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Title Page

Search for Novel Anti-Inflammatory Drugs by Screening Chiral Conjugates of the Endocannabinoid – NAGly

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Abstract

Throughout human history, people have been plagued by inflammation. Diseases that are characterized by chronic inflammation cause immense pain and suffering for those afflicted by them. These conditions have been especially difficult to develop effective treatments for, primarily due to the complexity of the inflammatory response. Marijuana, or *Cannabis sativa L.*, has been documented numerous times in recorded history for its ability to alleviate the symptoms of inflammation. Recent discoveries have revealed that the underlying connection responsible for the anti-inflammatory activities of cannabinoids lies in the endogenous cannabinoid system. Specifically, evidence suggests that one function of N-arachidonyl-glycine (NAGly) is to induce the resolution of inflammation. In this project, chiral analogs of NAGly were synthesized and screened to determine the role of chirality in our inflammatory model. To that end, D-EMA-2 (18:2) and L-EMA-2 (18:2) were selected for comparison. The results confirmed the stereo-selectivity, previously noted by Burstein and colleagues, favoring the D-isomers over the L-isomers. Therefore, further research projects seeking to design new pharmaceuticals should develop larger drug libraries of elmiric acids focusing on the D-isomers.

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Preface

The goal of this project was to discover novel anti-inflammatory agents based on NAGly, using a rational drug design approach. The list of the compounds as well as the logic for their selection will be detailed in the experimental design at the end of the Background section. However, it will first be necessary to cover all the background information that was required to undertake this project. The following section begins with a brief chronology of crucial events that lead to the discovery of the endocannabinoid system. After a detailed overview of the physiological and biochemical concepts that contribute to the inflammation, contemporary theories that attempt to explain the mechanisms responsible for the inflammatory effects of endocannabinoids will be presented. The rationale for the compounds chosen should be highly intuitive following this detailed review.

Background

Modern medicine (or most specifically "Western Medicine") is one most astonishing achievements in the history of our species. It is the culmination of scientific and technological progress applied towards a singular purpose: to understand and manipulate the biological malfunctions of the human body that occur in response to injury or disease. Modern therapeutic techniques utilize both surgical and pharmaceutical means in order to relieve symptoms, and in some cases, even restore "normal" function. Vaccinations and pharmaceuticals have facilitated the eradication, or at least suppression, of many of mankind's most vile biological threats. Life-threatening injuries and debilitating conditions, which would have meant certain death in the past, are now curable using routine procedures. These "simple" surgeries would not be possible without anesthesia, advanced surgical technology, and well-trained professionals.

The multidisciplinary field of modern medicine is indeed impressive; however, it is hardly a new concept. Almost every society throughout human history has had some sort of medical professional. These "doctors" were often viewed as mystical, and their potions used to heal usually were considered magical. In reality, their "magic" usually boiled down to the profound effects that some naturally occurring substances can have on human physiology. The drugs in any given potion were bound to be acquired from local plants and animals considered to be poisonous. Pharmacologically active compounds found in nature have been used by humans for thousands of years, and those origins have also provided the foundation upon which modern medicine is built. It is, therefore, crucial to look at the achievements of the past in order to fully appreciate the developments of the present.

Medical and Scientific History of Cannabis

Chronology of Ancient Scripture

Marijuana, or *Cannabis sativa L.*, has been one of the most extensively cultivated plants throughout human history[11, 18]. Appreciated primarily for its durable fibers, the earliest evidence of human use arguably dates back over 20,000 years[11]. Although the following chronology is limited to the medical uses and physiological effects of cannabis, the material-based value of the plant should not disregarded. In fact, from colonial times up until the 20th century, hemp was among the most abundant (and lucrative) crops grown in the United States. Medicinally, preparations containing cannabis have been used to treat many diverse conditions ranging from depression to kidney stones[11]. Despite the vast array of applications cited throughout ancient literature, only a select few are repeatedly affirmed. The documentation presented below illustrates reported uses of cannabis as a treatment for, among other things, inflammation (often referred to as "rheumatism").

The earliest accredited account of the pharmacological effects of cannabis dates back to approximately 2700 BCE. This is found in a text from ancient China, known as the "Pentsao Chin", which was written under Emperor Shun Nung. In one of the earliest pharmacopoeias known to man, cannabis is recommended for the treatment of many diseases including: constipation, malaria, gout, rheumatism, menstrual cramps, and poor memory. Several other sources reference marijuana dating back before the Common Era. The most definitive of these originate from ancient India, Egypt, and Assyria. The original version of the Atharva Veda is thought to have been written sometime between 2000 BCE and 1400 BCE in India. In this document, cannabis (or "bhang") was described as one of the five sacred plants of India[11]. Another record, found in the Egyptian pharmacopoeia "Ebers Papyrus" (circa 1500 BCE), suggests the use of cannabis as an analgesic. Around 650 BCE, the Assyrian ruler, Assurbanipal, recommended broad medical applications for the plant including: depression, impotence, kidney stones, swelling and bruising. Although these suggested treatments are by no means identical, they do illuminate some of the underlying biological activities of cannabinoids that will be explored in later sections.

Evidence of therapeutic use of cannabis first appears in Europe during the Greco-Roman era. *De Materia Medica* is, perhaps, the best source for ancient knowledge of the medicinal qualities of plants. Almost 2000 years ago, in 70 CE, Pedanius Dioscorides compiled one of the most complete pharmacopoeias in human history (rivaled only by those produced in the modern era). Kannabis agria (or cannabis root) was recommended by Dioscorides as a treatment for inflammation, edema, and joint pain[11]. His description, translated into English, is as follows: "The root being sodden, and so laid on hath ye force to assuage inflammations and to dissolve Oedemata, and to disperse ye obdurate matter about ye joints"[8]. During the dark ages, much of the ancient wisdom of the Greeks was lost. Not surprisingly, there is little documentation of medical use of cannabis from that time period in Europe. Interest in marijuana-based treatments seemed to reignite in post-Renaissance Europe, specifically in the British Empire. In the middle of the 17th century, British herbalist Nicholas Culpeper recognized the therapeutic potential of cannabis and administered it to his patients suffering from chronic inflammation. In *The English Physitian*, Culpeper reiterates the benefits previously reported by Dioscorides and, even earlier, in the Pentsao Chin. Minimal progress was made on the subject, however, until the Industrial Revolution. By the mid 1800's, marijuana was formally recognized for its medical value associated with inflammation.

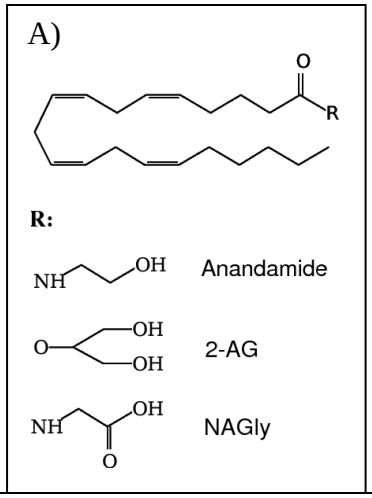
Modern or Western medicine came into existence due to the technological and scientific progress of the 19th century. Far from being the major social stigma that marijuana is today, cannabis or hemp was among the major crops grown in the United States at that time[9]. In 1850, marijuana was officially added to the US Pharmacopoeia as a treatment for nausea, rheumatism, and labor pains[15]. However, the advent of the hypodermic syringe facilitated the use of injectable opiates. Morphine and its derivatives quickly overshadowed cannabis as the analgesic of choice for severe pain. Finally, in 1894, the advent of Aspirin nearly eliminated the use of cannabis as an analgesic. Over the course of the next half century, social and political forces overrode the scientific and medical foundations of therapeutic cannabis. Leading to the 1937 Marijuana Tax Act, which formally made cannabis and all its constituents federally regulated[11]. Finally, due to propaganda based on scientific fallacies, marijuana was removed from the US Pharmacopoeia in 1941[9]. Despite the unfounded regulation in the US, research has continued internationally.

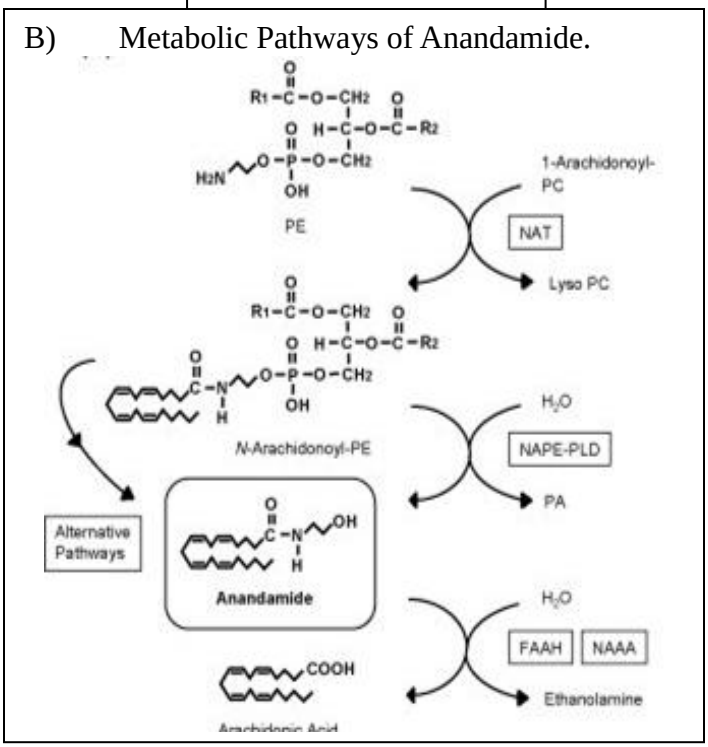
Modern Progress: Identification of Exogenous and Endogenous Cannabinoids

In 1963, cannabidiol (or CBD) was the first of many chemicals identified that would become known as "Cannabinoids". It was isolated by Mechoulam and colleagues at the Hebrew University of Jerusalem[13]. The following year, Mechoulam's group isolated tetrahydrocannabinol and determined its structure[14]. Δ^9-tetrahydrocannabinol, more commonly known as Δ^9-THC, is the primary psychoactive substance responsible for the "high" experienced by the marijuana user. The broad-spectrum of effects from cannabis reported throughout history (detailed in the chronology), are clearly not the results of this one chemical alone. In fact, there are as many as 538 natural compounds found in marijuana[9]. Of these, over 100 are unique to *Cannabis sativa L.*, therefore classified as cannabinoids. 25 years after Δ^9-THC was first identified, its biological target was finally uncovered. In 1988, the first cannabinoid receptor – CB1 – was discovered by a postdoc in Mechoulam's lab named Bill Devane[6]. Logically, the presence of a CB receptor indicated that there must be endogenous cannabinoids found in humans, which bind to that receptor. The first of these endocannabinoids to be discovered was *N*-arachidonylethanolamide, or anandamide[7]. The structure, shown in Figure 1A, was elucidated in 1992 by none other than Devane. The following year a second distinct receptor - CB2 - was found expressed not in the brain, but throughout the body. Progress greatly accelerated following these breakthroughs, and by the mid 1990's the therapeutic value of endocannabinoids in the treatment of inflammation was indisputable.

The documentation of ancient use guided the early scientific investigators to explore the effects of cannabinoids. Since cannabis was repeatedly recommended for the treatment of inflammation, it is not surprising that modern research has sought to explain its relationship with the inflammatory response. Following the identification of anandamide, another endogenous ligand of CB1 was isolated: 2-arachidonyl glycerol (2-AG). Despite the presence of endocannabinoids that bind to CB1/CB2, activation of these receptors does not account for the anti-inflammatory effects of cannabis. Research on the subject has been limited to THC analogs that target these pathways, yet Burstein and colleagues[6] have shown that the resolution of inflammation occurs independantly of CB1 or CB2. Most likely, the anti-inflamamtory effect of cannabis is a result of one of the hundreds of other naturally occuring cannabinoids.

Figure 1: Major Endocannabinoids – Structure & Biosythesis





A) Structures of major endocannabinoids. The different endocannabinoids vary depending on which functional group (R) is coupled with arachidonic acid (top structure). In the cause of NAGly, R is a glycine residue.

B) Metabolic pathways of anandamide are complicated by the alternative pathway of its synthesis. More importantly is the degradation pathways, mediated by fatty acid amide hydrolase (FAAH) that breaks anandamide down into arachidonic acid and ethanolamine. NAGly, unlike 2-AG and anandamide, is not

Jun Wang and Natsuo Ueda. Biology of endocannabinoid synthesis system, Prostaglandins & Other Lipid Mediators. *New Intercellular Lipid Mediators and Their Receptors*. 89: 112-119, 2009.

The Inflammatory Response

Physiology of Inflammation

Inflammation is a vital function of the immune system. It accelerates the effects of the immunological response by facilitating the access of cellular components of the immune system, such as macrophages, to their target sites. Despite this essential benefit, inflammation causes many adverse side effects that can have devastating repercussions. Although the damage resulting from these detrimental effects is typically reversible, excessive elicitation of the inflammatory response can cause permanent damage (e.g. in patients suffering from chronic inflammation). In cases of acute inflammation, on the other hand, the affected tissues are usually repaired by the natural healing process. Inflammation (both acute and chronic) is characterized by alterations in the function of smooth muscle cells causing vasodilatation and increased vascular permeability. These changes allow for the migration of phagocytes to the target site resulting in phagocytosis of the instigating agent[17]. There are many different triggers that can elicit this response including: cellular fragments released by simple trauma, immune complexes, and bacterial and viral byproducts recognized by macrophages[17]. The vast array of stimuli that induce inflammation has been described as a "tangled-web" of affecting molecules. Because there are so many different pathways that can cause inflammation, it is very difficult to develop selective treatments.

Overall, the inflammatory response is crucial for our survival, however it must be kept in check. The endogenous regulators of this process sometimes fail to do so. The consequences that result are painful and potentially debilitating. Diseases characterized by chronic inflammation are extremely difficult to treat effectively. In fact, common anti-inflammatory drugs are often ineffective. Some cases of chronic inflammation have even been reported, in which non-steroidal anti-inflammatory drugs (NSAIDs) actually made the condition worse[17]. Discovering new pharmaceuticals is critical for patients such as these. Because of the complexities involved with both the initiation and regulation of inflammation, a complete explanation of it is beyond the scope of this paper. However, the following section will contain pertinent information regarding molecular pathways involved with the inflammatory response.

Overview of the Biochemistry of Inflammation

Inflammation, as previously mentioned, may be induced by many different stimuli. Regardless of the cause, the biochemical cascades that follow are highly conserved. They usually occur within various components of the immune system, utilizing byproducts and side reactions of other immunological functions[17]. Mediators produced by these pathways fall into two major categories based on their effect; either pro-inflammatory or pro-resolution (i.e. anti-inflammatory). These compounds include peptides, cellular fragments, and other small signaling molecules. There are many effectors that play an important role in the process, but a complete explanation of all of them is not necessary to understand the results and implications of this project (for more details see Whicher and Evans 1992). Despite the fact that inflammation is a multi-functional response that requires the synchronized activation of many different cellular components, the remainder of this paper will focus in on a family of compounds called prostaglandins (PGs).

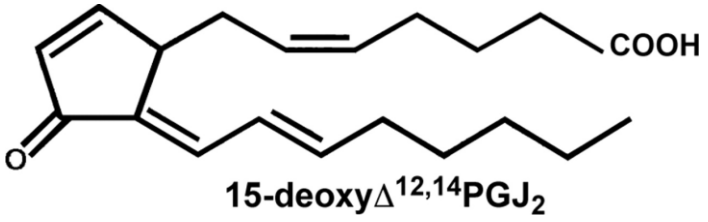
PGs are small, lipid-based signaling compounds ubiquitous to the inflammatory response. There are many sub-types that exhibit varying effects on inflammation. All PGs are synthesized by cylcooxygenase (COX) enzymes from free arachidonic acid liberated from cell membranes during both acute and chronic inflammation (shown in Figure 2B). PGs are divided into many different families based on the structure and function. The PGH series (denoted "other prostaglandins" in Figure 2B) is broken down into PGE and PGI which are pro-inflammatory. NSAIDs resolve inflammation by inhibiting the enzymatic activity of both COX-1 and COX-2. This therapeutic approach is effective because it prevents the production of PGs.

However, not all PGs exhibit the pro-inflammatory effects of the PGH derivatives (e.g. PGE). In fact, the PGD family has been linked to the resolution of inflammation, specifically 15d-PGJ_2 (structure shown in Figure 2A)[16]. This compound is produced, in vivo, by the dehydration of PGD_2 (see Figure 2B). It is thought to be synthesized by the same enzymes responsible for producing PGD_2[1,10,16]. Regulation of the inflammatory response is achieved endogenously by the selective synthesis of 15d-PGJ_2.

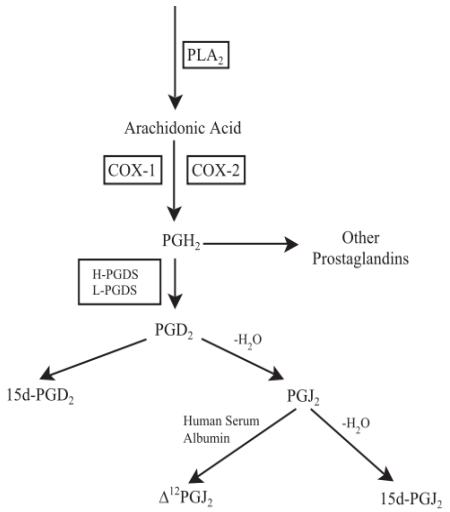
Exactly how this is accomplished is not yet fully understood. The ambiguity arises from the fact that COX-2 has been shown to produce PGE during the initiation phase of inflammation, and PGD during the resolution phase[1,10]. Even though the underlying basis for this process is remains a mystery, 15d-PGJ_2 levels are widely accepted as a useful model to test anti-inflammatory drugs both in vitro and in vivo[4]. Unlike COX-inhibitors, NAGly selectively increases the production of 15d-PGJ_2. This novel therapeutic approach provided the framework for experiments presented in the following investigation.

Figure 2: 15d-PGJ_2

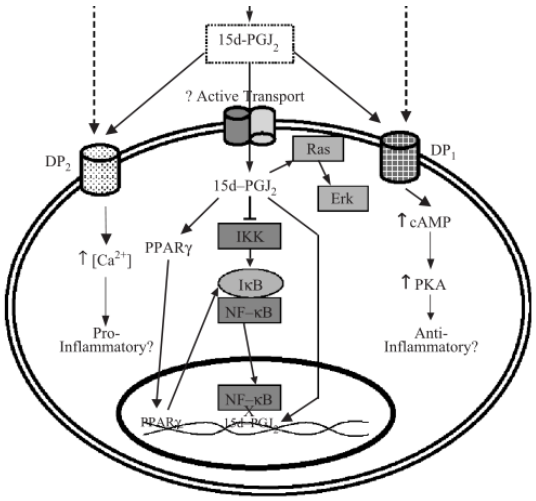
A) Chemical Structure of 15d-PGJ_2.



B) Biosynthetic Pathway of 15d-PGJ_2.



C) Mechanism of 15d-PGJ_2 Activity.



A) Chemical structure of 15d-PGJ_2.

B) Arachidonic acid, released from cell membranes, is targeted by COX enzymes to yield prostaglandins. In addition to producing the pro-inflammatory "other prostaglandins", the PGD derived PGJ_2 series is another down-stream product of this pathway.

C) Multiple pathways are postulated to explain the actions of 15d-PGJ_2 are illustrated by this diagram. Perhaps it undergoes active transport by some unknown extracellular transporter (? Active Transport), or else it goes through the same sites as PGD_2 (i.e. DP_2). Once inside the cell, knockout of NF-kB is achieved either by activation its inhibitor (IkB) through IKK or by activating PPAR-gamma. The inactivation of NF-kB leads to the resolution of inflammation.

J.U. Scher, M.H. Pillinger. 15d-PGJ2: Anti-inflammatory effects of Prostaglandins. *Clinical Immunology*. 114, 2005.

Experimental Design: Outline of Project Goals and Expectation

This project was designed to explore the pathway, by which NAGly induces the resolution of inflammation. Elucidating the way the body naturally regulates the inflammatory response will facilitate the development of more effective anti-inflammatory drugs that have few side effects. The connection between NAGly and 15d-PGJ_2 provides new insight into this process. To expand our understanding of this important interaction, synthetic analogs of NAGly were investigated in this project.

NAGly & Elmiric Acids: A Novel Class of Anti-Inflammatory Drugs

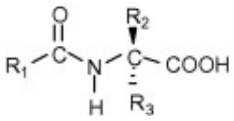
Although the absolute mechanism underlying the biological effects of NAGly is not certain, there are a few plausible explanations. One promising hypothesis, postulated by Burstein and colleagues, involves the activation an extracellular G-protein – GPR-18[4,5]. Interest in this receptor was sparked by the discovery that NAGly is the endogenous ligand for GPR-18 by Kohno and colleagues[12]. Along with the fact that GPR-18 is primarily expressed by immune cells, this evidence provides solid support for Burstein's theory[4,5,12]. Although not entirely conclusive, this receptor-mediated mechanism is the best available option to explain the anti-inflammatory effects of NAGly and the elmiric acids.

Elmiric acid describes any chemical composed of a fatty acid bonded to the nitrogen of an amino acid (see Figure 3A). The structures of the compounds selected for this project are shown in Figure 3B and C. The amino acid (alanine) was chosen because it has a chiral center. In order to demonstrate the stereo-selective nature of the anti-inflammatory action of elmiric acids, the activities of the D and L isomers were compared. Linoleic acid (18:2) was chosen to replace arachidonic acid (20:4), which is the fatty acid component of NAGly. With the removal of 2 out of 4 double bonds, linoleoyl dirivatives are far more stable and have been shown to maintain similar efficacy[*]. Alanine was selected for the amino acid moiety because it is the simplest analog of glycine containing a chiral center. The variation between the D and L isomers lies in the orientation of the methyl group (structures shown in Figure 3B and 3C).

[*] Burstein unpublished data.

Figure 3: Structure of Elmiric Acids

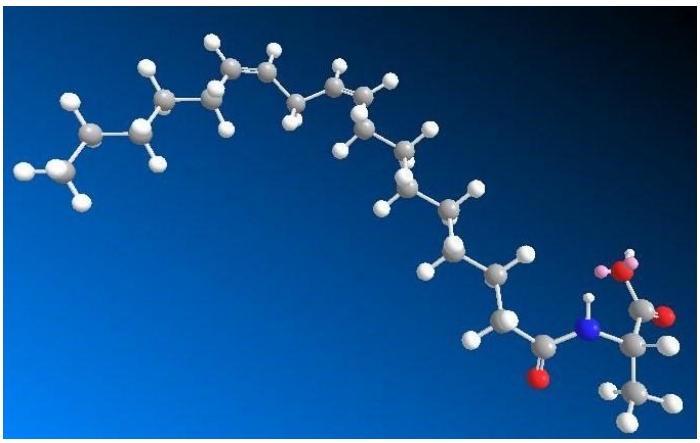
A) Genera Structure of Elmiric Acids.



R_1 = long chain saturated or unsaturated alkyl group.

R_2 or R_3 = various side chain or ring structures with or without heteroatom

B)



C)

A) The general form of elmiric acids is depicted with R groups representing the variable regions. In the case of the compounds synthesized in this project, $R_1 = C_{17}H_{34}$; in identities of R_2 and R_3 define the D and L isomers.

B) L-EMA-2 (18:2) or *N*-linoleoyl-L-alanine. R_2 = H and $R_3 = CH_3$.

C) D-EMA-2 (18:2) or *N*-linoleoyl-D-alanine. $R_2 = CH_3$ and R_3 = H.

Burstein SH, et al. Potential anti-inflammatory actions of the elmiric (lipoamino) acids. *Bioorg. Med. Chem.* 15:3345-3355, 2007.

Materials and Methods

Chemistry Procedures

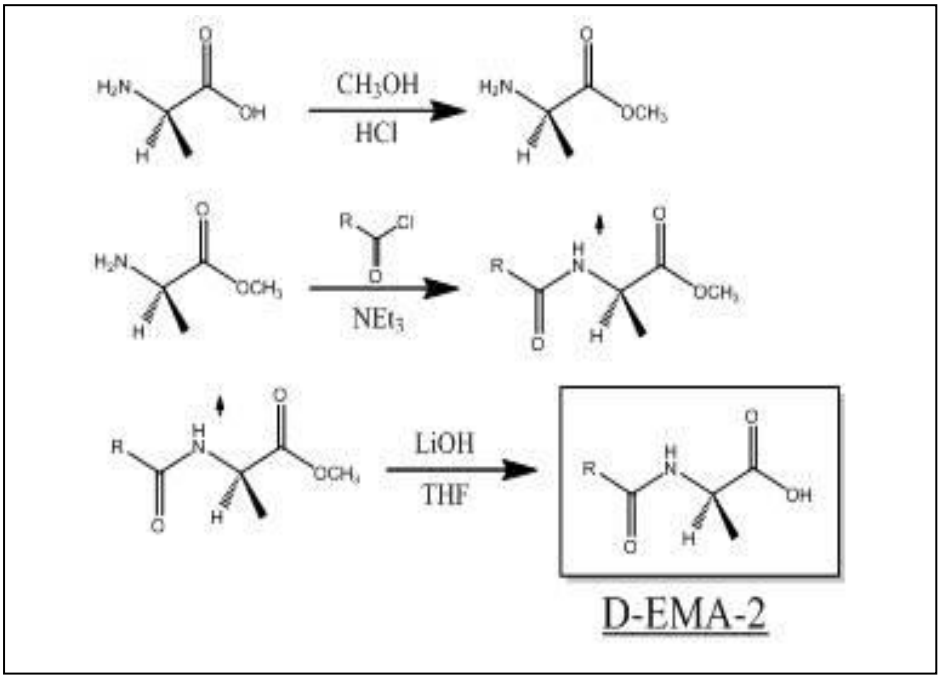
Synthetic Procedure

D-EMA-2 (18:2) and L-EMA-2 (18:2) were synthesized from chirally pure D-alanine and L-alanine, respectively. The procedure for L-EMA-2 (18:2) is identical to that used for D-EMA-2 (18:2), which is shown in Scheme 1. The amino acid was esterified by refluxing in saturated methanol/HCl for 6 hours. The alanine methyl ester was then dissolved in a mixture of methylene chloride and triethylamine (NEt_3). A solution of linoleoyl chloride diluted in methylene chloride was added drop-wise and allowed to react at room temperature for 20 hours. The coupled product was extracted with ethyl acetate and washed with 1N HCl, sodium bicarbonate, and water. In the last step, the crude material was saponified by refluxing for 4 hours in a solution of LiOH and THF yielding the final product.

Chemical Analysis

TLC was used to confirm the presence of the target compound prior to purification. The sample was separated on 100uM Whatman 60A TLC-plates and compared against a known compound, EMA-1 (18:1), as a standard. The solvent used for separation contained 10% methanol and 90% methylene chloride. The plate was then developed using iodine vapor. Appreciable quantities of purified products were harvested after separation using 500uM Whatman 60A plates. The chemical identities were confirmed by mass spectroscopy (MS) and proton nuclear magnetic resonance (H^1-NMR).

Scheme 1: Synthesis



Description:

The synthetic procedure for L-EMA-2 (18:2) is identical to that shown for D-EMA-2 (18:2), except the starting reactant is L-alanine instead of D-alanine. The process consisted of 3 reactions as follows. The first step involves the methyl esterification of the amino acid to protect the COOH group. This facilitates the coupling of linoleoyl chloride selectively to the N of the protected alanine. Finally the target compound is obtained through the saponification of the ester back to the COOH form.

Biology Procedures

Cell Culture Techniques

RAW264.7 macrophage cells are commonly used as a model of inflammation[4]. They were, therefore, used to measure the effects of the elmiric acids synthesized for this project. The cultures were grown in approximately 15mL of DMEM media containing 5% serum incubated at 37^{o}C and 5% CO_2. Since RAW cells adhere to the bottom of the flask, harvesting them was simply done by scraping them from the surface. Once the cells were suspended into the media, the contents of the flask was transferred into a 15mL centrifuge tube and spun for 5 minutes at 1250 rpm. The media was then aspirated off, leaving just the pellet of cells in the tube, which was then re-suspended in about 1mL of media. Finally, a new flask was seeded using a drop of the concentrated cells and 15mL of fresh media. Within the 1mL of media there were as many as 20,000,000 cells, so dilutions were made in order to carry out the assay described below.

Treatment of RAW Cells and Assay Protocols

48 well plates were seeded with 20,000 RAW cells / 500uL media and incubated for 20 hrs at 37oC and 5% CO2. After washing, 500uL of serum free DMEM media was added to each well. Cells were treated with each elmiric acid at 5 concentrations using dilutions of 1/3 (0.1uM, 0.3uM, 1.0uM, 3.0uM, and 10.0uM). Each dose was administered in triplicate (N=3) to provide reliable statistics. These concentrations were chosen because NAGly is effective starting around 1uM. The expectation, therefore, was that D-EMA-2 (18:2) would have a similar effective dose. 5 uL of LPS was then added to stimulate the inflammatory response from the RAW cells. After treatment, the cells were incubated for 2 hours. Media was then harvested, centrifuged and 50uL aliquots were assayed by ELISA with PGJ enzyme immunoassay kits. In order to normalize the PGJ results, the remaining cells were counted by MTT cell proliferation assay.

Results

Chemical Identification and Purification

Mass Spectroscopy and H^1-NMR

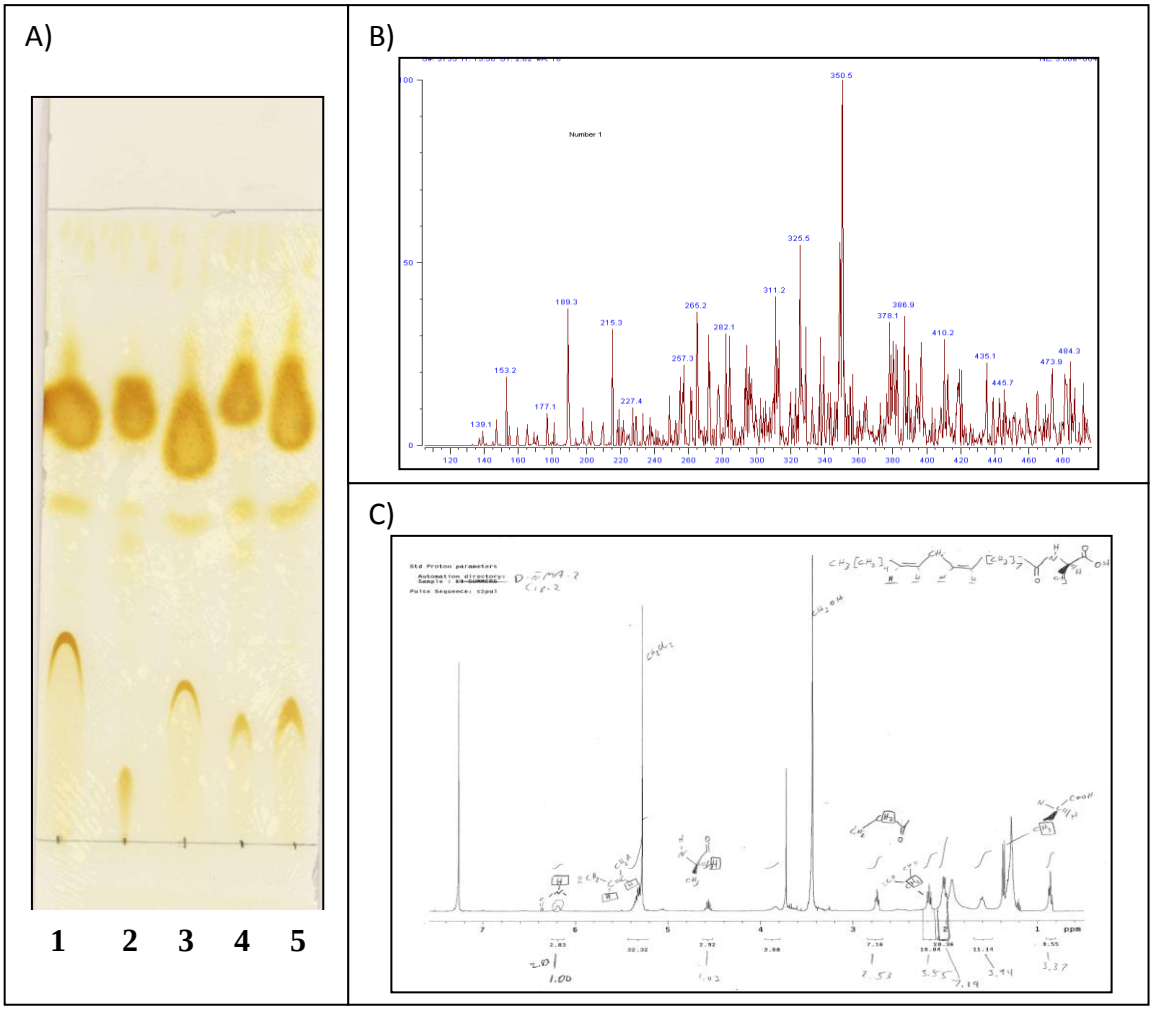
To learn more about the inflammatory effects of NAGly, two compounds were synthesized as described in the previous section (see Scheme 1). Before the assays could be preformed, the chemical identity of each one had to be confirmed. Initially, the crude synthetic products were compared against a standard on a TLC plate using a solvent containing 90% CH_2Cl_2 and 10% CH_3OH (see Figure 4A). Further analysis by mass spectroscopy is shown for D-EMA-2 (18:2) in Figure 4B. The expected (M+H) peak appears at 350.5, strongly supporting the presence of the correct final product (MW = 351.5g/mol). Due to the large amount of noise present in this scan, it was necessary to obtain more concrete evidence of the correct identity.

The additional results from the H^1-NMR provided the support needed. The scan shown in Figure 4B accounted for all of the primary functional groups found within D-EMA-2 (18:2). Because neither of these techniques detects stereochemistry, the scans of L-EMA-2 (18:2) were nearly identical to those shown in Figure 4B and 4C; so they were therefore omitted. Together, these scans provide enough certainty of the correct chemical identity to move on and screen for the biological activity. Next, appreciable amounts of each compound were obtained as follows.

Preparative Thin Layer Chromatography (Prep-TLC)

In order to obtain enough of each compound to accurately measure the mass (at least 10 mg), several prep-TLC plates were run the purified products were recollected. 27.6mg of D-EMA-2 (18:2) and 47.1mg of L-EMA-2 (18:2) were recovered and dissolved in 1mL of DMSO. From these concentrations, appropriate dilutions were done in order to prepare 500uL of each treatments ranging from 0.1uM to 10uM. The results of the screening, shown in the following section, demonstrate the role of chirality in our model system. The 0.1uM - 10uM range proved successful in generating a dose-response curve for the effective compounds.

Figure 4: Spectroscopy



A) Thin layer chromatography comparing the crude products of 4 different syntheses. **1** and 2 are both L-EMA-2 (18:2); 3 is the standard provided by Dr Burstein D-EMA-2 (18:1); 4 and 5 are both D-EMA-2 (18:2). 1 and 5 were selected for purification and use for the screening.
B) Mass spectroscopy result contains the major peak of 350.5 confirming the compound has the appropriate molecular weight.
C) Work up of H^1-NMR demonstrates the presence of the primary functional group expected for the target compound.

PGJ and MTT Assay Results

To quantify the anti-inflammatory effects of the elmiric acids synthesized in this project, RAW264.7 cells were treated with the dilutions prepared. The 15d-PGJ2 concentration was measured using ELISA, and the cells were counted by MTT. The data obtained from these asssays (shown in Table 1) illustrates the effectiveness of the D-EMA-2(18:2) and L-EMA-2(18:2). The D-isomer analog of NAGly demonstrated significantly greater efficacy than its L counterpart. This reveals the stereo-chemical preference in favor of D-isomers, which is an important feature of the anti-inflammatory pathway that is currently under investigation. The results in Table 1 were calculated from the raw PGJ data and used to generate the graphs in Figure 5. The concentration of 15d-PGJ2 was normalized for the cell count determined by MTT, in order to account for the potential change in the total number of cells per well. Recall that 20,000 cells were added to each well; however, the MTT cell proliferation assay proved this number did not remain constant. In fact, when high levels of the drugs were applied to the cells, there was a significant decrease in the cell counts. Therefore, the concentration of 15d-PGJ2 alone does not accurately reflect the cellular activity, and normalizing the data is necessary. Once the PGJ level / cell number values are calculated along with a standard deviation, statistical analysis (ANOVA) can be used to determine significant results.

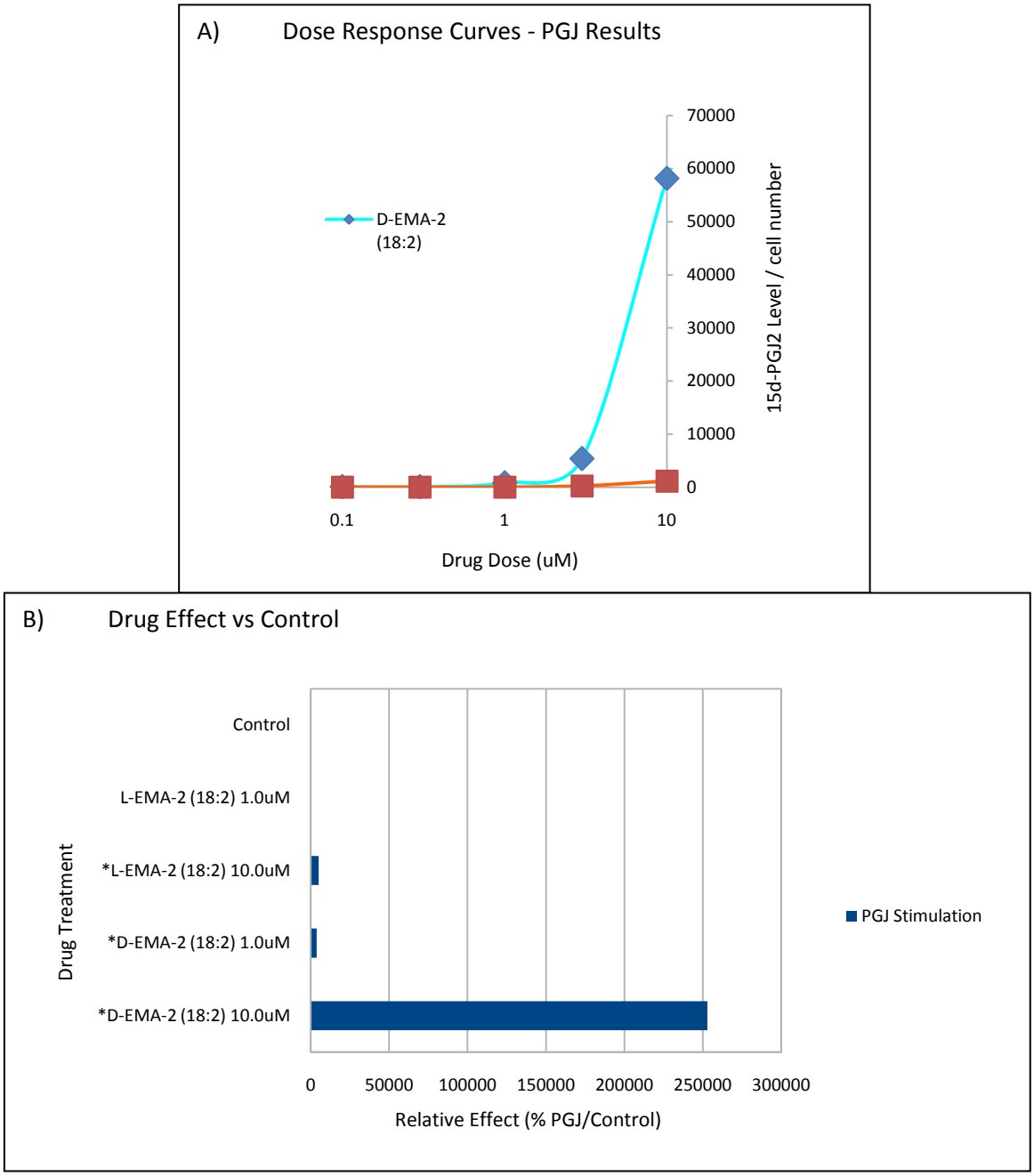
Table 1: PGJ Data & Statistical Analysis

Elmiric Acid (Treatment)	Drug Dose (uM)	[15d-PDJ2] (pg/mL)	PGJ Level / cell number	+/- SD	P value (n=3)
D-EMA-2 (18:2)					
	0.1	76	72	5	0.17
	0.3	101	74	1	0.13
	1	575	872	13	0.04
	3	1822	5377	409	0.02
	10	17757	58164	11579	0.01
L-EMA-2 (18:2)					
	0.1	15	24	4	0.37
	0.3	16	29	4	0.04
	1	33	44	5	0.38
	3	179	261	25	0.07
	10	648	1168	48	0.03
Control					
	DMSO	23	23	4	

Description:

Data used to generate the dose response curves in Figure 5. P-values under 0.05 indicate statistically significant results compared to the activity recorded for the Control (DMSO).

Figure 5: PGJ Assay Results



A) Dose response curves of D-EMA-2 (18:2) and L-EMA-2 (18:2) using [15d-PGJ_2]/cell number as the normalized measure of effect. At a dose of 0.1uM neither compound exhibits anti-inflammatory effect. D-EMA-2 (18:2) shows a dramatic increase in the production of 15d-PGJ_2 beginning around 1.0uM. The distinction becomes far more pronounced at 10.0uM.

B) To further illustrate this point, PGJ stimulation or Relative Effect was calculated to take into account the minimal levels of 15d-PGJ_2 produced by the Control (DMSO).

Discussion

The Counterintuitive Role of Chirality in our Anti-inflammatory Model

Astonishingly, D-EMA-2 (18:2) increased the prostaglandin concentrations dramatically more than L-EMA-2 (18:2) in every treatment given. The first statistically significant increase occurs for the unnatural D-isomer at a dose of only 1.0uM. On the other hand, the naturally derived L-isomer exhibits comparable effects at a dose of 10.0uM, 10-fold greater than its counterpart. The majority of the amino acids in the human body are the L-isomers, while their counterpart D forms are only found naturally in certain bacteria. The results of this project demonstrate the chiral selectivity of our anti-inflammatory model. The role of chirality is counterintuitive in that the favorable ligands contain the unnatural amino acids (i.e. D-isomers).

Possibilities for Future Research

Future research projects into the anti-inflammatory effects of elmiric acids could build on my results by further employing the chiral approach. Additional screens should include compounds with multiple functional groups attached to the methyl group of alanine. For example a prospective study could be focused on aromatic substitutions such as phenylalanine and tyrosine, which have both shown efficacy in previous experiments[†]. The structural similarity between the phenyl and phenol moieties provides an illuminating comparison, since the chemical properties of phenols are dramatically different from phenyl side chains. The results of a study such as this could provide greater insight into the electrochemical nature underlying the interaction of the elmiric acids, presumably, with GPR-18. Therefore, uncovering the details of this receptor-ligand bond will lead to the development of novel anti-inflammatory treatments inspired by the activity of NAGly.

[†] Burstein unpublished data.

Pharmaceutical Implications: Potential Development of Novel Anti-Inflammatory Drugs

Unlike the common treatment such as NSAIDs, which completely inhibit a multi-functional enzyme causing numerous side effects, anti-inflammatory drugs derived from NAGly induce the endogenous pathways to signal the resolution of inflammation. Although this research project presents only preliminary findings, the evidence is certainly compelling enough to initiate further studies. And, perhaps, this new approach to treating inflammation will lead to novel pharmaceuticals in the future that are highly effective and safe alternatives to the currently used techniques.

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