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Synthesis and Evaluation of Paracetamol Esters As Novel Fatty Acid Amide Hydrolase Inhibitors

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Fatty acid amide hydrolase (FAAH) is the key hydrolytic enzyme for the endogenous cannabinoid receptor ligand anandamide. The synthesis and evaluation for their FAAH inhibitory activities of a series of 18 paracetamol esters are described. Structure−activity relationship studies indicated that the ester (**33**) with a 2-(4-(2-(trifluoromethyl)pyridin-4-ylamino)phenyl)acetic acid substituent was the most potent analogue in this series. The compound inhibited FAAH activity in a competitive manner with a K_i value of 0.16 μM. The compound was also able to inhibit the FAAH activity in rat basophilic leukemia cells as assessed by measuring either the hydrolysis of anandamide, the FAAH-dependent cellular accumulation of anandamide, or the FAAH-dependent recycling of tritium to the cell membranes. The compound also inhibited the activity of monoacylglycerol lipase (MGL), the enzyme responsible for the hydrolysis of the endogenous cannabinoid receptor ligand 2-arachidonoylglycerol, with an IC_{50} value of 1.9 μM. It is concluded that the compound may be a useful template for the design of potent novel inhibitors of FAAH.

Introduction

Fatty acid amide hydrolase (FAAH[a])[1,2] is a membrane-bound serine hydrolase that catalyzes the deactivating hydrolysis of the fatty acid ethanolamide family of signaling lipids,[3,4] which includes endogenous ligands for cannabinoid receptors such as arachidonoylethanolamide (anandamide, AEA)[5] and peroxisome proliferator-activated receptors α (oleoylethanolamide[6,7] and palmitoylethanolamide[8,9]).

Endogenous FAAH substrates such as AEA serve key regulatory functions in the body and have been implicated in a variety of pathological conditions including pain,[10−12] inflammation,[13] sleep disorders,[14,15] anxiety, depression, and vascular hypertension,[16−18] and there has been an increasing interest in the development of inhibitors of this enzyme.[19−25] Different structural classes of FAAH inhibitors have been reported including α-ketoheterocycles,[26−33] (thio)hydantoins,[34] piperidine/piperazine ureas,[35] and carbamate derivatives.[34−42] When tested, these compounds have been shown to efficacious in models of inflammatory, visceral, and in some cases neuropathic pain without producing the central effects seen with directly acting cannabinoid receptor agonists.[20,21,36]

An intriguing aspect of FAAH inhibition is that some currently marketed nonsteroidal anti-inflammatory drugs (NSAIDs) have also been shown to inhibit FAAH. The NSAID ibuprofen is a weak inhibitor of FAAH,[43,44] but can be used as a template for the design of more potent compounds. In this respect, we have reported an analogue of ibuprofen, Ibu-am5 (*N*-(3-methylpyridin-2-yl)-2-(4′-isobutylphenyl)propionamide), that retaines the cyclooxygenase (COX) inhibitory properties of ibuprofen but is a more potent FAAH inhibitor.[45]

The contribution of the endocannabinoid system to paracetamol (acetaminophen) analgesia has been a subject of particular speculation. Paracetamol itself does not inhibit FAAH.[43] However, Hoegestaett et al.[46] recently described that paracetamol undergoes a FAAH-dependent two-step metabolic transformation in the brain, liver, and spinal cord to form the bioactive *N*-acylphenolamine AM404 (*N*-(4-hydroxyphenyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide). This compound inhibits FAAH by acting as a competing substrate, inhibits COX, affects the cellular accumulation of AEA, interacts with both CB_1 and TRPV1 (vanilloid) receptors, and has analgesic effects in vivo.[46−51] Three independent studies have shown that blockade of CB_1 receptors affects the antinociceptive properties of paracetamol,[52−54] suggesting that this metabolic pathway can at least contribute to the pharmacological properties of paracetamol. This may also be true of the paracetamol analogue *N*-(4-hydroxybenzyl)acetamide), which shows analgesic properties in vivo in the formalin test of persistent pain but does not inhibit FAAH or interact with CB receptors in vitro, while its equivalent arachidonoyl analogue is more potent than AM404 at these targets.[55] AM404 derivatives in which the 4-aminophenol moiety has been replaced by NSAIDs or in which mefenamic and flufenamic acid served as arachidonic acid mimetics were capable of interacting with TRPV1 and retained the COX inhibition properties of the parent NSAIDs.[56] Furthermore, FAAH inhibitors targeting also other proteins involved in pain tranduction migth be more efficacious than selective FAAH inibitors, as shown by the case of *N*-arachidonylserotonin, a dual FAAH/TRPV1 inhibitor, that has been reported to be efficacious against pain and anxiety.[57,58]

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[a] Abbreviations: AA-maleimide, *N*-arachidonoylmaleimide; AEA, anandamide; AM404, *N*-(4-hydroxyphenyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide; CB, cannabinoid; COX, cyclooxygenase; DMF-DMA, *N*,*N*-dimethylformamide dimethylacetal; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; FAAH, fatty acid amide hydrolase; HOBt, hydroxybenzotriazole; Ibu-am5, (*N*-(3-methylpyridin-2-yl)-2-(4′-isobutylphenyl)propionamide); MGL monoacylglycerol lipase; NSAIDs, nonsteroidal anti-inflammatory drugs; OTMK, oleoyl trifluoromethyl ketone; SAR structure−activity relationship; URB597, 3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate.

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The findings above that paracetamol (indirectly) and NSAIDs (directly) interact with FAAH raise the possibility that compounds containing elements of both structures may be a new source of FAAH inhibitors. In the present study, we report the synthesis and characterization of a novel series of paracetamol esters, designed as inhibitors of FAAH by progressive modification of the structure of 4-acetamidophenyl 2-(2-(trifluoromethyl)pyridin-4-ylamino)benzoate (**21**).

Chemistry

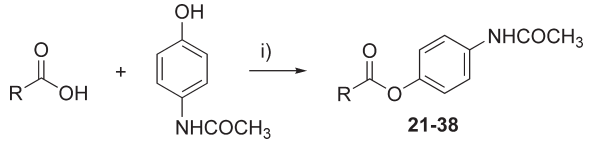
The synthesis of paracetamol esters was performed by condensation of paracetamol with the appropriate acid, using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), in the presence of hydroxybenzotriazole (HOBt) in acetonitrile (MeCN) solution. (Scheme 1). This method was found to be clean and high yielding.

The bulk of the syntheses required acids **6a**−**i** that were not commercially available. These were synthesized using our previously described procedure that allows the construction of the trifluoromethylpyridine moiety linked by an amino nitrogen to an aromatic ring.[59] According to Scheme 2, methyl esters **1a**−**i** were reacted with trifluoroacetylvinyl ether **2**, in a 1.5 molar ratio, in boiling MeCN solution affording high yields of intermediates **3a**−**i**. Treatment of **3a**−**i** with an excess of *N*,*N*-dimethylformamide dimethylacetal (DMF-DMA) in refluxing toluene provided 1,1,1-trifluorohexadienones **4a**−**i**. Intermediates **4a**−**i** readily underwent pyridine ring closure to give compounds **5a**−**i** upon treatment with ammonium acetate (NH_4OAc) in hot DMF. Sodium hydroxide catalyzed hydrolysis of compounds **5a**−**i** produced the required acids **6a**−**i**.

A chemistry similar to that described in the synthesis of acids **6a**−**h** was used for the preparation of acid **11**. As reported in Scheme 3, ester **7** was sequentially treated with compound **2**, then with DMF-DMA, NH_4OAc, and successively hydrolyzed to **11**.

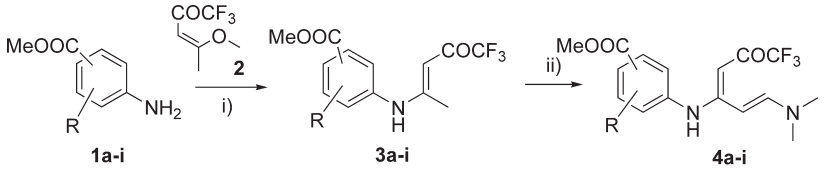
Carboxylic acids **17**−**20** were obtained by reaction of ethyl glycinate hydrochloride (**12**) with the appropriate acid **6e**−**h** by EDC method and subsequent hydrolysis of esters **13**−**16** (Scheme 4).

Scheme 1. Synthesis of Target Paracetamol Esters **21**−**37**[a]

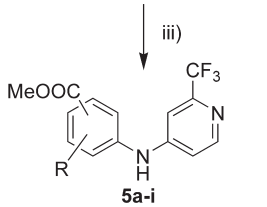


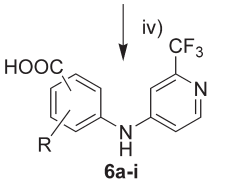
[a] Reagents and conditions: (i) EDC, HOBt, MeCN, rt, 24 h.

Scheme 2. Synthesis of Intermediates Compounds **3**−**6**[a]



1, 3-5a: 2-COOMe, R=4-Me
1, 3-5b: 2-COOMe,R= 4-Cl
1, 3-5c: 2-COOMe, R=5-Cl
1, 3-5d: 3-COOMe, R=H
1, 3-5e: 4-COOMe, R=H
1, 3-5f: 4-COOMe, R=2-Me
1, 3-5g: 4-CH_2-COOMe, R=H
1, 3-5h: 4-CH(CH_3)-COOMe, R=H
1, 3-5i: 4-CH_2CH_2COOMe, R=H





6a: 2-COOH, R=4-Me
6b: 2-COOH,R= 4-Cl
6c: 2-COOH, R=5-Cl
6d: 3-COOH, R=H
6e: 4-COOH, R=H
6f: 4-COOH, R=2-Me
6g: 4-CH_2-COOH, R=H
6h: 4-CH(CH_3)-COOH, R=H
6i: 4-CH_2CH_2-COOH, R=H

[a] Reagents and conditions: (i) MeCN, reflux, 2 h; (ii) DMF-DMA, PhMe, reflux, 1 h; (iii) NH_4OAc, DMF, reflux, 1.5 h; (iv) 10% aq NaOH, reflux, 30 min.

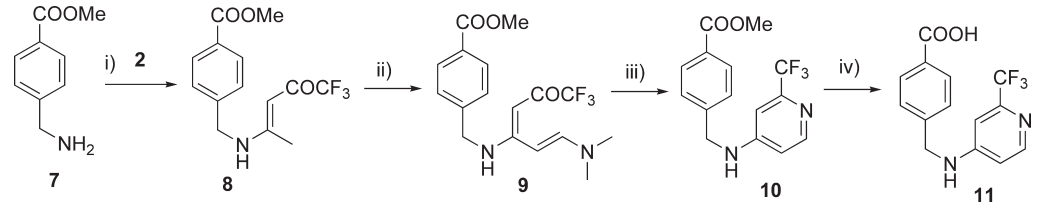
Biology

Compounds **21**−**38**, along with their parent compound paracetamol, were evaluated for their ability to inhibit [^{3}H]-AEA hydrolysis in rat brain homogenates. In all cases reported here, concentration−response curves were constructed from values from at least three experiments using at least five concentrations over the appropriate range (in half log units, i.e., 0.3, 1, 3, 10, 30, 100 μM) (for examples, see Figure 1), with the exception of (*R*,*S*)-ibuprofen, where four concentrations (30, 100, 300, and 500 μM) were used. The effects of paracetamol esters **21**−**38** upon the FAAH-catalyzed hydrolysis of AEA by rat brain membranes at an assay pH of 7.4 are shown in Tables 1 and 2. Experiments investigating FAAH activity in intact cells and effects upon MGL and COX were also performed for selected compounds.

Results and Discussion

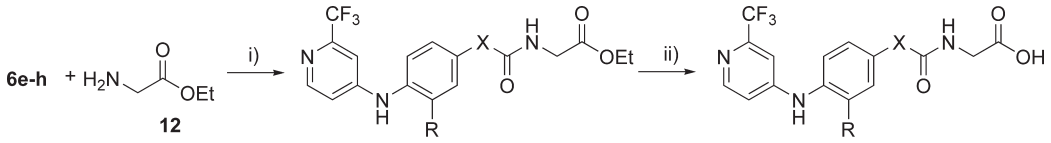
Although paracetamol was devoid of FAAH inhibitory activity (IC_{50} value >300 μM), a result consistent with the literature,[54,55] its conversion into esters resulted in generation of FAAH inhibitors. To address this issue, an SAR study on paracetamol esters, focused on aromatic nucleus variation, was systematically undertaken. Different groups on the anthranilic acid scaffold were first investigated (Table 1). In some cases, a complete inhibition of FAAH was not seen. This was usually the case for the weaker compounds and

Scheme 3. Synthesis of Intermediates Compounds **8**−**11**[a]



[a] Reagents and conditions: (i), (ii), (iii), (iv) steps (i), (ii), (iii), (iv) in Scheme 2.

Scheme 4. Synthesis of Intermediates Compounds **13**−**20**[a]



[a] Reagents and conditions: (i) EDC, HOBt, TEA, MeCN, rt, 4 h; (ii) 50% aq NaOH, rt, 24 h.

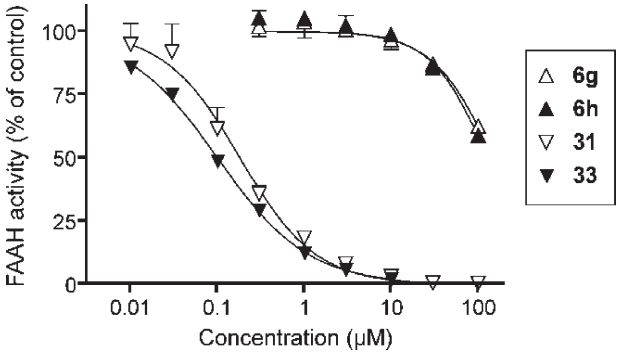


Figure 1. Inhibition of FAAH-catalyzed hydrolysis of 0.5 μM [^{3}H]AEA by rat brain homogenates by compounds **6g**, **6h**, **31**, and **33**. Data are means and SEM (when not enclosed by the symbols), n = 3−15.

presumably reflects a solubility issue with these lipophilic compounds (for discussion, see ref 60). However, the IC_{50} values here are comparable because they reflect the inhibitable component of the FAAH activity.

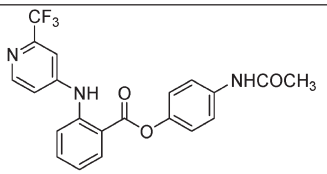
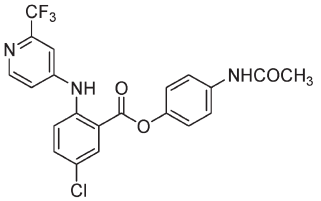
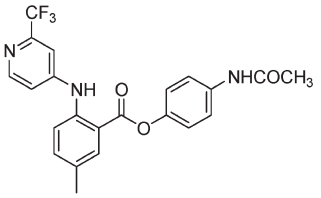
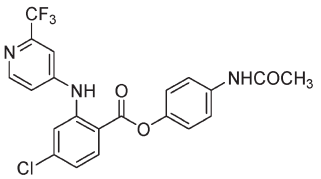
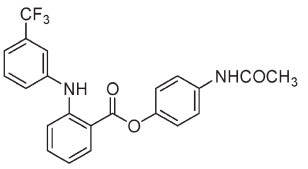
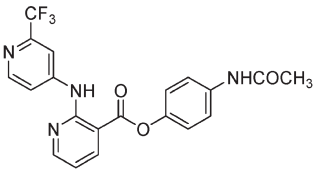
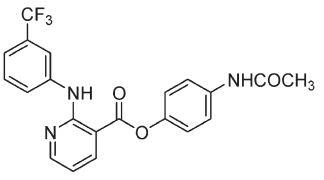
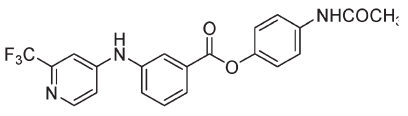
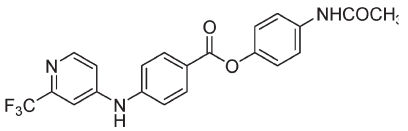
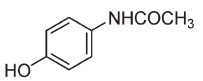
It was found that antranoyl ester (**21**) inhibited FAAH with an IC_{50} value of 64 μM. While the introduction of 5-Me (**23**) or 4-Cl (**24**) substituent resulted in about 2-fold increased activity, it was a chorine atom in 5-position (**22**) that produced a significant improvement in activity (IC_{50} 4.3 μM). Replacement of trifluoromethylpyridine mojety of **21** with a trifluoromethylbenzene to give the flufenamic acid ester (**25**) also led to significant enhancement in potency (IC_{50} 8.7 μM). In contrast, the substitution of benzene ring of **21** with a pyridine (ester **26**, IC_{50} 58 μM) did not change the activity with respect to ester **21**. Replacement of trifluoromethylpyridine mojety of **26** with a trifluoromethylbenzene to give the niflumic acid ester (**27**) resulted in a loss of activity. An important finding arising from the comparison of the IC_{50} values for esters **21**, **28**, and **29** was that going from ortho- (IC_{50} 64 μM) to meta- (IC_{50} 47 μM) to para-substituted (IC_{50} 2.7 μM) analogues resulted in gradual increase of potencies. This fact indicated potential favorable interaction of the para-substituted ester **29** with the enzyme. Therefore, ester and trifluoromethylpyridine-amino groups were kept in para for further SAR studies. Next, connecting patterns of these groups (Table 2) were evaluated. Introduction of an extra methylene linker between phenyl ring and trifluoromethylpyridine-amino group, such as in compound **32**, led to drop in potency (IC_{50} 22 μM). We observed the opposite effect by introduction of an extra methylene linker between phenyl ring and ester moiety (ester **31**, IC_{50} 0.18 μM) that caused an increase in activity with respect to **29**. Although less potent when compared to **31**, ester **38** bearing an ethylene linker between phenyl ring and ester moiety (IC_{50} 1.5 μM) showed increased activity as compared with **29**. Branching the methylene linker with a methyl produced further increase of activity (ester **33**, IC_{50} 0.10 μM). This compound contains structural elements of ibuprofen and Ibu-am5, and so data for these compounds are included in Table 2 for comparative purposes.

On the most active compounds introduction of an extra three atom linker was evaluated. Even though derivatives **34** and **35** were slightly stronger than the parent **29** and **30**, in the case of **31** and **33**, the same modification led to weaker compounds **36** and **37**. The substitution of the phenyl ring was also probed. Methylated derivatives **30** and **35** have essentially the same potency as that of **29**.

The stability of ester derivatives **31** and **33** was investigated both toward acid (0.1 M aq trifluoroacetic acid) and base (0.1 M aq NaOH) hydrolysis. After 48 h at room temperature, the formation of the corresponding acids **6g** and **6h** was monitored by TLC (ethyl acetate/*n*-hexane 1:1). In all cases, examination of TLC plates revealed a single spot with rf identical to starting compound. Thus, these paracetamol esters do not appear to be hydrolytically labile. Furthermore, acids **6g** and **6h** do not inhibit FAAH (IC_{50} values > 100 μM) (Figure 1), confirming that it is the paracetamol esters rather than the theoretical hydrolysis products that inhibit FAAH.

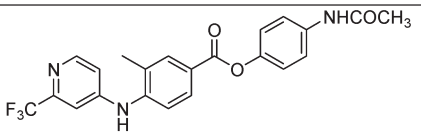
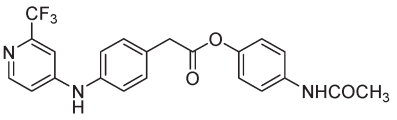
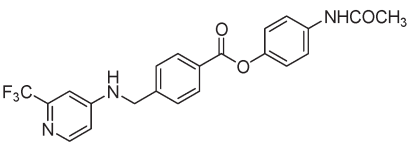
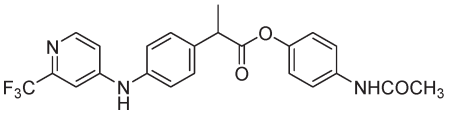
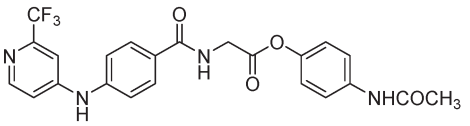
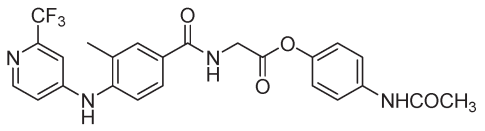
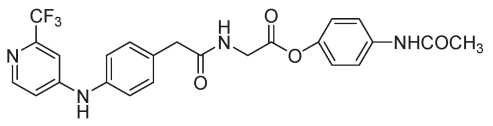
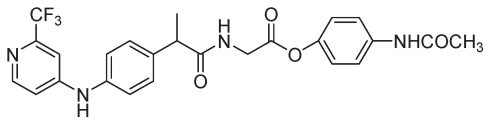
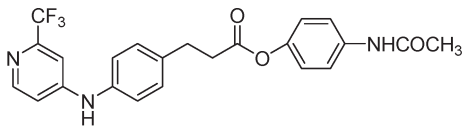
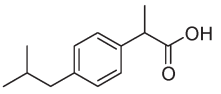
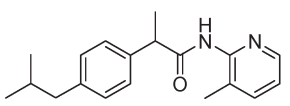
pH Sensitivity of [^{3}H]-AEA Hydrolysis by Compounds 22, 33, and 34. The FAAH inhibitory effect of acidic NSAIDs is sensitive to the assay pH used. Thus (*R*,*S*)-ibuprofen was more potent at pH 6 than at pH 8.[45] Rat brain membranes in assay buffer of pH 6 or pH 9 were preincubated with **22**, **33**, and **34** or (*R*,*S*)-ibuprofen prior to addition of [^{3}H]-AEA and

Table 1. IC_{50} Values for Inhibition of Rat Brain AEA Hydrolysis by Compounds **21**−**29** and Paracetamol

compd	structure	IC_{50} (μM) [pI_{50} values ± s.e.m.][a]
21		64 [4.19±0.08] (max. inhibition 74±7%)
22		4.3 [5.37±0.06] (max. inhibition 89±3%)
23		38 [4.42±0.05] (max. inhibition 100%)
24		31 [4.51±0.04] (max. inhibition 62±2%)
25		8.7 [5.06±0.04] (max. inhibition 91±2%)
26		58 [4.24±0.02] (max. inhibition 68±1%)
27		>300 (19±3% inhibition at 300 μM)
28		47 [4.33±0.03] (max. inhibition 73±2%)
29		2.7 [5.57±0.06] (max. inhibition 93±3%)
Paracetamol		>300 (4±1% inhibition at 300 μM)

[a] pI_{50} values (i.e., $-\log_{10}(IC_{50})$ values) are indicated because the analysis method returns these ± SE values, which are then antilogged to give the IC_{50} values for the inhibitable component (max inhibition) of the FAAH activity.

Table 2. IC_{50} Values for Inhibition of Rat Brain AEA Hydrolysis by Compounds **30**−**37**, Ibuprofen, and Ibu-am5

compd	structure	IC_{50} (μM) [pI_{50} values ± s.e.m.]
30		4.6 [5.34±0.01] (max. inhibition 100%)
31		0.18 [6.74±0.06] (max. inhibition 100%)
32		22 [4.66±0.07] (max. inhibition 84±5%)
33		0.10 [7.00±0.02] (max. inhibition 100%)
34		0.87 [6.06±0.02] (max. inhibition 97±2%)
35		3.0 [5.53±0.02] (max. inhibition 100%)
36		2.6 [5.59±0.02] (max. inhibition 100%)
37		0.73 [6.14±0.03] (max. inhibition 100%)
38		1.5 [5.82±0.04] (max. inhibition 100%)
(*R*,*S*)-Ibuprofen		260 [3.58±0.04] (max. inhibition 100%)
Ibu-am5		1.3 [5.88±0.03] (max. inhibition 100%)

incubation for 10 min at 37 °C. As expected, ibuprofen was more potent at pH 6 than at pH 9: at pH 9, the IC_{50} value was greater than the highest concentration tested (500 μM), whereas at pH 6, the lowest concentration tested (100 μM), produced 71 ± 2% inhibition (Figure 2). At pH 7.4, (*R*,*S*)-ibuprofen inhibited [^{3}H]AEA hydrolysis with an IC_{50} value of 240 μM (data not shown). The ionization properties of esters **22**, **33**, and **34** are very reduced as compared to acidic compounds such as ibuprofen. Consistent with this, these compounds showed little pH dependency, and if anything, the potencies were lower at pH 6 than pH 9 (see Figure 2 for data with **22**, **33**, and **34**).

Mode of Inhibition of FAAH by 31 and 33. To determine the likely mechanism of the inhibition, a time dependent inhibition study was performed using as reference compounds **31** and **33**. In theory, if a compound inhibits the enzyme via an irreversible mechanism, upon prolonged preincubation the potency should become greater; a constant IC_{50}, conversely, supports a reversible mechanism. Compounds **31** and **33** showed no time-dependent improvement of potency, arguing against an irreversible mechanism of action (Figure 3). The kinetics of inhibition were also investigated. The inhibition was essentially competitive in nature, with K_i values calculated from slope replots of 0.19 μM and 0.16 μM for **31** and **33**, respectively (Figure 3).

FAAH Inhibition in Intact Cells. The effects of **33** were investigated in RBL2H3 rat basophilic leukemia cells (Figure 4). Initially, cells were incubated with 100 nM [^{3}H]AEA (labeled in the ethanolamine part of the molecule) and the tritium recovered in the aqueous phase after methanol and chloroform extraction (corresponding to [^{3}H]ethanolamine) was determined. A significant reduction of tritium recovery was found at 1 and 3 μM of **33**, consistent with inhibition of FAAH. The inhibition seen with 3 μM of **33** was similar to that seen with 30 μM of Ibu-am5 (Figure 4A), which is consistent with the relative potencies of these compounds in cell free homogenates (Table 2 and ref 45). However, the blank values seen in this assay were high, and so two indirect models were used to corroborate the finding of Figure 4A, namely FAAH-driven accumulation and membrane processing of tritium in RBL2H3 cells incubated with AEA labeled in the arachidonate part of the molecule. In RBL2H3 cells, FAAH regulates the extra-intracellular concentration of AEA by metabolizing the intracellularly accumulated AEA. In consequence, inhibition of FAAH reduces the observed rate of uptake.[61,62] This is shown in Figure 4B, where 1 μM of the selective FAAH inhibitor 3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate (URB597) reduces the uptake of AEA by about $^2/_3$. Compound **33** behaved in a similar manner, consistent with its FAAH inhibitory properties. In RBL2H3 cells, arachidonic acid produced as a result of the FAAH-catalyzed hydrolysis of AEA is recycled back to the plasma membrane.[63] Measurement of membrane tritium accumulation following incubation of RBL2H3 cells with [^{3}H]AEA is thus a simple way of assessing the FAAH inhibitory properties of compounds.[64] As expected, URB597 almost completely blocked the accumulation of membrane tritium, whereas the monoacylglycerol lipase (MGL)-selective 4-nitrophenyl-4-(dibenzo[d]-[1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate[65] **39** (JZL184) was without effect (Figure 4C). Compound **33** produced a concentration-dependent inhibition of the tritium accumulation (Figure 4C). Thus, in all three models, **33** produces effects consistent with an inhibition of FAAH in intact RBL2H3 cells.

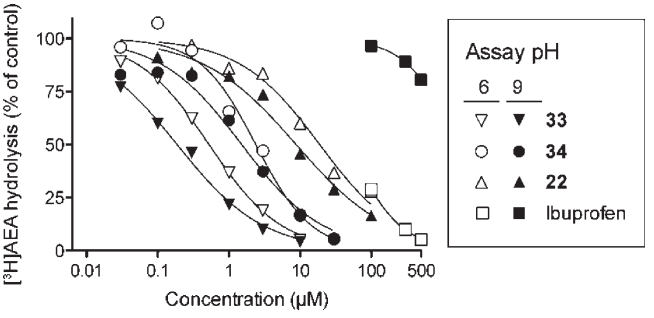


Figure 2. Inhibition of FAAH-catalyzed hydrolysis of 0.5 μM [^{3}H]AEA by rat brain homogenates by (*R*,*S*)-ibuprofen and compounds **22**, **33**, and **34**. Data are means, $n = 3$ for the assay pH values shown.

Inhibition of MGL. MGL is the primary enzyme responsible for the hydrolysis of the endocannabinoid 2-arachidonoylglycerol in the brain,[66,67] and its selective inhibition by **39** produces not only analgesic effects but effects upon body temperature and locomotion.[65] OMDM169, a potent inhibitor of 2-AG hydrolysis, has been reported to enhance 2-AG levels and to exert analgesic activity via indirect activation of cannabinoid receptors.[68]

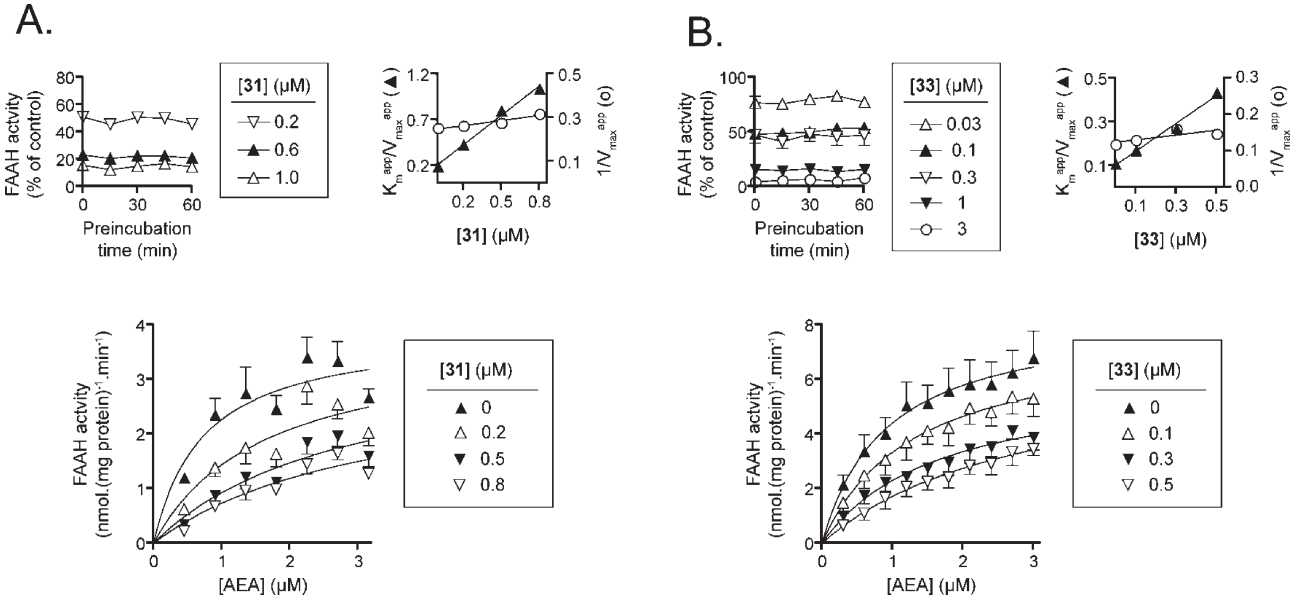


Figure 3. Mode of inhibition of rat brain FAAH by **31** (A) and **33** (B). Top left (for each panel): homogenates were preincubated with the compounds for the times shown prior to addition of 0.5 μM [3H]AEA and assay for FAAH activity (means and SEM, $n = 3-4$). Bottom (for each panel): AEA hydrolysis at the substrate and inhibitor concentrations shown (means and SEM, $n = 3$). Secondary slope (K_m^{app}/V_{max}^{app}) and intercept ($1/V_{max}^{app}$) replots are shown for the mean data at the top right (for each panel).

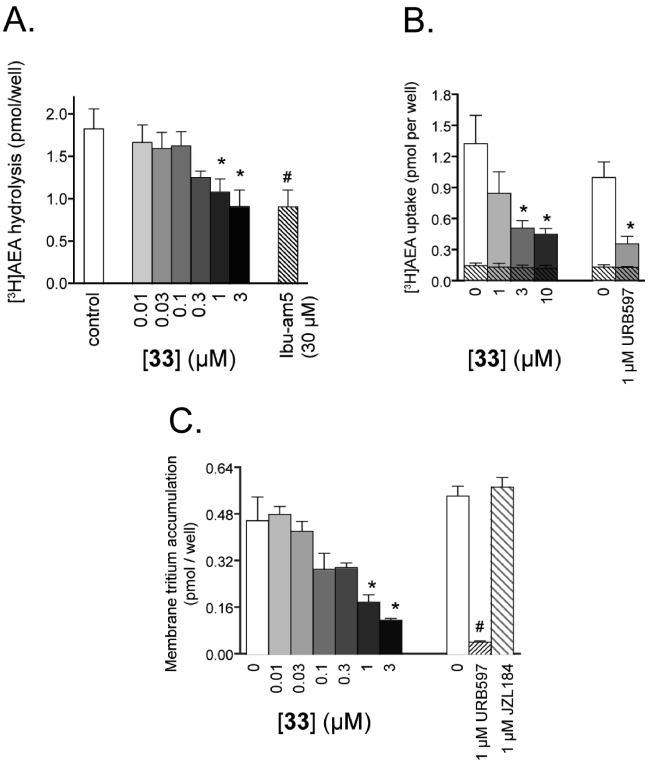


Figure 4. Inhibtion of [^{3}H]AEA (100 nM) hydrolysis in intact RBL2H3 cells by **33**. In (A) (hydrolysis), cells were preincubated with **33** or Ibu-am5 for 10 min prior to addition of [^{3}H-ethanolamine]-labeled AEA and incubation for a further 20 min. In (B) (uptake), cells or wells alone (shaded columns) were preincubated with **33** or Ibu-am5 for 10 min prior to addition of [^{3}H-arachidonoyl]-labeled AEA and incubation for a further 4 min. In (C) (membrane recycling) cells were preincubated with **33**, URB597, or **39** for 10 min prior to addition of [^{3}H-arachidonoyl]-labeled AEA and incubation for a further 5 min. Shown are means and SEM, n = 3−7. *Significantly different ($p < 0.05$ Dunnett's multiple comparison test) from the corresponding control (vehicle) value following significant one-way factorial ANOVA. $^{\#}p < 0.05$ vs the corresponding vehicle value, two-tailed t test.

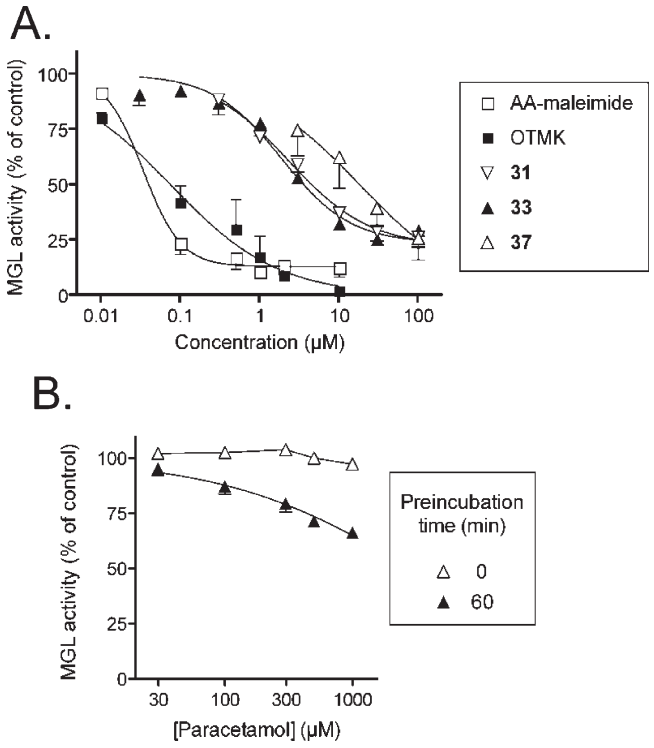


Figure 5. Inhibition of recombinant human MGL by: (A) **31**, **33**, **37**, and as positive controls AA-maleimide and OTMK; (B) paracetamol. The compounds were incubated together with enzyme and substrate (0.25 mM 4-nitrophenyl acetate) for 20 min. Unless otherwise indicated, a preincbuation phase between inhibitor and enzyme prior to addition of substrate was not used. Shown are means and SEM, n = 3−4.

The inhibition of MGL by compounds **31**, **33**, and **37** was investigated using recombinant human MGL and 4-nitrophenyl acetate as substrate.[69] (Figure 5A) As positive controls, oleoyltrifluoromethyl ketone (OTMK) and *N*-arachidonoylmaleimide (AA-maleimide) were used because these compounds are known to inhibit MGL.[70,71] In a subsequent experiment, the selective MGL inhibitor **39** was also tested and found potently to inhibit the hydrolysis of 4-nitrophenylacetate in a time-dependent manner.[72] Compounds **31**, **33**, and **37** all inhibited MGL activity. The IC_{50} values (with pI_{50} values and max inhibition attained given in brackets) were: **31**, IC_{50} 2.3 μM (pI_{50} 5.64 ± 0.11, max inhibition 78 ± 5%); **33**, IC_{50} 1.9 μM (pI_{50} 5.73 ± 0.09, max inhibition 76 ± 4%); **37**, IC_{50} 18 μM (pI_{50} 4.74 ± 0.16, max inhibition 100%). In contrast, indomethacin, Ibu-am5, and (*R*,*S*)-ibuprofen, at concentrations of 100 μM, were without effect upon the MGL activity (data not shown). Paracetamol itself produced no inhibition up to a concentration of 1 mM. However, when the compound was preincubated with MGL for 60 min prior to assay, some inhibition was seen (Figure 5B). In theory, inhibition of MGL could explain the CB receptor-mediated analgesic properties of paracetamol[52−54] because the effects of **39** are also blocked by a CB_1 receptor antagonist.[65] However, the potency of paracetamol toward recombinant human MGL is rather weak (20 and 34% inhibition at 300 and 1000 μM, respectively, following 60 min of preincubation, Figure 5B), and it is in any case unwise to overinterpret in vitro data in terms of the situation in vivo.

Although the IC_{50} values for **33** and **31** are about an order of magnitude higher than seen for inhibition of FAAH, the MGL and FAAH assays were undertaken in rather different conditions, so it is unwise to claim a FAAH selectivity for the compounds. However, the relative potencies of compounds within a given assay can be compared, and it is noted that the IC_{50} values for **33** and **37** relative to **31** are rather similar for FAAH (1.8 and 7.2, respectively) as for MGL (1.2 and 9.3, respectively, calculated from the mean pI_{50} values). Thus, in this limited series, the structural elements conferring an increased potency toward FAAH also increase the potency toward MGL.

Interaction of Compounds 22, 25, 32, and 33 with COX. The effects of using as reference compounds **22**, **25**, **32**, and **33** upon ovine COX-1 and human COX-2 were assessed using an oxygen electrode.[73] The assay was undertaken in two ways, either preincubating the enzyme and test compound for 5 min before starting the reaction with 10 μM arachidonic acid as substrate ("preincubation method") or mixing the test compound and the substrate and starting the assay by addition of the COX ("non-preincubation method"). Parts A and B of Figure 6 show the effects of (*R*)- and (*S*)-ibuprofen and **33** upon the activity of COX-1 using the preincubation method. As expected,[73,74] (*S*)-ibuprofen was more potent than the (*R*)-enantiomer (Figure 6A), and a concentration of 50 μM of (*S*)-ibuprofen produced a large

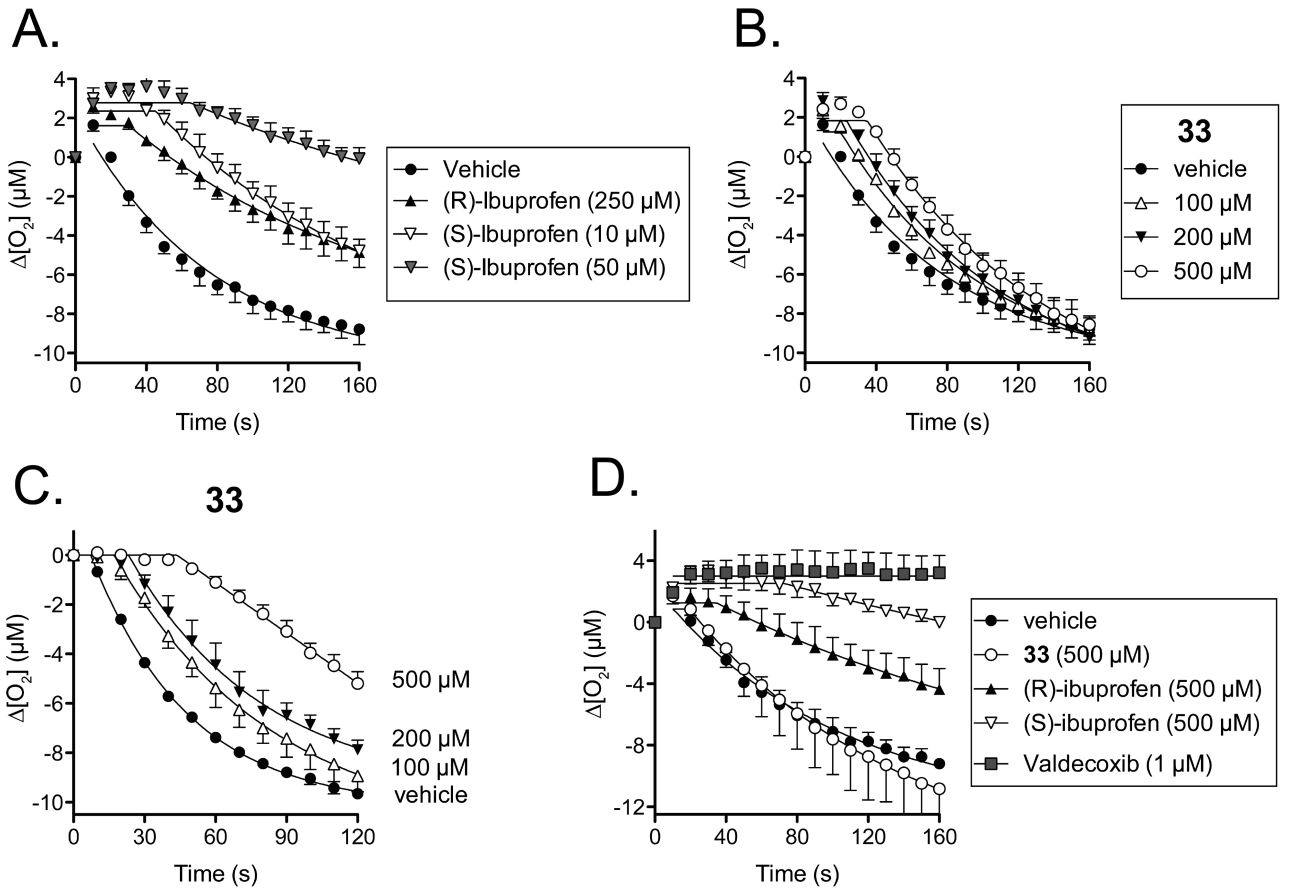


Figure 6. The effects of (*R*)- and (*S*)-ibuprofen (A) and **33** (B) upon the oxygenation of 10 μM arachidonic acid by ovine COX-1 using the preincubation assay. In (C), the effect of **33** upon the activity of COX-1 was measured using the nonpreincubation assay. In (D), the effects of **33**, (*R*)- and (*S*)-ibuprofen, indomethacin, and valdecoxib upon the activity of human recombinant COX-2 using the preincubation assay. Note that one of the three valdecoxib experiments was conduced using a different control to those shown in the graph. Shown are means and SEM (when not enclosed by the symbols), $n = 3-4$.

inhibition of oxygen consumption. In contrast, **33** at a concentration of 100 and 200 μM (i.e., 2−3 orders of magnitude higher than required for inhibition of FAAH) had very little effect upon COX-1 activity. The highest concentration tested (500 μM) produced a slight lag prior to the start of oxygen consumption by the enzyme (Figure 6B). With the nonpreincubation method, similar results were found for the ibuprofen enantiomers (data not shown), whereas the slight delay produced by the 500 μM concentration of **33** before the enzyme started to metabolize arachidonic acid was more pronounced (Figure 6C). Curve fitting of the data indicated that the plateau length was 7, 17, 23, and 44 s after addition of enzyme to the assay mixture to start the reaction at concentrations of **33** of 0, 100, 200, and 500 μM, respectively. This delay was still seen if the nonpreincubation assays were undertaken in the presence of detergent (0.3 mM Triton X-100) if the COX-1 was premixed with hematin rather than being added to the hematin solution or if the assays were undertaken at pH 8.0 rather than at pH 7.4 (data not shown). Compounds **22**, **25**, and **32** were without effect upon COX-1 activity at the concentrations tested (100, 100, and 500 μM, respectively, nonpreincubation method, data not shown). Human COX-2 is more expensive than ovine COX-1, and so the number of experiments undertaken was limited and the preincubation assay alone was used. However, under these conditions, the COX-2-selective inhibitor valdecoxib[75] (1 μM) produced a complete blockade of activity and the ibuprofen enantiomers produced a partial inhibition at 500 μM. In contrast, **33** was without effect upon the COX-2 activity at 500 μM (Figure 6D). These data indicate that **33** has at best minor effects upon COX enzymes that are only seen at very high concentrations relative to those needed for FAAH inhibition, which is perhaps not surprising given the weak potency of paracetamol toward COX-1 and COX-2.[76]

Conclusions

In the present study, a series of paracetamol esters with FAAH inhibitory activity have been described. The most potent compound, **33**, inhibits rat brain FAAH in homogenates in an essentially competitive manner with submicromolar potency and can inhibit the hydrolysis of AEA by intact RBL2H3 rat basophilic leukemia cells. The compound, however, can also inhibit MGL activity, and so selectivity vis á vis this enzyme is less than ideal. Nevertheless, **33** may be useful as a template for the design of novel reversible FAAH inhibitors derived from a much used, but incompletely understood, analgesic agent.

Experimental Section

Chemistry. Unless otherwise noted, all solvents, including anhydrous solvents and chemicals, were purchased from Aldrich Co. and/or AlfaAesar, and used without further purification. Melting points were recorded on a Stuart Scientific

melting point SMP1 apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 300 MHz (Varian Unity 300) using DMSO-d_6 as solvent and TMS as the internal standard. Splitting patterns are designated as singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m). Infrared spectra were obtained with a Bruker Vector 22 spectrophotometer. Elemental analyses were carried out with a Carlo Erba model 1106 elemental analyzer, and all values were within 0.4% of the calculated values, which indicates >95% purity of the tested compounds. Analytical thin layer chromatography (TLC) was carried out on E. Merck TLC plates coated with silica gel 60 F254 (0.25 mm layer thickness). TLC visualization was carried out using either a UV lamp. *N*-(2-(Trifluoromethyl) pyridin-4-yl)anthranilic acid,[59] 2-(2-(trifluoromethyl)pyridin-4-ylamino)nicotinic acid,[59] **1a**,[77] **1f**,[78] **1h**,[79] **1i**,[80] **2**,[81] and **7**[82] were obtained with previously described procedures.

General Procedure for the Preparation of Paracetamol Esters 21−38. A mixture of the appropriate acid (1 mmol), EDC (0.19 g, 1.1 mmol), and HOBt (0.13 g, 1 mmol) in dry MeCN (10 mL) was stirred at room temperature for 30 min and then treated with paracetamol (0.15 g, 1 mmol). The mixture was stirred at room temperature for an additional 24 h. Then the solution was evaporated to dryness in vacuo. The residue was dissolved in ethyl acetate (20 mL) and washed with brine (2× 5 mL), 5% aqueous sodium hydroxide (2× 5 mL), and water (2× 5 mL). The organic layer was dried over anhydrous magnesium sulfate. Concentration of the dried extract yielded a residue, which was triturated with isopropyl ether. The formed precipitate was filtered off and purified by crystallization from the adequate solvent to give the ester derivatives **21−38**.

Example: 4-Acetamidophenyl 2-(2-(trifluoromethyl)pyridin-4-ylamino)benzoate (21). Following the general procedure, the title compound was obtained from *N*-(2-(trifluoromethyl) pyridin-4-yl)anthranilic acid;[59] yield 87%; mp 149−150 °C (isopropyl ether). ^{1}H NMR (DMSO-d_6) δ 2.16 (s, 3H), 7.23 (m, 3H), 7.29 (m, 1H), 7.46 (m, 2H), 7.73 (m, 3H), 7.84 (m, 1H), 8.27 (d, J = 5.8, 1H), 8.48 (d, J = 4.2, 1H), 9.55 (s, 1H), 10.15 (s, 1H). IR (Nujol) 3314, 1692, 1666 cm^{-1}. Anal. ($C_{21}H_{16}F_3N_3O_3$) C, H, N.

General Procedure for the Synthesis of 3a−i, 8. A mixture of methyl esters **1a−i**, **7** (10 mmol), and enol ether **2** (2.52 g, 15 mmol) in anhydrous MeCN (10 mL) was refluxed for 2 h. After cooling, the formed precipitate was collected by filtration, washed with isopropyl ether, dried, and used without further purification.

Example: (*E*)-Methyl 5-Methyl-2-(5,5,5-trifluoro-4-oxopent-2-en-2-ylamino)benzoate (3a). Yield 90%; mp 114−116 °C (*n*-hexane). ^{1}H NMR (DMSO-d_6) δ 2.28 (s, 3H), 2.40 (s, 3H), 3.97 (s, 3H), 5.80 (s, 1H), 7.52 (d, J = 8.1, 1H), 7.77 (s, 1H), 8.01 (d, J = 8.6, 1H), 12.78 (s, 1H, NH). IR (nujol) 3142, 1702 cm^{-1}. Anal. ($C_{14}H_{14}F_3N_3O_3$) C, H, N.

General Procedure for the Synthesis of 4a−i, 9. A mixture of **3a−i**, **8** (5 mmol), and DMF-DMA (1.79 g, 15 mmol) in anhydrous toluene (20 mL) was refluxed for 1 h, then allowed to reach the room temperature and stirred for additional 24 h. The mixture was carefully concentrated in vacuo to give **4a−i**, **9**.

Example: Methyl 2-((1*E*,3*E*)-1-(Dimethylamino)-6,6,6-trifluoro-5-oxohexa-1,3-dien-3-ylamino)-5-methylbenzoate (4a). Yield 66%; mp 124−125 °C (2-PrOH). ^{1}H NMR (DMSO-d_6) δ 2.34 (s, 3H), 2.87 (s, 3H), 3.30 (s, 3H), 3.91 (s, 3H), 5.00 (d, J = 11.5, 1H), 5.84 (s, 1H), 7.46 (m, 1H), 7.68 (s, 1H), 8.02 (m, 2H), 12.86 (s, 1H). IR (nujol) 1719 cm^{-1}. Anal. ($C_{17}H_{19}F_3N_2O_3$) C, H, N.

General Procedure for the Synthesis of 5a−i, 10. To a solution of **4a−i**, **9** (2 mmol) in dry DMF (5 mL), ammonium acetate (0.308 g, 4 mmol) was added and the mixture was gently refluxed for 1.5 h. The mixture was carefully concentrated in vacuo, and then ice−water (15 mL) was added. The formed solid was filtered off, washed with water, air-dried, and then crystallized from the appropriate solvent to give **5a−i**, **10**.

Example: Methyl 5-Methyl-2-(2-(trifluoromethyl)pyridin-4-ylamino)benzoate (5a). Yield 85%; mp 90−92 °C (*n*-hexane). ^{1}H NMR (DMSO-d_6) δ 2.40 (s, 3H), 3.96 (s, 3H), 7.14 (m, 1H), 7.35 (s, 1H), 7.55 (m, 1H), 7.93 (m, 1H), 8.02 (m, 1H), 8.45 (d, J = 5.8, 1H), 9.04 (s, 1H). IR (nujol) 3328, 1698 cm^{-1}. Anal. ($C_{15}H_{13}F_3N_2O_2$) C, H, N.

General Procedure for Synthesis of Acids 6a−i, 11. A mixture of **5a−i**, **10** (1 mmol) in 10% aqueous sodium hydroxide (15 mL) was refluxed for 30 min, during which a homogeneous solution was formed. After cooling, the solution was acidified with 20% aqueous hydrochloric acid to pH 3−4. The formed solid was filtered off, washed with water, air-dried, and crystallized from ethanol to give **6a−i**, **11**.

Example: 5-Methyl-2-(2-(trifluoromethyl)pyridin-4-ylamino)-benzoic Acid (6a). Yield 94%; mp 248−250 °C. ^{1}H NMR (DMSO-d_6) δ 2.33 (s, 3H), 6.55 (m, 1H), 7.12 (m, 1H), 7.74 (m, 2H), 8.60 (d, J = 5.8 Hz, 1H), 9.83 (s, 1H, NH). IR (nujol) 3239, 2499, 1863, 1681 cm^{-1}. Anal. ($C_{14}H_{11}F_3N_2O_2$) C, H, N.

General Procedure for the Preparation of Compounds 13−16. A mixture of the acid **6e−h** (2 mmol), EDCI (0.39 g, 2.2 mmol), and HOBt (0.27 g, 2 mmol) in dry MeCN (10 mL) was stirred at room temperature. After 30 min, TEA (0.4 mL, 4 mmol) and **12** (0.56 g, 4 mmol) were added. The mixture was stirred at room temperature for an additional 4 h. Then the solution was evaporated to dryness in vacuo. The residue was dissolved in ethyl acetate (20 mL) and washed with brine (2× 5 mL), 10% aqueous hydrochloric acid (2× 5 mL), 5% aqueous sodium hydroxide (2× 5 mL), and water (2× 5 mL). The organic layer was dried over anhydrous magnesium sulfate. Concentration of the dried extract yielded a residue, which was triturated with isopropyl ether. The formed precipitate was filtered off and purified by crystallization from the adequate solvent to give derivatives **13−16**.

Example: Ethyl 2-(4-(2-(Trifluoromethyl)pyridin-4-ylamino)-benzamido)acetate (13). Yield 75%; mp 170−172 °C (2-PrOH). ^{1}H NMR (DMSO-d_6) δ 1.30 (t, J = 7.8, 3H), 4.12 (s, 2H), 4.16 (q, J = 7.8, 2H), 7.33 (m, 4H), 7.99 (d, J = 8.4 Hz, 2H), 8.44 (d, J = 5.8 Hz, 1H), 9.05 (s, 1H, NH). IR (nujol) 3360, 3303, 1729, 1636 cm^{-1}. Anal. ($C_{17}H_{16}F_3N_3O_3$) C, H, N.

General Procedure for the Synthesis of Acids 17−20. To a solution of **13−16** (1 mmol) in ethanol (10 mL), 50% aqueous sodium hydroxide (2 mL) and water (2 mL) were added. The mixture was stirred at room temperature for 24 h. The mixture was concentrated in vacuo, and then ice was added. Then the solution was acidified with 20% aqueous hydrochloric acid to pH 3−4. The formed solid was filtered off, washed with water, air-dried, and crystallized from ethanol to give **17−20**.

Example: 2-(4-(2-(Trifluoromethyl)pyridin-4-ylamino)benzamido)acetic Acid (17). Yield 90%; mp 210−212 °C. ^{1}H NMR (DMSO-d_6) δ 4.14 (s, 2H), 6.66 (m, 2H), 7.68 (m, 2H), 7.70 (m, 2H), 8.42 (d, J = 5.4 Hz, 1H), 9.42 (s, 1H), 10.03 (s, 1H). IR (nujol) 3411, 3278, 3183, 2518, 1895, 1713, 1638 cm^{-1}. Anal. ($C_{15}H_{12}F_3N_3O_3$) C, H, N.

Assay of FAAH Activity. Membrane preparations were obtained from brains (without cerebellum) from adult Wistar and Sprague−Dawley rats. The frozen brains were thawed on ice and homogenized with a glass homogenizator in 20 mM HEPES, 1 mM $MgCl_2$, pH 7.0. The homogenates were centrifuged at 17000 rpm for 20 min (4 °C), resuspended in 20 mL buffer, and recentrifuged at 17000 rpm for 20 min (4 °C). The pellets were resuspended in 10 mL of buffer and incubated at 37 °C for 15 min in order to remove any endogenous FAAH substrates which otherwise might interfere with the assay. After this, they were recentrifuged at 17000 rpm for 20 min (4 °C) and the pellets were resuspended in Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 3 mM $MgCl_2$. The FAAH assay was carried out essentially as described previously.[83] Membrane homogenates were diluted with buffer (10 mM Tris-HCl, 1 mM EDTA pH 7.4) to the appropriate protein concentrations so that initial velocities were measured. Test compounds (10 μL, in ethanol except for URB 597 (in dimethyl sulphoxide (DMSO)), 165 μL of homogenates and 25 μL of [^{3}H]AEA

labeled in the ethanolamine part of the molecule (American Radiolabeled Chemicals Inc., St. Louis, MO, in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, containing 1% w/v fatty acid-free bovine serum albumin, final substrate concentration of 0.5 μM) were added to the tubes. The tubes were incubated for 10 min at 37 °C. The reaction was stopped by placing the tubes on ice and adding 400 μL of activated charcoal mixture (80 μL of activated charcoal, 320 μL 0.5 M HCl). The samples were mixed and left at room temperature for about 30 min and then centrifuged at 2500 rpm for 10 min. Aliquots (200 μL) of the supernatants were removed from each tube and analyzed for titrium content by liquid scintillation spectroscopy with quench correction. Blanks contained buffer instead of the membrane homogenates.

Hydrolysis of AEA by Intact RBL2H3 Cells. Rat basophilic leukemia (RBL2H3) cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in MEM with Earl's salts, 2 mM L-glutamine, 15% fetal bovine serum, and 1% 100 U mL^{-1} penicilin + 100 μg mL^{-1} streptomycin. Hydrolysis of [^{3}H-ethanolamine]AEA was measured in 24-well culture plates.[84] Cells were plated at a density of 2×10^5 cells per well and incubated overnight at 37 °C in an atmosphere of 5% CO_2. After incubation, cells were washed with Krebs−Ringer HEPES (KRH) buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethyl-sulfonic acid, 0.12 mM KH_2PO_4, 0.12 mM $MgSO_4$ in Milli-Q deionized water, pH 7.4) containing 1% of BSA and once with KRH buffer alone. Cells were preincubated with KRH buffer containing 0.1% of fatty acid-free BSA and the test compounds for 10 min at 37 °C. After preincubation, 50 μL of [^{3}H]AEA, containing 1% fatty acid-free BSA (assay substrate concentration 100 nM), were added and incubated for 20 min. To stop the reaction, 400 μL of ice-cold MeOH was added and the wells were carefully scraped and then left on ice. Aliquots (400 μL) of the suspension were then transferred in to glass tubes and 200 μL of chloroform was added. After two vortex steps, the samples were left at room temperature for approximately 30 min with subsequent centrifugation at 2500 rpm for 5 min. Aliquots (200 μL) of supernatant were analyzed for titrium content by liquid scintillation spectroscopy with quench correction. Blanks were defined as the tritium recovered in samples without cells added.

Uptake of AEA by RBL2H3 Cells. The assays were undertaken as using a standard method[85] modified as described previously,[86] using 24-well culture plates, a preincubation time of 10 min, and an incubation time of 4 min with substrate (100 nM [^{3}H]AEA, labeled in the arachidonoyl part of the molecule, American Radiolabeled Chemicals). Parallel experiments were undertaken using wells alone to determine whether the test compounds affected the ability of AEA to be retained by inert surfaces.[87]

Assay of URB597-Sensitive Accumulation of Tritium in RBL2H3 Cell Membranes Following Incubation of Cells in Suspension with [^{3}H]AEA. A recently published method[64] was used. RBL2H3 cells were resuspended in medium in Eppendorf tubes to a concentration of 2×10^5 cells per tube (5×10^5 cells/ml) and preincubated with **33**, URB597, or **39** (both Cayman Chemical Co.) for 10 min at 37 °C prior to addition of [^{3}H]AEA (labeled in the arachidonoyl part of the molecule, assay concentration 100 nM) in medium and incubation for 5 min (final assay volume 225 μL). After incubation, the cells were sedimented using a microcentrifguge (1 min, 1000*g*) and washed with ice cold medium, and aliquots (200 μL) of the suspensions were placed in 96-well plates. The radioactivity retained by the cells was separated from that in the medium by filtration through polyethylenimine-coated FilterMAT filters (Skatron Instruments Inc., Sterling, VA) using a Micro cell harvester (Skatron Instruments Inc.) and a 30 s period of washing with deionized water to rupture the cells. The filter papers were analyzed for titrium content by liquid scintillation spectroscopy with quench correction. Blanks were defined as the tritium recovered in samples without cells added.

Assay of MGL Activity. Assays were carried out in a 96-well microtiter plate (100 μL total volume) using a slight modification of a recently reported method.[69] Human recombinant MGL (Cayman Chemical Co., Ann Arbor, MI) in 10 mM Tris-HCl, 1 mM EDTA, and test compounds were added to each well. To start the hydrolysis, 20 μL of 4-nitrophenylacetate (Sigma Chemical Co., St. Louis, MO), final concentration of 0.25 mM, were added. Blanks contained buffer alone. The concentration of MGL was chosen to ensure that initial velocities were measured. The samples were incubated at room temperature. The absorbance was measured at 405 nm after 0 min (to rule out effects of the compounds per se upon the absorbance), 20 and 40 min using a Thermomax microplate reader (ThermoMax Kinetic Microplate Reader, Molecular Devices, Sunnyvale, CA). The 20 min readings were used for the analyses.

Assay of COX Activity. An assay based on an oxygen electrode method[73] was used. For the preincubation assays, a buffer containing 1 μM hematin, 2 mM phenol, 5 mM EDTA, either ovine COX-1 (cat. no. 60100, Cayman Chemical Co., Ann Arbor, MI) or human recombinant COX-2 (cat. no. 60122, Cayman Chemical Co.) (200 U per assay) and 0.1 M tris-HCl, pH 7.4 (final assay volume 2 mL) at room temperature was added to an oxygen electrode chamber with an integral stirring unit (Oxygraph System, Hansatech Instruments, King's Lynn, U.K.) that had been calibrated with respect to air pressure and ambient temperature. After addition of test compound dissolved in DMSO (20 μL), a baseline was established, usually over a period of 5 min. Reactions were started by addition of arachidonic acid (10 μM, unless otherwise stated), and the oxygen consumption was followed for the next 5 min. For the nonpreincubation assays, the assay buffer contained the arachidonic acid, and the reactions were started by addition of the COX. Data is presented as the $\Delta[O_2]$ (μM) from the point of addition of the arachidonic acid (preincubation assay) or COX-1 (nonpreincubation assay).

Data Analyses. Inhibition curves were expressed as % of control and pI_{50}, and hence IC_{50} values were determined using the built-in equation "sigmoidal dose−response (variable slope)" in the GraphPad Prism computer program (GraphPad Software Inc., San Diego, CA). Top (uninhibited) values were set to 100, and bottom (residual activity) values were either set to 0 (eq 1) or not preset (eq 2). The equation best fitting the data was then chosen by use of Akaike's information criteria. In the case of eq 2, the max inhibition was defined as 100—the residual activity. K_m^{app} and V_{max}^{app} (i.e., the observed K_m and V_{max} values in the presence of a given concentration of test compound) were calculated using the built-in equation "one-site binding (hyperbola)" in the GraphPad Prism computer program. For the measurements of oxygen consumption shown in Figure 6, the data over the first 120 s after addition of COX was fitted to the built-in equation "plateau followed by one-phase decay" in the GraphPad Prism computer program.

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Supporting Information Available: Physical and spectral data for compounds **3b**−**3i**, **4b**−**4i**, **5b**−**5i**, **6b**−**6i**, **8**−**11**, **14**−**16**, and **18**−**20**. This material is available free of charge via the Internet at http://pubs.acs.org.

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