/18:1/18:2)-H	CL	0.80	0.02092	
CL(18:1/16:1/16:0/22:6)-H	CL	2.90	0.02092	
CL(18:1/18:0/20:3/20:3)-H	CL	1.66	0.02092	
CL(18:1/18:1/18:1/22:6)-H	CL	2.20	0.02092	
CL(18:2/16:0/18:1/20:4)-H	CL	1.44	0.02092	
CL(18:2/16:1/18:2/18:1)-H	CL	1.29	0.02092	
CL(18:2/18:0/20:3/18:1)-H	CL	1.53	0.02092	
CL(18:2/18:1/16:1/18:1)-H	CL	1.43	0.04331	
CL(18:2/18:1/18:1/18:1)-H	CL	1.34	0.02092	
CL(18:2/18:1/18:1/18:2)-H	CL	1.71	0.02092	
CL(18:2/18:1/18:1/20:4)-H	CL	1.68	0.02092	
CL(18:2/20:4/16:1/20:4)-H	CL	1.60	0.02092	

Supplementary Table 11 (cont.)

CL(18:2/20:4/18:1/20:4)-H	CL	1.54	0.02092
CL(18:2/20:4/18:2/20:4)-H	CL	1.61	0.02092
CL(18:2/20:4/20:4/20:4)-H	CL	1.50	0.02092
CL(18:2/20:4/22:6/20:4)-H	CL	1.13	0.02092
CL(18:2/20:4/22:6/22:6)-H	CL	1.83	0.02092
CL(18:2/22:6/20:4/22:6)-H	CL	1.70	0.02092
CL(18:4/20:4/16:1/20:4)-H	CL	1.52	0.04331
CL(18:4/20:4/22:6/20:4)-H	CL	1.88	0.04331
CL(20:0/18:0/18:0/18:0)-H	CL	1.43	0.02092
CL(20:2/18:1/18:1/22:6)-H	CL	1.64	0.02092
CL(20:2/18:1/20:4/20:4)-H	CL	2.14	0.02092
CL(20:4/16:0/18:1/18:1)-H	CL	2.12	0.04331
CL(20:4/16:1/16:1/20:4)-H	CL	1.62	0.04331
CL(20:4/16:1/16:1/22:6)-H	CL	1.86	0.04331
CL(20:4/16:1/18:1/20:4)-H	CL	1.69	0.02092
CL(20:4/18:1/16:1/18:1)-H	CL	1.50	0.02092
CL(20:4/18:1/18:1/18:1)-H	CL	1.49	0.04331
CL(20:4/18:1/18:1/20:4)-H	CL	2.19	0.02092
CL(20:4/18:1/20:4/20:4)-H	CL	1.65	0.02092
CL(20:4/20:4/16:1/20:4)-H	CL	1.47	0.02092
CL(20:4/20:4/22:6/18:2)-H	CL	2.12	0.02092
CL(20:5/16:1/22:6/18:2)-H	CL	1.57	0.02092
CL(20:5/18:0/18:1/20:4)-H	CL	1.74	0.04331

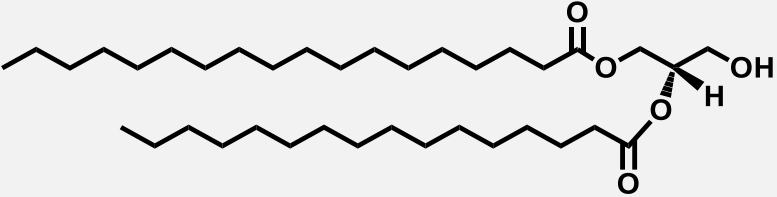


CL (18:2/18:1/16:1/18:1)

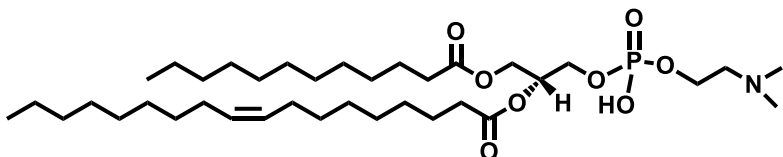
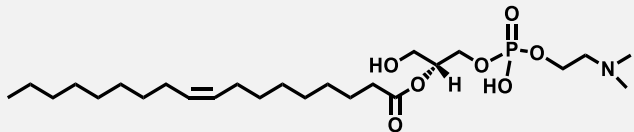
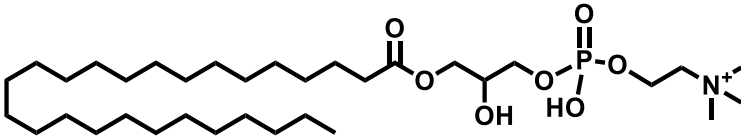
Supplementary Table 11 (cont.)

CL(20:5/20:4/18:2/20:4)-H	CL	1.83	0.02092	
CL(20:5/22:6/18:2/22:6)-H	CL	1.52	0.02092	
CL(21:0/16:0/18:1/22:6)-H	CL	1.49	0.02092	
CL(21:0/16:0/20:3/22:6)-H	CL	1.56	0.04331	
CL(21:0/22:6/16:0/18:1)-H	CL	2.49	0.02092	
CL(22:0/16:0/18:0/18:1)-H	CL	1.64	0.04331	
CL(22:1/18:0/18:0/18:0)-H	CL	1.59	0.02092	
CL(22:4/18:1/18:1/18:2)-H	CL	1.74	0.02092	
CL(22:6/16:0/16:0/16:0)-H	CL	2.04	0.04331	
CL(22:6/16:1/16:1/22:6)-H	CL	1.66	0.04331	
CL(22:6/18:0/20:0/22:4)-H	CL	1.64	0.02092	
CL(22:6/18:0/20:4/22:1)-H	CL	1.55	0.02092	
CL(22:6/18:1/16:1/18:1)-H	CL	1.65	0.02092	
CL(22:6/18:1/20:4/22:6)-H	CL	1.75	0.02092	
CL(22:6/20:4/16:1/20:4)-H	CL	1.91	0.04331	
CL(22:6/20:4/20:4/22:6)-H	CL	2.02	0.02092	
CL(22:6/22:6/22:6/18:2)-H	CL	1.71	0.02092	
CL(23:0/16:0/16:0/18:1)-H	CL	1.37	0.02092	
CL(23:0/16:0/20:4/22:6)-H	CL	1.60	0.02092	
CL(24:2/16:0/20:4/18:0)-H	CL	1.51	0.04331	
CL(77:4)-H	CL	1.47	0.04331	
CL(82:3)-H	CL	1.32	0.02092	
DG(16:0/20:4)+NH4	DG	1.78	0.02092	

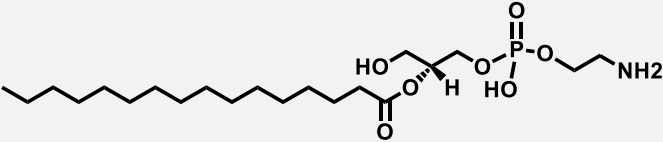
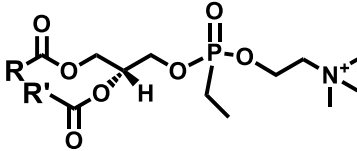
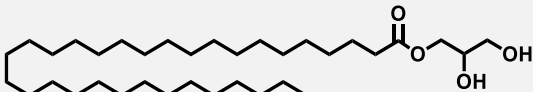
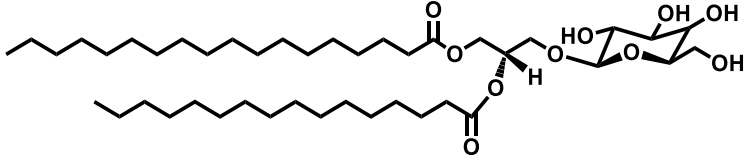
Supplementary Table 11 (cont.)

DG(16:0/22:6)+NH4	DG	2.60	0.04331	 DG (16:0/18:0)
DG(18:0/18:1)+NH4	DG	1.24	0.04331	
DG(18:0/18:2)+NH4	DG	1.92	0.02092	
DG(18:0/20:1)+NH4	DG	1.21	0.04331	
DG(18:0/20:2)+NH4	DG	2.18	0.04331	
DG(18:0/20:3)+NH4	DG	2.54	0.04331	
DG(18:0/20:4)+NH4	DG	2.08	0.02092	
DG(18:0/22:1)+NH4	DG	1.32	0.04331	
DG(18:0/22:4)+NH4	DG	2.42	0.04331	
DG(18:0/22:6)+NH4	DG	2.11	0.02092	
DG(18:1/18:1)+NH4	DG	2.67	0.02092	
DG(18:1/20:4)+NH4	DG	2.27	0.02092	
DG(18:1/22:1)+NH4	DG	3.99	0.04331	
DG(18:1/22:6)+NH4	DG	2.36	0.02092	
DG(18:1/23:0)+NH4	DG	2.41	0.02092	
DG(18:1/24:0)+NH4	DG	3.06	0.04331	
DG(18:1/24:1)+NH4	DG	1.68	0.02092	
DG(20:1/18:1)+NH4	DG	2.24	0.04331	
dMePE(16:0/18:1)-H	dMePE	1.34	0.04331	
dMePE(16:0/22:6)-H	dMePE	1.72	0.02092	
dMePE(16:0p/24:1)-H	dMePE	1.27	0.04331	
dMePE(16:1p/24:1)-H	dMePE	1.94	0.04331	
dMePE(18:0/18:1)-H	dMePE	1.31	0.04331	

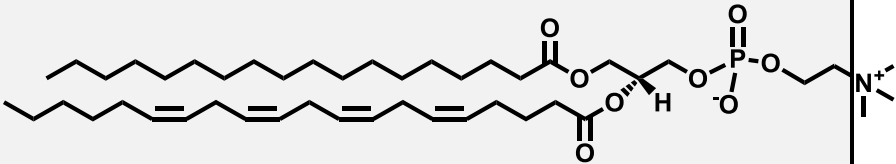
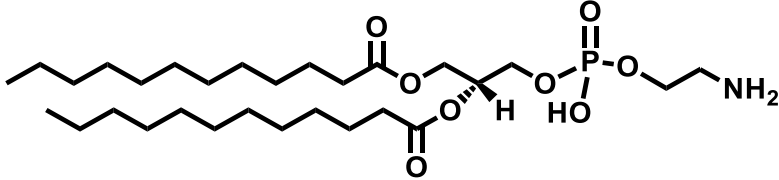
Supplementary Table 11 (cont.)

dMePE(18:0/22:5)-H	dMePE	1.43	0.02092	 dMePE (16:0/18:1)
dMePE(18:2p/20:4)-H	dMePE	1.31	0.04331	
dMePE(22:6/22:6)-H	dMePE	1.75	0.02092	
dMePE(38:0p)-H	dMePE	1.42	0.04331	
dMePE(38:4)-H	dMePE	1.41	0.02092	
dMePE(38:7)-H	dMePE	1.84	0.02092	
dMePE(54:5)-H	dMePE	1.74	0.04331	
LdMePE(18:1)-H	LdMePE	1.52	0.02092	 LdMePE (16:0/18:1)
LPC(12:0)+H	LPC	1.83	0.02092	 LPC (26:0)
LPC(16:0e)+H	LPC	1.42	0.02092	
LPC(18:0)+H	LPC	1.77	0.04331	
LPC(18:0e)+H	LPC	1.80	0.04331	
LPC(18:0p)+H	LPC	1.78	0.02092	
LPC(18:2)+H	LPC	1.80	0.04331	
LPC(22:0)+H	LPC	1.31	0.04331	
LPC(23:0)+H	LPC	1.55	0.02092	
LPC(24:0)+H	LPC	2.65	0.02092	
LPC(24:1)+H	LPC	2.77	0.02092	

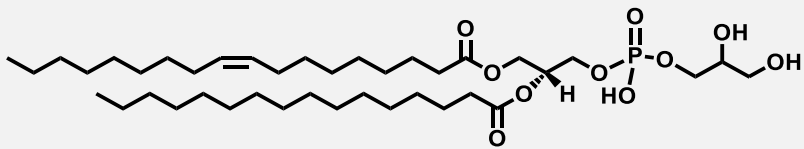
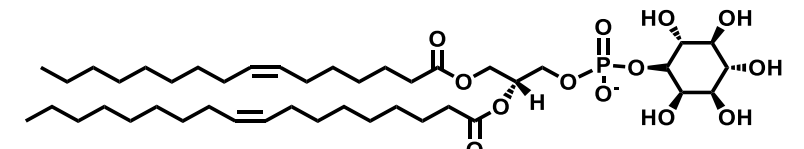
Supplementary Table 11 (cont.)

LPE(16:0)-H	LPE	0.69	0.02092	 LPE (16:0)
MePC(30:0)+NH4	MePC	1.99	0.02092	 MePC
MePC(32:3)+NH4	MePC	1.46	0.04331	
MePC(53:3)+NH4	MePC	1.79	0.04331	
MG(18:0)+H	MG	1.29	0.04331	 MG (32:0)
MG(32:0)+H	MG	1.35	0.04331	
MGDG(37:5)-H	MGDG	1.07	0.04331	 MGDG (16:0/18:0)
MGDG(39:5)-H	MGDG	1.48	0.02092	
MGDG(44:10)-H	MGDG	1.41	0.02092	
MGDG(46:4)-H	MGDG	1.38	0.02092	
PC(16:0p/22:6)+H	PC	2.23	0.04331	
PC(17:1/16:1)+H	PC	1.53	0.04331	
PC(18:1/22:6)+H	PC	1.99	0.04331	
PC(18:1p/16:0)+H	PC	1.41	0.02092	

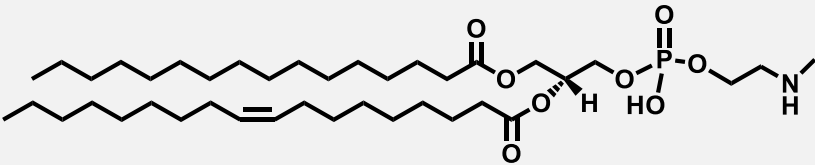
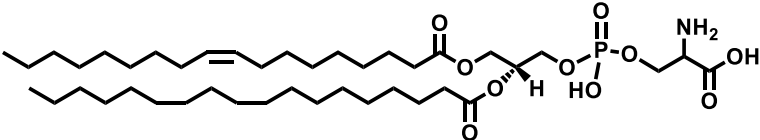
Supplementary Table 11 (cont.)

PC(20:1p/16:0)+H	PC	1.44	0.02092	 PC (18:0/20:4)
PC(23:0e)+H	PC	1.01	0.04331	
PC(31:3)+H	PC	1.33	0.04331	
PC(33:3)+H	PC	1.27	0.04331	
PC(34:2)+H	PC	1.98	0.04331	
PC(34:3)+H	PC	1.87	0.02092	
PC(34:3p)+H	PC	2.47	0.04331	
PC(36:4e)+H	PC	2.01	0.02092	
PC(36:5p)+H	PC	1.44	0.02092	
PC(38:4p)+H	PC	1.46	0.02092	
PC(38:6e)+H	PC	1.50	0.02092	
PC(40:6p)+H	PC	1.64	0.02092	
PC(40:8)+H	PC	1.51	0.04331	
PC(42:11)+H	PC	1.78	0.04331	
PC(46:5)+H	PC	2.58	0.04331	
PE(16:0/16:1)+H	PE	0.95	0.04331	 PE (12:0/12:0)
PE(18:0/18:2)+H	PE	1.61	0.04331	
PE(18:1/18:2)+H	PE	1.69	0.04331	
PE(18:1/18:3)+H	PE	1.42	0.02092	
PE(18:1/22:6)+H	PE	1.77	0.04331	
PE(18:1p/20:1)+H	PE	1.70	0.04331	
PE(18:2/20:4)+H	PE	1.27	0.04331	
PE(18:2/22:6)+H	PE	1.24	0.02092	

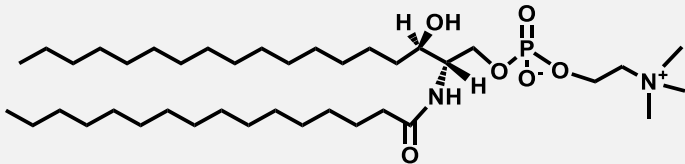
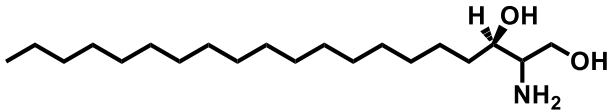
Supplementary Table 11 (cont.)

PE(18:2p/22:6)+H	PE	2.29	0.02092	
PE(18:3/22:6)+H	PE	2.06	0.04331	
PE(20:4e)+H	PE	1.17	0.04331	
PE(22:4/20:4)+H	PE	1.29	0.04331	
PE(38:4p)+H	PE	1.36	0.02092	
PE(38:5)+H	PE	1.42	0.02092	
PE(38:6e)+H	PE	2.27	0.04331	
PE(40:7)+H	PE	1.49	0.02092	
PG(12:0/14:0)-H	PG	1.54	0.04331	 PG (18:1/16:0)
PG(16:0/18:1)-H	PG	1.61	0.04331	
PG(16:0/18:2)-H	PG	1.63	0.04331	
PG(17:0/17:0)-H	PG	1.10	0.02092	
PG(18:1/18:2)-H	PG	1.41	0.02092	
PG(18:2/20:4)-H	PG	1.16	0.02092	
PI(16:0/18:1)-H	PI	1.52	0.04331	 PI (16:1/18:0)
PI(16:0/20:5)-H	PI	1.20	0.04331	
PI(16:1/18:1)-H	PI	1.52	0.02092	
PI(16:1/20:4)-H	PI	4.47	0.04331	
PI(18:1/20:4)-H	PI	1.96	0.02092	
PI(18:1/22:6)-H	PI	1.50	0.04331	
PI(18:1p/20:4)-H	PI	1.62	0.02092	
PI(18:2/20:4)-H	PI	1.62	0.02092	
PI(20:4/20:4)-H	PI	1.81	0.02092	

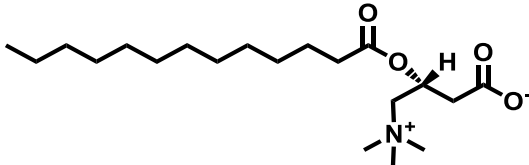
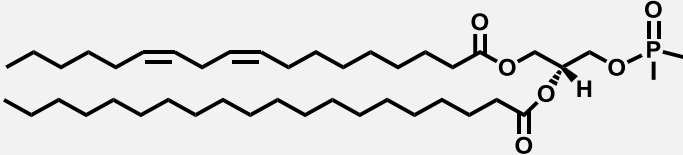
Supplementary Table 11 (cont.)

PI(20:4/22:6)-H	PI	2.30	0.02092	 PMe (16:0/18:1)
PI(37:1)-H	PI	2.21	0.02092	
PI(41:6)-H	PI	2.45	0.02092	
PMe(16:0/16:1)-H	PMe	2.20	0.02092	
PMe(16:0/18:1)-H	PMe	6.87	0.02092	
PMe(16:0/20:4)-H	PMe	9.83	0.02092	
PMe(18:0/18:1)-H	PMe	12.35	0.02092	
PS(12:0/14:0)-H	PS	1.59	0.02092	 PS (18:1/18:2)
PS(18:1/24:1)-H	PS	2.13	0.04331	
PS(20:1/18:1)-H	PS	1.34	0.02092	
PS(20:4/20:4)-H	PS	1.51	0.02092	
PS(22:6/22:6)-H	PS	1.63	0.04331	
PS(24:0/20:4)-H	PS	2.12	0.04331	
PS(37:2)-H	PS	1.37	0.04331	
PS(38:3p)-H	PS	1.55	0.04331	
PS(39:3)-H	PS	1.34	0.02092	
PS(41:5)-H	PS	1.35	0.02092	
PS(41:6)-H	PS	2.64	0.04331	
PS(45:1)-H	PS	1.43	0.04331	
SM(d18:1/22:1)+H	SM	1.49	0.02092	
SM(d37:1)+H	SM	1.68	0.04331	

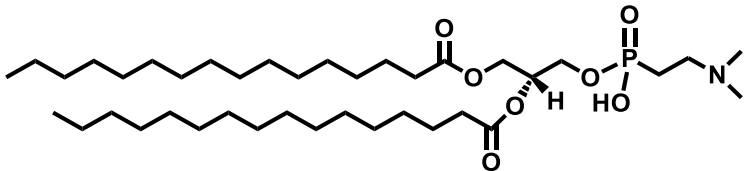
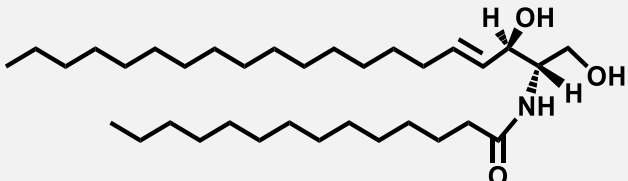
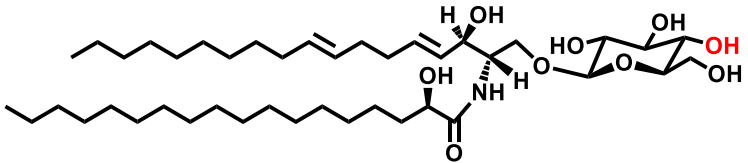
Supplementary Table 11 (cont.)

SM(d38:1)+H	SM	1.36	0.04331	 SM (d18:0/16:0)
SM(d40:2)+H	SM	2.48	0.04331	
So(d18:0)+H	So	1.62	0.02092	 So (18:0)
TG(16:1/16:1/18:2)+NH4	TG	1.16	0.02092	 TG (16:0/16:0/18:1(11E))

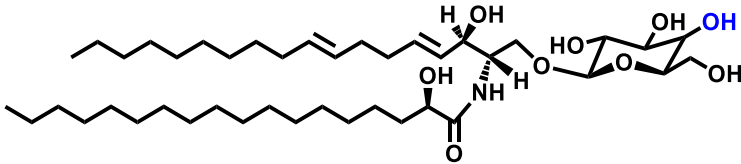
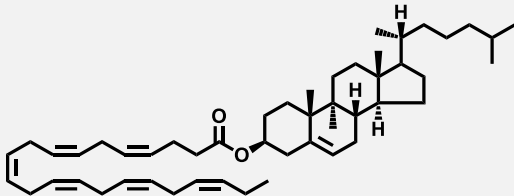
Supplementary Table 11: Differentially expressed lipids in the medial prefrontal cortex (mPFC) of IAV at day 8 vs PBS C57BL/6 mice. Lipid ion, major lipid class, fold change (IAV day 8 / PBS), p-value and representative structure are included.

Supplementary Table 12: mPFC (PBS vs. IAV day 16)				
<i>Lipid Ion</i>	<i>Lipid Class</i>	<i>Fold Change (IAV D16/PBS)</i>	<i>p-value</i>	<i>Representative Structures</i>
AcCa(12:0)+H	AcCa	1.99	0.03389	 AcCa (13:0)
AcCa(14:0)+H	AcCa	1.92	0.03389	
AcCa(14:1)+H	AcCa	1.84	0.03389	
AcCa(16:0)+H	AcCa	1.91	0.03389	
AcCa(16:1)+H	AcCa	1.86	0.03389	
AcCa(18:0)+H	AcCa	1.88	0.03389	
AcCa(18:1)+H	AcCa	2.22	0.03389	
AcCa(18:2)+H	AcCa	2.26	0.03389	
AcCa(20:1)+H	AcCa	1.62	0.03389	
AcCa(20:4)+H	AcCa	2.27	0.03389	
BisMePA(10:0p/10:4)+NH4	BisMePA	1.68	0.03389	 BisMePA (18:2/20:0)
BisMePA(16:0/20:5)+NH4	BisMePA	1.54	0.03389	
BisMePA(18:0/20:4)+NH4	BisMePA	1.60	0.03389	
BisMePA(18:0/20:5)+NH4	BisMePA	1.55	0.03389	
BisMePA(18:0/22:5)+NH4	BisMePA	1.39	0.03389	
BisMePA(18:1/20:5)+NH4	BisMePA	1.58	0.03389	
BisMePA(18:2p/16:0)+NH4	BisMePA	1.76	0.03389	
BisMePA(18:2p/18:1)+NH4	BisMePA	1.83	0.03389	
BisMePA(18:2p/20:3)+NH4	BisMePA	1.63	0.03389	
BisMePA(18:2p/22:6)+NH4	BisMePA	1.62	0.03389	
BisMePA(22:5/22:6)+NH4	BisMePA	1.77	0.03389	
BisMePA(22:6/22:6)+NH4	BisMePA	1.66	0.03389	
BisMePA(42:11)+NH4	BisMePA	1.64	0.03389	

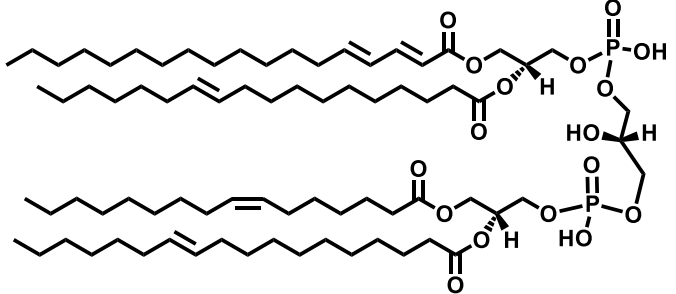
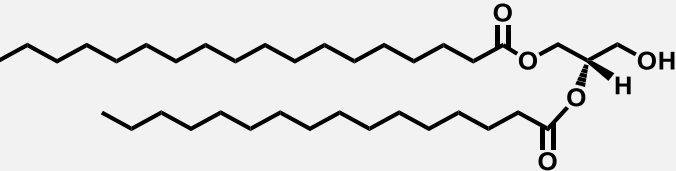
Supplementary Table 12 (cont.)

BisMePE(18:0/22:6)+H	BisMePE	2.05	0.03389	 BisMePE (16:0/16:0)
Cer(d18:0/12:0)+H	Cer	1.49	0.03389	 Cer (18:1/14:0)
Cer(d18:0/24:1)+H	Cer	2.03	0.03389	
Cer(d18:1/22:1)+H	Cer	1.65	0.03389	
Cer(d18:1/24:2)+H	Cer	1.58	0.03389	
Cer(d18:2/18:0)+H	Cer	1.75	0.03389	
Cer(d18:2/20:0)+H	Cer	2.01	0.03389	
Cer(d18:2/22:0)+H	Cer	1.73	0.03389	
Cer(d18:2/22:1)+H	Cer	1.97	0.03389	
Cer(d18:2/23:1)+H	Cer	1.80	0.03389	
Cer(d18:2/24:1)+H	Cer	2.05	0.03389	
Cer(d18:2/24:2)+H	Cer	2.06	0.03389	
CerG1(d18:0/18:0)+H	CerG1	2.23	0.03389	 Glucosylceramide (18:2/18:0)
CerG1(d18:0/24:1)+H	CerG1	1.93	0.03389	
CerG1(d18:1/16:0)+H	CerG1	1.89	0.03389	
CerG1(d18:1/18:0)+H	CerG1	1.69	0.03389	
CerG1(d18:1/18:1)+H	CerG1	1.42	0.03389	
CerG1(d18:1/22:0)+H	CerG1	1.88	0.03389	
CerG1(d18:1/22:1)+H	CerG1	1.64	0.03389	
CerG1(d18:1/23:1)+H	CerG1	1.51	0.03389	
CerG1(d18:1/24:2)+H	CerG1	1.63	0.03389	
CerG1(d18:1/24:3)+H	CerG1	1.90	0.03389	
CerG1(d18:1/26:3)+H	CerG1	1.70	0.03389	
CerG1(d18:2/18:0)+H	CerG1	2.60	0.03389	

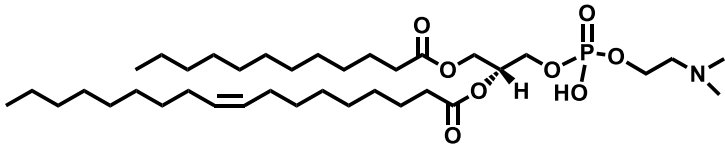
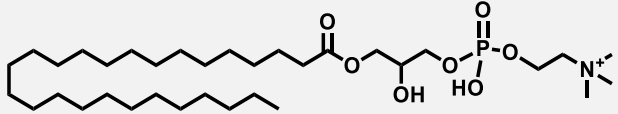
Supplementary Table 12 (cont.)

CerG1(d18:2/18:1)+H	CerG1	1.79	0.03389	 Galactosylceramide (18:2/18:0)
CerG1(d18:2/22:1)+H	CerG1	1.91	0.03389	
CerG1(d18:2/23:0)+H	CerG1	2.08	0.03389	
CerG1(d18:2/23:1)+H	CerG1	1.86	0.03389	
CerG1(d18:2/24:0)+H	CerG1	2.15	0.03389	
CerG1(d18:2/24:1)+H	CerG1	2.10	0.03389	
CerG1(d36:2)+H	CerG1	2.55	0.03389	
CerG1(d39:4)+H	CerG1	1.60	0.03389	
CerG1(d40:3)+H	CerG1	1.75	0.03389	
CerG1(d41:4)+H	CerG1	1.43	0.03389	
CerG1(d41:5)+H	CerG1	1.68	0.03389	
CerG1(d41:6)+H	CerG1	1.81	0.03389	
CerG1(d42:4)+H	CerG1	1.79	0.03389	
CerG1(d42:8)+H	CerG1	1.42	0.03389	
CerG1(d44:5)+H	CerG1	1.66	0.03389	
ChE(24:6)+NH4	ChE	0.07	0.03389	 ChE (22:6)
CL(17:1/18:2/20:4/18:1)-H	CL	1.36	0.03389	
CL(18:1/16:1/16:0/22:6)-H	CL	1.34	0.03389	
CL(18:1/18:1/18:1/22:6)-H	CL	1.70	0.03389	
CL(18:1/20:3/20:4/20:4)-H	CL	1.94	0.03389	
CL(18:2/14:0/14:0/14:0)-H	CL	0.04	0.03389	
CL(18:2/16:0/18:1/20:4)-H	CL	1.29	0.03389	
CL(18:2/20:4/18:2/20:4)-H	CL	1.49	0.03389	
CL(18:2/20:4/22:6/18:2)-H	CL	1.55	0.03389	

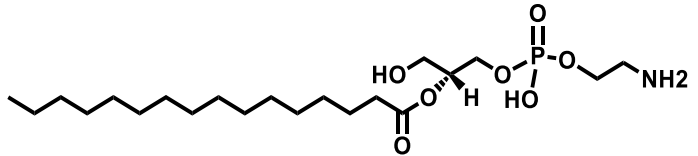
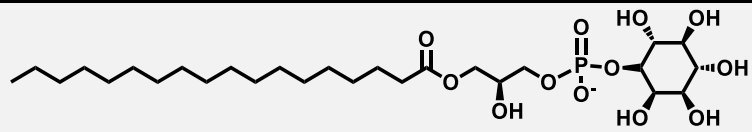
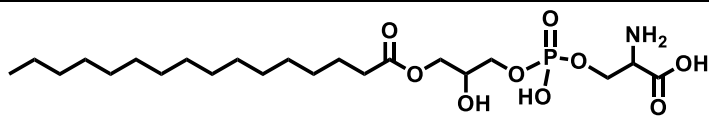
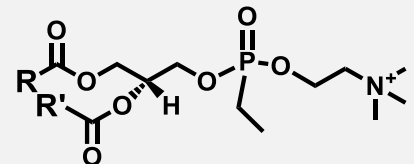
Supplementary Table 12 (cont.)

CL(18:4/22:6/20:4/22:6)-H	CL	1.27	0.03389	 CL (18:2/18:1/16:1/18:1)
CL(20:0/18:0/18:0/18:0)-H	CL	1.75	0.03389	
CL(20:2/18:1/20:4/20:4)-H	CL	1.67	0.03389	
CL(20:4/16:1/16:1/22:6)-H	CL	1.60	0.03389	
CL(20:4/18:1/18:1/20:4)-H	CL	1.89	0.03389	
CL(20:4/18:1/18:1/22:6)-H	CL	1.79	0.03389	
CL(20:4/18:1/20:4/20:4)-H	CL	1.29	0.03389	
CL(20:4/18:1/20:4/22:6)-H	CL	1.39	0.03389	
CL(20:4/20:4/16:1/20:4)-H	CL	1.39	0.03389	
CL(20:4/20:4/22:6/18:2)-H	CL	1.58	0.03389	
CL(22:1/18:0/18:0/18:0)-H	CL	1.64	0.03389	
CL(22:4/18:1/18:1/18:2)-H	CL	1.46	0.03389	
CL(22:6/16:0/20:0/20:4)-H	CL	1.39	0.03389	
CL(22:6/16:0/20:1/20:4)-H	CL	1.56	0.03389	
CL(22:6/20:4/20:4/22:6)-H	CL	1.71	0.03389	
CL(23:0/16:0/20:4/22:6)-H	CL	1.49	0.03389	
CL(75:6)-H	CL	1.01	0.03389	
DG(16:0/18:1)+NH4	DG	1.15	0.03389	 DG (16:0/18:0)
DG(16:0/20:3)+NH4	DG	1.60	0.03389	
DG(16:0/20:4)+NH4	DG	1.52	0.03389	
DG(16:0/22:6)+NH4	DG	1.59	0.03389	
DG(18:0/18:2)+NH4	DG	1.58	0.03389	
DG(18:0/20:3)+NH4	DG	1.96	0.03389	
DG(18:0/20:4)+NH4	DG	1.76	0.03389	
DG(18:0/22:1)+NH4	DG	1.10	0.03389	
DG(18:0/22:4)+NH4	DG	1.94	0.03389	
DG(18:0/22:6)+NH4	DG	2.03	0.03389	
DG(18:1/18:1)+NH4	DG	2.08	0.03389	
DG(18:1/20:4)+NH4	DG	2.27	0.03389	

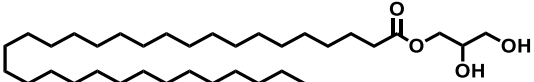
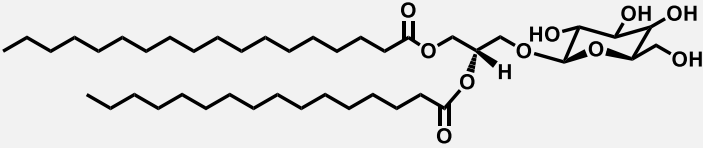
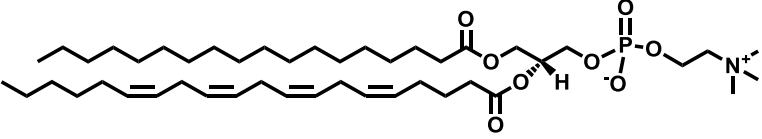
Supplementary Table 12 (cont.)

DG(18:1/22:6)+NH4	DG	1.93	0.03389	
DG(18:1/23:0)+NH4	DG	2.48	0.03389	
DG(18:1/24:0)+NH4	DG	2.12	0.03389	
DG(18:1/24:1)+NH4	DG	2.15	0.03389	
DG(20:1/18:1)+NH4	DG	1.84	0.03389	
DG(20:4/22:6)+NH4	DG	2.29	0.03389	
dMePE(12:0/12:0)-H	dMePE	0.93	0.03389	 dMePE (16:0/18:1)
dMePE(16:0/18:1)-H	dMePE	1.52	0.03389	
dMePE(16:1p/24:1)-H	dMePE	1.60	0.03389	
dMePE(18:0/16:0)-H	dMePE	1.44	0.03389	
dMePE(18:0/20:4)-H	dMePE	2.17	0.03389	
dMePE(20:4/22:6)-H	dMePE	1.50	0.03389	
dMePE(22:6/22:6)-H	dMePE	1.87	0.03389	
dMePE(52:2)-H	dMePE	1.94	0.03389	
dMePE(54:5)-H	dMePE	2.00	0.03389	
LPC(14:0)+H	LPC	1.53	0.03389	 LPC (26:0)
LPC(16:0e)+H	LPC	0.78	0.03389	
LPC(16:0p)+H	LPC	2.02	0.03389	
LPC(16:1)+H	LPC	1.70	0.03389	
LPC(18:0p)+H	LPC	1.48	0.03389	
LPC(18:1p)+H	LPC	1.31	0.03389	
LPC(18:3)+H	LPC	1.46	0.03389	
LPC(20:0)+H	LPC	1.51	0.03389	
LPC(20:1)+H	LPC	1.87	0.03389	
LPC(20:4)+H	LPC	1.27	0.03389	
LPC(22:0)+H	LPC	1.61	0.03389	
LPC(22:1)+H	LPC	2.19	0.03389	
LPC(22:6)+H	LPC	1.59	0.03389	
LPC(23:0)+H	LPC	1.88	0.03389	

Supplementary Table 12 (cont.)

LPC(24:0)+H	LPC	2.14	0.03389	 LPE (16:0)
LPC(24:1)+H	LPC	2.36	0.03389	
LPE(16:0)-H	LPE	1.12	0.03389	
LPE(18:0p)-H	LPE	0.96	0.03389	
LPE(18:1)-H	LPE	1.85	0.03389	
LPE(22:6)-H	LPE	2.21	0.03389	
LPI(18:0)-H	LPI	1.78	0.03389	 LPI (18:0)
LPS(22:6)-H	LPS	2.05	0.03389	 LPS (16:0)
MePC(30:0)+NH4	MePC	1.96	0.03389	 MePC
MePC(32:1)+NH4	MePC	1.76	0.03389	
MePC(33:4)+NH4	MePC	1.63	0.03389	
MePC(34:2)+NH4	MePC	1.57	0.03389	
MePC(38:7)+NH4	MePC	1.58	0.03389	
MePC(38:8)+NH4	MePC	1.46	0.03389	
MePC(51:2)+NH4	MePC	1.46	0.03389	
MePC(51:3)+NH4	MePC	2.37	0.03389	
MePC(53:3)+NH4	MePC	2.01	0.03389	
MG(18:2p)+H	MG	1.92	0.03389	

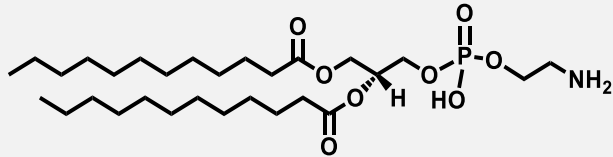
Supplementary Table 12 (cont.)

MG(32:0)+H	MG	1.74	0.03389	 MG (32:0)
MG(34:1)+H	MG	1.64	0.03389	
MGDG(15:0/22:4)-H	MGDG	1.75	0.03389	 MGDG (16:0/18:0)
MGDG(37:5)-H	MGDG	3.76	0.03389	
MGDG(39:5)-H	MGDG	1.68	0.03389	
MGDG(40:5)-H	MGDG	2.02	0.03389	
MGDG(42:7)-H	MGDG	1.44	0.03389	
MGDG(46:4)-H	MGDG	1.42	0.03389	
PC(15:0/22:6)+H	PC	1.36	0.03389	 PC (18:0/20:4)
PC(16:0/14:0)+H	PC	1.00	0.03389	
PC(16:0e/22:5)+H	PC	1.34	0.03389	
PC(16:0p/18:1)+H	PC	1.40	0.03389	
PC(16:1/13:0)+H	PC	1.31	0.03389	
PC(17:0/22:6)+H	PC	1.22	0.03389	
PC(18:0/22:4)+H	PC	1.17	0.03389	
PC(18:0p/16:0)+H	PC	1.27	0.03389	
PC(18:0p/18:0)+H	PC	1.10	0.03389	
PC(18:1/13:0)+H	PC	1.19	0.03389	
PC(18:1p/18:0)+H	PC	1.28	0.03389	
PC(19:1/18:1)+H	PC	1.40	0.03389	
PC(20:0/22:6)+H	PC	1.31	0.03389	
PC(20:0p/20:4)+H	PC	1.34	0.03389	

Supplementary Table 12 (cont.)

PC(22:0/20:3)+H	PC	1.26	0.03389
PC(24:0/20:4)+H	PC	0.80	0.03389
PC(32:4)+H	PC	1.39	0.03389
PC(33:2)+H	PC	1.24	0.03389
PC(34:1)+H	PC	1.39	0.03389
PC(34:2)+H	PC	1.47	0.03389
PC(34:4)+H	PC	0.81	0.03389
PC(36:2)+H	PC	1.37	0.03389
PC(36:4p)+H	PC	1.27	0.03389
PC(37:2)+H	PC	1.32	0.03389
PC(38:2)+H	PC	1.48	0.03389
PC(38:2e)+H	PC	1.71	0.03389
PC(38:4e)+H	PC	1.30	0.03389
PC(38:5)+H	PC	1.53	0.03389
PC(39:7)+H	PC	1.15	0.03389
PC(40:2)+H	PC	1.19	0.03389
PC(40:6)+H	PC	1.10	0.03389
PC(40:6p)+H	PC	1.43	0.03389
PC(40:7p)+H	PC	1.34	0.03389
PC(41:2)+H	PC	1.71	0.03389
PC(41:4)+H	PC	1.32	0.03389
PC(42:2)+H	PC	1.83	0.03389
PC(42:4)+H	PC	1.26	0.03389
PC(43:4)+H	PC	1.60	0.03389
PC(44:1)+H	PC	1.40	0.03389
PC(44:11)+H	PC	1.36	0.03389
PC(44:2)+H	PC	1.33	0.03389
PC(44:4)+H	PC	1.71	0.03389
PC(44:5)+H	PC	1.95	0.03389
PC(44:7)+H	PC	2.19	0.03389

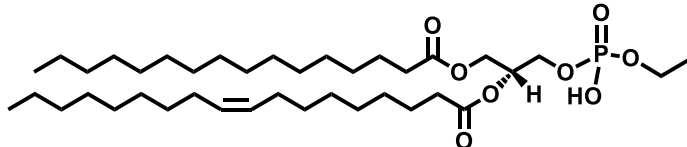
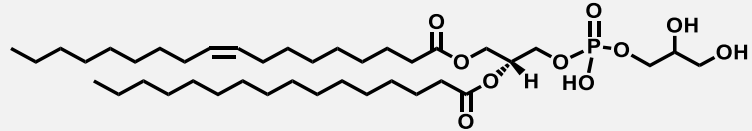
Supplementary Table 12 (cont.)

PC(45:5)+H	PC	1.96	0.03389	 PE (12:0/12:0)
PC(46:5)+H	PC	1.94	0.03389	
PE(10:0e/10:4)+H	PE	1.61	0.03389	
PE(12:0/14:0)+H	PE	0.90	0.03389	
PE(14:0/20:4)+H	PE	1.23	0.03389	
PE(14:0/22:6)+H	PE	0.91	0.03389	
PE(16:0/18:1)+H	PE	1.24	0.03389	
PE(16:0/20:1)+H	PE	1.30	0.03389	
PE(16:0/20:4)+H	PE	1.53	0.03389	
PE(16:0/22:4)+H	PE	1.54	0.03389	
PE(16:0/22:6)+H	PE	1.61	0.03389	
PE(16:0e)+H	PE	1.21	0.03389	
PE(16:0p/18:1)+H	PE	1.49	0.03389	
PE(16:0p/19:1)+H	PE	1.56	0.03389	
PE(16:0p/20:3)+H	PE	1.68	0.03389	
PE(16:0p/22:4)+H	PE	1.48	0.03389	
PE(16:0p/22:5)+H	PE	1.56	0.03389	
PE(16:1/20:4)+H	PE	1.04	0.03389	
PE(16:1/22:6)+H	PE	1.52	0.03389	
PE(17:0/18:1)+H	PE	1.31	0.03389	
PE(17:1/22:6)+H	PE	1.65	0.03389	
PE(18:0/18:1)+H	PE	1.80	0.03389	
PE(18:0/18:2)+H	PE	1.62	0.03389	
PE(18:0/20:4)+H	PE	1.40	0.03389	
PE(18:0/22:4)+H	PE	1.37	0.03389	
PE(18:0/22:6)+H	PE	1.66	0.03389	
PE(18:0p/18:1)+H	PE	1.60	0.03389	
PE(18:0p/18:2)+H	PE	1.57	0.03389	
PE(18:0p/20:3)+H	PE	0.94	0.03389	

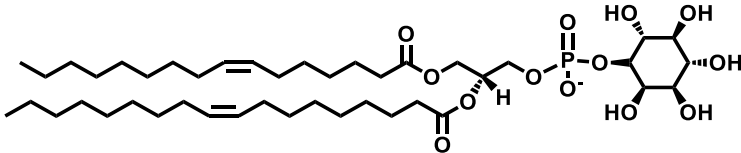
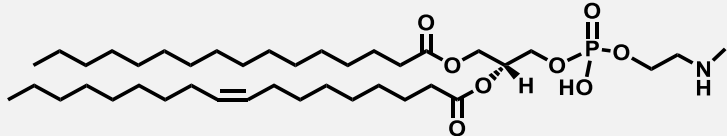
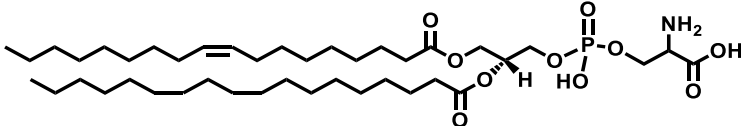
Supplementary Table 12 (cont.)

PE(18:0p/20:4)+H	PE	1.36	0.03389	
PE(18:0p/21:1)+H	PE	1.20	0.03389	
PE(18:0p/22:3)+H	PE	1.26	0.03389	
PE(18:0p/22:4)+H	PE	1.32	0.03389	
PE(18:0p/22:5)+H	PE	1.57	0.03389	
PE(18:0p/22:6)+H	PE	1.44	0.03389	
PE(18:0p/24:1)+H	PE	1.56	0.03389	
PE(18:1/18:1)+H	PE	1.58	0.03389	
PE(18:1/20:4)+H	PE	1.41	0.03389	
PE(18:1/22:1)+H	PE	1.61	0.03389	
PE(18:1/22:4)+H	PE	1.42	0.03389	
PE(18:1/22:6)+H	PE	1.67	0.03389	
PE(18:1p/17:1)+H	PE	1.55	0.03389	
PE(18:1p/18:1)+H	PE	1.77	0.03389	
PE(18:1p/19:1)+H	PE	1.35	0.03389	
PE(18:1p/20:1)+H	PE	1.98	0.03389	
PE(18:1p/20:5)+H	PE	1.34	0.03389	
PE(18:1p/22:3)+H	PE	1.48	0.03389	
PE(18:1p/22:6)+H	PE	1.46	0.03389	
PE(18:1p/23:1)+H	PE	1.46	0.03389	
PE(18:1p/24:2)+H	PE	1.31	0.03389	
PE(18:2/20:4)+H	PE	1.73	0.03389	
PE(18:2/22:6)+H	PE	2.02	0.03389	
PE(18:2p/20:4)+H	PE	1.77	0.03389	
PE(18:2p/22:6)+H	PE	1.74	0.03389	
PE(20:0/22:6)+H	PE	1.26	0.03389	
PE(20:0p/22:6)+H	PE	1.43	0.03389	
PE(20:1/18:1)+H	PE	1.62	0.03389	
PE(20:1/22:6)+H	PE	1.18	0.03389	
PE(20:1p/22:6)+H	PE	1.31	0.03389	

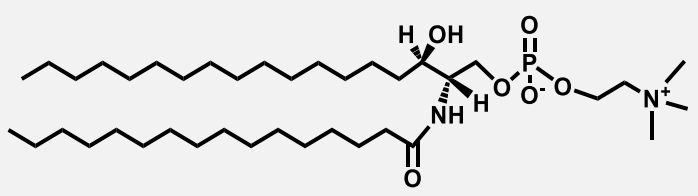
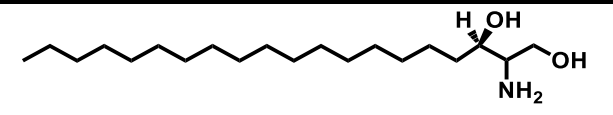
Supplementary Table 12 (cont.)

PE(20:3/22:6)+H	PE	4.60	0.03389	
PE(20:4/22:6)+H	PE	1.54	0.03389	
PE(22:4/20:4)+H	PE	1.72	0.03389	
PE(22:4/22:6)+H	PE	1.61	0.03389	
PE(22:5/22:6)+H	PE	1.68	0.03389	
PE(22:6/22:6)+H	PE	1.58	0.03389	
PE(35:0)+H	PE	1.79	0.03389	
PE(38:4e)+H	PE	1.47	0.03389	
PE(38:4p)+H	PE	1.52	0.03389	
PE(38:5)+H	PE	1.50	0.03389	
PE(38:6e)+H	PE	1.61	0.03389	
PE(40:1)+H	PE	1.59	0.03389	
PE(40:5e)+H	PE	1.55	0.03389	
PE(40:7p)+H	PE	1.52	0.03389	
PE(42:4p)+H	PE	1.32	0.03389	
PE(44:2p)+H	PE	1.64	0.03389	
PEt(42:4)-H	PEt	0.94	0.03389	 PEt (16:0/18:1)
PG(12:0/14:0)-H	PG	1.61	0.03389	 PG (18:1/16:0)
PG(16:0/16:0)-H	PG	3.49	0.03389	
PG(16:0/18:2)-H	PG	1.27	0.03389	
PG(17:0/17:0)-H	PG	1.48	0.03389	

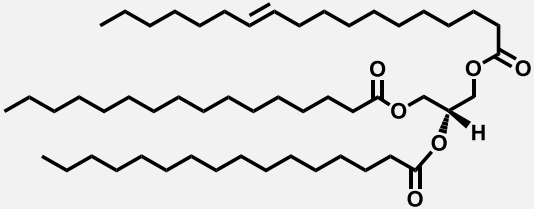
Supplementary Table 12 (cont.)

PG(18:1/18:1)-H	PG	1.91	0.03389	 PI (16:1/18:0)
PI(16:0/22:4)-H	PI	1.70	0.03389	
PI(16:1/18:1)-H	PI	1.55	0.03389	
PI(18:1/20:3)-H	PI	1.40	0.03389	
PI(18:1p/20:4)-H	PI	1.50	0.03389	
PI(35:0)-H	PI	1.74	0.03389	
PMe(16:0/16:1)-H	PMe	4.16	0.03389	 PMe (16:0/18:1)
PMe(16:0/18:1)-H	PMe	5.99	0.03389	
PMe(16:0/20:4)-H	PMe	6.80	0.03389	
PMe(18:0/18:1)-H	PMe	9.67	0.03389	
PMe(42:10)-H	PMe	5.87	0.03389	
PS(12:0/14:0)-H	PS	1.94	0.03389	 PS (18:1/18:2)
PS(18:1/22:0)-H	PS	1.00	0.03389	
PS(18:1/22:6)-H	PS	1.17	0.03389	
PS(20:0p/18:1)-H	PS	1.45	0.03389	
PS(20:1/18:1)-H	PS	1.36	0.03389	
PS(20:4/22:6)-H	PS	2.05	0.03389	
PS(22:4/22:6)-H	PS	1.79	0.03389	
PS(22:6/22:6)-H	PS	1.53	0.03389	
PS(24:0/20:4)-H	PS	1.58	0.03389	
PS(30:3)-H	PS	1.85	0.03389	
PS(37:0)-H	PS	1.24	0.03389	
PS(39:1)-H	PS	1.43	0.03389	
PS(39:3)-H	PS	1.40	0.03389	
PS(39:4)-H	PS	1.80	0.03389	

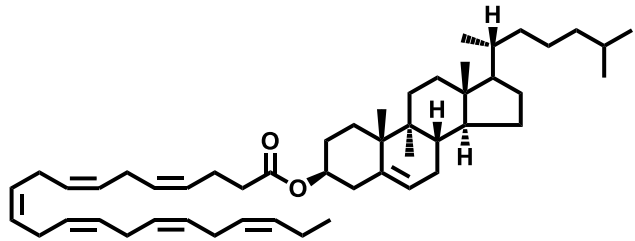
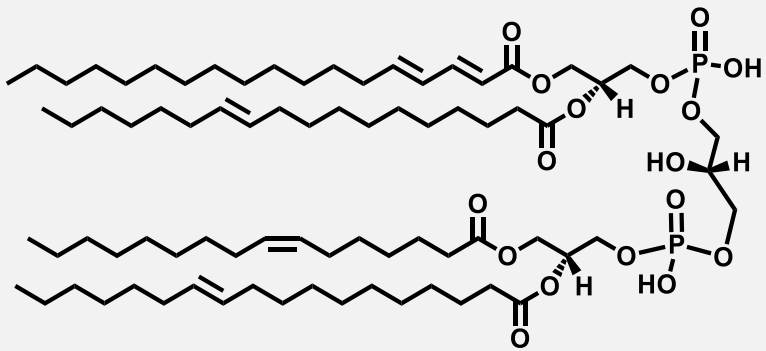
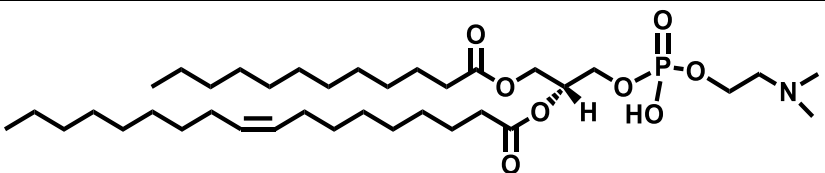
Supplementary Table 12 (cont.)

PS(41:0)-H	PS	1.42	0.03389	
PS(41:1)-H	PS	1.58	0.03389	
PS(43:6)-H	PS	1.32	0.03389	
PS(45:1)-H	PS	1.38	0.03389	
SM(d18:0/18:1)+H	SM	1.39	0.03389	 SM (d18:0/16:0)
SM(d18:0/23:1)+H	SM	1.56	0.03389	
SM(d18:1/14:0)+H	SM	1.73	0.03389	
SM(d18:1/18:3)+H	SM	1.91	0.03389	
SM(d18:1/20:1)+H	SM	1.61	0.03389	
SM(d18:1/22:1)+H	SM	1.47	0.03389	
SM(d18:1/23:1)+H	SM	1.42	0.03389	
SM(d18:1/24:1)+H	SM	1.48	0.03389	
SM(d18:1/24:2)+H	SM	1.81	0.03389	
SM(d18:1/26:3)+H	SM	1.55	0.03389	
SM(d18:1/28:1)+H	SM	1.96	0.03389	
SM(d18:2/23:0)+H	SM	1.65	0.03389	
SM(d33:1)+H	SM	1.91	0.03389	
SM(d34:0)+H	SM	1.59	0.03389	
SM(d34:1)+H	SM	1.56	0.03389	
SM(d35:2)+H	SM	1.31	0.03389	
SM(d36:0)+H	SM	1.58	0.03389	
SM(d36:1)+H	SM	1.63	0.03389	
SM(d38:1)+H	SM	1.75	0.03389	
SM(d40:1)+H	SM	1.57	0.03389	
SM(d40:2)+H	SM	3.01	0.03389	
SM(d44:3)+H	SM	1.80	0.03389	
So(d18:1)+H	So	1.56	0.03389	 So (18:0)

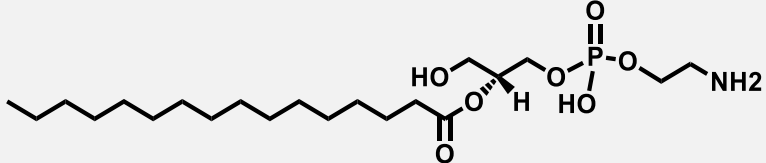
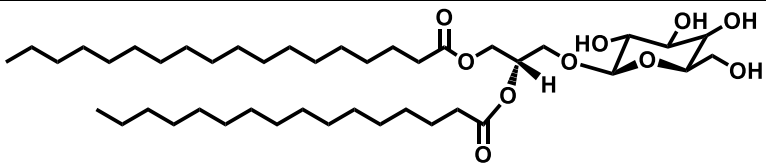
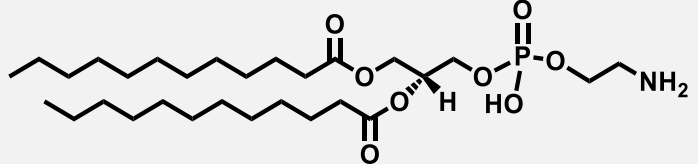
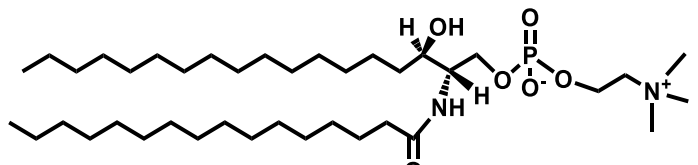
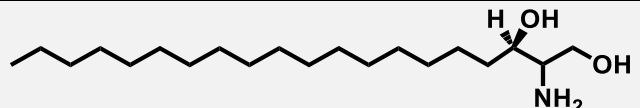
Supplementary Table 12 (cont.)

TG(15:0/16:0/22:6)+NH4	TG	1.11	0.03389	 TG (16:0/16:0/18:1(11E))
TG(16:0/18:1/18:1)+NH4	TG	1.29	0.03389	
TG(16:0e/16:0/22:6)+NH4	TG	1.29	0.03389	
TG(18:0/18:0/20:1)+NH4	TG	1.14	0.03389	
TG(18:0/18:0/22:4)+NH4	TG	0.95	0.03389	
TG(18:0/18:1/20:0)+NH4	TG	1.53	0.03389	
TG(8:0/8:0/10:0)+NH4	TG	1.71	0.03389	

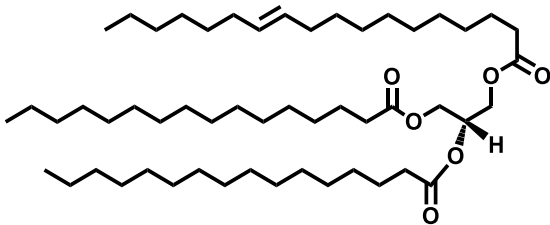
Supplementary Table 12: Differentially expressed lipids in the medial prefrontal cortex (mPFC) of IAV at day 16 vs PBS C57BL/6 mice. Lipid ion, major lipid class, fold change (IAV day 16 / PBS), p-value and representative structure are included.

Supplementary Table 13: mPFC (IAV day 8 vs. IAV day 16)				
Lipid Ion	Lipid Class	Fold Change (IAV D16/IAV D8)	p-value	Representative Structures
ChE(24:6)+NH4	ChE	0.02	0.03389	 ChE (22:6)
CL(18:2/14:0/14:0/14:0)-H	CL	0.01	0.03389	 CL (18:2/18:1/16:1/18:1)
CL(20:4/18:1/16:1/18:1)-H	CL	1.01	0.03389	
dMePE(16:0/18:1)-H	dMePE	1.14	0.03389	 dMePE (16:0/18:1)

Supplementary Table 13 (cont.)

LPE(18:0p)-H	LPE	0.50	0.03389	 LPE (16:0)
MGDG(15:0/22:4)-H	MGDG	0.93	0.03389	 MGDG (16:0/18:0)
PE(17:0/18:1)+H	PE	1.50	0.03389	 PE (12:0/12:0)
PE(18:1/18:3)+H	PE	1.08	0.03389	
PE(35:0)+H	PE	1.05	0.03389	
SM(d35:2)+H	SM	1.26	0.03389	 SM (d18:0/16:0)
SM(d37:1)+H	SM	1.13	0.03389	
SM(d40:7)+H	SM	1.24	0.03389	
So(d18:0)+H	So	1.05	0.03389	 So (18:0)

Supplementary Table 13 (cont.)

TG(16:1/16:1/16:1)+NH4	TG	1.07	0.03389	 TG (16:0/16:0/18:1(11E))
TG(16:1/16:1/18:2)+NH4	TG	1.11	0.03389	
TG(68:4)+NH4	TG	0.63	0.03389	

Supplementary Table 13: Differentially expressed lipids in the medial prefrontal cortex (mPFC) of IAV at day 8 vs IAV day 16 C57BL/6 mice. Lipid ion, major lipid class, fold change (IAV day 16 / IAV day 8), p-value and representative structure are included.

APPENDIX F: SUPPLEMENTARY MOVIES – ANTHRACYCLINE DERIVATIVES INHIBIT CARDIAC CYP2J2

The supplementary files “Supplementary_Movie_1.mp4,” “Supplementary_Movie_2.mp4,” and “Supplementary_Movie_3.mp4” contain movies for supplemental movies referenced in Chapter 3 and Appendix D in the dissertation. All Supplementary Movies are listed below.

Supplementary Movie 1. *CYP2J2 Active Site.* Visualization of CYP2J2 active site. Red indicates the heme iron, and yellow indicates the lipid membrane CYP2J2 is embedded in.

Supplementary Movie 2. *Aclarubicin Docking to CYP2J2.* All poses of aclarubicin from 4 docking clusters in relation to the heme iron. Each subsequent MD simulation was run for 50 ns.

Supplementary Movie 3. *Occupation of CYP2J2 Active Site.* Visualization of aclarubicin occupying active site of CYP2J2 in relation to the heme iron. Red indicates the heme iron, and yellow indicates the lipid membrane CYP2J2 is embedded in.

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