

This is the postprint (accepted) version of the final paper, published afterwards, here the link:
<https://doi.org/10.1021/acs.jmedchem.0c00191>

We thank ACS for the opportunity.

N-Acylethanolamine Acid Amidase (NAAA): Structure, Function and Inhibition

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KEYWORDS: Palmitoylethanolamide, anandamide, peroxisome proliferator-activated receptor type- α (PPAR- α), inflammation, resolution.

ABSTRACT

N-Acylethanolamine acid amidase (NAAA) is an N-terminal cysteine hydrolase primarily found in the endosomal-lysosomal compartment of innate and adaptive immune cells. NAAA catalyzes the hydrolytic deactivation of palmitoylethanolamide (PEA), a lipid-derived peroxisome proliferator-activated receptor- α (PPAR- α) agonist that exerts profound anti-inflammatory effects in animal models. Emerging evidence points to NAAA-regulated PEA signaling at PPAR- α as a critical control point for the induction and the resolution of inflammation, and to NAAA itself as a target for anti-inflammatory medicines. The present perspective discusses three key aspects of this hypothesis: the role of NAAA in controlling the signaling activity of PEA; the structural bases for NAAA function and inhibition by covalent and non-covalent agents; and, finally, the potential value of NAAA-targeting drugs in the treatment of human inflammatory disorders.

INTRODUCTION

Mammalian host-defense cells produce a diverse array of lipid-derived signaling molecules that enable the initial escalation of the inflammatory response. For example, macrophages and neutrophils release cyclooxygenase metabolites of arachidonic acid that cause local vasodilation and sensitization of nociceptive nerve fibers, two key signs of acute inflammation (for review, see ref. 1). The history of cyclooxygenase inhibitors proves that interrupting the formation of these pro-inflammatory mediators can have remarkable therapeutic benefits. But host-defense cells also generate signals that *counter* inflammation, either by heightening the intrinsic resistance of tissues to injury and infection, or by facilitating the transition toward resolution and healing. Lipid

messengers involved in these defensive and reconstructive actions include products of oxidative polyunsaturated fatty acid metabolism (e.g., lipoxins and resolvins; for review, see ref. 1, 2) as well as amides of polyunsaturated and saturated fatty acids, such as anandamide and palmitoylethanolamide (PEA) (**Figure 1**).

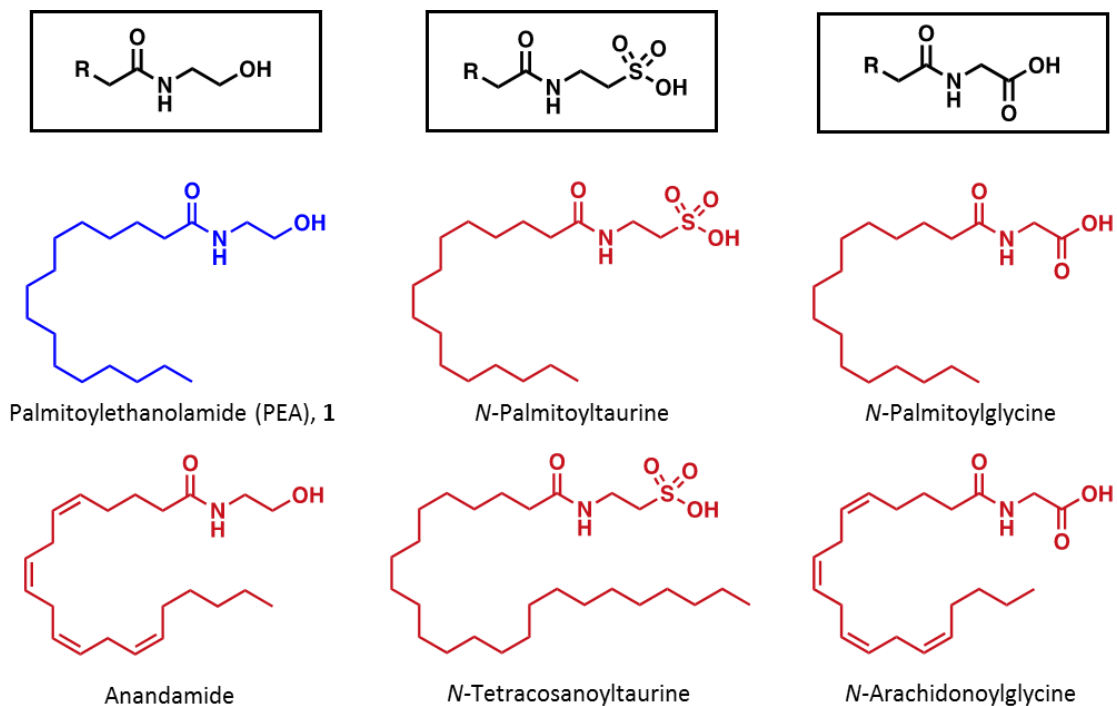


Figure 1. Bioactive amides of long-chain fatty acids that are preferred substrates either for NAAA (blue) or FAAH (red). Fatty acid ethanolamides (also known as *N*-fatty acyl ethanolamines, left) include endogenous agonists for the ligand-operated transcription factor PPAR- α (e.g., PEA, oleoylethanolamide) and for G protein-coupled cannabinoid-1 receptor (e.g., anandamide). In animal models, *N*-fatty acyl taurines (center) accelerate skin wound healing³ and modulate feeding and glucose homeostasis,^{4,5} while *N*-fatty acyl glycines (right) exert analgesic and anti-inflammatory effects.⁶ The receptor mechanisms underpinning the latter effects are only partially understood.

Anandamide and PEA are generated, either constitutively or upon demand, by various types of host-defense cells.⁷ They ligate cannabinoid receptors⁸ and peroxisome proliferator-activated receptor- α (PPAR- α),^{9,10,11} respectively, to attenuate nociceptive responding,⁷ diminish production of pro-inflammatory cytokines and eicosanoids,¹² and stimulate macrophages to remove invading bacteria and apoptotic neutrophils.^{13,14} The two intracellular enzymes responsible for terminating these actions, *N*-acylethanolamine acid amidase (NAAA) and fatty acid amide hydrolase (FAAH), share the ability to cleave lipid amides but differ in virtually every other respect (**Figure 2**). FAAH uses the side chain of an internal serine as catalytic nucleophile to attack amide bonds in a diverse set of fatty acid amides that can access its relatively spacious substrate-binding cavity (for review, see ref. 15). An integral membrane protein, FAAH functions as a homodimer and is found in the endoplasmic reticulum and mitochondria of most mammalian cells. NAAA, on the other hand, utilizes as nucleophile an N-terminal cysteine exposed during protein maturation and recognizes substrates that, like PEA, are able to fit into the narrow hydrophobic pocket that lies at the core of its active site. It is a soluble heterodimeric protein and is primarily, albeit not exclusively, localized to the lysosomal compartment of innate and adaptive immune cells (for earlier reviews on NAAA, see ref. 16,17,18).

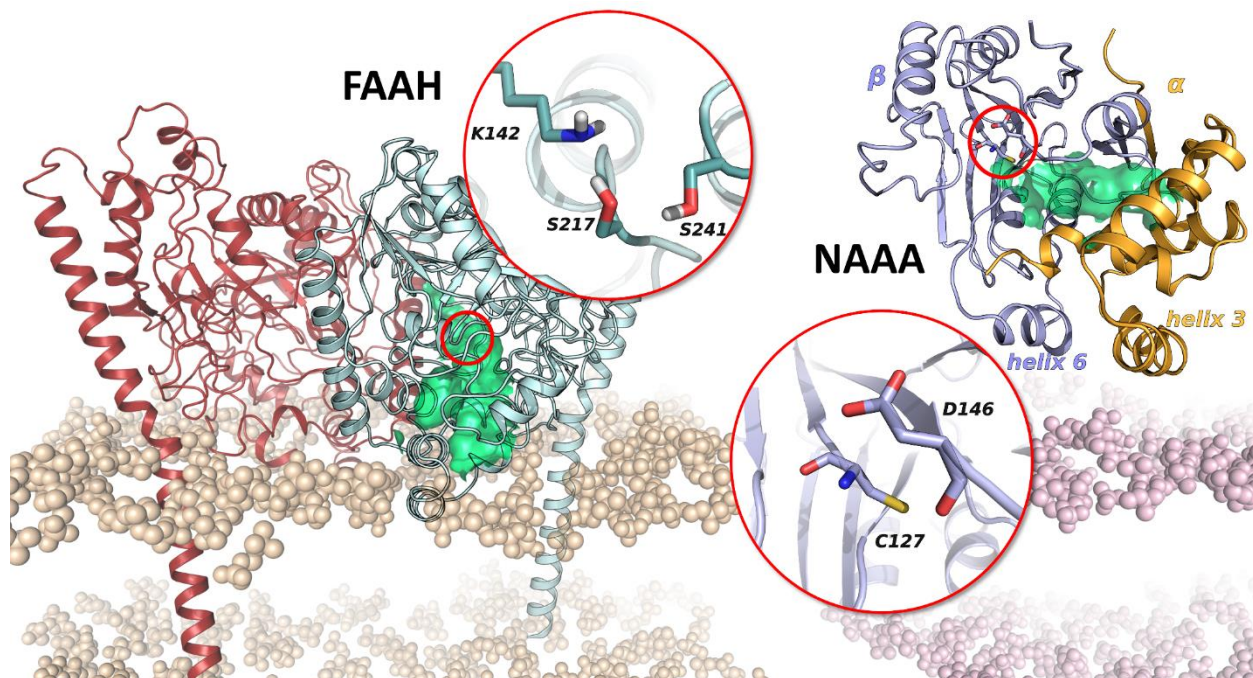


Figure 2. Structural and functional differences between FAAH and NAAA. Left: structure of the FAAH homodimer modeled from crystal coordinates (PDB: 1MT5),¹⁹ adding the membrane-spanning helix and a model phospholipid bilayer (polar heads shown as spheres). The two monomers (identified in cyan and red) are oriented in parallel, each with an α -helix spanning the membrane and pointing their large substrate-binding cavities (green in the monomer to the right) in the same direction, towards the membrane. The red circle marks the position of the catalytic triad (Ser241, Lys142 and Ser217), which is magnified in the inset. Right: representation of NAAA based on the crystal structure of its rabbit orthologue (PDB: 6DY1).²⁰ The closely juxtaposed α (orange) and β (grey) subunits shape a rectilinear and relatively narrow substrate-binding pocket (green). Substrate recruitment may occur through an opening created by the interaction of α -helices 3 and 6 with the lipid membrane. The N-terminal catalytic nucleophile (Cys127 for rabbit NAAA; the residues are numbered following the primary sequence deposited in UniProt)²¹ and Asp146, which interacts with the amine group of the N-terminal cysteine, are highlighted in the inset.

Since its characterization as an anandamide amide hydrolase in 1995^{22,23,24} (for a review of previous work see ref. 25), FAAH has been the object of intensive study and is now undergoing clinical evaluation as target for social anxiety, post-traumatic stress disorder and painful peripheral neuropathies. NAAA, which was first identified by Natsuo Ueda, Shozo Yamamoto and collaborators in 1999,²⁶ has attracted comparatively less attention. Nevertheless, the discovery of several classes of small-molecule inhibitors has revealed potentially important functions for NAAA in the tissue response to injury. In particular, recent studies have shed new light on three areas, which are the focus of the present review: first, the role of NAAA in the regulation of PEA-mediated signaling at PPAR- α ; second, the structural requirements for NAAA inhibition by covalent and non-covalent agents; and, finally, the potential utility of NAAA-targeting drugs in the treatment of human inflammatory disorders.

PEA-MEDIATED SIGNALING AND THE ROLE OF NAAA

PEA was identified in 1957 as an anti-inflammatory lipid constituent of egg yolk, soybeans and peanuts.^{27,28} Preclinical and clinical studies in the 1960s and 1970s documented its ability to dampen allergic and inflammatory responses in animal models^{29,30,31,32} and to alleviate symptoms of viral respiratory tract infection in young and adult humans.^{33,34} In the 1990s, after a hiatus of more than ten years, renewed interest in PEA led to a further appraisal of its anti-inflammatory effects,^{35,36,37,38,39} to the discovery of its neuroprotective⁴⁰ and antinociceptive^{41,42} properties, and eventually to the identification of the ligand-operated transcription factor PPAR- α as its primary molecular target.^{9,10,11} The last decade of preclinical research has firmly established PEA as an analgesic and anti-inflammatory agent, and has confirmed that the majority of its actions are rooted

in the ability to engage PPAR- α (for review, see ref. 43). Strengthening the translational significance of these findings, preliminary clinical evidence indicates that PEA – which is now commercially available in many countries as cream for topical use (Mimyx®) and dietary supplement – may be beneficial in inflammatory pathologies such as atopic dermatitis⁴⁴ and irritable bowel syndrome⁴⁵ as well as in painful disorders such as fibromyalgia,⁴⁶ chemotherapy-induced neuropathy,⁴⁷ chronic prostatitis⁴⁸ and the side effects of interferon- γ treatment in persons with multiple sclerosis⁴⁹ (for a systematic review and meta-analysis, see ref. 50).

Shortly after its isolation from food sources²⁷ PEA was also discovered in mammalian tissues⁵¹ and efforts were soon undertaken to elucidate the mechanisms responsible for its formation and degradation. Early studies suggested (for review, see ref. 25) and subsequent work confirmed (for review, see ref. 52), that PEA and its fatty acid ethanolamide congeners are produced through a pathway consisting of two enzyme-catalyzed steps (Figure 3). First, the calcium-dependent phospholipase A₂ PLA2G4E catalyzes the transfer of a fatty acyl group (e.g. palmitate) from the *sn*-1 position of phosphatidylcholine to the free amine of phosphatidylethanolamine (PE).⁵³ This reaction produces a set of *N*-acyl PE species with varying *N*-, *sn*-1, *sn*-2 substituents, which are then cleaved by the zinc-containing hydrolase *N*-acyl PE-selective phospholipase D (NAPE-PLD)^{54,55,56} to generate PEA and other fatty acid ethanolamides (also known as *N*-acyl ethanolamines). This NAPE-PLD-dependent (or ‘canonical’) pathway is critical for PEA mobilization in mouse macrophages⁵⁷ but, as illustrated in Figure 3, other multi-step mechanisms of *N*-acyl PE cleavage may exist (for review, see ref. 52). Like its formation, PEA degradation involves more than one enzyme since, as mentioned earlier, both NAAA and FAAH can degrade the compound in intact cells.⁵⁸ Even though the biological significance of this catabolic redundancy is unclear, the different subcellular and cellular distributions of the two enzymes –

along with the lack of compensation by FAAH after genetic deletion of NAAA⁵⁹ – imply that they might act on topologically and functionally distinct pools of PEA.

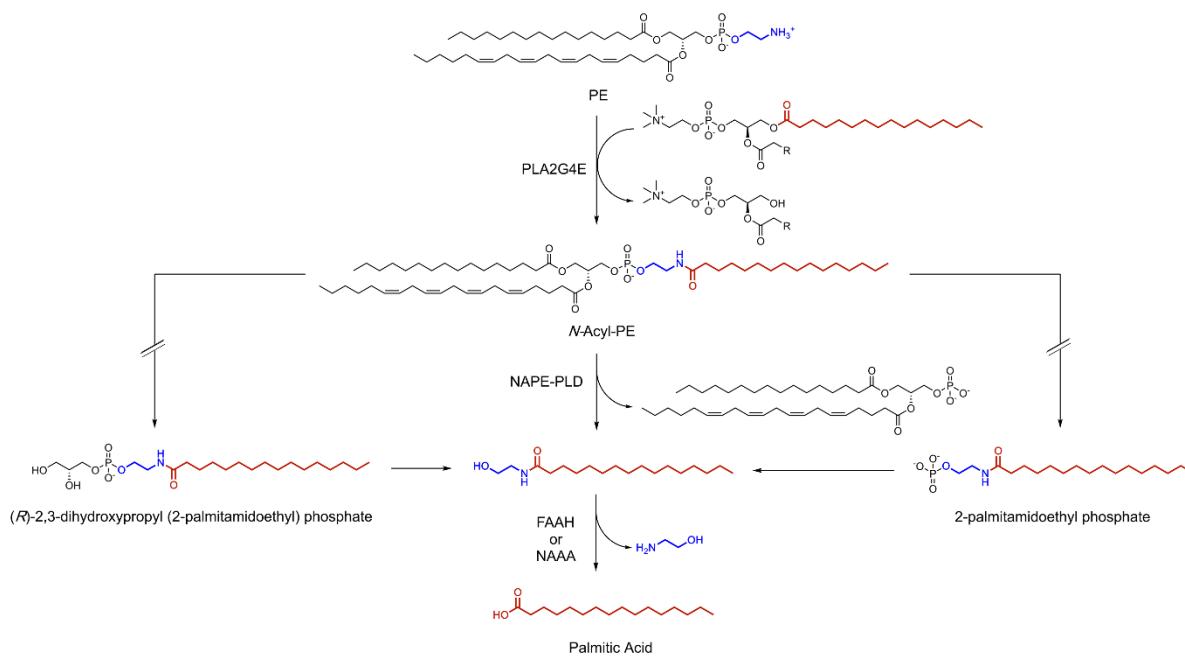


Figure 3. Formation and degradation of PEA. The ‘canonical’ pathway of PEA production, shown in the middle, proceeds through two steps: first, the transfer of palmitate from the *sn*-1 position of phosphatidylcholine (PC) to the free amine group of phosphatidylethanolamine (PE), catalyzed by PLA2G4E, yields *N*-palmitoyl PE;⁵³ second, NAPE-PLD-mediated cleavage of *N*-palmitoyl PEs generates PEA.^{54,55,56} The acyl chain giving rise to PEA is highlighted in red. Multi-step ‘non-canonical’ routes also transit through *N*-palmitoyl PE, but involve different enzymes and metabolic intermediates.⁵² The hydrolytic deactivation of PEA may be catalyzed either by NAAA or FAAH, which have broadly different subcellular and cellular distributions.

What controls PEA mobilization? Unlike anandamide, which is primarily generated upon demand *via* activity- or receptor-dependent mechanisms, PEA appears to be constitutively produced in macrophages and other peripheral cells.⁷ This process is suppressed in inflammatory states, when the formation and degradation of PEA are altered in opposite directions. NAPE-PLD expression is lowered whereas NAAA activity and/or expression is heightened in mouse macrophages incubated with gram-negative bacterial endotoxin (lipopolysaccharide),⁵⁷ paws and dorsal root ganglia of rats challenged with complete Freund's adjuvant,⁶⁰ synovial membrane and lumbar spinal cord of rats treated with mono-iodoacetate,⁶¹ and intestinal biopsies of persons suffering from ulcerative colitis.⁶² Furthermore, marked decreases in PEA content have been observed in inflammatory exudates of endotoxin- or carrageenan-exposed mice,^{63,64,65} skin of mice challenged with a phorbol ester⁹ and small intestine of mice treated with croton oil.^{66,67} Strikingly low PEA levels were also found in synovial fluid of patients with rheumatoid arthritis or osteoarthritis, compared to healthy subjects.⁶⁸

The possibility, raised by these findings, that NAAA might contribute to the induction and maintenance of inflammation is supported by three additional lines of evidence. First, the enzyme is primarily expressed in cell lineages that mediate innate and adaptive immune reactions – including tissue-resident macrophages, circulating monocytes, B-lymphocytes and CD8+ cytotoxic T cells.^{69,70,71} Second, mice in which the *Naaa* gene is deleted by homologous recombination fail to develop allergic contact dermatitis when challenged with the sensitizing hapten 1-fluoro-2,4-dinitrobenzene.⁷² Lastly, as discussed in a subsequent section of this review, small-molecule NAAA inhibitors exert beneficial effects in animal models used to investigate acute and chronic inflammation. The fact that such effects depend, in a majority of cases, on the activation of PPAR- α by endogenously produced PEA^{61,63,70,72,73} prompted the suggestion that

NAAA activity might promote inflammation and delay resolution by suppressing PEA-dependent PPAR- α activation (Figure 4). It should be noted that palmitic acid, one of the two products of PEA hydrolysis, disables the obligatory PPAR- α co-activator PGC-1 α ^{74,75} and heightens the ability of macrophages to respond to inflammatory triggers.⁷⁶ It is thus possible, though remains to be demonstrated, that NAAA could serve as a ‘rheostat’ to balance the availability of these metabolically linked but functionally antagonistic lipid substances.

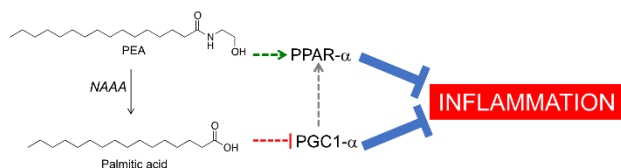


Figure 4. Hypothetical model of the role of NAAA in inflammation. NAAA catalyzes the hydrolytic cleavage of PEA into palmitic acid and ethanolamine. PEA is an endogenous agonist of the anti-inflammatory nuclear receptor PPAR- α . Conversely, palmitic acid may promote inflammation, at least in part, by suppressing expression of the transcription co-activator PGC1- α ,^{74,75} which enables PPAR- α (dashed arrow) and other transcriptional regulators of inflammation (e.g., PPAR- γ).⁷⁷ The model posits that NAAA activity might promote and sustain inflammation (i.e., slow down or prevent resolution) by removing the protective influence of PEA while concomitantly accelerating the generation of palmitic acid.

STRUCTURE AND FUNCTION OF NAAA

NAAA belongs to the N-terminal nucleophile (Ntn) superfamily of enzymes but shares significant sequence homology (35% identity) with only one other member of that group, acid ceramidase (*ASAH1*), a lysosomal cysteine hydrolase that converts various ceramide species into

fatty acid and sphingosine.⁷⁸ Ntn enzymes owe their name to a shared catalytic mechanism in which the nucleophilic side chain of the protein's amino terminal – cysteine, serine or threonine – attacks susceptible carbonyl groups in a variety of structurally diverse substrates.^{79,80} Translated as inactive precursors, Ntn proteins are activated by removal of the initiator methionine or by self-catalyzed cleavage of an internal peptide bond. The latter reaction involves the same amino acid residue responsible for catalysis proper, and yields a mature protein whose two subunits (conventionally termed α and β) remain physically attached.^{79,80}

The sequence alignment of NAAA across three mammalian species is reported in **Figure 5**. Its signal peptide is removed during translation and self-proteolysis gives rise to a heterodimer in which a relatively small α -subunit is closely associated with a larger β -subunit that starts with the catalytic nucleophile – Cys126 for human NAAA, Cys131 for mouse and rat, and Cys127 for rabbit and Cys123 for guinea pig (**Figure 6**).^{20,81}

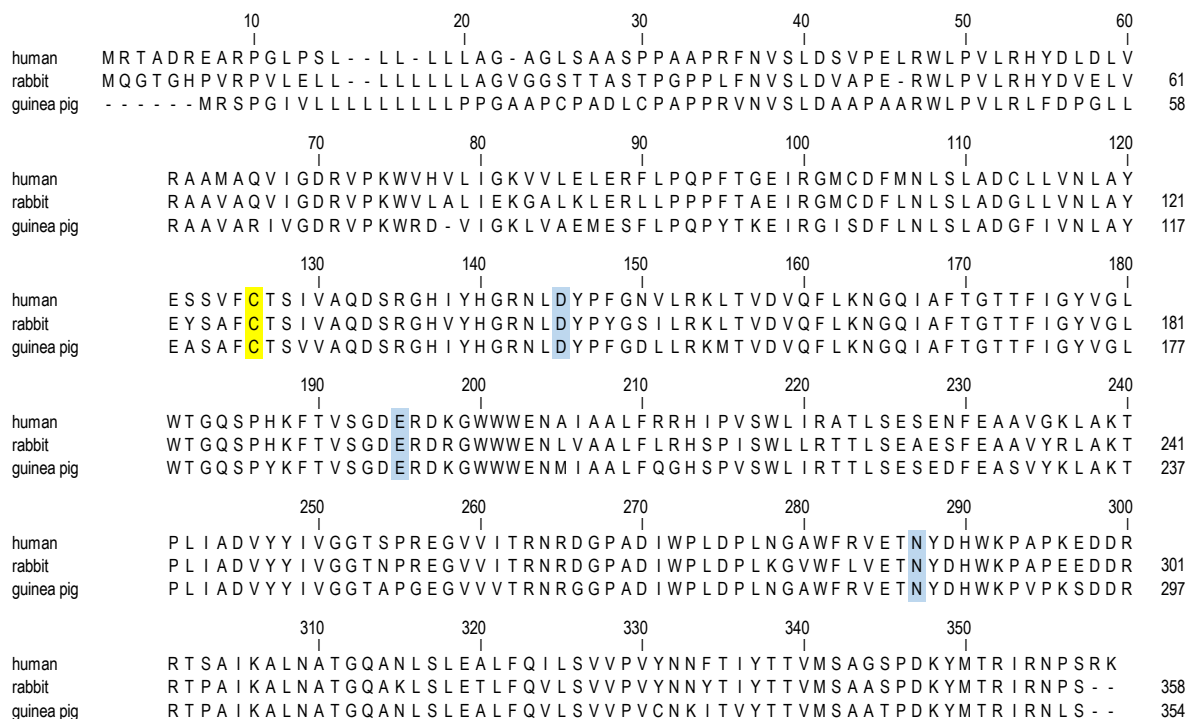


Figure 5. Sequence alignment of crystallized NAAA orthologues discussed in this review.

Numbers on the top of the graph refer to human NAAA, numbers on the right refer to rabbit or guinea-pig NAAA. The catalytic cysteine residues (Cys126 for human NAAA, corresponding to Cys127 in rabbit and Cys123 in guinea pig) are highlighted in yellow. Other residues involved in catalysis are highlighted in cyan.

Consistent with its postulated (but not yet fully proven) subcellular localization to lysosomes,^{58,69} which maintain an internal pH of 4.6 to 5,⁸² NAAA undergoes self-cleavage only in an acidic environment.⁸¹ This presumably involves the attack by Cys126 to the carbonyl group in the preceding peptide bond (with Phe125) to generate a catalytically competent dimer.²⁰ It is possible, but remains to be tested, that self-proteolysis might enable the recruitment of NAAA by specific cellular signals (e.g., inflammatory triggers) and/or within appropriate subcellular compartments in which signaling-competent PEA might also be found (e.g., in acidic late-stage endosomes and lysosomes *versus* pH-neutral early-stage endosomes). Before self-cleavage, NAAA undergoes *N*-glycosylation.^{20,81,83} This modification occurs at four highly conserved sites, two in each subunit of the mature protein,⁸³ and may contribute to its subcellular targeting and structural stabilization.^{83,84}

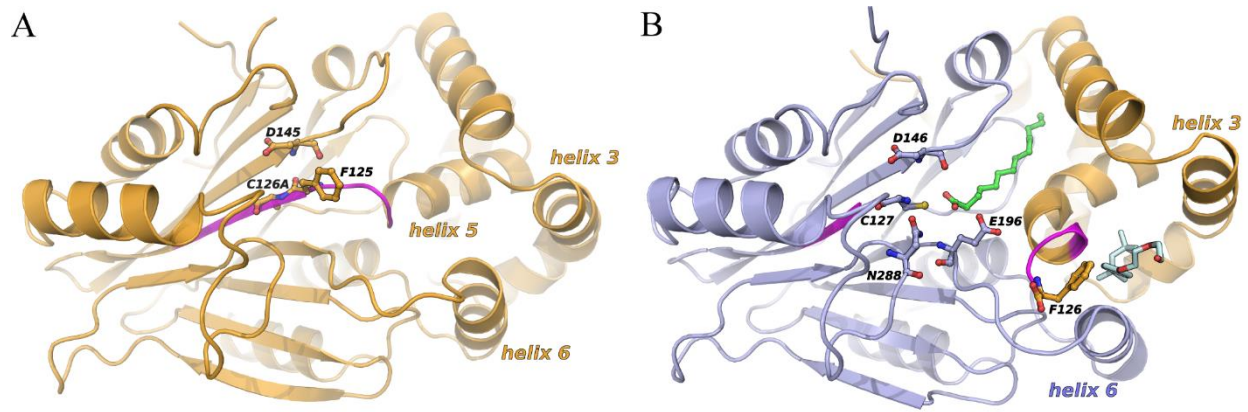


Figure 6. Structures of (A) human NAAA precursor (PDB: 6DXW)²⁰ and (B) mature rabbit NAAA (PDB: 6DY1).²⁰ (A) Maturation occurs via self-catalyzed cleavage of the peptide bond between Phe125 and Cys126 (mutated to alanine in the crystallized protein and labelled as C126A). Residues Phe125, Ala126 and Asp145 are represented as balls and sticks. The backbone of residues 123-128 is colored in magenta. (B) Mature rabbit NAAA in complex with myristic acid (green), which occupies the binding pocket shaped by subunits α (orange) and β (grey). The catalytic nucleophile Cys127 (corresponding to Cys126 in the human orthologue; residues are numbered following the primary sequence deposited in UniProt)²¹ is located at the N-terminal end of the β subunit, within a calix-shaped cavity exposed to the solvent. Several amino acids are involved in the catalytic process: Asp146 interacts with the amine group of the N-terminal cysteine, while Asn288 and Glu196 form the oxyanion hole with their side-chain amide and backbone NH group, respectively. Self-catalyzed cleavage may cause a conformational change in the C-terminal α -helix 5 of NAAA α -subunit, as well as in α -helices 3 (α -subunit) and 6 (β -subunit), which are presumed to interact with the membrane. A molecule of the detergent TritonX-100 is co-crystallized between α -helices 3 and 6 (light cyan carbons).

Structural studies have provided important insights into the association of NAAA with the lipid bilayer as well as into the topography of its substrate-binding site²⁰ (**Figure 6**). NAAA is a soluble protein²³ but its α -helices 3 and 6 shape a largely hydrophobic and positively charged surface that may enable binding to the specialized anionic lipids that compose lysosomal luminal vesicles [e.g., bis(monoacylglycero)phosphate].⁸⁵ Once embedded in the membrane, NAAA might undergo a conformational change that exposes its previously hidden substrate-binding site and thus allows catalysis to occur at an optimal velocity. Partial support for this hypothetical model comes from

structural data²⁰ as well as from experiments showing that certain phospholipid species (e.g., PE and sphingomyelin) are able to stimulate NAAA activity *in vitro*.⁵⁹

The crystal structure of rabbit NAAA in complex with myristic acid suggests that PEA may position its 16-carbon saturated tail in the site's narrow hydrophobic pocket, at the interface between the α - and β -subunits, and its polar ethanolamine head in a calyx-shaped cavity open to the solvent (**Figure 6B**).²⁰ It is currently unknown whether this arrangement permits naturally occurring lipid amides with polar heads bulkier than ethanolamine, such as taurine and glycine (**Figure 1**), to serve as substrates for NAAA.

How does NAAA effect catalysis? Structural considerations²⁰ and comparison with acid ceramidase and other Ntn enzymes^{20,79,80} point to four probable steps: (i) transfer of a proton from the thiol group of Cys126 (for human NAAA) to the α -amino group of the same residue, resulting in the formation of a reactive thiolate anion; (ii) nucleophilic attack by the thiolate on the carbonyl carbon of PEA and stabilization of the resulting oxyanion by a network of hydrogen bonds with Glu195 and Asn287; (iii) protonation and exit of PEA's ethanolamine moiety with concomitant formation of the acyl-enzyme intermediate; and, finally, (iv) regeneration of the initial enzyme. This scenario is plausible, but significant details are still missing. For example, we do not know whether catalysis might be influenced by the protonation state of critical amino acid residues, - which is not revealed by current structural data. Given the role played by ambient pH in NAAA activation, filling this gap appears to be especially important.

DISCOVERY OF NAAA INHIBITORS

The resolution of NAAA's structure in various activation states and in complex with different ligands²⁰ has offered invaluable insights into the enzyme's inner working, and will undoubtedly

accelerate the discovery of new inhibitors. But efforts to identify potent and selective NAAA-targeting agents began well before structural information had become available. Early studies adopted one of two strategies: they either utilized PEA as a starting point for chemical modulation or searched for covalent modifiers of the catalytic cysteine.

As illustrated in **Figure 7**, systematic changes to the polar head of PEA (**1**) led to the identification of *N*-pentadecyl-cyclohexanecarboxamide (**2**), which was initially reported to inhibit rat NAAA with a median effective concentration (IC_{50}) of 4.5 μ M.⁸⁶ This effect was not readily reproducible, however,⁶³ and subsequent work suggested that it may have been caused by the synthetic intermediate pentadecylamine (**3**; IC_{50} = 5.7 μ M on rat NAAA).⁸⁷ Consistent with this possibility, another lipophilic amine, tridecyl 2-aminoacetate (**4**), was also found to inhibit rat NAAA with micromolar potency (IC_{50} = 11.8 μ M).⁸⁷ Comparable pharmacological activities were reported for cyclopentyl palmitate (**5**) (IC_{50} = 10 μ M on human NAAA)⁸⁸ and, more recently, for *N*-pentadecyl-3-hydroxypropanamide (**6**) (IC_{50} = 34 μ M on human NAAA)⁸⁹ and 2-pentadecyl-2-oxazoline (IC_{50} not determined, but probably > 30 μ M on human NAAA).⁹⁰ All these compounds retain the two most salient structural features of PEA – its long-chain carbon tail and small polar head – and possibly compete with it for binding to NAAA’s hydrophobic pocket. However, their weak potencies are suggestive of minimal productive interactions with other components of the substrate-binding site.

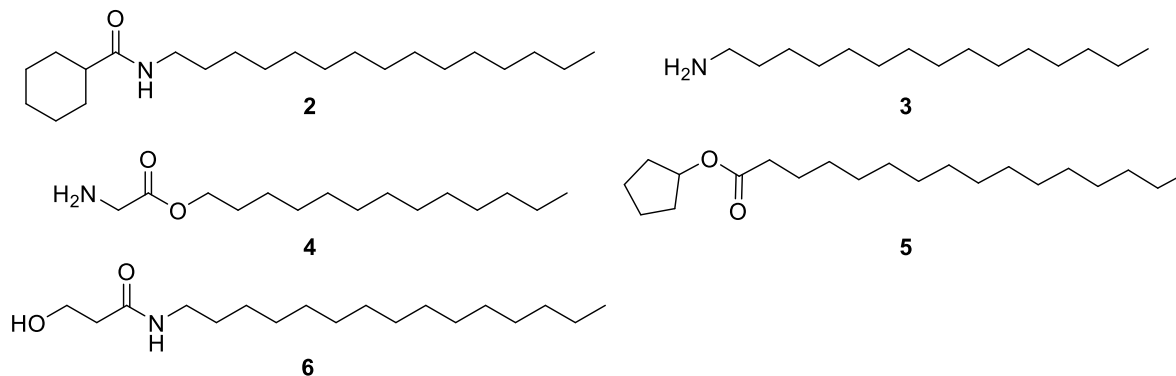


Figure 7. First-generation NAAA inhibitors designed to mimic the two salient structural features of PEA, namely, its long-chain carbon tail and small polar head. The micromolar activity initially reported for compound **2** is now attributed to its synthetic intermediate **3**. The lipophilic amine **4**, the ester **5** and the retroamide **6** inhibit NAAA with double-digit micromolar potencies.

The search for covalent modifiers proved to be more fruitful. Guided by a homology model of NAAA built using the crystallographic coordinates of the Ntn enzyme, conjugated bile acid hydrolase (CBAH),⁹¹ Solorzano and collaborators screened a library of cysteine-reactive probes and identified the β -lactone **7** (**Figure 8A**) as a single-digit micromolar NAAA inhibitor ($IC_{50} = 3 \mu M$ on rat NAAA).⁶³ The compound had been previously shown to react with the catalytic cysteine of hepatitis A virus proteinase 3C *via* its β -lactone ring,⁹² whose essential role in NAAA inhibition was confirmed by demonstrating that analogs in which the ring was opened or replaced with a cyclobutanone or cyclobutene were inactive. Importantly, isosteric substitution of the *N*-benzyl-carbamate with an amide and separation of the two enantiomers led to *N*-[(3*S*)-2-oxo-3-oxetanyl]-3-phenylpropanamide (*S*-OOPP; **8**), which inhibited rat NAAA with submicromolar potency ($IC_{50} = 420 \text{ nM}$) through a partially reversible and noncompetitive mechanism.⁶³

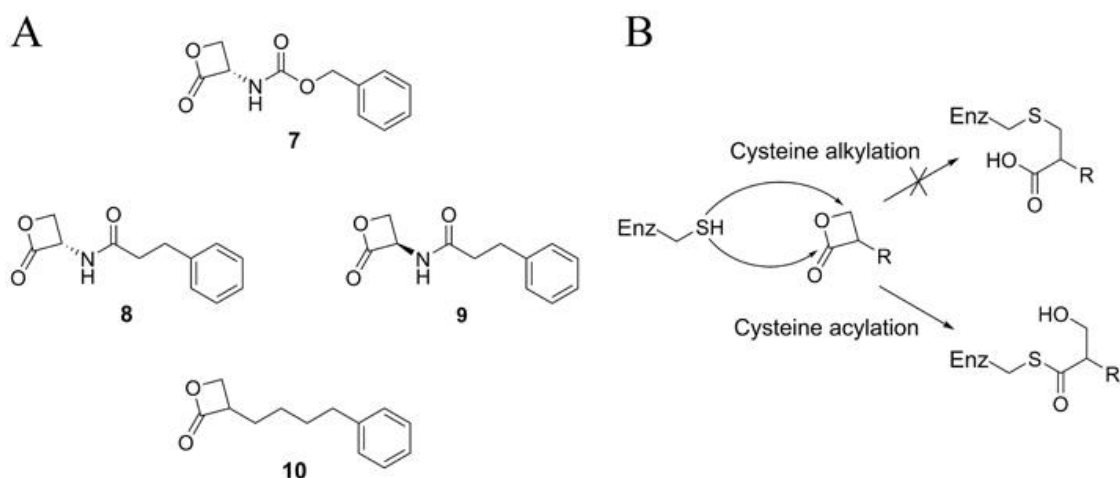


Figure 8. First-generation NAAA inhibitors designed to interact with the catalytic cysteine *via* a β -lactone warhead. (A) Structures of the hepatitis A virus proteinase 3C inhibitor **7**,⁹² a single-digit micromolar NAAA inhibitor; **8**, the first sub-micromolar NAAA inhibitor;⁶³ **9**, weakly active enantiomer of **8**; and **10**, inactive derivative of **8** that lacks the side chain amide-like fragment. (B) Theoretical mechanisms for the covalent inhibition of NAAA by β -lactones: (top) attack on the 4-methylene carbon produces a hydrolysis-resistant thioether, whereas (bottom) attack on the 2-carbonyl carbon yields a hydrolysable thioester. Kinetic and mass spectrometry studies⁹⁵ support the latter mechanism.

Despite its propensity for hydrolytic deactivation (due to opening of the β -lactone ring), compound **8** had two properties that encouraged its use as a tool for biological studies. First, it was selective for NAAA over other structurally (acid ceramidase) or functionally (FAAH) related enzymes.⁶³ Second, it was substantially more potent at inhibiting NAAA than its *R*-enantiomer **9** ($IC_{50} = 6 \mu\text{M}$ on rat NAAA), which could thus be used as a negative control in pharmacological experiments. Indeed, when incubated with RAW264.7 macrophages stimulated with bacterial endotoxin, compound **8** normalized PEA levels, which were reduced by exposure to the toxin,

whereas its enantiomer **9** had no such effect.⁶³ Furthermore, subdermal application of compound **8**, but not **9**, prevented carrageenan-induced neutrophil infiltration and plasma extravasation in mice, two effects that were abrogated by targeted PPAR- α deletion and were mimicked by administration of PEA or a synthetic PPAR- α agonist.⁶³ These findings pointed for the first time to NAAA as a druggable anti-inflammatory target, and identified its N-terminal nucleophile as an accessible site for intervention. Efforts were soon undertaken to test these ideas further.

INHIBITORS TARGETING THE CATALYTIC CYSTEINE

In theory, NAAA's catalytic nucleophile could attack the β -lactone ring of compound **8** at two distinct positions: the 2-carbonyl carbon, producing a hydrolysable thioester, and the 4-methylene carbon, yielding a hydrolysis-resistant thioether (**Figure 8B**). The literature offers support to both scenarios,^{92,93,94} but the finding that overnight dialysis of the enzyme-inhibitor complex reversed in part the effect of **8** tentatively implicated cysteine acylation rather than alkylation.⁶³ Guided by this hypothesis, which was later confirmed in mass spectrometry experiments,⁹⁵ Solorzano and collaborators carried out a systematic structure-activity relationship (SAR) exploration aimed at elucidating the roles played by the β -lactone ring and by the shape and size of its side chain (**Figure 9**). Computational studies with the CBAH homology model mentioned above suggested that the β -lactone and amide moieties engage in favorable polar interactions with amino acid residues critical for enzyme function.⁹⁶ The importance of the side chain amide was confirmed by the potency loss caused by its removal, as in the phenylbutyl lactone **10** ($IC_{50} = 11 \mu\text{M}$ on rat NAAA). The model also offered a tentative explanation for the stereoselectivity observed with *syn*- β -methyl lactones, which were either completely inactive ($\alpha R, \beta S$ enantiomer **11**) or weakly active ($\alpha S, \beta R$ enantiomer **12**; $IC_{50} = 3.2 \mu\text{M}$ on rat NAAA). Exploration of SARs with lipophilic substituents,

including rigid scaffolds with various substitution patterns, showed higher inhibitory potencies for straight hydrophobic chains and highlighted, among aromatic amides, the good performance of 2-naphthamide, (1,1'-biphenyl)-4-carboxamide and 4-(benzyloxy)benzamide derivatives (**13-15**)($IC_{50} = 160, 115$ and 90 nM, respectively, on rat NAAA).

The propensity to undergo hydrolytic deactivation hindered broad experimental use of the first β -lactone NAAA inhibitors. Searching for more stable molecules, Duranti and collaborators investigated further the *N*-(2-oxo-3-oxetanyl)amide core (**Figure 9**) and found that replacing the amide group with a carbamate or introducing a *syn*-methyl group at the β position of the ring improved stability without affecting potency.⁹⁷ Among these threonine- β -lactones, compound **16** (URB913, ARN077) stood out for its improved chemical stability and strong inhibitory potency ($IC_{50} = 50$ nM and 7 nM on rat and human NAAA, respectively). Further SAR investigations of its side chain led to the biphenyl derivative **17**,⁹⁸ whose strong activity ($IC_{50} = 7$ nM on both rat and human enzymes) was the starting point for a 3D-QSAR analysis, which confirmed that aromatic or aliphatic structures designed to rigidify the flexible carbon tail of PEA enhance target recognition.⁹⁹ As discussed below, the presence of a rigid and straight lipophilic chain turned out to be a critical feature also for the design of other covalent and non-covalent inhibitors. Local application of **16** alleviated skin inflammation⁷² and pain-related responses^{100,101} in mouse and rat models. Furthermore, the compound was found to be selective for NAAA when assessed in a broad panel of potential off-targets, and to be safe in preclinical toxicology studies. Advanced to clinical testing, **16** was shown to be safe in healthy volunteers and in persons with eczematous skin lesions. However, as discussed in a later section, its clinical development was prematurely terminated.

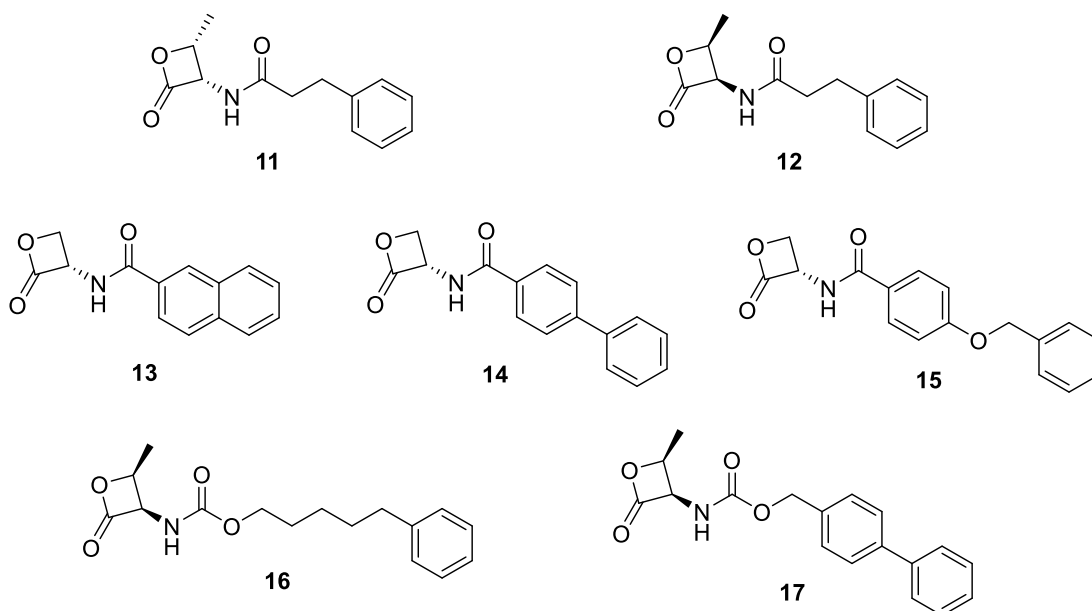


Figure 9. Second-generation β -lactone NAAA inhibitors with enhanced NAAA inhibitory potency and improved chemical and metabolic stability. The high inhibitory potency demonstrated by compounds **13-14** and **17** reveals that replacement of the flexible carbon chain of PEA with straight and rigid structures leads to increased activity.

Despite the significant steps forward made with compounds **16** and **17**, the inherently low stability of the β -lactone ring posed an unresolved challenge to the systemic use of this class of inhibitors. In an effort to overcome this problem, Fiasella and colleagues adopted a core-hopping strategy and substituted the β -lactone with a β -lactam.¹⁰² Their SAR studies showed that, similarly to β -lactones, opening the four-term β -lactam ring or modifying its endocyclic nitrogen by substitution or methylation was detrimental to activity. The *S* configuration of the α -acylamino group was strongly favored over the *R* configuration, and the amide could be replaced by a carbamic acid ester, resulting in accrued potency. Once again, the shape and length of the lipophilic side chain proved to be a critical determinant for activity. More importantly, β -lactam derivatives

such as **18** (ARN726, **Figure 10**) were found to be potent at inhibiting human NAAA ($IC_{50} = 27$ nM), relatively stable to chemical and enzymatic hydrolysis, and systemically active.⁷⁰

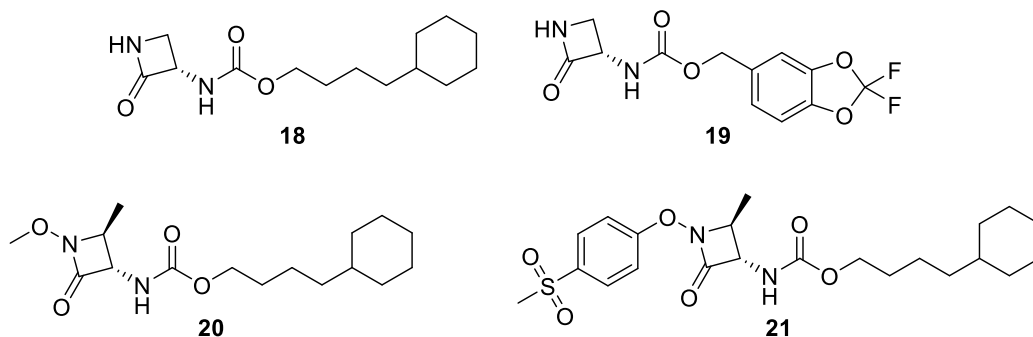


Figure 10. Structures of representative β -lactam NAAA inhibitors. Compound **18** (ARN726) is a potent and systemically active NAAA inhibitor.⁷⁰

Subsequent structural studies showed that **18** forms a covalent adduct with NAAA's N-terminal cysteine and positions the carbonyl fragment of its carbamate group in close proximity of the oxyanion hole formed by NH dipoles in the side chain of Asn288 and the backbone of Glu196 in rabbit NAAA (**Figure 11A**).²⁰ Superseding the CBAH homology model,⁶³ the X-ray structure of rabbit NAAA demonstrated that the open β -lactam ring of **18** is located in the solvent-exposed cavity of the active site, and engages in stabilizing polar interactions with both the catalytic cysteine and Asp146.²⁰ Pharmacokinetic and pharmacodynamic studies further showed that, despite its rapid elimination *in vivo*, compound **18** effectively inhibited NAAA in peripheral mouse and rat tissues.^{60,70} (Its brain penetration was limited, however, see ref. 103) Additional SAR investigation of the β -lactam side chain yielded compound **19**, which maintained considerable potency ($IC_{50} = 85$ nM on human NAAA) while displaying improved stability compared to **18**.¹⁰⁴

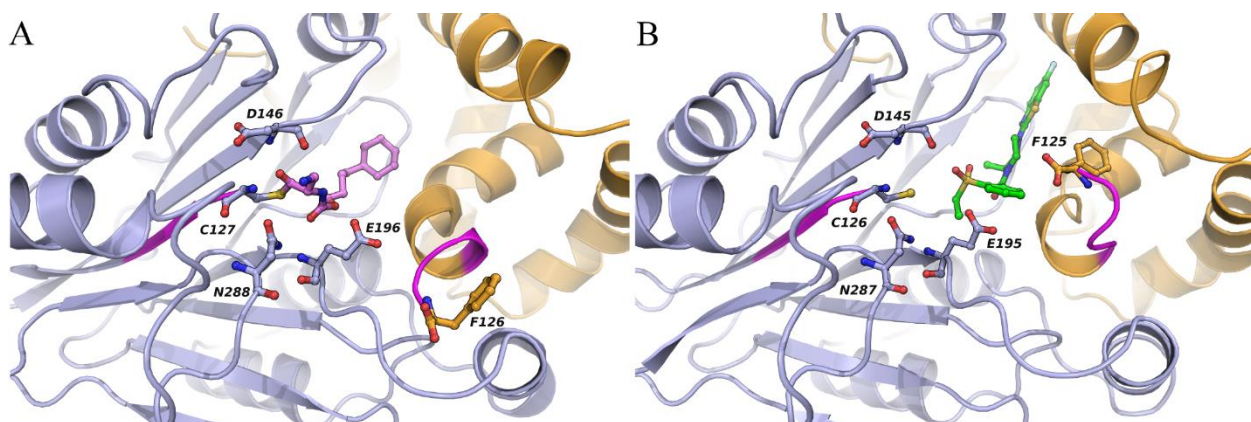


Figure 11. Interaction of NAAA with covalent (A) and non-covalent (B) inhibitors. (A) Detail of rabbit NAAA (PDB: 6DY0) with the sulfur atom of the N-terminal cysteine acylated by the β -lactam **18** (ARN726)⁷⁰ (light pink carbons). The amino-propionate portion of the β -lactam ring points toward the solvent-accessible region lined by N-terminal Cys127 and the backbone of Asp146 (residues are numbered following the primary sequence deposited in UniProt). The carbamate carbonyl points toward the oxyanion hole formed by the side-chain amide of Asn288 and the backbone of Glu196 while the cyclohexylbutyl group lies within the lipophilic pocket that is likely occupied by the acyl chain of PEA (see **Figure 2**). (B) Detail of the substrate-binding site of human NAAA (PDB: 6DXX)²⁰ bound to the non-covalent inhibitor **37** (ARN19702)¹⁰³ (green carbons). The sulfonyl group of **37** lies within the solvent-accessible cavity with an oxygen positioned 3.9 Å away from the sulfur atom of the catalytic cysteine (Cys126). The benzothiazole nucleus is inserted within the lipophilic pocket. Other interactions that may stabilize the enzyme-inhibitor complex (e.g. between the benzoyl carbonyl and Trp181, the fluorine atom and the backbone of Met64) are not shown for clarity.

More recently, Petracca and colleagues investigated the role of *N-O*-aryl and *N-O*-alkyl substituents at the endocyclic nitrogen of the β -lactam nucleus as well as the impact of introducing a methyl group at its C3 position.¹⁰⁵ The researchers found that addition of a methyl, acetyl or methylcarbamoyl group on the nitrogen attenuated or abolished activity, whereas insertion of a methoxy group had an opposite effect. Interestingly, among the four stereoisomeric *N-O*-methyl threonine derivatives, only the isomer with (2*S*,3*S*) configuration (**20**) maintained adequate potency and target selectivity ($IC_{50} = 69$ nM on human NAAA). Focusing on this structure, Petracca and collaborators examined the effects of bulkier *N-O*-alkyl and *N-O*-aryl groups, and found that aryl moieties with polar substituents conferred greater potency, as shown by the methylsulfonyl-containing inhibitor **21** ($IC_{50} = 6$ nM on human NAAA) (**Figure 10**).

The progress outlined above laid the foundation for the discovery of chemical probes that should facilitate the investigation of NAAA in broken cell preparations (e.g., in activity-based profiling studies), intact cell cultures, and live animals (**Figure 12**). The first such probe, **22** (ARN14686), combined key features of **18** with a terminal alkyne tag enabling the copper-catalyzed ‘click’ cycloaddition reaction with azide-bearing reporter molecules (e.g., biotin and rhodamine).^{106,107} The compound’s nanomolar potency ($IC_{50} = 6$ nM and 13 nM on human and rat NAAA, respectively) and selectivity over other cysteine hydrolases¹⁰⁶ prompted its use *in vivo* to quantify catalytically active NAAA in inflamed paws of arthritic rats.⁶⁰ The *N-O*-aryl β -lactam **21** provided the starting point for the design of two fluorescent molecules that contained either a boron-dipyrromethene (BODIPY; **23**) or norbornene moiety (**24**).¹⁰⁸ The latter’s ability to cross cell membranes may allow its application to monitor, for example, the acid-catalyzed generation of active NAAA in its natural setting.

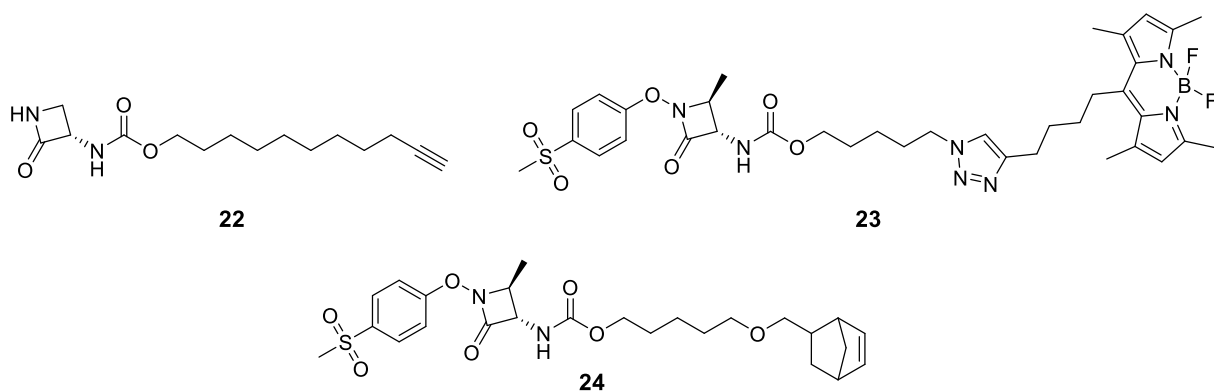


Figure 12. Chemical probes for the study of NAAA *in vitro* and *in vivo*. The figure shows three β -lactam-based molecules: **22** bears a terminal alkyne group that allows the ‘click’ reaction with an appropriate azide-bearing reporter; **23** and **24**, whose design is based on **21** (see **Figure 10**), contain fluorescent moieties and may be used to tag NAAA in broken (**23**) or intact (**24**) cell preparations.

The general strategy based on merging a mimic of PEA’s carbon tail with a cysteine modifier was also exploited by the laboratory of Alexandros Makriyannis in a series of compounds bearing an isothiocyanate warhead¹⁰⁹ (**Figure 13**). Despite the known propensity of this moiety to form covalent adducts with biological thiols,¹¹⁰ the pentadecyl isothiocyanate **25** (AM9023) was found to inhibit NAAA in a reversible manner.¹¹¹ Introduction of a terminal phenyl ring on its carbon chain yielded compound **26** (AM9053),¹⁸ which was reported in a published patent¹⁰⁹ as being potent ($IC_{50} = 30$ nM on the human enzyme) and selective for NAAA over acid ceramidase. Tested in a mouse model of colitis, AM9053 was effective at restoring normal PEA levels (which were reduced in inflamed colon) and alleviating inflammatory symptoms.¹¹²

Efforts aimed at modulating the reactivity of the isothiocyanate group led to the discovery of the first class of NAAA-targeting agents containing a cyanamide functionality (**Figure 13**), whose

chemical affinity toward thiols had been previously exploited to create reversible cathepsin K inhibitors.¹¹³ Building on this knowledge and on the pharmacophoric model emerged from the β -lactone experience, Malamas and colleagues^{109,114} attached a biphenyl scaffold to an azetidine-nitrile nucleus that incorporated the cyanamide function, obtaining the single-digit nanomolar inhibitor **27** ($IC_{50} = 3$ nM on human NAAA). Covalent and induced-fit docking simulations, informed by structural data, confirmed that the cyanamide moiety can form an isothioureia adduct with the cysteine nucleophile, while the lipophilic portion of the inhibitor can maintain the binding pose assumed before formation of the covalent bond. To enhance selectivity for NAAA and reduce cytochrome P₄₅₀-mediated oxidation of the benzylic fragment, bulky groups and conformational restrictions were inserted in proximity of the cyanamide warhead. In particular, a cyclopropyl substituent was aimed at filling an unoccupied pocket of the enzyme close to the azetidine ring. The modification (**28**) produced a significant improvement in potency, but did not remove cross-reactivity with FAAH ($IC_{50} = 0.3$ nM and 1 nM on human NAAA on FAAH, respectively). By contrast, adding a 3-methyl group on the azetidine nucleus and a methoxy group on the distal phenyl ring, while replacing the flexible benzyloxy linker with a rigid benzamide fragment, resulted in the potent NAAA-preferring inhibitor **29** ($IC_{50} = 1.6$ nM; >100-fold selectivity for human NAAA over FAAH and cathepsin K). Of note, addition of a sulfonamide linker fully restored activity toward FAAH, yielding an interesting dual inhibitor (**30**) that targets both human NAAA and FAAH with balanced high potency ($IC_{50} = 3$ nM and 10 nM, respectively). Lastly, cyanamide-based agents were found to be stable in rat and human plasma ($t_{1/2} > 120$ min), but also to undergo rapid metabolism when incubated with liver microsomal preparations ($t_{1/2}$ values ranging from 5 to 15 min).¹¹⁴

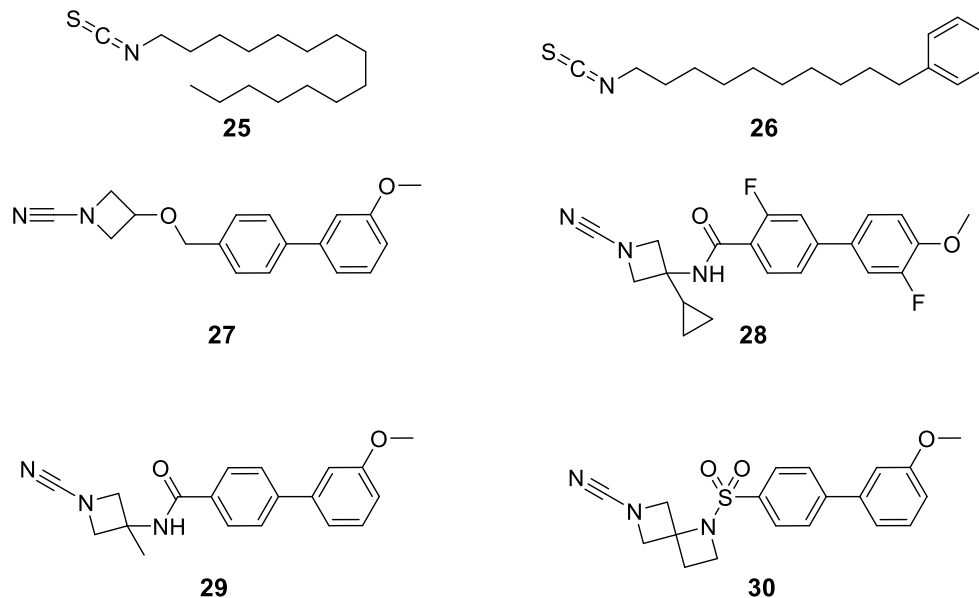


Figure 13. NAAA inhibitors containing isothiocyanate (**25**, **26**) and cyanamide (**27-30**) warheads. Compound **29** is potent and selective for NAAA, whereas compound **30** is a balanced high potency dual inhibitor of NAAA and FAAH activities.

BEYOND THE CATALYTIC CYSTEINE

In addition to targeting the N-terminal nucleophile, the search for NAAA inhibitors continued to focus on agents inspired by the scaffold of PEA or, alternatively, turned to the identification of entirely novel scaffolds by chemical library screening. We have seen before that simple changes to PEA's structure – in analogs like cyclopentyl palmitate (**5**)⁸⁸ and *N*-pentadecyl-3-hydroxypropanamide (**6**)⁸⁹ – had led to an apparent dead end. In an effort to break this impasse, Li and colleagues replaced the ethanolamine head of PEA with a pyrrolidine nucleus and rigidified its hydrophobic tail by introducing a biphenyl group. This led to the micromolar inhibitor **31** ($IC_{50} = 2.1 \mu M$ on human NAAA)¹¹⁵ (**Figure 13**), which was further modified to produce the 4-

phenylcinnamoyl analog **32**, which showed improved selectivity toward FAAH and anti-inflammatory activity in the mouse lipopolysaccharide model of acute lung injury.¹¹⁶ A similar approach led to the potent and *in vivo* active oxazolidinone imide **33** (F96; IC₅₀ = 267 nM on human NAAA).⁷³ Further work on the alkyl-phenyl tail of this molecule and, more specifically, addition of a chlorine atom in the *meta* position of its phenyl ring, yielded the potent (IC₅₀ = 9 nM on rat NAAA), reversible and *in vivo* active inhibitor **34** (F215).^{61,117}

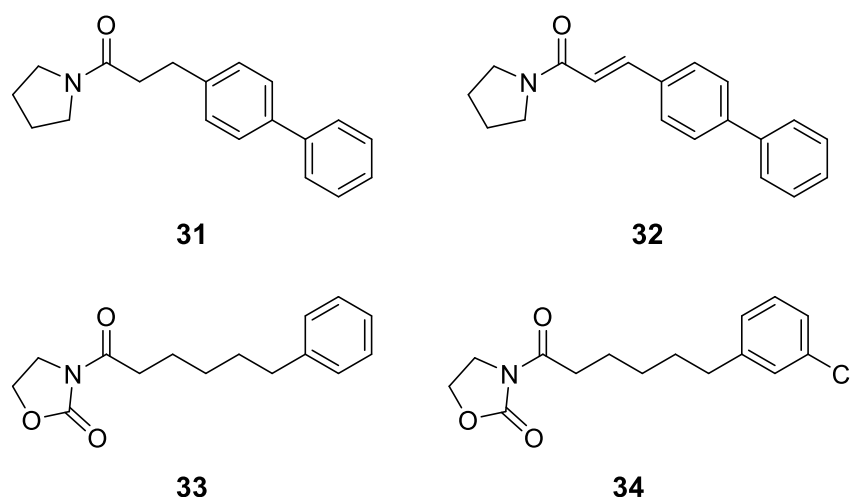


Figure 14. N-acyl pyrrolidine (**31-32**) and oxazolidinone imide (**33-34**) derivatives targeting NAAA. Compounds **32** and **34** (F215) are potent, reversible and *in vivo* active NAAA inhibitors.

Advances in unexpected directions were made by screening chemical libraries, which was combined in some cases with SAR-based optimization (**Figure 15**). Testing a collection of known anti-inflammatory drugs, Petrosino and collaborators found that the anthraquinone derivative diacerein (EPT4900, **35**) inhibited human NAAA with moderate potency (IC₅₀ = 0.7 μ M) *via* a non-competitive and time-dependent mechanism.¹¹⁸ Diacerein, which is approved in some countries to treat osteoarthritis,^{119,120} also reduced paw inflammation and normalized paw skin

PEA levels in rats that had received intraplantar injections of carrageenan.¹¹⁸ Another clinically used drug, the antineoplastic agent carmofur (**36**), was shown to inhibit NAAA with nanomolar potency, but also to display comparable activities toward FAAH and acid ceramidase.^{121,122}

A random screening campaign of a library comprising $\approx 58,000$ molecules identified 45 active hits, including the benzothiazole derivative **37**¹⁰³ (**Figure 15**). Starting from this low-potency inhibitor, Migliore and colleagues carried out an SAR investigation to evaluate the effects of substituents at the benzamide and benzothiazole groups, and to determine the role of the piperazine ring. Replacing the *ortho* methyl group of the benzamide with an ethylsulfonyl moiety was sufficient to achieve a remarkable increase in potency, while other modifications at the same position were either less favorable or not tolerated. Removal of the benzothiazole fluorine, or insertion of halogen atoms at various positions of the benzothiazole nucleus had minor effects, while substituting piperazine with piperidine was detrimental to the activity. Conversely, introducing a methyl group at a strategic position of the piperazine ring produced compound **38** (ARN19702), which inhibited human NAAA in a reversible manner and with an IC_{50} of 230 nM.¹⁰³ Structural analysis of the enzyme-inhibitor complex^{Errore. Il segnalibro non è definito.} revealed that the ethylsulfonyl group of the inhibitor protrudes toward the solvent-accessible portion of the catalytic site, with the closest sulfonyl oxygen located at a distance of approximately 4 Å from the cysteine sulfur atom and the benzene ring accommodated in a nearby pocket. The benzothiazole and the piperazine, with its methyl group in an axial arrangement, are lodged within the lipophilic pocket and participate in a number of weak interactions that likely concur to the compound's overall potency (**Figure 11B**). Metabolically stable, selective in a broad panel of potential off-targets and orally active, **38** was also found to partially cross the blood-brain barrier (brain-to-plasma ratio = 0.2) and to elevate the levels of PEA and oleoylethanolamide (but not anandamide) in brain tissue.

Pharmacological studies further showed that subchronic administration of **38** alleviated neural inflammation and motor dysfunction in the experimental allergic encephalomyelitis model of multiple sclerosis.¹⁰³ Lastly, the same screening campaign that had led to the discovery of **38** yielded a second promising hit, which was modified to produce the azabicyclo-octane derivative **39** (ARN16186). Data reported in a published patent suggest that this inhibitor may be potent (IC_{50} = 23 nM on human NAAA), reversible and active *in vivo*.¹²³

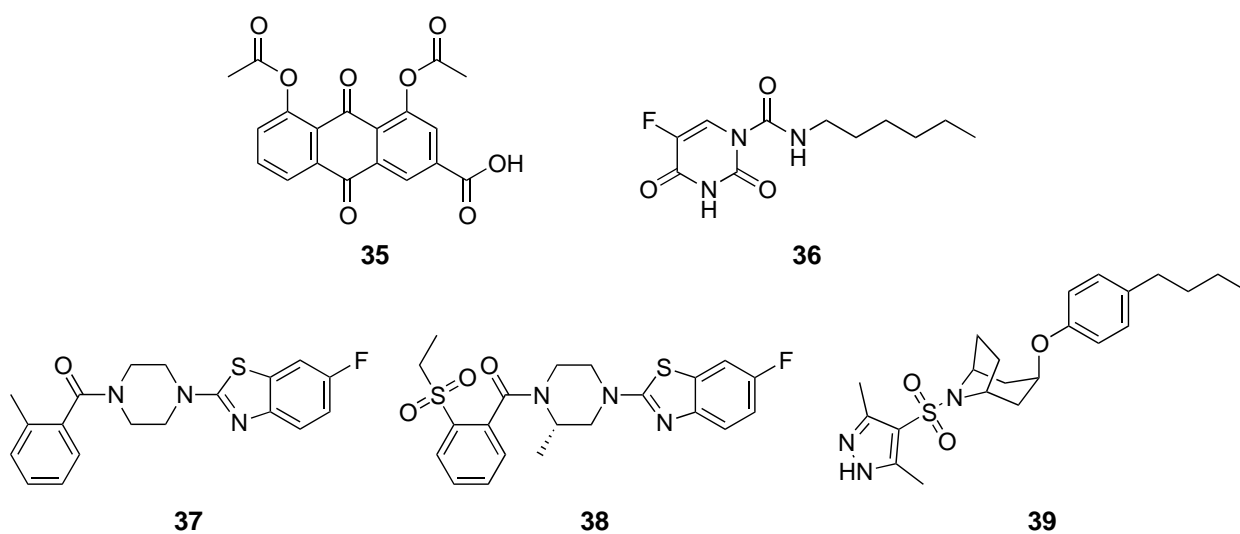


Figure 15. NAAA inhibitors identified by testing compounds used in the clinic (diacerein, **35** and carmofur **36**) or by high-throughput screening of chemical libraries (**37**) followed by SAR-driven optimization (**38**, **39**).

SUMMARY OF LEAD DISCOVERY

The qualitative pharmacophore model illustrated in **Figure 16** summarizes the SARs documented thus far for NAAA inhibitors. While early compounds (e.g. **4**) closely mimicked the structure of PEA, introduction of a cysteine-reacting group and replacement of the flexible carbon

chain with more rigid and rectilinear hydrophobic fragments led to the identification of potent and selective covalent inhibitors (e.g. **14**, **16**, **18**, **27** and **29**). Subsequent studies exploited additional interactions at the binding site, such as the lipophilic pocket targeted by the cyclopropyl group of **28**, and lead to the discovery of NAAA-targeting agents in which a balanced combination of various pharmacophoric elements (e.g. in **38**) results in potent inhibitory activity even in the absence of a cysteine-reactive warhead.

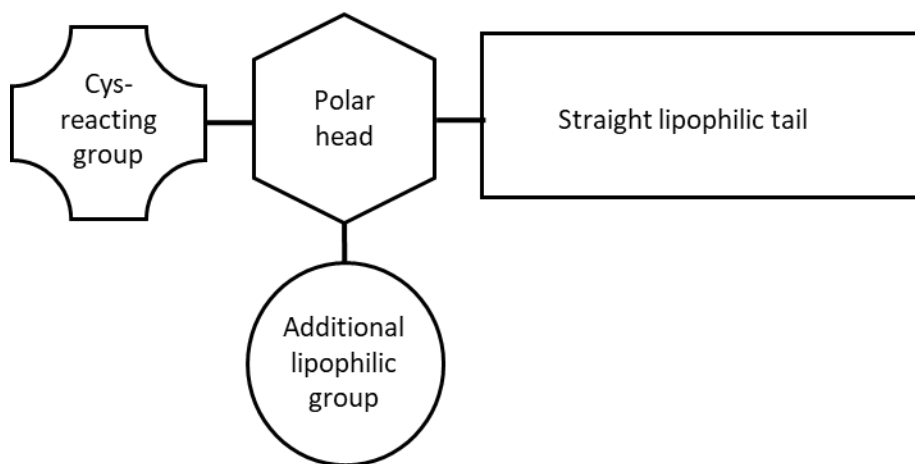


Figure 16. Pharmacophore elements common to current NAAA inhibitors.

NAAA AS A TARGET FOR ANTI-INFLAMMATORY DRUGS

Translational evidence for a role of NAAA inhibition in the treatment of chronic inflammation – an unmet medical need of gigantic proportions – remains sparse. As pointed out at the beginning of this article, deficits in PEA availability have been documented for only two human inflammatory disorders, arthritis (synovial fluid) and ulcerative colitis (active inflammatory foci).^{62,68} Nevertheless, the fact that chemically diverse classes of NAAA-targeting agents display clear anti-inflammatory efficacy in animal models gives grounds for cautious optimism. Indeed, while no animal model captures the complexity of human inflammation, the mechanistic diversity

of the paradigms investigated thus far offers intriguing clues about possible applications of NAAA inhibitors in therapy.

Topical application of the β -lactone **16** (ARN077, URB913) (**Figure 9**) suppressed cutaneous inflammatory reactions evoked by exposure to phorbol ester in mice and to ultraviolet B-radiation in rats.¹⁰¹ The inhibitor also reversed redness and edema, attenuated pruritus and normalized circulating levels of interleukin 4 and immunoglobulin E in sensitized mice challenged with the hapten 1-fluoro-2,4-dinitrobenzene.⁷² In those studies, the effects of **16** were strictly dependent on PPAR- α activation and were accompanied by restoration of normal PEA content in inflamed skin tissue.^{72,101} Because of its potency, target selectivity and soft-drug properties, **16** was advanced to clinical development as topical treatment for atopic dermatitis (eczema). However, after a successful Phase 1a safety trial, a subsequent Phase 1b study in patients with eczematous skin lesions failed to provide initial indications of efficacy. It is unclear whether this failure was due to true lack of activity, insufficient target engagement, acuity of the disease state or, possibly, underpowering or bias in the design of the study, which included only \approx 10 subjects. Despite this setback, the favorable selectivity and safety profile demonstrated by **16** in preclinical and clinical tests, its striking effects on critical mediators of the allergic response (e.g., interleukin 4 and immunoglobulin E),⁷² and the preliminary efficacy demonstrated by PEA as an adjuvant treatment for eczema,⁴⁴ encourage further evaluation of NAAA as a target for either ‘soft drugs’¹²⁴ (i.e., drugs that undergo predictable metabolic deactivation after exerting their effects) or systemically active drugs to treat disorders of the atopic diathesis – a broad rubric that encompasses eczema, bronchial asthma, hay fever and allergic rhinitis.

The improved metabolic stability of the β -lactam NAAA inhibitor **18** (ARN726) (**Figure 10**) provided the opportunity of examining the role of NAAA in models of lung or joint inflammation,

which do not readily lend themselves to topical drug application. When administered orally to mice, **18** prevented carrageenan- and endotoxin-induced pleural damage in a dose-dependent manner, whereas its weakly active (*R*)-enantiomer had no such effect. Importantly, *in vitro* experiments showed that **18** also suppressed inducible nitric oxide synthase (iNOS) expression and tumor necrosis factor- α (TNF- α) release from CD68+ human macrophages stimulated with endotoxin,⁷⁰ a critical translational finding that needs to be replicated and expanded. In a subsequent study, Bonezzi and collaborators reported that compound **18** was as effective as the potent steroid dexamethasone at attenuating edema and hyperalgesia evoked in rats by intraplantar injection of complete Freund's adjuvant.⁶⁰ Other NAAA inhibitors that have been successfully tested in rodent models used to study inflammation are the pyrrolidine amide **32** (mouse lipopolysaccharide-induced acute lung injury),¹¹⁶ the oxazolidinone imide **34** (F215; rat monoiodoacetate model of osteoarthritis),⁶¹ the benzothiazole **38** (ARN19702, mouse model of multiple sclerosis)¹⁰³ and the isothiocyanate derivative **26** (AM9053, mouse models of colitis).¹¹² The available data are still limited in scope but, when weighed together with evidence from human studies,^{62,68,70} they do point to osteoarthritis and inflammatory bowel disease as potential therapeutic targets for systemic NAAA inhibition.

In addition to peripheral inflammation, evidence suggests that NAAA blockade might also ameliorate neural inflammation and persistent pain states. As mentioned above, administration of the reversible inhibitor **38** (ARN19702) alleviated symptoms of neuroinflammation in a mouse model of multiple sclerosis.¹⁰³ Moreover, the β -lactone derivative **16** (ARN077) demonstrated analgesic efficacy in mouse and rat models of inflammation and nerve damage,¹²⁵ while the oxazolidinone imide **33** (F96) attenuated acetic acid-induced writhing and tactile allodynia evoked by sciatic nerve injury in mice.¹²⁶

CONCLUSIONS

Building a strong case for NAAA as a viable target for anti-inflammatory (or pro-resolution) medicines will largely depend on our ability to elucidate the precise functions served by this enzyme – and the signaling complex it controls – in the induction and resolution of human inflammation. With only a small number of studies published on this subject, we are still a long way from achieving this goal. Critical aspects of NAAA’s basic biology remain unclear, including the localization of precursor *versus* mature NAAA within the endosome/lysosomal compartment, the mechanisms through which competing signals regulate its expression and activity in response to tissue damage and infection, the role it may play in resolution and tissue healing, and its possible function as a ‘rheostat switch’ to generate a signaling-competent pool of pro-inflammatory palmitic acid. Our understanding of NAAA’s place in human pathology is also rudimentary and worthy of additional study, especially considering the enzyme’s striking localization to cells of the innate and adaptive immune systems. Informed by animal data, translational experiments should probe the contributions of NAAA to inflammatory disorders such as eczema, osteoarthritis and ulcerative colitis. In these pathologies, selective inhibition of intracellular NAAA activity might offer greater efficacy than exogenously administered PEA – whose range of clinical applications is restricted by poor solubility, rapid metabolism and limited bioavailability – and greater safety than direct-acting high-affinity PPAR- α agonists – whose unfavorable side-effect profile has led to multiple late-stage development failures.¹²⁷ The chemical tools already available to researchers will facilitate these investigations, but efforts should also be made to expand our armamentarium

of small-molecule NAAA inhibitors and to develop new agents with improved potency, target selectivity and *in vivo* activity.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding Sources

This work was partially supported by the Italian Ministry for University and Research (MIUR, PRIN 2017, 20175SA5JJ project, to MM) and the University of Parma (Progetti di Ateneo FIL Quota Incentivante 2019, to AL).

Notes

The following authors are inventors in patents and patent applications (owned by the University of California, the University of Urbino ‘Carlo Bo’, and the University of Parma) that protect composition of matter and use of various NAAA inhibitor classes: DP, WO 2009049238, WO 2013078430, WO 2014144547, WO 2014144836; GT and MM, WO 2009049238, WO 2013078430, WO 2014144547, WO 2014144836.

Biographies

Daniele Piomelli received his PhD in pharmacology at Columbia University and carried out postdoctoral work in neuroscience at the Rockefeller University (both in New York, NY). He is distinguished professor and Louise Turner Arnold Chair in Neurosciences at the University of California, Irvine, as well as visiting professor at the University of Parma, Italy, under the TeachinParma project. Research in his laboratory is focused on the functions of endocannabinoid molecules and other lipid-derived signals.

Laura Scalvini received her PhD at the University of Parma, where she is a postdoctoral scholar in the Drug Design and Discovery group led by Prof. Marco Mor. In her work, Dr. Scalvini uses computational free-energy approaches to investigate the function of NAAA and other lipid-metabolizing enzymes.

Yannick Fotio completed his Pharm D and PhD degrees at the University of Camerino, Italy. He is currently a postdoctoral scholar at the University of California, Irvine, where he studies the role of lipid-derived mediators in pain and inflammation.

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Gilberto Spadoni is full professor of Medicinal Chemistry at the University of Urbino ‘Carlo Bo’, Italy. His research is focused on the design and synthesis of selective ligands for melatonin receptors, small-molecules with endocannabinoid-modulating activity and multitarget compounds for the treatment of diseases ranging from cancer to psychiatric and neurodegenerative disorders.

Giorgio Tarzia graduated in Organic Chemistry at the University of Rome and did post-doctoral research at the Syntex Research Institute in Palo Alto (CA) and the Research Institute for Medicine and Chemistry in Cambridge (MA). Back in Italy, he joined the Medicinal Chemistry Research Departments first of Dow-Lepetit (Milan) and subsequently of Glaxo (Verona). Eventually he became full professor of Medicinal Chemistry at the University of Urbino (Italy), where is now Professor Emeritus. Over the past 20 years, his research activity has focused on the design and synthesis of endocannabinoid signaling modulators and selective melatonin ligands.

Marco Mor received his degree in Pharmaceutical Chemistry and Technology from the University of Parma and did postdoctoral studies at the Glaxo Research Center in Verona. He is full professor of Medicinal Chemistry, director of the Ph.D. course in Drug Sciences and head of the Drug Design Discovery group and at the University of Parma. Research in his group is centered on computer-aided design and SAR analysis of endocannabinoid modulators, GPCR ligands and tyrosine kinase inhibitors.

ACKNOWLEDGMENT

The authors are grateful to Mr. Andrea Ghidini for help with illustrations.

ABBREVIATIONS

CBAH, conjugated bile acid hydrolase; FAAH, fatty acid amide hydrolase; NAAA, N-acylethanolamine acid amidase; NAPE-PLD, N-acylphosphatidylethanolamine phospholipase D; Ntn, N-terminal nucleophile; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEA, palmitoylethanolamide; PLA2, phospholipase A2; PPAR, peroxisome proliferator-activated receptor; PGC-1 α , peroxisome proliferator-activated receptor γ coactivator-1 α ; SAR, structure-activity relationship; TNF- α , tumor necrosis factor- α .

REFERENCES

1 Dennis, E. A.; Norris, P. C. Eicosanoid storm in infection and inflammation. *Nat. Rev. Immunol.* **2015**, *15*, 511-523.

2 Serhan, C. N.; Levy, B. D. Resolvins in inflammation: emergence of the pro-resolving superfamily of mediators. *J. Clin. Invest.* **2018**, *128*, 2657-2669.

3 Sasso, O.; Pontis, S.; Armirotti, A.; Cardinali, G.; Kovacs, D.; Migliore, M.; Summa, M.; Moreno-Sanz, G.; Picardo, M.; Piomelli, D. Endogenous N-acyl taurines regulate skin wound healing. *Proc. Natl. Acad. Sci. U S A.* **2016**, *113*, 4397-4406.

4 Aichler, M.; Borgmann, D.; Krumsiek, J.; Buck, A.; MacDonald, P. E.; Fox, J. E. M.; Lyon, J.; Light, P. E.; Keipert, S.; Jastroch, M.; Feuchtinger, A.; Mueller, N. S.; Sun, N.; Palmer, A.; Alexandrov, T.; Hrabe de Angelis, M.; Neschen, S.; Tschöp, M. H.; Walch, A. N-acyl taurines and acylcarnitines cause an imbalance in insulin synthesis and secretion provoking β cell dysfunction in type 2 diabetes. *Cell. Metab.* **2017**, *25*, 1334-1347.

5 Grevenkoed, T. J.; Trammell, S. A. J.; McKinney, M. K.; Petersen, N.; Cardone, R. L.; Svenningsen, J. S.; Ogasawara, D.; Nexøe-Larsen, C. C.; Knop, F. K.; Schwartz, T. W.; Kibbey, R. G.; Cravatt, B. F.; Gillum, M. P. N-acyl taurines are endogenous lipid messengers that improve glucose homeostasis. *Proc. Natl. Acad. Sci. U S A.* **2019**, *116*, 24770-24778.

6 Anderson, R. L.; Merkler, D. J. N-fatty acylglycines: underappreciated endocannabinoid-like fatty acid amides? *J. Biol. Nat.* **2017**, *8*, 156-165.

7 Piomelli, D.; Sasso, O. Peripheral gating of pain signals by endogenous lipid mediators. *Nat. Neurosci.* **2014**, *17*, 164-174.

8 Devane, W. A.; Hanus, L.; Breuer, A.; Pertwee, R. G.; Stevenson, L. A.; Griffin, G.; Gibson, D.; Mandelbaum, A.; Etinger, A.; Mechoulam, R. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* **1992**, *258*, 1946-1949.

9 Lo Verme, J.; Fu, J.; Astarita, G.; La Rana, G.; Russo, R.; Calignano, A.; Piomelli, D. The nuclear receptor peroxisome proliferator-activated receptor- α mediates the anti-inflammatory actions of palmitoylethanolamide. *Mol. Pharmacol.* **2005**, *67*, 15-19.

10 Lo Verme, J.; La Rana, G.; Russo, R.; Calignano, A.; Piomelli, D. The search for the palmitoylethanolamide receptor. *Life Sci.* **2005**, *77*, 1685-1698.

11 Lo Verme, J.; Russo, R.; La Rana, G.; Fu, J.; Farthing, J.; Mattace-Raso, G.; Meli, R.; Hohmann, A.; Calignano, A.; Piomelli, D. Rapid broad-spectrum analgesia through activation of peroxisome proliferator-activated receptor- α . *J. Pharmacol. Exp. Ther.* **2006**, *319*, 1051-1061.

12 Chiurchiù, V.; Rapino, C.; Talamonti, E.; Leuti, A.; Lanuti, M.; Gueniche, A.; Jourdain, R.; Breton, L.; Maccarrone, M. Anandamide suppresses proinflammatory T cell responses in vitro

through type-1 cannabinoid receptor-mediated mTOR inhibition in human keratinocytes. *J. Immunol.* **2016**, *197*, 3545-3553.

13 Redlich, S.; Ribes, S.; Schütze, S.; Nau, R. Palmitoylethanolamide stimulates phagocytosis of Escherichia coli K1 by macrophages and increases the resistance of mice against infections. *J. Neuroinflammation.* **2014**, *11*:108.

14 Rinne, P.; Guillamat-Prats, R.; Rami, M.; Bindila, L.; Ring, L.; Lyytikäinen, L. P.; Raitoharju, E.; Oksala, N.; Lehtimäki, T.; Weber, C.; van der Vorst, E. P. C.; Steffens, S. Palmitoylethanolamide promotes a proresolving macrophage phenotype and attenuates atherosclerotic plaque formation. *Arterioscler. Thromb. Vasc. Biol.* **2018**, *38*, 2562-2575.

15 McKinney, M. K.; Cravatt, B. F. Structure and function of fatty acid amide hydrolase. *Annu. Rev. Biochem.* **2005**, *74*, 411-432.

16 Tuo, W.; Leleu-Chavain, N.; Spencer, J.; Sansook, S.; Millet, R.; Chavatte, P. Therapeutic potential of fatty acid amide hydrolase, monoacylglycerol lipase, and N-acylethanolamine acid amidase inhibitors. *J. Med. Chem.* **2017**, *60*, 4-46.

17 Ueda, N.; Tsuboi, K.; Uyama, T.; N-acylethanolamine metabolism with special reference to N-acylethanolamine-hydrolyzing acid amidase (NAAA). *Prog. Lipid Res.* **2010**, *49*, 299-315.

18 Botteman, P.; Muccioli, G. G.; Alhouayek, M. N-acylethanolamine hydrolyzing acid amidase inhibition: tools and potential therapeutic opportunities. *Drug Discov. Today* **2018**, *23*, 1520-1529.

19 Bracey, M. H.; Hanson, M. A.; Masuda, K. R.; Stevens, R. C.; Cravatt, B. F. Structural adaptations in a membrane enzyme that terminates endocannabinoid signaling. *Science* **2002**, *298*, 1793-1796.

20 Gorelik, A.; Gebai, A.; Illes, K.; Piomelli, D.; Nagar, B. Molecular mechanism of activation of the immunoregulatory amidase NAAA. *Proc. Natl. Acad. Sci. U S A.* **2018**, *115*, E10032-E10040.

21 The UniProt Consortium. UniProt: a worldwide hub of protein knowledge. *Nucleic Acids Res.* **2019**, *47*, D506-D515.

22 Désarnaud, F.; Cadas, H.; Piomelli, D. Anandamide amidohydrolase activity in rat brain microsomes. Identification and partial characterization. *J. Biol. Chem.* **1995**, *270*, 6030-6035.

23 Ueda, N.; Kurahashi, Y.; Yamamoto, S.; Tokunaga, T. Partial purification and characterization of the porcine brain enzyme hydrolyzing and synthesizing anandamide. *J. Biol. Chem.* **1995**, *270*, 23823-23827.

24 Hillard, C. J.; Wilkison, D. M.; Edgemond, W. S.; Campbell, W. B. Characterization of the kinetics and distribution of N-arachidonyl ethanolamine (anandamide) hydrolysis by rat brain. *Biochim. Biophys. Acta.* **1995**, *1257*, 249-256.

25 Schmid, H. H. O.; Schmid, P. C.; Natarajan, V.; N-Acylated glycerophospholipids and their derivatives. *Prog. Lipid Res.* **1990**, *29*, 1-43.

26 Ueda, N.; Yamanaka, K.; Terasawa, Y.; Yamamoto, S. An acid amidase hydrolyzing anandamide as an endogenous ligand for cannabinoid receptors. *FEBS Lett.* **1999**, *454*, 267-270.

27 Kuehl Jr., F. A.; Jacob, T. A.; Ganley, O. H.; Ormond, R. E.; Meisinger, M. A. P. The identification of N-(2-hydroxyethyl)-palmitamide as a naturally occurring anti-inflammatory agent. *J. Am. Chem. Soc.* **1957**, *79*, 5577-5578.

28 Ganley, O. H.; Robinson, H. J. Antianaphylactic and antiserotonin activity of a compound obtained from egg yolk, peanut oil and soybean lecithin. *J. Allergy* **1959**, *30*, 415-419.

29 Mašek, K., and Raskova, H. Pharmacology of palmitoylethanolamide. In *Drugs of animal origin*, Leonardi, A., Walsh J., Eds.; Ferro Edizioni: Milan, 1967; pp. 199-209.

30 Benvenuti, F.; Lattanzi, F.; De Gori, A.; Tarli, P. Activity of some derivatives of palmitoylethanolamide on carragenine-induced edema in the rat paw. *Boll. Soc. Ital. Biol. Sper.* **1968**, *44*, 809-813.

31 Perlík, F.; Rasková, H.; Elis, J. Anti-inflammatory properties of N-(2-hydroxyethyl) palmitamide. *Acta Physiol. Acad. Sci. Hung.* **1971**, *39*, 395-400.

32 Perlík, F.; Krejčí, J.; Elis, J.; Pekárek, J.; Svejcar, J. The effect of N-(2-hydroxyethyl)-palmitamide on delayed hypersensitivity in guinea-pig. *Experientia* **1973**, *29*, 67-68.

33 Masek, K.; Perlík, F.; Klíma, J.; Kahlich, R. Prophylactic efficacy of N-2-hydroxyethyl palmitamide (impulsin) in acute respiratory tract infections. *Eur. J. Clin. Pharmacol.* **1974**, *7*, 415-419.

34 Kahlich, R.; Klíma, J.; Cihla, F.; Franková, V.; Mašek, K.; Rosický, M.; Matousek, F.; Bruthans, J. Studies on prophylactic efficacy of N-2-hydroxyethyl palmitamide (Impulsin) in acute respiratory infections. Serologically controlled field trials. *J. Hyg. Epidemiol. Microbiol. Immunol.* **1979**, *23*, 11-24.

35 Aloe, L.; Leon, A.; Levi-Montalcini, R. A proposed autacoid mechanism controlling mastocyte behaviour. *Agents Actions* **1993**, *39*, 145-147.

36 Mazzari, S.; Canella, R.; Petrelli, L.; Marcolongo, G.; Leon, A. N-(2-hydroxyethyl)hexadecanamide is orally active in reducing edema formation and inflammatory hyperalgesia by down-modulating mast cell activation. *Eur. J. Pharmacol.* **1996**, *300*, 227-236.

37 Berdyshev, E. V.; Boichot, E.; Germain, N.; Allain, N.; Anger, J. P.; Lagente, V. Influence of fatty acid ethanolamides and Δ^9 -tetrahydrocannabinol on cytokine and arachidonate release by mononuclear cells. *Eur. J. Pharmacol.* **1997**, *330*, 231-240.

38 Ross, R. A.; Brockie, H. C.; Pertwee, R. G. Inhibition of nitric oxide production in RAW264.7 macrophages by cannabinoids and palmitoylethanolamide. *Eur. J. Pharmacol.* **2000**, *401*, 121-130.

39 Farquhar-Smith, W. P.; Rice, A. S. A novel neuroimmune mechanism in cannabinoid-mediated attenuation of nerve growth factor-induced hyperalgesia. *Anesthesiology* **2003**, *99*, 1391-1401.

40 Skaper, S. D.; Buriani, A.; Dal Toso, R.; Petrelli, L.; Romanello, S.; Facci, L.; Leon, A. The ALIamide palmitoylethanolamide and cannabinoids, but not anandamide, are protective in a delayed postglutamate paradigm of excitotoxic death in cerebellar granule neurons. *Proc. Natl. Acad. Sci. U S A.* **1996**, *93*, 3984-3989.

41 Calignano, A.; La Rana, G.; Giuffrida, A.; Piomelli D. Control of pain initiation by endogenous cannabinoids. *Nature* **1998**, *394*, 277-281.

42 Calignano, A.; La Rana, G.; Piomelli, D. Antinociceptive activity of the endogenous fatty acid amide, palmitylethanolamide. *Eur. J. Pharmacol.* **2001**, *419*, 191-198.

43 Pontis, S.; Ribeiro, A.; Sasso, O.; Piomelli, D. Macrophage-derived lipid agonists of PPAR- α as intrinsic controllers of inflammation. *Crit. Rev. Biochem. Mol. Biol.* **2016**, *51*, 7-14.

44 Eberlein, B.; Eicke, C.; Reinhardt, H. W.; Ring, J. Adjuvant treatment of atopic eczema: assessment of an emollient containing N-palmitoylethanolamine (ATOPA study). *J. Eur. Acad. Dermatology Venereol.* **2008**, *22*, 73-82.

45 Cremon, C.; Stanghellini, V.; Barbaro, M. R.; Cogliandro, R. F.; Bellacosa, L.; Santos, J.; Vicario, M.; Pigrau, M.; Alonso Cotoner, C.; Lobo, B.; Azpiroz, F.; Bruley des Varannes, S.; Neunlist, M.; DeFilippis, D.; Iuvone, T.; Petrosino, S.; Di Marzo, V.; Barbara G. Randomised clinical trial: the analgesic properties of dietary supplementation with palmitoylethanolamide and polydatin in irritable bowel syndrome. *Aliment. Pharmacol. Ther.* **2017**, *45*, 909-922.

46 Schweiger, V.; Martini, A.; Bellamoli, P.; Donadello, K.; Schievano, C.; Balzo, G. D.; Sarzi-Puttini, P.; Parolini, M.; Polati, E. Ultramicronized palmitoylethanolamide (um-PEA) as add-on treatment in fibromyalgia syndrome (FMS): retrospective observational study on 407 patients. *CNS Neurol. Disord. Drug Targets* **2019**, *18*, 326-333.

47 Truini, A.; Biasiotta, A.; Di Stefano, G.; La Cesa, S.; Leone, C.; Cartoni, C.; Federico, V.; Petrucci, M. T.; Cruccu, G. Palmitoylethanolamide restores myelinated-fibre function in patients with chemotherapy-induced painful neuropathy. *CNS Neurol. Disord. Drug Targets* **2011**, *10*, 916-920.

48 Giammusso, B.; Di Mauro, R.; Bernardini R. The efficacy of an association of palmitoylethanolamide and alpha-lipoic acid in patients with chronic prostatitis/chronic pelvic pain syndrome: A randomized clinical trial. *Arch. Ital. Urol. Androl.* **2017**, *89*, 17-21.

49 Orefice, N. S.; Alhouayek, M.; Carotenuto, A.; Montella, S.; Barbato, F.; Comelli, A.; Calignano, A.; Muccioli, G. G.; Orefice, G. Oral palmitoylethanolamide treatment is associated with reduced cutaneous adverse effects of interferon- β 1a and circulating proinflammatory cytokines in relapsing-remitting multiple sclerosis. *Neurotherapeutics* **2016**, *13*, 428-438.

50 Artukoglu, B. B.; Beyer, C.; Zulooff-Shani, A.; Brener, E.; Bloch, M. H. Efficacy of palmitoylethanolamide for pain: a meta-analysis. *Pain Physician* **2017**, *20*, 353-362.

51 Bachur, N. R.; Masek, K.; Melmon, K. L., Udenfriend, S. Fatty acid amides of ethanolamine in mammalian tissues. *J. Biol. Chem.* **1965**, *240*, 1019-1024.

52 Tsuboi, K.; Uyama, T.; Okamoto, Y.; Ueda, N. Endocannabinoids and related N-acylethanolamines: biological activities and metabolism. *Inflamm. Regen.* **2018**, *38*, 28.

53 Ogura, Y.; Parsons, W. H.; Kamat, S. S.; Cravatt, B. F. A calcium-dependent acyltransferase that produces N-acyl phosphatidylethanolamines. *Nat. Chem. Biol.* **2016**, *12*, 669-671.

54 Okamoto, Y.; Morishita, J.; Tsuboi, K.; Tonai, T.; Ueda, N. Molecular characterization of a phospholipase D generating anandamide and its congeners. *J. Biol. Chem.* **2004**, *279*, 5298-5305.

55 Wang, J.; Okamoto, Y.; Morishita, J.; Tsuboi, K.; Miyatake, A.; Ueda, N. Functional analysis of the purified anandamide-generating phospholipase D as a member of the metallo-beta-lactamase family. *J. Biol. Chem.* **2006**, *281*, 12325-12335.

56 Magotti, P.; Bauer, I.; Igarashi, M.; Babagoli, M.; Marotta, R.; Piomelli, D.; Garau G. Structure of human N-acylphosphatidylethanolamine-hydrolyzing phospholipase D: regulation of fatty acid ethanolamide biosynthesis by bile acids. *Structure* **2015**, *23*, 598-604.

57 Zhu, C.; Solorzano, C.; Sahar, S.; Realini, N.; Fung, E.; Sassone-Corsi, P.; Piomelli, D. Proinflammatory stimuli control N-acylphosphatidylethanolamine-specific phospholipase D expression in macrophages. *Mol. Pharmacol.* **2011**, *79*, 786-792.

58 Sun, Y. X.; Tsuboi, K.; Zhao, L. Y.; Okamoto, Y.; Lambert, D. M.; Ueda, N. Involvement of N-acylethanolamine-hydrolyzing acid amidase in the degradation of anandamide and other N-acylethanolamines in macrophages. *Biochim. Biophys. Acta* **2005**, *1736*, 211-220.

59 Tai, T.; Tsuboi, K.; Uyama, T.; Masuda, K.; Cravatt, B. F.; Houchi, H.; Ueda, N. Endogenous molecules stimulating N-acylethanolamine-hydrolyzing acid amidase (NAAA). *ACS Chem. Neurosci.* **2012**, *3*, 379-385.

60 Bonezzi, F. T.; Sasso, O.; Pontis, S.; Realini, N.; Romeo, E.; Ponzano, S.; Nuzzi, A.; Fiasella, A.; Bertozzi, F.; Piomelli, D. An important role for N-acylethanolamine acid amidase in the complete Freund's adjuvant rat model of arthritis. *J. Pharmacol. Exp. Ther.* **2016**, *356*, 656-663.

61 Zhou, P.; Xiang, L.; Yang, Y.; Wu, Y.; Hu, T.; Liu, X.; Lin, F.; Xiu, Y.; Wu, K.; Lu, C.; Ren, J.; Qiu, Y.; Li, Y. N-Acylethanolamine acid amidase (NAAA) inhibitor F215 as a novel therapeutic agent for osteoarthritis. *Pharmacol. Res.* **2019**, *145*, 104264.

62 Suárez, J.; Romero-Zerbo, Y.; Márquez, L.; Rivera, P.; Iglesias, M.; Bermúdez-Silva, F. J.; Andreu, M.; Rodríguez de Fonseca, F. Ulcerative colitis impairs the acylethanolamide-based anti-

inflammatory system reversal by 5-aminosalicylic acid and glucocorticoids. *PloS One* **2012**, *7*, e37729.

63 Solorzano, C.; Zhu, C.; Battista, N.; Astarita, G.; Lodola, A.; Rivara, S.; Mor, M.; Russo, R.; Maccarrone, M.; Antonietti, F.; Duranti, A.; Tontini, A.; Cuzzocrea, S.; Tarzia, G.; Piomelli, D. Selective N-acylethanolamine-hydrolyzing acid amidase inhibition reveals a key role for endogenous palmitoylethanolamide in inflammation. *Proc. Natl. Acad. Sci. U S A.* **2009**, *106*, 20966-20971.

64 De Filippis, D.; D'Amico, A.; Cinelli, M. P.; Esposito, G.; Di Marzo, V.; Iuvone, T. Adelmidrol, a palmitoylethanolamide analogue, reduces chronic inflammation in a carrageenin-granuloma model in rats. *J. Cell. Mol. Med.* **2009**, *13*, 1086-1095.

65 De Filippis, D.; D'Amico, A.; Cipriano, M.; Petrosino, S.; Orlando, P.; Di Marzo, V.; Iuvone, T. Levels of endocannabinoids and palmitoylethanolamide and their pharmacological manipulation in chronic granulomatous inflammation in rats. *Pharmacol. Res.* **2010**, *61*, 321-328.

66 Capasso, R.; Izzo, A. A.; Fezza, F.; Pinto, A.; Capasso, F.; Mascolo, N.; Di Marzo, V. Inhibitory effect of palmitoylethanolamide on gastrointestinal motility in mice. *Br. J. Pharmacol.* **2001**, *134*, 945-950.

67 Izzo, A. A.; Capasso, R.; Aviello, G.; Borrelli, F.; Romano, B.; Piscitelli, F.; Gallo, L.; Capasso, F.; Orlando, P.; Di Marzo, V. Inhibitory effect of cannabichromene, a major non-psychoactive cannabinoid extracted from *Cannabis sativa*, on inflammation-induced hypermotility in mice. *Br. J. Pharmacol.* **2012**, *166*, 1444-1460.

68 Richardson, D.; Pearson, R. G.; Kurian, N.; Latif, M. L.; Garle, M. J.; Barrett, D. A.; Kendall, D. A.; Scammell, B. E.; Reeve, A. J.; Chapman, V. Characterisation of the cannabinoid receptor system in synovial tissue and fluid in patients with osteoarthritis and rheumatoid arthritis. *Arthritis Res. Ther.* **2008**, *10*, R43.

69 Tsuboi, K.; Zhao, L. Y.; Okamoto, Y.; Araki, N.; Ueno, M.; Sakamoto, H.; Ueda, N. Predominant expression of lysosomal N-acylethanolamine-hydrolyzing acid amidase in macrophages revealed by immunochemical studies. *Biochim. Biophys. Acta* **2007**, *1771*, 211-220.

70 Ribeiro, A.; Pontis, S.; Mengatto, L.; Armirotti, A.; Chiurchiù, V.; Capurro, V.; Fiasella, A.; Nuzzi, A.; Romeo, E.; Moreno-Sanz, G.; Maccarrone, M.; Reggiani, A.; Tarzia, G.; Mor, M.; Bertozzi, F.; Bandiera, T.; Piomelli, D. A potent systemically active N-acylethanolamine acid amidase inhibitor that suppresses inflammation and human macrophage activation. *ACS Chem. Biol.* **2015**, *10*, 1838-1846.

71 ImmGen, <http://www.immgen.org> (accessed Jan 28, 2020).

72 Sasso, O.; Summa, M.; Armirotti, A.; Pontis, S.; De Mei, C.; Piomelli, D. The N-acylethanolamine acid amidase inhibitor ARN077 suppresses inflammation and pruritus in a mouse model of allergic dermatitis. *J. Invest. Dermatol.* **2018**, *138*, 562-569.

73 Yang, L.; Li, L.; Chen, L.; Li, Y.; Chen, H.; Li, Y.; Ji, G.; Lin, D.; Liu, Z.; Qiu, Y. Potential analgesic effects of a novel N-acylethanolamine acid amidase inhibitor F96 through PPAR- α . *Sci. Rep.* **2015**, *5*, 13565.

74 Zheng, B.; Liao, Z.; Locascio, J. J.; Lesniak, K. A.; Roderick, S. S.; Watt, M. L.; Eklund, A. C.; Zhang-James, Y.; Kim, P. D.; Hauser, M. A.; Grünblatt, E.; Moran, L. B.; Mandel, S. A.;

Riederer, P.; Miller, R. M.; Federoff, H. J.; Wüllner, U.; Papapetropoulos, S.; Youdim, M. B.; Cantuti-Castelvetri, I.; Young, A. B.; Vance, J. M.; Davis, R. L.; Hedreen, J. C.; Adler, C. H.; Beach, T. G.; Graeber, M. B.; Middleton, F. A.; Rochet, J. C.; Scherzer, C. R.; Global PD Gene Expression (GPEX) Consortium. PGC-1 α , a potential therapeutic target for early intervention in Parkinson's disease. *Sci. Transl. Med.* **2010**, *2*, 52ra73.

75 Su, X.; Chu, Y.; Kordower, J. H.; Li, B.; Cao, H.; Huang, L.; Nishida, M.; Song, L.; Wang, D.; Federoff, H. J. PGC1 α promoter methylation in Parkinson's disease. *PloS One* **2015**, *10*, e0134087.

76 Korbecki, J.; Bajdak-Rusinek, K. The effect of palmitic acid on inflammatory response in macrophages: an overview of molecular mechanisms. *Inflamm. Res.* **2019**, *68*, 915-932.

77 Handschin, C.; Spiegelman, B. M. Peroxisome proliferator-activated receptor gamma coactivator 1 coactivators, energy homeostasis, and metabolism. *Endocr. Rev.* **2006**, *27*, 728-735.

78 Gebai A.; Gorelik A.; Li Z.; Illes K.; Nagar B. Structural basis for the activation of acid ceramidase. *Nat. Commun.* **2018**, *9*, 1621.

79 Brannigan, J. A.; Dodson, G.; Duggleby, H. J.; Moody, P. C.; Smith, J. L.; Tomchick, D. R.; Murzin, A. G. A protein catalytic framework with an N-terminal nucleophile is capable of self-activation. *Nature* **1995**, *378*, 416-419.

80 Oinonen, C.; Rouvinen, J. Structural comparison of Ntn-hydrolases. *Protein Sci.* **2000**, *9*, 2329-2337.

81 Tsuboi, K.; Sun, Y. X.; Okamoto, Y.; Araki, N.; Tonai, T.; Ueda, N. Molecular characterization of N-acylethanolamine-hydrolyzing acid amidase, a novel member of the

choloylglycine hydrolase family with structural and functional similarity to acid ceramidase. *J. Biol. Chem.* **2005**, *280*, 11082-11092.

82 Mellman, I.; Fuchs, R.; Helenius, A. Acidification of the endocytic and exocytic pathways. *Annu. Rev. Biochem.* **1986**, *55*, 663-700.

83 Zhao, L. Y.; Tsuboi, K.; Okamoto, Y.; Nagahata, S.; Ueda, N. Proteolytic activation and glycosylation of N-acylethanolamine-hydrolyzing acid amidase, a lysosomal enzyme involved in the endocannabinoid metabolism. *Biochim. Biophys. Acta* **2007**, *1771*, 1397-1405.

84 West, J. M.; Zvonok, N.; Whitten, K. M.; Wood, J. T.; Makriyannis, A. Mass spectrometric characterization of human N-acylethanolamine-hydrolyzing acid amidase. *J. Proteome Res.* **2012**, *11*, 972-981.

85 Kolter, T.; Sandhoff, K. Lysosomal degradation of membrane lipids. *FEBS Lett.* **2010**, *584*, 1700-1712.

86 Tsuboi, K.; Hilligsmann, C.; Vandevoorde, S.; Lambert, D. M.; Ueda, N. N-cyclohexanecarbonylpentadecylamine: a selective inhibitor of the acid amidase hydrolysing N-acylethanolamines, as a tool to distinguish acid amidase from fatty acid amide hydrolase. *Biochem. J.* **2004**, *379*, 99-106.

87 Yamano, Y.; Tsuboi, K.; Hozaki, Y.; Takahashi, K.; Jin, X. H.; Ueda, N.; Wada, A. Lipophilic amines as potent inhibitors of N-acylethanolamine-hydrolyzing acid amidase. *Bioorg. Med. Chem.* **2012**, *20*, 3658-3665.

88 Saturnino, C.; Petrosino, S.; Ligresti, A.; Palladino, C.; De Martino, G.; Bisogno, T.; Di Marzo, V. Synthesis and biological evaluation of new potential inhibitors of N-acylethanolamine hydrolyzing acid amidase. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 1210-1213.

89 Vago, R.; Bettiga, A.; Salonia, A.; Ciuffreda, P.; Ottria, R. Development of new inhibitors for N-acylethanolamine-hydrolyzing acid amidase as promising tool against bladder cancer. *Bioorg. Med. Chem.* **2017**, *25*, 1242-1249.

90 Petrosino, S.; Campolo, M.; Impellizzeri, D.; Paterniti, I.; Allarà, M.; Gugliandolo, E.; D'Amico, R.; Siracusa, R.; Cordaro, M.; Esposito, E.; Di Marzo, V.; Cuzzocrea, S. 2-Pentadecyl-2-oxazoline, the oxazoline of PEA, modulates carrageenan-induced acute inflammation. *Front. Neurol.* **2017**, *8*, 233.

91 Rossocha, M.; Schultz-Heienbrok, R.; von Moeller, H.; Coleman, J. P.; Saenger, W. Conjugated bile acid hydrolase is a tetrameric N-terminal thiol hydrolase with specific recognition of its cholyl but not of its tauryl product. *Biochemistry* **2005**, *44*, 5739-5748.

92 Lall, M. S.; Karvellas, C.; Vederas, J. C. Beta-lactones as a new class of cysteine proteinase inhibitors: inhibition of hepatitis A virus 3C proteinase by N-Cbz-serine beta-lactone. *Org. Lett.* **1999**, *1*, 803-806.

93 Greenspan, M. D.; Bull, H. G.; Yudkovitz, J. B.; Hanf, D. P.; Alberts, A.W. Inhibition of 3-hydroxy-3-methylglutaryl-CoA synthase and cholesterol biosynthesis by beta-lactone inhibitors and binding of these inhibitors to the enzyme. *Biochem. J.* **1993**, *289*, 889-895.

94 Pojer, F.; Ferrer, J. L.; Richard, S. B.; Nagegowda, D. A.; Chye, M. L.; Bach, T. J.; Noel, J. P. Structural basis for the design of potent and species-specific inhibitors of 3-hydroxy-3-methylglutaryl CoA synthases. *Proc. Natl. Acad. Sci. U S A.* **2006**, *103*, 11491-11496.

95 Armirotti, A.; Romeo, E.; Ponzano, S.; Mengatto, L.; Dionisi, M.; Karacsonyi, C.; Bertozzi, F.; Garau, G.; Tarozzo, G.; Reggiani, A.; Bandiera, T.; Tarzia, G.; Mor, M.; Piomelli, D. β -Lactones inhibit N-acylethanolamine acid amidase by S-acylation of the catalytic N-terminal cysteine. *ACS Med. Chem. Lett.* **2012**, *3*, 422-426.

96 Solorzano, C.; Antonietti, F.; Duranti, A.; Tontini, A.; Rivara, S.; Lodola, A.; Vacondio, F.; Tarzia, G.; Piomelli, D.; Mor, M. Synthesis and structure–activity relationships of N-(2-oxo-3-oxetanyl)amides as N-acylethanolamine-hydrolyzing acid amidase inhibitors. *J. Med. Chem.* **2010**, *53*, 5770-5781.

97 Duranti, A.; Tontini, A.; Antonietti, F.; Vacondio, F.; Fioni, A.; Silva, C.; Lodola, A.; Rivara, S.; Solorzano, C.; Piomelli, D.; Tarzia, G.; Mor, M. N-(2-Oxo-3-oxetanyl)carbamic acid esters as N-acylethanolamine acid amidase inhibitors: synthesis and structure–activity and structure–property relationships. *J. Med. Chem.* **2012**, *55*, 4824-4836.

98 Ponzano, S.; Berozzi, F.; Mengatto, L.; Dionisi, M.; Armirotti, A.; Romeo, E.; Berteotti, A.; Fiorelli, C.; Tarozzo, G.; Reggiani, A.; Duranti, A.; Tarzia, G.; Mor, M.; Cavalli, A.; Piomelli, D.; Bandiera, T. Synthesis and structure–activity relationship (SAR) of 2-methyl-4-oxo-3-oxetanylcarbamic acid esters, a class of potent N-acylethanolamine acid amidase (NAAA) inhibitors. *J. Med. Chem.* **2013**, *56*, 6917-6934.

99 Ponzano, S.; Berteotti, A.; Petracca, R.; Vitale, R.; Mengatto, L.; Bandiera, T.; Cavalli, A.; Piomelli, D.; Bertozzi, F.; Bottegoni, G. Synthesis, biological evaluation, and 3D QSAR study of

2-methyl-4-oxo-3-oxetanylcarbamic acid esters as N-acylethanolamine acid amidase (NAAA) inhibitors. *J. Med. Chem.* **2014**, *57*, 10101-10111.

100 Khasabova, I. A.; Xiong, Y.; Coicou, L. G.; Piomelli, D.; Seybold, V. Peroxisome proliferator-activated receptor α mediates acute effects of palmitoylethanolamide on sensory neurons. *J. Neurosci.* **2012**, *32*, 12735-12743.

101 Sasso, O.; Moreno-Sanz, G.; Martucci, C.; Realini, N.; Dionisi, M.; Mengatto, L.; Duranti, A.; Tarozzo, G.; Tarzia, G.; Mor, M.; Bertorelli, R.; Reggiani, A.; Piomelli, D. Antinociceptive effects of the N-acylethanolamine acid amidase inhibitor ARN077 in rodent pain models. *Pain*, **2013**, *154*, 350-360.

102 Fiasella, A.; Nuzzi, A.; Summa, M.; Armirotti, A.; Tarozzo, G.; Tarzia, G.; Mor, M.; Bertozzi, F.; Bandiera, T.; Piomelli, D. 3-Aminoazetididin-2-one derivatives as N-acylethanolamine acid amidase (NAAA) inhibitors suitable for systemic administration. *ChemMedChem* **2014**, *9*, 1602-1614.

103 Migliore, M.; Pontis, S.; Fuentes de Arriba, A. L.; Realini, N.; Torrente, E.; Armirotti, A.; Romeo, E.; Di Martino, S.; Russo, D.; Pizzirani, D.; Summa, M.; Lanfranco, M.; Ottonello, G.; Busquet, P.; Jung, K. M.; Garcia-Guzman, M.; Heim, R.; Scarpelli, R.; Piomelli, D. Second-generation non-covalent NAAA inhibitors are protective in a model of multiple sclerosis. *Angew. Chem. Int. Ed. Engl.* **2016**, *55*, 11193-11197.

104 Nuzzi, A.; Fiasella, A.; Ortega, J. A.; Pagliuca, C.; Ponzano, S.; Pizzirani, D.; Bertozzi, S. M.; Ottonello, G.; Tarozzo, G.; Reggiani, A.; Bandiera, T.; Bertozzi, F.; Piomelli, D. Potent α -amino- β -lactam carbamic acid ester as NAAA inhibitors. Synthesis and structure-activity relationship (SAR) studies. *Eur. J. Med. Chem.* **2016**, *111*, 138-159.

105 Petracca, R.; Ponzano, S.; Bertozzi, S. M.; Sasso, O.; Piomelli, D.; Bandiera, T.; Bertozzi, F. Progress in the development of β -lactams as N-acylethanolamine acid amidase (NAAA) inhibitors: synthesis and SAR study of new, potent N-O-substituted derivatives. *Eur. J. Med. Chem.* **2017**, *126*, 561-575.

106 Romeo, E.; Ponzano, S.; Armirotti, A.; Summa, M.; Bertozzi, F.; Garau, G.; Bandiera, T.; Piomelli, D. Activity-based probe for N-acylethanolamine acid amidase. *ACS Chem. Biol.* **2015**, *10*, 2057-2064.

107 Romeo, E.; Pontis, S.; Ponzano, S.; Bonezzi, F.; Migliore, M.; Di Martino, S.; Summa, M.; Piomelli, D. Preparation and in vivo use of an activity-based probe for N-acylethanolamine acid amidase. *J. Vis. Exp.* **2016**, *117*, 54652.

108 Petracca, R.; Romeo, E.; Baggelaar, M. P.; Artola, M.; Pontis, S.; Ponzano, S.; Overkleeft, H. S.; van der Stelt, M.; Piomelli, D. Novel activity-based probes for N-acylethanolamine acid amidase. *Chem. Commun.* **2017**, *53*, 11810-11813.

109 Malamas, M.; Makriyannis, A.; Subramian, K. V.; Whitten, K. M., N-acylethanolamine hydrolyzing acid amidase (NAAA) inhibitors and their use thereof. WO2015179190A1, November 26, 2015.

110 Tarozzi, A.; Marchetti, C.; Nicolini, B.; D'Amico, M.; Ticchi, N.; Pruccoli, L.; Tumiatti, V.; Simoni, E.; Lodola, A.; Mor, M.; Milelli, A.; Minarini, A. Combined inhibition of the EGFR/AKT pathways by a novel conjugate of quinazoline with isothiocyanate. *Eur. J. Med. Chem.* **2016**, *117*, 283-291.

111 West, J. M.; Zvonok, N.; Whitten, K. M.; Vadivel, S. K.; Bowman, A. L.; Makriyannis, A. Biochemical and mass spectrometric characterization of human N-acylethanolamine-hydrolyzing acid amidase inhibition. *PloS One* **2012**, *7*, e43877.

112 Alhouayek, M.; Botteman, P.; Subramanian, K. V.; Lambert, D. M.; Makriyannis, A.; Cani, P. D.; Muccioli, G.G. N-Acylethanolamine-hydrolyzing acid amidase inhibition increases colon N-palmitoylethanolamine levels and counteracts murine colitis. *FASEB J.* **2015**, *29*, 650-661.

113 Falgoutyret, J. P.; Oballa, R. M.; Okamoto, O.; Wesolowski, G.; Aubin, Y.; Rydzewski, R. M.; Prasit, P.; Riendeau, D.; Rodan, S. B.; Percival, M. D. Novel, nonpeptidic cyanamides as potent and reversible inhibitors of human cathepsins K and L. *J. Med. Chem.* **2001**, *44*, 94-104.

114 Malamas, M. S.; Farah, S. I.; Lamani, M.; Pelekoudas, D. N.; Perry, N. T.; Rajarshi, G.; Miyabe, C. Y.; Chandrashekhar, H.; West, J.; Pavlopoulos, S.; Makriyannis, A. Design and synthesis of cyanamides as potent and selective N-acylethanolamine acid amidase inhibitors. *Bioorg. Med. Chem.* **2020**, *28*, 115195.

115 Li, Y.; Yang, L.; Chen, L.; Zhu, C.; Huang, R.; Zheng, X.; Qiu, Y.; Fu, J. Design and synthesis of potent N-acylethanolamine hydrolyzing acid amidase (NAAA) inhibitor as anti-inflammatory compounds. *PloS One* **2012**, *7*, e43877.

116 Zhou, P.; Xiang, L.; Zhao, D.; Ren, J.; Qiu, Y.; Li, Y. Synthesis, biological evaluation, and structure activity relationship (SAR) study of pyrrolidine amide derivatives as N-acylethanolamine acid amidase (NAAA) inhibitors. *MedChemComm* **2019**, *10*, 252-262.

117 Li, Y.; Chen, Q.; Yang, L.; Li, Y.; Zhang, Y.; Qiu, Y.; Ren, J.; Lu, C. Identification of highly potent N-acylethanolamine acid amidase (NAAA) inhibitors: Optimization of the terminal phenyl moiety of oxazolidone derivatives. *Eur. J. Med. Chem.* **2017**, *139*, 214-221.

118 Petrosino, S.; Ahmad, A.; Marcolongo, G.; Esposito, E.; Allarà, M.; Verde, R.; Cuzzocrea, S.; Di Marzo, V. Diacerein is a potent and selective inhibitor of palmitoylethanolamide inactivation with analgesic activity in a rat model of acute inflammatory pain. *Pharmacol. Res.* **2015**, *91*, 9-14.

119 Fidelix, T. S.; Macedo, C. R.; Maxwell, L. J.; Fernandes Moça Trevisani, V. Diacerein for osteoarthritis. *Cochrane Database Syst. Rev.* **2014**, *2*, CD005117.

120 Pavelka, K.; Bruyere, O.; Cooper, C.; Kanis, J. A.; Leeb, B. F.; Maheu, E.; Martel-Pelletier, J.; Monfort, J.; Pelletier, J. P.; Rizzoli, R.; Reginster, J. Y. Diacerein: benefits, risks and place in the management of osteoarthritis. An opinion-based report from the ESCEO. *Drugs Aging* **2016**, *33*, 75-85.

121 Realini, N.; Solorzano, C.; Pagliuca, C.; Pizzirani, D.; Armirotti, A.; Luciani, R.; Costi, M. P.; Bandiera T.; Piomelli, D. Discovery of highly potent acid ceramidase inhibitors with in vitro tumor chemosensitizing activity. *Sci. Rep.* **2013**, *3*, 1035.

122 Wu, K.; Xiu, Y.; Zhou, P.; Qiu, Y.; Li, Y. A new use for an old drug: carmofur attenuates lipopolysaccharide (LPS)-induced acute lung injury via inhibition of FAAH and NAAA activities. *Front. Pharmacol.* **2019**, *10*, 818.

123 Bertozzi, F.; Bandiera, T. Modulation of N-acylethanolamine-hydrolysing acid amidase (NAAA) for disease treatment. US 20190135802 A1, May 9, 2019.

124 Bodor, N.; Buchwals, P. Soft drug design: General principles and recent applications. *Med. Res. Rev.* **2000**, *20*, 58-101.

125 Sasso, O.; Moreno-Sanz, G.; Martucci, C.; Realini, N.; Dionisi, M.; Mengatto, L.; Duranti, A.; Tarozzo, G.; Tarzia, G.; Mor, M.; Bertorelli, R.; Reggiani, A.; Piomelli, D. Antinociceptive effects of the N-acylethanolamine acid amidase inhibitor ARN077 in rodent pain models. *Pain* **2013**, *154*, 350-360.

126 Yang, L.; Li, L.; Chen, L.; Li, Y.; Chen, H.; Li, Y.; Ji, G.; Lin, D.; Liu, Z.; Qiu, Y. Potential analgesic effects of a novel N-acylethanolamine acid amidase inhibitor F96 through PPAR- α . *Sci. Rep.* **2015**, *5*, 13565.

127 Rubenstrunk, A.; Hanf, R.; Hum, D. W.; Fruchart, J. C.; Staels, B. Safety issues and prospects for future generations of PPAR modulators. *Biochim. Biophys. Acta* **2007**, *177*, 1065-1081.

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