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## Potential anti-inflammatory actions of the elmiric (lipoamino) acids

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### Abstract

A library of amino acid-fatty acid conjugates (elmiric acids) was synthesized and evaluated for activity as potential anti-inflammatory agents. The compounds were tested *in vitro* for their effects on cell proliferation and prostaglandin production and compared with their effects on *in vivo* models of inflammation. LPS stimulated RAW 267.4 mouse macrophage cells was the *in vitro* model and phorbol ester-induced mouse ear edema served as the principal *in vivo* model. The prostaglandin responses were found to be strongly dependent on the nature of the fatty acid part of the molecule. Polyunsaturated acid conjugates produced a marked increase in media levels of i15-deoxy-PGJ<sub>2</sub> with minimal effects on PGE production. It is reported in the literature that prostaglandin ratios in which the J series predominates over the E series promote the resolution of inflammatory conditions. Several of the elmiric acids tested here produced such favorable ratios suggesting that their potential anti-inflammatory activity occurs *via* a novel mechanism of action. The ear edema assay results were generally in agreement with the prostaglandin assay findings indicating a connection between them.

### Keywords

endocannabinoid; anti-inflammatory; ear edema; prostaglandin

### 1. Introduction

Acid congeners of anandamide or lipoamino acids, here named elmiric acids, exist as endogenous substances and may have a role in regulating tissue levels of anandamide<sup>1</sup>. This hypothesis received support from a study that showed that such a substance, N-arachidonylglycine (NAGly, Fig.1), is indeed an endogenous constituent of many tissues and occurs at higher concentrations than anandamide<sup>2</sup>. An interesting property of NAGly is its potent inhibitory effect on FAAH, the enzyme primarily responsible for the termination of anandamide action *via* hydrolysis. As could be expected, NAGly treatment in both *in vitro* and

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*in vivo* models, leads to a robust increase in anandamide concentrations<sup>3</sup>. It was also observed that several of the elmiric acids profoundly reversed the prostaglandin (PG) profile from a pro-inflammatory to an anti-inflammatory state in a cell culture model. This has formed the basis for a hypothesis on the mechanism for the potential anti-inflammatory action of these compounds. At this point, it is assumed that these two effects occur independently of one another, however, the possibility that the amidase inhibition and PG ratio effects are somehow interrelated cannot be ruled out.

The older literature on this topic is mainly concerned with lipoamino acids of bacterial origin<sup>4–8</sup>. These involve amino acid conjugation with complex and unusual fatty acids and little is known about their function in bacteria. More recently, attention has been given to the lipoamino acids present in mammalian species in part because of their possible relationships to the endocannabinoids. The origin of NAGly *in vivo* is not well understood, however, it is an endogenous substance found in rat brain and other sites that occurs in amounts greater than the closely related endocannabinoid, anandamide<sup>2</sup>. In this same report, it was suggested that NAGly might have analgesic properties similar to those reported for anandamide<sup>9,10</sup> but would be inactive in assays for psychotropic action such as the "ring test"<sup>11</sup>. The latter was in agreement with a report showing a lack of affinity by NAGly for the cannabinoid receptor, CB1<sup>12</sup>. This activity profile is reminiscent of that observed for THC-11-oic acid and its analog ajulemic acid<sup>13</sup>. Based on preliminary data, NAGly appears to be the endogenous ligand for the orphan G-protein-coupled receptor GPR18<sup>14,15</sup>. Like anandamide, NAGly is also a substrate for COX-2 giving rise to amino acid conjugates of the prostaglandins<sup>16</sup>. Other amino acid conjugates have been found in diverse tissues<sup>17</sup> and N-arachidonoyl-L-serine has recently been isolated from rat<sup>17</sup> and from bovine brain and reported to have vasodilatory effects in rat mesenteric arteries<sup>18</sup>. Finally, NAGly inhibits the glycine transporter, GLYT2a, which is expressed in pre synaptic glycinergic neurons<sup>19</sup>.

We have defined the term elmiric acids as compounds that conform to the general structure shown in Figure 2 for which a short hand nomenclature system is proposed. Using this system N-arachidonoylglycine would be written as: EMA-1 (20:4). EMA stands for elmiric acid; each amino acid constituent is assigned a number, e.g. 1=glycine; 2=alanine, etc. The identity of the acyl substituent is indicated in parentheses; e.g. (20:4)=arachidonoyl; (16:0)=palmitoyl, etc. This system would also accommodate any number of unnatural amino acids. We are proposing this nomenclature to simplify the naming of these compounds; it has not been approved or adopted by any official body.

In this report, we have screened *in vitro* a library of elmiric acids consisting of glycine and l-alanine conjugates with a series of ten fatty acids. The objective was to determine whether any of these compounds are candidates for further study as potential anti-inflammatory agents. Positive findings would support further studies in which the amino acid moieties would be expanded not only to include naturally occurring molecules but also synthetic examples. The ultimate goal is to discover a potent and efficacious molecule for the treatment of conditions characterized by acute and chronic inflammation.

## 2. Chemistry

Several of the elmiric acids were synthesized in the university laboratories using the procedures described below and shown in Scheme 1. Briefly this involved condensation of the acid chloride with the amino acid methyl ester in the presence of triethylamine followed by saponification with lithium hydroxide to yield the elmiric acid. However, the majority of the compounds came from a commercially available library of endocannabinoids obtained from Biomol International LP (Plymouth Meeting PA 19462) that contains sixty examples, half of which would fall into the category of elmiric acids. Table 1 is a partial list of these compounds. They

are composed of glycine, L-alanine and  $\gamma$ -aminobutyric acid (GABA) conjugates with ten different naturally occurring long-chain fatty acids ranging from palmitic to docosahexaenoic acid.

### 3. Results and discussion

#### 3.1. Effects on cell proliferation

Initially, the library shown in Table 1 was screened for anti-proliferative activity in an *in vitro* assay using the mouse macrophage cell line RAW264.7 as the drug target. Table 2 shows the results of this study in which each compound was tested at three concentrations, 0.1, 1.0, and 10  $\mu$ M, and compared with vehicle treated cells as a control. Based on these limited data, it is not possible to assign a rank order for this group of substances, however, it is clear that many produced around 90% reduction of cell numbers with increasing concentrations between 1.0 to 10  $\mu$ M. In the glycine series this amounted to 6 out of 10 compounds whereas in the l-alanine series 9 out of 10 compounds were inhibitory at 10 $\mu$ M. A cross series comparison showed general agreement with one exception, namely N-palmitoyl glycine, which was inactive while its l-alanine counterpart showed 89% inhibition at 10 $\mu$ M. Palmitoyl glycine is an abundant component of the lipid fraction from rat skin and has significant effects on calcium levels in F11 cells, considered to be a model for dorsal root ganglion neurons (Bradshaw et al., unpublished data). It also can reduce heat stimulated firing of nociceptive neurons. The results obtained with the  $\gamma$ -aminobutyric acid (GABA) conjugates were similar to most of the other elmiric acids tested (data not shown).

#### 3.2. Anti-inflammatory action *in vitro*

Data obtained with selected examples of elmiric acids from our library are shown in Table 3. These were chosen to provide a variety of fatty acid conjugates with either glycine or alanine to test for a possible structure-activity relationship. With the latter, examples of both enantiomers were included to detect possible stereoselectivity. For comparison, the widely used NSAID naproxen, a COX inhibitor, was included. All of the elmiric acids were screened at two concentrations, 1 and 10  $\mu$ M. A clear pattern emerged from this experiment in which compounds with saturated fatty acids have little or no effect on the ratio of PGJ/PGE while all of those with polyunsaturated fatty acids induced large increases. The control substance naproxen failed to cause a measurable increase even at a concentration of 50 $\mu$ M. This suggests a novel mechanism of action for the active elmiric acids. A modest degree of stereoselectivity was observed between the alanine containing D and L-EMA-2(20:4) isomers; interestingly, the D isomer was somewhat more active. Unlike the cell proliferation assay shown above, a possible structure-activity pattern seems to be present, suggesting that the PGJ/PGE ratio in RAW cells may be a useful preliminary screening procedure to identify anti-inflammatory activity.

Several literature reports suggest that an elevation of tissue concentrations of 15d-PGJ<sub>2</sub> is associated with the resolution of an inflammatory condition<sup>20–23</sup>. This is thought to come about through the binding and activation of the transcription factor PPAR- $\gamma$  followed by increased expression of anti-inflammatory factors. Previous studies in our lab showed that the synthetic cannabinoid analog ajulemic acid elevates i15d-PGJ<sub>2</sub> in similar models, i.e. LPS-challenged fibroblast-like synovial cells (Stebulis et al. unpublished data).

The murine macrophage cell line RAW 264.7 has been used widely as a model for inflammatory responses *in vitro*. Moreover, we and others have reported data on cannabinoid induced responses in these cells<sup>24–26</sup>. LPS is a potent inducer of inflammatory responses including, the arachidonic acid cascade, and has been used in a wide variety of models including some used to evaluate drug candidates. Our model consisted of a cultured monolayer of RAW

cells in which media concentrations of prostaglandins were measured by ELISA following LPS stimulation. The use of immunoassay procedures always raises questions of specificity. Therefore, we have indicated that we measured immunoreactive prostaglandin as defined by the cross reactivities of the antiserum used by the manufacturer. It is also necessary to monitor PGE<sub>2</sub> concentrations in our model since they may increase as well. In view of the fact that PGE<sub>2</sub> is a pro inflammatory eicosanoid, a sharp increase in its level following drug treatment would possibly offset any benefit resulting from an increase in PGJ concentration. It is suggested that PGJ/PGE ratio measurements may be a useful initial test for anti inflammatory activity.

### 3.3. Anti-inflammatory effects *in vivo*

*In vivo* data on a small group of the elmiric acids have been obtained and are shown in Figures 3–7. These specific examples were chosen on the basis of their responses in the *in vitro* assay for the PGJ/PGE in mouse macrophages (Table 3). Both active and inactive examples were selected to determine whether or not an SAR could be demonstrated in these whole animal models. Several examples containing unnatural amino acids were also tested to study the effect of steric factors. The *in vivo* assay models used have been previously described in a variety of applications including proof of principle studies for anti inflammatory agents<sup>27–32</sup>. However, complete dose response studies and a much larger series of compounds will be needed to confirm the findings shown here.

Suppression of chemically induced paw edema in mice is a long-standing assay for assessment of an agent's potential anti-inflammatory activity<sup>33–39,32</sup>. In prior studies, several cannabinoids<sup>40</sup> and a number of NSAIDs tested in this model exhibited inhibitory effects on chemically induced edema. Figure 3 shows a single dose comparison of three glycine derivatives in this assay with arachidonate as the edema inducer. Some suppression was seen with the polyunsaturated compounds, however the effects were barely statistically significant. Thus, further studies using this assay were not carried out. Induction of inflammation by carrageenan in the paw edema assay gave an SAR different than that predicted by the prostaglandin ratio screen with EMA-1(16:0) being a potent anti-inflammatory agent (data not shown). At this time we cannot explain these differences, however, it seems clear that the nature of the assay will influence the relative activities of the elmiric acids in reducing an inflammatory reaction.

Figure 4 shows several examples of the effects of elmiric acids in the subcutaneous pouch assay using two methods for inducing an inflammatory response, namely, a combination of cytokines in panel A and carrageenan in panel B. The subcutaneous air pouch simulates a synovium lined joint space by providing a blind connective tissue cavity without a mesothelial basement membrane; the pouch lining has the two cell types (fibroblasts and macrophage-like cells) common to synovia<sup>41–43</sup>. While the precision of the assay was improved over the paw edema, an unexpected discrepancy arose with the palmitoyl derivatives when carrageenan was the inducer. There is no obvious explanation for this finding and further studies are needed to address this question. Thus, pending a better understanding of this issue, it was decided not to use this assay.

In Figure 5 we show the results of a preliminary experiment comparing two methods, using either arachidonic acid or phorbol ester, for inducing ear edema in the mouse. Both procedures yielded useful data, however, we found that the phorbol ester data were more precise and more easily obtained. This may be due to the transient nature of the induction produced by arachidonic acid. Nevertheless a favorable comparison between EMA-1(20:4) and dexamethasone, a potent anti-inflammatory agent, was demonstrated (Panel A). All subsequent studies used phorbol ester as the inducing agent. In Panel B ear edema was induced by the topical application of phorbol ester in acetone. EMA-1(20:4) gave a comparable reduction in

ear thickness of about 40–50% as that obtained in the arachidonate model. The response obtained with EMA-23(20:4) was similar suggesting that substituted amino acids do not lose activity.

The four examples of elmiric acids tested at three doses shown in Figure 6 suggests that the anti-inflammatory response is sensitive to both fatty acid and amino acid structure. Comparing 6B and 6D, namely, oleoyl and arachidonoyl show a preference for greater unsaturation in reducing ear edema. When 6A and 6B are compared it appears that the substitution pattern at the  $\alpha$  position can be an important factor. However, in 6C and 6D, the two  $\alpha$  disubstituted arachidonoyl examples are active as are the compounds shown in Figure 5.

The data in Figure 7 were obtained using dimethylacetamide rather than acetone as the formulation vehicle. This was necessary due to poor solubility of the fully saturated elmiric acids in acetone. As predicted by the results of the prostaglandin ratio screen, the palmitoyl (7B) and steroyl (7C) congeners showed little or no activity as anti-inflammatory agents. The results in 7A suggest a possible biphasic response, however, this needs a more detailed study before such a conclusion is made. As positive controls<sup>44</sup>, indomethacin (20 mg/ml), phenidone (40 mg/ml) and dexamethasone (1 mg/ml) were studied using the conditions described in Figure 7. Phenidone, a lipoxygenase inhibitor, had no effect whereas indomethacin and dexamethasone produced some decrease in ear thickness (data not shown) demonstrating a favorable comparison for the elmiric acids with established anti-inflammatory agents.

### 3.4. Possible anti-inflammatory mechanisms

In animal models of acute vs chronic inflammation (carrageenan-induced pleurisy and collagen induced arthritis, respectively), COX inhibition suppresses endogenous 15d-PGJ<sub>2</sub> production and prolongs the inflammatory phase whereas administration of 15d-PGJ<sub>2</sub> has been shown to promote resolution of inflammation<sup>45,21</sup>. Prostaglandins released during an inflammatory response derive from the sequential activity of COX-2 on free arachidonic acid released from cell membranes and the activity of specific terminal synthases on the prostaglandin endoperoxide, PGH<sub>2</sub>. This process is depicted in Scheme 2. The dehydration product of PGD<sub>2</sub>, 15d-PGJ<sub>2</sub>, antagonizes inflammatory signaling pathways mediated by the transcription factors NF kappa-B, AP-1, and STAT, suppresses production of matrix metalloproteinases (MMPs) and other mediators of inflammation, prevents joint damage in rats with adjuvant arthritis, and generally acts to facilitate resolution of inflammation serving as a negative regulator of inflammation.

The mechanisms responsible for the anti-inflammatory and joint protective properties of the synthetic cannabinoid ajulemic acid are currently being studied in our lab<sup>46</sup>. Although the elmiric acids are structurally dissimilar to ajulemic acid, there are certain similarities in their actions. In preliminary experiments, addition of ajulemic acid to human fibroblast-like synovial cells (FLS) *in vitro* increased arachidonic acid release and COX-2 expression with a subsequent marked increase in 15d-PGJ<sub>2</sub> production without an appreciable change in PGE<sub>2</sub> synthesis (Stebulis et al. unpublished findings). The stimulatory effect of ajulemic acid could be reduced by pretreatment with the CB2 antagonist SR144728 but not with the CB1 antagonist SR141716a. These remarkable results are potentially important in light of accumulating evidence that the timing of COX-2 expression may determine its potential to promote either initiation or resolution of inflammation by differential production of prostaglandins, depending on the stage and progression of the inflammatory response<sup>47</sup>. Moreover, we have observed that EMA-1(20:4) causes the mobilization of free arachidonic acid in RAW cells from sources other than the elmiric acid (unpublished data). These considerations provided the rationale for the current studies on the effects of the elmiric acids on prostaglandin ratios described above.

## 4. Summary and conclusions

The goal of this project was to discover a structure-activity relationship for the elmiric (lipoamino) acids as potential anti-inflammatory agents. To this end, a screening procedure was developed to allow the selection of molecules for testing in intact animal models for anti-inflammatory activity. The procedure is based on the concept that prostaglandins can mediate either pro or anti-inflammatory actions depending on their molecular composition. While PGE<sub>2</sub> is dominant during the development of inflammation, prostaglandins of the J series are dominant during the resolution of an inflammatory condition. Thus, compounds that promote the synthesis of the latter without significantly raising the level of the former could be considered as good candidates for the treatment of inflammation. The findings reported here support this hypothesis. Specifically, elmiric acids with unsaturated structures generally produced a favorable prostaglandin ratio and were effective in reducing *in vivo* responses to pro inflammatory agents. Analogs with saturated structures did not enhance the J prostaglandins and had only minor effects *in vivo*. These observations can now be applied to the search for drug candidates in larger libraries of elmiric acids and possibly in other families of compounds.

## 5. Experimental

### 5.1. General chemical methods

All materials were obtained from commercial sources and used without further purification. For TLC, Silica Gel 60 F<sub>254</sub> plates from Merck were used with detection by UV light or iodine vapor chamber. Acid chlorides were obtained from Nu-chek Prep, Inc. (Elysian, MN). Amino acids and esters were from Sigma-Aldridge.

### 5.2. General procedure for synthesis

Compounds not available commercially in the Biomol library were prepared as follows.

*Esterification:* The amino acid (200 mg) is dissolved in 30 ml of methanol saturated with HCl and refluxed for 6 hrs. The solvent is then removed under vacuum and the crude product dissolved in ethyl acetate and partitioned with saturated bicarbonate to remove traces of unreacted amino acid. *Conjugation:* the ester is dissolved in methylene chloride / triethylamine and cooled in ice. Commercially available fatty acid chloride (100 mg) (Nucheck) in methylene chloride is added and the mixture allowed to react for 4 hrs. on ice. The mixture is then quenched on ice and extracted with ethyl acetate. After washing with dilute HCl, saturated bicarbonate and water, the solution is dried over sodium sulfate and evaporated to a clear oil.

*Saponification:* the ester is dissolved in THF and treated under nitrogen with an equal volume of 1N LiOH for 24 hrs. at room temperature with stirring. Silica gel chromatography is used to purify the crude product whose identity is confirmed by mass spectral and NMR analysis.

### 5.3. N-arachidonoyl-1,1-dimethylglycine; EMA-22 (20:4)

Waxy low melting off-white solid. TLC: 10% methanol/methylene chloride; R<sub>f</sub> 0.42. <sup>1</sup>H NMR (250 MHz CDCl<sub>3</sub>) δ 5.93 (bs, 1H), 5.25–5.45 (m, 8H), 2.72–2.90 (m, 6H), 2.18–2.29 (t, 2H), 2.00–2.17 (m, 4H); <sup>13</sup>C NMR (CDCl<sub>3</sub>)

δ 176.3, 174.6, 130.8, 129.3, 129.1, 128.9, 128.5, 128.3, 128.0, 127.7, 77.6, 77.4, 77.2, 76.9, 36.2, 31.7, 29.5, 27.5, 26.7, 25.9, 25.4, 25.2, 22.8, 14.3, 0.23; HRMS calcd. C<sub>24</sub>H<sub>39</sub>NO<sub>3</sub> (M +H); 390.3003, found; 390.3008 (Δ mass –0.0005).

#### 5.4. N-arachidonoyl-1-aminocyclopropane carboxylic acid; EMA-23 (20:4)

Waxy low melting off white solid. TLC: 10% methanol/methylene chloride; Rf 0.44. <sup>1</sup>H NMR (250 MHz CDCl<sub>3</sub>) δ 5.95 (s, 1H), 5.25–5.45 (m, 8H), 2.72–2.90 (m, 6H), 1.98–2.32 (m, 6H), 1.66–1.80 (dt, 2H), 1.57–1.65 (m, 2H), 1.25–1.46 (m, 6H), 1.14–1.22 (m, 2H), 0.83–0.96 (t, 3H); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 175.1, 130.8, 129.2, 129.1, 128.9, 128.5, 128.4, 128.1, 127.7, 35.9, 31.7, 29.5, 27.5, 26.8, 25.9, 25.8, 25.4, 22.8, 18.2, 14.3. HRMS calcd. C<sub>24</sub>H<sub>37</sub>NO<sub>3</sub> (M+H); 388.2846, found; 388.2855 (Δ mass–0.0009).

#### 5.5. N-arachidonoyl-1-aminocyclohexane carboxylic acid; EMA-25 (20:4)

Waxy low melting off-white solid. TLC: 10% methanol/methylene chloride; Rf 0.48. <sup>1</sup>H NMR (250 MHz CDCl<sub>3</sub>) δ 5.57 (s, 1H), 5.25–5.45 (m, 8H), 2.72–2.90 (m, 6H), 2.23–2.34 (t, 2H), 1.85–2.20 (m, 8H), 1.52–1.80 (m, 6H), 1.20–1.48 (m, 8H), 0.83–0.93 (t, 3H); <sup>13</sup>C NMR

(CDCl<sub>3</sub>) δ 175.4, 175.3, 130.8, 129.4, 128.9, 128.8, 128.6, 128.3, 128.0, 127.7, 77.6, 77.4, 77.2, 76.9, 60.2, 42.2, 36.2, 32.2, 31.7, 29.5, 27.4, 26.7, 25.9, 25.6, 25.2, 22.8, 21.5, 14.3. HRMS calcd. C<sub>27</sub>H<sub>43</sub>NO<sub>3</sub> (M+H); 430.3316, found; 430.3320 (Δ mass–0.0004).

#### 5.6. N-steroyl-L-alanine; L-EMA-2 (18:0)

Crystalline, white solid; m.p. 103–105°C. <sup>1</sup>H NMR (400 MHz CDCl<sub>3</sub>) 5.97–5.99 (1H), 4.53–4.58 (1H), 2.22–2.26 (2H), 1.61 (2H), 1.45–1.47 (3H), 1.25–1.30 (29H), 0.86–0.89 (3H); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 175.0, 174.5, 48.6, 36.6, 32.2, 29.9, 29.89, 29.83, 29.69, 29.60, 29.53, 29.40, 25.7, 22.9, 17.9, 14.4. HRMS calcd. C<sub>21</sub>H<sub>41</sub>NO<sub>3</sub> (M+H); 356.3159, found; 356.3187 (Δ mass–0.0028).

#### 5.7. N-palmitoyl methionine; EMA-8 (16:0)

Crystalline white solid; m.p. 75–77°C. <sup>1</sup>H NMR (400 MHz CDCl<sub>3</sub>) 6.34–6.36 (1H), 4.67–4.72 (1H), 2.56–2.60 (2H), 2.11–2.22 (3H), 2.02–2.10 (1H), 1.62–1.65 (2H), 1.25–1.29 (25H), 0.86–0.89 (3H); <sup>13</sup>C NMR (CDCl<sub>3</sub>)

δ 174.8, 174.6, 77.6, 77.4, 77.2, 76.9, 52.2, 36.7, 32.2, 31.0, 30.3, 29.94, 29.92, 29.89, 29.86, 29.72, 29.60, 29.5, 29.45, 25.8, 22.9, 15.7, 14.4; HRMS calcd. C<sub>21</sub>H<sub>41</sub>NO<sub>3</sub>S (M+H); 388.2880, found; 388.2899 (Δ mass–0.0019).

#### 5.8. Enantiomeric alanine analogs

Chirally pure alanine enantiomers, D-EMA-2 and L-EMA-2, were prepared as previous reported by Cascio et al.<sup>48</sup>.

#### 5.9. Pharmacological assays

**5.9.1 Cell proliferation assay**—Cell numbers were estimated using a commercially available kit (Promega). The CellTiter-Glo™ Luminescent Cell Viability Assay kit used here is based on the measurement of ATP, which signals the presence of metabolically active cells<sup>49,50</sup>. The assay uses luciferase as the detection enzyme because of the absence of endogenous luciferase activity in mammalian cells. An equal volume of CellTiter-Glo™ Reagent is added to the cell culture and luminescence is measured. The light signal is proportional to the amount of ATP present, which correlates with the number of viable cells present. The Veritas™ Microplate Luminometer used detects as little as 1.5X10<sup>–15</sup> moles ATP and values are linear from 760 fg to 5.1 ng of ATP. The supplier's instructions were followed in our studies reported here.

**5.9.2 Measurement of the ratio of iPGJ/iPGE in RAW cell media**—48 well plates were seeded with 20,000 RAW cells / 500ul media and incubated for 20 hrs at 37° and 5%

CO<sub>2</sub>. After washing, 500ul of serum free RPMI media was added to each well. Cells were treated with 5ul in DMSO of each of the elmiric acids; final concentrations 1 or 10 uM (N = 3). After 30 minutes, 5ul LPS (1 ug/ml) was added to each well and the incubation continued for 2 hours. Media were then harvested, centrifuged and 50ul from each tube assayed by ELISA with iPGE (Cayman) and iPGJ<sub>2</sub> (Assay Design) enzyme immunoassay kits.

**5.9.3 The mouse paw edema model**—The compounds were dissolved in safflower oil and administered orally to male CD-1 mice that weighed between 35–40 g. After 60 min, 50 ul of the 0.5% arachidonate in pH 8.3 carbonate buffer was injected into the plantar surface of the left (ipsilateral) hind paw of each mouse. The right (contra lateral) paws were injected with 50 ul of the buffer vehicle. This step was done with mice under deep halothane anesthesia. After 2 hours, mice were euthanized with halothane and both hind paws severed at the ankle joint, placed in a tared weighing pan, and the weights recorded. Plots and statistical analyses were done using Prism by GraphPad.

**5.9.4. The mouse subcutaneous pouch assay**—Subcutaneous pouches were generated in male CD-1 mice (30–35g) by injection of air on two or three successive occasions. Drugs were given orally in safflower oil (50ul) at a dose of 20mg/kg. One hour later inflammation is induced by injection into the pouch cavity of 10 ng rHuIL-1-beta plus 0.25 ng rHuTNF-alpha in 3 ml of 1% carboxymethylcellulose. Inflammation is quantified 90 min. later by determination of pouch exudate leukocyte counts. Alternatively, after 60 min, 2% carrageenan was injected into the pouches. The pouch contents are allowed to incubate for 4 hrs following which the mice were euthanized with halothane and the cells harvested and counted. Plots and statistical analyses were done using Prism by GraphPad. N=4 mice/group. P<0.05 by ANOVA

#### 5.9.5. Mouse ear edema assay

**Arachidonic acid induced edema** CD-1 male mice between 34 and 40g were obtained from Charles River and maintained on standard feed and tap water. Treatment was initiated by the application of vehicle (10ul acetone), or drug in 10ul acetone to the ipsi lateral ears and acetone to all contra lateral ears. After 30 min, ear edema was induced by the topical application of 10ul of free arachidonic acid (20mg/ml) to all ipsi lateral ears and 10ul acetone to all contra lateral ears. One hour later, the mice were euthanized with halothane and three replicate ear thickness measurements made using a digital micrometer. Data are expressed as the differences between the ipsi and contra lateral ears in microns.

**Phorbol ester induced ear edema** Conditions were as above up until the induction of edema. 30 min after drug treatment 10ul of the PMA (phorbol ester) solution in acetone was applied to all ipsi lateral ears and 10ul of acetone to all of the contra lateral ears. After 4 hrs, all ear thicknesses were measured using a digital micrometer. Compounds with saturated fatty acid groups were only sparingly soluble in acetone in which case dimethylacetamide (DMA) was substituted for acetone.

**5.9.6. Animals**—The animals were housed in the central facility of the school that is approved and under the supervision of a licensed veterinarian. The experiments were done with regard to all of the regulations of the University Animal Care Committee. The injection of pro inflammatory agents was done with mice under deep halothane anesthesia.

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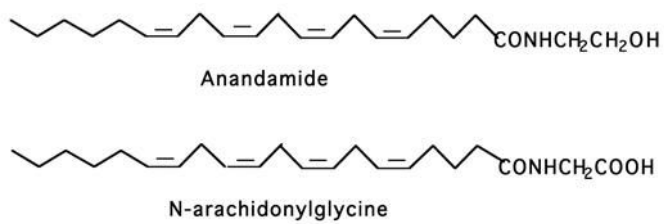
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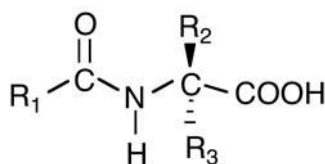
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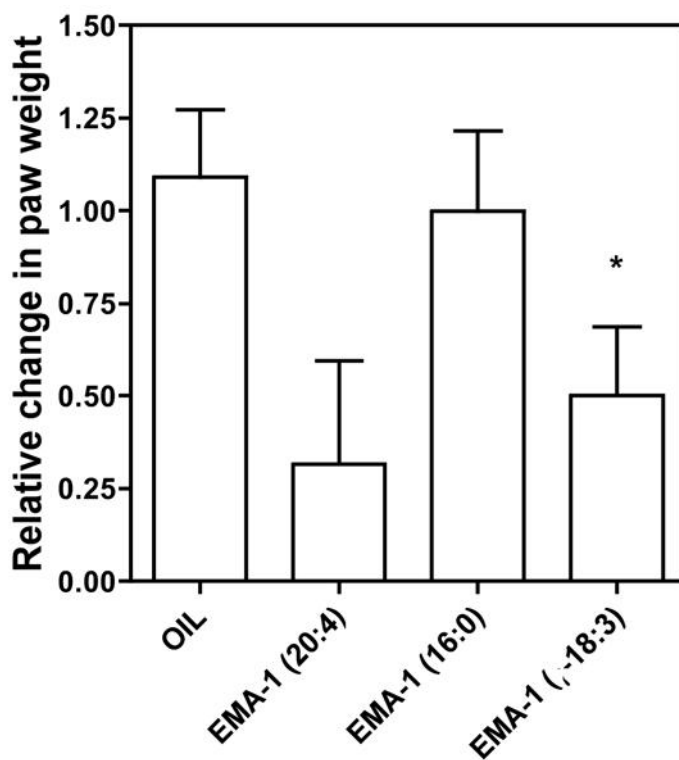
**Fig 1.** The structure of the endocannabinoid anandamide and its endogenous analog, N-arachidonylglycine (NAGly)

Several amino acid – fatty acid combinations have been found in tissue extracts <sup>2</sup>.



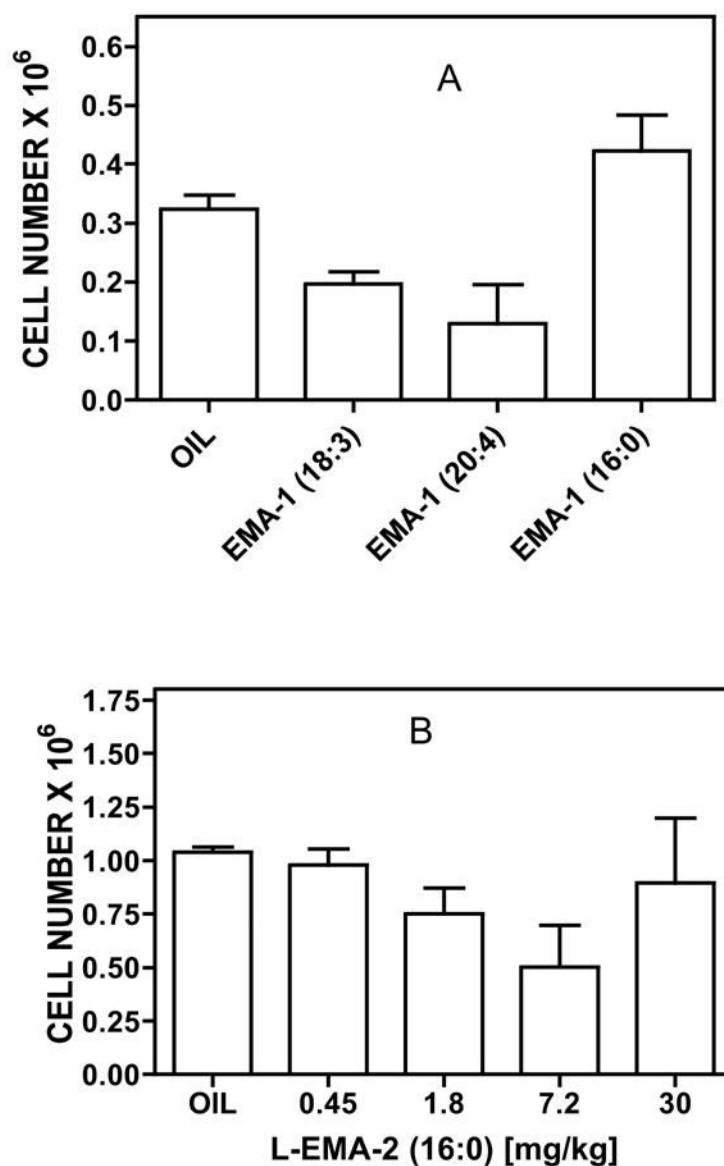
**Fig 2. The general structure for the elmirc acids**

This name has been chosen to designate the entire family of molecules that includes both the naturally occurring lipoamino acids such as NAGly and synthetic analogs where R<sub>1</sub> is a long chain alkyl group and R<sub>2</sub> and R<sub>3</sub> can be a wide range of substituents.

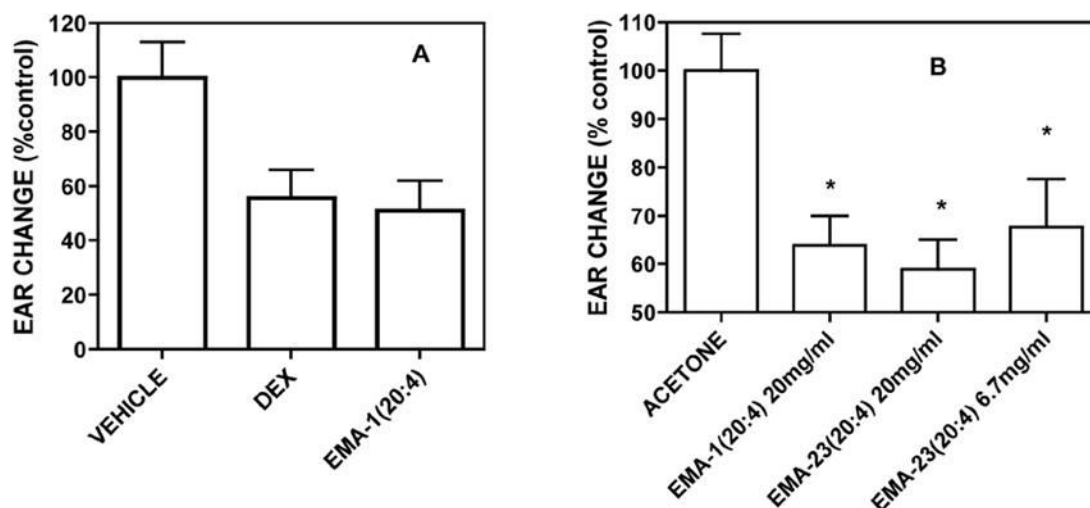


**Figure 3. Effects of the elmiric acids in the mouse paw edema model**

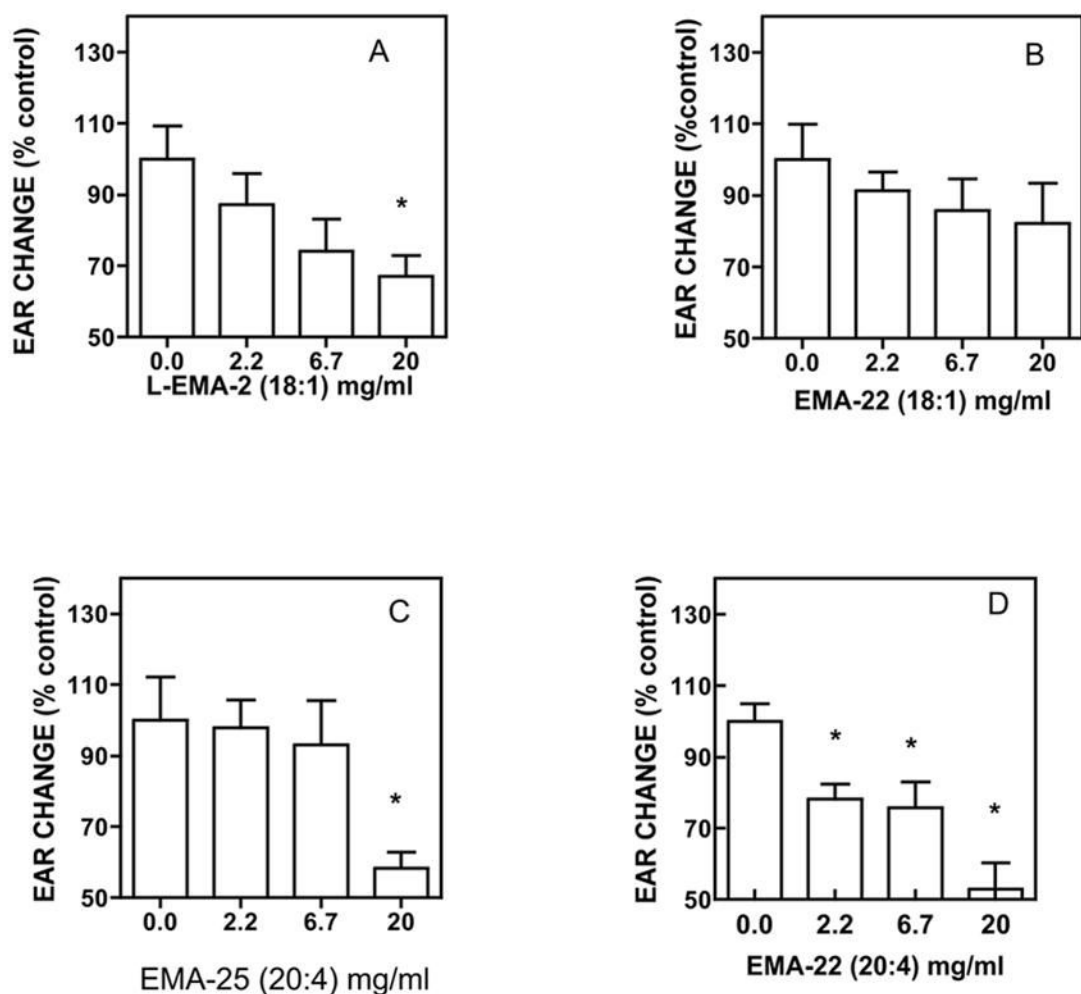
The compounds were dissolved in safflower oil and administered orally at 30 mg/kg. After 60 min, 50  $\mu$ l of the 0.5% arachidonate in pH 8.3 carbonate buffer was injected into the plantar surface of the left (ipsilateral) hind paw of each mouse. The right (contra lateral) paws were injected with 50  $\mu$ l of the buffer vehicle. This step was done with mice under deep halothane anesthesia. After 2 hours, mice were euthanized with halothane, both hind paws severed at the ankle joint and the weights recorded. Values shown are the means  $\pm$  s.e.m.  $p < 0.05$ . Plots and statistical analyses were done using Prism by GraphPad.



**Figure 4. Effects of elmiric acids in the mouse subcutaneous pouch assay**  
Subcutaneous pouches were generated in male CD-1 mice (30–35g) by injection of air on two-three successive occasions. Panel A. Drugs were given orally in safflower oil (50ul) at a dose of 20mg/kg. After 60 min, cytokines TNF-a and IL1-b were injected into the pouches with mice under halothane anesthesia. After 90 min, mice were euthanized with halothane, cells harvested and counted. Panel B. One hour after oral dosing at the indicated levels, 2% carrageenan was injected into the pouches. The pouch contents were allowed to incubate for 4 hrs following which the mice were euthanized with halothane and the cells harvested and counted. Plots and statistical analyses were done using Prism by GraphPad. N=4 mice/group. P<0.05 by ANOVA



**Figure 5. Reduction of arachidonic acid (A) phorbol ester (B) induced ear edema in the mouse** CD-1 male mice between 34 and 40g were treated as described in **Experimental**. Treatment was initiated by the application of vehicle (10ul), dexamethasone (10ug/ear) or EMA-1(20:4) and EMA-23(20:4) (200ug/ear) in 10ul acetone. Values shown are expressed as the differences between the ipsi lateral (induced) and contra lateral (vehicle control) ears in microns. Paired t-test results were:  $p < 0.02$  for vehicle vs. dexamethasone and  $p < 0.007$  for vehicle vs. EMA-1 (20:4). Plots and statistical analyses were done using Prism by GraphPad. N = 8.  
Note: EMA-23 = 1-aminocyclopropane-carboxylic acid

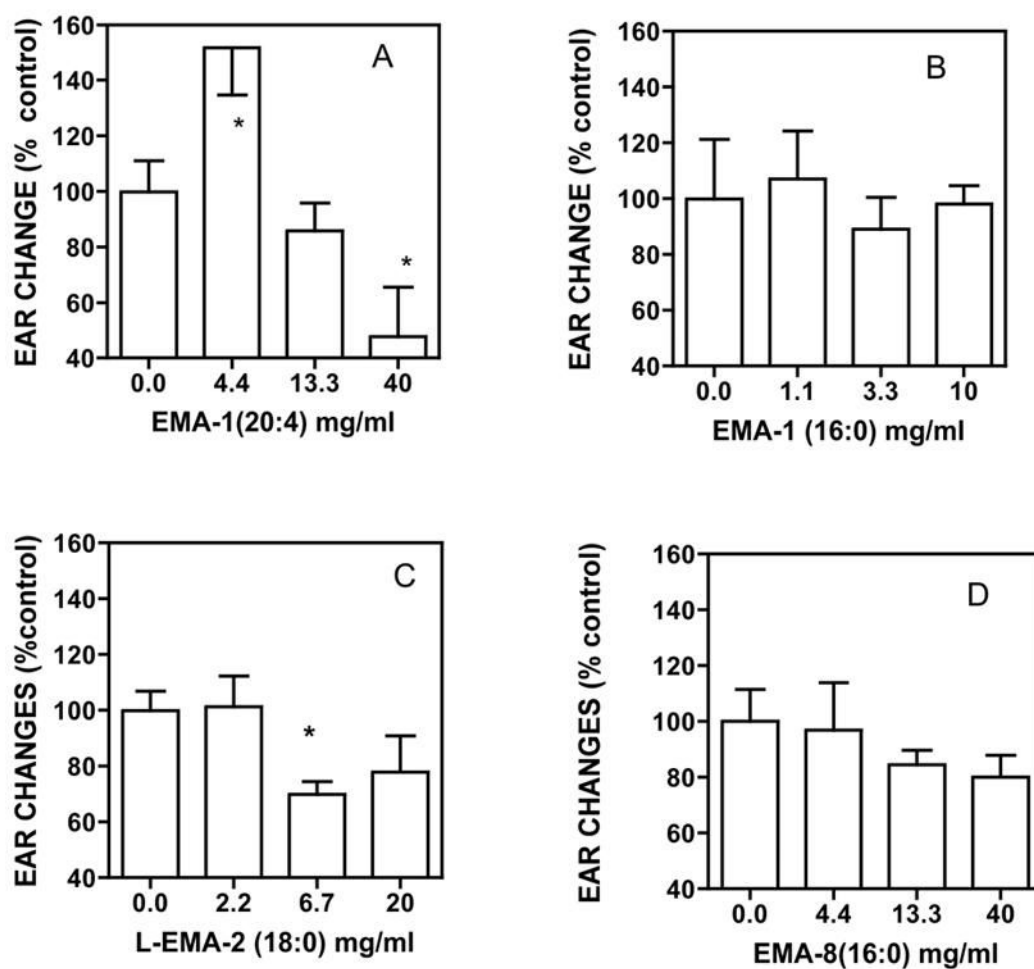


**Figure 6. Dose related reduction of phorbol ester induced ear edema in the mouse**

Treatment was initiated by the application of vehicle (10ul acetone), or drug in 10ul acetone to the ipsi lateral ears and only acetone to all contra lateral ears. 30 min after drug treatment 10ul of the PMA (phorbol ester) solution in acetone was applied to all ipsi lateral ears and 10ul of acetone to all of the contra lateral ears. After 4 hrs, all ear thicknesses were measured using a digital micrometer. Data are expressed as the differences between the ipsi and contra lateral ears in microns. Plots and statistical analyses were done using Prism by GraphPad. N = 4; p<0.05.

Note: EMA-22 = 1,1-dimethylglycine; EMA-25 = 1-aminocyclohexane-carboxylic acid.

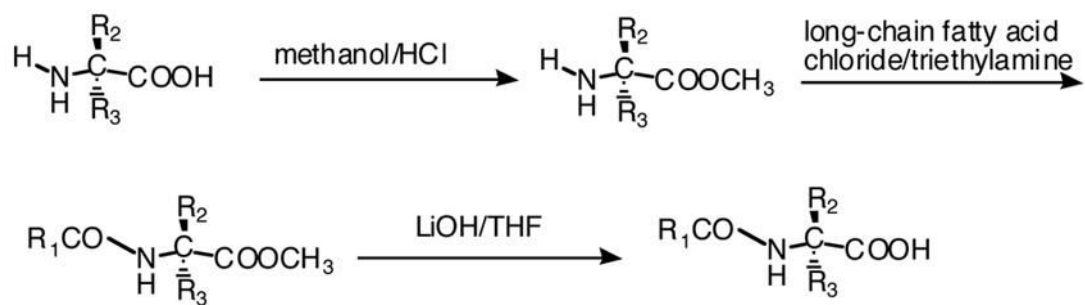




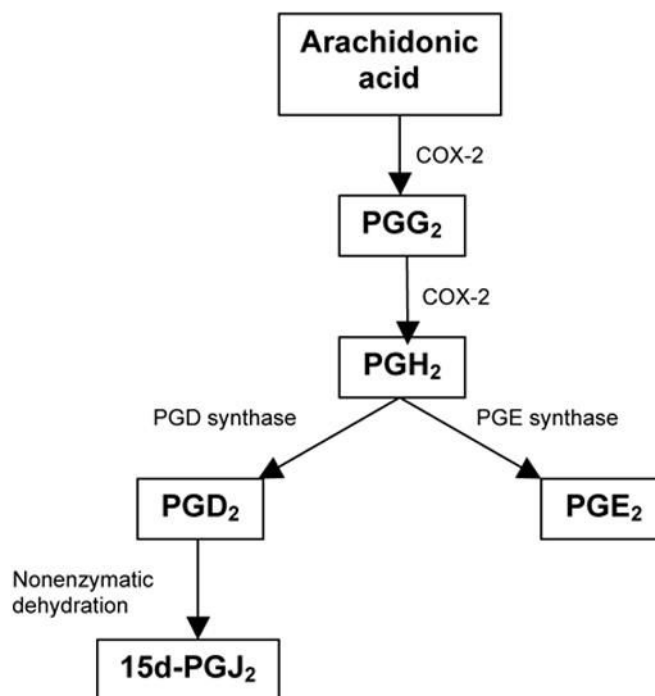
**Figure 7. Dose related reduction of phorbol ester induced ear edema in the mouse**

All of the conditions were the same as in Figure 6 except that the drugs were dissolved in dimethylacetamide (DMA) instead of acetone.  $p < 0.05$ .

Note: EMA-8 = methionine.



**Scheme 1.**  
General synthetic procedure for the elmiric acids.

**Scheme 2. Putative mechanism of action for the elmiric acids**

During an inflammatory response, cells are activated to release arachidonic acid. Activity of the bi functional enzyme COX-2 oxidizes arachidonic acid to PGG<sub>2</sub> and a subsequent peroxidase reaction yields PGH<sub>2</sub>. Then, under the influence of terminal synthases, PGD<sub>2</sub> and PGE<sub>2</sub> are formed. PGD<sub>2</sub> is then converted to the PGJ series in an unregulated manner. Resolution of inflammation occurs when the PGJ series predominates over the PGE series. The elmiric acids, which are not COX-2 inhibitors, apparently up regulate PGD synthase without affecting PGE synthase.

Table 1

## Library of Compounds

Chemical Name	EMA NAME
Palmitoyl glycine	EMA-1 (16:0)
Oleoyl glycine	EMA-1 (18:1)
Linoleoyl glycine	EMA-1 (18:2)
gamma- Linolenoyl glycine	EMA-1 (γ-18:3)
Eicosa-11Z,14Z-dienoyl glycine	EMA-1 (20:2)
Dihomo-gamma-linolenoyl glycine	EMA-1 (γ-20:3)
Arachidonoyl glycine	EMA-1 (20:4)
Eicosapentaenoyl glycine	EMA-1 (20:5)
Docosatetra-7Z,10Z,13Z,16Z-enoyl glycine	EMA-1 (22:4)
Docosahexaenoyl glycine	EMA-1 (22:6)
Palmitoyl l-alanine	L-EMA-2 (16:0)
Oleoyl l-alanine	L-EMA-2 (18:1)
Linoleoyl l-alanine	L-EMA-2 (18:2)
gamma- Linolenoyl l-alanine	L-EMA-2 (γ-18:3)
Eicosa-11Z,14Z-dienoyl l-alanine	L-EMA-2 (20:2)
Dihomo-gamma-linolenoyl l-alanine	L-EMA-2 (γ-20:3)
Arachidonoyl l-alanine	L-EMA-2 (20:4)
Eicosapentaenoyl l-alanine	L-EMA-2 (20:5)
Docosatetra-7Z,10Z,13Z,16Z-enoyl l-alanine	L-EMA-2 (22:4)
Docosahexaenoyl l-alanine	L-EMA-2 (22:6)
Palmitoyl GABA	EMA-9 (16:0)
Oleoyl GABA	EMA-9 (18:1)
Linoleoyl GABA	EMA-9 (18:2)
gamma- Linolenoyl GABA	EMA-9 (γ-18:3)
Eicosa-11Z,14Z-dienoyl GABA	EMA-9 (20:2)
Dihomo-gamma-linolenoyl GABA	EMA-9 (γ-20:3)
Arachidonoyl GABA	EMA-9 (20:4)
Eicosapentaenoyl GABA	EMA-9 (20:5)
Docosatetra-7Z,10Z,13Z,16Z-enoyl GABA	EMA-9 (22:4)
Docosahexaenoyl GABA	EMA-9 (22:6)

**Table 2****Effects of elmiric acids on the proliferation of mouse macrophage RAW cells**

96 well plates were seeded with 500 RAW cells / 100ul media/well. The cells were incubated for 24hrs at 37°C and 5% CO<sub>2</sub> removed from the incubator and a 1ul solution of each of the above compounds added with an N of 4. After 1hr., 1ul LPS (1 ug/ml) was added to each well and the cells returned to the incubator for 48 hrs. Cell numbers were determined with the CellTiter-Glo assay kit (Promega). Luminometer units are linearly proportional to the number of cells in each well and the values shown are the means of four wells.

Acyl group	Conc.*	'GLYCINE SERIES			L-ALANINE SERIES		
		MEAN	SD	T/C**	MEAN	SD	T/C**
DMSO+LPS	0.0	4866	326	1.000	3889	369	1.000
Palmitoyl	0.1	5120	480	1.052	4541	249	1.168
	1.0	5524	207	1.135	4670	248	1.201
	10	5108	851	1.050	432	46	0.111
Oleoyl	0.1	5172	239	1.063	3637	339	0.935
	1.0	5557	269	1.142	4533	535	1.166
	10	425	6	0.087	422	45	0.109
Linoleoyl	0.1	5979	179	1.229	3955	236	1.017
	1.0	4595	1793	0.944	4233	635	1.088
	10	713	24	0.147	621	55	0.160
$\gamma$ -Linolenoyl	0.1	5435	157	1.117	4247	636	1.092
	1.0	5700	190	1.171	4557	173	1.172
	10	6602	1935	1.357	2696	511	0.693
Eicosa-11Z,14Z-dienoyl	0.1	2945	982	0.605	3641	612	0.936
	1.0	5738	179	1.179	4397	280	1.131
	10	609	3	0.125	605	4	0.156
Dihomo- $\gamma$ -linolenoyl	0.1	5486	494	1.127	4326	487	1.112
	1.0	5563	122	1.143	4680	389	1.203
	10	647	20	0.133	637	12	0.164
Arachidonoyl	0.1	5630	245	1.157	4781	530	1.229
	1.0	6156	68	1.265	4735	398	1.218
	10	591	20	0.121	2110	1327	0.543
Eicosapentaenoyl	0.1	5659	282	1.163	4270	758	1.098
	1.0	5161	771	1.061	4798	518	1.234
	10	5937	367	1.220	4672	222	1.201
Docosatetra-7Z,10Z,13Z,16Z-enoyl	0.1	5131	282	1.055	4668	382	1.200
	1.0	5310	127	1.091	4415	734	1.135
	10	3574	655	0.734	618	22	0.159
Docosahexaenoyl	0.1	5708	387	1.173	4452	419	1.145
	1.0	5675	288	1.166	4176	251	1.074
	10	2103	888	0.432	1010	153	0.260

**Table 3****Effects of elmiric acids on the ratio of iPGJ/iPGE in RAW cell media**

48 well plates were seeded with 20,000 RAW cells / 500ul media and incubated for 20 hrs at 37° and 5% CO<sub>2</sub>. After washing, 500ul of serum free RPMI media was added to each well. Cells were then treated with 5ul in DMSO of each of the elmiric acids; final concentrations of agents were 1 or 10 uM (N = 3). After 30 minutes, 5ul LPS (1 ug/ml) was added to each well and the incubation continued for 2 hours. Media were then harvested, centrifuged at 800xg and 50ul from each tube assayed by ELISA with iPGE (Cayman) and iPGJ<sub>2</sub> (Assay Design) enzyme immunoassay kits. Prostaglandin concentrations are in pg/ml.

Treatment	Conc.	iPGJ <sub>2</sub>	S.D.	iPGE <sub>2</sub>	S.D.	J/E Ratio
DMSO+LPS	-	136	84	120	3	1
Naproxen	2	57	51	106	6	1
Naproxen	10	70	38	75	9	1
Naproxen	50	119	44	81	2	1
EMA-1(16:0)	1	219	215	103	23	2
EMA-1(16:0)	10	401	194	128	12	3
EMA-1(18:0)	1	201	159	121	20	1
EMA-1(18:0)	10	159	26	126	1	1
EMA-1(18:3)	1	5042	1672	127	6	39
EMA-1(18:3)	10	80205	7534	289	19	277
EMA-1(20:4)	1	40407	10331	273	125	147
EMA-1(20:4)	10	97830	9805	425	180	229
EMA-1(20:5)	1	1729	249	126	14	13
EMA-1(20:5)	10	44729	8848	355	46	125
D-EMA-2(16:0)	1	281	46	256	35	1
D-EMA-2(16:0)	10	3079	3936	319	18	9
L-EMA-2(16:0)	1	261	55	251	21	1
L-EMA-2(16:0)	10	4177	1352	232	16	17
D-EMA-2(20:4)	1	1496	427	124	5	12
D-EMA-2(20:4)	10	28224	5201	269	27	104
L-EMA-2(20:4)	1	2345	252	241	18	9
L-EMA-2(20:4)	10	41059	3834	652	92	62
L-EMA-2(22:6)	1	3156	317	229	22	13
L-EMA-2(22:6)	10	62176	12969	437	153	142