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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-$\Delta^{13,14}$-PGJ_2. 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-$\Delta^{13,14}$-PGJ_2 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.

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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 molecules[3] that act as physiological regulators of pain and inflammation.[4] 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 mice.[5] Subsequent studies identified several naturally occurring EMAs in rat brain extracts.[6] 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 effects,[2] selective inhibition of cancer cell proliferation,[9] vasodilation,[10] cell migration[11,12] and calcium ion mobilization.[13,14]

The prototypic EMA, *N*-arachidonylglycine (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, anandamide.[4] 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 receptor,[17] however, it appears 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 (Fig. 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 Ref. 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

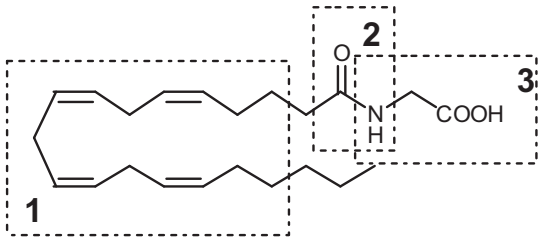

Figure 1. The structure of NAgly. There are three regions of the molecule that are of pharmacological interest. Region 1 confers a high degree of specificity of action. Polyunsaturated residues produce molecules with analgesic and anti-inflammatory action whereas saturated structures are inactive. Region 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.

Abbreviations: EMA, elmiric acids; GPCR, G-protein coupled receptor; LXA_4, lipoxin A_4; LINgly, *N*-linoleoylglycine; LINphe, *N*-linoleoylphenylalanine; LINtyr, *N*-linoleoyltyrosine; NAgly, *N*-arachidonoylglycine; PALgly, palmitoylglycine; PGJ, 15-deoxy-$\Delta^{13,14}$-PGJ_2.

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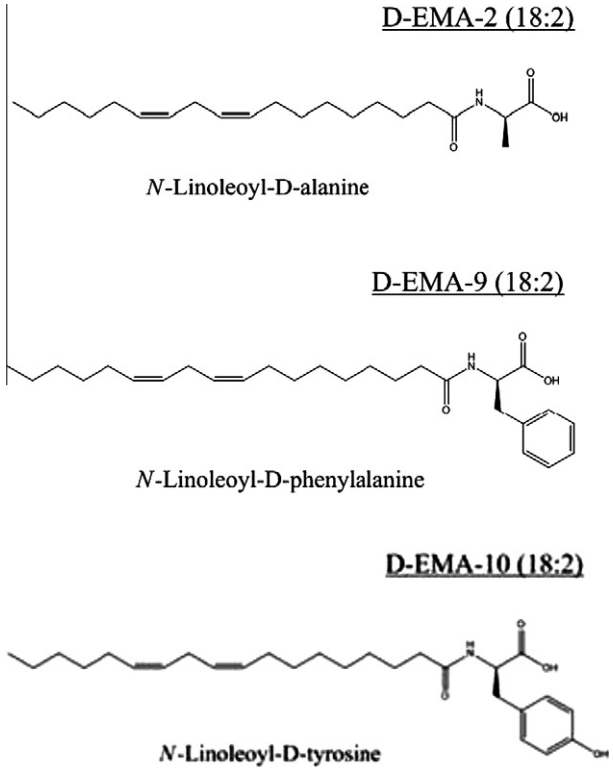

Figure 2. Structures of the novel elmiric acids.

a compound. Figure 3 Panel A shows the results obtained when LINgly[24] 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 response.[19] 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

Table 1
LINgly shows similar potency compared to NAgly in the RAW cell model

Treatment	Concentration (μM)	[PGJ][a] (pg/ml)	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	–

[a] Cells were treated as in Figure 4. $N = 4$.

of PGJ by LINgly was observed in RAW cells over a concentration range of 0.5–10 μM (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 neurons.[20] 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 LXA_4. 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

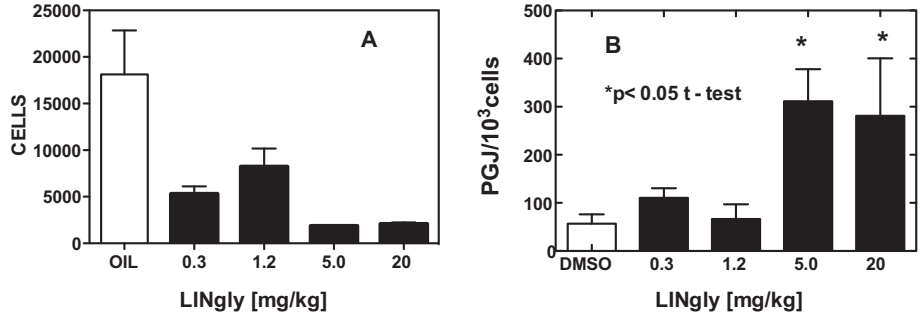

Figure 3. *N*-Linoleoylglycine inhibits leukocyte migration (A) and increases PGJ production (B) in the mouse peritonitis model. (A) The indicated treatments were administered by mouth and after 30 min, the mice were injected ip with 1 ml (sterile filtered) 8% BBL Fluid Thioglycollate Medium. Cells were harvested from the peritoneal cavity after 3 h, exposed to lysing buffer for 2 min 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 h. 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.

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