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N-acylethanolamine hydrolyzing acid amidase inhibition: tools and potential therapeutic opportunities

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_N_-acylethanolamines (NAEs) (e.g., _N_-palmitoylethanolamine, _N_-arachidonoylethanolamine, _N_-oleoylethanolamine) are bioactive lipids involved in many physiological processes including pain, inflammation, anxiety, cognition and food intake. Two enzymes are responsible for the hydrolysis of NAEs and therefore regulate their endogenous levels and effects: fatty acid amide hydrolase (FAAH) and _N_-acylethanolamine-hydrolyzing acid amidase (NAAA). As discussed here, extensive biochemical characterization of NAAA was carried out over the years that contributed to a better understanding of NAAA enzymology. An increasing number of studies describe the synthesis and pharmacological characterization of NAAA inhibitors. Recent medicinal chemistry efforts have led to the development of potent and stable inhibitors that enable studying the effects of NAAA inhibition in preclinical disease models, notably in the context of pain and inflammation.

N-acylethanolamines: targets, general activities and metabolism

Targets and general activities

N-acylethanolamines (NAEs) are endogenous bioactive lipids consisting of an acyl chain linked by an amide bond to ethanolamine. They differ in the length and unsaturation degree of the acyl chain. Although sharing the same basic scaffold, NAEs can bind to several different receptors and exert a plethora of biological effects. One of the most-studied NAEs is the endocannabinoid *N*-arachidonoylethanolamine (anandamide or AEA), which was first discovered as the endogenous ligand of the cannabinoid receptors CB_1 and CB_2 on which it acts as a partial agonist. Since then, AEA has also been shown to be an agonist of transient receptor potential cation channel subfamily V (TRPV)1 and peroxisome proliferator-activated receptor (PPAR) (Fig. 1a) [1,2]. AEA has also been suggested as a putative ligand for G-protein-coupled receptor 18 (GPR18), PPARα and transient receptor potential cation channel subfamily M (TRPM)8, although this remains to be confirmed [3–5]. AEA is known to be anti-inflammatory, analgesic and neuroprotective. It also has anxiolytic, antidepressive and cognitive effects [2,6–8]. Moreover, whereas AEA stimulates appetite and lipogenesis, its structural congener *N*-oleoyethanolamine (OEA) is anorexigenic and stimulates lipolysis via PPARα, through which it could also exert neuroprotective effects [5,9,10]. Some of the effects of OEA on hunger suppression could also be mediated by TRPV1 [11]. OEA also stimulates glucagon-like peptide (GLP)-1 secretion in a GPR119-dependent manner [12]. Another well-studied NAE is *N*-palmitoylethanolamine (PEA) which has anti-inflammatory, analgesic and neuroprotective effects generally mediated by PPARα [13]. Owing to its interesting properties and its low toxicity, PEA has been the subject of several clinical trials studying its anti-inflammatory effects [14,15].

Besides these well characterized NAEs, NAEs derived from omega-3 fatty acids such as *N*-docosahexaenoylethanolamine (DHEA) and *N*-eicosapentaenoylethanolamine (EPEA) were recently shown to reduce inflammatory cytokine expression in adipocytes [16], to induce autophagy in breast cancer cells in culture in a PPAR-dependent manner [17] and to have antiproliferative effects in prostate cancer cell lines [18]. These NAEs were also reported to bind to the cannabinoid receptors [18]. Moreover, DHEA reduces macrophage activation and exhibits anti-inflammatory effects *in vivo* [19–21]. The orphan receptor GPR110 was also proposed as a receptor for DHEA, mediating its effects on brain development and function [22]. Other NAEs, although found in tissues, are less well

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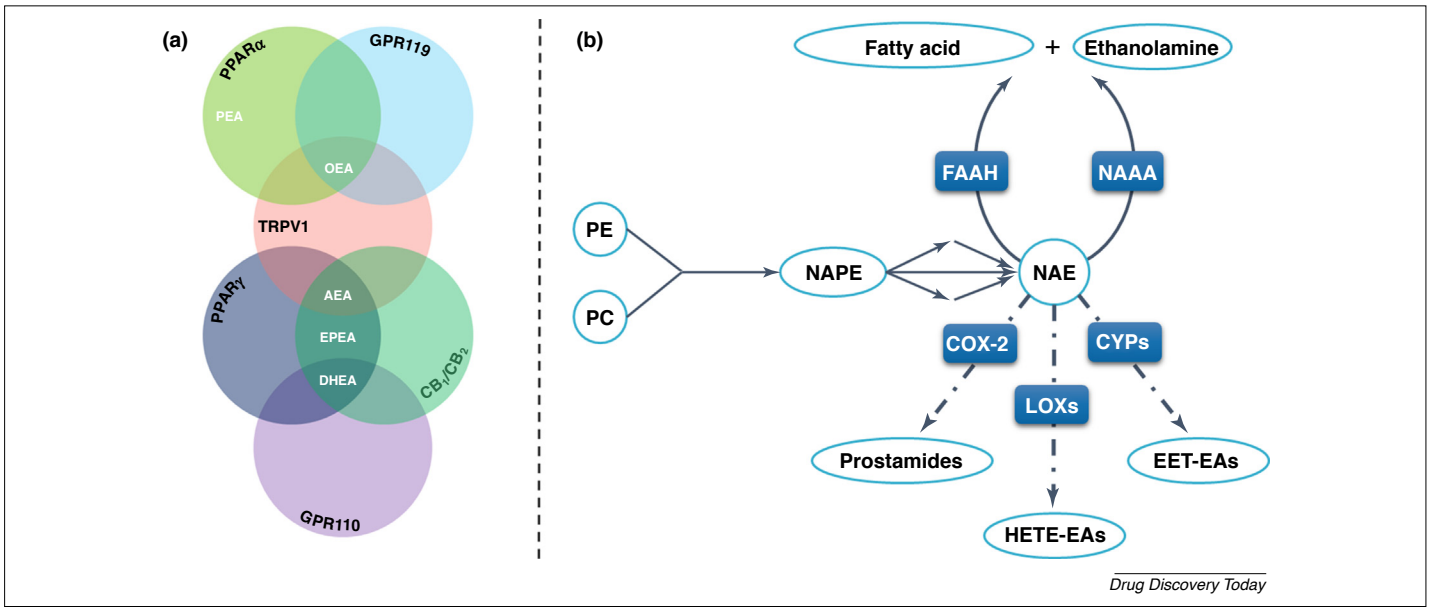

FIGURE 1

Simplified view of (a) receptors activated by *N*-acylethanolamines (NAEs) and (b) NAE metabolism. (a) Representation of the molecular targets for the main NAEs studied to date. As illustrated, *N*-oleoyethanolamine (OEA) binds to peroxisome proliferator-activated receptor (PPAR)α, the G-protein-coupled receptor GPR119 and to the ion channel transient receptor potential cation channel subfamily V (TRPV)1. *N*-palmitoylethanolamine (PEA) is mostly a PPARα ligand. *N*-arachidonoylethanolamine (AEA), *N*-eicosapentaenoylethanolamine (EPEA) and *N*-docosahexaenoylethanolamine (DHEA) are endocannabinoids (i.e., they activate the CB_1 and CB_2 cannabinoid receptors) that also bind to PPARγ. (b) NAEs are synthetized following the actions of an *N*-acyltransferase that leads to the production of *N*-acylphosphatidylethanolamines (NAPEs) from membrane glycerophospholipids. The acyl donor is most often a phosphatidylcholine (PC) although 1-acyl-lyso-PC, cardiolipin and phosphatidylethanolamine (PE) are also possible donors. The acceptor is an ethanolamine phospholipid (PE, alkylacyl-PE and plasmenylethanolamine). NAPEs are then metabolized into NAEs via several possible pathways not described here [28]. NAEs are hydrolyzed by two enzymes: fatty acid amide hydrolase (FAAH) and *N*-acylethanolamine-hydrolyzing acid amidase (NAAA), into fatty acids and ethanolamine. NAEs with a long-chain unsaturated fatty acid such as arachidonic acid or docosahexaenoic acid can also be metabolized by the same enzymes that metabolize these fatty acids, leading to the formation of diverse bioactive lipids. Metabolism of these NAEs by cyclooxygenase (COX)-2 will lead to ethanolamide derivatives of the classical prostaglandins, known as prostamides. Metabolism of NAEs by lipoxygenases (LOXs) or cytochrome P450 enzymes (CYPs) will lead to the formation of ethanolamides of hydroxyeicosatetraenoic acids (HETE-EAs) and epoxyeicosatrienoic acids (EET-EAs), respectively.

studied. For instance, *N*-stearoylethanolamine (SEA) is anorexigenic and known to exert proapoptotic effects through as yet uncharacterized receptors [23,24] and *N*-docosahexatetraenoylethanolamine (DTEA) was recently shown to reduce macrophage activation [21].

Metabolism

Unlike many other mediators, NAEs are not stored in the cells but rather synthetized in a stimuli-dependent manner. Their levels are tightly regulated by their biosynthetic and degradation enzymes. Accordingly, these enzymes are considered as potential drug targets and their inhibitors could unravel the pathophysiological roles of NAEs and enable the pharmacological modulation of NAE levels to take advantage of their effects.

NAEs are synthetized from membrane phospholipids through the sequential actions of several enzymes (Fig. 1b). This canonical pathway involves two steps with the first being catalyzed by a calcium-dependent *N*-acyltransferase (NAT), recently annotated as PLA2G4E [25,26], that produces *N*-acylphosphatidylethanolamines (NAPEs) from membrane glycerophospholipids. A family of calcium-independent NATs was also shown to contribute to NAPE formation in mammalian cells [27]. The second step involves a NAPE phospholipase D that generates NAEs from NAPEs. Several alternative pathways involving different enzymes have also been put forth, highlighting the intricate biosynthesis of NAEs. For a recent and comprehensive review on NAE biosynthesis see Ref. [28].

Contrary to the large number of enzymes involved in their production, only two enzymes have been consistently involved in the hydrolysis of NAEs so far: fatty acid amide hydrolase (FAAH) and *N*-acylethanolamine-hydrolyzing acid amidase (NAAA). FAAH-2, an amidase that shares 20% sequence identity with FAAH, was cloned in 2006 and shown to hydrolyze NAEs although with different preferences to FAAH [29]. FAAH-2 expression was found in humans and several primates but not in mouse and rat; therefore, although the described FAAH inhibitors also inhibit FAAH-2, much less is known about its implication in NAE metabolism. FAAH is a membrane-bound serine hydrolase localized to the endoplasmic reticulum that was first characterized in the 1990s with extensive work being done since then on its function and inhibition [30]. NAAA by contrast is a lysosomal NAE-hydrolyzing enzyme that was characterized largely by Ueda *et al.* [31,32]. NAAA is highly expressed in macrophages, notably alveolar macrophages, and peripheral tissues [33].

In addition to hydrolysis, AEA and other NAEs with long-chain polyunsaturated fatty acid chains such as DHEA can be metabolized by several enzymes involved in the metabolism of arachidonic acid and polyunsaturated fatty acids, such as cyclooxygenase-2, lipoxygenases and cytochrome P450 enzymes owing to the presence of their polyunsaturated side chain [34].

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Although they do not seem to significantly alter NAE levels, these pathways lead to the synthesis of other bioactive lipids that exhibit unique biological activities.

Overview of NAAA properties

Contrary to the other hydrolytic enzymes of the endocannabinoid system, NAAA is not a serine hydrolase but an N-terminal nucleophile (Ntn)-hydrolase, more specifically an N-terminal cysteine hydrolase of the choloylglycine hydrolase family. It has no sequence homology with FAAH, but rather with acid ceramidase (AC), which explains why most inhibitors are also tested against AC. The human and murine enzyme are similar with 76% sequence identity [32]. Several splice variants of NAAA are expressed in human cells. Among these, two code for catalytically inactive proteins and can represent up to 20% of the expressed NAAA in certain cell lines (e.g., the human prostate cancer cell line VCAP) [35].

In line with its presence in lysosomes, NAAA is highly active at acidic pH and only marginally around pH 7. NAAA is produced as an inactive pro-enzyme that is autocatalytically activated by cleavage at acidic pH before the catalytic cysteine residue (Cys-126 in human NAAA). This cleavage leads to a heterodimer where the α-subunit is formed by the chain on the N-terminal side preceding Cys-126 and the β-subunit by the chain on the C-terminal side starting with Cys-126 that serves as the catalytic nucleophile of the enzyme [36–38]. Absence of the Cys-126 residue prevents the formation of the active enzyme. The two other catalytic residues: Arg-142 and Asp-145, are also located in the β-subunit [37]. Additional important residues for the activity have been identified as Asn-287, and more recently Leu-325 and Thr-335 [35]. Point mutation of Thr-335 results in an inactive enzyme probably owing to the absence of conversion of the precursor enzyme into active NAAA [35]. Modeling studies, based on the crystal structure of choloylglycine hydrolase, suggested that Asn-204 and Asn-287 form the oxyanion hole [39].

In early studies, glycosylation of the enzyme seemed necessary to reach its full activity, with four glycosylation sites identified in human NAAA [32]. However, a recent study found that glycosylation was most probably necessary for trafficking of the enzyme to the lysosomes and its maturation. Indeed, deglycosylation of the purified mature enzyme had minimal effects on enzyme activity [40]. Interestingly, NAAA activity is enhanced by the presence of nonionic detergents and dithiothreitol (DTT) [32]. It was also shown that several endogenous lipids, such as phosphatidylcholines and phosphatidylethanolamines, were able to increase NAAA activity to a similar extent as the classically used nonionic detergents [41,42]. In terms of substrates, when assessed *in vitro*, NAAA has a strong preference for saturated NAEs, with PEA being the preferred substrate, although AEA and OEA are also substrates of the enzyme [32]. Importantly, exogenously added radiolabeled substrate was hydrolyzed by NAAA in intact cells, supporting the notion that NAEs can reach the lysosomal compartment to be hydrolyzed [21,32]. However, because FAAH and NAAA inhibitors do not have the same effects on NAE levels (see below), the question remains whether these enzymes have access to different pools of NAEs or whether this difference occurs as a result of the pathologies or models considered. Based on the biochemical properties of NAAA summarized here, several assays described in Box 1 have been developed to assess its activity.

NAAA inhibitors

The early work on NAAA inhibitors by Lambert's and Ueda's groups was based on the development of substrate analogs. Although the activity of *N*-cyclohexanecarbonylpentadecylamine (CCP) is now questioned [39,43], these early inhibitors were useful in the initial characterization of NAAA activity in macrophages [44]. Further improvements of the substrate-based inhibitors led to compounds such as 1-(2-biphenyl-4-yl)ethyl-carbonyl pyrrolidine (pyrrolidine derivative 16, see Table 1 and Fig. 2) with a low micromolar potency against NAAA [45]. Makriyannis's research group developed a series of isothiocyanate-based NAAA inhibitors. Despite their electrophilic isothiocyanate moiety, AM9023 and AM9053 were shown to interact reversibly with the enzyme [46,47]. Interestingly, AM9053 is active following systemic administration *in vivo* [48].

Other series of potent NAAA inhibitors were developed by Piomelli and co-workers around β-lactone and β-lactam moieties. The first compound characterized was the β-lactone (*S*)-OOPP that interacts in a noncompetitive manner with NAAA [39]. SAR studies have shown that the β-lactone motif is key to the interaction of this series with NAAA, whereas the lipophilic side chain can be modulated to improve NAAA inhibition [39,49]. Modeling studies on the enzyme and (*S*)-OOPP analogs suggest that, in addition to lipophilicity, specific molecular recognition in the lipophilic pocket is important for explaining the compound activity [50]. Although essential in the characterization of NAAA as an anti-inflammatory target, further development of (*S*)-OOPP is hampered by poor plasma stability [39]. To improve the chemical stability of the β-lactone, several avenues have been explored. The first consists of introducing a methyl group in position 2 on the β-lactone ring resulting in 4–5-times improved chemical stability [51]. Noteworthy, bulkier substituents were also assessed with positive effects on the stability but detrimental effects on the activity [52]. The other strategy was the replacement of the amide link of (*S*)-OOPP by a carbamate moiety also resulting in a 4-times improved chemical stability. This optimization led to the development of ARN077 which, in addition to improved potency, has an improved chemical, but not plasma, stability [51,53]. Within this series, the modulation of the lipophilic side-chain also led to further improved potency (e.g., the *p*-biphenylmethyl analog has an IC_{50} of 7 nM) [53]. These 2-methyl-4-oxo-3-oxetanylcarbamate derivatives were shown to covalently react with the N-terminal cysteine residue of the enzyme leading to a partially reversible inhibition [38,53]. In the absence of a crystal structure for NAAA, 3D QSAR studies were also performed with this series of inhibitors, further confirming the crucial roles of size and shape of the lipophilic side chain. The obtained 3D QSAR model (correlation of 0.93 between the predicted and the actual pIC_{50} values) was validated by synthesizing and testing novel compounds with a good predicted activity [54]. Scaffold-hopping from a β-lactone to a β-lactam resulted in the synthesis of ARN726 which shows excellent potency and improved plasma stability ($t_{1/2} \approx 40$ min using mouse plasma *in vitro*) compared with the β-lactone inhibitors [55–57]. Despite a short half-life ($t_{1/2} \approx 15$ min after i.v. administration to mice), ARN726 was shown to effectively inhibit

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BOX 1

Assays for assessing NAAA activity

Several *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) activity assays were developed; most based on *N*-acylethanolamine (NAE) hydrolysis into ethanolamine and a fatty acid. Additionally, NAAA requires stringent conditions (e.g., acidic pH, presence of sulfhydryl-reducing DTT and of nonionic detergents) for optimal activity [42].

NAE hydrolysis assays use a NAE [most often *N*-palmitoylethanolamine (PEA)] incubated with a preparation containing NAAA and then measure the products (i.e., either the increase in fatty acid or ethanolamine, or the decrease in NAE).

The first described assay used PEA labeled on the first carbon of palmitic acid: [1′-^{14}C]-PEA (Figure Ia). After allowing the substrate and enzyme to react, a liquid–liquid extraction is performed and the organic phase, containing the [^{14}C]-palmitic acid and the non-hydrolyzed [1′-^{14}C]-PEA, is spotted on a silica gel thin layer plate. Following chromatography to separate the two, radioactivity is measured either directly on the plate or following scraping of the silica and mixing with scintillation liquid [42]. Alternatively, the ethanolamine moiety carries the radioactivity (Figure Ib). In this case [1,2-^{3}H]-PEA, with tritium on the ethanolamine, is most often used although [1,2-^{14}C]-PEA is also used [21,65,72]. After incubation of the radiolabeled PEA with the enzyme preparation followed by a liquid–liquid extraction, the aqueous phase containing the radiolabeled ethanolamine is recovered and counted using a liquid scintillation counter.

LC–MS analyses are also used to assay NAAA activity. The enzyme preparation is incubated with known amounts of PEA and then, following lipid extraction, the amount of PEA left in the reaction mixture is quantified by HPLC-MS [73]. A mimetic substrate, such as heptadecanoylethanolamide (Figure Ic), is sometimes used instead of PEA and the production of heptadecanoic acid monitored by LC–MS [39,56,61,64,66]. Another method uses *N*-(4-methyl-2-oxo-chromen-7-yl)-hexadecanamide (PAMCA, Figure Id) as a substrate. Hydrolysis of PAMCA leads to the release of palmitic acid and the fluorescent compound 7-amino-4-methylcoumarin [46,58,73].

With these methods, it is important to have cell or tissue protein preparations that do not contain fatty acid amide hydrolase (FAAH) because the hydrolysis assay in itself will not distinguish between the two enzymes. In this context, working at acidic pH will favor NAAA activity and abrogate FAAH activity. An additional control using a FAAH inhibitor to determine the FAAH activity of the preparation is also a safe precaution. These issues are less relevant when working with purified preparations of NAAA.

Recently, an assay based on the activity-based protein profiling (ABPP) assay described for serine hydrolases [74] was described for NAAA [75,76]. The first described probe, ARN14686 (Figure Ie), was based on the NAAA inhibitor ARN726, and requires addition of the reporting group to the probe via click chemistry after the probe reacted with the proteome [76]. Additional probes (for example Figure If) that do not require this two-step labeling procedure were developed [77]. Among the advantages of ABPP is the possibility to test potency and selectivity of an enzyme within a single experiment and to detect the enzyme in its active form *ex vivo* after administration of the probe to animals. It also circumvents the potential interference of FAAH because the chemical probes used for NAAA will not react with FAAH.

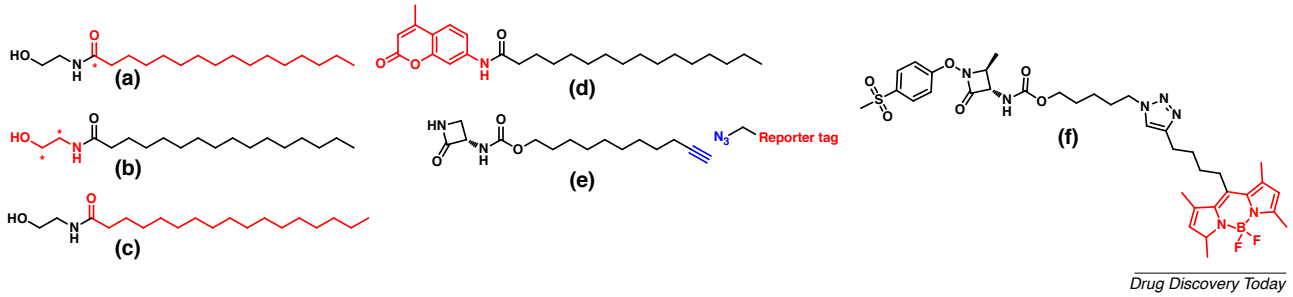

FIGURE I

Substrates (a-d) and probes (e-f) used in NAAA activity assays.

NAAA in several tissues such as spleen and lung after i.p. administration (between 3 and 30 mg/kg) and even after oral administration (30 mg/kg) [56]. Part of the explanation can be found in the covalent bond formed by ARN726 with the N-terminal cysteine residue as shown by Ribeiro *et al.* [56]. It is of interest to note that introduction of fluorine atoms on the cyclohexyl moiety improved the microsomal stability of the molecule. The most recent structural modification of this class of inhibitors consisted of the introduction of an *N*-substituent on the β-lactam ring of ARN726 [58]. The *N*-*O*-aryl-substituted derivative 37 [58] (see Table 1 and Fig. 2) is highly active against NAAA but appears less stable than ARN726 ($t_{1/2} < 5$ min using mouse plasma *in vitro*). However, its topical administration in the rat paw was able to reduce carrageenan-induced edema and hyperalgesia [58].

The oxazolidone is another motif that progressively appears suitable for NAAA inhibition. Starting from poorly active pyrrolidine derivatives [e.g., 1-pentadecanyl-carbonyl pyrrolidine (IC_{50} = 2 μM)] [45], subsequent SAR studies led to the identification of F96 with a 10–100-times improved potency against NAAA and bioactivity *in vivo* after i.p. and oral administration [59,60]. Introduction of a chlorine in position 2 on the phenyl ring led to F215 which shows nanomolar potency against NAAA [61]. The results of biochemical studies suggest a noncompetitive inhibition via reversible covalent binding to the enzyme [61].

A potentially important step forward in the field is the recent characterization of a series of NAAA inhibitors based on a benzothiazole-piperazine scaffold that are devoid of a warhead (such as a β-lactone or β-lactam). Indeed, such inhibitors are expected to be more easily optimized in terms of stability and selectivity. The prototypical compound of this series noncovalently inhibits NAAA with an IC_{50} value of 230 nM [62]. Interestingly, it has one of the highest reported stabilities in plasma ($t_{1/2} > 120$ min

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TABLE 1

Characteristics of representative NAAA inhibitors[a]

Inhibitor[b]	*In vitro*			In vivo			
	NAAA inhibition	**FAAH inhibition**	**Effect on NAE levels**	**Model**	**Dose**	**Effect on NAE levels**	**Refs**
Cyclopentyl hexadecanoate	IC_{50} = 10 μM (hNAAA) [78]	FAAH: no significant effect (at 50 μM) [78]	Increases PEA levels in NAAA-expressing HEK cells [78]				
Pyrrolidine derivative 16	IC_{50} = 2 μM (rNAAA) [45]	FAAH: IC_{50} >100 μM [45]	Increases PEA levels in RAW264.7 cells [45]				
AM9023	IC_{50} = 0.600 μM (hNAAA) [46]	FAAH: IC_{50} >10 μM [46]					
AM9053	K_i = 0.030 μM (hNAAA) [21,47]	FAAH: IC_{50} ≈ 100 μM [47]	Increases NAE levels in J774 cells [21]	DSS-induced colitis	10 mg/kg, b.i.d. (i.p.)	Increases PEA levels in the colon	[48]
(*S*)-OOPP	IC_{50} = 0.420 μM (rNAAA) [39]	FAAH: IC_{50} >100 μM [39]	Increases PEA levels in RAW264.7 cells and in NAAA-expressing HEK cells (at 10 μM) [39]	Carrageenan-instilled sponges to recruit leukocytes	25 μg/implanted sponge	Increases PEA levels in carrageenan-recruited leukocytes	[39]
				Spinal cord injury in mice	30 μg/mice (intrathecally)	Not reported	[39]
ARN077	IC_{50} = 0.01–0.130 μM (rNAAA) [51,53,64] IC_{50} = 0.007 μM (hNAAA) [53]	FAAH: no significant effect (at 10 μM) [53]		Chronic constriction injury	20 μl of a 10% suspension topically administered on the paw skin	Increases PEA levels in the sciatic nerve	[64]
				DNFB-induced dermatitis	3% topically administered on the ear skin	Increases PEA levels in the DNFB-treated ear	[63]
ARN726	IC_{50} = 0.063 μM (rNAAA) [56] IC_{50} = 0.027–0.073 μM (hNAAA) [56,57]	FAAH: IC_{50} >100 μM [56]		Carrageenan-induced lung inflammation	30 mg/kg (*per os*)	Increased PEA and OEA levels	[56]
				CFA-injected paw	30 mg/kg (i.p.)	Increased PEA and OEA levels	[66]
N-*O*-aryl-substituted derivative 37	IC_{50} = 0.006 μM (hNAAA) [58]	FAAH: IC_{50} >50 μM [58]		Carrageenan-induced paw inflammation	5–50 μg/paw (intraplantar)	Not reported	[58]
F96	IC_{50} = 0.270 μM (rNAAA) [59,60]	FAAH: IC_{50} = 41 μM [59]		TPA-induced ear edema	10 mg/kg (i.p.)	Increased PEA and OEA levels in TPA-treated ears	[59]
F215	IC_{50} = 0.009 μM (rNAAA) [61]	ND					

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TABLE 1 (*Continued*)

Inhibitor[b]	*In vitro*			*In vivo*			
	NAAA inhibition	FAAH inhibition	Effect on NAE levels	Model	Dose	Effect on NAE levels	Refs
EPT4900 (diacerein)	IC_{50} = 0.700 μM (hNAAA) [65]	FAAH: IC_{50} >50 μM [65]	Increases PEA levels in NAAA-expressing HEK cells (at 10 μM) [65]	Carrageenan-induced paw inflammation	10 mg/kg to 25 mg/kg (i.p.)	Increased PEA levels (at 25 mg/kg)	[65]
Benzothiazole-piperazine derivative 8	IC_{50} = 0.230 μM (hNAAA) [62]	FAAH: IC_{50} ≈ 10 μM [62]		Naive mice	30 mg/kg (per os)	Increased PEA and OEA levels	[62]

[a] The table reports the *in vitro* properties of the inhibitors against *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) activity as well as fatty acid amide hydrolase (FAAH) activity. When available, the effect on *N*-acylethanolamine (NAE) levels in *in vitro* cell systems is also shown. The right part of the table reports the *in vivo* model and the dose tested for which an effect on NAE levels was described. If the compounds were tested *in vivo* but the effect on NAE levels are not described — we discuss them in the manuscript but not in this table. Abbreviations: CFA, complete Freund's adjuvant; DNFB, 2,4-dinitrofluorobenzene; DSS, dextran sulfate sodium; OEA, *N*-oleoylethanolamine; PEA, *N*-palmitoylethanolamine.

[b] Chemical names are: AM9023, 1-isothiocyanatopentadecane; AM9053, (10-isothiocyanatodecyl)benzene; ARN077, 5-phenylpentyl *N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl] carbamate; ARN726, 4-cyclohexylbutyl-*N*-[(*S*)-2-oxoazetidin3-yl]-carbamate; benzothiazole-piperazine derivative 8, (2-ethylsulfonylphenyl)-[(2*S*)-4-(6-fluoro-1,3-benzothiazol-2-yl)-2-methyl-piperazin-1-yl]methanone; EPT4900, 4,5-diacetyloxy-9,10-dioxo-anthracene-2-carboxylic acid; F96, 3-(6-phenylhexanoyl)oxazolidin-2-one; F215, 3-(6-(3-chlorophenyl)hexanoyl)oxazolidin-2-one; *N*-*O*-aryl-substituted derivative 37, 4-cyclohexylbutyl-((2*S*,3*S*)-2-methyl-1-(4-(methylsulfonyl)phenoxy)-4-oxoazetidin-3-yl)carbamate; pyrrolidine derivative 16, 1-(2-biphenyl-4-yl)ethyl-carbonyl pyrrolidine; (S)-OOPP, *N*-[(3*S*)-2-oxo-3-oxetanyl]-3-phenylpropanamide.

using mouse plasma *in vitro*) and against mouse liver microsomes ($t_{1/2}$ > 60 min). *In vivo*, after i.v. administration to mice, the reported half-life is significantly improved over that of ARN726 ($t_{1/2}$ ≈ 70 min vs 15 min) [62]. Importantly, this compound is the first for which increased PEA levels have been found in the brain following oral administration [62].

Despite major advances in the development of NAAA inhibitors, medicinal chemistry efforts have been hampered by the lack of NMR or crystal structures of NAAA. The recent production, purification and deglycosylation of recombinant human NAAA [40] will hopefully enable crystallographic characterization and NMR ligand-binding studies. These new inhibitors that can raise NAE levels following systemic administration are precious tools to study the role of NAAA *in vivo* as discussed in the following section.

NAAA inhibition and its potential therapeutic implications

Owing to the many effects of NAEs, inhibitors of their hydrolysis have been proposed as therapeutic tools in several pathophysiological conditions. Because NAAA is highly expressed in macrophages and is active at acidic pH, it was first proposed as an interesting target in inflammatory processes. Moreover, because NAAA is believed to primarily control PEA levels [32,41], its inhibition would make use of the anti-inflammatory, analgesic and neuroprotective effects of PEA [13]. Accordingly, NAAA inhibitors have been studied in the context of inflammation and immune disorders, as well as pain. Nevertheless, NAAA inhibition also increases other NAE levels, typically OEA and, depending on the model and tissue, AEA, and therefore this has to be considered when interpreting the effect of NAAA inhibitors.

Inflammation

Owing to the role of macrophages during inflammation and the expression of NAAA by those cells, NAAA inhibitors are often tested on macrophages *in vitro*. Several NAAA inhibitors such as (*S*)-OOPP, 1-(2-biphenyl-4-yl)ethyl-carbonyl-pyrrolidine and AM9053 have been shown to increase PEA levels in murine macrophage cell lines activated with lipopolysaccharides (LPS) and to decrease expression of proinflammatory markers [21,39,45]. ARN726 decreased LPS-induced activation of monocyte-derived human macrophages [56]. However, (*S*)-OOPP had no effect on AEA levels in LPS-activated RAW264.7 cells [39], whereas AM9053 also increased the levels of the other NAEs measured, namely AEA, OEA, SEA and DHEA, in J774 cells [21].

NAAA inhibitors have also been tested *in vivo* in a variety of disease models with an inflammatory component. For instance, (*S*)-OOPP reduces tissue injury as well as inflammation and apoptosis markers in a model of spinal cord injury in mice [39]. ARN077 reduces inflammation in 2,4-dinitrofluorobenzene (DNFB)-induced allergic dermatitis. In this case, NAAA deletion protected mice against DNFB-induced dermatitis [63]. As mentioned, one drawback of these inhibitors was their poor stability *in vivo*, therefore they were mostly used in topical applications, which complicated the study of NAAA inhibition in other models. The subsequent development of more-stable inhibitors enables a more thorough study of the implication of NAAA in inflammation. For instance, AM9053 administered intraperitoneally to mice increased PEA levels in the colon and extensively decreased colon

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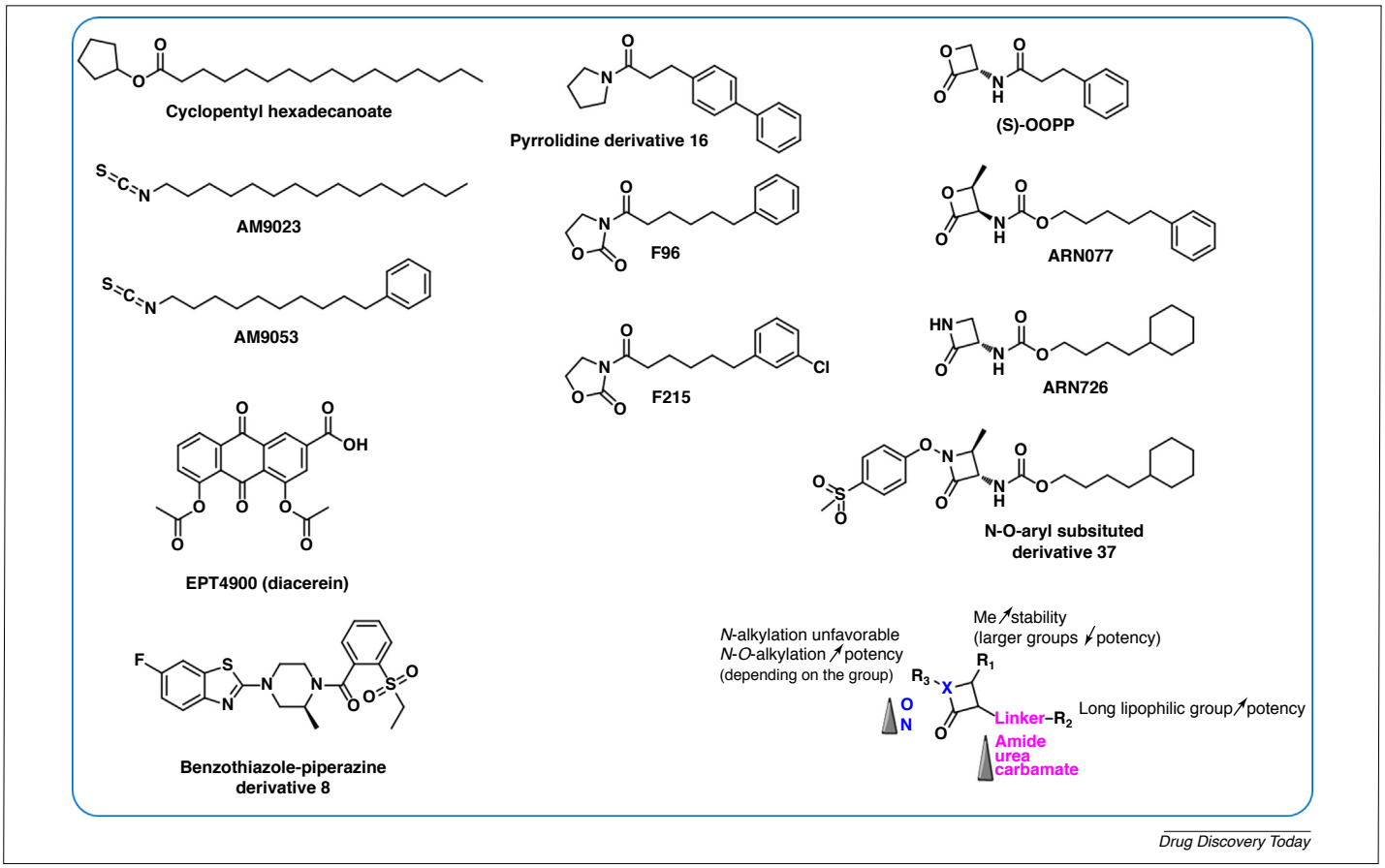

FIGURE 2

Representative *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) inhibitors. The structure of representative NAAA inhibitors is presented. Their pharmacological properties are described in Table 1. A summary of the SAR described for the β-lactone and β-lactam inhibitors is also presented. The carbamate moiety as the linker favors the potency and stability of the compounds.

inflammation in the trinitrobenzene sulfonic acid (TNBS)-induced model of inflammatory bowel diseases [48]. Oral administration of ARN726 decreased carrageenan-induced and LPS-induced lung inflammation in mice [56]. Interestingly, in several of these studies, the effects of the inhibitors were not present in PPARα$^{-/-}$ mice, suggesting that the effects of NAAA inhibition were mediated by a NAE acting on PPARα, probably PEA [39,56,63].

NAAA inhibitors were also assessed in a murine model of multiple sclerosis, a neurodegenerative disease with an inflammatory component. Indeed, compound 8, a benzothiazole-piperazine derivative [62] developed by Piomelli and co-workers (see Table 1 and Fig. 2), increased PEA and OEA levels in mouse brain, and delayed disease onset and reduced symptoms in experimental autoimmune encephalomyelitis [62]. Interestingly, in the same model, ARN726 had no effect on the clinical signs of the disease [62]. The superiority of the benzothiazole-piperazine inhibitor is not the result of a higher potency against NAAA (Table 1) but, more likely, because of improved pharmacokinetic properties.

Pain

Because PEA is known to have analgesic properties, NAAA inhibitors were assessed in several models of inflammatory or neuropathic pain. ARN077 applied topically reduced hyperalgesia in the carrageenan-induced model of inflammatory pain as well as mechanical allodynia in the chronic nerve ligation model of neuropathic pain in mice [64]. F96 given intraperitoneally decreased hyperalgesia in the acetic acid model of inflammatory pain and nerve-injury-induced mechanical allodynia [59]. In both cases, the effects were absent in PPARα$^{-/-}$ mice pointing again to an effect mediated by PPARα-activating NAEs (Fig. 1a) [59,64]. Other inhibitors were also tested in the same settings. Intraperitoneal administration of diacerein increased PEA levels in the rat paw and decreased carrageenan-induced hyperalgesia [65], whereas ARN726 increased PEA and OEA levels and decreased paw edema and hyperalgesia induced by complete Freund's adjuvant (CFA) injection in the rat paw [66]. These elements clearly support the interest of further studying NAAA inhibition as a potential analgesic treatment. FAAH inhibitors are also highly active in preclinical models of pain [67], but whether NAAA inhibition has more potential than FAAH inhibition remains to be established.

Cancer

NAAA expression and activity were reported in human prostate cancer cell lines leading the authors to suggest it could potentially be a biomarker of prostate cancer [68]. More recently, NAAA and tyrosine kinase 7 were associated with aggressiveness of prostate cancer in a glycoproteomic analysis of human prostate cancer tissues, with NAAA expression being higher in nonaggressive

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tumors [69]. By contrast, NAAA was among the genes overexpressed in highly aggressive ovarian cancer in mice [70]. One study using new NAAA inhibitors consisting of amide derivatives of hexadecylamine (e.g., *N*-hexadecyl-2-hydroxybenzamide) found that these inhibitors decreased proliferation and migration of bladder cancer cell lines [71]. However, to date the physiological role of NAAA in this setting remains unknown. Further studies using the novel, more-potent and -selective inhibitors coupled to genetic silencing of the enzyme will help unravel the role, if any, of NAAA in cancer development.

Control of NAE levels following NAAA inhibition

Because NAAA has not one substrate but several substrates that activate different receptors, understanding how NAAA inhibition exerts its effects requires the quantification of its substrates in the model assessed. Indeed, NAAA inhibition in the same setting could have different effects on individual NAE levels [21,44]. However, limited amounts of data are available in this respect, because most studies with NAAA inhibitors only report PEA levels, and sometimes OEA, but not the levels of the other NAEs. This is because NAAA is classically considered as preferentially hydrolyzing PEA over other NAEs. Although this is true when the purified enzyme is considered, the situation could be more complex in cells or *in vivo*, as described above. We and others have shown that, in macrophages in culture, NAAA inhibition has more effect on AEA levels than PEA levels whereas the opposite is true for FAAH [21,44]. However, the situation is different *in vivo*. Indeed, when authors reported the levels of other NAEs in addition to PEA, NAAA inhibition affected PEA and sometimes OEA, but not AEA, levels in inflammatory settings *in vivo* [39,48,63,64]. In this case it is important to consider that NAE levels as well as the expression of their metabolic enzymes can be altered differently depending on the tissue or the pathology considered. Therefore, inhibitors of FAAH or NAAA could have different effects in a given pathology, as we have shown in colon inflammation where NAAA inhibition increases PEA but not AEA levels in the colon but FAAH inhibition has the opposite effect [48]. Moreover, the effect of one inhibitor can be different depending on the tissue considered. For instance, in the same study, we showed that FAAH and NAAA inhibition increased PEA and AEA levels in the liver despite not having that effect in the colon [48].

The effect of NAAA inhibitors on PEA and other NAE levels was often not the same in resting cells or in cells stimulated with LPS or ionomycin [21,39,45]. The same was observed in lungs from naive mice and mice exposed to the inflammatory compound carrageenan [56]. This suggests that post-translational modifications could be involved in NAAA activity or that the inflammatory trigger could affect the access of NAAA to the cellular NAE pools. Another possibility could be that the biosynthesis of NAEs is altered in these conditions. However, and surprisingly, there are very few reports of the effect of inflammation on NAAA expression and activity. One study reported an increase in NAAA expression and activity in the paws of rats injected with complete Freund's adjuvant [66]. By contrast, we saw a slight decrease in NAAA expression and activity in murine J774 macrophages activated with LPS [21], whereas another study found no alteration in LPS-activated murine RAW264.7 macrophages [39].

Concluding remarks

As discussed here, the discovery of potent inhibitors for NAAA is stepping up and should favor the study of NAAA in disease models. To date, the studies outlined here suggest that NAAA could be a potential therapeutic target for pain treatment or in inflammatory diseases such as inflammatory bowel diseases, chronic obstructive pulmonary disease and arthritis. The development of more-stable and -potent inhibitors is excellent news that will contribute to further demonstrating the druggability of NAAA. Nevertheless, this does not mean the end of the game for potential drugs for the earlier short-lived inhibitors. Indeed, NAAA being an attractive target in inflammation, conditions characterized by localized inflammation might benefit from the use of potent NAAA inhibitors where poor systemic stability would prevent them from leaving the site of administration.

Several intriguing questions remain open. Because NAEs are substrates for FAAH and NAAA, but the regulation, substrate preference and localization of the enzymes differ, studying NAAA and FAAH inhibitors in parallel in the same setting should bring interesting information from a fundamental and translational point of view. Another open question is the extent to which NAAA should be inhibited to induce an effect. This is even more relevant because we now have access to systemically active, reversible NAAA inhibitors. Furthermore, the fact that FAAH and NAAA have distinct subcellular localization and have access to potentially different substrate pools suggests that the concomitant inhibition of FAAH and NAAA could lead to a further increase of NAE levels and potentially stronger effects. This hypothesis, of potential interest from a drug discovery perspective, remains to be tested.

Conflicts of interest

The authors have no conflicts of interest to declare.

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