Bioorganic & Medicinal Chemistry Letters 20 (2010) 1210–1213

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and biological evaluation of new potential inhibitors of *N*-acylethanolamine hydrolyzing acid amidase

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ARTICLE INFO

Article history:
Received 2 October 2009
Revised 26 November 2009
Accepted 30 November 2009
Available online 4 December 2009

Keywords:
N-Acylethanolamines
Palmitoylethanolamide
Inflammation
Degradation
Enzyme
Endocannabinoid
Vanilloid
NAAA

ABSTRACT

N-Acylethanolamines, including *N*-palmitoyl-ethanolamine (PEA), are hydrolyzed to the corresponding fatty acids and ethanolamine by fatty acid amide hydrolase (FAAH). Recently, *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) was identified as being able to specifically hydrolyze PEA. In order to find selective and effective inhibitors of this enzyme, we synthesized and screened several amides, retroamides, esters, retroesters and carbamates of palmitic acid (**1–21**) and esters with C15 and C17 alkyl chains (**22–27**). Cyclopentylhexadecanoate (**13**) exhibited the highest inhibitory activity on NAAA (IC_{50} = 10.0 μM), without inhibiting FAAH up to 50 μM. Compound **13** may become a useful template to design new NAAA inhibitors.

N-Palmitoyl-ethanolamine (PEA) is an endogenous lipid produced by most mammalian cells[1,2] well known for its anti-inflammatory and analgesic properties.[3–5] It is biosynthesized from a phospholipid precursor, *N*-palmitoyl-phosphatidyl-ethanolamine, through the catalytic action of *N*-acyl-phosphatidyl-ethanolamine-selective phospholipase D (NAPE–PLD).[6] Its inactivation to palmitic acid and ethanolamine is catalyzed by fatty acid amide hydrolase (FAAH)[7] and, more selectively, also by *N*-acylethanolamine-hydrolyzing acid amidase (NAAA).[8] The molecular mechanism of action of PEA is still controversial and several hypotheses have been put forward to explain its anti-inflammatory and analgesic effects. These include: (1) an Autacoid Local Inflammation Antagonism (ALIA) mechanism through which PEA acts by down-regulating mast-cell degranulation;[9] (2) the direct stimulation of an as-yet uncharacterized cannabinoid CB_2 receptor-like target;[3,10,11] (3) an 'entourage effect',[12–14] through which PEA would enhance the anti- inflammatory and anti-nociceptive effects exerted by another fatty acid ethanolamide, anandamide (AEA), which is often produced together with PEA, and activates cannabinoid CB_1 and CB_2 receptors or the transient receptor potential vanilloid receptor type 1 (TRPV1) channel;[15–18] (4) the direct activation of the nuclear peroxisome proliferator-activated receptor-α (PPAR-α), which clearly mediates many of the anti-inflammatory effects of this compound;[19] and (5) the direct activation of the orphan receptor G-protein coupling, GPR55.[20] Whatever its mechanism of action, based on its pharmacological properties, and on the fact that its tissue concentrations are altered during several pathological conditions,[5,21] PEA has been proposed to act as a protective endogenous mediator produced 'on demand' during inflammatory and neurodegenerative conditions to counteract inflammation, neuronal damage and pain. However, the lack of pharmacological tools able to selectively modulate its tissue levels (such as specific inhibitors of its biosynthesis or degradation), has negated so far the conclusive demonstration of such protective functions for endogenous PEA. Indeed, at the present time, there is almost no report of NAAA inhibitors. The screening of different esters, retroesters and retroamides of palmitic acid[22] allowed to identify three compounds that inhibited NAAA: *N*-(3-hydroxy-propionyl)pentadecanamide, cyclohexylhexadecanoate (**15**) and hexadecyl propionate (Fig. 1). These compounds were selective for NAAA, with no inhibitory activity on FAAH,[22] and exhibited IC_{50} values of 32, 19, and 54 μM, respectively.[23] Tsuboi et al. reported two additional compounds, *N*-pentadecylbenzamide and *N*-pentadecylcyclohexanecarboxamide (**28**) (Fig. 1), which exhibited

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doi:10.1016/j.bmcl.2009.11.134

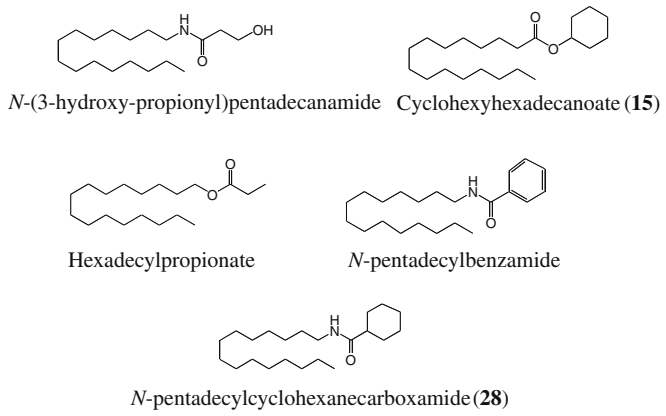


Figure 1. Chemical structures of the previously reported esters, retroesters and retroamides of palmitic acid and NAAA inhibitors.

stronger inhibitory activity, with IC_{50} values of 8.3 μM and 4.5 μM, respectively.[24] Compound **28** is, therefore, the most efficacious and selective inhibitor of NAAA reported so far, and its mechanism of action is reversible and non-competitive.[24]

The aim of the present work was to gain new insights into the structure–activity relationships of NAAA inhibition, and possibly to obtain new NAAA inhibitors able to increase the endogenous levels of PEA, to be used as pharmacological tools to corroborate the suggested role of this compound in various pathological conditions.[21] With this purpose, we established a specific enzymatic assay of NAAA activity, using human embryonic kidney (HEK)-293 cells in which the cDNA encoding the human enzyme was stably overexpressed (HEK-NAAA cells). NAAA activity was measured as the capability of membranes from these cells to hydrolyze [^{14}C]PEA to palmitic acid and [1,2-^{14}C]ethanolamine, as quantified using a liquid scintillation analyzer. In parallel, we designed and synthesized several new PEA analogues, to be tested as potential NAAA inhibitors in this assay. We carried out the synthesis of new amides (**1–9**), retroamides (**10–12**), esters (**13–16**), retroesters (**17–19**) and carbamates (**20–21**) of palmitic acid, and of esters with C15 and C17 alkyl chains (**22–24** and **25–27**, respectively) (Scheme 1).

In agreement with the findings previously obtained in various cells and mammalian tissues,[23,25] we found that significantly higher NAAA activity is found at pH 5 in the membranes (submitted to two cycles of freezing and thawing) of HEK-NAAA cells, whereas no appreciable activity was detected in HEK293 wild-type (HEK-WT) cells (data not shown).[26] Consequently, we concluded that HEK-NAAA cell membrane fractions could be used for our subsequent experiments. To validate the assay, we next tested the most potent selective NAAA inhibitor identified so far, compound **28**, and found that the inhibition was concentration-dependent with an IC_{50} value under our conditions (17.0 ± 1.4 μM) higher than that previously reported by Tsuboi et al., possibly because these authors used a different source of enzyme from another species (the rat lung) to measure NAAA activity.[24] The screening of the new analogues synthesized here allowed to identify a compound more active than **28**, in which the amide bond and the cyclohexyl group were changed to an ester bond and a cyclopentyl group, respectively.

The new compound, cyclopentylhexadecanoate (**13**), inhibited NAAA by 85% at 50 μM. The inhibition was concentration-dependent and the IC_{50} was calculated as 10.0 ± 2.1 μM (Fig. 2). It should be noted that among the ester derivatives, the nature of the cycle inserted was important to observe an inhibitor activity. In fact, although much less than **13**, cyclobutylhexadecanoate (**16**) also inhibited NAAA activity to some extent (41% inhibition at the maximal concentration tested). Increasing the steric hindrance or

Equation 1

$$CH_3(CH_2)_{14}\text{-}COCl \xrightarrow[\text{Toluene, } Na_2CO_3]{H_2N\text{-}R^1} CH_3(CH_2)_{14}\text{-}CONH\text{-}R^1$$

(1-9)

Equation 2

$$CH_3(CH_2)_{15}\text{-}NH_2 \xrightarrow[R^2\text{-}COCl]{\text{TEA, DCM}} CH_3(CH_2)_{15}\text{-}NHCO\text{-}R^2$$

(10-12)

Equation 3

$$CH_3(CH_2)_n\text{-}COOH \xrightarrow[\text{DCM, } R^3\text{-}OH \text{ or } R^6\text{-}OH]{\text{DCC, DMAP}} CH_3(CH_2)_n\text{-}COO\text{-}R$$

n=14 (13-16) with R^3
n=13 (22-24) with R^6
n=15 (25-27) with R^6

Equation 4

$$CH_3(CH_2)_{14}\text{-}CH_2OH \xrightarrow[\text{DCM, TEA}]{R^4\text{-}COCl} CH_3(CH_2)_{14}\text{-}CH_2\text{-}OCO\text{-}R^4$$

(17-19)

Equation 5

$$CH_3(CH_2)_{14}\text{-}CH_2OH \xrightarrow[\text{Toluene}]{NCO\text{-}R^5} CH_3(CH_2)_{14}\text{-}CH_2\text{-}OCONH\text{-}R^5$$

(20-21)

Scheme 1. Chemical synthesis of compounds (**1–27**).

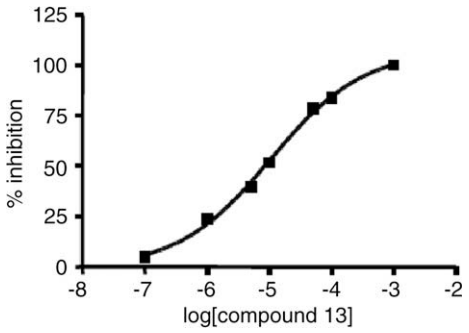


Figure 2. Concentration-dependent inhibition of human recombinant NAAA by **13**. Mean values ± S.D. are shown. $N = 3$ separate determinations were carried out for these experiments.

inserting an aromatic ring also considerably reduced or even erased the inhibitory activity. In fact, **15** and 4-methoxyphenyl hexadecanoate (**14**) exhibited, respectively, 18% inhibition at the maximal concentration tested and no activity (Table 1).

All other compounds were significantly less active or inactive, with IC_{50} values always higher than 50 μM (Table 1). In particular, among the amide derivatives, only the insertion of a *p*-nitrophenyl substituent (**1**) introduced some NAAA inhibitory activity (15% inhibition at the maximal concentration tested), whereas all other amide derivatives were significantly inactive. We also observed that all the ester derivatives with an alkyl chain length of C15 or C17 exhibited a modest activity or were inactive, thus suggesting that the length of alkyl chain also influences the potency of these compounds (Table 1).

Compound **13** exhibited no inhibitory activity on FAAH, nor any binding activity at CB_1 and CB_2 receptors, or any functional activity at TRPV1 receptors, at concentrations as high as 50 μM (data not shown).[26] The type of inhibition of NAAA by **13** was examined by incubating different concentrations of PEA with HEK-NAAA membranes in the presence or absence of 20 μM **13**. As indicated by

Table 1
Inhibition of NAAA by new analogues of palmitic acid

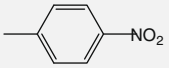
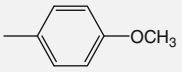
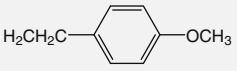
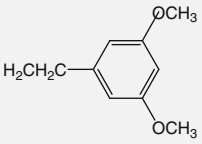
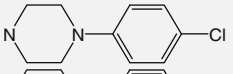
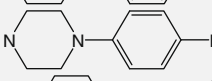
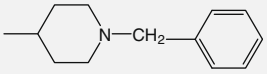
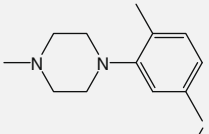
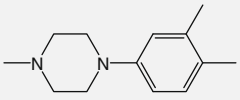
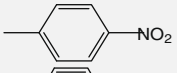
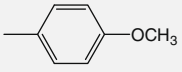
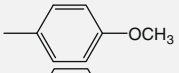
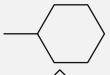
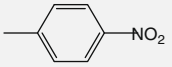
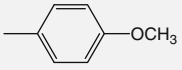
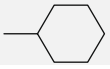
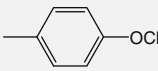
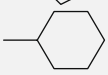
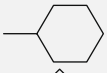
Compound	R^1	Maximal inhibition of NAAA at 50 μM
$CH_3(CH_2)_{14}$–CONH–R^1		
1		15%
2		Inactive
3		Inactive
4		Inactive
5		Inactive
6		Inactive
7		Inactive
8		Inactive
9		Inactive
$CH_3(CH_2)_{15}$–NHCO–R^2		
	R^2	
10		8%
11		4%
12		Inactive
$CH_3(CH_2)_{14}$–COO–R^3		
	R^3	
13		85%
14		Inactive
15		18%
16		41%
$CH_3(CH_2)_{14}$–CH_2–OCO–R^4		
	R^4	
17		17%
18		26%
19		Inactive
$CH_3(CH_2)_{14}$–CH_2–OCONH–R^5		
	R^5	
20		Inactive

Table 1 (*continued*)

	R^5	
21		Inactive

Compound	*n*	R^6	Maximal inhibition of NAAA at 50 μM
$CH_3(CH_2)_n$–COO–R^6			
22	13		25%
23	13		28%
24	13		29%
25	15		Inactive
26	15		Inactive
27	15		Inactive

Data are means of three separate determinations and are expressed as IC_{50} (μM) or, when this was higher than 10 μM, as maximum inhibition observed at a 50 μM concentration.

Lineweaver–Burk plots (not shown), the inhibition appeared to be of a competitive nature, since, in the presence of **13**, the apparent K_m value for PEA was significantly enhanced from 118 ± 2.0 μM to 198 ± 1.0 μM, with no significant change in the V_{max} value (11.1 ± 3.6 nmol min^{-1} mg $proteins^{-1}$ and 14.1 ± 3.4 nmol min^{-1} mg $proteins^{-1}$, respectively). Finally, **13** also appeared to inhibit NAAA in intact HEK-NAAA cells since it selectively increased the levels of PEA (as determined by isotope-dilution liquid chromatography-mass spectrometry)[27,28] in these cells from 2.39 ± 0.21 pmol/mg lipid to 3.96 ± 0.03 pmol/mg lipid, without influencing the levels of the other *N*-acylethanolamines, AEA and OEA, nor of the other endocannabinoid, 2-arachidonoylglycerol.

In summary, **13** is a selective and competitive inhibitor of NAAA with potency, under our assay conditions using a human recombinant enzyme, nearly two-fold higher than that of the previously described **28**. Compound **13** inhibits NAAA both in the cell-free preparations and intact cells, and will be useful both as a template to design new and more potent NAAA inhibitors and as a pharmacological tool to investigate the consequences of pharmacological elevation of endogenous PEA levels in cells and tissues.

Acknowledgments

This work was partly supported by Epitech Group S.r.l. The authors are grateful to Marco Allarà for technical assistance.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.11.134.

References and notes

1. Di Marzo, V.; De Petrocellis, L.; Sepe, N.; Buono, A. *Biochem. J.* **1996**, *316*, 977.
2. Bisogno, T.; Maurelli, S.; Melck, D.; De Petrocellis, L.; Di Marzo, V. *J. Biol. Chem.* **1997**, *272*, 3315.
3. Calignano, A.; La Rana, G.; Giuffrida, A.; Piomelli, D. *Nature* **1998**, *394*, 277.
4. Costa, B.; Conti, S.; Giagnoni, G.; Colleoni, M. *Br. J. Pharmacol.* **2002**, *137*, 413.
5. Darmani, N. A.; Izzo, A. A.; Degenhardt, B.; Valenti, M.; Scaglione, G.; Capasso, R.; Sorrentini, I.; Di Marzo, V. *Neuropharmacology* **2005**, *48*, 1154.
6. Okamoto, Y.; Morishita, J.; Tsuboi, K.; Tonai, T.; Ueda, N. *J. Biol. Chem.* **2004**, *279*, 5298.

7. Cravatt, B. F.; Giang, D. K.; Mayfield, S. P.; Boger, D. L.; Lerner, R. A.; Gilula, N. B. *Nature* **1996**, *384*, 83.
8. Ueda, N.; Yamanaka, K.; Yamamoto, S. *J. Biol. Chem.* **2001**, *276*, 35552.
9. Aloe, L.; Leon, A.; Levi-Montalcini, R. *Agents Actions.* **1993**, *39 Spec No*, C145.
10. Calignano, A.; La Rana, G.; Piomelli, D. *Eur. J. Pharmacol.* **2001**, *419*, 191.
11. Conti, S.; Costa, B.; Colleoni, M.; Parolaro, D.; Giagnoni, G. *Br. J. Pharmacol.* **2002**, *135*, 181.
12. Ben-Shabat, S.; Fride, E.; Sheskin, T.; Tamiri, T.; Rhee, M. H.; Vogel, Z.; Bisogno, T.; De Petrocellis, L.; Di Marzo, V.; Mechoulam, R. *Eur. J. Pharmacol.* **1998**, *353*, 23.
13. Lambert, D. M.; Di Marzo, V. *Curr. Top. Med. Chem.* **1999**, *6*, 757.
14. Di Marzo, V.; Melck, D.; Orlando, P.; Bisogno, T.; Zagoory, O.; Bifulco, M.; Vogel, Z.; De Petrocellis, L. *Biochem. J.* **2001**, *358*, 249.
15. De Petrocellis, L.; Davis, J. B.; Di Marzo, V. *FEBS Lett.* **2001**, *506*, 253.
16. Smart, D.; Jonsson, K. O.; Vandevoorde, S.; Lambert, D. M.; Fowler, C. J. *Br. J. Pharmacol.* **2002**, *136*, 452.
17. Ho, W. S.; Barrett, D. A.; Randall, M. D. *Br. J. Pharmacol.* **2008**, *155*, 837.
18. Costa, B.; Comelli, F.; Bettoni, I.; Colleoni, M.; Giagnoni, G. *Pain* **2008**, *139*, 541.
19. Lo Verme, J.; Fu, J.; Astarita, G.; La Rana, G.; Russo, R.; Calignano, A.; Piomelli, D. *Mol. Pharmacol.* **2005**, *67*, 15.
20. Ryberg, E.; Larsson, N.; Sjögren, S.; Hjorth, S.; Hermansson, N. O.; Leonova, J.; Elebring, T.; Nilsson, K.; Drmota, T.; Greasley, P. J. *Br. J. Pharmacol.* **2007**, *152*, 1092.
21. Re, G.; Barbero, R.; Miolo, A.; Di Marzo, V. *Vet. J.* **2007**, *173*, 21.
22. Vandevoorde, S.; Tsuboi, K.; Ueda, N.; Jonsson, K. O.; Fowler, C. J.; Lambert, D. M. *J. Med. Chem.* **2003**, *46*, 4373.
23. Ueda, N.; Tsuboi, K.; Lambert, D. M. *Neuropharmacology* **2005**, *48*, 1079.
24. Tsuboi, K.; Hilligsmann, C.; Vandevoorde, S.; Lambert, D. M.; Ueda, N. *Biochem. J.* **2004**, *379*, 99.
25. Tsuboi, K.; Sun, Y. X.; Okamoto, Y.; Araki, N.; Tonai, T.; Ueda, N. *J. Biol. Chem.* **2005**, *280*, 11082.
26. For a detailed description of materials and methods, synthetic procedures and characterization of the novel synthesized compounds see online Supplementary data.
27. Marsicano, G.; Wotjak, C. T.; Azad, S. C.; Bisogno, T.; Rammes, G.; Cascio, M. G.; Hermann, H.; Tang, J.; Hofmann, C.; Zieglgansberger, W.; Di Marzo, V.; Lutz, B. *Nature* **2002**, *418*, 530.
28. Di Marzo, V.; Goparaju, S. K.; Wang, L.; Liu, J.; Batkai, S.; Jarai, Z.; Fezza, F.; Miura, G. I.; Palmiter, R. D.; Sugiura, T.; Kunos, G. *Nature* **2001**, *410*, 822.