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A structure–activity relationship study on *N*-arachidonoyl-amino acids as possible endogenous inhibitors of fatty acid amide hydrolase

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Abstract

N-arachidonoyl-glycine (NAGly) has been recently identified in rodent tissues and found to exhibit analgesic activity in vivo. NAGly is a potent inhibitor of the fatty acid amide hydrolase (FAAH), the enzyme primarily responsible for the degradation of the endocannabinoid *N*-arachidonoyl-ethanolamine (anandamide), and was shown recently to elevate the blood levels of the this analgesic compound. We have synthesized several *N*-arachidonoyl-amino acids of potential natural occurrence, as well as the D- and L-isomers of *N*-arachidonoyl-alanine, and have tested their activity on FAAH preparations from mouse, rat, and human cell lines, and from mouse or rat brain. The results indicate that the relative potency and enantioselectivity of *N*-arachidonoyl-amino acids as FAAH inhibitors depend on the animal species. Thus, whilst NAGly is the most potent compound on the rat and mouse enzymes, *N*-arachidonoyl-isoleucine is active only on human FAAH and *N*-arachidonoyl-alanine enantiomers show a varying degree of potency. Taken together, these data support the view that an enhancement of endogenous anandamide levels underlies in part the analgesic effects of NAGly in rodents.

The enzyme "fatty acid amide hydrolase" (FAAH, previously known as *N*-acyl-ethanolamine amidohydrolase) was first identified in the 1980s ([1], for review) and cloned from several mammalian species in the late 1990s [2–4]. FAAH catalyses the hydrolysis of several bioactive fatty acid amides and esters (see [5] for review), including: (1) the endogenous ligands of cannabinoid receptors, anandamide, and 2-arachidonoylglycerol [6–8]; (2) the anandamide congeners, *N*-palmitoyl-ethanolamine and *N*-oleoylethanolamine, which do not bind to cannabinoid receptors but nevertheless exert important biological effects [9–12]; and (3) the sleep inducing factor, *cis*-9-octadecenamide (oleamide, [13]), and its congeners. At the transcriptional level, FAAH is up-regulated by several stimuli, including bacterial lipopolysaccharides [14], progesterone, and leptin [15,16], and is down-regulated by estrogens and glucocorticoids [17]. Since transgenic mice lacking a functional gene encoding for FAAH (the FAAH knock-out mice) [18] exhibit less sensitivity to several pain stimuli, and selective FAAH inhibitors show analgesic activity [19,20], this enzyme has been suggested to control tonically the levels of endogenous analgesic fatty acid amides, such as anandamide and *N*-palmitoylethanolamine. These findings indirectly suggest that endogenous mediators with strong inhibitory action on FAAH may act as analgesic and anti-inflammatory compounds by elevating the levels of endogenous cannabinoids. These putative mediators might act as additional regulatory factors for FAAH, and one possible example of such a compound was recently identified in several rodent tissues, namely *N*-arachidonoyl-glycine (NAGly) [21].

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NAGly is synthesized from arachidonic acid and glycine, possibly by the enzyme acylCoA:glycine *N*-acyltransferase (ACGNAT), or by an as yet unidentified isoform of this enzyme, and is inactivated by FAAH. However, NAGly is inactive on both cannabinoid CB_1 and CB_2 receptors, nevertheless it exerts both analgesic and anti-inflammatory activities [21,22]. In bovine brain two analogues of NAGly, *N*-arachidonoyl-alanine and *N*-arachidonoyl-γ-amino-butyric acid, have been identified [21]. In the report, the chirality of the alanine congener was not established, however, it is very likely, but not unequivocally, the L-alanine derivative since D-alanine is uncommon in mammalian systems. Recently, systemic administration of NAGly to rats was found to lead to a significant elevation of blood anandamide levels, and treatment of intact cells with this compound also resulted in the finding of higher levels of the endocannabinoid in the culture medium [23]. These findings, and the lack to the present date of other molecular targets for this compound, suggest that interaction with FAAH underlies the analgesic and anti-inflammatory effects of NAGly [21], and that these effects are mediated by an increased concentration of compounds, like anandamide and *N*-palmitoylethanolamine, which appear to exert these effects tonically (see [24], for review).

This study was designed based on four considerations: (1) the likely occurrence in mammalian tissues of several arachidonoylated amino acids, suggested by the finding of *N*-arachidonoyl-alanine and *N*-arachidonoyl-γ-amino-butyric acid along with NAGly; (2) the possibility that FAAH is a molecular target for NAGly as well as of other possible naturally occurring *N*-arachidonoyl-amino acids (NAAs); (3) the necessity of a better understanding of the structure–activity relationships for the inhibition of FAAH by NAAs; and (4) the previous observation of species differences in the affinity of FAAH for fatty acid amide substrates [3]. Therefore, we synthesized a series of *N*-arachidonoyl-amino acids and investigated their inhibitory effect on FAAH prepared from either tissues or cell lines from three different mammalian species, i.e., rat, mouse, and human. We report that, within the NAAs investigated, NAGly was the most potent FAAH inhibitor on the mouse and rat, but not human, enzymes, and that the potency and enantioselectivity of NAAs as FAAH inhibitors show a remarkable species selectivity.

Materials and methods

Synthesis of N-arachidonoyl-amino acids. Briefly, the compounds with general structure shown in Fig. 1, were obtained from commercially available amino acid *tert*-butyl esters by acylation with arachidonic acid with the DEPC (diethyl cyanophosphonate)/TEA (triethylamine) protocol, followed by de-protection with TFA (trifluoroacetic acid) in CH_2Cl_2.The compounds were purified by gravity column chromatography, and their structure and purity was assessed by proton nuclear magnetic resonance.

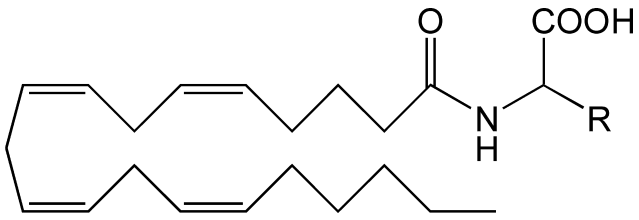

Fig. 1. General chemical structure of the *N*-arachidonoyl-amino acids synthesized and tested in this study. R denotes the type of amino acidic residue (i.e., those in Gly, L- and D-Ala, Val, Leu, Ile, Glu, Gln. Asp, Tyr, Phe, and L-DOPA).

The alanine congeners were prepared from chirally pure amino acid methyl esters (Aldridge Chemicals) using a different method. Arachidonoyl chloride (Nucheck) in methylene chloride was reacted with alanine methyl ester in methylene chloride containing 5% triethylamine for 4 h at room temperature. The mixture was partitioned between ethyl acetate and dilute HCl, washed, dried, and evaporated to an oily residue. This was dissolved in tetrahydrofuran and saponified by stirring under nitrogen with 1 N LiOH for 5 h at room temperature. The product was extracted as above and subjected to thin layer chromatography. The principal product was identified as *N*-arachidonoyl-alanine by mass spectrometric analysis and exhibited an MH^+ of 376.1. Circular dichroism measurements showed mirror image spectra between 220 and 240 nm. The [α] values were—16.6×10^5 for the D-isomer and $+16.5 \times 10^5$ for the L-isomer.

FAAH assays. The effect of increasing concentrations of the synthetic compounds on the enzymatic hydrolysis of anandamide was studied as described previously [25], by using membranes prepared from mouse brain and neuroblastoma N18TG2 cells, rat brain and RBL-2H3 cells, and human MCF-7, EFM-19, and HEK-293 cells. Membranes were prepared as described previously [25]. In brief, tissues or cells were homogenized at 4 °C in 50 mM Tris–HCl buffer, pH 7.0, by using an ultraturrax and a dounce homogenizer, respectively. Homogenates were first centrifuged at 900*g* to get rid of debris and the supernatant was centrifuged at 10,000*g*. The pellets from this latter centrifugation were used for the assay. Membranes were incubated with increasing concentrations (1–5–10–25–50 μM) of the test compounds and [^{14}C]EtOH-arachidonoyl-amide ([^{14}C]AEA, 8 μM, 20,000 cpm) in 50 mM Tris–HCl, pH 9, for 30 min at 37 °C. [^{14}C]Ethanolamine produced from [^{14}C]AEA hydrolysis was used to calculate FAAH activity and was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of $CHCl_3/CH_3OH$ 2:1 (by vol.). Control FAAH activities of the various preparations are shown in Table 1.

Results and discussion

The fatty acid amide, NAGly, is an endogenous compound with a widespread but nonetheless heterogeneous distribution in bovine and rat tissues, and with a potent analgesic and anti-inflammatory activity [21]. This compound seems to accompany the endocannabinoid anandamide in many tissues, and preliminary observations carried out in rat uterus indicate that its levels as well as those, normally lower, of anandamide might be regulated by estrogens [26]. Whilst no receptor has been identified for NAGly, its analgesic actions seem to be due, at least in part, to the capability of this

Table 1
FAAH inhibitory activity of *N*-arachidonoyl-glycine

FAAH species and preparation	Control activity (nmol mg protein^{-1} min^{-1})	Inhibitory activity of NAGly (IC_{50}, μM)
Mouse brain membranes	0.10 ± 0.02	27.0 ± 4.1
Mouse N18TG2 cell membranes	0.16 ± 0.03	29.0 ± 5.2
Rat brain membranes	0.14 ± 0.02	26.0 ± 3.4
Rat RBL-2H3 cell membranes	0.21 ± 0.05	8.5 ± 1.9
Human MCF-7 cell membranes	0.39 ± 0.08	50.0 ± 5.3
Human EFM-19 cells	0.64 ± 0.11	27.1 ± 3.9
Human HEK-293 cells	0.15 ± 0.04	23.3 ± 3.5

The enzymatic activity for each preparation in the absence of inhibitor is also shown, and no correlation between starting enzyme activity and inhibitory potency can be observed. Data are means ± SD of $n = 3$ separate experiments.

compound to act as a substrate/inhibitor of FAAH, the enzyme primarily involved in anandamide hydrolysis [23]. This, and the occurrence in mammalian tissues of other NAAs with unknown activity at FAAH [21], prompted us to screen a series of *N*-arachidonoyl-amino acids as FAAH inhibitors.

The first finding of this study is that, of the twelve NAAs synthesized and tested, only NAGly was capable of inhibiting FAAH, irrespective of the brain species or cell used for the enzyme preparation. On the other hand, its potency was dramatically dependent on the brain species and the type of preparation (tissue or cell) used to assay FAAH activity (Table 1). Thus, while NAGly was equipotent on FAAH from rat and mouse brain ($IC_{50} = 26.0$ and 27.0 μM, respectively), it was significantly more potent on FAAH from rat RBL-2H3 cells than mouse N18TG2 cells ($IC_{50} = 8.5$ and 29.0 μM, respectively). Most surprisingly, with all three human cell lines used, NAGly was overall a weaker FAAH inhibitor than with rat FAAH (23.3, 27.1, and 50 μM with HEK-293, EFM-19, and MCF-7 cells, respectively). It can be concluded that although variations due to the use of crude enzymatic preparations are likely, as shown by the occasionally different behaviour of the compound when using membranes obtained by either whole brain or isolated cells, NAGly is definitively a potent inhibitor of rat, but not of human, FAAH. NAGly is also an FAAH substrate and, as shown in Fig. 2, a competitive inhibitor of the enzyme. In fact, in the presence of the inhibitor, only the K_m for AEA was increased (from 19 to 39 μM), whereas the V_{max} was not changed (from 2.5 to 2.4 nmol mg protein^{-1} min^{-1}), when using the enzyme from N18TG2 cells. Therefore, our findings indicate a different substrate selectivity of FAAH for this compound and are in agreement with the previously described different selectivity of FAAH from various species for NAE and primary amide substrates [3].

Of the other NAAs tested in this study, two compounds that also exhibited dose-related inhibitory activity on FAAH were *N*-arachidonoyl-alanine (NAAla), which has been found to occur in mammalian tissues [21], and *N*-arachidonoyl-isoleucine (NAIle) (Table 2). The potency of NAAla (IC_{50}'s ranging between 20 and >50 μM) was strongly dependent on its stereochemistry. In fact, L-NAAla was more potent than D-NAAla with the enzyme from rat brain and rat RBL-2H3 cells, and both enantiomers were very weak inhibitors with the enzyme from mouse brain and mouse N18TG2 cells. Nevertheless, the D-enantiomer exhibited a distinctive trend for higher efficacy (in terms of maximal inhibitory effect achieved at the highest concentration tested). Interestingly, when using human FAAH preparations, both enantiomers were almost equipotent and quite good inhibitors. As to NAIle, this compound, in contrast to NAGly, was strikingly more potent on human FAAH than on the enzyme preparations from either rat or mouse brain or cells. In particular, the potency of NAIle on FAAH from both EFM-19 and MCF-7 cells ($IC_{50} = 18$–34 μM) was comparable to, or slightly lower than, NAGly potency on rat FAAH, and this suggests that this compound, much in the same way as NAGly in rats [23], might exert biological (e.g., antinociceptive) actions in humans by inhibiting anandamide degrada-

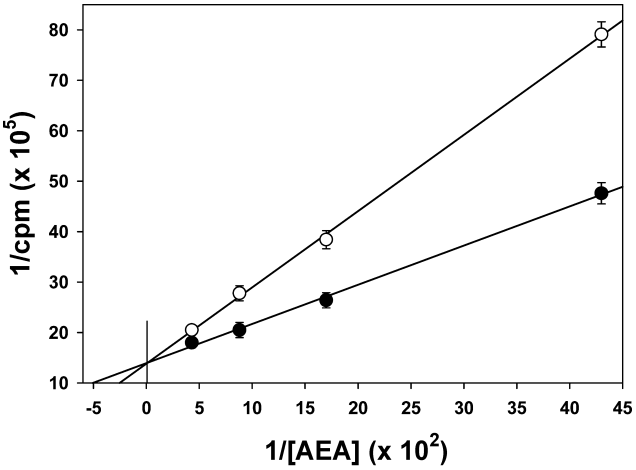

Fig. 2. Lineweaver–Burk curves for the inhibition by *N*-arachidonoyl-glycine (NAGly) of FAAH from mouse N18TG2 cells. Different concentrations (1.0, 2.3, 5.7, 11.4, and 23 μM) of the [^{14}C]AEA substrate were tested, in the absence (closed circles) or presence (open circles) of 25 μM NAGly, but data for the 1 μM are not shown in order to show the detail of the curves at the highest concentrations. Data are means ± SEM of three experiments.

Table 2
FAAH inhibitory activity of the novel most potent *N*-arachidonoyl-amino acids synthesized in this study

Amino acid	Mouse brain	Mouse N18TG2 cells	Rat brain	Rat RBL-2H3 cells	Human MCF-7 cells	Human EFM-19 cells	Human HEK-293 cells
L-Ala	>50 (25.0 ± 1.1)	>50 (25.5 ± 1.4)	38.1 ± 2.2	35.0 ± 1.8	50.0 ± 3. 2	35.1 ± 2.3	20.0 ± 2.1
D-Ala	>50 (43.1 ± 2.1)	>50 (42.6 ± 1.9)	>50 (23.0 ± 2.1)	>50 (31.1 ± 2.2)	>50 (23.0 ± 2.5)	39.5 ± 2.1	38.1 ± 2.0
L-Val	>50 (11.7 ± 3.4)	>50 (11.5 ± 3.9)	>50 (9.0 ± 2.9)	N.T.	N.E.	N.E.	N.T.
L-Leu	>50 (4.0 ± 1.9)	>50 (15.7 ± 3.1)	>50 (16.1 ± 3.5)	N.T.	N.T.	N.E.	N.T.
L-Ile	>50 (33.2 ± 3.2)	>50 (25.1 ± 2.7)	>50 (30.3 ± 2.9)	>50 (34.0 ± 2.2)	34.0 ± 2.9	18.2 ± 1.7	N.T.

Data are means ± SD of $n = 3$ separate experiments and are expressed as IC_{50}, μM, or, when IC_{50}>50 μM, as the percentage inhibition (between brackets) observed at 50 μM. N.T., not tested. N.E., no inhibition up to 50 μM. The amino acid amidated with arachidonoic acid (see Fig. 1) is shown.

tion. For the same reason, also the naturally occurring L-NAAla might exert biological (e.g., analgesic) activity in rats and humans. It is interesting to note that neither the NAIle isomer, *N*-arachidonoyl-leucine, nor *N*-arachidonoyl-valine, which differs from NAIle and L-NAAla only for one less and one more methylene group, respectively, exhibited any FAAH inhibitory activity, irrespective of the brain species or cell used to test the enzyme activity. This observation, together with the different potency of L- and D-NAAla, indicates that the structural requirements for NAA interaction with FAAH are very stringent.

This view was further supported by the finding that the addition of a free carboxylic function to NAAla, as in *N*-arachidonoyl-aspartate, totally abolished its capability to inhibit FAAH. Nor the introduction of a further methylene group in this compound, as in *N*-arachidonoyl-glutamate, or the further amidation, as in *N*-arachidonoyl-glutamine, could restore the FAAH inhibitory activity. Finally, of the aromatic NAAs tested in this study, only *N*-arachidonoyl-phenylalanine exhibited some inhibitory activity, but only at the highest concentration tested (data not shown).

Taken together, the data presented in Tables 1 and 2, and summarized in Fig. 3, while describing the structure–activity relationships within a series of arachidonoylated proteogenic amino acids, also highlight the strong dependence of their activity on the species and source (brain or cell type) of the membranes used to assay the enzyme activity, warning against the extrapolation of data from different assays.

In conclusion, our data have revealed the existence of: (1) an interesting species-dependent potency/selectivity; and (2) stringent structural requirements, for NAA inhibitory action on FAAH. These data might be useful to anticipate the activity of other, yet-to-be found NAAs, making it possible to predict that only few of these compounds might act as substrates of FAAH or as FAAH inhibitors. Though our data contribute to a better understanding of the structure/activity relationships underlying the interaction of long-chain fatty acids with FAAH, their translation into analgesic and anti-inflammatory potency depends on the definitive recognition of the role of FAAH as the molecular target for NAGly. This compound differs from other bioactive fatty acid amide substrates of FAAH due to the presence of a negatively charged functional group, and the discovery of its FAAH binding properties [21] was quite surprising, even though our data show that this activity is rather species-selective. Nevertheless, the recognition of remarkable differences of FAAH inhibiting properties in a series of arachidonoylated proteogenic amino acids suggests that the FAAH active site has a rather narrow ligand tolerance. A similar, though possibly less stringent, situation was recently shown to occur for cyclooxygenase-2, whose active site can recognize anandamide, 2-arachidonoylglycerol, and NAGly [27], but not other arachidonoyl-amides (L. Marnett and V. Di Marzo, unpublished observations).

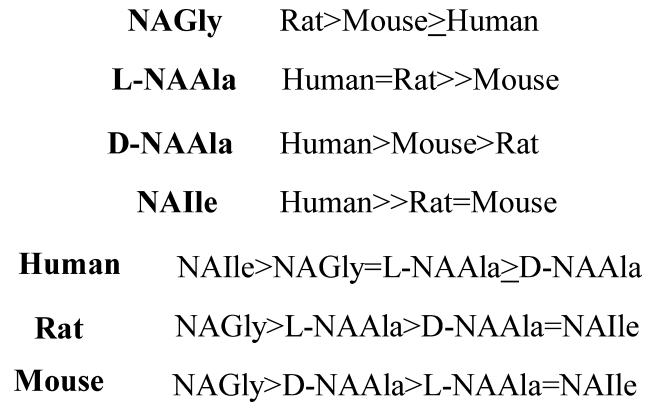

Fig. 3. Different selectivities of FAAH from different species for *N*-arachidonoyl-amino acids. The rank of affinity of each FAAH species for each compound (upper panel) and the rank of potency of each compound for the different FAAH species (lower panel) are shown. Other *N*-arachidonoyl-amino acids with weak or null activity at FAAH are not included.

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