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Therapeutic potential of fatty acid amide hydrolase, monoacylglycerol lipase, and *N*-acylethanolamine acid amidase inhibitors

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Abstract: Fatty acid ethanolamides (FAEs) and endocannabinoids (ECs) have been shown to alleviate pain and inflammation, regulate motility and appetite, and produce anti-cancer, anxiolytic, and neuroprotective efficacies *via* cannabinoid receptor type 1 (CB_1) or type 2 (CB_2), or *via* peroxisome proliferator-activated receptor α (PPAR-α) stimulation. FAEs and ECs are synthesized by a series of endogenous enzymes, including *N*-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD), diacylglycerol lipase (DAGL), or phospholipase C (PLC), and their metabolism is mediated by several metabolic enzymes, including fatty acid amide hydrolase (FAAH), monoacylglycerol lipase (MAGL), *N*-acylethanolamine acid amidase (NAAA), or cyclooxygenase-2 (COX-2). Over the last decades, increasing the concentration of FAEs and ECs through the inhibition of degrading enzymes has been considered to be a viable therapeutic approach to enhance their anti-nociceptive and anti-inflammatory effects, as well as protecting the nervous system.

Introduction

Fatty acid ethanolamides (FAEs) and endocannabinoids (ECs) are biosynthetic ligands, which are widely distributed in the central nervous system (CNS), the immune system, and the peripheral tissues of mammals (e.g. human, monkey, dog, mouse and rat).[1]

Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are two of the most studied members of the endocannabinoid family. Indeed, there is a basal amount of ECs in tissues. The balance and circulation of ECs in different tissues are regulated by related synthases, degrading enzymes, and transporters. The rapid accumulation of ECs is observed in tissues during injury.[1-3] AEA was identified as an agonist of CB_1 receptors by Devane in 1992.[4] Furthermore, 2-AG was demonstrated to be another biosynthetic agonist of CB_1 receptors in 1995.[5] Although AEA levels are much lower than 2-AG levels in the brain, AEA has been determined to show greater *in vitro* affinity for CB_1 receptors (K_i = 50-100 nM) than 2-AG (K_i = 1-10 μM) in a number of binding assays.[6] Nevertheless, AEA showed only partial agonist effects, whereas 2-AG exerted full efficacy in a [^{35}S]GTPγS assay. Additionally, 2-AG has been repored to bind to CB_2, another cannabinoid receptor subtype. Although AEA was also identified as a CB_2 ligand, it exerts lower affinity for CB_2 than CB_1.[6] Aside from CB_1 and CB_2 receptors, AEA and 2-AG also serve as ligands for non-cannabinoid targets, including the transient receptor potential cation channel subfamily V member 1 (TRPV1).[1]

Palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) belong to a large family of FAEs, a class of lipid mediators which consist of saturated FAEs, monounsaturated FAEs, and polyunsaturated FAEs. The formation of FAEs is tissue-dependent during the course of pro-inflammatory stimuli or tissue damage. For instance, inflammation suppresses the production of PEA and OEA in macrophages, whereas the elevation of AEA levels is detected in the same cells.[1] In addition, the formation of PEA and OEA appears to be less stimulus-dependent in comparison with AEA in dorsal root ganglion neurons and skin cells.[1,7] PEA was identified as a peroxisome proliferator-activated receptor α (PPAR-α) agonist by Lo Verme and co-workers in 2004.[8] OEA was described as a PPAR-α agonist in 2003.[9] To date, there is sufficient evidence to demonstrate that PEA and OEA do not bind CB receptors with significant affinity.

Biosynthesis of FAEs and ECs

FAEs are derived from a membrane precursor, termed *N*-acylphosphatidylethanolamine (NAPE), which is formed from phosphatidylcholine and phosphatidylethanolamine by *N*-acyltransacylase (NAT). On the one hand, NAPE is transformed into FAEs and phosphatidic acid by NAPE-phospholipase D (NAPE-PLD) directly.[10-12] On the other hand, NAPE is hydrolyzed by phospholipase C (PLC) to generate phospho-FAE (P-FAE), which is further dephosphorylated to yield FAEs by phosphatase (e.g. protein tyrosine phosphatase, non-receptor type 22 (PTPN22) in lymphoid cells).[11-13] Alternatively, NAPE can also be deacylated by α/β-hydrolase 4 (ABHD4) to generate lyso-NAPE, which is followed by lyso-PLD-mediated removal of acylphosphatidyl moiety to give FAEs directly, or ABHD4-mediated deacylation to yield glycerophospho-FAE (GP-FAE). Afterwards, GP-FAE is catabolized into FAEs by a metal-dependent phosphodiesterase GDE1 (Figure 1).[11,12]

Conversely, 2-AG is obtained from a membrane phospholipid, termed phosphatidylinositol-4,5-bisphosphate (PIP_2). PIP_2 is hydrolyzed by the β-isoform PLC (PLC-β) to yield 1,2-diacylglycerol (DAG), which is further degraded into 2-AG through the action of diacylglycerol lipase (DAGL), initiated by calcium signaling.[10] Additionally, 2-AG can be also synthesized from phosphatidyl-inositol (PI) *via* phospholipase A1 (PLA1) and lyso-PLC. First, PI is degraded by PLA1 to produce a lysophospholipid, and then lyso-PLC converts this lysophospholipid into 2-AG (Figure 2).[10]

Biodegradation of FAEs and ECs

Fatty acid amide hydrolase (FAAH) was first identified as an enzyme which hydrolyzes AEA into arachidonic acid (AA) and ethanolamine.[14] Additionally, AEA and AA can be oxidized by cyclooxygenase-2 (COX-2) to generate proalgesic prostamides, such as prostaglandin H_2 ethanolamide (PGH_2-EA) and prostaglandin H_2 (PGH_2), respectively.[1] There is growing evidence that cytochrome P450 participates in the catabolism of AEA.[15] Although FAAH is confirmed as the primary hydrolytic enzyme

for hydrolysis of AEA, *N*-acylethanolamine acid amidase (NAAA) has been identified as another hydrolase for AEA (Figure 1).[16]

Monoacylglycerol lipase (MAGL) has been demonstrated to be the major hydrolase responsible for the transformation of 2-AG into AA and glycerol. COX-2-regulated oxidations also convert 2-AG into proalgesic lipids, such as prostaglandin H_2 glycerol (PGH_2-G).[1,15] Apart from MAGL and FAAH, the degradation of 2-AG is attributed to α/β-hydrolase 6 and 12 (ABHD6 and 12).[17] In addition, 2-AG levels can be affected by FAAH *in vitro* but not in intact cells.[17,18] Particularly, MAGL is responsible for approximately 85% of 2-AG hydrolysis in mouse brain, while ABHD6 and ABHD12 were shown to mediate around 9% and 4% of 2-AG degradation, respectively. Less than 2% of 2-AG metabolism is attributed to other ezymes (Figure 2).[17]

PEA and OEA are both substrates of FAAH and NAAA. Particularly, FAAH principally metabolizes PEA and OEA into palmitic acid and oleic acid, respectively, in neurons, while NAAA plays a crucial role in downregulating PEA levels in macrophages (Figure 1).[1] Furthermore, fatty acid amide hydrolase-2 (FAAH-2), an isozyme of FAAHidentified only in primates, not in rodents, exhibits superior capacity to hydrolyze OEA compared to PEA or AEA.[19]

FAEs and ECs-mediated effects

The damage of nerves or tissues results in the activation of macrophages by stimulation of toll-like receptor-4 (TLR4), the activation of mast cells, and the entry of calcium ions into neurons.[1,20] Next, pro-inflammatory cytokines (such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)) are produced by macrophages and mast cells. The release of neurotransmitters such as acetylcholine (ACh) is initiated by Ca^{2+} in neurons.[20] Additionally, activation of TLR4 is also accompanied by the production of AEA and platelet-activating factor (PAF) by macrophages. PAF further activates PAF receptors, which induces the formation and release of 2-AG. The synthesis of AEA and 2-AG by neurons is Ca^{2+} and/or neuroreceptor-dependent.[1,21,22] Newly formed AEA and 2-AG bind to CB_1/CB_2 receptors on macrophages

and mast cells and to the CB_1 receptor on neurons. Activation of these CB receptors is followed by the inhibition of pro-inflammatory cytokines produced by macrophages and mast cells, the production of anti-inflammatory cytokines (such as IL-4 and IL-10) by macrophages, the suppression of calcium signaling, and the inhibition of neurotransmitter release in neurons, thereby blocking action potential and excitation transmission (Figure 3a).[1,20]

Alternatively, TNF-α and IL-1β released from macrophages and mast cells decrease PEA levels through the modulation of NAPE-PLD-dependent FAEs synthesis[8] and activate the nuclear factor kappa B (NF-κB).[23] Then, NF-κB further upregulates the expression of COX-2 and pro-inflammatory cytokine (e.g. TNF-α, IL-1, IL-6, and IL-8).[23,24] PEA-mediated anti-inflammation is mainly caused by the activation of PPAR-α, associated with negative activation of NF-κB, and thereby inhibition of pro-inflammatory cytokines formation and COX-2 expression (Figure 3b).[23,24]

Although PEA and OEA are not endogenous ligands for cannabinoid receptors CB_1 and CB_2, it is argued that they enhance AEA-dependent activities through purported "entourage" effects.[25] In 1998, Calignano and colleagues discovered an interesting phenomenon that the PEA-mediated anti-nociceptive effect was suppressed by a CB_2 receptor antagonist **1** (SR144528, Figure 4) in an *in vivo* assay after a 0.1 mg/kg intravenous injection in mice.[26] Indeed, AEA and PEA have been identified as two effective substrates for FAAH.[14,27] As a competitive substrate for FAAH, PEA has been shown to attenuate the biodegradation of AEA. OEA has also been demonstrated to modulate the concentrations of AEA in a similar mechanism.[28]

FAAH

FAAH was identified and cloned by Cravatt and co-workers in 1996.[27] It is an integral membrane-bound enzyme, belonging to the serine hydrolase family.[10] Its first X-ray crystal structure was disclosed in 2002.[29] Its binding site comprises three functional channels: the membrane access channel (MAC), the acyl-chain binding pocket (ABP), and the cytosolic port (CP), which are relevant to transporting the

substrate to the catalytic site, providing the accommodation of the acyl chain during catalysis, and removing the leaving group after hydrolysis, respectively.[30] Notably, the substrate is surrounded by the catalytic triad Ser241-Ser217-Lys142[31] and the oxyanion hole residues (including Ser241, Gly240, Gly239, and Ile238)[30] during catalysis (Figure 5). Firstly, Lys142 deprotonates Ser217, which then deprotonates Ser241. The activated hydroxyl group of Ser241 further interacts with the electrophilic carbonyl group of the substrate, which is accompanied by the formation of an acylated enzyme adduct and the release of ethanolamine.[31] At the end of the hydrolysis, the acyl group of the substrate is released from the acyl-enzyme complex through the action of a water molecule (Figure 6).[30,31] The oxyanion hole has been demonstrated to stabilize the intermediate during the catalytic triad-mediated catalysis.[32] Recently, the residues Phe432 and Trp531 were identified to form a "gate" in the MAC. The rotation of Phe432 was demonstrated to result in an "open" or "closed" state of the MAC, which is accompanied by a size alteration of the MAC and the ABP (Figure 7).[33] The "open" state is proposed to favor the access of substrate into binding cavities. Afterwards, the "closed" state provides enlarged ABP, which allows the easy access of substrate from the MAC into the ABP.[34] The shift of the conformational equilibrium in favor of "open" or "closed" state is due to the hydrophobic interaction between Phe432 and substrate. Molecular dynamics simulations identified that unsaturated substrates (i.e. AEA and OEA) rather than saturated substrates (i.e. PEA) are more likely to trigger the rotation of Phe432, which might be attributed to the fact that the hydrophobic interaction with C=C is stronger than the interaction with C–C. This finding explains why unsaturated substrates are preferred for FAAH rather than saturated substrates (i.e. hydrolysis rates: AEA (4 C=C) > OEA (1 C=C) > PEA (0 C=C)).[34] Hence, inhibitors, which are designed based on this "gate", might be more selective over other serine hydrolases with a different "gate" composition. As another two important amino acid residues in the MAC, Asp403 and Arg486 might facilitate the access of substrate into binding cavities (Figure 5).[33]

FAAH is first responsible for the degradation of several FAEs (such as AEA, PEA and OEA). Thus, FAAH inhibitors can alter signaling of FAEs and thereby induce numerous biological responses (such as

pain relief, anti-inflammation, injury repair, and neuroprotection) by stimulation of CB_1/CB_2 receptors and/or PPAR-α.[1,8,21,22.]

NAAA

NAAA, an amidohydrolase of the *N*-terminal cysteine hydrolase family, was isolated from rat lung in 2001[35] and cloned from rat lung in 2005.[36] NAAA is highly expressed in macrophages and peripheral tissues.[37] NAAA-mediated hydrolysis is initiated by self-proteolysis (from 48 kDa form to 30 kDa active form) at pH ~4.5 in the extracellular medium.[38] Despite the absence of a crystal structure, Cys131 and Cys126 were identified as catalytic residues of NAAA in rat and human, respectively.[7,39] Exemplified by human NAAA, its catalytic site comprises three principal residues Cys126, Arg142 and Asp145.[37] In particular, as the reactive nucleophilic moiety of NAAA, the thiol group of Cys126 interacts with the electrophilic carbonyl group of the substrate to generate an acyl-enzyme adduct.[39,40] Mutation of any one of these three residues leads to a total loss of catalytic activity, which means that Cys126, Arg142 and Asp145 play a crucial role in the interaction with the substrate.[41] Unlike FAAH, which exerts optimal activity over a wide pH range from 8.5 to 10, NAAA is more pH-sensitive. NAAA is almost inactive at neutral, alkaline, or strong acidic pH and its catalytic activity occurs only at pH 4.5-5.[37,39,41]

Recently, NAAA inhibitors have been regarded as a novel, potentially-effective treatment for inflammation, pain, and immune disorders, since they enhance PPAR-α-mediated anti-inflammatory, anti-nociceptive and immunomodulatory capacities through the increase of related FAEs levels.[8,22]

MAGL

MAGL, a membrane-associated hydrolase, is another important member of serine hydrolase family. It was purified from rat adipose tissue and cloned from mouse adipocytes by Karlsson in 1997.[42] The first X-ray crystal structures of MAGL were independently reported by Bertrand et al and by Lambert and co-workers in 2009.[43,44] Ser122-Asp239-His269 has been identified as the catalytic triad of MAGL.[42] Especially, the nucleophilic hydroxyl group of Ser122 was identified to interact with the electrophilic

carbonyl group of the substrate.[44,45] The residues Leu174 and Leu176 form the entrance to the catalytic site, whereas Phe93 and Phe209 display "gate"-like functions (Figure 8).[34,43] Interestingly, three cysteines (Cys201, Cys208, Cys242), which are close to the catalytic triad, were hypothesized to stabilize MAGL conformation and additionally interact with some substrates (such as maleimides).[44,46,47] These cysteines were once considered as beneficial targets for the development of selective MAGL inhibitors over other serine hydrolases.[45] The functional site of MAGL comprises the ABP, the alcohol-binding channel and the glycerol exit channel, which serve to accommodate the acyl chain, the glycerol moiety, and the leaving group, respectively (Figure 8).[43] Especially, mild alkaline pH (pH 8.0) is preferred for MAGL-mediated hydrolysis.[45] Unsaturated monoacylglycerols (MAGs) are preferred substrates for MAGL in comparison with diacylglycerols (DAGs), triacylglycerols (TAGs) and saturated MAGs.[45,48] Moreover, MAGL exerts a tissue-dependent control of monoglyceride metabolism. MAGL inhibition brings about an increase of 2-AG levels in the brain, whereas the dramatic elevation of other MAGs (such as mono-oleoylglycerol) after MAGL blockade mostly occurs in peripheral tissues.[48]

Since MAGL was reported to be responsible for the degradation of 2-AG, inhibition of MAGL has been viewed as a new approach to produce analgesic, anti-inflammatory, and neuroprotective effects. [1,17,18]

Accordingly, over several decades, enhancing the levels of ECs and FAEs *via* the inhibition of relevant endogenous metabolic enzymes has been considered as an interesting approach to suppress inflammation, pain, and other disorders. In this context, several pathways to terminate the catabolism of endogenous lipids will be reviewed.

Enzyme inhibitors

According to their interactive mechanisms, enzyme inhibitors can be classified as reversible, slowly/partially reversible and irreversible inhibitors.[49] As illustrated in Figure 9, the inhibitor initially interacts with the enzyme to form a transition state complex EI. Afterwards, the EI either reverses to

release free enzyme E or undergoes a tightening interaction (e.g. covalent binding and/or conformational change of enzyme). Irreversible inhibitors induce a permanent tightening form EI^* (K_d = 0), whereas slowly/partially reversible inhibitors undergo a conversion from tightening form EI^* to transition state form EI (K_d > 0).[49,50] The EI complex can be reversed by a rapid dilution for reversible inhibitors, whereas reversal of a slowly/partially reversible inhibitor-enzyme complex EI^* requires specific treatments (e.g. dialysis).[49]

FAAH inhibitors

The first-generation FAAH inhibitors were designed with reactive groups able to form a covalent bond with the catalytic residue Ser241.[51] For example, the widely known serine hydrolase inhibitor, phenylmethylsulfonylfluoride **2** (PMSF) (Figure 10) was demonstrated to completely block FAAH-mediated activities and increase concentrations of AEA *in vitro*.[14] Moreover, fluorophosphonate derivatives **3** (MAFP)[52] and trifluoromethyl ketones **4** and **5**[53] (Figure 10) were disclosed to inhibit FAAH. The replacement of a trifluoromethyl group by a methyl group brought about a dramatic decrease of FAAH inhibitory potency. This result revealed that the electrophilicity of the carbonyl group exerted a notable effect on the molecule's binding to FAAH.[54] Although these first generation inhibitors were capable of blocking FAAH in *in vitro* pharmacological assays, their lack of selectivity made them poor candidates for preclinical applications. Nevertheless, the disclosure of these inhibitors inspired the further exploration of selective FAAH inhibitors.

Over the last decades, the development of FAAH inhibitors has significantly progressed and a plethora of selective FAAH inhibitors have been designed and synthesized. Hereafter, recent FAAH inhibitors will be reviewed.

1. α-Ketoheterocycle derivatives

The first α-ketoheterocycles developed as selective FAAH inhibitors were reported by Boger and co-workers in 2000.[55,56] Their work represented a significant breakthrough in the development of selective

FAAH inhibitors. The α-ketoheterocycles were derived from the trifluoromethyl oleyl ketone **5** (Figure 10). Different heterocycles were introduced to replace the trifluoromethyl group.[54] Researchers argued that oxazolopyridines give rise to enhanced potency against FAAH in comparison with other heterocycles (such as benzoxazoles, compound **6**, K_i = 370 nM) (Figure 11). Of note, oxazolo[4,5-*b*]pyridine (**7**) and oxazolo[5,4-*c*]pyridine (**8**) afforded the most obvious improvement in FAAH inhibition (K_i = 2.3 and 3.7 nM, respectively). Oxazolo[4,5-*c*]pyridine (**9**) and oxazolo[5,4-*b*]pyridine (**10**) gave rise to an approximately 7 to 11-fold increase in potency (K_i = 7.2 and 11 nM, respectively) in comparison with **5**.[55] It was shown that oxazolopyridines enhanced the electrophilicity and reactivity of carbonyl groups of α-ketoheterocycle derivatives. Additionally, the heterocyclic nitrogen of the pyridine ring contributes to the improvement of FAAH inhibitory efficacy, which is probably due to its property to form an H-bond with Thr236. Indeed, an X-ray co-crystal structure (PDB: 3PPM) of non-covalently bound state of α-ketoheterocycle-FAAH complex identified that an H-bond was established between the pyridine ring and Thr236,[57] which was proposed to lock the inhibitor and favor the formation of tetrahedral intermediate with Ser241. Furthermore, different alkyl and phenylalkyl groups were introduced instead of the oleyl moiety. Structure-activity relationships illustrated that alkyl α-ketoheterocycles with 7 to 11 carbons displayed the best FAAH inhibition ($0.57 \leq K_i \leq 0.75$ nM, Figure 11). Particularly, the introduction of a phenyl ring at the end of the acyl chain gave rise to a significant enhancement of FAAH inhibition of the molecules ($0.20 \leq K_i \leq 0.52$ nM, Figure 11).[55] Moreover, optimization of the alkyl chain linker between the oxazolopyridine and the phenyl ring was carried out, indicating an optimal chain length of 5 carbons (**11**, K_i = 0.20 nM, Figure 11).[54,55]

An extensive development of α-ketoheterocycle derivatives was continued by Boger and co-workers. In 2004, compound **12** (OL-135, K_i = 4.7 nM, Figure 11) was reported to be the best studied FAAH inhibitor.[58] It was discovered following structural modifications on α-keto heterocycle-based derivatives. The preliminary pharmacological data showed that the introduction of 5-substituted oxazoles on the ketone provided a significant improvement in FAAH inhibition by the molecules.[58] Specifically,

pyrimidinyl (**13**, K_i = 16 nM), pyridazinyl (**14**, K_i = 14 nM), pyridinyl (**15**, K_i = 18 nM), oxazolyl (**16**, K_i = 12 nM), and thiazolyl (**17**, K_i = 16 nM) substituents at the C5 position of an oxazole ring lead to potent FAAH inhibitors (Figure 11). At the same time, research on phenylalkyl-substituted α-keto oxazole derivatives was undertaken. It was disclosed that compounds with 5 to 8 carbons between the phenyl and the oxazole ring exhibited good inhibitory potency for FAAH, and a C6 chain was determined to be the optimal chain length (Figure 11).[59] These 5-phenylalkyl(pyridin-2-yl)oxazole derivatives displayed potent selectivity for FAAH over triacylglycerol hydrolase (TGH) and the membrane-associated hydrolase KIAA1363.[58,60] According to these results, compound **12** was identified as a potential candidate for preclinical development with potent inhibition and selectivity for FAAH.[58] Moreover, compound **12** was demonstrated to elevate AEA levels in the brain and spinal cord and induced CB_1-dependent analgesia *in vivo*,[61] and was unable to activate cannabinoid receptors (CB_1 and CB_2) directly or affect P450-dependent activities *in vivo*.[54] A co-crystal structure of **12**-FAAH revealed that the phenylalkyl group of **12** was located in the ABP, while its carbonyl function interacted with Ser241 to form a tetrahedral adduct (Figure 12a).[33]

Furthermore, structure-activity relationships on **12** were undertaken. The pyridinyl group at the C5 position of the oxazole ring was replaced by different non-aromatic or aromatic substituents. The pharmacological assay data revealed that removal of the nitrogen atom of the pyridine led to an appreciable loss of inhibition (Figure 12b).[62] Structure-activity relationships indicated non-aromatic substituents (such as cyano, trifluoromethyl, and methyl carboxylate groups) with electron-withdrawing properties were found to enhance the inhibitory potency of the molecules (Figure 12b).[62] Boger and co-workers found a fundamental relationship between the K_i values and the Hammett σ_p constant of non-aromatic substituents. They made a good correction plot of -Log K_i (μM) *versus* σ_p (Figure 12c)[62], which could be useful for the design of α-ketoheterocycles-based FAAH inhibitors and the prediction of K_i values. Overall, most derivatives with a 2-pyridinyl substituent at the C5 position of the oxazole ring exhibited the favorable selectivity for FAAH over other serine enzymes (e.g. TGH, KIAA1363).[62]

Modifications of the acyl moiety of **12** were performed. The terminal phenyl ring of the acyl group was replaced by a series of aromatic groups. Almost all compounds possessed effective potency except for 2-pyridinyl (**18**, K_i = 120 nM) and carboxyphenyl (**19**, **20** and **21**, K_i > 600 nM) derivatives that manifested a dramatic loss of activity (Figure 12b).[63] In addition, a phenyl ring substituted with electron-withdrawing groups (such as chloro or trifluoromethyl) enhanced FAAH inhibition in comparison with **12** (Figure 12b).[63] It was noteworthy that the 2,3-dichlorophenyl derivative **22** (K_i = 0.9 nM, Figure 12b) exerted approximately a 5-fold better affinity for FAAH than **12**.[63] The biphenyl-4-ethyl chain derivative **23** (K_i = 0.75 nM, Figure 12d) provided around a 6-fold stronger affinity for FAAH, as compared to **12**.[63,64] The five-membered ring of **12** was also modified by introducing additional heteroatoms. 1,2,4-Oxadiazoles **24** and **25** (K_i = 0.34 nM and 1.1 nM, respectively, Figure 12d) and 1,3,4-oxadiazole **26** (K_i = 0.29 nM, Figure 12d) showed dramatically enhanced inhibition with respect to **12**.[65]

A systematic exploration of phenylhexyl-α-keto oxazole derivatives has been carried out over the last few years. Several molecules displayed potent FAAH inhibitory capacity. Typically, a compound with a phenoxyphenylhexyl side chain (**27**, CE-12, K_i = 4.4 nM, Figure 12d) elevated AEA levels in brain with duration of action more than 10 h.[66] It was determined to significantly relieve pain in a chronic constriction injury (CCI) model post a 50 mg/kg oral dose.

Recently, a new series of extended α-keto oxazole derivatives were reported to target a new binding site: the Cys269 of the CP of FAAH.[67] These compounds were obtained by introducing different groups at the C5 position of the pyridine ring of **12**. Derivatives with electrophilic groups (such as compound **28**, Figure 13a) were shown to increase FAE levels in mouse brain. Activity-based protein profiling (ABPP) assays showed that these newly developed molecules possessed high selectivity for FAAH over other serine hydrolases, including TGH, KIAA1363, MAGL, and ABHD6. In addition to interacting with Ser241, these new α-keto oxazole-based FAAH inhibitors were determined to bind covalently and irreversibly to the Cys269 by dialysis and X-ray crystal structure studies (Figures 13b and 13c).[67,68] Additional binding to Cys269 was argued to endow these novel inhibitors with improved selectivity and

prolonged inhibitory activity. Compared to the reversible FAAH inhibitor **12**, these irreversible inhibitors had a greater ability to enhance AEA signaling over longer periods in mice.[67]

2. Carbamates

The first class of aryl carbamate derivatives as FAAH inhibitors was disclosed in 2003.[69] Compound **30** (URB597, IC_{50} = 4.6 nM, Figure 14) was shown to inhibit FAAH in the nanomolar range and elevate brain AEA levels after a 0.3 mg/kg intraperitoneal injection in rats.[69,70] Compound **30** was devoid of activity in a panel of receptors (e.g. CB_1/CB_2, NOP1 receptor), ion channels (e.g. Na^+, K^+, and Ca^{2+} channels), transporters (e.g. AEA transporter), and enzymes (e.g. COX1/2 and MAGL) at 10 μM.[71] Nevertheless, compound **30** was shown to inactivate carboxylesterases and block the expression of tyrosine hydroxylase as off-target effects.[72,73] It manifested potent anxiolytic properties without affecting appetite or body temperature in models of elevated zero-maze and the isolation-induced ultrasonic emission in rats (0.1-0.3 mg/kg intraperitoneal injection) and produced pain relief in the complete Freund's adjuvant (CFA) model of arthritis pain in rats (0.5 mg/kg intraperitoneal injection).[69,71] The X-ray crystal structure of a **30**-FAAH adduct illustrated that the rotatable *N*-cyclohexyl ring occupied the ABP. Adjacent to the cyclohexyl group, the carbamate group was confirmed to form a covalent bond with Ser241 (Figures 15a and 15b).[74] The biphenyl moiety, which serves as a leaving group, was located in the CP.

Although compound **30** had excellent selectivity for FAAH in brain without affecting other serine hydrolases, it was found to interfere with the function of several liver carboxylesterases.[72] To reinforce the selectivity for FAAH and reduce carboxylesterase off-target effects, a further extension was performed, based on the scaffold of **30**. As a representative second generation aryl carbamate-based FAAH inhibitor, compound **31** (URB694, IC_{50} = 30 nM, Figure 14), which was obtained by the modification of the substituents of the biphenyl moiety, had weakened affinity for carboxylesterases in comparison with **30**.[75] The introduction of polar and electron-donating groups lowered the electrophilicity of the carbamate group , which reduced its hydrogen bond donor interactions, but resulted in improved

selectivity for FAAH.[75] As a result of focusing on the attributes of **30** and **31**, a novel carbamate derivative **32** (URB937, IC_{50} = 26.8 nM, Figure 14), containing both carbamoyl and hydroxyl substituents on the biphenyl moiety, was designed. Compound **32** is a selective FAAH inhibitor over MAGL *in vitro*, which produces inhibitory effect in peripheral tissues (e.g. liver) rather than CNS (e.g. brain).[76] It attenuated inflammation-related and neural injury-induced pain in mice after a 1 mg/kg intraperitoneal dose through CB_1-dependent activity.[76]

In addition to the *O*-aryl carbamates, other series of carbamate derivatives, such as oxime[77] and enol[78] carbamates, have been developed over the last decades. Particularly, compound **33** (ST4070, IC_{50} = 9 nM, Figure 14) was disclosed as a reversible inhibitor in dialysis studies and a highly selective FAAH inhibitor versus other targets in the endocannabinoid system, including CB_1, CB_2, TRPV1, NAPE-PLD, MAGL, and DAGL.[78] Due to a lack of an X-ray co-crystal structure of a FAAH-**33** adduct, the reversible mechanism remains unclear. Generally speaking, given its structural similarity with **30**, the biphenyl enol moiety of **33** is expected to act as a leaving group, which is accompanied by the covalent carbamoylation of Ser241.[78] As illustrated in Figure 16, the groups forming covalent bonds with Ser241 differ between **30** and **33**, and are *N*-cyclohexylformamide (secondary amide) and piperidine-1-formyl (tertiary amide), respectively. The co-crystal structure of FAAH-**30,** showed that the polar hydrogen of *N*-cyclohexylformamide forms an H-bond with Ser193, which stabilizes the carbamonyl-FAAH adduct. Conversely, due to the lack of a suitable hydrogen, piperidine-1-formyl of **33** is not expected to establish an H-bond connection with the enzyme. The lack of such an H-bond renders the FAAH-**33** adduct unstable and hydrolyzable, which confers reversibility to **33**. In addition, the latter was able to elevate brain AEA and PEA levels in mice at a 30 mg/kg oral dose and was capable of alleviating neuropathic pain and anti-anxiety at 30 mg/kg oral doses in models of streptozotocin-induced diabetes and CCI-induced pain in mice.[78,79] As a reversible FAAH inhibitor, compound **33** can be regarded as a lead for the development of a clinical candidate.

Recently, the replacement of phenyl group in **30** by a triazolylmethyl group or the introduction of a pyrazole moiety between the two phenyl groups was performed.[80,81] Since a triazolylmethoxy group acts as a poorer leaving group than phenoxy, such a replacement was deemed to confer higher stability to the molecules. This hypothesis was confirmed by pharmacokinetics studies. *O*-Substituted triazolylmethyl carbamates (such as **34** and **35**, Figure 17) were detected in rat plasma 7 hours after administration, whereas *O*-substituted 4-biphenyl carbamate **29** (URB524, IC_{50} = 25.6 nM, Figure 14) was undetectable at the same time.[80] Hence, several *O*-substituted triazolylmethyl carbamates were prepared by Colombano and co-workers. Particularly, *N*- and *O*-disubstituted 1,2,3-triazolylmethyl carbamates (such as **36**, IC_{50} = 5.8 nM, Figure 17) exerted optimal inhibitory potency. The replacement of 1,2,3-triazolylmethyl (**35**, IC_{50} = 26.2 nM, Figure 17) with 1,2,4-triazolylmethyl (**37**, IC_{50} = 51500 nM, Figure 17) resulted in a dramatic decrease of potency against FAAH, which might be due to the altered electrophilic reactivity of the carbamate.[80] This research on metabolic stability of the molecules in plasma can be considered as an encouraging starting point for prolonging the duration of action. Moreover, a series of *O*-pyrazolylphenyl carbamates bearing a 3-alkyloxycarbonyl group on the pyrazole moiety also exerted potent capacity to inhibit FAAH and high selectivity over MAGL (compounds **38**, **39**, **40**, **41**, and **42**, 11 nM $\leq IC_{50} \leq$ 29 nM, selectivity index (SI) > 1000, Figure 18).[81] The newly introduced alkyloxycarbonyl function at the 3-position of the pyrazole ring was expected to form an H-bond with the hydroxyl group of Thr488, since this latter interaction was previously identified as a means to improve reactivity and selectivity of the molecules toward FAAH.[70,81] These compounds were determined to reversibly bind to FAAH by dialysis studies. This observation is meaningful for the further development of novel selective and reversible FAAH inhibitors.[81]

Most recently, Lehr and co-workers disclosed a novel series of ω-imidazolyl and ω-tetrazolyl alkyl carbamates as potent FAAH inhibitors.[82] Although compound **43** (IC_{50} = 5.3 nM, Figure 19) manifested optimal potency against FAAH at single-digit nanomolar concentrations, its instability in plasma made it a poor candidate for systemic administration. Compounds **44** and **45** (IC_{50} = 11 and 81 nM, respectively,

Figure 19) exerted potent FAAH inhibition in the two-digit nanomolar range. However, they were shown to be degraded by carboxylesterases in porcine plasma.[82] Specifically, compounds **46** and **47** (IC_{50} = 110 and 200 nM, respectively, Figure 19) possessed moderate FAAH inhibitory activities, but suitable stability in the presence of liver fractions, plasma or albumin.[82] Further investigation of structure-activity relationships indicated that a C6 to C8 alkyl chain, notably a C7, appended to the tetrazolyl ring was favorable for FAAH inhibitory potency.[82]

3. Sulfonylfluorides

The first covalent but nonselective sulfonylfluoride FAAH inhibitor disclosed was compound **2**.[14] To improve selectivity over other serine enzymes, an extension of the acyl chain was carried out.[83] Sulfonylfluorides bearing a C14, C16, or C18 length chains exhibited weak affinity for CB_1 receptor and blocked the hydrolysis of AEA.[83] For instance, the representative alkylsulfonylfluoride **48** (AM374, IC_{50} = 13 nM, Figure 20a) with a C16 length side chain, manifested approximately 20-fold more potency than compound **2** (IC_{50} = 290 nM).[83] Nevertheless, compound **48** was previously disclosed to affect outer-membrane phospholipase A-dependent activities.[84] To improve selectivity for FAAH over other serine enzymes, a further modification of the acyl chain was performed. The carbon chain between the phenyl ring and the sulfonylfluoride group of compound **2** was extended from C1 to C8 length. Particularly, phenylalkyl sulfonylfluorides with five to seven carbons exhibited potent inhibitory potency and selectivity for FAAH.[85] The introduction of a hydroxyl or benzyloxy group at the 4-position of the phenyl ring appeared to enhance the inhibitory potency for FAAH.[85] Specifically, 4-hydroxyphenylpentyl sulfonylfluoride **49** (AM3506, IC_{50} = 48 nM, Figure 20a) exerted a potent inhibitory effect for FAAH, without triggering other serine hydrolase-mediated degradation at a dose of 0.1 mg/kg in mouse brain and liver. Nevertheless, it inhibited MAGL and ABHD6 as off-targets at a dose of 1 mg/kg.[86] Moreover, compound **49** was disclosed to have a favorable property in suppressing blood pressure, and depressing heart rate in a hypertensive model by stimulating CB_1 receptor. In addition, the administration of **49** was identified to be devoid of hyperglycemia effects.[86] The absence of inhibitory activities for several serine

hydrolases in liver at a dose of 1 mg/kg made **49** a favorable lead compound, since the inhibition of hepatic FAAH could result in the elevation of hepatic AEA signaling associated with diet-dependent fatty liver.[87,88] A molecular docking study illustrated that the two oxygen atoms of the sulfonyl group formed H-bonds with Ser241 and the oxyanion hole of FAAH, respectively, the phenylalkyl side chain occupied the ABP and the phenol interacted with Thr488 (Figure 20b).[85] In particular, the additional formation of a hydrogen bond between the hydroxyl group of **49** and the Thr488 of FAAH was expected to be advantageous for the improvement of selectivity and inhibitory activity.[70,85]

4. Ureas

The first generation urea-based FAAH inhibitors were a result of serendipity. Indeed, compound **50** (LY-2183240, Figure 21), which belongs to the earliest urea-based FAAH inhibitors, was previously developed to upregulate AEA levels by preventing AEA from transporting across the plasma membrane.[2,3] In 2006, compound **50** was found to strongly inhibit FAAH and other brain serine hydrolases.[89] Its carbamoyl moiety was shown to covalently and irreversibly interact with Ser241 of FAAH as shown using mass spectrometry and dilution analysis.[89] Switching AEA regulation from transporter inhibition to FAAH inhibition is a favorable starting point for the development of urea-based FAAH inhibitors. During the last decade, several major series of urea-based FAAH inhibitors have been explored by Johnson & Johnson, Pfizer and Takeda.

In 2006, Johnson & Johnson described a series of piperazinyl and piperidinyl ureas as FAAH inhibitors.[90] Typically, compound **51** (JNJ-1661010, Figure 21) exerted potent inhibitory efficacy for both rat FAAH and human FAAH (IC_{50} = 34 and 33 nM, respectively).[91] Compound **51** was discovered to be a FAAH inhibitor by high-throughput screening (HTS) of Johnson & Johnson's chemical library.[91] Afterwards, an exploration of *N*-substitutions and an introduction of different functions on the phenyl ring appended to the 1,2,4-thiadiazole ring was performed. Structure-activity relationships illustrated (Figure 22): (1) The introduction of a fluoro or chloro group at the 2-position of the *N*-phenyl ring provides an obvious improvement of inhibitory activity for rat FAAH, and a moderate increase of inhibitory capacity

for human FAAH, compared to **51**. (2) The introduction of different substituents on the phenyl ring appended to the 1,2,4-thiadiazole ring leads to a dramatic loss of potency against FAAH. (3) The replacement of the *N*-phenyl ring with heteroaryl groups (such as 3-/4-pyridinyl, 4-pyrimidinyl, 6-methoxypyrimidine-4-yl or benzo[*c*][1,2,5]thiadiazole-4-yl groups) result in a remarkably enhanced inhibitory potency for both rat and human FAAH with respect to **51**.[91] Dialysis and mass spectrometry studies identified that the phenyl-1,2,4-thiadiazol-5-piperazinyl moiety of compound **51** covalently and reversibly bound to FAAH, while the aniline moiety acted as a leaving group during hydrolysis (Figure 23).[91] Compound **50**, but not compound 51 inactivated liver esterase.[91] The absence of inhibitory effects toward liver esterases makes **51** a potential attractive lead compound.

Replacement of the phenylthiadiazole and the *N*-phenyl moieties of **51** with arylmethyl and aromatic groups, respectively, was carried out in 2012. Overall, compound **52** (JNJ-40355003, Figure 21) manifested optimal capacity to elevate three related FAEs (AEA, PEA, OEA) levels in rat, dog, and cynomolgus monkey plasma *via* the inhibition of FAAH (IC_{50} (hFAAH) = 1.4 nM, IC_{50} (rFAAH) = 33 nM in a irreversible manner (dialysis studies).[92] It was noteworthy that **52** did not affect FAAH-2 (IC_{50} > 10 μM), whereas another two potent FAAH inhibitors **12** and **30** were shown to inactivate FAAH-2 at nanomolar concentrations (IC_{50} = 13.4 and 5 nM, respectively).[19] Compound **52** dramatically increased FAE levels in monkey plasma after a 5 mg/kg oral administration.[19,92]

Recently, Janssen Research, part of Johnson & Johnson, disclosed a series of spirocyclic diamine core-based urea FAAH inhibitors, which were obtained through the modification of the aromatic amine and piperazine moieties of **52**.[93] Compounds **53**, **54**, and **55** (Figure 24) exhibited remarkable FAAH inhibition activities (IC_{50} (hFAAH) = 27, 80 and 125 nM, respectively).[93] A further screening of different aromatic amine functions was performed. The pharmacological assays indicated that **56** (JNJ-42119779, Figure 24), acted as an irreversible inhibitor in dialysis studies, displaying optimal inhibitory efficacy against both rat and human FAAH (IC_{50} (hFAAH) = 8 nM, IC_{50} (rFAAH) = 9 nM) and produced analgesia in a spinal nerve ligation (SNL) model after a 20 mg/kg oral dose. Replacement of a 2,7-

diazaspiro[3.5]nonane group with a 2,6-diazaspiro[3.3]heptane group (**57**, IC_{50} (hFAAH) = 18 nM, IC_{50} (rFAAH) = 42 nM, Figure 24) resulted in a slight loss of inhibitory potency against FAAH, but almost complete loss of ability to raise central FAEs (AEA, OEA and PEA) levels *in vivo*.[93] More recently, compound **58** (JNJ-42165279, Figure 24), a selective and slowly reversible (dialysis assays) piperazine urea-based FAAH inhibitor developed by Johnson & Johnson, was reported to be in phase II clinical trials.[94] Compound **58** has been reported to time-dependently inhibit rat and human FAAH (IC_{50} (hFAAH) = 70 nM, IC_{50} (rFAAH) = 313 nM) and to elevate brain FAEs (AEA, PEA, and OEA) levels until 24 h post-dose in rats. Moreover, it exhibited high selectivity versus a panel of enzymes, transporters, receptors, and ion-channels at 10 μM.[94] Compound **58** was demonstrated to produce analgesia in a rat SNL model (22 mg/kg oral dose).[94]

Pfizer discovered a series of piperidine/piperazine-based urea-FAAH inhibitors through a HTS screening campaign. Of note, compounds **59** (PF-622, Figure 21) and **60** (PF-750, Figure 21) were reported to dramatically inhibit FAAH (IC_{50} (hFAAH) = 33 and 12.5 nM, respectively).[50] Furthermore, a time-dependent inhibitory activity assay was performed.[50] Human FAAH was preincubated with inhibitors and the substrate was added after a certain time. IC_{50} values for **59** and **60** were dramatically decreased from 0.991 μM to 0.033 μM and 0.595 μM to 0.0162 μM respectively after a longer preincubation time (60 min instead of 5 min). Compound **30** also exerted similar time-dependent properties (IC_{50} = 0.986 μM after 5 min, IC_{50} = 0.109 μM after 60 min). Conversely, the prolonged preincubation time did not affect the FAAH inhibitory capacity of **12**.[50] Time-dependent properties of irreversible FAAH inhibitors are probably due to their ability to form a covalent substrate-FAAH complex or induce a conformational change in the enzyme. Hence, the inhibitory activities of irreversible FAAH inhibitors were assessed with k_{inact}/K_i values (k_{inact}: maximal rate of enzyme deactivation at a saturating concentration of inhibitor) instead of IC_{50} values by Pfizer.[50] The higher the value, the better the activity. k_{inact}/K_i values were determined for the irreversible FAAH inhibitors **30** and **60**. Although these two FAAH inhibitors were reported with remarkable IC_{50} values, they only showed modest k_{inact}/K_i values (k_{inact}/K_i =

1,590 $M^{-1}s^{-1}$ [1] and 791 $M^{-1}s^{-}$for **30** and **60**, respectively).[95] This observation indicated that **30** and **60** might possess only moderate binding affinity for FAAH or inactivate FAAH at a moderate rate.

Compounds **59** and **60** displayed no detectable activity for other serine enzymes at a 500 μM concentration *in vitro*, and at a dose of 30 mg/kg *in vivo* in ABPP assays. However, compounds **30** and **12** had off-target serine enzyme interactions under the same experimental conditions.[50] The co-crystal structure of **60**-FAAH indicated: the urea functions of **60** covalently reacted with Ser241 of FAAH, the piperidine moiety was positioned in the ABP of FAAH (Figure 25), and the aniline group attached to the CP serving as the leaving group after hydrolysis.[50,96] Compounds **59** and **60** were shown to irreversibly bind to FAAH, since the hydrolysis capacity of FAAH did not recover after a rapid dilution.[50]

Inspired by the structures of **59** and **60**, a series of benzothiophene-based piperazine/piperidine ureas were described as FAAH inhibitors. The replacement of the *N*-phenyl ring of benzothiophene urea derivatives with the *N*-pyridine-3-yl group led to enhanced inhibition by the molecules (Figure 26).[97] Moreover, the introduction of additional substituents on the pyridine ring was reported to markedly alter the inhibitory potency against FAAH. In particular, structure-activity relationships showed that a substituent at the 6-position led to the dramatic loss of inhibition potency, except for a methoxy group (Figure 26). For instance, a diethylamino substituent at the 6-position of the pyridine ring resulted in around a 20-fold loss of inhibitory activity for human FAAH, in comparison with the amino substituent. It seems that steric effects limit the interaction of the molecule with FAAH.[97] Furthermore, substituent optimization on the benzothiophene was carried out (Figure 26). The methyl group at the 3-position of the benzothiophene seemed to be essential for the FAAH inhibitory capacity of the molecule. Indeed, the replacement of this methyl group with an ethyl group was shown to slightly decrease the inhibitory efficacy and the remove of this substituent dramatically reduce the inhibitory activity (k_{inact}/K_i = 2401 $M^{-1}s^{-1}$, 1402 $M^{-1}s^{-1}$ and 695 $M^{-1}s^{-1}$, respectively). The introduction of small groups at the 4-/5-positions appeared to have a slight impact on inhibitory efficacy. However, the introduction of an ethoxy group at the 6-position of the benzothiophene brought about a complete loss of inhibitory activity (k_{inact}/K_i = 14 M^{-}

$^{1}s^{-1}$).[97] The binding model revealed that the 6-ethoxy benzothiophene substituent was too large to be suitably placed in the binding channel of FAAH according to the mutual exclusion between the ethoxy group and Met436.[97] Most of these developed benzothiophene urea derivatives were determined to be highly selective as shown using competitive ABPP. Compound **61** (PF-465, Figure 26) alleviated pain response in the complete Freund's adjuvant (CFA) model of inflammatory pain in rats after a 10 mg/kg dose, with efficacy comparable to that of the nonsteroidal anti-inflammatory drug (NSAID) naproxen at the same dose.[97]

As previously described, compound **60** only showed moderate affinity for FAAH. To improve the binding affinity for FAAH, replacement of the quinoline with different biaryl ether functions was performed and the pharmacological activities were profiled as k_{inact}/K_i values.[95] These modifications led to the discovery of a potent, selective, covalent, irreversible FAAH inhibitor, compound **62** (PF-3845, Figure 27).[95] The profile of inhibitory potency indicated that **62** possessed an impressive k_{inact}/K_i value of 12600 $M^{-1}s^{-1}$ for hFAAH, which was 8 times and 16 times more than **30** and **60**, respectively. Indeed, compound **62** showed rapid elevation of brain AEA, PEA, and OEA levels in mice brain. The significant increase of brain AEA levels was still detected 24 hours post a 10 mg/kg intraperitoneal administration. Conversely, the action of **30** in improving brain AEA levels was not observable 7 hours post a 10 mg/kg intraperitoneal dose.[95] Compound **62** produced CB_1-/CB_2-dependent pain relief in various models (such as mechanical allodynia model and CFA-induced inflammatory pain) at a 10 mg/kg oral dose.[95] Selectivity profiling by competitive ABPP showed that **62** exhibited high selectivity for FAAH without affecting other serine enzymes. Apart from the high selectivity, compound **62** also exerted prolonged duration of enzyme inactivation (> 24 h) and high oral bioavailability (> 80%).[95] These advantageous characteristics make **62** a useful tool for the further exploration and investigation of endocannabinoid system-mediated multiple biological responses.[95] Recently, compound **62** was demonstrated to promote neuronal survival and alleviate brain damage in a traumatic brain injury (TBI) model in mice post a 5 mg/kg intraperitoneal dose.[98]

Further determination of the X-ray crystal structure of a **62**-FAAH complex showed that the carboxamide moiety of **62** covalently interacted with the hydroxyl group of Ser241, the 3-aminopyridine group was positioned in the CP and acted as a leaving group during catalysis, and the biaryl ether piperidine moiety was bound to the ABP.[95] Notably, the conformation of FAAH was variable according to the rotation of Phe432 and Met436. The data reported showed that the inhibitors induced conformational changes.[77,95] As exemplified by **62**, overlay of X-ray co-crystal structures of **62**-FAAH and **60**-FAAH indicated that the trifluoromethyl group induced a conformational shift of Phe432 from the ABP to the MAC. (Figures 28a and 28b) This conformational switching is accompanied by a rotation of Met436, and results in the expansion of the ABP and the easier access of inhibitors or substrates from MAC into the ABP.[95] In fact, the replacement of the trifluoromethyl group of **62** with smaller substituents (such as methyl, fluoro) resulted in a sudden decrease of inhibitory potency, because the van der Waals interactions between the small substitutions and Phe432/Met436 was too weak to alter the conformation of the enzyme.[95] Additionally, switching a trifluoromethyl group from the 5-position of pyridine ring to the 3/6-position also impaired inhibitory capacity, which was possibly due to the steric clash between the inhibitor molecular and the enzyme.[95]

To improve the inhibitory efficacy, a C=C linker was introduced into the basic scaffold of **62**.[99] In particular, the introduction of a C=C linker between the piperidine ring and the aromatic group confers several advantageous properties. For instance, the C=C moiety provides a rigid conformation with fewer rotatable bonds, lower conformational energy, and improves the likelihood of interactions with the ABP (e.g. Phe432).[99] Indeed, pharmacological assay results indicated that piperidine derivatives with the C=C linker (**63**, k_{inact}/K_i (hFAAH) = 21600 $M^{-1}s^{-1}$) exerted enhanced inhibitory potency in comparison with **62**. Additionally, the replacement of *N*-pyridin-3-yl by pyrazin-2-yl (**64**) or pyridazine-3-yl (**65**) dramatically improved the inhibitory efficacy (Figure 27), and also decreased the hydrophobicity of the molecular and alter physicochemical parameters (such as clog *P*).[99] Subsequently, compound **65** (PF-04457845, Figure 27), a novel potent, highly selective, covalent, and irreversible FAAH inhibitor was identified as a clinical

candidate. Compound **65** was shown by competitive ABPP to possess high selectivity over numerous serine enzymes in kidney, spleen, and testis at 100 μM and exhibit no off-target activity against a panel of receptors, ion channels, and transporters.[99,100] It possessed remarkable pharmacokinetic properties in dogs and humans, including rapid absorption after oral administration, a low clearance rate, a high bioavailability of 88% and 58% in rats and dogs, respectively, a low probability of drug-drug interaction based on *in vitro* cytochromes P450 (CYPs) inhibition investigations, and a long duration of action lasting until 24 hours post-dose.[99] Compound **65** was found to elevate FAEs (e.g. AEA and OEA) levels and alleviate inflammation-related pain in a CFA model of inflammatory pain in rats (0.1 mg/kg oral dose) through CB_1/CB_2-dependent activities without affecting motility or body temperature.[99,100] Although oral administration of 0.5-40 mg **65** also brought about the elevation of plasma FAEs levels, it failed to produce analgesia in patients with pain induced by osteoarthritic knee in clinical trials.[101] The complete loss of analgesic potency in humans might be attributed to the difference in biological responses after FAAH inhibition between humans and rodents. Two hypotheses were suggested for this clinical trial failure by Pfizer. Firstly, complex human emotions related to pain are not observable in rodent models.[102] Indeed, human pain observation was demonstrated to be significantly influenced by emotionality. Pain sensation can be attributed to heightened anxiety or depression. Secondly, there is sufficient evidence that CB receptor activation induced by AEA elevation after FAAH inhibition is equivalent to that induced by exogenous CB receptor agonists in rodents. However, this has not been confirmed in humans.[101] Overall, pain and its related symptoms are complicated and might differ in various species. How to translate pain symptoms into rational and reliable data needs be considered carefully. Moreover, pain can be induced by multiple conditions (e.g. inflammation, cancer, or injury), therefore, analgesic mechanisms might differ depending on the types of pain. Nevertheless, clinical trials of compound **65** in other disorders (e.g. post-traumatic stress disorders and Tourette syndrome) are ongoing.

[^{11}C-carbonyl] **65** (Figure 27) was shown to be a suitable candidate for FAAH positron emission tomography (PET), which can be used for the neuroimaging study of FAAH and the clinical research.[103]

Furthermore, optimization of the core between the urea function and the aromatic group of **65** was performed. The pharmacological assay identified two series of novel potent urea-based FAAH inhibitors bearing a 1-oxa-8-azaspiro[4.5]decane or a 7-azaspiro[3.5]nonane core instead of a piperidine.[104] Three compounds (**66**, **67**, and **68**, Figure 29) from these two series were profiled and assessed for their selectivity *versus* more than 200 human serine hydrolases. The competitive ABPP assay indicated that these three compounds were devoid of off-targets at 100 μM. They were further determined to exert low clearance and a low risk of drug-drug interactions.[104] To further improve potency against FAAH and optimize CNS-dependent drug properties, the exploration and screening of aromatic moieties led to the identification of a 7-azaspiro[3.5]nonane urea **69** (PF-04862853, k_{inact}/K_i value = 4190 and 5820 $M^{-1}s^{-1}$ towards human and rat FAAH, respectively, Figure 29) as one of the optimal FAAH inhibitors to date.[105] In addition, compound **69** was determined to possess suitable physiochemical (*c*log*P* = 3.8) and pharmacokinetic properties (a half-life of 7.1 h and 53% bioavailability in rats, a half-life of 7.8 h and 33% bioavailability in dogs), display a pronounced anti-inflammatory effect at 0.3 mg/kg, almost totally inhibit FAAH in brain at 0.3 mg/kg, and substantially increase AEA levels to reach the maximum concentration in brain at 1 mg/kg in a CFA model of inflammatory pain in rats.[105] Given its advantageous drug-like properties, compound **69** was selected as a lead compound.[105]

Recently, Takeda developed a series of heterocyclic piperazine ureas as FAAH inhibitors.[106,107] These novel inhibitors were inspired by two identified FAAH inhibitors **51**[91] and **70** (Figure 30). It was hypothesized that the replacement of the phenyl ring of **51** with benzoisoxazolyl might improve the inhibitory potency.[106] In this case, **71** (Figure 30) was discovered by the hydridization of **51** and **70**. Compound **71** was demonstrated to exhibit remarkable inhibitory capacity against human FAAH (IC_{50} = 4.8 nM). However, this *N*-benzoisoxazolyl substituted urea derivative has poor aqueous solubility and disappointing bioavailability (5%) in rats.[106] Hence, the replacement of benzoisoxazolyl with isoxazole, pyridazine, or pyridine was performed to improve aqueous solubility and other physicochemical parameters. Particularly, *N*-(3,4-dimethylisoxazol-5-yl) substituted derivative **72** (Figure 30) exerted

enhanced inhibitory efficacy (IC_{50} = 1.7 nM) and moderate aqueous solubility (1.6 μg/mL) with respect to **71**.[106] Moreover, the replacement of the 1,2,4-thiadiazole group with a thiazole ring seemed to improve both inhibitory potency and aqueous solubility (Figure 30, IC_{50} = 0.92 nM, solubility of 38 μg/mL). Additionally, the exploration of substituents on the terminal phenyl ring was carried out. Notably, the 2-/4-monosubstituted fluoro group and 2,4-disubstituted fluoro group on the terminal phenyl ring appeared to be favorable for inhibition and for maintaining modest aqueous solubility (Figure 30).[106] It is noteworthy that mono- or di-substituted fluoro groups at the 2/4 position of the terminal phenyl group enabled potent oral bioavailability (70-98%) in rats and the ability to cross the BBB.[106] Compound **73** (Figure 30) not only possessed the optimal inhibitory activity for human and rat FAAH (IC_{50} = 0.43 and 0.46 nM, respectively), but also exerted remarkable analgesic potency in the acetic acid-induced writhing model in mice at doses of 10 and 30 mg/kg (oral administration).[106]

To improve the aqueous solubility, pyridazine and pyrimidine groups were introduced to replace the 3,4-dimethylisoxazole group and the thiazole ring, respectively.[107] Notably, compound **74** (Figure 30) was exposed to exert potent inhibitory activity against human/rat FAAH (IC_{50} = 0.72/0.28 nM) and produce analgesic potency in both the spared nerve injury (SNI)-induced neuropathic pain and CFA-induced inflammatory pain models in rats at 3 mg/kg doses (oral administration).[107]

5. Boronic acids

Boronic acid-based derivatives have been considered as potential pharmaceutical candidates and demonstrated to potently and reversibly inhibit several serine enzymes.[108] In 2008, boronic acids were determined to inhibit FAAH.[109] The para-substituted hydrophobic or electron-withdrawing groups of phenylboronic acids were shown to be advantageous for the inhibitory activity against FAAH. Notably, (4-nonylphenyl) boronic acid **75** (Figure 31a) exerted potent FAAH inhibitory capacity (IC_{50} (rFAAH) = 9.1 nM). Exemplified by compound **75**, molecular modeling with FAAH assumed that the two hydroxyl groups of **75** were able to form hydrogen bonds with Met191 and Ser193, respectively. The electrophilic boron atom was hypothesized to interact covalently with the nucleophilic hydroxyl group of Ser241

because of its trigonal planar sp^2-hybridization and its empty p orbital.[109] The covalent interaction between **75** and Ser241 was presumed to form a reversible tetrahedral intermediate (Figure 31b).[109] According to recent research on molecular binding simulation of FAAH,[30,34] it was hypothesized that 4-nonylphenyl might be located in the ABP. Although the binding mechanism between boronic acid-based inhibitors and FAAH is still not clear, the remarkable inhibitory efficacy makes boronic acids useful chemical tools to further elucidate the catalytic mechanism of FAAH. However, boronic acid derivatives have been shown to suppress multiple serine enzymes, which could be a disadvantageous property for pre-clinical applications.[109]

6. Arylthioheterocycles

Most recently, a series of arylthioheterocycle-based non-covalent FAAH inhibitors were disclosed by Merck.[110,111] According to the creation of a short-lived complex between non-covalent inhibitors and FAAH, they are expected to decrease potential risk factors over the formation of a long-lived covalent adduct. The development of arylthioheterocycle-based FAAH inhibitors was derived from an HTS hit **76** (Figure 32). However, compound **76** manifested moderate FAAH inhibitory activity (IC_{50} = 119 nM) and poor pharmacokinetic parametres.[111] To improve the inhibitory potency and pharmacokinetic properties, structural modifications of **76** were carried out.[111] Structure-activity relationships indicated that use of an ether, sulfone, or sulfoxide instead of a thioether as the linker between the aromatic group and heterocycle ring resulted in a dramatic loss of inhibitory activity (Figure 32).[111] The introduction of a phenyl, a *para*-fluorophenyl or a pyridine group to the *N*-1 of the pyrazole and the replacement of the dioxane ring with a methyl sulfone or a 1,2,4-oxadiazole led to an improvement to the FAAH inhibitory potency ($17 \leq IC_{50} \leq 32$ nM, Figure 32). Finally, the oxazole analogue **77** (MK-4409, Figure 32) bearing the optimized substituents, a 4-pyridyl tertiary alcohol, a *para*-fluorophenyl and a thiopyridine, drastically improved the pharmacokinetic properties. The profile of analgesic effects in numerous models illustrated that the oxazole-based derivative **77** reduced inflammatory pain in a CFA model at a 10 mg/kg oral dose and attenuate neuropathic pain in a SNL model at a 3 mg/kg oral dose with a long duration of action until 24

h post-dose, and no apparent side effects were observed up to a dose of 100 mg/kg.[111] The clinical trials data of **77** will be reported in due course. Compound **77** has been presumed to reversibly interact in a non-covalent manner with FAAH. It can be used for further studies of non-covalent binding mechanisms with FAAH.[111]

In addition, arylthioheterocycle derivatives were developed as PET tracers, which could be used for imaging FAAH in brain.[110] The exploration was based on a lead compound **78** (Figure 33), which was previously identified as a potent FAAH inhibitor.[110] However, compound **78** possessed an unfavorable lipophilicity (LogD = 4.2 at pH 7.3) complicating the *in vivo* PET studies in rhesus monkeys. To optimize lipophilicity and brain penetration, several modifications were performed.[110] Notably, compound **79** (MK-3168, Figure 33), possessing an ideal hydrophilic parameter (Log D = 3.3 at pH 7.3), was revealed to remarkably inhibit rat, rhesus, and human FAAH (IC_{50} = 1.0, 5.5 and 1.7 nM, respectively).[110] The isotope labeled compound **79** could be used for the study of FAAH by imaging and for pharmacokinetics.

7. 3-Carboxamido-5-arylisoxazoles

A new series of FAAH inhibitors based on 3-carboxamido-5-aryl-isoxazole scaffold was disclosed in 2011.[112, 113] The pharmacological results indicated that a 4-biphenyl ring at the 5-position of the isoxazole and *N*-substituted 2-(benzo[*d*][1,3]dioxol-5-yl)ethyl provided the optimal inhibitory efficacy.[112] Compound **80** (Figure 34) exerted potent capacity to suppress FAAH (IC_{50} = 88 nM) and produced protective efficacy and anti-inflammatory potency in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice at a 10 mg/kg oral dose.[112] The molecular modeling of compound 80 suggested that its isoxazole and carboxamide moieties interact with the oxyanion hole, and form hydrogen bonds with Ile138 and Lys142, respectively. The rigid biphenyl group was assumed to establish a π-π interaction with Phe194. Additionally, the benzodioxole group was presumed to serve as a hydrogen bond acceptor and bind to Gln273.[112]

It was noteworthy that a series of CB_2 agonists were concurrently explored based on the same 3-carboxamido-5-arylisoxazole scaffold.[113] Interestingly, during the exploration of CB_2 agonists, some compounds were found to be more potent as FAAH inhibitors.[113] For instance, compound **81** (Figure 34) was demonstrated as a potent CB_2 agonist. Nevertheless, modification of the phenyl ring substituents revealed that a shift of the pentoxy group from the ortho position to the para position gave rise to a complete loss of CB_2 agonism. Correspondingly, compound **82** (Figure 34) with a para-pentoxy substituent was discovered to drastically inhibit FAAH, compared with a modest FAAH inhibition of its meta analogue.[113] Moreover, compound **82** was determined to exhibit anti-inflammatory efficacy in dextran sulfate sodium (DSS)-induced acute colitis in mice at a 10 mg/kg intraperitoneal dose.[113] Overall, the 3-carboxamido-5-arylisoxazole could be a favorable scaffold used for the exploration of FAAH inhibitors or CB_2 agonists.

8. 1,3,4-Oxadiazol-2-ones

1,3,4-Oxadiazol-2-ones were initially reported to reversibly inhibit several EC hydrolases, such as FAAH and MAGL.[114] For example, compound **83** (Figure 35) exhibited remarkable FAAH inhibitory potency at nanomolar doses (IC_{50} = 6.1 nM), and approximately 20-fold selectivity over MAGL (IC_{50} = 0.11-0.38 μM).[114] To further improve the potency and selectivity, the exploration of *N*- and *O*-substituted phenyl groups was carried out.[115] It was demonstrated that 1,3,4-oxadiazol-2-ones could be modified to fit either FAAH or MAGL by focusing on the different distinct characteristics of the catalytic center between FAAH and MAGL.[115] Recently, Patel and co-workers made a breakthrough in the improvement of the selectivity of 1,3,4-oxadiazol-2-ones. They hypothesized that the poor selectivity of previously prepared 1,3,4-oxadiazol-2-ones could be attributed to the directly linked phenyl-oxadiazole moiety.[116] In this case, a chiral carbon linker was introduced between the phenyl ring and oxadiazole group to give molecular flexibility.[116] Structure-activity relationships illustrated that the chiral carbon linker (**84**, IC_{50} = 23 nM) provided enhanced inhibitory potency against FAAH in comparison with the achiral methylene linker (**85**, IC_{50} = 91 nM). Moreover, the *S*-isomer (**86**, IC_{50} = 11 nM) was determined to show superior

potency, as compared to its enantiomer (IC_{50} = 240 nM).[116] Typically, compound **86** (JZP-327A, Figure 35) manifested optimal FAAH inhibitory potency and high selectivity over MAGL and COX isoenzymes. An ABPP assay indicated that **86** showed no off-target activity against other serine hydrolases (such as MAGL, ABHD6/12).[116] Compound **86** was identified as a reversible FAAH inhibitor, according to the recovery of enzyme activity after a rapid dilution.[50,116] Molecular modeling revealed the 1,3,4-oxadiazol-2-one core establishes a strong H-bond network with the oxyanion hole of FAAH.[116] The introduction of a chiral carbon linker could encourage the development of new selective FAAH inhibitors. The 1,3,4-oxadiazol-2-one might be considered as a privileged scaffold for the further development of FAAH inhibitors, given the favorable strong interactions between 1,3,4-oxadiazol-2-ones and FAAH.

9. Other FAAH inhibitors

Apart from these interesting scaffolds mentioned above, a number of miscellaneous structures were disclosed to inhibit FAAH.

Compound **87** (Figure 36), discovered at Abbott, was hypothesized to reversibly and non-covalently inhibit FAAH in the nanomolar range (IC_{50} (rFAAH) = 18 nM).[117] The determination of selectivity by ABPP indicated that compound **87** exerted no off-target activity against numerous enzymes (such as TGH, KIAA1363, and carboxy esterases).[117]

A series of non-covalent and reversible FAAH inhibitors was disclosed by Amgen in 2011.[118] Typically, compound **88** (Figure 36) exerted potent capacity to disable FAAH-mediated hydrolysis. As exemplified by compound **88**, the X-ray crystal structure of **88**-FAAH adduct indicated that the 4-(2,3-dihydrobenzofuran-2-yl) substituent was located in the MAC, while piperidine moiety occupied the ABP.[118] Additionally, van der Waals interactions were present between the pyrimidine and several hydrophobic residues of FAAH, including Phe432, Leu433, Met436, Thr488, Ile491, and Trp531. (Figure 37)[118,119] Compound **88** was determined to possess favorable pharmacokinetic parameters, including low

clearance rate, suitable bioavailability (92%) in rats and reasonable residence time (5.5 h after a 2 mg/kg dose).[118]

Renovis, Inc. developed a series of tetrahydropyridopyridines as non-covalent and reversible FAAH inhibitors.[120] Amongst these compounds, compound **89** (RN-450, Figure 36) exerted attractive FAAH inhibitory potency with IC_{50} of 13 nM and 25 nM towards human and rat FAAH, respectively.[120]

Compound **90** (Figure 36) was disclosed to potently inhibit FAAH, but exhibited poor metabolic stability due to rapid reduction by a carbonyl reductase.[121]

Compound **91** (MM-433593, Figure 36), a potent and selective FAAH inhibitor with an IC_{50} in the nanomolar range, was disclosed by Ironwood Pharmaceuticals Inc.[122] It was determined to only slightly antagonize CB_1 (IC_{50} > 1 μM) and be devoid of effects on CB_2, COX1/2, FAAH-2, other serine hydrolases and a broad panel of receptors and channels.[122] Although compound **91** has been shown to interact with FAAH in a non-covalent fashion, it was proposed to be an irreversible FAAH inhibitor.[122] It is interesting to know the binding mechanism between **91** and FAAH. Probably, the non-covalent but irreversible property is attributed to the strong H-bonds interactions established between **91** and FAAH and/or the conformational change of FAAH induced by **91**. The detailedbinding mechanism of **91** is expected to be disclosed. Moreover, compound **91** was shown to possess excellent pharmacokinetic parameters, moderate oral bioavailability (14-21%) in monkeys and a long half-life (17-20 h) post a 10 mg/kg oral dose or a 1 mg/kg intravenous dose.[123] The detailed metabolic pathways and at least 18 metabolites of **91** in monkey were described. Compound **91** will be further studied in human clinical trials.[123] The discovery of **91** and its metabolic pathways *in vivo* is meaningful for the design and development of drug candidates.

Moreover, a series of 1-aryl-2-((6-aryl)pyrimidin-4-yl)amino)ethanol derivatives as competitive FAAH inhibitors were reported by Johnson & Johnson.[124] The extension of this series of FAAH inhibitors was encouraged by the HTS hit **92** (Figure 36). Interestingly, unlike α-ketoheterocycles, carbamates, or ureas, compound **92** does not possess any covalently reactive function that is intended to interact with

FAAH.[124] The *R*-isomer of compound **92**, rather than its *S*-isomer, was demonstrated to provide the principal inhibitory efficacy against FAAH (IC_{50} = 17 nM and 10 μM, respectively). Indeed, the hydroxyl group of the *R*-isomer was identified to be amenable to forming an H-bond with Thr488. In this case, the exploration was performed based on the *R*-isomer. Numerous aromatic groups were introduced to replace the trifluoromethoxyphenyl moiety appended to the pyrimidine ring.[124] Generally, the compounds with substituted groups at the 2-position of the phenyl ring appended to the pyrimidine ring suffered from a substantial reduction of inhibitory capacity, whereas 3-/4-substituted hydrophobic groups contributed to the enhancement of inhibitory potency.[124] Several compounds (such as compounds **93**, **94**, **95**, and **96**, Figure 36) exhibited remarkable capacity to block rat and human FAAH, although poor oral bioavailability and high clearance rate were detected in rats. Specifically, 3-chloro-4-trifluoromethylphenyl substituted derivative **97** (JNJ-40413269, Figure 36) was shown to exert potent FAAH inhibition activity (IC_{50} = 5.3 nM) and advantageous pharmacokinetic properties including good oral bioavailability (94%), good brain penetration (brain/plasma ratio ~1), and prolonged half-life ($t_{1/2}$ = 4.1 h) in rats.[124] Moreover, compound **97** was determined to produce a dose-dependent analgesic effect in a SNL model of neuropathic pain in rats post a 2/6/20 mg/kg oral administration.[124] Compound **97** was modeled in a crystal structure of humanized rat FAAH, which indicated that the hydroxyl group appended to the chiral carbon possibly formed a hydrogen bond with Thr488.[124] Given its advantageous pharmacokinetic properties, compound **97** might inspire the further development of reversible FAAH inhibitors.

NAAA inhibitors

1. Substrate inspired inhibitors

Inspired by the structure of natural substrates, a series of PEA analogues were initially disclosed to inhibit NAAA. The PEA analogue-based inhibitors contain a hydrophobic side chain and a reactive functionality, such as an amide, reverse amide, ester, reverse ester, or amino group. *N*-Pentadecylcyclohexanecarboxamide **98** (Figure 38) and *N*-pentadecylbenzamide **99**, belonging to first

generation NAAA inhibitors, were revealed to inhibit rat lung NAAA.[125] However, compound **98** was found to be unable to inhibit NAAA at 100 μM concentration, when it was resynthesized by Yamano and co-workers.[126] Eventually, pentadecylamine **100**, an impurity detected in the first batch of **98**, was identified to strongly inhibit rat lung NAAA (around 90% inhibition) at 100 μM. Thus, the inhibitory activity of **99** is also uncertain and needs to be reevaluated, due to its similar chemical structure to **98**. According to this interesting discovery, a series of lipophilic amines derived from **100** was developed by Yamano and co-workers.[126] Especially, tridecyl 2-aminoacetate **101** (Figure 38) showed approximately 90% inhibition of rat lung NAAA at 100 μM *via* competitive mechanism (examined by a Lineweaver–Burk plot).[126] The lipophilic amines might be protonated in solution while they were determined to inhibit NAAA at pH 5, the optimal catalytic pH of NAAA. Since the protonated and non-protonated molecules interact with enzymes through different mechanisms, the selectivity over FAAH was evaluated at both pH 5 and pH 8.5, a suitable pH for FAAH. The result indicated that there was no detectable inhibitory activity against FAAH at pH 8.5, whereas compounds **100** and **101** slightly inhibited rat FAAH at pH 5 at a concentration of 100 μM.[126]

Moreover, the cyclobutyl palmitate **102** and cyclopentyl palmitate **103** compounds (Figure 38) were determined to block human recombinant NAAA at a concentration of 50 μM with 41% and 85% inhibition of NAAA activity, respectively.[127] Compound **103** was observed to drastically elevate PEA levels in intact HEK-NAAA cells without affecting the AEA and OEA signaling at 50 μM.[128] Furthermore, topical administration of compound **103** (200-400 μg/sponge, 1.5 cm^3) in a model of carrageenan-induced inflammation in rats, was found to reduce inflammation.[128]

Compound **104** (Figure 38) was reported to be a weak dual inhibitor against NAAA and FAAH.[129] The replacement of the pyrrolidine ring with other cyclic functions led to a dramatic decrease of inhibitory potency against NAAA. The introduction of a biphenyl group on the acyl chain produced compound **105** (Figure 38), which potently inhibited rNAAA selectively *vs.* rFAAH (IC_{50} = 2.12 μM and > 100 μM, respectively).[129] Furthermore, the optimal substrate-based NAAA inhibitor **106** (AM9023, IC_{50} = 0.6 μM,

Figure 38) to date was revealed.[130] The isothiocyanate moiety of **106** was determined to interact reversibly with the cysteine of the enzyme catalytic triad by kinetic studies and using mass spectrometry. Compound **106** displayed moderate selectivity for human NAAA over human MAGL and rat FAAH (IC_{50} > 10 μM).[130] Indeed, the electrophilicity of isothiocyanate enables it to interact with serines, cysteines, and amino groups in biomolecules. Therefore, isothiocyanate can be considered as an interesting scaffold for drug design. However, off-target effects of isothiocyanates should be considered with attention due to their highly reactive properties.

Overall, most substrate-based NAAA inhibitors only exert modest inhibitory activity and selectivity. Typically, compound **106** exhibits the optimal inhibitory potency, which could be used for the further research of interactive mechanisms with NAAA.

2. β-Lactones

The presence of the catalytic triad in NAAA has encouraged the development of a series of electrophilic function-dependent NAAA inhibitors. Since the nucleophilic reactive sulfhydryl cysteine group of NAAA was found to interact with the electrophilic carbonyl group of substrates during catalysis, the presence of the electrophilic function was proposed to provide inhibitory potency through the formation of a covalent bond with the thiol group. In this case, compounds with electrophilic functions were expected to be developed.

Compound **107** (Figure 39), a serine-derived β-lactone, was initially reported to inhibit the cysteine protease hepatitis A Virus 3C protease. Afterwards, compound **107** was discovered to inhibit NAAA.[7] In this case, the β-lactone ring rather than the carbamate moiety appears to covalently bind to the NAAA active site, based on the structure-activity relationships showing that the removal of the β-lactone gives rise to a complete loss of potency.[7,131] Removal of the oxygen atom of the carbamate moiety produced compound **108** ((*S*)-OOPP, Figure 39), which exerted an approximately 7-fold increase in inhibition, in comparison with compound **107** (IC_{50} = 0.42 and 2.96 μM, respectively).[7,131] Interestingly, the (*R*)-

enantiomer of **108** displayed a markedly lower inhibition with respect to its (*S*)-enantiomer (IC_{50} = 6 μM). Kinetic analyses disclosed that **108** interacted with NAAA through a non-competitive mechanism.[7] Compound **108** showed high selectivity for human NAAA over acid ceramidase and one kind of cysteine hydrolase that is very similar to the structure and function of NAAA. In addition, compound **108** was determined to be devoid of inhibitory potency against FAAH or other serine hydrolases.[7] Furthermore, compound **108** was shown to reduce neutrophil migration and alleviate injury in a model of traumatic spinal cord injury in mice (30 μg per mouse, intrathecal injection).[7] However, lack of stability in plasma (half-life < 1 min)[7] makes **108** a poor drug candidate for systemic application. To further improve potency and stability, the exploration of β-lactone-based NAAA inhibitors was performed (Figure 40). The replacement of the phenethyl ring with a hexyl chain maintained the capacity to inhibit NAAA (compound **109**, IC_{50} = 460 nM).[131] The introduction of lipophilic groups, such as naphthyl or biphenyl groups, was shown to be advantageous for inhibitory potency. Specially, compound **110** (Figure 40) exerted the optimal inhibitory efficacy against NAAA. Compound **110** was purported to covalently and reversibly bind to NAAA according to a slow recovery of enzyme activity following 12-hour dialysis. In addition, compound **110** was reported to possess non-competitive inhibitor property that the increase of **110** concentration did not affect K_m but induced a decrease of V_{max}.[131] Compound **110** was shown to reduce carrageenan-induced inflammation post a 10 μL/sponge (1 cm^3) topical administration.[131] Mass spectrometry studies supposed that the nucleophilic thiol group of the Cys131 of rat NAAA interact the carbonyl of β-lactone group to form an acyl-NAAA intermediate.[7,131,132]

The β-lactone group has been shown to react readily with nucleophiles, and can be rapidly hydrolyzed in aqueous solution. In fact, most amide-based β-lactone derivatives were rapidly hydrolyzed in aqueous buffer at pH 7.4 with half-lives of less than 20 minutes, whereas the β-substituted methyl group endowed the β-lactone derivatives with improved stability (half-life > 70 min).[133] Afterwards, urea- and carbamate-based β-lactone derivatives were identified to possess drastically increased chemical stability at both pH 5 and pH 7.4 in comparison with the corresponding amide-based β-lactone derivatives.[133]

Structure-activity relationships revealed that the (*R*)-configuration of carbamate-based β-lactones was favorable for inhibitory capacity, whereas the (*S*)-configuration is suitable for urea-based β-lactones (Figure 40).[133] Although these urea/carbamate-based β-lactones manifested improved chemical stability in buffer solution, they were rapidly eliminated in the presence of bovine serum albumin. In particular, *tert*-butoxycarbonyl substitution increased the compounds' stability in the presence of bovine serum albumin ($t_{1/2}$ ~2 h) and retained moderate inhibitory potency.[133] Nevertheless, they still possessed poor stability in rat plasma ($t_{1/2}$ < 1 min). Chemical structure optimization gave rise to a potent NAAA inhibitor, compound **111** (URB913/ARN077, Figure 39), which manifested the optimal inhibitory efficacy against rat and human NAAA (IC_{50} = 0.05 and 0.007 μM, respectively) and enhanced chemical stability in buffer solution ($t_{1/2}$ > 110 min).[133,134] Furthermore, compound **111** was shown to protect PEA and OEA from hydrolysis in mice ear skin post a topical administration (10% in acetone). It attenuated inflammation in a model of carrageenan-induced edema post a topical administration (30% in petrolatum/5% lauric acid) through the stimulation of PPAR-α, due to the blockage of anti-inflammatory effect in PPAR-/- deficient mice.[135] Compound **111** rapidly, non-competitively, covalently and reversibly combined with rat NAAA, whereas the inhibition of human NAAA was only partially reversible.[132] The binding mechanism studies indicated that the thiol group of the active cysteine (Cys131 for rat NAAA, Cys126 for human NAAA) interacted with the carbonyl of β-lactone moiety of **111** to form a hydrolysable thioester bond (Figure 41).[132] Further investigation of the selectivity of **111** indicated that it was devoid of off-target effects in a panel of several targets including GPCRs, enzymes, transporters, and channels at a 10 μM concentration.[40] Due to the instability of compound **111** in plasma, it was administrated topically rather than systemically in mouse models of inflammation and nerve injury. The compound (10% in acetone) was effective in TPA-induced edema and CCI mouse models.[135]

Modification of the phenylpentyl moiety of **111** was performed. Removal of the phenyl ring or modification of the carbon chain length of the phenylpentyl group appeared to result in a notable decease of inhibition (Figure 42).[134] However, the extension of the alkyl chain after the removal of the phenyl ring

brought about a recovery of inhibitory ability. Especially, the aliphatic chains with 7-8 carbon atoms length (**112** and **113**, IC_{50} (rNAAA) = 0.04 and 0.03 μM, respectively, Figure 42) provided a slight increase of inhibitory potency in comparison with **111**. The replacement of the phenyl ring with the cyclohexyl group (**114**, Figure 42) led to an enhancement of inhibitory efficacy (IC_{50} (rNAAA) = 0.013 μM). Moreover, the replacement of a carbon in the alkyl chain of **111** with an oxygen atom was tolerated.[134] It was noteworthy that **115** (Figure 42) displayed optimal inhibitory potency in the single-digit nanomolar range against both rat and human NAAA (IC_{50} = 0.007 μM), whereas its *m*-biphenylmethyl analogue was determined to only slightly inhibit rat NAAA (IC_{50} = 23 μM).[134] Compound **115** showed no inhibitory activity against rat FAAH at 10 μM concentrations, whereas, it was observed to slightly inhibit rat acid ceramidase with 30% inhibition at the same concentration.[134]

Further modification of the β-substituent on the β-lactone ring of NAAA inhibitors was carried out to improve their stability.[136] In general, carbamate-based β-lactones exhibited enhanced inhibitory potency and improved chemical stability in comparison to urea-based β-lactone derivatives.[136] Bulky alkyl substituents (such as isopropyl and *tert*-butyl) at the β-position of the β-lactone ring resulted in improved chemical stability in buffer solution, although this resulted in a decrease of inhibitory efficacy against human NAAA (IC_{50} = 0.063 and 0.302 μM, respectively, Figure 42).[136] Most β-lactone derivatives exerted poor plasma stability with a half-life less than 5 min. Notably, compounds **116** and **117** (IC_{50} (hNAAA) = 0.234 and 0.507 μM, respectively) (Figure 42) possessed improved stability in plasma with a half-life greater than 5 min and exerted modest inhibitory potency against NAAA.[136] Although the result indicated that it was difficult to find a functional group providing both desirable stability and potent NAAA inhibitory potency for β-lactone derivatives, this investigation of stability could be considered as a favorable starting point for further development of NAAA inhibitors with suitable chemical and metabolic stability.

Most recently, the first three dimensional quantitative structure-activity relationship (3D-QSAR) model was established based on the (2-methyl-4-oxooxetan-3-yl)carbamate-derived NAAA inhibitors to

predict chemical structures that probably inhibit NAAA.[137] This model was established using a training set comprising 33 compounds and the Atomic Property Fields method. The squared correlation coefficients between the experimental pIC_{50} and the predicted pIC_{50} is 0.93 and the leave one-out squared correlation coefficients Q^2 is 0.69. Afterwards, 5 predicted compounds were used to valid this model. The experimental pIC_{50} of these 5 compounds were found to be very close to the predicted values.[137] This model has already been used to discover several potent NAAA inhibitors (such as **118** and **119**, Figure 39)., Compound **118** blocks the catalytic activity of NAAA in the nanomolar range.[137] From this 3D-QSAR model, the linear symmetrical structure of the aliphatic side chain appears to be advantageous for the inhibitory capacity against NAAA. This 3D-QSAR model is meaningful for the further design and prediction of potent NAAA inhibitors.

3. β-Lactams

Exploration of β-lactams as NAAA inhibitors was inspired by the structure of β-lactone-based inhibitors. Since the β-lactone moiety was identified to be unstable in plasma, its replacement with a more stable functionality was required and this was demonstrated to dramatically enhance the stability in plasma.[138] Structure-activity relationships indicated that the linear acyl chain seemed to be favorable for the inhibitory capacity of β-lactams against NAAA. For instance, the amide-based β-lactams with a 9 to 11 carbon length acyl chain (**120**, **121**, **122** and **123**) and the carbamate-based β-lactam with a 7-carbon length *O*-alkyl chain (**124**) exerted potent activity against NAAA (Figure 43).[138] The (*S*)-configuration rather than the (*R*)-configuration at the C3 position of the amide function is suitable for β-lactam-based NAAA inhibitors.[138] For example, compound **120** exerted potent inhibitory activity against human NAAA without affecting human FAAH-mediated hydrolysis and possessed good oral bioavailability (67%) in rats.[138] Overall, the significantly improved stability affords β-lactams the potential for systemic application.

Furthermore, compound **124** encouraged the development of carbamated-based β-lactams as potent NAAA inhibitors. Most recently, compound **125** (ARN726, Figure 43) was shown to markedly inhibit

both rat and human NAAA (IC_{50} = 63 and 27 nM, respectively) with approximately 9-fold enhanced activity in comparison with compound **124** (IC_{50} (hNAAA) = 0.24 μM).[139] Nevertheless, the *R*-isomer of **125** showed weak affinity for NAAA (IC_{50} = 3.1 μM). Compound **125** was determined to covalently and irreversibly interact with NAAA by mass spectrometry and dialysis studies, which indicated that the cysteine thiol group of NAAA interacted with the amide group of β-lactam moiety to generate the acyl-NAAA adduct (Figure 44).[139] Compound **125** was devoid of effects against rat FAAH and only marginally inactivated rat acid ceramidase. Compound **125** was further determined to have no effect on a panel of 28 biological targets including lipid-hydrolyzing enzymes and 79 common targets comprising ion channels, membrane transporters and receptors (such as cannabinoid receptors) at a concentration of 10 μM.[139] Pharmacokinetic analyses revealed that **125** was rapidly eliminated *in vivo* (C_{max} = 1608 ng/kg in plasma with a $t_{1/2}$ ~15 min, and clearance = 139 mL min^{-1} kg^{-1}) post a 3 mg/kg intravenous dose in mice. These data were consistent with the *ex vivo* assay that **125** was efficiently eliminated in plasma (a $t_{1/2}$ of 41 min in mice and 12 min in rats) and in liver microsomes (a $t_{1/2}$ less than 5 min in both rats and mice). Nevertheless, the inhibition of NAAA-mediated hydrolysis in several organs was detected after intraperitoneal administration of **125** (3-30 mg/kg). For instance, the covalent adducts of NAAA-**125** were detected by mass spectrometry in mice lungs[139] Notably, compound **125** was disclosed as the first β-lactam-derived NAAA inhibitor for systemic application. It reduced inflammation (30 mg/kg oral dose) in a model of carrageenan-induced inflammation in mice. Compound **125** possessed a short plasma $t_{1/2}$ but exerted profound anti-inflammatory efficacy *in vivo*, which might indicate that NAAA-dependent modulation of anti-inflammation is efficient. The disclosure of **125** may encourage the exploration of β-lactam-based NAAA inhibitors with suitable properties for systemic administration.

More recently, the optimization of the β-lactam moiety or the side chain of carbamate-based β-lactams was carried out.[140] The investigation of structure activity-relationships disclosed that a linear and lipophilic side chain is favorable for NAAA inhibitory capacity, and (*S*)-configuration is preferred rather than its (*R*)-isomer. Specifically, compounds **126**, **127** and **128** (Figure 43) possessed encouraging

inhibitory potency towards NAAA (IC_{50} = 12 nM, 7 nM, and 22 nM, respectively, evaluated by UPLC/MS-based assay). These three compounds were determined to be devoid of activity against FAAH and display a moderate selectivity ratio over human acid ceramidase (50 < SI < 400) at 10 μM. In addition, compound **128** manifested a superior stability (half-life ≥ 25 min in rat plasma and rat/mouse liver) in comparison to **125**. Its metabolic stability is probably attributed to the phenyl ring adjacent to carbamate moiety. Its pharmacological properties will be disclosed in due course.[140]

Generally speaking, β-lactams can be considered as a more suitable scaffold than β-lactones for the investigation of NAAA inhibitors, according to their relatively stable chemical structure. Although some β-lactam-derived NAAA inhibitors described herein (e.g. compounds **126**, **127** and **128**) have been demonstrated not to affect FAAH, β-lactam derivatives have also been described as FAAH inhibitors.[141] Thus, the selectivity of β-lactams over NAAA or FAAH needs to be investigated. According to related data, structure-activity relationships are summarized in Figure 45. As illustrated, the R^2 group plays a crucial role in selectivity over NAAA or FAAH. Alkylation of the N-H function of β-lactam brings about a complete loss of NAAA inhibitory activity, whereas removal of R^2 group results in a sharp decrease of FAAH inhibitory activity. The different structural preference for a β-lactam-derived NAAA and FAAH inhibitor is probably due to their different binding mechanism. Indeed, the β-lactam moiety was predicted to form H-bonds with FAAH, whereas the β-lactam was identified to covalently react with NAAA.[139,141] Hence, the reactive secondary β-lactam function rather than tertiary β-lactam is preferred for NAAA.

4. Other NAAA inhibitors

In a recently published paper, compound AM9053 (structure not disclosed, IC_{50} = 30 nM) was revealed as one of the first systemically active NAAA inhibitors that increased PEA rather than AEA levels in a model of inflammatory bowel disease (IBD) in mice.[142] It showed excellent selectivity over FAAH (IC_{50} > 100 μM) and produced PPAR-α-dependent anti-inflammatory effect, including the inhibition of pro-inflammatory cytokines (e.g. TNF-α, IL-1β) expression, in a model of TNBS-induced colitis post a 10 mg/kg intraperitoneal dose in mice.[142]

Compound **129** (diacerein, Figure 46), is an approved medicine for alleviating osteoarthritis (swelling and pain in the joints) through the inhibition of IL-1β.[143] Recently, it was shown to display time-dependent inhibition of human NAAA (IC_{50} = 0.7-7.2 μM) in both the cell-free enzyme and intact cells.[144] Compound **129** showed no interaction with rat FAAH, human cannabinoid receptors (CB_1 and CB_2), and human TRPV1 receptors at a 50 μM concentration.[144] It was determined to alleviate inflammation and hyperalgesia with a long duration of action (> 6 h) in a carrageenan-induced acute inflammatory pain model in rats post a 25 mg/kg oral dose.[144] Although **129** has been shown to inhibit NAAA *in vitro*, produce anti-inflammation and elevate PEA levels in a carrageenan-induced acute inflammatory pain model, further investigated is needed in order to ascertain whether **129**-mediated anti-inflammation and increase of PEA levels can be specifically attributed to NAAA inhibition. In summary, the discovery of **129** as a potent NAAA inhibitor might encourage the development of new scaffold-based NAAA inhibitors with suitable duration of action for systemic administration. Compound **129** could be a useful tool for the investigation of other probable mechanisms in addition to the inhibition of IL-1β-dependent activities *in vivo*.

Most recently, compound **130** (F96, Figure 46) was revealed to be a potent inhibitor of NAAA and have excellent chemical stability in buffer solution with a half-life of more than 24 hours, and dramatically improved metabolic stability in rat plasma with a half-life of nearly 200 min.[145] Compound **130** exhibited high selectivity for NAAA over FAAH and almost no activity against MAGL or acid ceramidase at a 10 μM concentration *in vitro* and was devoid of affinity for cannabinoid receptors and PPAR-α. The anti-inflammatory and anti-nociceptive effects of **130** can be reversed by a PPAR-α antagonist (1-[(4-chlorophenyl)methyl]-3-[(1,1-dimethylethyl)thio]-α,α-dimethyl-5-(1-methylethyl)-1*H*-indole-2-propanoic acid, MK886) but not by cannabinoid receptor antagonists **1** and rimonabant (**131**, Figure 47), which led to the conclusion that **130** produced anti-inflammation and analgesia through a PPAR-α-dependent activity rather than cannabinoid receptors.[145] It is noteworthy that **130** elevated PEA levels *via* inactivation of NAAA without the alteration of AEA signaling. Compound **130** alleviated pain and

inflammation (10 mg/kg intraperitoneal injection) in several animal models including 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema, sciatic nerve injury-induced neuropathic pain, and acetic acid-induced visceral pain in mice.[145]

Compound **130** showed less toxicity than ibuprofen (a widely used anti-inflammatory and anti-nociceptive drug) in zebrafish models.[145] Further investigation is needed to establish whether **130** is devoid of severe side effects in other *in vivo* animal models (e.g. mammals). In general, compound **130** possesses potent NAAA inhibitory efficacy and suitable metabolic stability in plasma. The advantageous properties of **130** might encourage the further development of NAAA inhibitors with a long duration of action *in vivo*. The investigation of the binding mechanism between **130** and NAAA is expected to be carried out in the future.

MAGL inhibitors

Generally, MAGL is sensitive to some early described serine hydrolases inhibitors, such as compounds **2-4**,[45] although these compounds usually manifest off-target potency against other serine hydrolases (such as FAAH). With respect to other serine hydrolases, MAGL has been demonstrated to additionally interact with sulfhydryl-sensitive substrates in line with its structural features as it contains several active cysteine residues (such as Cys201, Cys208, and Cys242).[46] This property was regarded as the starting point for the development of selective MAGL inhibitors over other serine hydrolases.

1. Maleimides

First generation MAGL inhibitors were described several decades ago. Some inhibitors, such as *N*-ethylmaleimide **132** (Figure 48a), comprise mercapto-specific functions. Moreover, a series of maleimide derivatives were identified to inhibit MAGL *via* a Michael addition with cysteine residue thiol groups by mass spectrometry and mutational analysis.[46,146] Structure-activity relationships indicated that *N*-substituted hydrophobic groups of maleimide derivatives have improved inhibition against MAGL in comparison with *N*-hydrophilic substituents.[46] Especially, compounds **133** (IC_{50} = 0.14 μM) and **134** (IC_{50}

= 9.2 µM) exerted approximately 6-fold and 380-fold enhanced potency against MAGL-like enzyme in rat cerebellar membranes, respectively, compared to **132 (**IC_{50} = 53 µM, Figure 48a).[46] The sulfhydryl group of cysteine residues was shown to interact covalently and irreversibly with the maleimide moiety through a Michael addition to yield a *S*-alkylated MAGL adduct (Figure 48b).[146] Moreover, the modification of the *N*-substituent of **133** led to the identification of **135** (Figure 48a) as a moderate and selective inhibitor with an IC_{50} of 0.79 µM against hMAGL over hFAAH (SI > 20-fold).[147]

2. Disulfides

Compound **136** (disulfiram), an approved drug to treat alcoholism *via* suppression of aldehyde dehydrogenase, was reported to inhibit human MAGL at submicromolar concentrations (IC_{50} = 0.36 µM, Figure 49).[148] Additionally, two disulfide derivatives, compounds **137** (IC_{50} = 1.6 µM) and **138** (IC_{50} = 0.13 µM), were shown to inactivate MAGL-mediated activity in the micromolar range (Figure 49).[148] These disulfide derivatives were hypothesized to interact with the cysteine residues of MAGL to form a mixed adduct or a disulfide bond.[148] The exploration of numerous analogs of 136 with different substitutions led to discovering a series of selective MAGL inhibitors. Typically, compound **139** (Figure 49) exerted optimal inhibitory capacity against hMAGL with an IC_{50} of 0.11 µM and more than 1000-fold selectivity over hFAAH.[45,149] Compound **139** was presumed to interact irreversibly with MAGL, which might be accompanied by the formation of disulfide bond with cysteine.[149]

3. Isothiazolinones

Compound **140** (octhilinone, IC_{50} = 88 nM, Figure 50a) was reported to be a potent MAGL inhibitor by King and co-workers, when they investigated a series of sulfhydryl-specific candidates.[150] It was shown to disable MAGL through a partially reversible mechanism, due to a recovery of enzyme activity after dilution. The introduction of a *N*-substituted long hydrophobic alkyl group (such as **141**, IC_{50} = 43 nM) or replacement of isothiazolinone moiety with benzisothiazolinone (**142**, IC_{50} = 20 nM) brought about a slight improvement of inhibitory potency (Figure 50a).[150] To investigate a probable interactive

mechanism, studies using a reducing agent dithiothreitol (DTT) were performed. The addition of DTT appeared to block **140**-induced inhibition of MAGL, but did not affect **3**-mediated inhibition of MAGL. This finding indicated that **140,** rather than **3,** might form a reducible bond with MAGL, which was not consistent with the formation of a Michael addition product. Additionally, compound **142** exerted a comparable inhibitory activity to that of **140**, which supported that isothiazolinone-induced inhibition of MAGL was not attributed to their ability to undergo a Michael addition. Compound **140** was proposed to form a disulfide adduct with MAGL (Figure 50b). Furthermore, mutation studies showed that Cys208 rather than Cys201 or Cys242 played a crucial role in the reaction with compound **140**.[150] Isothiazolinone could be regarded as a favorable scaffold for the further exploration of potent MAGL inhibitors.

4. Ureas

Compound **50**, which was originally reported as an AEA transporter inhibitor, was also shown to exert off-target effects against several carboxylases and serine hydrolases (such as FAAH and MAGL).[3,89] Indeed, a 2,5-regioisomer of **50** (compound **143**, Figure 51) exhibited approximately 400 times more potent inhibition against MAGL-like enzyme in comparison to **50** (IC_{50} = 0.02 and 8.10 μM, respectively).[151] Unfortunately, compound **143** exerted poor selectivity over FAAH expressed in rat brain membranes (IC_{50} = 0.033 μM). The investigation of its binding mechanism by mass spectrometry and mutational analysis showed that the hydroxyl group of Ser122 interacted with the urea moiety of **143** to produce a carbamylated-enzyme adduct, accompanied by the release of the biphenylmethyl tetrazole moiety (Figure 52).[146] Moreover, Sanofi-Aventis disclosed a triazole urea **144** (SAR629, Figure 51) as a potent covalent MAGL inhibitor. Similar to compound **143**, the urea moiety of **144** was determined by X-ray to interact with Ser122 of MAGL followed by the formation of a carbamylated-enzyme adduct (Figure 53), associated with the release of the triazole moiety. Compound **144**-induced inhibition of MAGL is proposed to be quasi-irreversible due to the possible hydrolysis of **144**-MAGL adduct by a water molecule. This hypothesis needs to be further examined.[44] Over the last few years, the exploration of numerous urea-based MAGL inhibitors has been performed. For example, compound **145** (ML30, IC_{50}

(hMAGL) = 0.54 nM, Figure 51) exerted a strong ability to irreversibly inhibit hMAGL in the subnanomolar range with more than a 1000-fold selectivity over FAAH.[152] Recently, a series of piperazine/piperidine triazole ureas were described as highly selective MAGL inhibitors.[153] Structure-activity relationships illustrated that the Y-type scaffold of triazole ureas favors selectivity over FAAH.

The replacement of the triazole moiety with a 4-nitrophenoxy group or the removal of a nitrogen of the triazole resulted in a dramatic decrease of inhibitory potency (Figure 54).[153] Furthermore, compounds **146** (JJKK-046) and **147** (JJKK-048) (Figure 54) were determined to inhibit hMAGL in the subnanomolar range (IC_{50} = 0.562 and 0.363 nM, respectively) and exert appreciable selectivity over several serine hydrolases, as shown using ABPP assays, including FAAH (SI > 1200 and 11200 fold, respectively), ABHD6 (SI > 150 and 600 fold, respectively). In addition, these two compounds exhibited no detectable potency against ABHD12 at a 1 μM concentration.[153] In addition, compound **147,** unlike **146,** was disclosed to selectively elevate 2-AG levels without affecting AEA signaling at a concentration of 1 μM in rat brain.[153] Indeed, the presence of a C=C bond was shown to favor the interaction with FAAH.[34] The presence of a further C=C bond might make **146** less selective than **147** over FAAH. The discovery of **147** could inspire the further development of highly selective MAGL inhibitors.

More recently, Patel's group disclosed a novel series of urea-based MAGL inhibitors derived from loratadine (Figure 51), a histamine H_1 receptor antagonist.[154] Specially, compounds **148** and **149** (JZP-361, Figure 51) were determined to optimally and selectively inactivate human MAGL at submicromolar concentrations over human FAAH (SI > 140 folds and > 150 folds, respectively), human ABHD6 (SI ≈ 38-fold and ≈ 19-fold, respectively), and human ABHD12 (no detectable inhibitory potency at a 10 μM concentration). Their inhibitory effects were determined to be slowly reversed after dilution.[154] In addition, compound **149** exhibited no observable affinity for cannabinoid CB_1 and CB_2 receptors at 10 μM concentration. However, both **148** and **149** maintained histamine H_1 receptor antagonistic activities comparable to loratadine. As a novel dual-action agent, compound **149** is desirable to be profiled in a model of inflammation.[154]

5. Carbamates

In 2005, Hohmann and co-workers profiled a series of *N*-biphenyl carbamates and revealed **150** (URB602, Figure 55) as an inhibitor against MAGL with weak potency (IC_{50} = 28 μM),[155] it was further identified to inhibit FAAH as an off-target.[156] The finding that **150** elevates 2-AG levels without affecting AEA levels at a 100 μM concentration in rat brain slice cultures[155] is probably due to the fact that more than 80% inhibition of FAAH is required for a detectable increase of AEA level.[157] Compound **150** was determined to covalently but partially reversibly bind to MAGL by dialysis experiments. The *O*-substituent of **150** was assumed to serve as the leaving group after hydrolysis.[158]

The disclosure of a new series of carbamate-based MAGL inhibitors by Cravatt and co-workers was regarded as one of the most encouraging breakthroughs in this area.[159] The profile of selectivity by ABPP assays led to the identification of **151** (JZL184, Figure 55) as a potent and selective MAGL inhibitor. Compound **151** was determined to inactivate MAGL-mediated activity at nanomolar levels and be devoid of off-target effect towards cannabinoid receptors and several serine enzymes (such as FAAH and ABHD6) at 1 μM concentration (IC_{50} (mMAGL) = 8 nM, IC_{50} (FAAH) = 4 μM).[159] Despite exerting the suppression of FAAH at a 10 μM concentration *in vitro* and 16 mg/kg *in vivo*, compound **151** still showed no detectable effect on the accumulation of AEA levels.[159] Indeed, this result is consistent with the previous demonstration that the observable elevation of AEA levels occurs in the case of more than 80% inhibition of FAAH.[157] However, multidimensional protein identification technology (ABPP-MudPIT) assays implied that **151** inactivated esterase 1, esterase 1-like, and triacylglycerol hydrolase 2 as off-targets.[48] In addition, compound **151** was found to exert a long duration of action such that the increase of 2-AG signaling was detectable even 24 h post administration.[159] The administration of **150** was shown to produce analgesic effects in several pain models, including acute thermal pain (16 mg/kg intraperitoneal injection), acetic acid-induced visceral pain writhing (16 mg/kg intraperitoneal injection), and formalin-induced edema pain (40 mg/kg oral dose) in mice. Compound **151**-induced anti-nociception was reversed by a CB_1 antagonist (**131**). This observation indicated that **151**-induced pain relief was mediated by CB_1-

dependent activities.[159] The administration of **151** at high doses (40 mg/kg) rather than low doses (4 mg/kg) gave rise to elevation of both AEA and 2-AG levels, which was accompanied by a decrease of body temperature and motility. These adverse effects following high-dose **151** treatment might be attributed to its off-target inhibition of FAAH, consistent with the previous finding that dual FAAH/MAGL inhibition produced cannabimimetic side effects.[159,160] Furthermore, the administration of **151** was demonstrated to produce gastroprotective and antidepressant-like effects post a 4 mg/kg intraperitoneal injection.[160,161] The determination of the binding mechanism indicated that **151** covalently and irreversibly bound to MAGL through the formation of the carbamylated-enzyme adduct (Figure 56). Overall, compound **151** is a selective MAGL inhibitor that exerts multiple CB_1-dependent protective and anti-nociceptive effects through the elevation of 2-AG levels in the nervous system. Compound **151** could be regarded as a useful tool for the further investigation of the functional roles of MAGL. Nevertheless, the selectivity of **151** is still improvable. The further repeated administration of high-dose **151** resulted in the alteration of both 2-AG and AEA levels.[162]

Recently, Cravatt's team reported a novel series of *O*-hexafluoropropyloxy carbamates as MAGL inhibitors.[163] In comparison with **151**, compounds **152** (KML29), **153** (JW618), and **154** (JW642) exerted superior selectivity over mFAAH in ABPP assays (Figure 55). Specifically, compound **154** only slightly inhibited mFAAH at a 10 μM concentration, whereas compound **151** almost completely inhibited mFAAH at the same concentration.[159,163] Nevertheless, compound **154**, unlike compounds **152** and **153**, markedly inhibited mFAAH at a high concentration of 50 μM. Compound 152 inhibited ABHD6 at a 10 μM concentration, whereas compounds **153** and **154** resulted in a significant inhibition of ABHD6 at a 100 nM concentration. Thus, compounds **153** and **154** appear to be dual FAAH/ABHD6 inhibitors.[163] It was noteworthy that **152** did not affect any serine hydrolases at a high dose of 40 mg/kg in mouse brain in ABPP studies. Moreover, compound **152** was determined to exhibit no off-target effects against any carboxylesterases even at 40 mg/kg concentrations in mouse liver in ABPP studies. On the contrary, compound **151** was observed to inhibit several carboxylesterases including esterase 1 and esterase 1-

like.[48,163] Compound **152** only slightly inhibited carboxylesterase 1 as an off-target in mouse lung. Furthermore, compound **152** was determined to give maximal inhibition of MAGL in the brain by 1 h post-dose and such inhibitory action was still detectable 24 h post-administration.[163] Like most of carbamate-based MAGL inhibitors, compound **152** was shown to covalently and irreversibly interact with MAGL along with the formation of the carbamylated-enzyme adduct and the release of a hexafluoropropyloxy group. Compound **152** exerted antinociceptive effects in a wide range of pain models (such as carrageenan-induced inflammatory pain and CCI neuropathic pain) post a 20-40 mg/kg intraperitoneal injection without resulting in cannabimimetic side effects including hypomotility, hypothermia and catalepsy.[164] Its administration was accompanied by a dramatic elevation of 2-AG levels and was devoid of alteration of AEA, PEA, OEA levels in mouse brain. In addition, it was shown to alleviate NSAID (e.g. diclofenac sodium)-induced gastric injury (haemorrhages) after a 40 kg/mg intraperitoneal injection. Compound **152**-induced gastro-protective effects were later identified to be CB_1 rather than CB_2-dependent, since this protective action was blocked by a CB_1 antagonist **131** but not by the CB_2 antagonist **1** (3 kg/mg intraperitoneal injection)[164] More recently, compound **152**-induced accumulation of 2-AG was revealed to be tissue-dependent. Indeed, the most pronounced elevation of 2-AG levels was observed in the brain and spinal cord.[165] This observation indicated that **152**-mediated activity was more CNS-dependent. Compound **152** could be regarded as a promising agent for the therapy of CNS-related disorders.[165]

In 2013, the screening of *O*-substituents of benzhydrylpiperazine carbamates by Cravatt and co-workers led to the identification of **155** (JW651) and **156** (MJN110) as potent selective MAGL inhibitors as shown using *in vitro*/*in vivo* competitive and click chemistry ABPP (Figure 55).[166,167] Compound **155** was determined to markedly inhibit MAGL at a 0.1 μM concentration *in vitro* and almost completely inactivate MAGL at low doses (5 mg/kg) in mouse brain. Apart from the inhibition of ABHD6 at 10 μM concentrations *in vitro* and 40 mg/kg doses in *vivo*, no off-target effect was detected even at high concentrations of 100 μM and a high dose of 40 mg/kg.[166] Replacement of the hexafluoropropyloxy group

of **155** by a succinimidyl functionality produced another potent and selective MAGL inhibitor **156**.[166,167] The latter was assessed to almost completely inhibit MAGL at 1 μM concentrations. By either oral or intraperitoneal administration in mice, compound **156** manifested an impressive inhibitory capacity against MAGL at a low dose of 1 mg/kg in ABPP studies. The inhibition of MAGL induced by **156** was observed up to 24 h post-dose. In addition, compound **156** showed no detectable off-target effects at a 10 μM concentration except ABHD6.[166,167] Moreover, it was identified to relieve chronic pain in a model of diabetes induced by a combination of high fat diet and streptozotocin in rats (5 mg/kg intraperitoneal injection).[167] Recently, compound **156** was found to produce anti-allodynia and anti-hyperalgesia through the increase of 2-AG levels along with the activation of cannabinoid receptors and mu opioid receptors in a CCI model of neuropathic pain (0.43 mg/kg intraperitoneal injection), and to be devoid of opioid/cannabinoid-dependent side effects (such as constipation).[168] Compounds **155** and **156** can be further investigated for their clinical applications. The disclosure of these two potent, selective and irreversible MAGL inhibitors could inspire the development of novel selective and irreversible MAGL inhibitors.

Most recently, a novel potent and selective MAGL inhibitor, compound **157** (SAR127303, Figure 55), was revealed to inactivate MAGL at nanomolar levels (IC_{50} (mMAGL) = 3.8 nM), markedly elevate 2-AG levels in mice and produce analgesic effects in several models of inflammation and pain.[169] Notably, compound **157** displayed high selectivity over most human serine enzymes except ABHD6 and was devoid of effects on 170 kinases, ion channels, transporters, and cannabinoid receptors, including CB_1 and CB_2. The administration of **157** resulted in a significant increase of 2-AG levels without affecting AEA, OEA, and PEA levels at a dose of 8 mg/kg. In addition, compound **157**-induced activity was detectable until 24 h post-dose.[169] Compound **157** elicited anti-nociceptive activities in several models of pain and inflammation (such as phenylbenzoquinone (PBQ)-induced writhing, 3 mg/kg oral dose). Moreover, compound **157** was identified to attenuate epilepsy symptoms. Interestingly, compound **157**-induced analgesic effects can be reversed by a CB_1 (**131**) but not a CB_2 (**1**) antagonist, which indicated

that **157** mainly produced analgesia through a CB_1-dependent mechanism.[169] Although the administration of **157** was demonstrated to be devoid of inducing hypothermia, catalepsy, and hypomotility, it resulted in an alteration of learning and memory performance. This adverse property might limit the application of **157** in a clinical setting.[169] The investigation of its binding mechanism indicated that the Ser132 of MAGL could interact with the carbamate moiety of **157** accompanied by the release of hexafluoropropyloxy group. The carbonyl oxygen of the carbamate moiety was suggested to serve as an H-bond acceptor and bind to Ala61/Met133. The piperidine moiety made contact with one side of Leu251 through van der Waals interactions. One oxygen atom of the sulfonyl moiety was found to interact with the main chain of Asn162 through the formation of H-bond as well as additionally make contact with the side chain of Asn162 and the main chain of Leu251 through water-mediated interactions (Figure 57).[169] Like most *O*-hexafluoropropyloxy carbamates, the hexafluoropropyloxy moiety of **157** served as the leaving group during catalysis.[169] Furthermore, [^{11}C]-**157** has been synthesized as a radiotracer for investigating the density and distribution of MAGL, which could be used for the diagnosis of CNS-dependent disorders (such as nerve injury).[170]

6. Other MAGL inhibitors

Tetrahydrolipstatin (THL, Figure 58), also termed orlistat, is an approved drug to treat obesity through the inhibition of lipases.[171] In 2003, THL was shown to additionally target DAGLα/β.[172] Furthermore, Di Marzo's group developed a series of THL derivatives as modulators of 2-AG levels.[173] Notably, the screening of these synthesized THL analogues led to the identification of **158** (OMDM169, IC_{50} = 2.8 μM, Figure 58) as a selective MAGL inhibitor over DAGLα (approximately 7-fold) and FAAH (> 7-fold). Compound **158** also exerted poor affinity for CB_1 and CB_2 receptors.[173] Interestingly, compound **158** exhibited varied inhibitory potency against MAGL according to the different enzyme sources and species.[174] In fact, compound **158** produced superior potency in rat brain and rat cerebellum rather than in the corresponding mouse tissues.[174] Compound **158** was detected to produce anti-nociception and elevate 2-AG levels without affecting AEA concentrations in mice pre-treated with

formalin. However, the elevation of 2-AG levels was only observed in the ipsilateral paw that influenced by the administration of formalin, but not in the contralateral paw.[174] This result is consistent with the fact that 2-AG is produced "on demand". Like most β-lactone-based enzyme inhibitors, the β-lactone moiety of **158** was suggested to covalently and reversibly interact with the Ser122 of MAGL to form an acyl-enzyme adduct (Figure 59).[173]

In 2008, Poupaert and co-workers disclosed a series of arylthioamides derivatives as MAGL inhibitors. Although most of these derivatives only inhibited MAGL at millimole levels, logP values made arylthioamide a favorable scaffold for the further exploration of MAGL inhibitors.[175] Moreover, the modification of the amide moiety and the introduction of the sulfur atom appended to the thioamide group was performed based on an arylthioamide scaffold.[176] Especially, compounds **159** (CK16) and **160** (CK37) exerted weak activity against MAGL at submicromolar concentrations (IC_{50} = 0.355 and 0.155 μM, respectively, Figure 58). Additionally, compound **159** possessed superior selectivity over FAAH (> 2810 fold) in comparison with **160** (SI = 11).[176] This result is consistent with the hypothesis that the Y-type scaffold favors selectivity of MAGL inhibitors over other serine hydrolases. Interestingly, although compounds **159** and **160** manifested similar potencies against MAGL *in vitro*, compound **159** only slightly elevated 2-AG levels while the administration of **160** resulted in a dramatic increase of 2-AG levels *in vivo*. The different effects on the alteration of 2-AG levels could be attributed to the differences in stability of **159** and **160**.[176] Dilution studies indicated that **160** might covalently and irreversibly inactivate MAGL.[176]

In 2009, two natural terpenoids **161** (pristimerin) and **162 (**euphol) (Figure 58) were reported to inhibit MAGL at submicromolar concentrations (IC_{50} = 0.093 and 0.315 μM, respectively).[47] Specifically, compound **161** rather than **162** was shown to elevate 2-AG levels without alteration of AEA concentrations. Moreover, the administration of **161** led to an obvious increase of 2-AG levels in brain neurons. Compounds **161** and **162** were hypothesized to reversibly interact with Cys208 and Cys201 of

MAGL, respectively. The discovery of **161** and **162** might inspire the exploration of novel reversible agents against MAGL.[47]

As previously described, numerous 1,3,4-oxadiazol-2-one derivatives were disclosed as dual MAGL/FAAH inhibitors. Among these derivatives, compound **163** (Figure 58) exerted optimal potency against human recombinant MAGL (IC_{50} = 0.085 μM), however it also inactivated FAAH with IC_{50} of 0.23 μM.[114,115] Moreover, another 1,3,4-oxadiazol-2-one derivative **164** (CAY10499, Figure 58) was shown to irreversibly and time-dependently inhibit human recombinant MAGL in the nanomolar range (IC_{50} = 0.092 μM).[177] Nevertheless, compound **164** was determined to lack selectivity over FAAH (IC_{50} = 0.076 μM). Although **164** comprises two possible reactive groups, i.e. carbamate and 1,3,4-oxadiazol-2-one, the latter functional group is assumed to be responsible for the principal interaction with MAGL.[177]

Perspectives

Following the observation that EC and FAE-mediated biological responses produce analgesia, anti-inflammation, neuroprotection, gastroprotection, and other protective effects, the elevation of endogenous ligand levels through the inhibition of corresponding hydrolases (FAAH, NAAA, and MAGL) has been regarded as a potential therapeutic approach for pain, inflammation, depression, anxiety, and gastroprotection. Over recent decades, the development of selective FAAH, NAAA, and MAGL inhibitors has made many significant strides. Numerous molecules have been shown to inhibit relevant endogenous hydrolases at single digit-/sub-nanomolar concentrations and exert attractive analgesic, anti-inflammatory or tissue-protective efficacy in animal models. Although MAGL inhibitors (such as **151**, a moderately selective inhibitor) rather than FAAH inhibitors were previously reported to induce CB_1-dependent side effects (hypomotility and hypothermia),[178] a highly selective MAGL inhibitor **152** was recently identified to be devoid of these adverse effects. Compound **151**-induced side effects following high-dose treatment might be attributed to its off-target activity against FAAH, consistent with the previous finding that dual FAAH/MAGL inhibition resulted in cannabimimetic side effects. In this case, highly selective MAGL inhibitors are required for further investigation.

Several FAAH inhibitors have been evaluated in clinical trials: compound **58** developed by Johnson & Johnson (phase II, treatment for depressive and anxiety disorders), compound **65** synthesized by Pfizer (Phase II, treatment for pain, post-traumatic stress disorders and Tourette syndrome, failed to relieve osteoarthritic knee induced pain in clinical trials), SSR-411298 (structure not disclosed) developed by Sanofi-Aventis (Phase II, treatment for cancer pain and depressive disorders), compound **77** synthesized by Merck (phase I, treatment for pain), and V158866 (structure not disclosed) developed by Vernalis (Phase II, failed to treat neuropathic pain) (Table 1).[179] The lack of analgesia in clinical trials brings a new challenge in the development of FAAH inhibitors as antinociceptive agents. Producing analgesia in rodents but failing to treat pain in clinical trials may be attributed to the following reasons: (1) the discrepancies between animal pain models and human pain disorders. For instance, pain in an animal model is usually induced by external stimuli (e.g. pro-inflammatory compound injection, tissue damage), whereas human pain in clinical settings is due to complex factors and associated with other disorders (e.g. osteoarthritis, colitis, cancer). (2) Emotionality significantly influences pain observation in humans rather than in rodents. Anxiety and depression status should be considered in the treatment of patients with pain. (3) Biological responses might differ in various species. Is the activation of CB receptors induced by AEA elevation after FAAH inhibition equivalent to that induced by exogenous CB receptors agonists in humans? Does AEA elevation after FAAH inhibition induce CB-independent effects in humans rather than in rodents? Relevant research needs to be carried out in order to answer these issues. Additionally, detailed clinical data are often not reported, which also makes reasons for failure unclear. Preclinical pain models that are predictive of human pain should be reconsidered if these clinical trial failures are really attributed to species differences. In adition to pain, FAAH inhibitors have shown therapeuticpotential in inflammation, anxiety and depression in numerous preclinical assays. Of note, clinical trials of FAAH inhibitors in other disorders (e.g. depression, anxiety, post-traumatic stress disorders and Tourette syndrome) are in progress. Hence, FAAH inhibition can be considered as an interesting target for neuroprotection and/or anti-inflammation.

Recently, a drug clinical trial tragedy in Bial-Portela company occurred when oral administration of an irreversible FAAH inhibitor **165** (BIA 10-2474, Figure 60) induced severe side effects (i.e. one man died, five other participants had different degrees of brain injuries), which raises suspicions of FAAH inhibitor safety. In late April, a report of the French National Agency for Medicines and Health Products Safety (ANSM)[179] indicated that this tragedy might be attributed to **165**-induced off-target effects in the brain. Other pharmaceutical companies, including Johnson & Johnson, Pfizer, Sanofi-Aventis, Vernalis, and Merck, have developed several FAAH inhibitors which were administered to a large number of patients without observing such severe side effects. After this tragedy, Vernalis disclosed the detailed report of a reversible FAAH inhibitor V158866 (IC_{50} ~60 nM) in clinical trials.[180] V158866 was rapidly absorbed with a $t_{1/2}$ of 9.6-18.3 h post a 5-500 mg oral dose. There were no severe or serious adverse effects observed post a 5-300 mg daily dose and a 50-500 mg repeated dose. Mild adverse effects (i.e. headache, dizziness, somnolence and fatigue) were mainly observed at the highest doses (500 mg daily).[180] Overall, V158866 was tolerated and did not trigger severe adverse effects even post a high dose (500 mg daily). Accordingly, FAAH can be still regarded as a safe target for therapeutic intervention.

The selectivity and potential off-targets of enzyme inhibitors used for clinical trials should be investigated and addressed carefully. In this case, PET ligands could be considered as useful tools for distribution and potential off-target studies in humans. To the best of our knowledge, an irreversible inhibitor seems to increase potential safety concerns due to its long duration of action.[49,111] Therefore, development of reversible rather than irreversible inhibitors appears to be more favorable for clinical application. Nevertheless, their ability to tightly interact with enzyme makes irreversible inhibitors useful tools for the investigation of enzyme function.

Although there is no MAGL or NAAA inhibitor in clinical trials reported to date, several inhibitors of NAAA (such as AM9053 and **130**) or MAGL (such as **152**, **155**, and **156**) were disclosed to manifest attractive drug-like properties. These compounds could be further investigated for their potential preclinical or clinical applications in pain remission, anti-inflammation, and neuroprotection.

Additionally, the metabolic stability of β-lactam-based NAAA inhibitors and the selectivity of carbamate-based MAGL inhibitors are still improvable for the development of potential systemic application.

As another target in the endocannabinoid system, MAGL inhibitor studies might also be helpful for finding the explanation of clinical treatment failure with FAAH inhibitors and investigating potential roles of ECs in different disorders (such as inflammation, pain, anxiety and depression). Do AEA and 2-AG contribute equally to anti-inflammation, analgesia, anti-anxiety, or anti-depression? If not, how is the equilibrium between AEA and 2-AG regulated? More studies are required to answer these questions, since some controversy remains.

Unlike FAAH or MAGL, NAAA inhibitor-induced PEA elevation appears to activate PPAR-α rather than cannabinoid receptors, which confers advantageous properties, such as lack of cannabinoid-dependent adverse effects (e.g. hypomotility and hypothermia). Additionally, PPAR-α activation after NAAA inhibition is followed by negative modulation of NF-κB thereby suppressing the pro-inflammatory cytokines and COX-2 expression, which makes NAAA an ideal target for anti-inflammation and inflammatory pain relief due to the use of COX-2 and pro-inflammatory cytokine (e.g. IL-1β, TNF-α) inhibitors as approved anti-inflammatory medicines over several years.

Biographies

Wei Tuo received his Master's degree in Medicinal Chemistry on the design, synthesis and biological evaluation of potential anti-cancer agents in 2013 at Wuhan University, China, followed by a Ph.D. in Medicinal Chemistry at the University of Lille 2, France, under the supervision of Profs Philippe Chavatte and Régis Millet. His research is focused on the development of new FAAH inhibitors and CB_2 agonists against inflammatory bowel diseases.

Natascha Leleu-Chavain received her Ph.D. degree in Medicinal Chemistry at the University of Lille 1 in 2008. She published her Ph.D. thesis in the "Editions universitaires européennes" and received a Ph.D. award « International Research » from the European Doctoral College of Lille in 2009. After 2

postdoctoral positions at the Rega Institute for Medical Research (KU Leuven, Pr. Herdewijn) and at the ICPAL (Lille, Pr. Millet), she was appointed Assistant Professor of Medicinal Chemistry at the University of Lille 2 and undertakes her research in the Lille Inflammation Research International Center (U995–Inserm). Her research involves mainly the design and synthesis of anti-inflammatory molecules. She received in 2011 a research award and 2 poster awards in national and international symposiums.

John Spencer is a graduate of Sussex, PhD (Michel Pfeffer, ULP, Strasbourg) with a postdoctoral stay at ETH (Togni). He spent 10 years in the pharmaceutical sector as a medicinal chemist, in small pharmaceutical organizations including the James Black Foundation. After, he embarked on an academic career and was recruited as a Reader in Organic Chemistry, first at the University of Greenwich and, in 2012, at the University of Sussex, where he is now Professor of Bioorganic Chemistry. JS has authored over 120 publications, is inventor on 10 patents and editorial board member for *Fut. Med. Chem.* and *Scientific Reports*. His research interests are on the synthesis of chemical probes in medicinal chemistry, mainly cancer. Such efforts employ parallel synthesis, microwave assisted organic synthesis (MAOS) and palladium catalysis.

Supojjanee Sansook graduated with her B.Sc. and M.Sc. in Chemistry from the Prince of Songkla University and after became a lecturer in the Faculty of Science and Technology at the Princess of Naradhiwas University, Thailand. She received a Scholarship from The Royal Thai Government to carry out a PhD with Prof. John Spencer at the University of Sussex and has been working on ferrocene-based bioinorganic probes.

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Abbreviations used

2-AG, 2-arachidonoylglycerol; 3D-QSAR, three dimensional quantitative structure-activity relationship; AA, arachidonic acid, ABHD12, α/β-hydrolase 12; ABHD4, α/β-hydrolase 4; ABHD6, α/β-hydrolase 6; ABP, acyl binding pocket; ABPP, activity-based protein profiling; AEA, anandamide; CCI, chronic constriction injury; COX-2, cyclooxygenase-2; CNS, central nervous system; CFA, complete Freund's adjuvant; CP, cytosolic port; DAG, 1,2-diacylglycerol; DAGL, diacylglycerol lipase; DSS, dextran sulfate sodium; DTT, dithiothreitol; ECs, endocannabinoids; FAAH, fatty acid amide hydrolase; FAAH-2, fatty acid amide hydrolase-2; FAEs, fatty acid ethanolamides; GPCR, G protein-coupled receptor; IL-1β, interleukin-1β; lyso-PLC, lyso-phospholipase C; lyso-PLD, lyso-phospholipase D; MAC, membrane

access channel; MAG, monoacylglycerol; MAGL, monoacylglycerol lipase; MudPIT, multidimensional protein identification technology; *N*-arachidonyl lysophosphatidylethanolamine; NAAA, *N*-acylethanolamine acid amidase; NAPE-PLD, *N*-acylphosphatidylethanolaminephospholipase D; NAT, *N*-acyltransacylase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; OEA, oleoylethanolamide; PAF, platelet-activating factor; PBQ, phenylbenzoquinone; PEA, palmitoylethanolamide; PGH_2, prostaglandin H_2; PGH_2-EA, prostaglandin H_2 ethanolamide; PGH_2-G, prostaglandin H_2 glycerol; PI, phosphatidylinositol; PIP_2, phosphatidylinositol-4,5-bisphosphate, PLA1, phospholipase A1; PLC, phospholipase C; PLC-β, β-isoform phospholipase C; PPAR, peroxisome proliferatoractivated receptor; PTPN22, protein tyrosine phosphatase non-receptor type 22; SI, selectivity index; SNI, spared nerve injury; SNL, spinal nerve ligation; TAGs, triacylglycerols; TBI, traumatic brain injury; TGH, triacylglycerol hydrolase; THL, tetrahydrolipstatin; TLR-4, toll-like receptor-4; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNF-α, tumor necrosis factor-α; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TRPV1, transient receptor potential cation channel subfamily V member 1.

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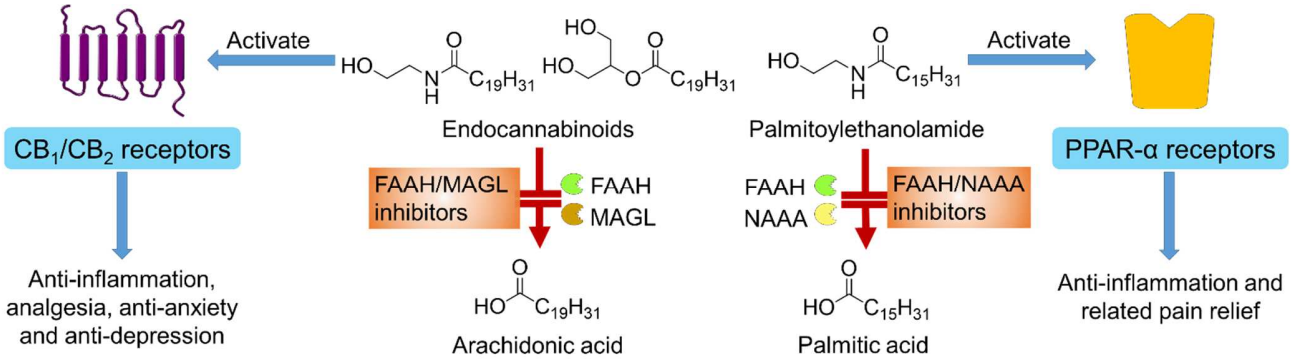

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