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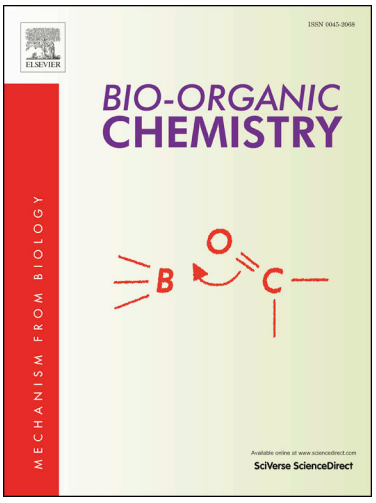

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The fatty acid amide hydrolase and cyclooxygenase-inhibitory properties of novel amide derivatives of carprofen

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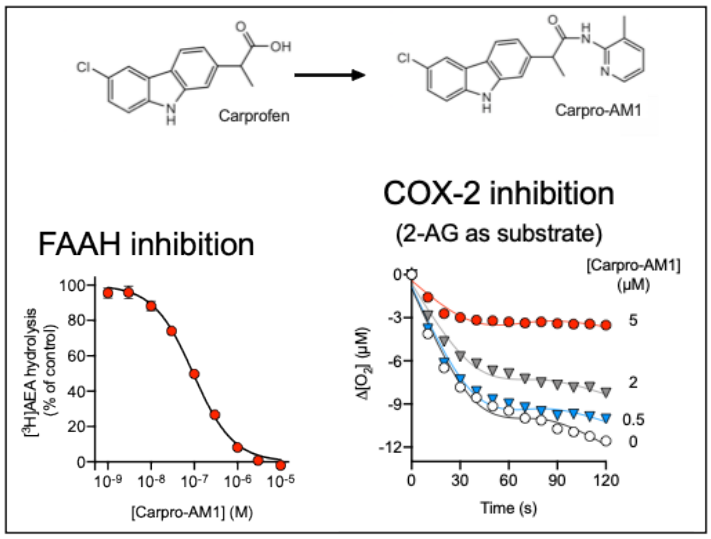
Highlights

* New profen amides were designed and synthesized.
* Profen amides were tested as FAAH and COX inhibitors.
* Some Carprofen amides show high activity against FAAH
* Some Carprofen amides potent are inhibitors of human recombinant COX-2
* Some Carprofen amides are dual-acting FAAH/substrate-selective COX inhibitors representing leads for pharmacological applications.

Keywords:

Carprofen amides; FAAH inhibition; fatty acid amide hydrolase; endocannabinoid; cyclooxygenase; carprofen; non-steroidal anti-inflammatory drugs

Graphical abstract



Abstract

In experimental animals, inhibition of fatty acid amide hydrolase (FAAH) reduces the gastrointestinal damage produced by non-steroidal anti-inflammatory agents that act by inhibition of cyclooxygenase (COX). This suggests that compounds able to inhibit both enzymes may be potentially useful therapeutic agents. In the present study, we have investigated eight novel amide analogues of carprofen, ketoprofen and fenoprofen as potential FAAH/COX dual action inhibitors. **Carpro-AM1** (2-(6-Chloro-9*H*-carbazol-2-yl)-*N*-(3-methylpyridin-2-yl)propenamide) and **Carpro-AM6** (2-(6-Chloro-9*H*-carbazol-2-yl)-*N*-(3-chloropyridin-2-yl)propenamide) were found to be fully reversible inhibitors of the hydrolysis of 0.5 μM [^{3}H]anandamide in rat brain homogenates with IC_{50} values of 94 and 23 nM, respectively, i.e. 2-3 orders of magnitude more potent than carprofen in this respect. Both compounds inhibited the cyclooxygenation of arachidonic acid by ovine COX-1, and were more potent inhibitors of human recombinant COX-2 when 2-arachidonoylglycerol was used as substrate than when arachidonic acid was used. It is concluded that **Carpro-AM1** and **Carpro-AM6** are dual-acting FAAH/substrate-selective COX inhibitors.

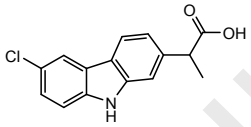
1. Introduction

FAAH catalyses the hydrolysis of the endogenous cannabinoid (endocannabinoid) ligand anandamide (AEA, arachidonoylethanolamide) [1] FAAH inhibition has been shown to be associated with increased levels of AEA and other fatty acid amides (FAAs), producing analgesic effects in various animal models of inflammation and chronic pain primarily via activation of endocannabinoid CB receptors, but without the side effects seen with cannabinoid receptor agonists [2]. FAAH inhibitors show efficacy in preclinical models of various disease

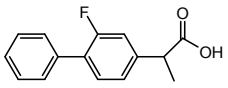
states that range from pain, gastrointestinal, cardiovascular, cancer to neuropsychiatric disorders [3-6].

The primary mode of action of non-steroidal anti-inflammatory agents (NSAIDs) such as ibuprofen and naproxen is the inhibition of cyclooxygenase (COX)-catalysed production of prostaglandins from arachidonic acid (AA) [7]. However, there is good evidence in animal models that NSAIDs also involve the endocannabinoid system in their pharmacological effects [8-11]. In brief, in its simplest form the endocannabinoid system, which is involved in regulatory mechanism as diverse as control of pain and of bone turnover, comprises two G-protein coupled cannabinoid receptors, their endogenous ligands 2-arachidonoylglycerol (2-AG) and anandamide (arachidonoylethanolamide, AEA) and the metabolic machinery required for their synthesis and catabolism [12]. AEA and 2-AG are both substrates for COX-2, but not COX-1 mediated catabolism. *In vitro* experiments have indicated that the (*R)*-profens inhibit the cyclooxygenation of the AEA and 2-AG by COX-2 at lower concentrations than required for the inhibition of the cyclooxygenation of arachidonic acid [13] and achiral profen analogues which retain this property have been designed [14]. This property may contribute to the efficacy of (*R)*-flurbiprofen in an animal model of neuropathic pain [15].

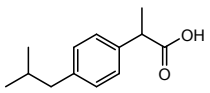
In 1997, one of us (C.J.F.) reported that ibuprofen inhibited the hydrolysis of AEA in rat brain homogenates [16]. This property is shared by other profens, albeit with a wide range of potencies (Figure 1). Since the profens behave as mixed-type or competitive reversible inhibitors of fatty acid amide hydrolase (FAAH), the enzyme responsible for AEA hydrolysis in the brain [17], their IC_{50} value will be dependent upon the AEA substrate concentration.



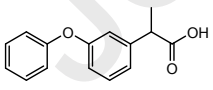
Carprofen (**1**)
IC_{50} 28 μM[b]
IC_{50} 59 μM[c]



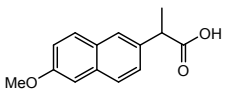
Flurbiprofen
IC_{50} 29, 51 μM[d]
IC_{50} 55 μM[e]



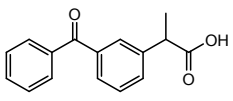
Ibuprofen
IC_{50} 260 μM[f]
IC_{50} 270 μM[g]



Fenoprofen (**2**)
IC_{50} 480 μM[g]



Naproxen
38% inh at 100 μM[h]



Ketoprofen (**3**)
IC_{50} 650 μM[g]

Figure 1. Structures and inhibitory potencies[a] of six NSAIDs of the profen class towards rat brain FAAH.
[a]The assay pH was in the range 7.3 – 7.6 in all cases. [b]Determined using 0.5 μM AEA [18]. [b]Determined using 0.5 μM AEA [19]. [d]Determined using 0.5 μM AEA [20, 21]. [e]Determined using 2 μM AEA the value is the average for the two enantiomers assayed *per se* (60 and 50

μM, for the *R*- and *S*-enantiomers, respectively [22]. [f]Determined using 0.5 μM AEA [21]. [g]Determined using 2 μM AEA [16]. [h]At 0.5 μM AEA, the highest concentration used was 100 μM, and the % inhibition at this concentration is shown [20].

The FAAH inhibitory potencies of the profens are admittedly rather modest when compared with their abilities to inhibit COX, but the compounds form a useful starting point for the design of compounds with an improved potency towards FAAH (fatty acid amide hydrolase, the primary hydrolytic enzyme for AEA while retaining the COX-inhibitory properties of the original profens. There is an important scientific rationale for the design of such compounds, namely that in animal models, inhibition of FAAH prevents gastric mucosal damage produced by NSAIDs [23,24], which is a serious problem associated with the clinical use of these drugs [25,26]. So far, most studies have used ibuprofen as a template [27-31], and **Ibu-AM5**, the *N*-(3-methylpyridin-2-yl)amide derivative of ibuprofen (Figure 2), was found to inhibit rat brain FAAH with an IC_{50} in the range of 0.52-1.2 μM using the same assay conditions as in the present paper, whilst retaining the substrate-selective inhibition of COX-2 seen with the parent profen [21,30]. **Ibu-AM5** is active in a mouse model of visceral pain, without producing the gastric mucosal damage seen with ibuprofen. [32]. A similar increase in potency of about 2-3 orders of magnitude compared to the parent profen was seen with the *N*-(3-methylpyridin-2-yl)amide derivatives of flurbiprofen (**Flu-AM1**, IC_{50} 0.44 μM), [20] and naproxen (**Napro-AM1**, IC_{50} 0.74 μM) [20] (Figure 2).

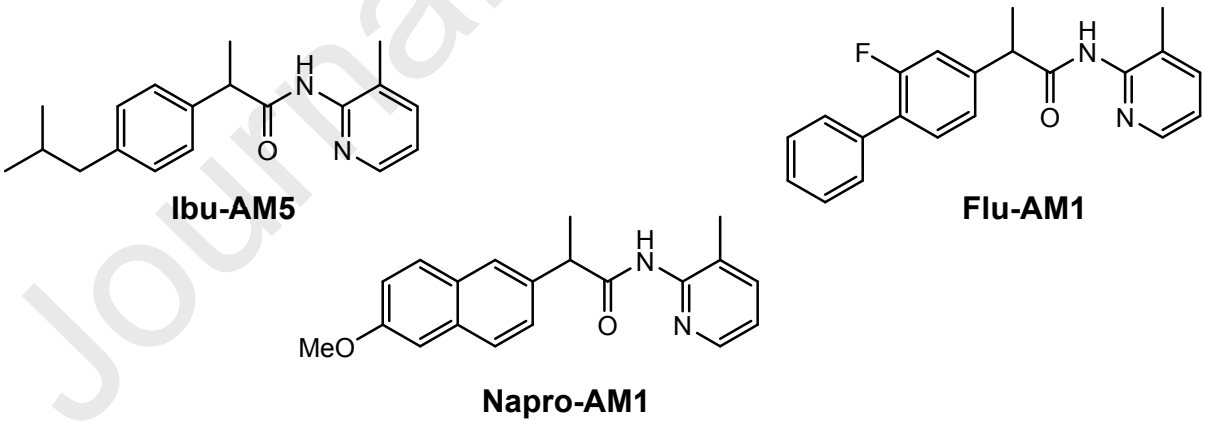


Figure 2. Structures of the profen amides **Ibu-AM5**, **Flu-AM1** and **Napro-AM1**

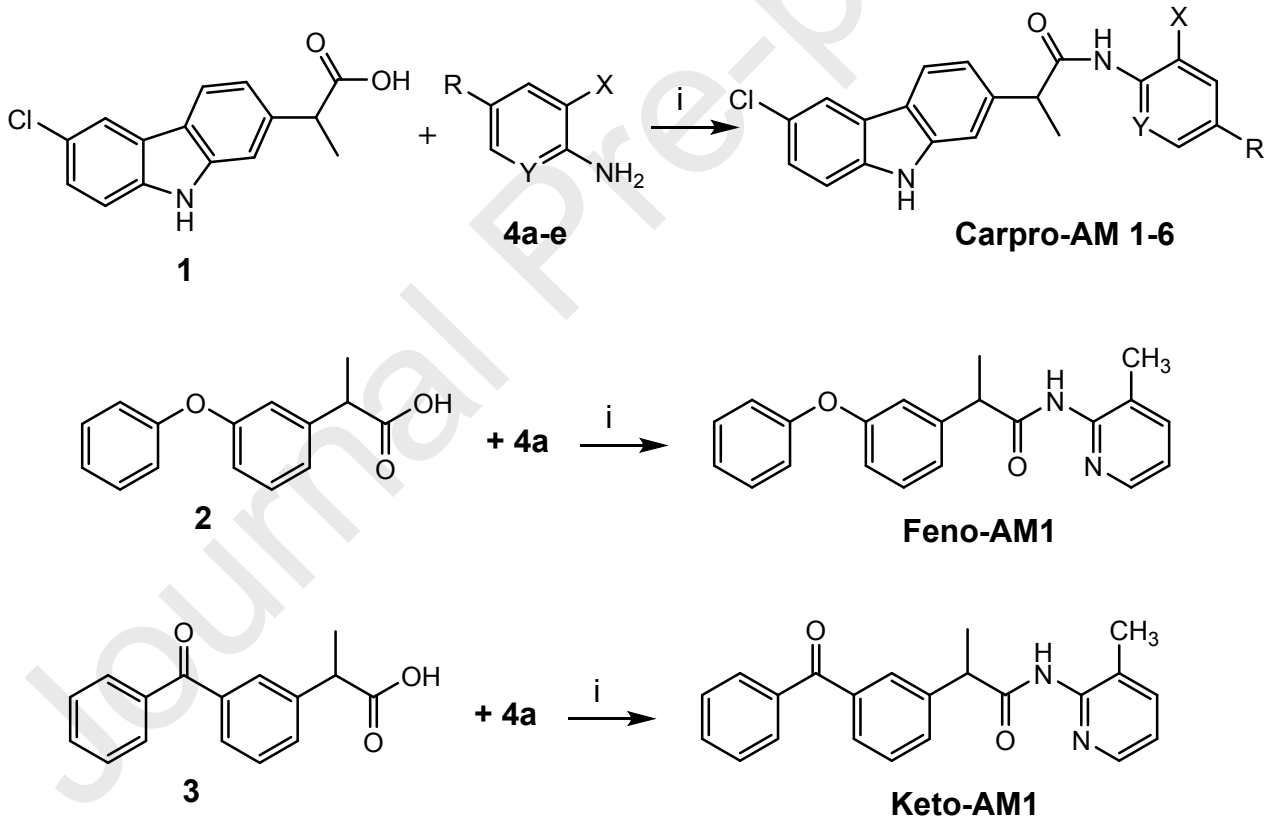
Given that carprofen is the most potent of the NSAIDs towards FAAH [18,19], it is a potentially useful template for the design of novel dual-action FAAH / substrate-selective COX inhibitors. To our knowledge, the only available data in this regard was by Favia *et al*. [19], who identified compounds with an FAAH inhibitory potency up to ~17-fold more potent than

carprofen. Unfortunately, however, the compounds did not inhibit COX. In the present study, we identify novel carprofen derivatives with sub-micromolar potencies towards rat brain FAAH and which retain their substrate-selective inhibition of COX.

2. Results and discussion

2.1. Chemistry

The synthesis of the new carprofen amides is reported in Scheme 1. Coupling of Carprofen (**1**) with substituted 2-aminopyridines or 2-methyl-4-hydroxyaniline **4** gave the **CarproAM1-6** in moderate to good yields. The reaction was performed in dry acetonitrile solution (CH_3CN), in the presence of 1-hydroxybenzotriazole hydrate (HOBt) using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) as coupling agent. The same synthetic pathway was used for the synthesis of **Feno-AM1** and **Keto-AM1** starting from 2-amino-3-methylpyridine (**4a**) and Fenoprofen (**2**) and Ketoprofen (**3**) respectively (Scheme 1).



4a Y = N, X = Me, R = H
4b Y = CH, X = Me, R = OH
4c Y = N, X = I, R = H
4d Y = N, X = Br, R = H
4e Y = N, X = CF_3, R = H
4f Y = N, X = Cl, R = H

Scheme 1. General synthetic procedure for Carprofen, Fenoprofen and Ketoprofen amides. Reagents and conditions: (i) EDCI, HOBt, dry CH_3CN r.t. 36h.

2.2. FAAH inhibition

Given that the conversion of ibuprofen, flurbiprofen and naproxen into their amides with 2-amino-3-methylpyridine increases the inhibitory potency towards rat brain FAAH by 2-3 orders of magnitude, [18,20,27,30], we investigated whether a similar increase in potency was seen for the corresponding *N*-(3-methylpyridin-2-yl)amide derivatives of carprofen, fenoprofen and ketoprofen. The data are shown in Figure 3 and summarized in Table 1. In all three cases, the potency was increased by at least one order of magnitude as compared to the corresponding profen. Of the three, the most potent was **Carpro-AM1**, with an IC_{50} of 94 nM, i.e. a ~300-fold than seen for carprofen when assayed under the same conditions. **Feno-AM1** and **Keto-AM1** were much less potent, with IC_{50} values of 51 and 19 µM, respectively.

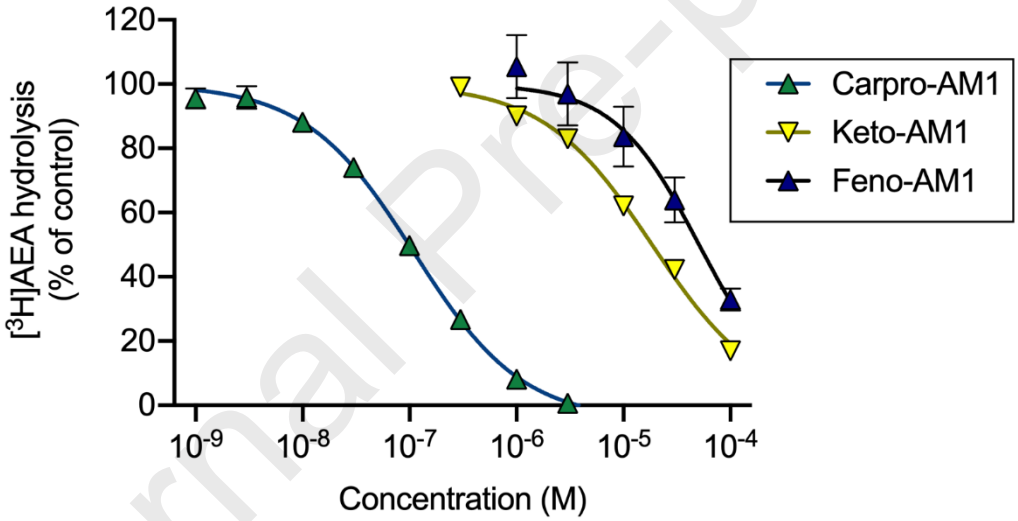
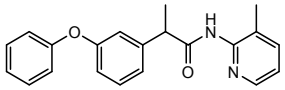
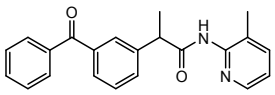
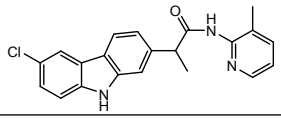


Figure 3. Inhibition of rat brain [^{3}H]AEA hydrolysis by **Carpro-AM1**, **Feno-AM1** and **Keto-AM1.** The data shows the means ± SEM (when not covered by the symbols), N=3 for the inhibition by the compounds shown of the hydrolysis of 0.5 µM [^{3}H]AEA by rat brain homogenates.

Table 1. IC_{50} values for the inhibition by novel profen amides of the hydrolysis of 0.5 µM [^{3}H]AEA by rat brain homogenates.[a]

Compound	Formula	IC_{50} (µM)	95% confidence limits of the IC_{50}
Feno-AM1		51	33-87

Keto-AM1		19	17-21
Carpro-AM1		0.094	0.083-0.11

[a]The values are for the data presented in Figure 3. The 95% confidence limits are the profile likelihood values returned by the computer program (see experimental).

In view of the potency of **Carpro-AM1**, we investigated a series of five analogs in order to determine whether or not the potency could be further improved. The new carprofen amides were designed on the basis of our previous studies on Ibu-AM5 analogs [31] indicating that the replacement of the 2-amino-3-methylpyridine moiety with a 2-methyl-4-hydroxyaniline or with 2-amino3-halolpyridine caused activity increase. The FAAH inhibition data of **Carpro-AM2-6** are shown in Figure 4 and summarized in Table 2.

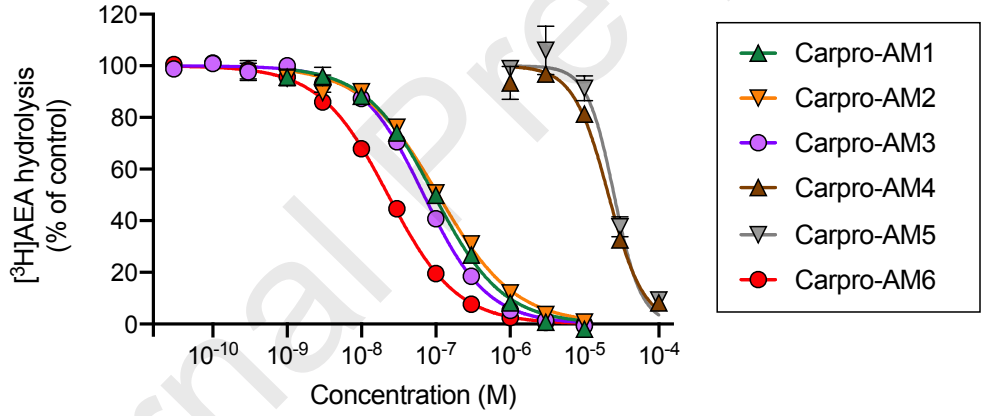
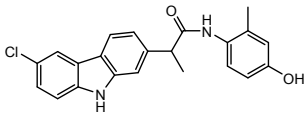
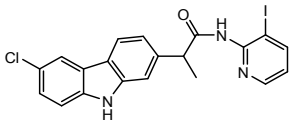
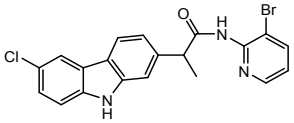
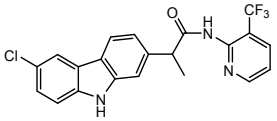
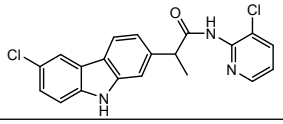


Figure 4. Inhibition of rat brain [^{3}H]AEA hydrolysis by Carpro-AM compounds.
The data shows the means ± SEM (when not covered by the symbols), N=3-5 for the inhibition by the compounds shown of the hydrolysis of 0.5 µM [^{3}H]AEA by rat brain homogenates. Note that the data for **Carpro-AM1** is the same as in Figure 3 and is included here for comparative purposes

Table 2. IC_{50} values for the inhibition by novel profen amides of the hydrolysis of 0.5 µM [^{3}H]AEA by rat brain homogenates.[a]

Compound	Formula	IC_{50} (µM)	95% confidence limits of the IC_{50}
Carpro-AM2		0.11	0.095-0.12

Carpro-AM3		0.069	0.064-0.074
Carpro-AM4		21	18-24
Carpro-AM5		25	21-30
Carpro-AM6		0.023	0.021-0.025

[a]The values are for the data presented in Figures 4A and 4B. The 95% confidence limits are the profile likelihood values returned by the computer program (see experimental).

Replacement of the 3-methyl group of the pyridine ring with either a bromine atom (**Carpro-AM4**) or a trifluoromehyl group (**Carpro-AM5**) greatly reduced the potency (IC_{50} values 21 and 25 μM, respectively). However, potency was increased slightly with a replacement with iodine atom (**Carpro-AM3**, IC_{50} value 69 nM) and more notably with a chlorine atom (**Carpro-AM6**, IC_{50} value 23 nM). The replacement of methylpyridine ring of **Carpro-AM1** with a 2-methyl-4-hydroxyphenyl to give **Carpro-AM2** that had essentially the same potency as **Carpro-AM1** (the IC_{50} value for **Carpro-AM2** was 110 nM, but the 95% confidence limits of this value overlapped with those for **Carpro-AM1**). The SAR of Carprofen amides resembles that of Ibuprofen amides with the exception of the bromine that in Ibu-AM series showed about the same activity of the iodine analog [31].

The nature of the inhibition (reversible or irreversible) was investigated for **Carpro-AM1** and **Carpro-AM6**. For an irreversible inhibitor, the enzyme inhibition will be time-dependent, and, following a preincubation period with the homogenate, should remain upon dilution of the enzyme-inhibitor complex. A freely reversible compound, on the other hand, should show a reduced inhibition upon such dilution. The data for **Carpro-AM1** and **Carpro-AM6** are shown in Figure 5. Taking **Carpro-AM1** as an example, the observed inhibition produced by 30, 100 and 300 nM was not increased by a preincubation period of up to 60 min prior to addition of substrate (Figure 5A). Preincubation of a concentrated homogenate with 120 nM of **Carpro-AM1** for 60 min followed by a 20-fold dilution (to reduce the free concentration to 6 nM) prior to addition of substrate gave a similar degree of inhibition to that seen with 6 nM without preincubation and dilution, and was much less than that seen with 120 nM without preincubation and dilution (Figure 5B). The same pattern was seen with 180 nM (vs. 9 and 180

nM) and 240 nM (vs. 12 and 240 nM) (Figure 5B). These data indicate that the inhibition of [^{3}H]AEA **by Carpro-AM1** is fully reversible in nature. A similar result was seen with **Carpro-AM6** (Figures 5C and 5D).

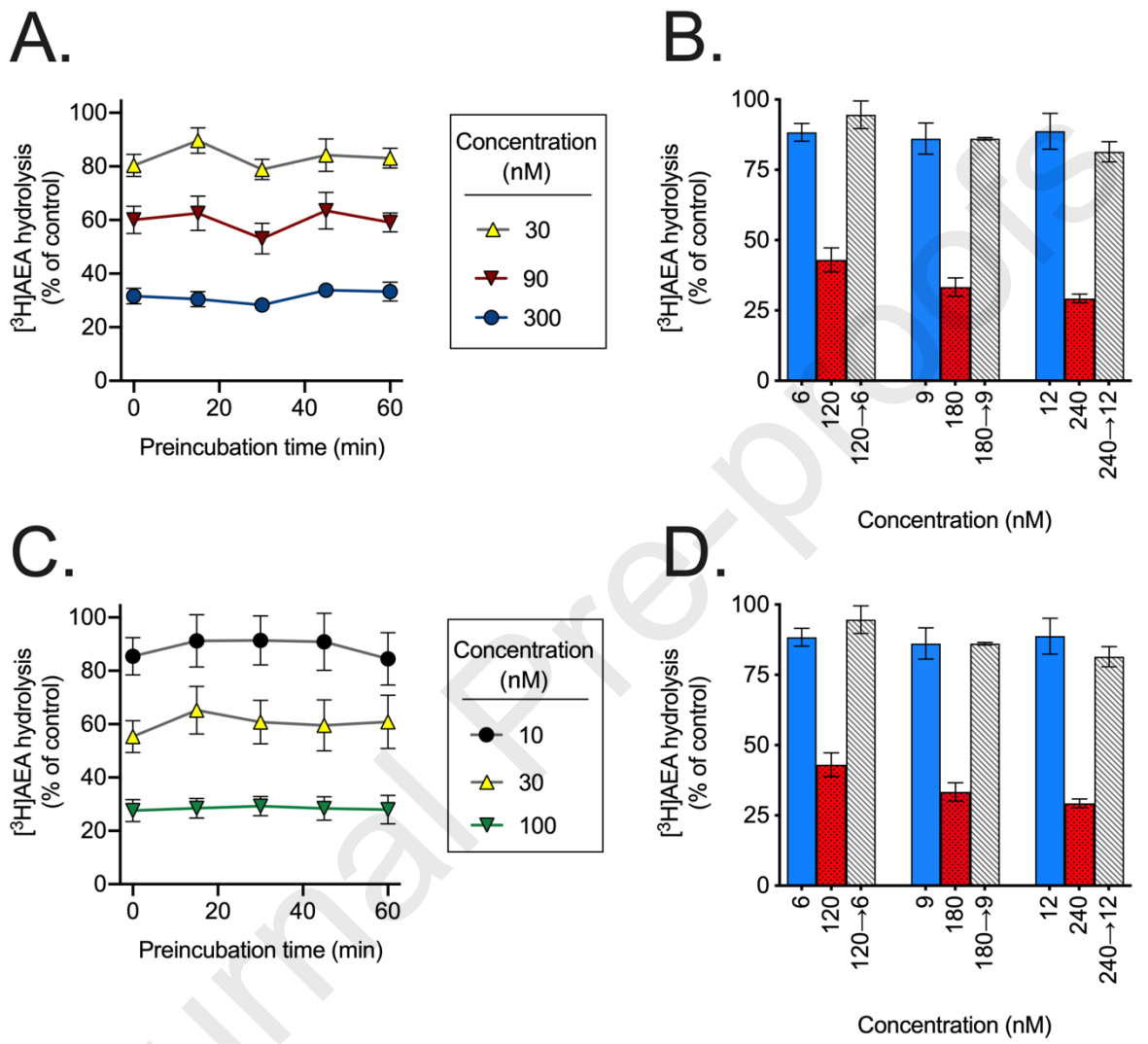


Figure 5. Reversibility of the inhibition of [^{3}H]AEA inhibition by A, B: **Carpro-AM1** and C, D: **Carpro-AM6**

In (A) and (C), the homogenates were preincubated with **Carpro-AM1** (A) and **Carpro-AM6** (C) for the times shown prior to addition of 0.5 mM [^{3}H]AEA. In (B) and (D), homogenates were preincubated with either vehicle, 120, 180 or 240 nM **Carpro-AM1** (B) and **Carpro-AM6** (D) for 60 min. Aliquots were then diluted 20-fold and assayed for FAAH activity with 0.5 mM [^{3}H]AEA. These are shown as 120→6, 180→9 and 240→12 in the figure. Concomitantly, the Carpro-AM were added to vehicle-preincubated aliquots to give concentrations of 6, 9 and 12 nM (representing free concentrations after a 20-fold dilution), 120, 180 and 240 nM (representing the concentrations added prior to preincubation) concentrations. Data are means ± SEM (when not covered by the symbols), N=3.

*2.3. Inhibition of COX isoenzymes by **Carpro-AM1** and **Carpro-AM6***

The ability of **Carpro-AM1** and **Carpro-AM6** to inhibit the cyclooxygenation of AA and 2-AG by COX-1 and COX-2 was investigated (Figure 6). In the case of **Carpro-AM1**, the compound clearly inhibited the cyclooxygenation of AA by COX-1 at concentrations of 10 and 30 µM, whereas concentration of 50 and 100 µM were required for the inhibition of the cyclooxygenation of this substrate by COX-2 (Figure 6A). In contrast, robust inhibition was seen with 5 µM **Carpro-AM1** when 2-AG was used as a substrate for COX-2 (COX-1 does not metabolise this substrate). A similar pattern was seen with **Carpro-AM6** (Figure 6B). This substrate-selective inhibition of COX-2, first reported by Marnett *et al.* [13] for *R*-profens, has also been seen for **Ibu-AM5**, **Flu-AM1** and **Naprox-AM1** [20,21,30]. Marnett *et al.* [13] suggested that the substrate selectivity may reflect the ability of the *R*-profen to bind allosterically to one of the homodimeric sites of COX-2, and that this binding is sufficient to prevent 2-AG, but not AA hydrolysis. It is possible that a similar mechanism is operative here.

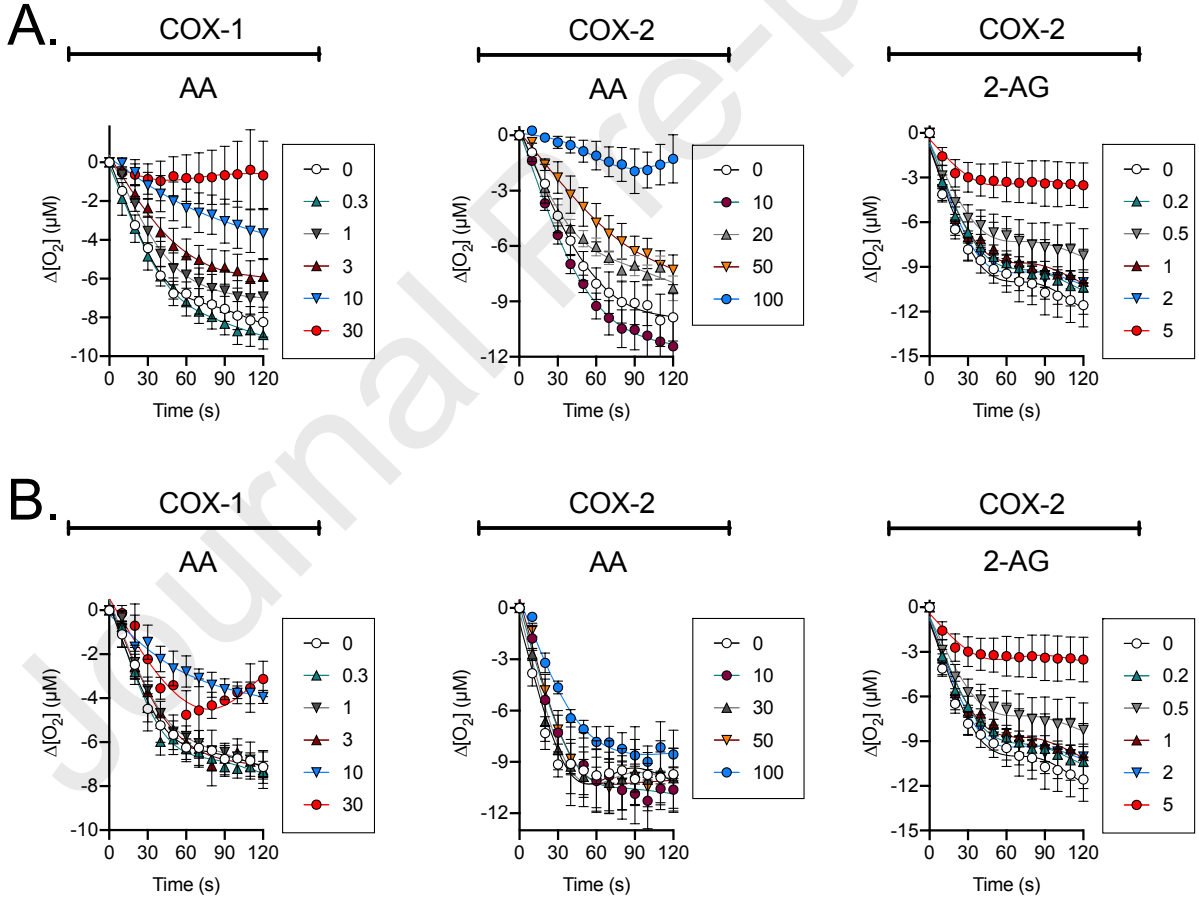


Figure 6. The influence of A. **Carpro-AM1** and B. **Carpro-AM6** upon the cyclooxygenation of 10 µM arachidonic acid (AA) and 2-arachidonoylglycerol (2-AG) by COX-1 and COX-2. Shown are means ± SEM, N=3-4 in each graph for the changes in oxygen tension following addition of enzyme in the presence of the Carpro-AM compound. The concentrations of the

Carpro-AM compounds, in μM, are shown on the right of each panel. The enzyme isoform and substrate used is given above each panel.

3. Conclusion

In conclusion, the present study has characterized *in vitro* analogues of carprofen that are reversible inhibitors of FAAH with nanomolar inhibitory potencies and which retain the substrate-selective inhibition of COX seen with the profens. The nanomolar potency towards FAAH puts the compound in the same potency region as ARN2508, a dual-action functionally irreversible FAAH-COX inhibitor based on the structural elements of the irreversible FAAH inhibitor URB597 and of flurbiprofen [33]. Despite the similar potencies, carprofen amides and ARN2508 differ in their modes of inhibition, being reversible and irreversible, respectively. The irreversible inhibition of ARN2508 is due to the carbamate moiety and its removal or modification afforded lost in activity. On the contrary the lack of carbamate moiety in carprofen amides did not produces FAAH reduction in activity as in urea analog of ARN2508 [33]. The Carpro-AM compounds were tested as racemates, so it is in theory possible that one of the enantiomers may have an even higher potency towards FAAH than the racemate as the (*S*)-Ibu-AM5 or that the two enantiomers show no difference in potencies as Flu-AM1 [18]. Given that ARN2508 is active in the 2,4,6-trinitrobenzene sulfonic acid colon inflammation model at doses that do not produce deleterious effects upon stomach integrity [33], the most potent compounds identified in the present study should be investigated further in vitro with respect to the potential importance of chirality [18] and in vivo in animal models.

4. Experimental section

4.1. Chemistry

All commercially available solvents and reagents were used without further purification and were purchased from Sigma-Aldrich (Milan, Italy). NMR spectra were recorded on an Inova 500 spectrometer (Varian, Palo Alto, CA). The chemical shifts (δ) are reported in part per million downfield from tetramethylsilane (TMS), which was used as internal standard, and the spectra were recorded in hexadeuteriodimethylsulphoxide (DMSO-d_6). Infrared spectra were recorded on a Vector 22 spectrometer (Bruker, Bremen, Germany) in Nujol mulls. The main bands are given in cm^{-1}. Positive-ion electrospray ionisation (ESI) mass spectra were recorded on a double-focusing MAT 95 instrument (Finnigan, Waltham, MA) with BE geometry.

Melting points (mp) were determined on a SMP1 Melting Point apparatus (Stuart Scientific, Stone, UK) and are uncorrected. All products reported showed ^{1}H NMR spectra in agreement with the assigned structures. The purity of the tested compounds was determined by combustion elemental analyses conducted by the Microanalytical Laboratory of the Department of Chemical and Pharmaceutical Sciences of the University of Ferrara with a MT-5 CHN recorder elemental analyser (Yanagimoto, Kyoto, Japan) and the values found were within 0.4% of theoretical values.

4.1.1. General procedure for the synthesis of ***Feno-AM1****,* ***Keto- AM1****, and* ***Carpro-AM1-6****.*

The solution of the appropriate acid (1 mmol), EDCI (0.19 g, 1.1 mmol) and HOBt (0.13 g, 1 mmol) in anhydrous MeCN (10 mL) was stirred at r.t., after 30 minutes the appropriate amine was added (1 mmol). The mixture was stirred at r.t. for 36 hours after which time the solvent was removed under vacuum. The residue was dissolved in AcOEt (20 mL) and washed sequentially with brine (2x5 mL), 10% aqueous citric acid (2 x 5 mL), saturated $NaHCO_3$ aqueous solution (2 x 5 mL) and water (2 x 5 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated under vacuum.

*4.1.1.1. N-(3-Methylpyridin-2-yl)-2-(3-phenoxyphenyl)propanamide (****Feno-AM1****)*

Obtained following the general procedure by the condensation between fenoprofen (**2**) and 2-amino-3-methylpyridine (**4a**). Yield 82 %. Oil. ^{1}H NMR (DMSO-d_6) δ 1.33 (d, J = 7.0 Hz, 3H, CH_3), 2.03 (s, 3H, CH_3), 3.66 (q, J = 7.0 Hz, 1H, CH), 5.66 (s, 1H, NH), 6.45-7.77 (m, 12H, Ar). IR (Film) 2946, 1685, 1588 cm^{-1}. Elemental analysis: calculated for $C_{21}H_{20}N_2O_2$ (332.40) % C 75.88; H 6.06; N 8.43; found % C 75.96; H 6.04; N 8.46.

*4.1.1.2. 2-(3-Benzoylphenyl)-N-(3-methylpyridin-2-yl)propanamide (****Keto-AM1****)*

Obtained following the general procedure by the condensation between ketoprofen (**3**) and 2-amino-3-methylpyridine (**4a**). Yield 87 %. Oil. ^{1}H NMR (DMSO-d_6) δ 1.46 (d, J = 7.0 Hz, 3H, CH_3), 2.03 (s, 3H, CH_3), 3.81 (q, J = 7.0 Hz, 1H, CH), 7.16-8.22 (m, 12H, Ar), 10.23 (s, 1H, NH). IR (Film) 3323, 3201, 3060, 2973, 1659, 1596 cm^{-1}. Elemental analysis: calculated for $C_{22}H_{20}N_2O_2$ (344.41) % C 76.72; H 5.85; N 8.13; found % C 76.78; H 5.84; N 8.16.

*4.1.1.3. 2-(6-Chloro-9H-carbazol-2-yl)-N-(3-methylpyridin-2-yl)propanamide (****Carpro-AM1****)*

Obtained following the general procedure by the condensation between carprofen (**1**) and 2-amino-3-methylpyridine (**4a**). Yield 44 %. m.p. 206-208 °C. ^{1}H NMR (DMSO-d_6) δ 1.48 (d, J = 6.5 Hz, 3H, CH_3) 1.95 (s, 3H, CH_3) 4.06 (q, J = 6.5 Hz, 1H, CH), 7.07-7.58 (m, 6H, Ar), 8.05-8.19 (m, 3H, Ar), 10.12 (s, 1H, NH), 11.35 (s, 1H, NH). IR (Nujol) 3204, 1650, 1516 cm^{-1}. m/z 364 $(M + H)^+$. Elemental analysis: calculated for $C_{21}H_{18}ClN_3O_2$ (363.84) % C 69.32; H 4.99; N 11.55; found % C 69.38; H 5.01; N 11.52.

*4.1.1.4. 2-(6-Chloro-9H-carbazol-2-yl)-N-(4-hydroxy-2-methylphenyl)propanamide (**Carpro-AM2**)*

Obtained following the general procedure by the condensation between carprofen (**1**) and 4-hydroxy-2-methylaniline (**4b**). Yield 26 %. m.p. 125-126 °C. ^{1}H NMR (DMSO-d_6) δ 1.48 (d, *J* = 6.5 Hz, 3H, CH_3), 1.93 (s, 3H, CH_3), 3.98 (q, *J* = 6.5 Hz, 1H, CH), 6.52 (m, 2H, Ar), 6.96 (m, 1H, Ar), 7.23 (d, *J* = 3.5 Hz, 1H, Ar), 7.35 (d, *J* = 6.0 Hz, 1H, Ar), 7.49 (m, 2H, Ar), 8.09 (d, *J* = 3.5 Hz, 1H, Ar), 8.16 (s, 1H, Ar), 9.15 (s, 1H, OH), 9.21 (s, 1H, NH), 11.35 (s, 1H, NH). m/z 379 $(M + H)^+$. IR (Nujol) 3286, 1659, 1569 cm^{-1}. Elemental analysis: calculated for $C_{22}H_{19}ClN_2O_2$ (378.85) % C 69.75; H 5.06; N 7.39; found % C 69.81; H 5.08; N 7.42.

*4.1.1.5. 2-(6-Chloro-9H-carbazol-2-yl)-N-(3-iodopyridin-2-yl)propanamide (**Carpro-AM3**)*

Obtained following the general procedure by the condensation between carprofen (**1**) and 2-amino-3-iodopyridine (**4c**). Yield 37 %. m.p. 190-191 °C. ^{1}H NMR (DMSO-d_6) δ 1.52 (d, *J* = 6.5 Hz, 3H, CH_3), 4.04 (q, *J* = 6.5 Hz, 1H, CH), 7.04 (m, 1H, Ar), 7.23 (d, *J* = 7.5 Hz, 1H, Ar), 7.35 (d, *J* = 8.0 Hz, 1H, Ar), 7.47 (d, *J* = 8.0 Hz, 1H, Ar), 7.54 (s, 1H, Ar), 8.09 (d, *J* = 8.0 Hz, 1H, Ar), 8.17 (s, 1H, Ar), 8.28 (d, *J* = 8.0 Hz, 1H, Ar), 8.40 (d, *J* = 3.5 Hz, 1H, Ar), 10.25 (s, 1H, NH), 11.35 (s, 1H, NH). IR (Nujol) 3290, 1659, 1569 cm^{-1}. m/z 476 $(M + H)^+$. Elemental analysis: calculated for $C_{20}H_{15}ClIN_3O$ (475.71) % C 50.50; H 3.18; N 8.83; found % C 50.44; H 3.19; N 8.79.

*4.1.1.6. 2-(6-Chloro-9H-carbazol-2-yl)-N-(3-bromopyridin-2-yl)propanamide (**Carpro-AM4**)*

Obtained following the general procedure by the condensation between carprofen (**1**) and 2-amino-3-bromopyridine (**4d**). Yield 43 %. m.p. 116-118 °C. ^{1}H NMR (DMSO-d_6) δ 1.43 (d, *J* = 6.5 Hz, 3H, CH_3), 3.82 (q, *J* = 6.5 Hz, 1H, CH), 6.58 (d, *J* = 6.0 Hz, 1H, Ar), 7.10 (d, *J* = 7.5 Hz, 1H, Ar), 7.34 -7.48 (m, 4H, Ar), 8.08 (m, 3H, Ar), 8.16 (s, 1H, NH), 11.34 (s, 1H, NH). IR (Nujol) 3314, 1648, 1562 cm^{-1}. m/z 429 $(M + H)^+$. Elemental analysis: calculated for $C_{20}H_{15}BrClN_3O$ (428.71) % C 56.03; H 3.53; N 9.80; found % C 55.96; H 3.54; N 9.84.

*4.1.1.7. 2-(6-Chloro-9H-carbazol-2-yl)-N-(3-(trifluoromethyl)pyridin-2-yl)propanamide (**Carpro-AM5**)*

Obtained following the general procedure by the condensation between carprofen (**1**) and 2-amino-3-(trifluoromethyl)pyridine (**4d**). Yield 41 %. m.p. 115 °C. ^{1}H NMR (DMSO-d_6) δ 1.44 (d, *J* = 6.5 Hz, 3H, CH_3), 3.82 (q, *J* = 6.5 Hz, 1H, CH), 6.58 (d, *J* = 5.5 Hz, 1H, Ar), 7.10 (d, *J* = 7.5 Hz, 1H, Ar), 7.35 -7.49 (m, 4H, Ar), 8.08 (m, 3H, Ar), 8.17 (s, 1H, NH), 11.34 (s, 1H, NH). IR (Nujol) 3330, 1688, 1648, 1561 cm^{-1}. m/z 418 $(M + H)^+$. Elemental analysis: calculated for $C_{21}H_{15}ClF_3N_3O$ (417.81) % C 60.37; H 3.62; N 10.06; found % C 60.44; H 3.60; N 10.10.

*4.1.1.8. 2-(6-Chloro-9H-carbazol-2-yl)-N-(3-chloropyridin-2-yl)propanamide (**Carpro-AM6**)*
Obtained following the general procedure by the condensation between carprofen (**1**) and 2-amino-3-chloropyridine (**4f**). Yield 61 %. m.p. 174-175 °C. ^{1}H NMR (DMSO-d_6) δ 1.48 (d, *J* = 6.5 Hz, 3H, CH_3), 4.07 (q, *J* = 6.5 Hz, 1H, CH), 7.08-7.51 (m, 5H, Ar), 7.92-8.34 (m, 4H, Ar), 10.33 (s, 1H, NH), 11.40 (s, 1H, NH). IR (Nujol) 3213, 1664, 1581 cm^{-1}. m/z 385 $(M + H)^+$. Elemental analysis: calculated for $C_{20}H_{15}Cl_2N_3O$ (384.26) % C 62.51; H 3.93; N 10.94; found % C 62.45; H 3.94; N 10.90.

4.2. Pharmacology

4.2.1. Materials

Anandamide [ethanolamine-1-^{3}H] ([^{3}H]AEA, specific activity 2.22 TBq mmol^{-1}) was purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO). Ovine COX-1 (cat. no. 60100), human recombinant COX-2 (cat. no. 60122), non-radioactive AEA, arachidonic acid (AA) and 2-AG were purchased from the Cayman Chemical Co. (Ann Arbor, MI, USA).

4.2.2. FAAH assay

Frozen (-80 °C) brains (minus cerebella) from adult rats (Wistar or Sprague-Dawley) were thawed and homogenised in 20mM HEPES, 1mM $MgCl_2$, pH 7.0. Homogenates were centrifuged (~35000 x g for 20 min at 4 °C) followed by washing (by recentrifugation and by resuspension in the buffer) twice before incubation at 37 °C for 15 min in order to hydrolyse all endogenous FAAH substrates. Following a further centrifugation, pellets were resuspended in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 3 mM $MgCl_2$, and frozen at -80 °C in aliquots until used for the assay. The FAAH assay was conducted as described by Boldrup *et al.* [34]. Test compounds, homogenates and [^{3}H]AEA (diluted with non-radioactive AEA to give a substrate concentration of 0.5 μM) in 10mM Tris- HCl, 1mM EDTA, pH 7.4, containing 1% w/v fatty acid-free bovine serum albumin were incubated for 10min at 37°C. Following addition of activated charcoal in 0.5M HCl to adsorb the unmetabolized [^{3}H]AEA, samples were mixed, briefly cooled on ice and left at r.t. for ~30 min. Following centrifugation (2000 *g* for 10 min), aliquots of the supernatants, containing the [^{3}H]ethanolamine produced by hydrolysis of [^{3}H]AEA, were analysed for tritium content by liquid scintillation spectroscopy with quench correction. Blank values were obtained by the use of buffer in place of homogenate. The amount of homogenate used per assay was in general 1 μg protein. In the dilution experiments, a stronger concentration was used initially (10 μg protein /assay tube).

For all compounds, data were expressed as % of vehicle control and analysed using the algorithm log(inhibitor) vs. response – variable slope (four parameters) built into the GraphPad

Prism computer program v8.3 for the Macintosh (GraphPad Software Inc., San Diego, CA). The 95% confidence limits (profile likelihood) for the IC_{50} values returned by the programme are in the results.

Time dependency of FAAH inhibition was assayed as described above with the addition of a preincubation phase of homogenate with substance (0-60 min) at 37°C is added prior to addition of 0.5 μM [^{3}H]AEA and a further incubation of 10 min.

Reversibility of FAAH inhibition is assayed by preincubation for 60 min of homogenate and substance (20x concentration) prior to dilution (20x) in buffer (10mM Tris- HCl, 1mM EDTA, pH 7.4, containing 1% w/v fatty acid-free bovine serum albumin). The diluted enzyme and substance is assayed at standard homogenate concentration with 0.5 μM [^{3}H]-AEA for 10 min and compared to the hydrolysis by samples of diluted and concentrated (20x) substance that has not been preincubated.

4.2.3. *COX-1 and 2 assay*

COX assays were undertaken essentially according to the method of Meade *et al.* [35]. An oxygen electrode chamber with integral stirring (Oxygraph System, Hansatech Instruments, King´s Lynn, U.K.) was calibrated daily to the ambient temperature and air pressure. The assay buffer contained 0.1 M Tris-HCl buffer pH 7.4, 1 μM haematin, 2 mM phenol, 5 mM EDTA, 10 μM substrate (AA for both COX isoenzymes, or 2-AG for COX-2) in a final assay volume of 2 ml. After addition of test compound, a baseline was established for 5 min. Thereafter reactions were initiated by addition of 200 units ovine COX-1 or human recombinant COX-2. The change in oxygen consumption was then monitored for approximately 5 min.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.