REVIEW

Fatty Acid Amide Hydrolase: From Characterization to Therapeutics

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Fatty acid amide hydrolase (FAAH) is an integral membrane enzyme within the amidase-signature family that terminates the action of several endogenous lipid messengers, including oleamide and the endocannabinoid anandamide. The hydrolysis of such messengers leads to molecules devoid of biological activity, and, therefore, modulates a number of neurobehavioral processes in mammals, including pain, sleep, feeding, and locomotor activity. Investigations into the structure and function of FAAH, its biological and therapeutic implications, as well as a description of different families of FAAH inhibitors are the topic of this review.

1. Introduction. – In the early 1990s, *Matsuda* and colleagues [1] identified a DNA sequence encoding a G-protein-coupled receptor that soon revealed to be an endogenous target for Δ^9-tetrahydrocannabinol (Δ^9-THC), the well-known major psychoactive constituent of *Cannabis sativa L.* [2], as well as for other related compounds. This receptor was named 'CB_1 cannabinoid receptor', and its identification constituted one of the major steps towards the characterization of a new signal-transmission system termed 'endocannabinoid system'. Three years later, the molecular characterization of a second receptor, the CB_2 cannabinoid receptor, was reported. These discoveries led to the quest of endogenous molecules able to recognize and/or activate the cannabinoid receptors, assuming that Δ^9-THC binding to this central target, associated with psychotropic effects, cannot be the first role for a receptor selected through evolution. And, indeed, several endogenous lipids, the so-called endocannabinoids (*Fig. 1*), were soon discovered and shown to play a key role in this complex system.

The first part of this special issue of *Chemistry & Biodiversity* is devoted to the discovery of the endocannabinoids and, consequently, we will not describe here extensively all the endocannabinoids. In 1992, *Devane et al.* [3] identified *N*-arachidonoylethanolamine (AEA; *Fig. 1*), known as anandamide, as the first endogenous ligand for the CB_1 receptor [3]. Some 15 years later, anandamide still appears as a major actor of the endocannabinoid system, even though homologues and analogues have been isolated or suggested. Anandamide is now thought to act as a partial agonist of the cannabinoid receptors, without any significant selectivity between the CB_1 and CB_2 cannabinoid receptors. Today, it is widely accepted that anandamide may bind to

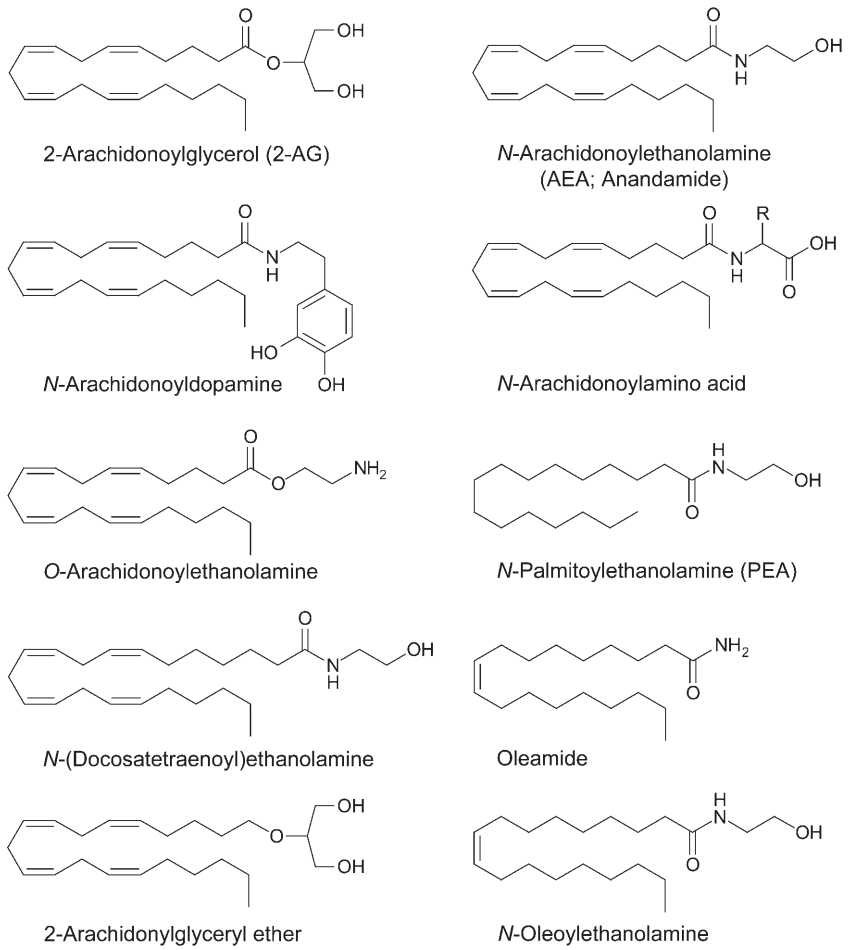


Fig. 1. *Structure of selected endocannabinoids and related compounds*

additional pharmacological targets, including, *e.g.*, TRPV1 vanilloid receptors, the PPAR nuclear receptor, or calcium channels.

In 1995, a known monoglyceride, 2-arachidonoylglycerol (2-AG; *Fig. 1*), has been proposed by two independent teams as an endogenous cannabinoid ligand [4][5]. Unlike anandamide, 2-arachidonoylglycerol acts as a full agonist at both cannabinoid-receptor subtypes, and the detected amounts of 2-arachidonoylglycerol found in different tissues, including brain, are much higher than those of anandamide [6]. Other lipid candidates have also been suggested, including the sleep-inducing compound oleamide, some ether derivatives, and amino acid conjugates [7][8].

Despite much progress in the field, the majority of messengers and targets, as well as the chemical diversity of the endocannabinoids still raise several fundamental questions. Why is this system so diverse and how has it evolved to regulate adequately local levels of endocannabinoids?

2. The Role of Fatty Acid Amide Hydrolase in the Endocannabinoid System. – By the use of a linked version of oleoyl trifluoromethyl ketone, *Cravatt* and co-workers [9] cloned in 1996 a gene encoding fatty acid amide hydrolase (FAAH), a 63 kDa hydrolase. The molecular characterization of this membrane enzyme, whose existence had already been suggested in 1985 in a rat liver microsomal preparation, constituted the first milestone to the discovery of a complex enzymatic system devoted to the regulation of endocannabinoid signaling. To date, in addition to FAAH, at least three enzymes are known to be involved in the termination of endocannabinoid signaling: monoacylglycerol lipase (MAGL), *N*-acylethanolamine acid amidase (NAAA) [10], and fatty acid amide hydrolase-2 (FAAH-2) [11]. Moreover, there is evidence for the involvement of additional hydrolases in regulating endocannabinoid tone, giving rise to the crucial question of the biological meaning of such a diversity of metabolic pathways [12–14]. Indeed, these proteins are able to hydrolyze, at least *in vitro*, a wide and overlapping panel of endocannabinoids, but the precise role played by each enzyme has remained so far unclear. To date, the lack of pharmacological tools, selective inhibitors, as well as knock-out-mice models impede the thorough characterization of the functions of these enzymes.

The complex nature of degradation pathways of endocannabinoids is highlighted when considering the *in vitro* and *in vivo* metabolism of 2-arachidonoylglycerol. *In vitro*, 2-arachidonoylglycerol constitutes a good substrate for FAAH, which, indeed, hydrolyzes this ester much more efficiently than anandamide. *In vivo*, however, it has been shown that neither a disruption of the FAAH gene nor a pharmacological inhibition of FAAH resulted in enhanced 2-arachidonoylglycerol levels (see below) [15][16]. The observation that the levels of 2-arachidonoylglycerol are not modified in mice lacking FAAH highlights the intricate nature of the events controlling the catabolism of endocannabinoids in general, and this complexity likely rationalizes the apparent redundancy of enzymatic pathways.

The tissular distribution of the different enzymes might constitute a first argument to justify, at least in part, the co-existence of different and apparently overlapping routes of degradation of endocannabinoids. For example, following disruption of the FAAH gene, *N*-palmitoylethanolamine (PEA; *Fig. 1*) levels are increased by *ca.* 1000% in brain, but only by 150% in kidney, possibly just reflecting different expression levels of FAAH and NAAA – two enzymes responsible for PEA degradation – in these tissues [17]. Second, active-site accessibility might also be of major concern in the ability of these enzymes to regulate the endocannabinoid levels. For instance, the post- *vs.* presynaptic localization of FAAH and MAGL, respectively, or the intracellular distribution of the protein differ following the enzyme considered. Indeed, FAAH-1 and FAAH-2 are located in the cytosolic and luminal sides of intracellular membranes, respectively, while NAAA is present in the lysosomes or the *Golgi* apparatus. Some of the molecular messengers might need an efficient and still hypothetic transport system to access the catalytic site of the enzyme. Another key point could be that different degradation pathways might regulate signaling mediated through different targets by the same endocannabinoid, *i.e.*, CB_1 or CB_2 cannabinoid receptor at the cellular membrane *vs.* nuclear PPAR receptors. In the *Table*, a literature summary of the localization and substrate-recognition properties of the enzymes of the endocannabinoid system is given.

Table. *Comparison of Metabolic Pathways of Different Endocannabinoids*

	FAAH-1	FAAH-2	NAAA	MAGL	Novel MGL
Substrate selectivity[a])	2-AG > AEA (17) > oleamide (9.7) > OEA (5.6) > PEA (2.1)	oleamide (8.4) > OEA (1.9) > PEA (0.20) ≅ AEA (0.46)	PEA (8.1) > MEA (2.2) > LEA (0.3) > SEA (0.3) > AEA (0.2) > OEA (0.2)	2-AG, 2-OG (AEA not hydrolyzed)	2-AG
Organ distribution[b])	Brain, kidney, liver, small intestine, lung, prostate, testis	Kidney, liver, lung, prostate, (heart), (ovary)	Lung, thymus, spleen, colon, *caecum*, (brain, heart, kidney)	Adipose tissue, adrenal gland, kidney, testis > brain, heart, lung, liver, skeletal muscle	BV-2 (mouse microglial cell line), mouse primary microglia
Pre- *vs.* postsynaptic	Postsynaptic [21]	Unknown	Unknown	Presynaptic [19]	Unknown
Subcellular localization	*Golgi* apparatus/endoplasmic reticulum: cytosolic side of the membrane [11][22][23]	Cellular organelles: luminal side of the membrane [11]	*Golgi apparatus*/lysosome [10]	In cytosolic and particulate fraction after expression in HeLa cells [19]	Mitochondrial fraction [14]

[a]) Values in parentheses refer to activities (in nmol/min/mg protein) measured after recombinant enzyme expression. Data for FAAH-1 and FAAH-2 taken from [11], excepted for 2-arachidonoylglycerol (2-AG) *in vitro* hydrolysis [18]. Note that 2-AG levels are not enhanced in $FAAH^{-/-}$ mice. For acylethanolamine acid amidase (NAAA), data are from [10]. For monoacylglycerol lipase (MAGL), data are from [19]. For the novel monoacylglycerol lipase (MGL), data are from [14]. [b]) FAAH-1 and FAAH-2 (human, RT-PCR) [11]; NAAA (rat, RT-PCR) [10]; MAGL (rat, RT-PCR) [20]. Novel MGL (enzyme activity) [14]. Only the organs for which the expression was tested are indicated. FAAH-1 and FAAH-2 data came from the same study and hatched tissues are organs where no expression was found.

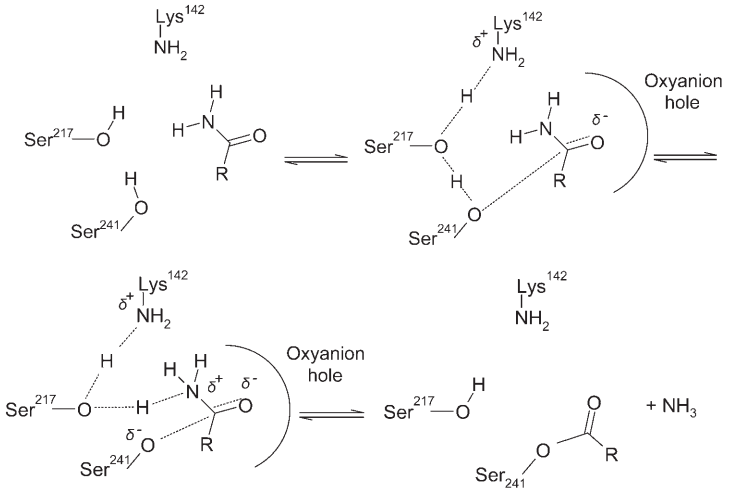
In 2002, a study on a $FAAH^{-/-}$ knock-out-mice model constituted a first breakthrough in the dissection of the pathways regulating endocannabinoid signaling. Results by *Cravatt* and co-workers led to the conclusion that FAAH is the major enzyme responsible for the *in vivo* degradation of anandamide. Anandamide levels enhanced by a factor of 15 were found in the brain of mice invalidated for the FAAH gene. Moreover, these rodents were more sensible to exogenous anandamide than wild-type littermates, and exhibited a phenotypic hypoalgesia [24].

Further studies, in particular those using mice invalidated for the other enzymes belonging to the endocannabinoid system, would undoubtedly constitute a major advance in the understanding of the dynamics that govern the regulation of endocannabinoid levels.

3. Biochemical and Catalytic Properties of FAAH. – The FAAH gene was cloned in 1996 from rat liver plasma membrane. It belongs to a protein family called amidase-signature (AS) family, whose members share a common, conserved amino acid sequence comprising *ca.* 130 residues, the so-called amidase-signature sequence. The AS family is mainly represented among the bacteria and fungi kingdoms, and FAAH was, until recently, the only known mammalian representative of this class of proteins. Transfection of the FAAH gene in Cos-7 cells resulted in the expression of a 63 kDa protein with 579 amino acids, which is able to hydrolyze oleamide (*Fig. 1*) and anandamide, as well as other fatty acid based primary amides [9]. In these transfection experiments, rat FAAH was found to be strongly associated with the membranes of the endoplasmic reticulum/*Golgi* apparatus [22]. Further studies were, therefore, conducted, aimed at the identification of the primary-sequence region of FAAH, allowing anchoring to the membrane. Although the amino acids in positions 9–29 were predicted with the aid of a sequence-analysis software to constitute the FAAH transmembrane domain, deletion of this segment did not release FAAH from the membranes [23][25]. Noteworthy, this so-called transmembrane domain, while not necessary for hydrolase activity, seems to be involved in the self-association of FAAH, as a mutant, lacking the first 30 amino acids, showed a lower level of oligomerization.

The catalytic mechanism of FAAH was first studied by mutagenesis and affinity labeling, emphasizing the role of Ser^{241} as a nucleophile. Interestingly, mutation of each conserved His residue in FAAH did not result in inactive enzymes, indicating that FAAH does not exert its activity through the Ser-His-Asp triad, as commonly shared by other serine hydrolases. Instead, Lys^{142} was reported to serve as a general acid/base catalyst, activating the nucleophile and amino leaving group [26][27]. Although its real contribution remained unclear, Ser^{217} was also found to play a key role in the catalytic mechanism of FAAH, as the S217A mutant displayed a decrease in FAAH activity and reactivity by two to three orders of magnitude. Based on the structural organization of the catalytic triad, as well as on the measure of the activity, nucleophilic strength, pH dependence, and amidase/esterase selectivity of three FAAH mutants, a mechanism was proposed in which Ser^{217} and Lys^{142} cooperate to activate the nucleophilic Ser^{241} (*Scheme 1*). In this scenario, Ser^{217} serves as a bridge between Lys^{142} and Ser^{241} [28]. Subsequent H^+ transfer between Lys^{142} and the N-atom of the substrate allows for the release of the amino group, Ser^{217} acting, once again, as bridging residue. Moreover, these protonation steps seem to occur through a series of concerted events.

Scheme 1. *Proposed Mechanism for the Acylation Step of Amide Hydrolysis Catalyzed by FAAH*



Most interestingly, the role played by Lys^{142}/Ser^{217} in the activation of the Ser^{241} nucleophile seems to constitute the key to rationalize how FAAH has acquired the ability to hydrolyze less-reactive amides at the same rate than their structurally related, far more-abundant and -reactive esters. This triad, therefore, allowed FAAH to evolve into a hydrolase able to access its favorite amide substrates, thereby regulating anandamide/oleamide signaling in an environment mostly populated by esters. The enzymatic mechanism, the concertedness of this process, and the selectivity of FAAH have been deeply explored by a mixed quantum/molecular mechanics study [29].

Very recently, a second membrane-associated AS enzyme, displaying FAAH activity through the Ser-Ser-Lys triad, was identified in two human cell lines, and termed FAAH-2 (originally designated as FLJ31204) [11]. The FAAH-2 enzyme shares 20% sequence identity with FAAH-1, and exhibits overlapping, but distinct, tissue-distribution, substrate-selectivity, and inhibitor-sensitivity profiles compared to FAAH-1. Analysis of genome and gene-expression databases revealed that the gene encoding FAAH-2 is present in primates, as well as in a variety of distantly related vertebrates, but not in murids (mice and rats). These results suggest differences in the enzymatic complexity of fatty acid amide catabolism across mammalian species. This finding opens, therefore, new insights for the design of novel inhibitors of endocannabinoid degradation.

4. Three-Dimensional Structure of FAAH. – In 2002, the structures of three AS enzymes – FAAH [30], malonamidase E2 (MAE2) [31], and peptide amidase (PAM) from *Stenotrophomonas maltophilia* [32] – were reported, allowing a comparison of the structural features of these enzymes and a detailed view on their respective active sites and catalytic mechanisms. All three enzymes share a common core fold comprised of a twisted β-sheet consisting of eleven mixed strands surrounded by a number of α-helices (*Fig 2*,*a*).

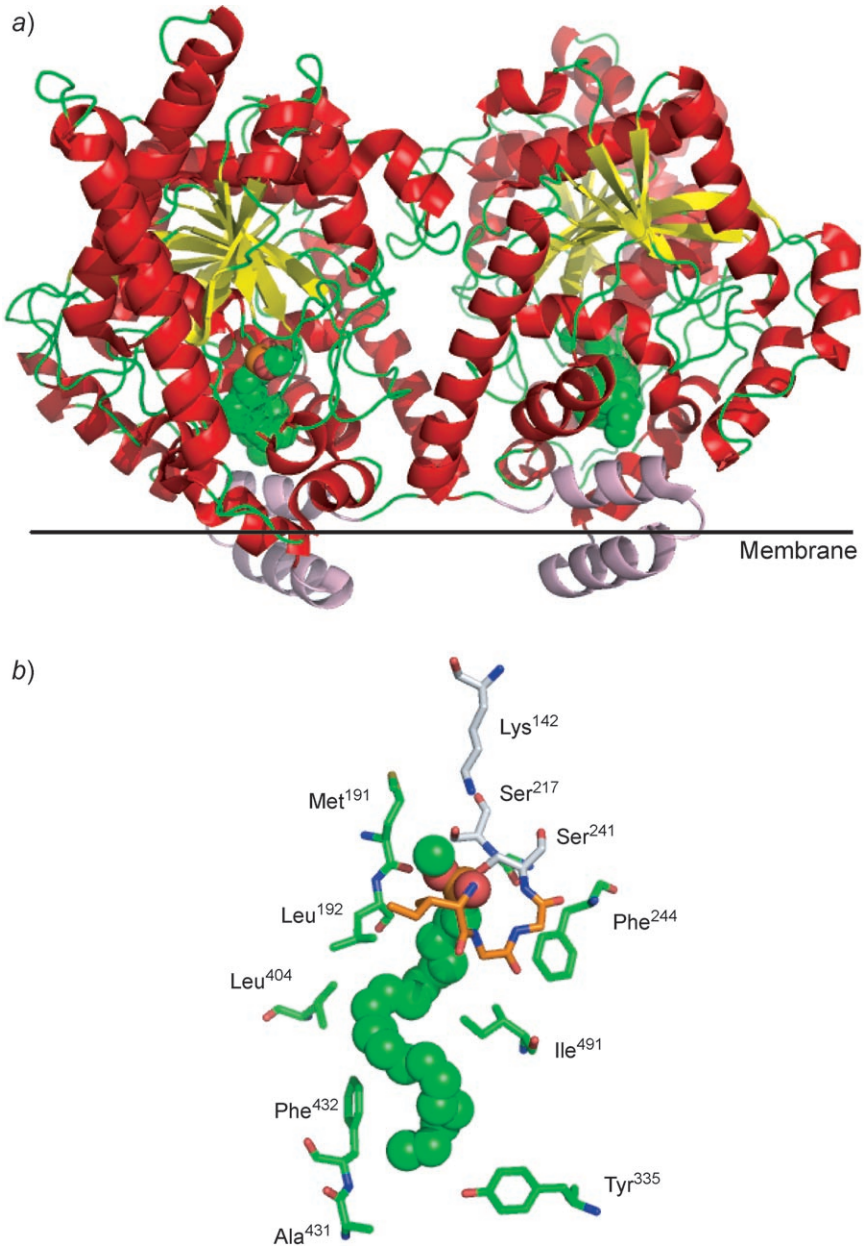


Fig. 2. a) *Crystal structure of the FAAH dimer* (helices in red, sheets in yellow) *with a bound methyl arachidonyl fluorophosphonate* (MAFP; green) *as inhibitor* (*PDB* entry: 1MT5). The membrane-binding domain of FAAH is shown in light pink). b) *Active site of FAAH in the complex with MAFP.* The catalytic triad and the oxyanion hole (Gly^{240}-Gly^{239}-Ile^{238}) are shown in grey and orange, resp.

The X-ray crystal structure of the truncated N-terminal rat FAAH variant (residues 30–579; 2.8 Å resolution; *Fig. 2,b*), in complex with the active-site-directed inhibitor methoxy arachidonyl fluorophosphonate (MAFP; see also *Scheme 2* below) revealed some interesting features, particularly the dimeric nature of FAAH and the presence of two crucial channels. The so-called 'membrane channel' or 'acyl chain binding channel' (ACB) is thought to accommodate the hydrophobic moieties of the substrates, while the other, the 'cytoplasmic-access channel', allows the more hydrophilic ethanolamine leaving group to reach the cytosolic compartment.

In their interesting discussion, *Bracey* and co-workers [30] proposed a scenario recounting how – through incorporation of a lipophilic plateau constituted of α-18 and α-19 helices – FAAH evolved from the soluble MAE2 to a membrane enzyme able to cleave lipophilic substrates. Indeed, the above-mentioned membrane channel, mainly bordered by lipophilic amino acids, is in close communication with the hydrophobic plateau, likely anchoring FAAH to the membrane, thereby allowing direct access of the substrates to the active site. Moreover, the three-dimensional structure highlights the catalytic triad and an oxyanion hole made of Ile^{238}, Gly^{239}, Gly^{240}, and Ser^{241} backbone residues stabilizing the negatively charged O-atom from the amide at the tetrahedral-intermediate step.

5. FAAH in Obesity and Drug/Alcohol Addiction. – Genetic polymorphism has received a great attention recently, since the progress in molecular biology made easier the detection of mutations and, especially, of single-nucleotide polymorphism. Such polymorphisms are tracked to delineate sensibility to pathologies, behavior, and/or impaired or enhanced drug metabolisms or drug responses. The relation between the genetic polymorphism of FAAH and drug/alcohol addiction or obesity was first studied by *Sipe et al.* [33][34].

It is known since several years that the endocannabinoid system is implicated in the pathways that lead to addiction. Δ^9-THC activates the mesolimbic dopaminergic transmission in a similar way as heroin does [35]. Besides this, $CB_1^{-/-}$ mice exhibit reduced withdrawal symptoms, when deprived of morphine [36], and HU210 (a CB_1- and CB_2-receptor agonist) and SR141716A (a CB_1-receptor antagonist) provoke or attenuate relapse to cocaine dependence after withdrawal, respectively [37]. In a similar way, CB_1 signaling is involved in alcohol intake in rodents, as evidenced by studies using SR141716A [38–40] or CB_1-receptor knock-out mice [41]. The implication of CB_1 receptors, therefore, suggests that endocannabinoid tone and, thus, FAAH might modulate these events.

Indeed, *Sipe et al.* [34] observed the higher occurrence in street-drug users and drug/alcohol addicts of a single nucleotide polymorphism (cytosine 385 → adenosine) in the FAAH gene. This resulted in a P129T mutation, in a region of FAAH primary sequence otherwise highly conserved between species. Only homozygous FAAH 385A/385A subjects exhibited a clinical phenotype. However, no correlation between C385A mutation and other behavioral or psychiatric disorders (*i.e.*, schizophrenia, depression, alcohol problem alone, *etc.*) could be found.

After expression in *Escherichia coli*, this mutant and wild-type FAAH exhibited similar kinetics parameters. Similarly, heat- and urea-denaturation studies did not reveal any difference in the stability of the two enzymes. Taken together, these results

suggested that neither catalytic properties nor global stability of FAAH were affected by the P129T mutation. However, Pro^{129} is an amino acid present at the surface of the protein, and after expression in Cos-7 cells, mutant FAAH displayed an increased proteolytic sensitivity to trypsin compared to the wild-type enzyme. In an in-depth study on P129T mutant, the same group reported some interesting biochemical properties of the protein [42]. First, T-lymphocytes of wild-type patients displayed lower amounts of anandamide hydrolase activity than wild-type subjects. This fact is correlated with the reduction of FAAH expression, as assessed by Western blot. The same results were observed in Cos-7 cells. Moreover, in these cells, the levels of mRNA were the same for the two cDNAs, an evidence that transcription could not account for the reduced FAAH levels. Second, use of an *in vitro* translation system indicated that translation was achieved with similar efficiencies for both mRNA transcripts. As transcriptional and translational steps are unlikely to modify global FAAH quantities, it was postulated that a post-translational mechanism is involved. However, the turnover of FAAH was measured using [^{35}S]methionine, and found to be equivalent for both mutant and wild-type enzymes, although an increased trypsin sensitivity of the P129T protein had been reported by the same authors in a previous study. Therefore, the reduced cellular FAAH activities were suggested to be due to a post-translational event preceding the complete folding of the protein.

Other behavior involving a rewarding component might be subject to regulation by the endocannabinoid system and, therefore, by FAAH. In particular, this system has been shown to upregulate feeding behavior at peripheral (adipose tissue and gastrointestinal tract) but also at central levels. In the *hypothalamus*, endocannabinoid signaling modulates the level of several (an)orexigenic mediators, and in the mesolimbic system, activation of dopaminergic reward/craving circuits leads to a strengthening of the motivation to eat (for a review, see [43]). On the other hand, the *European Medicines Agency* has recently approved the use of the CB_1-receptor antagonist rimonabant (SR141716A; *Acomplia*®) in the field of obesity treatment [44a][1]). Indeed, a study conducted with 2,667 subjects led to the observation of a strong correlation between the FAAH 385A/385A genotype and overweight/obesity. This effect was observed in both white and black populations, but, interestingly, P129T mutation was not associated to weight disorders in the Asian subjects. When considering the entire population, the median body mass index was significantly higher in the FAAH 385A/385A homozygous group than in the heterozygous/wild-type group ($p < 0.0001$) [33].

In 2007, the implication of FAAH in alcohol consumption was documented in a study on $FAAH^{-/-}$ knock-out mice [45]. In this model, the mice exhibited a greater preference for alcohol and consumed more ethanol compared to wild-type mice. These effects were neither due to a decrease of alcohol-aversive properties nor to a difference in the severity of acute withdrawal. Administration of URB597 evidenced that the effects on alcohol intake were imputable to the loss of FAAH activity, and not to putative changes in the expression of other genes, a phenomenon likely to occur as a compensatory mechanism following any gene deletion. In a concomitant study,

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[1]) For a discussion, see the review by *Muccioli* in the first part of this special issue of *Chemistry & Biodiversity* [44b].

Basavarajappa et al. [46] reported a sex-related enhanced ethanol consumption in female FAAH knock-out mice [46]. To further study the mechanism underlying this phenomenon, they studied CB_1 signaling in the limbic forebrain, a region known to be important for alcohol addiction. While the functional CB_1-receptor density decreased after ethanol exposure in both male and female WT mice as well as in male $FAAH^{-/-}$ mice, it remained unchanged in female $FAAH^{-/-}$ mice. Endocannabinoid and dopaminergic systems work in concert to activate the reward process (for a review, see [47]), and there is evidence that sex hormones modulate dopaminergic functions [48]. On the other hand, progesterone and estrogen were reported to enhance anandamide signaling and to modulate FAAH expression [49][50]. These data, thus, suggest the presence of secondary sex-linked mechanisms – whether or not related to the endocannabinoid system – that may influence the regulation of addiction/reward pathways by FAAH.

Taken together, these results further confirmed the role of the endocannabinoid system in the neural events leading to overweight and drug abuse or alcoholism. Although it is clear that such problems result from a combination of genetic as well as environmental and social factors, the endocannabinoid system may constitute a key to the development of pharmaceutical tools to treat such disorders.

6. FAAH and Reproduction. – Intake of Δ^9-THC, *e.g.*, by chronic marijuana (cannabis) smoking, impairs the reproductive physiology [51–53]. Therefore, since the discovery of the endocannabinoid system in the early 1990s, great efforts were made to study the way the endocannabinoid system is involved in reproduction. Soon after its discovery, anandamide was shown to diminish the sperm-fertilizing capacity in sea urchins [54]; and in 1996, *Yang* and co-workers [55] observed that CB_1-receptor activation interfered with pre-implantation mouse-embryo development *in vitro*. Endocannabinoid signaling was soon identified as crucial for uterine receptivity for embryo implantation in mouse, with high anandamide levels associated to an impairment of the implantation process. This effect was inhibited by the antagonist SR141716A, indicating that anandamide is acting through the CB_1 receptor [56]. Moreover, anandamide has an inhibitory activity on the secretion of pituitary hormones (luteinizing hormone and prolactin) in rodents, an effect mediated through CB_1 receptors in the *hypothalamus* [57].

The importance of the, mainly deleterious, effects of anandamide on early pregnancy and on neuroendocrine function underlined the possible leading role played by FAAH in reproduction. Indeed, in a first study by *Paria et al.* [58], decreased anandamide hydrolase activity was reported in the uterus on day 5 of pseudopregnancy (a period where uterus is refractory to embryo implantation), compared to days 1–4. Accumulation of FAAH mRNA was found in the uterus during the first four days of pregnancy, as well as in the implanting blastocyst, suggesting the need to control anandamide levels during this period [59].

In their comprehensive review, *Maccarrone* and *Finazzi-Agro* [60] presented FAAH as a '*key integrator of fertility signals*', modulating the dual action of anandamide, which promotes uterine epithelium changes needed for normal gestation, whereas permitting embryo implantation when present at lower levels. Indeed, FAAH and, therefore, also anandamide seem to be one of the targets of progesterone, which

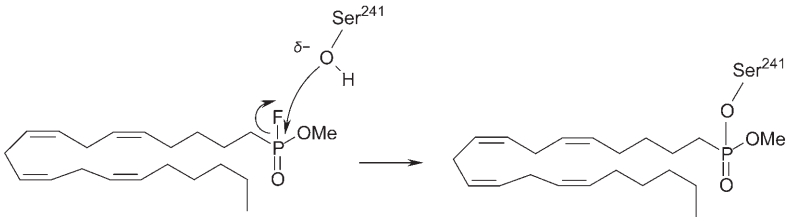
upregulates FAAH expression in human T-cells through the transcription factor Ikaros, an effect enhanced or decreased by the well-known pro-fertility Th2 and anti-fertility Th1 cytokines, respectively. Reduction of leukemia inhibitory factor release (an interleukin promoting embryo implantation and survival), following CB_1-receptor activation at the surface of T-cells, seems to account for the main part of the deleterious effects of anandamide on implantation (for more information on FAAH and reproduction, see [60][61]).

In humans, decreased FAAH expression and activity, as well as elevated anandamide concentrations are correlated with spontaneous abortion [62]. Accordingly, the recent article by *Wang* and co-workers [63] on $FAAH^{-/-}$ mice outlined the capital importance of FAAH in the (pre-)implantation process and fertility. Indeed, an increase in anandamide level in knock-out mice resulted in altered oviductal embryo transport and expression of genes required for differentiation, blastocyst implantation, and reduced fertility.

The intimate relationship between endocannabinoid signaling and reproductive physiology has important implications for the development of new drugs targeting this enzyme (and the endocannabinoid system in general). Actually, considering the pronounced deleterious effects caused by a decrease of anandamide hydrolase activity, and given that medicinal-chemistry efforts have been mainly directed toward the conception of inhibitors of this enzyme, a careful examination of the risks presented by such compounds on patients should be made. Beside this, the positive effect of FAAH at different stages of early pregnancy opens new prospects in the treatment of fertility problems.

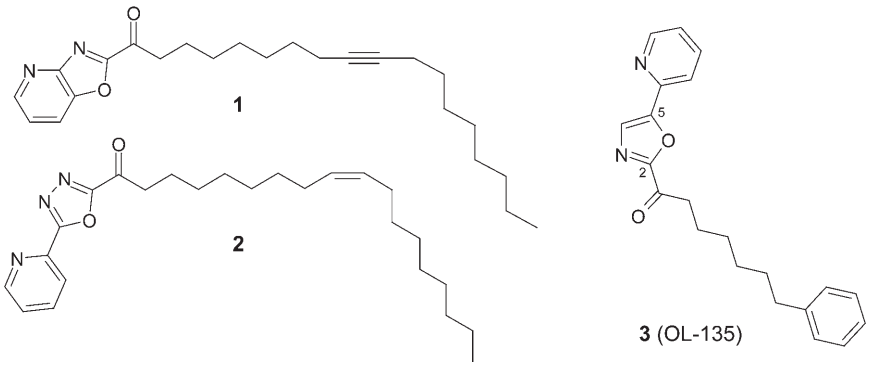
7. FAAH Inhibitors and Structure–Activity Relationships. – Since the discovery of FAAH and the awareness of its therapeutic potential, much attention has been paid to the design of selective and/or potent FAAH inhibitors. These include reversible inhibitors such as arachidonoyl trifluoromethyl ketone [64] or '2-octyl γ-bromoacetoacetate' [65], and irreversible inhibitors like fatty acid sulfonyl fluorides (*e.g.*, AM374) [66] or methyl arachidonyl fluorophosphonate (MAFP, *Scheme 2*) [67]. Mixed inhibitors of FAAH, like diazomethyl derivatives of fatty acid [68] and arachidonoyl serotonin [69], have also been developed. Further, since 2000, several new classes of FAAH inhibitors were described in publications and patents.

Scheme 2. *Inhibition of FAAH by MAFP*



7.1. *Heterocyclic Inhibitors with α-Keto Groups.* – Based on *Edwards*' 'α-keto-heterocycle' protease inhibitors [70], potent reversible competitive inhibitors were developed, combining an unsaturated acyl chain and an 'α-keto-*N*4-oxazolopyridine',

with incorporation of a second weakly basic N-atom. This system showed potency in the subnanomolar range, with K_i values dropping below 200 pM [71]. These compounds are two to three orders of magnitude more potent than the corresponding trifluoromethyl ketones. For example, one of these compounds, the Δ^9-octadecynoic acid derivative **1**, exhibited a K_i value of 140 pM [72], which led to two worldwide patents [73][74].



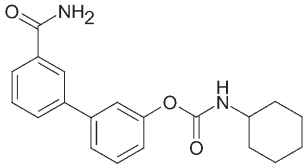
The electrophilic C=O group and the degree of α-substitution are essential for enzyme inhibition [75]. Indeed, deoxo analogues did not show any inhibition at a concentration of 100 μM, and the α-mono- and dimethyl derivatives lowered the potency 10- and 100-fold, respectively. Moreover, the potency is strongly dependent on the hydrophobicity of the flexible acyl chain. The inhibitors and their analogues were found to be quite selective towards other serine hydrolases, based on a proteomic assay, the most potent and selective ones being 2-pyridyl-subsituted 1,3,4-oxadiazoles such as compound **2** [76][77]. In the same family, the inhibitor **3** (OL-135) displayed an exceptional combination of potency ($K_i = 0.0047$ μM towards rat-recombinant FAAH) and *in vivo* selectivity [72][78].

Molecular-modeling studies based on Monte Carlo simulations [78][79] demonstrated that incorporation of pyridine at C(5) of the 2-keto-oxazole and 2-keto-1,3,4-oxadiazole derivatives significantly enhances binding affinity by formation of a H-bonded array between the pyridyl N-atom and Lys^{142} and Thr^{236}. Moreover, the oxazolyl O-atom is H-bonded to the OH group of Ser^{217} of the catalytic triad, which accepts a H-bond from the protonated N-atom of Lys^{142}. Following the attack of Ser^{241} on the C=O group of **1**, the negatively charged O-atom is H-bonded to the backbone N-atoms of Ile^{238}, Gly^{239}, Gly^{240}, and Ser^{241}. Further, the lipid chain is surrounded by numerous hydrophobic and aromatic residues. These results also attribute the activity boost upon substitution of the oxazole by an oxadiazole moiety to reduced steric interactions in the active site and a lower torsional-energy penalty upon binding.

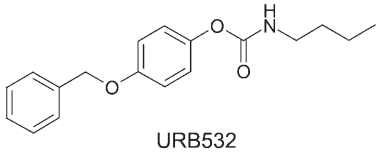
It was also shown that the increased electrophilic character of the C=O group in 2-position, imparted by the electron-withdrawing substituent at C(5), increasing the strength of the covalent bond formed with the OH group of the catalytic Ser^{241} residue, enhanced the stability of the tetrahedral adduct and lowered the K_i value [80]. A study targeting the 5-position of the oxazole moiety of **3** showed that the *meta*-position is optimal for substitution. Substituents on the pyridine ring were also examined. A series of small, non-aromatic substituents in 5-position revealed that the K_i follows a well-

defined correlation with the *Hammett* constants, in which electron-withdrawing substituents enhance potency, leading to inhibitors with K_i values as low as 400 pM [81].

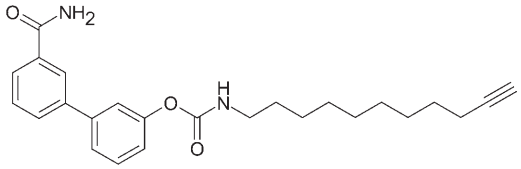
7.2. *Carbamate-Type Inhibitors.* FAAH is blocked by a variety of serine hydrolase inhibitors, including compounds with activated C=O groups [71] or carbamate esters such as analogues of the anticholinesterase agent carbaryl, which resulted in a new class of drugs [16][82]. URB597, the most-potent member [83–85], inhibited FAAH activity with an IC_{50} value of 4 nM in brain membranes, and with 0.5 nM in intact neurons, but did not affect three other serine hydrolases. The potency of carbamates is modulated by the shape of the rigid aromatic moiety. Indeed, when the biphenyl unit is replaced with a 5-phenylpentyl group, representing the most effective acyl chain in the 'α-keto-heterocycle' series, activity was lost. Moreover, a 3D-QSAR analysis on the alkylcarbamic acid aryl esters showed that the size and shape of the *O*-aryl moieties are correlated with FAAH inhibitory potency [86]. In addition, introduction of small polar groups in *meta*-position of the distal phenyl ring, and in *para*-position of the proximal phenyl ring, improved inhibition [87][88].



URB597



URB532

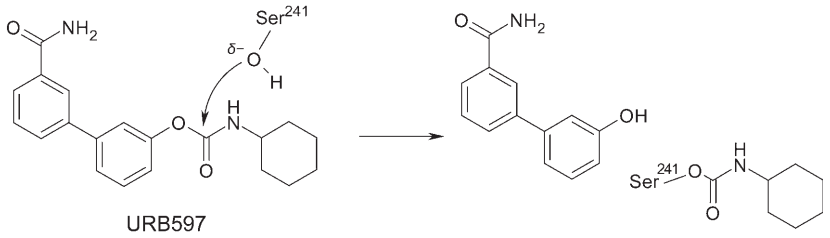


JP104

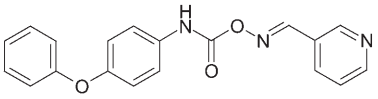
Unlike the 'α-keto-heterocycle' inhibitors, the carbamates inhibit FAAH activity through irreversible interaction based on nucleophilic attack of the active Ser^{241}, as shown in *Scheme 3* for URB597. Biochemical evidence [89] showed that these inhibitors covalently modify the active site by adopting an orientation opposite of that originally predicted from modeling [87]. Indeed, the *O*-biaryl substituents would reside in the cytoplasmic-access channel (rather than in the acyl-chain-binding channel), where they would be susceptible to enzyme-catalyzed protonation to enhance their function as leaving groups. Based on these results, a series of carbamates were designed, in which the *N*-cyclohexyl unit was replaced with various *N*-alkyl groups mimicking the acyl chains of anandamide. These compounds generally exhibited enhanced potency (*e.g.*, JP104; see chemical formula above).

Bristol-Myers Squibb has designed oxime carbamoyl derivatives as FAAH inhibitors, and compounds **4** and **5** exhibited IC_{50} values <10 nM [90]. Compound **5**, at a dose of 40 mg/kg, suppressed the development of thermal hyperalgesia induced by

Scheme 3. *Inhibition of FAAH by URB597*



URB597



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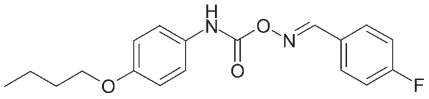

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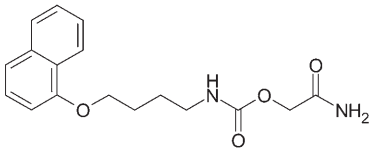

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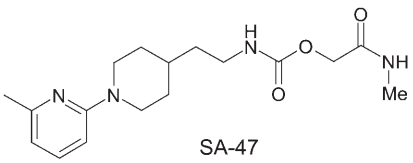
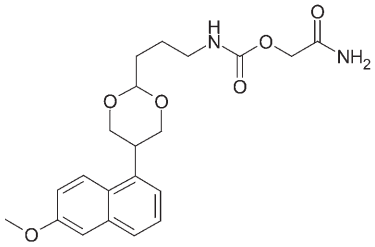
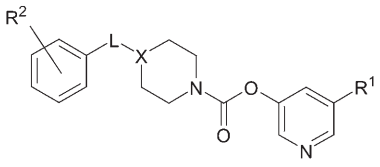


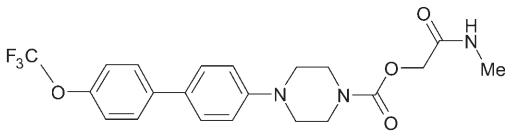
SA-47



SA-72



X = N, CH



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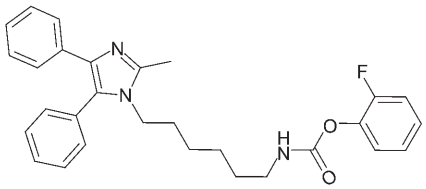


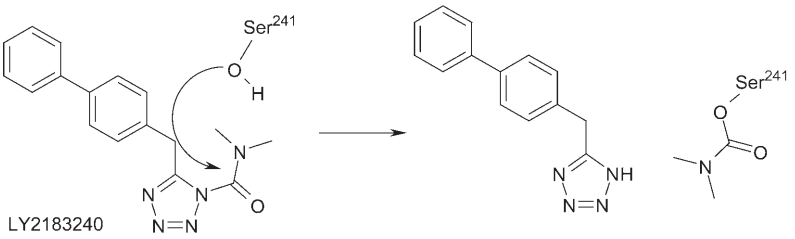
BMS-1

paw carrageenan. Other carbamate compounds from *Sanofi-Aventis*, including SA-72 and **6**, showed IC_{50} values of 47 nM [91–93].

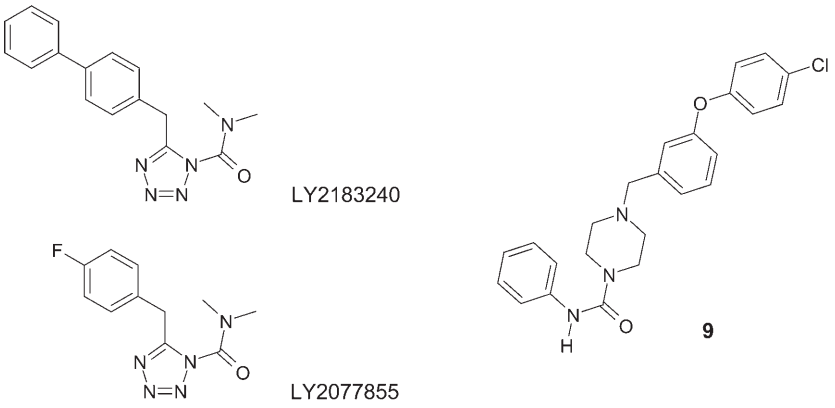
Carbamate compounds such as SA-47 or **7**, with piperazine or piperidine moieties, were recently developed as potent FAAH inhibitors [91–95]. Compound **7**, for example, has an IC_{50} value of 7 nM. Scientists at *Bristol-Myers Squibb* reported a distinct set of carbamate inhibitors comprising a bisarylimidazolyl group [96], promoting analgesia in both acute- and chronic-pain models in rodents. One representative, BMS-1, has an IC_{50} value <10 nM.

7.3. *Urea-Based Inhibitors.* The inhibitor LY2183240 (*Scheme 4*), a heterocyclic urea inhibitor of the putative anandamide transporter, was found to be a potent covalent inhibitor of FAAH, with an IC_{50} value of 12.4 nM, promoting analgesia *in vivo* [97]. This compound inactivates FAAH by carbamoylation of the nucleophilic Ser^{241}, in analogy to the mechanism of action of URB597.

Scheme 4. *Inhibition of FAAH by LY2183240*

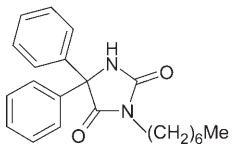


Unlike carbamate-based inhibitors, LY2183240 and its analogue LY2077855 are non-selective, and also inhibit other brain serine hydrolases *in vivo* [98]. More-global screens using activity-based proteomic probes identified several additional serine hydrolases that are also inhibited by LY2183240. Piperazinyl and piperidinyl urea inhibitors of FAAH were developed by *Janssen Pharmaceutica* [99], an example being **9**, which showed an IC_{50} value of 0.9 nM.



7.4. *2-Thioxoimidazolidin-4-ones and Imidazolidine-2,4-diones as Inhibitors.* Derivatives of 2-thioxoimidazolidin-4-one and imidazolidine-2,4-dione were evaluated as

FAAH inhibitors [100]. For instance, 3-heptyl-5,5-diphenylimidazolidine-2,4-dione (**10**) and 5,5-diphenyl-3-tetradecyl-2-thioxo-imidazolidin-4-one (**11**) showed pI_{50} values of 5.12 and 5.94, respectively, and are devoid of affinity for the cannabinoid receptors. They act reversibly as competitive inhibitors of FAAH, without being hydrolyzed by the enzyme. Molecular-modeling studies indicated that the alkyl chains replace the lipid chain of MAFP in the acyl-chain-binding channel [101]. One of the phenyl rings points toward the catalytic triad, and the other fills a small lipophilic pocket (composed of Ile^{491}, Phe^{194}, Leu^{404}, Leu^{192}, Gly^{485}, and Leu^{401}); and the 2-thioxo or C=O group, respectively, points just above Phe^{381}.



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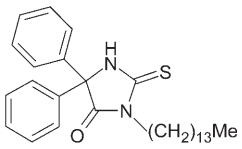

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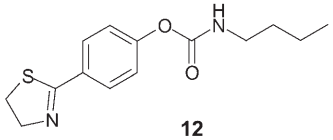
7.5. *Other Inhibitors.* Recently, *Saario et al.* [102] identified some new FAAH inhibitors, with IC_{50} values in the range 0.5–22 μM, from virtual screening of the endocannabinoid system. The compounds were docked to the FAAH crystal structure to investigate their binding mode. The most-frequent interaction partners appeared to be Gly^{239}, Ile^{238}, Met^{191}, and the catalytic residues Ser^{217} and Ser^{241}. In addition, Lys^{142} was close to almost all ligands. Compound **12** (IC_{50} = 0.52 μM) contains a carbamate group. Similarly, compound **13**, which does not possess a C=O moiety, inhibits FAAH with an almost identical IC_{50} value of 0.69 μM. This last result seems to suggest that a C=O group is not essential for inhibiting FAAH.



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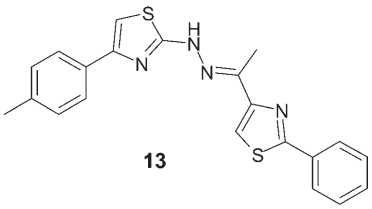

13

Beside substrate analogues and numerous potent inhibitors, other molecules were surprisingly found to be active on FAAH. Indeed, Δ^9-THC, cannabidiol, and cannabinol, compounds found in *Cannabis sativa* L., inhibit FAAH, although with relatively low potencies [103][104]. Other weak inhibitors include the non-steroidal anti-inflammatory drugs suprofen, ketorolac, ibuprofen, or indomethacin, as well as the general anesthetic propofol [105–108].

The selectivity of representative FAAH inhibitors was determined using a proteomic strategy known as activity-based protein profiling (ABPP) [98]. Several inhibitors, including the carbamates SA-47 and SA-72 (see above), were found to be exceptionally selective, while others, such as URB597, BMS-1, **3** (OL-135), and LY2077855 (see above) are less selective, displaying multiple off-targets like carboxylesterase isozymes. Considering that many carboxylesterases hydrolyze a

variety of ester-containing drugs and prodrugs, this suggests that several FAAH inhibitors could exhibit drug–drug interactions when developed as therapeutic agents.

8. *In vivo* Effects of FAAH Inhibitors. – The 'on-demand' nature of endocannabinoid biosynthesis [109–111] has led to the idea that a compound enhancing the endocannabinoid tone through FAAH inhibition could permit a pharmacological action to arise 'where needed', without the CB_1-associated central side effects. Along this line, several FAAH inhibitors have been tested *in vivo* for their analgesic, anti-inflammatory, as well as anxiolytic and antidepressive properties.

First, URB597 (see above) showed interesting therapeutic properties in rodent anxiety models. Indeed, although administration of URB597 (0.3 mg/kg, i.p.) to rats decreased FAAH activity and enhanced brain anandamide levels, it did not produce hypothermia, catalepsy, hyperphagia, or hypomobility, effects classically observed following cannabinoid-agonist administration [16][83]. In the elevated zero-maze test and isolation ultrasonic-emission test, URB597 (0.05–0.1 mg/kg, i.p.) elicited anxiolytic-like responses that were antagonized by rimonabant. Thus, the question arises of how these anxiolytic effects are mediated.

It is known that in the *amygdala*, a brain region involved in the control of emotional states, anandamide levels are enhanced in response to anxiogenic situations [111]. A hypothesis to rationalize the anxiolytic effect of URB597 is that increased anandamide tone leads to an inhibition of glutamatergic and/or GABAergic interneurons, which results in an inhibition of glutamatergic efferent structures from *amygdala* [112]. Beside this, in the forebrain, binding of anandamide to presynaptic CB_1 receptors could downregulate the release of the anxiogenic peptide cholecystokinin-8 by GABAergic neurons in the synapse [16].

URB597 also gives rise to an antidepressant activity in the tail-suspension and the forced-swim test, two widely used tests for antidepressants. Although the mechanism underlying these antidepressive effects are not fully elucidated, it is thought to be mediated through enhancement of noradrenergic and serotoninergic transmission in the midbrain [113].

Besides its anxiolytic and antidepressant activities, URB597 has also been tested for its analgesic properties in rodents. In the hot-plate test, a mild analgesic effect is observed [16]. On the other hand, URB597 reduces allodynia and hyperalgesia in an inflammatory, but not neuropathic, model of pain, *via* a CB_1/CB_2-dependant mechanism [85]. And last, URB597 enhances stress-induced analgesia (SIA), an adaptive response to stress mediated at the periaqueductal gray level, involving endocannabinoids and endogenous opioids [114]. URB-597 is currently being tested in Phase-I clinic by *Kadmus Pharmaceuticals* under the name *KDS-4103* (http://www.kadmuspharma.com; online access, as of February 13, 2007).

Besides, FAAH inhibitors could constitute a promising strategy in the regulation of mood disorders, and the analgesic and anti-inflammatory properties of such compounds are also well-documented. On the one hand, pain and inflammation are largely under the control of the endocannabinoid system, through mechanisms involving activation of both central and peripheral CB_1 and CB_2 receptors (for a review, see [115]). On the other hand, anandamide and related endogenous messengers only produce weak and transient effects due to the rapid action of metabolic enzymes [116]. This suggests that

pharmacological or genetic disruption of FAAH activity would be of great interest in the treatment of pain/inflammation processes. Indeed, *Lichtman* and co-workers reported a phenotypic hypoalgesia in $FAAH^{-/-}$ mice, compared to wild-type animals. This effect was observed in the hot-plate, tail-immersion, and formalin tests, as well as in the carrageenan model of inflammation [24]. The highly potent and selective FAAH inhibitor **3** (OL-135; see above), developed by the same group, allowed a profound increase of anandamide levels in the brain and spinal cord, and displayed CB_1-dependant antinociceptive effects in the hot-plate, tail-immersion, and formalin tests [72].

Taken together, the absence of the side effects classically associated with CB_1 agonists, and the interesting pharmacological properties elicitated by FAAH inhibitors, could give rise to a novel and innovative approach in the field of pain management, as well as for patients suffering from stress and depressive disorders.

9. Conclusions. – The membrane-based serine hydrolase FAAH, a protein from the amidase-signature family, is one of the enzymes responsible for the metabolism of endocannabinoids and lipid messengers, and is, thus, involved in the control of a wide range of physiological responses. Despite the fact that some important breakthroughs have led to an in-depth knowledge of FAAH as well as to the discovery and design of potent inhibitors – some of them being promising therapeutic tools currently in clinical trials – several important questions remain to be answered. Two major such questions concern the true physiological specificities of FAAH-1 and FAAH-2 *vs.* the other enzymes of the endocannabinoid system, as well as the dynamics driving the hydrolysis of FAAH substrates. No doubt, the story will continue.

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