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N-amino acid linoleoyl conjugates: anti-inflammatory activities

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Abstract

Several N-linked amino acid-linoleic acid conjugates were studied for their potential as anti inflammatory agents. The parent molecule, N-linoleoylglycine was tested in an *in vivo* model, the mouse peritonitis assay where it showed activity in reducing leukocyte migration at doses as low as 0.3 mg/kg when administered by mouth in safflower oil. Harvested peritoneal cells produced elevated levels of the inflammation- resolving eicosanoid 15-deoxy- $\Delta^{13,14}$ -PGJ₂. These results are similar to those obtained in earlier studies with N-arachidonoylglycine. An *in vitro* model using mouse macrophage RAW cells was used to evaluate a small group of structural analogs for their ability to stimulate 15-deoxy- $\Delta^{13,14}$ -PGJ₂ production. The D-alanine derivative was the most active while the D-phenylalanine showed almost no response. A high degree of stereo specificity was observed comparing the D and L alanine isomers; the latter being the less active. It was concluded that linoleic acid conjugates could provide suitable templates in a drug discovery program leading to novel agents for promoting the resolution of chronic inflammation.

Keywords

Elmiric acid; endocannabinoid; anti inflammatory; mouse peritonitis; prostaglandin

A class of lipoamino acids, long chain fatty acids covalently coupled to amino acids, which we have termed elmiric acids (EMA)^{1, 2}, are emerging as an important family of endogenous signaling molecules³ that act as physiological regulators of pain and inflammation⁴. The existence of these endogenous substances was first predicted in 1997 when synthetic examples were produced and shown to exhibit anti inflammatory and analgesic activity in mice⁵. Subsequent studies identified several naturally occurring EMAs in rat brain extracts⁶. To date, about 50 naturally occurring members as well as several synthetic analogs of the EMA family have been identified^{15, 16, 2}. Their actions include analgesia^{4, 7, 8}, anti-inflammatory effects², selective inhibition of cancer cell proliferation⁹, vasodilation¹⁰, cell migration^{11, 12} and calcium ion mobilization^{13, 14}.

The prototypic EMA, N-arachidonoylglycine (NAGly) shown in Figure 1, is found in rat brain, spinal cord, and other tissues where it occurs in amounts greater than the closely related endocannabinoid, anandamide⁴. Early reports^{4, 5}, suggested that NAGly possessed analgesic properties but lacked the psychotropic activity of the cannabinoids. It has been shown that NAGly has low affinity for the cannabinoid CB1 receptor¹⁷, however, it appears

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to activate the orphan G-protein coupled receptor (GPCR), GPR18^{14, 18}. In this report, we have focused on lipoamino acids containing a linoleoyl residue for the purpose of discovering a template structure better suited toward the design of promising drug candidates (Figure 2). The simplest member of this series is N-linoleoylglycine (LINGly) or, using the elmiric acid system, EMA-1 (18:2). The notations used in this report are as follows: Amino acid EMA names are: Glycine, EMA-1; Alanine, EMA-2; Phenylalanine, EMA-9 and Tyrosine, EMA-10. Fatty acid abbreviations are: Palmitic, 16:0; Linoleic, 18:2; Arachidonic, 20:4, For a more complete list see reference 1.

A widely used test for anti inflammatory action is the mouse peritonitis assay. This test is based on the migration of leukocytes into the peritoneal cavity following the injection of a pro inflammatory agent into the cavity. The inhibition of this migration is considered to be a measure of the anti inflammatory potential of a compound. Figure 3 Panel A shows the results obtained when LINGly²⁴ at doses ranging from 0.3 to 20 mg/kg was subjected to this test. The values obtained suggest that the ED-50 is less than 0.3 mg/kg, which would make it a relatively potent anti inflammatory agent. Since it was administered orally, it is also indicative of its stability and good bioavailability. Thus the prototypic structure for this study, LINGly, appears to have been well chosen. The data shown in Panel B relates to a putative mechanism of action for the anti-inflammatory activity of LINGly and will be discussed in below.

Literature reports indicate that the elevations of tissue concentrations of PGJ are associated with the resolution of an inflammatory response¹⁹. This was suggested to come about through the binding and activation of the transcription factor PPAR- γ followed by increased expression of anti inflammatory factors. Our previous studies showed a positive correlation between PGJ levels and an anti inflammatory action of the EMAs *in vivo*¹. A robust stimulation of PGJ by LINGly was observed in RAW cells over a concentration range of 0.5 to 10 μ M (Table 1). The response was comparable to that shown by NAgly indicating no sacrifice of potency when going from four to two double bonds and decreasing the chain length by two carbon atoms. However, earlier reports showed that the palmitoyl analog PALgly had no anti inflammatory effect either *in vitro*¹ or *in vivo*¹⁸ representing a dramatic change with the saturated, shorter chain analog. This is in contrast to an earlier report that describes PALgly as a modulator of calcium influx and nitric oxide production in sensory neurons²⁰. An explanation for such differences could be related to the binding affinities of these EMAs to GPR18, however, these data are currently not available.

The available data allow some comments to be made on a possible mechanism for the anti inflammatory effects of the linoleoyl sub family of EMAs reported on here. In previous publications, we have proposed a putative mechanism of action involving the activation of the arachidonic acid cascade leading to an elevation of eicosanoid products^{1, 2, 18, 21}. The initial step is the stimulation of the release of free arachidonic acid that can then promote the synthesis of specific anti inflammatory eicosanoids such as PGJ and LXA₄. How these eicosanoids bring about a resolution of chronic inflammation is uncertain and is a topic currently being studied^{19, 22, 23}. A possible role for GPR18 in the EMA promoted release reaction should be considered.

Stereoisomeric preferences in a response are often an indication of receptor binding involvement in a particular response. Thus, we have compared the D and L enantiomers of N-linoleoylalanine in the PGJ model for anti inflammatory activity. The data shown in Table 2 indicate that there is, in fact, a considerable difference in activity with the D isomer being the more active. The decreases in cell count with increased drug are probably due to a decrease in proliferation rather than cell viability since we did not see many dead cells in the culture media. This high level of chiral preference suggests that a specific receptor may

mediate the observed response. Again, based on our recent report ¹⁸, we would speculate that GPR18 is a likely mediator for this response.

Figure 4 depicts a dramatic loss of activity when N-linoleoyl-D-phenylalanine (D-EMA-9(18:2)), and to a lesser extent N-linoleoyl-D-tyrosine, (D-EMA-10(18:2)) is substituted for N-linoleoyl-D-alanine (EMA-2 (18:2)). This could be attributed to steric volume increases or, possibly, to electronic effects. Further studies would be needed to distinguish between these possibilities.

To summarize, a rank order (N-linoleoyl-D-alanine > N-linoleoylglycine > N-arachidonylglycine > N-linoleoyl-D-tyrosine > N-linoleoyl-L-alanine > N-linoleoyl-D-phenylalanine) of anti inflammatory action showing a complete range of responses was found even with the limited series of analogs studied in this report (Figure 5). Moreover, it may be concluded that linoleic acid analogs of NAgly can serve as lead molecules in a drug discovery program aimed at producing a stable and easily accessible drug candidate. By comparison, arachidonoyl derivatives are expensive and inherently less stable than linoleoyl derivatives and show a greater tendency to be non crystalline in nature

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Abbreviations

EMA	elmiric acids
GPCR	G-protein coupled receptor
LXA₄	lipoxin A ₄
LINgly	N-linoleoylglycine
LINphe	N-linoleoylphenylalanine
LINTyr	N-linoleoyltyrosine
NAgly	N-arachidonylglycine
PALgly	palmitoylglycine
PGJ	15-deoxy- $\Delta^{13,14}$ -PGJ ₂

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24. Materials. N-linoleoylglycine and N-linoleoyl-L-alanine and N-arachidonoylglycine were obtained from Medchem 101 (Conshohocken, PA). The other amide conjugates were synthesized according to published procedures (Burststein, Sumner H.; Zurier, Robert B. Lipid-amino acid conjugates and methods of use. US Pat Appl Publ. 2006US 2006014820 A1 20060119.). All materials were obtained from commercial sources and used without further purification. For TLC, Silica Gel 60 F254 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. RAW cells were from ATCC.

Figure 1. The structure of NAgly

There are three regions of the molecule that are of pharmacological interest. Region 1 confers a high degree of specificity of action. Polyunsaturated residues produce molecules with analgesic and anti-inflammatory action whereas saturated structures are inactive. Region 2 is related to metabolic stability since the EMAs are degraded by FAAH (fatty acid amide hydrolase) activity. Region 3, the amino acid residue, can modulate the analgesic/anti-inflammatory activities depending on steric factors and the chiral nature of the amino acid.

Figure 2.
Structures of the novel elmiric acids

Figure 3. N-linoleoylglycine inhibits leukocyte migration (A) and increases PGJ production (B) in the mouse peritonitis model

A. The indicated treatments were administered by mouth and after 30 min, the mice were injected i.p. with 1 ml (sterile filtered) 8% BBL Fluid Thioglycollate Medium. Cells were harvested from the peritoneal cavity after 3 hours, exposed to lysing buffer for 2 minutes to remove erythrocytes, suspended in PBS/BSA and differential cell counts obtained. Control mice were given safflower oil. N=8.

B. Peritoneal cells were collected and maintained in culture for 18 hrs. The media were then harvested and their PGJ levels measured by ELISA assay. N=4

Study A was carried out by BRM, Inc., (Worcester, MA). All animal studies were performed according to institutional, local, state, federal and NIH guidelines for the use of animals in research under Institutional Animal Use and Care Committee (IACUC)-approved protocols at BRM and The University of Massachusetts Medical School.

Figure 4. Electronic and steric effects on PGJ responses in RAW cells

Cells used were RAW264.7 and were obtained from ATCC. The base medium is Gibco DMEM with 10% fetal bovine serum added. Cells are grown in a T-75 flask in 15 ml of medium; medium is replaced on day four and sub cultured on day seven. Cells are removed by scraping without the aid of trypsin. A sub cultivation ratio of 1:3 to 1:6 was used. Elisa assay kits for PGJ were obtained from Assay Designs, Inc., (Ann Arbor, MI). The identity of the PGJ analyte in the culture medium was confirmed by mass spectrometry (Wood and Makriyannis, unpublished data). Treatments were carried out in 48 well plates with 50,000 cells/500ul DMEM+FCS media/well and TNF α (10 nM) added. Cells were incubated for 20 hrs at 37°C and 5% CO₂. Media were changed to 500ul of serum free DMEM, treated for 2 hrs and 100 ul removed for assays. NAgly [10 μ M] control; 16,300 pg/ml. DMSO control: <16.0 pg/ml. N=4

Figure 5. Estimated rank order responses in PGJ RAW cell model

Cells were treated as in Figure 4. All values were obtained at 10uM concentrations of each EMA analog. Control: 1% DMSO vehicle. N=4

*ANOVA gave a p value of less than 0.05.

Table 1

LINGly shows similar potency compared to NAGly in the RAW cell model.

Treatment	Concentration (μ M)	[PGJ] (pg/mL) ^a	SD
LINGly	0.5	< 16.0	-
LINGly	1	177	-
LINGly	2.5	1843	431
LINGly	5	3907	293
LINGly	10	7315	1444
NAGly	1	< 16.0	-
NAGly	10	6847	625
DMSO	-	< 16.0	-

^aCells were treated as in Figure 4. N=4

Table 2

Stereospecificity of EMA action in the RAW cell model.

Treatment ^a	Drug Concentration (μM)	Cell Count (% control)	PGJ (pg/ml)	PGJ/cell count	P Value
D-LINala	0.3	136.0	< 16.0	-	-
D-LINala	1	65.9	18.0	1186	0.08
D-LINala	3	33.9	233	7901	0.04
D-LINala	10	30.5	1580	48203	0.01
L-LINala	0.3	55.6	< 16.0	-	-
L-LINala	1	74.3	< 16.0	-	-
L-LINala	3	68.5	< 16.0	-	-
L-LINala	10	55.5	30.3	1682	0.07
DMSO	-	100	< 16.0	-	-

^a Cells were treated as in Figure 4. N=4