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Inhibitory properties of ibuprofen and its amide analogues towards the hydrolysis and cyclooxygenation of the endocannabinoid anandamide

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Abstract

A dual-action cyclooxygenase (COX) - fatty acid amide hydrolase (FAAH) inhibitor may have therapeutic usefulness as an analgesic, but a key issue is finding the right balance of inhibitory effects. This can be done by the design of compounds exhibiting different FAAH/COX inhibitory potencies. In the present study, eight ibuprofen analogues were investigated. Ibuprofen (**1**), 2-(4-Isobutylphenyl)-N-(2-(3-methylpyridin-2-ylamino)-2-oxoethyl)propanamide (**9**) and N-(3-methylpyridin-2-yl)-2-(4'-isobutylphenyl)propionamide (**2**) inhibited FAAH with IC₅₀ values of 134, 3.6 and 0.52 μM respectively. The corresponding values for COX-1 were ~29, ~50 and ~60 μM, respectively. Using arachidonic acid as substrate, the compounds were weak inhibitors of COX-2. However, when anandamide was used as COX-2 substrate, potency increased, with approximate IC₅₀ values of ~6, ~10 and ~19 μM, respectively. **2** was confirmed to be active *in vivo* in a murine model of visceral nociception, but the effects of the compound were not blocked by CB receptor antagonists.

Keywords

fatty acid amide hydrolase; cyclooxygenase; ibuprofen; analgesia; cannabinoid

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen (**1**, Figure 1) are used widely for the treatment of pain. The primary mechanism of action of NSAIDs, inhibition of prostaglandin production by cyclooxygenase (COX) enzymes, has been known for almost forty years.¹ However, their use is associated with an unacceptably high incidence of gastrointestinal and cardiovascular adverse events, resulting in the need for safer NSAIDs.

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Declaration of interest

The authors report no conflicts of interest with respect to the content in the current paper. The authors alone are responsible for the content and writing of the paper.

In 2009, it was reported that blockade of fatty acid amide hydrolase, the enzyme responsible for the hydrolysis of the endogenous cannabinoid receptor ligand anandamide (arachidonylethanolamide, AEA), reduced by almost an order of magnitude the dose of the NSAID diclofenac required to produce analgesia in a model of visceral pain.² The authors also demonstrated that the combination of an FAAH inhibitor* (or genetic deletion of the enzyme) and diclofenac was less ulcerogenic than the NSAID alone.² The authors concluded that "a combination of FAAH inhibitors and NSAIDs may have great utility to treat visceral pain, with reduced gastric toxicity".²

A disadvantage of treatment with a combination of drugs, either given separately or as a multicomponent formulation, is that different rates and variabilities in metabolism of the two component compounds can lead to a very wide variation in delivered dosages and thereby variation in treatment outcome.³ An alternative approach, termed "designed multiple ligands", is the use of compounds with efficacy towards both targets.³ This is advantageous inasmuch as the pharmacokinetic issues are resolved, but of course the main issue is obtaining optimal balance of actions towards the two targets. One way of addressing this approach is the design of compounds exhibiting different relative potencies towards the two targets in question. Such compounds can be used in predictive models to identify the ideal ratio of activities, which can then form the basis of design and choice of lead compound(s) for pharmaceutical development.

Ibuprofen, like other acidic NSAIDs, is a weak inhibitor of FAAH.^{4,5} In 2007, we reported that the ibuprofen analogue *N*-(3-methylpyridin-2-yl)-2-(4'-isobutylphenyl)-propionamide (ibu-am5, **2**, Fig. 1) was 2–3 orders of magnitude more potent as an inhibitor of FAAH than ibuprofen, but retained its COX inhibitory properties, as assessed using a simple COX assay kit.⁶ This dual-activity raises the possibility that novel compounds related to ibuprofen and ibu-am5 may, together with these two compounds, provide structures with different FAAH/COX inhibitory potencies for achieving the ideal balance of effects *in vivo*. Consequently in the present study we designed novel compounds related to **1** and **2**, and investigated their activity with respect to their FAAH-inhibitory potencies. The best novel derivative as well as **1**, **2** were further investigated with respect to their COX-inhibitory potencies using an oxygen electrode assay using arachidonic acid (COX-1 and -2) and anandamide (COX-2) as substrates. The endocannabinoid component of the effect of **2** was also investigated *in vivo* using a murine visceral pain model.

Materials and Methods

Materials

Anandamide [ethanolamine-1-³H] (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), arachidonic acid and non-radioactive anandamide were obtained from the Cayman Chemical Co (Ann Arbor, MI, USA). Rimonabant (CB₁ receptor antagonist) and SR144528 (CB₂ receptor antagonist) were obtained from NIDA (Rockville, MD).

Chemistry

Melting points were determined on a Stuart Scientific Melting point SMP1 and are uncorrected. Proton NMR spectra were recorded on a Varian Unity 300 spectrometer. The chemical shift are reported in part per million (δ , ppm) downfield from tetramethylsilane

*linguistic note: whether to write "an FAAH" or "a FAAH" is dependent upon whether FAAH is considered as an abbreviation (i.e. F-A-A-H, where the F is pronounced "ef") or a word (where the F is pronounced as f). In this paper, the abbreviation is used.

(TMS), which was used as internal standard. Infrared spectra were obtained with a Bruker Vector 22 spectrophotometer. Elemental analyses were carried out with a Carlo Erba model 1106 Elemental Analyzer and the values found were within 0.4% of theoretical values. Analytical thin layer chromatography (TLC) was carried out on E. Merck TLC plates coated with silica gel 60 F254 (0.25mm layer thickness). TLC visualization was carried out using an UV lamp. Ibuprofen and all reagents and solvents were purchased from the Sigma Chemical Co (St. Louis, MO, USA). Ibu-am5 was synthesised as described previously.⁷

Synthesis of 3-methylpyridin-2-yl 2-(4-isobutylphenyl)propanoate (3)—CDI

(0.39 g, 2.4 mmol) was added to a solution of **1** (0.41 g, 2 mmol) in 10 mL of dichloromethane. After the reaction mixture was stirred at r.t. for 30 min 2-iodoxy-3-methylpyridine (0.22 g, 2 mmol) was added and the reaction mixture was heated at reflux until disappearance of starting material detected by analytical TLC (72 h). After cooling the dichloromethane solution was washed consecutively with water, saturated sodium hydrogen carbonate and water. The organic layer was dried over sodium sulfate, filtered, and concentrated in vacuo. The residue was triturated with isopropyl ether to give title compound which was isolated by filtration and air dried. Yield 54%; mp 40–42 °C; ¹H NMR (DMSO-*d*₆) δ 0.91 (d, J=6.6, 6H), 1.30 (t, J=7.8, 3H), 1.56 (d, J= 7.0, 2H), 1.82 (m, 1H), 2.20 (s, 3H), 3.78 (m, 1H), 6.27, 7.13, 7.19, 7.30, 7.35 (m, 7H). IR (nujol) 3146, 1733, 1642 cm⁻¹. Anal. Calcd for C₁₉H₂₃N₂O: C, 76.74; H, 8.70; N, 4.71. Found: C, 76.80; H, 8.68; N, 4.74.

General procedure for the preparation of compounds 4, 5

A mixture of **1** (2 mmol), EDC (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 glycine ethyl ester hydrochloride (0.56 g, 4 mmol) or ethyl 3-aminopropanoate (0.61 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 **4, 5**.

Ethyl 2-(4-isobutylbenzamido)acetate (4)—Yield 70%; mp 132–133 °C (2-PrOH); ¹H NMR (DMSO-*d*₆) δ 0.91 (d, J=6.6, 6H), 1.30 (t, J=7.8, 3H), 1.52 (d, J= 7.0, 2H), 1.82 (m, 1H), 2.18 (m, 2H), 2.43 (m, 1H), 3.52 (m, 1H), 4.13 (q, J=7.8, 2H), 4.16 (s, 2H), 7.13 (m, 2H), 7.30 (m, 2H), 8.35 (s, 1H). IR (nujol) 3230, 2766, 1712, 1653 cm⁻¹. Anal. Calcd. for C₁₇H₂₅NO₃: C, 70.07; H, 8.65; N, 4.81. Found: C, 70.01; H, 8.66; N, 4.84.

Ethyl 3-(4-isobutylbenzamido)propanoate (5)—Yield 85%; mp 128–130 °C (2-PrOH); ¹H NMR (DMSO-*d*₆) δ 0.92 (d, J=6.6, 6H), 1.28 (t, J=7.8, 3H), 1.49 (d, J= 7.0, 2H), 1.82 (m, 1H), 2.19 (m, 2H), 2.40 (m, 1H), 2.65 (m, 2H), 3.52 (m, 1H), 4.12 (q, J=7.8, 2H), 4.15 (s, 2H), 7.17 (m, 2H), 7.33 (m, 2H), 8.32 (s, 1H). IR (nujol) 3244, 2754, 1718, 1647 cm⁻¹. Anal. Calcd. for C₁₈H₂₇NO₃: C, 70.79; H, 8.91; N, 4.59. Found: C, 70.84; H, 8.88; N, 4.56.

General procedure for the synthesis of acids 6 and 7

To a solution of **4, 5** (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 compounds **6** and **7**.

2-(2-(4-isobutylphenyl)propanoylamino)acetic acid (6)—Yield 76%; mp 160–161 °C (2-PrOH); ¹H NMR (DMSO-*d*₆) δ 0.91 (d, J=6.6, 6H), 1.52 (d, J= 7.0, 2H), 1.82 (m, 1H), 2.19 (m, 2H), 2.43 (m, 1H), 3.52 (m, 1H), 4.14 (s, 2H), 7.13 (m, 2H), 7.30 (m, 2H), 8.35 (s, 1H), 10.82 (s, 1H). IR (nujol) 3317, 1770, 1661 cm⁻¹. Anal. Calcd. for C₁₅H₂₁NO₃: C, 68.42; H, 8.04; N, 5.32. Found: C, 68.37; H, 8.06; N, 5.35.

3-(2-(4-isobutylphenyl)propanoylamino)-propanoic acid (7)—Yield 84%; mp 147–149 °C (2-PrOH); ¹H NMR (DMSO-*d*₆) δ 0.91 (d, J=6.5, 6H), 1.52 (d, J= 6.9, 2H), 1.84 (m, 1H), 2.19 (m, 2H), 2.42 (m, 1H), 2.66 (m, 2H), 3.52 (m, 1H), 4.16 (s, 2H), 7.17 (m, 2H), 7.35 (m, 2H), 8.40 (s, 1H), 10.78 (s, 1H). IR (nujol) 3307, 3061, 1698, 1643 cm⁻¹. Anal. Calcd. for C₁₆H₂₃NO₃: C, 69.29; H, 8.36; N, 5.05. Found: C, 69.24; H, 8.38; N, 5.02.

Synthesis of 4-acetamidophenyl 2-(2-(4-isobutylphenyl)propanamido)acetate (8)—A mixture of the acid **6** (0.28 g, 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 (0.15g, 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 sequentially with brine (2 × 5 mL), 5% aqueous sodium hydroxyde (2 × 5 mL), and water (2 × 5 mL). The organic layer was dried over anhydrous magnesium sulfate. Concentration of the dried extracts yielded the title compound in analytically pure form. Yield 58%; mp 94–97 °C; ¹H NMR (DMSO-*d*₆) δ 0.96 (d, J=6.3, 6H), 1.45 (d, J=6.9, 3H), 1.91 (m, 1H), 2.14 (s, 3H), 2.20 (s, 2H), 3.43 (m, 1H), 4.18 (s, 2H), 7.10, 7.20, 7.33, 7.68 (m, 7H), 8.62 (d, *J*=5.8 Hz, 1H), 8.34 (s, 1H), 10.13 (s, 1H). IR (nujol) 3307, 1779, 1669, 1650 cm⁻¹. Anal. Calcd. for C₂₃H₂₈N₂O₄: C, 69.68; H, 7.12; N, 7.07. Found: C, 69.64; H, 7.10; N, 7.04.

General procedure for the preparation of ibuprofen amides (9–13)

A mixture of the appropriate acid **6**, **7** (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 the appropriate amine (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 sequentially with brine (2.5 mL), 10% aqueous sodium carbonate (2.5 mL), 10% aqueous citric acid (2.5 mL), and water (2.5 mL). The organic layer was dried over anhydrous magnesium sulfate. Concentration of the dried extracts yielded amides **9–13** in analytically pure form without additional purification if not indicated otherwise.

2-(4-Isobutylphenyl)-N-(2-(3-methylpyridin-2-ylamino)-2-oxoethyl)propanamide (9, ibu-am14)—Yield 68%; oil; ¹H NMR (DMSO-*d*₆) δ 0.95 (d, J=6.6, 6H), 1.45 (d, J=7.0, 3H), 1.90 (m, 1H), 2.14 (s, 2H), 2.20 (s, 3H), 2.50 (s, 3H), 3.60 (m, 1H), 4.04 (m, 2H), 7.19, 7.30, 7.40, 7.74 (m, 7H), 8.34 (s, 1H), 10.05 (s, 1H). IR (nujol) 3264, 1660 cm⁻¹. Anal. Calcd. for C₂₁H₂₇N₃O₂: C, 71.36; H, 7.70; N, 11.89. Found: C, 71.41; H, 7.68; N, 11.93.

2-(4-Isobutylphenyl)-N-(2-(6-methylpyridin-2-ylamino)-2-oxoethyl)propanamide (10)—Yield 55%; mp 121–123 °C (Cyclohexane); ¹H NMR (DMSO-*d*₆) δ 0.96 (d, J=6.3, 6H), 1.44 (d, J=6.9, 3H), 1.92 (m, 1H), 2.42 (m, 2H), 2.50 (s, 3H), 3.54 (m, 1H), 4.10 (s, 2H), 7.07, 7.19, 7.35, 7.77, 7.93 (m, 7H), 8.44 (d, J=5.8 Hz, 1H), 8.34 (s, 1H), 10.43 (s, 1H). IR (nujol) 3284, 3069, 1682, 1645 cm⁻¹. Anal. Calcd. for C₂₁H₂₇N₃O₂: C, 71.36; H, 7.70; N, 11.89. Found: C, 71.30; H, 7.72; N, 11.86.

2-(4-Isobutylphenyl)-N-(3-(3-methylpyridin-2-ylamino)-3-oxopropyl)propanamide (11)—Yield 62%; oil; $^1\text{H NMR}$ (CDCl_3) δ 0.85 (d, $J=5.5$, 6H), 1.45 (d, $J=7.3$, 3H), 1.79 (m, 1H), 2.14 (m, 1H), 2.42 (m, 2H), 2.48 (m, 2H), 2.70 (m, 2H), 3.50 (m, 3H), 6.98–7.78 (m, 7H), 8.11 (s, 1H), 9.13 (s, 1H). IR (nujol) 3307, 1779, 1669, 1650 cm^{-1} . Anal. Calcd. for $\text{C}_{22}\text{H}_{29}\text{N}_3\text{O}_2$: C, 71.90; H, 7.95; N, 11.43. Found: C, 71.96; H, 7.92; N, 11.45.

2-(4-Isobutylphenyl)-N-(2-oxo-2-(pyridin-2-ylmethylamino)ethyl)propanamide (12)—Yield 68%; mp oil; $^1\text{H NMR}$ ($\text{DMSO}-d_6$) δ 0.95 (d, $J=6.5$, 6H), 1.44 (d, $J=7.0$, 3H), 1.89 (hept, $J=6.5$, 1H), 2.49 (m, 2H), 3.54 (m, 1H), 4.10 (s, 2H), 4.48 (m, 2H), 7.16, 7.40, 7.88, 8.37, 8.50 (m, 8H), 8.60 (s, 1H), 10.45 (s, 1H). IR (nujol) 3320, 1704, 1672, 1645 cm^{-1} . Anal. Calcd. for $\text{C}_{21}\text{H}_{27}\text{N}_3\text{O}_2$: C, 71.36; H, 7.70; N, 11.89. Found: C, 71.43; H, 7.71; N, 11.85.

2-(4-Isobutylphenyl)-N-(2-oxo-2-(pyridin-3-ylmethylamino)ethyl)propanamide (13)—Yield 75%; oil; $^1\text{H NMR}$ (CDCl_3) δ 0.84 (d, $J=5.9$, 6H), 1.41 (d, $J=6.5$, 3H), 1.92 (sept, $J=6.5$, 2H), 2.38 (t, $J=7.2$, 2H), 3.56 (m, 1H), 4.14 (s, 2H), 4.32 (s, 2H), 7.3, 7.13, 7.27, 7.65 (m, 8H), 8.43 (s, 1H), 8.47 (s, 1H). IR (nujol) 3298, 3069, 1692, 1654 cm^{-1} . Anal. Calcd. for $\text{C}_{21}\text{H}_{27}\text{N}_3\text{O}_2$: C, 71.36; H, 7.70; N, 11.89. Found: C, 71.31; H, 7.73; N, 11.94.

FAAH assay

Frozen ($-80\text{ }^\circ\text{C}$) brains (minus cerebella) from adult Wistar or Sprague-Dawley rats were thawed and homogenised in 20 mM HEPES, 1 mM MgCl_2 , pH 7.0. and thereafter centrifuged at $\sim 35000 \times g$ for 20 min at $4\text{ }^\circ\text{C}$. Homogenates were washed (by centrifugation at $\sim 35000 \times g$ for 20 min at $4\text{ }^\circ\text{C}$ followed by resuspension in buffer) twice and incubated at $37\text{ }^\circ\text{C}$ for 15 minutes in order to hydrolyse all endogenous FAAH substrates. After a further centrifugation, the pellets were resuspended in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 3 mM MgCl_2 and frozen at $-80\text{ }^\circ\text{C}$ in aliquots until used for assay. For FAAH assay⁸ test compounds, homogenates (0.5–0.8 μg protein per assay, diluted with 10 mM Tris-HCl, 1 mM EDTA pH 7.4) and 25 μL of [^3H]AEA 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 incubated for 10 min at $37\text{ }^\circ\text{C}$ (final assay volume 200 μL). Reactions were stopped by placing the tubes on ice. Final assay concentrations of the solvents used for the compounds (ethanol or DMSO) were in the range 1–5%. Activated charcoal (80 μL + 320 μL 0.5 M HCl) was added and the samples were mixed and left at room temperature for about 30 min. Following centrifugation at 2500 rpm for 10 min, aliquots (200 μL) of the supernatants were analyzed for tritium content by liquid scintillation spectroscopy with quench correction. Blank values were obtained by the use of buffer rather than homogenate.

COX assay

An assay based on the oxygen electrode method of Meade *et al.*⁹ was used.¹⁰ A buffer containing 1 μM hematin, 2 mM phenol, 5 mM EDTA, substrate (AA or AEA, 10 μM , as indicated) 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 vehicle (DMSO or ethanol, as indicated, 20 μL), a baseline was established over a period of 5 min. Reactions were started by addition of the appropriate COX isoform (200 U per assay, usually in a volume of 8–15 μL), and the oxygen consumption was followed for the next 5 min. Data are presented as the change in oxygen consumption (μM) from the point of

addition of the enzyme. We regard this assay as more “robust” than the assay kit we used previously⁶, simply because the oxygen electrode assay has been very well characterised in the literature with respect to effects of NSAIDs⁹, whereas the kit used in our previous study⁶ measured the peroxidase activity of COX and was characterised by the manufacturers with respect to sensitivity to two experimental compounds (SC-560 for COX-1, DuP-697 for COX-2) rather than towards NSAIDs.

Acetic acid-induced abdominal stretching test

Male C57Bl/6J mice (Jackson Laboratory, Bar Harbor, ME) weighing between 20 and 25 g and were housed four mice per cage in a temperature-controlled (20–22 °C) facility, with food and water available ad libitum. All animals were acclimatized to the laboratory environment for at least 2 h before testing and the acetic acid-induced stretching assay was carried out as previously described.¹¹ In brief, mice were injected intraperitoneally with 10 ml/kg of 0.6 % acetic acid in normal saline and the number of stretches (constriction of abdomen, turning of trunk (twist) and extension of the body and hind limbs) per mouse was counted for a 20-min period beginning 3 min after the administration of acetic acid. Mice were given s.c. injections of **2** (30 mg/kg) 30 min before acetic acid. The cannabinoid receptor antagonists were given s.c. 10 min before **2**. All the drugs were dissolved in a vehicle consisting of ethanol, alkamuls-620 (Rhône-Poulenc, Princeton, NJ), and saline in a ratio of 1:1:18. The drugs were administered in a volume of 10 µl/g body weight. All animal protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and were in concordance with the National Institutes of Health guide for the care and use of Laboratory animals.

Statistical analyses

For determinations of pI₅₀ and IC₅₀ values, the GraphPad Prism computer programme (GraphPad Software Inc., San Diego, CA) was used. The built-in programme “sigmoidal dose-response (variable slope)” calculated pI₅₀ and hence IC₅₀ values from the data expressed as % of control using top (i.e. uninhibited) values of 100% and bottom (residual activity) values that were either set to zero or allowed to float. The two curves were compared using Akaike’s informative criteria and the curve of best fit was then used. In vivo data were analyzed by two-way ANOVA, with agonist (vehicle or **2**) and antagonist (vehicle, rimonabant, or SR144528), as the two factors.

Results

Synthesis of ibuprofen analogues

The chemistry work was undertaken in the laboratory of Onnis and Congiu. The target ester and amides of ibuprofen (Table 1) were synthesized as reported in Schemes 1 and 2. Treatment of **1** with 2-hydroxy-3-methylpyridine in the presence of 1,1-carbonyldiimidazole (CDI) in CH₂Cl₂ solution gave ester **3**. Ibuprofen was coupled with glycine ethyl ester or ethyl 3-aminopropanoate, in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and hydroxybenzotriazole (HOBt) in MeCN solution, followed by hydrolysis to give 2-(2-(4-isobutylphenyl)propanoylamino)acetic acid (**6**) and 3-(2-(4-isobutylphenyl)propanoylamino)propanoic acid (**7**) respectively. The ester **8** was obtained by reaction of acid **6** with paracetamol by EDC method. Similarly, the amide derivatives **9–13** were obtained coupling the acids **6,7** with the appropriate 2-aminopyridine or picolylamine. This synthetic pathway was found to be clean and high yielding.

Pharmacological studies

FAAH inhibition—The in vitro pharmacology studies were undertaken in the laboratory of Fowler and Björklund. The ability of compounds **3**, **8–13**, with **1** and **2** as references, to inhibit FAAH activity was determined using $0.5 \mu\text{M}$ [^3H]AEA as substrate and rat brain homogenates as enzyme source (Table 1). Since some of the compounds used DMSO and others ethanol as vehicle, compounds **1** and **2** were tested in both vehicles, with very similar results (Table 1). Consistent with our previous data,⁶ **2** was approximately 250-fold more potent than **1** as an FAAH inhibitor (Table 1). This increase in potency was completely lost when the amido moiety of **2** was replaced with an ester moiety (ester **3**, Table 1). Introduction of an extra three/four atom linker between ibuprofen core and pyridine ring or esterification with paracetamol did not improve FAAH inhibitory potency with respect to **2**. However, the paracetamol ester (**8**) and the analog bearing an extra three atom linker (ibu-am14, **9**) had IC_{50} values in the low micromolar range, with **9** being approximately 40-fold more potent than **1** (Table 1). Concentration-response curves for **1**, **2** and **9** are shown in Figure 2A.

Of the novel compounds, the two most potent were **8** and **9**. However, compound **8** did not produce a complete inhibition of the FAAH activity, and so **9** was chosen for further investigation. The compound did not show time-dependent inhibition of FAAH (Figure 2B), suggesting that it interacts in a non-covalent manner with the enzyme and that the compound is stable during the preincubation period. In experiments undertaken with ten AEA concentrations ($0.3\text{--}3 \mu\text{M}$; Figure 2C), the mean K_m^{app} values in the presence of 0, 1, 3 and 5 of **9** were 0.56, 1.18, 2.04 and $2.56 \mu\text{M}$, respectively. The corresponding $V_{\text{max}}^{\text{app}}$ values were 2.09, 2.32, 2.42 and $2.40 \text{ nmol.mg protein}^{-1}.\text{min}^{-1}$, respectively, with overlapping 95% confidence intervals, i.e. a competitive mode of inhibition. A secondary replot of $K_m^{\text{app}}/V_{\text{max}}^{\text{app}}$ vs. [**9**] gave a K_i value of $2.0 \mu\text{M}$.

The reversibility of **9** was investigated by dilution experiments. In these experiments, **9** ($12 \mu\text{M}$) was preincubated with homogenates for 60 min at 37°C , and the homogenates were then diluted 20-fold in buffer. The inhibition produced by **9** was thereafter compared with concomitant data where **9** (0.6 or $12 \mu\text{M}$) was added after the preincubation phase. For a fully reversible compound, the inhibition produced by $12 \mu\text{M}$ prior to dilution (shown as $12 \rightarrow 0.6$ in Figure 2D) should be the same as that produced by $0.6 \mu\text{M}$ added after the inhibition. For a non-reversible inhibitor, on the other hand, the inhibition seen with $12 \rightarrow 0.6 \mu\text{M}$ should be greater than seen with $0.6 \mu\text{M}$ and closer to that produced by $12 \mu\text{M}$ added after the preincubation phase. The data shown in Figure 2D indicates that **9** is fully reversible. Experiments were also undertaken using $6 \mu\text{M}$ (and $6 \rightarrow 0.3 \mu\text{M}$) of **9**, but in this case the observed inhibition with **9** was lower than expected from the concentration-response experiments and conclusions with respect to reversibility at this concentration could not be made (data not shown).

COX inhibition

Given that our original study with **2** used a kit for estimation of COX activity based upon the peroxidase rather than cyclooxygenase activity of the enzyme,⁶ the present study employed a more robust measure of inhibitory potency. Consequently **1** and **2** were tested together with **9** using an oxygen electrode assay for COX, with commercially available ovine COX-1 and human recombinant COX-2 as enzyme sources. Arachidonic acid (AA) was used as substrate for both forms. COX-2 was also assayed using AEA as substrate in view of a recent report that the inhibitory potency of **1** towards COX-2 is dependent upon the substrate used for assay.¹² AEA is not a substrate for COX-1, and so this combination was not tested.

The results, with ethanol as vehicle, are shown in Figure 3. As expected, **1** inhibited the activity of ovine COX-1 towards AA (10 μ M). The initial activities (between 10 and 30 s after addition of COX isoform) determined from the data shown in Fig. 3A and from additional experiments, expressed as % of vehicle control, are summarised in Table 2. The number of concentrations that could be tested was constrained both by assay capacity and particularly assay cost, and so only approximate IC₅₀ values can be determined. With this caveat, an IC₅₀ value of ~29 μ M was found for **1**. A slightly lower potency was seen when DMSO was used as vehicle for **1** (IC₅₀ value ~77 μ M). Compound **2** was less potent than **1** with respect to COX-1, with an IC₅₀ value of ~60 μ M being found with ethanol as vehicle and ~240 μ M with DMSO as vehicle. Compound **9** inhibited COX-1 with an IC₅₀ value of ~50 μ M (ethanol vehicle). **1** (as expected), **2** and **9** were poor inhibitors of the cyclooxygenation of AA by COX-2, producing 36, 41 and 18% inhibition at the highest concentrations tested (300, 300 and 100 μ M, respectively). However, when AEA was used as substrate, the compounds were potent inhibitors of COX-2, with IC₅₀ values of ~6, ~19 and ~10 μ M being found for **1**, **2** and **9**, respectively (Figure 3, Table 2). Experiments with flurbiprofen indicated that a similar pattern of substrate selectivity was seen, indicating that it is not a phenomenon limited to ibuprofen and ibuprofen analogues alone (data not shown).

Effect of cannabinoid receptor blockade on the *in vivo* activity of compound **2** in a mouse model of visceral nociception

The *in vivo* studies were undertaken in the laboratory of Lichtman and Naidu. Compound **2** was chosen for three reasons: 1) The compound was the most potent of the series towards FAAH; 2) Sufficient compound was available for *in vivo* studies (not the case for compound **9**); 3) We have previously shown that **2** at doses of 10 and 30 mg/kg s.c. (mice) and 20 mg/kg i.p. (rats) has analgesic activity in the acetic acid-induced abdominal stretching test,^{7,13} but it is not known whether this effect involves activation of cannabinoid receptors. We investigated this here using the CB₁ receptor antagonist rimonabant and the CB₂ receptor antagonist SR144528. The doses chosen block CB receptor-mediated effects of FAAH inhibition or genetic ablation *in vivo* in mice.^{2,14,15} The data in Figure 4 indicate that although **2** decreased the number of abdominal stretches at the 30 mg/kg dose [$F_{(1,42)} = 72.7$, $p < 0.0001$], neither rimonabant nor SR144528 diminished these antinociceptive effects.

Discussion

In the present study, we have explored compounds related to **1** with respect to their FAAH-inhibitory properties, and compared **1**, **2** and **9** with respect to their actions upon COX isoforms. An *in vivo* investigation of **2** in a visceral pain model has also been presented. The main results are discussed in turn below.

Compounds with different FAAH/COX inhibitory ratios have been identified

Relatively little work has been undertaken upon the FAAH inhibitory properties of ibuprofen analogues. In our initial study,⁶ we explored a series of heterocyclic amide analogues of **1** and found that a 6-methyl-pyridin-2-yl substituent (compound **2**) gave the best activity, with the compound showing similar activity towards both rat and mouse FAAH. More recently, a 1,3-dithian-2-yl derivative of **1** has been described with a potency towards FAAH in the low micromolar region.¹⁶ This compound, which was almost two orders of magnitude more potent than its analogue lacking the 4-isobutyl substituent on the phenyl ring, showed anti-inflammatory activity in an *in vitro* model (interleukin-1 μ stimulated interleukin-8 secretion from Caco-2 cells).¹⁶ The ability of the compound to interact with COX enzymes, however, is not known. Finally, 4-acetamidophenyl 2-(4-(2-(trifluoromethyl)pyridin-4-ylamino)phenyl)propanoate in which the carboxyl moiety of **1** is

esterified with paracetamol and a 2-(trifluoromethyl)pyridin-4-ylamino replaced the isobutyryl chain was a very potent inhibitor of FAAH (IC₅₀ value of 0.1 μM). However, this compound did not inhibit either COX isoform.¹⁰

In the present study, we have not been able to improve upon the potency of **2**. Although this at first sight might be considered a limitation of the importance of the study, this is not in fact the case: the main aim, to produce dual-action compounds with different FAAH/COX potencies, has been achieved. Compound **9** shares with **1** and **2** a mode of inhibition that is not time-dependent. However **9** is a competitive inhibitor of rat brain AEA hydrolysis whereas **1** is a mixed-type inhibitor³ and **2** acts non-competitively.⁶ These observations would suggest that the compounds interact differently with the enzyme dependent upon the nature of the substituent of ibuprofen carboxyl moiety. This suggestion may in turn help to provide an explanation for the lack of activity of the ester (compound **3**): given that FAAH can metabolise both amides (such as AEA) and esters (such as 2-aracydonoylglycerol¹⁷), activity of **3** might have been expected.

Since FAAH and COX assays use different assay conditions, it is difficult to compare potencies directly. However, the approximate ratio of potencies of FAAH/COX-1 can be compared between compounds. Using this ratio and setting the FAAH/COX-1 ratio for **1** to unity, the ratios for **2** and **9** are ~500 and ~60, respectively, where the higher number reflects increased FAAH inhibitory potency relative to the COX-1 potency. In other words, **1**, **2** and **9** cover a wide range of FAAH inhibitory potencies relative to the COX-1 inhibitory potencies. A similar pattern is seen for COX-2 when assayed with AEA as substrate.

AEA cyclooxygenation by COX-2 is more sensitive to **1, **2** and **9** than AA cyclooxygenation**

When using AA as substrate, compounds **1** (as expected), **2** and **9** were clearly COX-1 selective. The difference in potency seen with ethanol and DMSO vehicles may reflect the way the lipophilic inhibitor presents itself to COX in the assay buffer – vehicle mixture. Whatever the reason, it underscores the need to use the same vehicle when comparing COX inhibitory potencies of different compounds. All three compounds were more potent as inhibitors of AEA cyclooxygenation by COX-2 than of AA cyclooxygenation by either COX isoform. Thus, for example, at a concentration of 10 μM, **1** produced a 19% inhibition of COX-1, but a 64% inhibition of AEA cyclooxygenation by COX-2, whilst a 100 μM concentration of this NSAID produced only 13% inhibition of COX-2 activity with arachidonic acid as substrate. Given that the K_m value of COX-2 towards AEA as substrate is slightly higher than for AA,¹⁸ these data indicate a considerable substrate-dependency of inhibition of COX-2. Our data are consistent with the recent finding that both **1** and other COX inhibitors, including mefamic acid and naproxen, were also better inhibitors of the cyclooxygenation of the other main endocannabinoid, 2-arachidonoylglycerol (2-AG) than of AA.^{12,19} Indeed, even the *R*-enantiomers of **1**, naproxen and flurbiprofen, were able to inhibit COX-2-catalysed oxygenation of 2-AG, whereas these compounds do not inhibit the cyclooxygenation of AA by this enzyme.¹⁹ Using site-directed mutagenesis and crystallography, the authors showed that the *R*-profens interact with the Arg¹²⁰ residue in the COX-2 active site.¹⁹ COX-2 is a dimer, and although both monomers are capable of oxygenating AA, the dimer displays half of sites reactivity, i.e. binding of AA to one monomer precludes binding to the second monomer.²⁰ Prusakiewicz et al.¹² suggested that in order to block AA oxygenation, the inhibitor needed to bind to both monomers, whereas binding to a single monomer was sufficient for blockade of 2-AG oxygenation. Given that the binding of AEA within the active site of COX-2 is different to that for AA,^{21,22} it is reasonable to conclude that a similar mechanism may be operative here.

The high sensitivity of COX-2 to ibuprofen with AEA as substrate, which was also seen with flurbiprofen, together with the ability, albeit modest, of acidic NSAIDs (including *R*-enantiomers) to inhibit FAAH,^{4,5,23,24} indicate that there is a considerable overlap in the cyclooxygenase and endocannabinoid systems. This has also been seen *in vivo* in several studies investigating the role of endocannabinoids in NSAID function. Thus, blockade or genetic deletion of cannabinoid CB₁ receptors: a) blocks the anti-nociceptive effects of spinally administered indomethacin and flurbiprofen in the formalin test in rats,^{25,26} b) blocks the effects of *R*-flurbiprofen upon heat hyperalgesia, mechanical hyperalgesia and cold allodynia in the spared nerve injury model of neuropathic pain,²⁴ c) antagonises the effects of the COX-2 inhibitor L-745,337 (5-methanesulphonamido-6-(2,4-difluorothiophenyl)-1-indanone) upon spinal hyperexcitability induced by knee joint inflammation,²⁷ and d) blocks the ability of spinally administered nimesulide to reduce mechanically-evoked responses of dorsal horn neurons.²⁸ In contrast, the analgesia produced by diclofenac in the acetic acid-induced abdominal stretching model is not blocked by the CB₁ receptor antagonist rimonabant.² Local injection into the paw of ibuprofen reduces the second phase of the behavioural response to local formalin injection, and although this is not blocked by a CB₁ receptor antagonist/inverse agonist, the antinociceptive effect of the combination of AEA and ibuprofen, which is greater than seen when either compound is given per se, is entirely sensitive to CB₁ receptor antagonist treatment.²⁹ Treatment of cultured dorsal root ganglia with inflammatory mediators, which results in an increased COX-2 expression, causes them to release COX-2 derived products of AEA and 2-AG upon ionomycin-stimulation, and that this is blocked (with a concomitant increase in endocannabinoid levels) by *R*-profens.¹⁹ These findings led the authors to conclude that the analgesic effects of *R*-flurbiprofen²⁴ may be related to blockade of endocannabinoid depletion by induced COX-2.¹⁹ Further studies are needed to determine the extent to which inhibition of FAAH and of the cyclooxygenation of endocannabinoids by COX-2 contribute to the *in vivo* observations described above.

The analgesic activity of Compound 2 in a visceral pain model is not blocked by CB receptor antagonists

Previous data^{7,13} demonstrated that **2** is active in the acetic acid-induced abdominal stretching test. In the rat, **2** at the dose of 20 mg/kg i.p. gave a significantly greater reduction in stretching than the same dose of **1**,⁷ whilst in the mouse, the effect of 30 mg/kg s.c. of **2** was greater than for a dose of 10 mg/kg s.c. and for 30 mg/kg of diclofenac.¹³ The acute ulcerogenesis produced by **2** was also much lower than seen with **1**.⁷ In order to determine whether the activity of **2** in the mouse model involved the endocannabinoid system, we investigated whether the CB₁ receptor antagonist rimonabant or the CB₂ receptor antagonist SR144528 could block the response at doses known to be efficacious in this species.^{2,14,15} Neither compound produced any antagonism of the effect of **2**. AEA itself can reduce acetic acid-induced stretching in a rimonabant-sensitive manner, and the alternate endogenous FAAH substrate palmitoylethanolamide is also active, in a manner blocked by SR144528,³⁰ although the latter effect may reflect an off-target action of SR144528 upon peroxisome proliferator-activated receptor- α .³¹ The reduction in the abdominal stretching response seen in mice treated with potent FAAH inhibitors and in mice lacking FAAH compared to their wild-type littermates is blocked by rimonabant but not SR144528.² It is possible that at the dose and in the model used, the contribution of analgesia due to the COX-inhibitory properties of **2** dominates, and that the additional endocannabinoid-mediated component is too small to detect. This pattern may not be the case in other pain models. A good analogy of this scenario is the case of paracetamol that has been reported to produce an active metabolite, AM404, which in turn acts both to inhibit the cellular accumulation and the FAAH-catalysed breakdown of AEA as well as being an inhibitor of COX isoenzymes.³² The analgesic effects of paracetamol have been shown to have an endocannabinoid

component (i.e. to be blocked by CB₁ receptor antagonists) in some pain tests, such as the formalin test,³³ but not in the phenyl-p-quinone stretch test³⁴ whereas both model systems show endocannabinoid-mediated effects of FAAH inhibitors.^{34,35} An alternative explanation is that *in vivo*, **2** is metabolised to a compound that retains (or even improves upon) its COX inhibitory properties, but loses its efficacy towards inhibition of FAAH. Such a metabolite is unlikely to be **1**, given that in rats a dose of 20 mg/kg of **1** was significantly less effective than 20 mg/kg of **2** in the abdominal stretching test.⁷ Clearly, further studies with this compound, and with other ibuprofen analogues with different COX:FAAH inhibitory properties, are needed in other pain model systems, particularly in those where NSAIDs involve the endocannabinoid system in their effects.

Conclusions

The present study demonstrate that some heterocycle amide analogues of ibuprofen are a useful source of compounds with biological activity *in vivo* and with varying FAAH/COX inhibitory ratios, particularly with respect to the effects of the latter upon AEA cyclooxygenation. Such compounds are of great potential value, given that they will prevent loss of the increased AEA due to cyclooxygenation¹⁹ following FAAH inhibition. Although an endocannabinoid component of **2** is not visible in a model of visceral pain, compounds **2** and **9**, together with **1** may be useful tools with which to explore the optimum balance of inhibitory effects in other experimental pain models.

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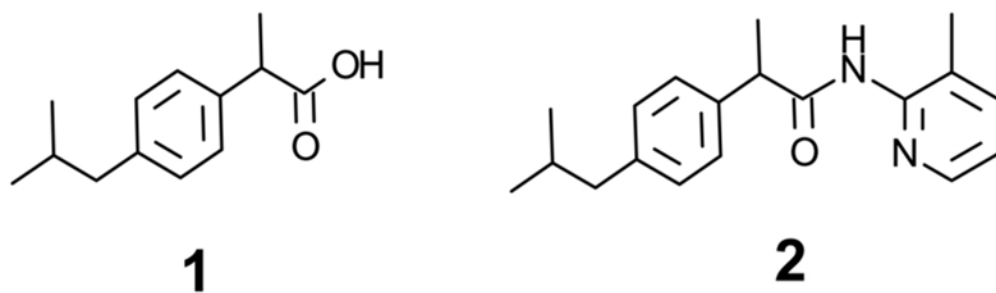
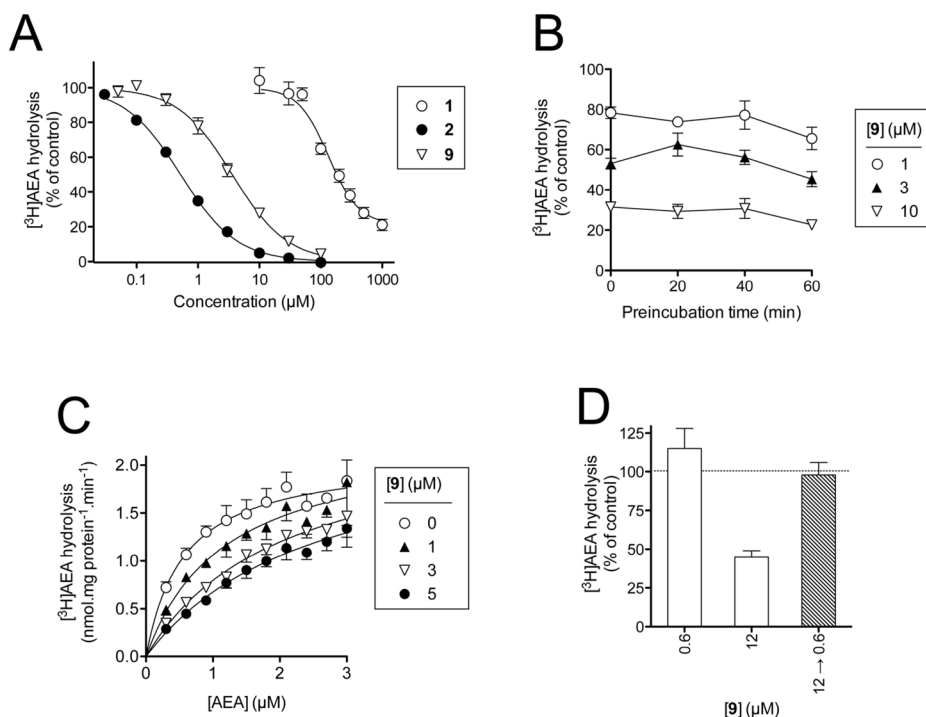


Figure 1.
Ibuprofen (1) and ibuprofen-5-aminopyridine (2)

**Figure 2.**

Panel A. Concentration-response curves for the inhibition of rat brain [^3H]AEA hydrolysis by the compounds shown. Shown are means \pm s.e.m. (when not enclosed by the symbols), $n=3-9$. Panel B. Lack of dependency upon the preincubation time for the inhibition by **9** of rat brain [^3H]AEA hydrolysis. For each test concentration, one-way factorial ANOVA gave a p value >0.05 . Shown are means \pm s.e.m., $n=3$. Panel C. Rates of AEA hydrolysis in the absence and presence of **9** at ten different concentrations of AEA. Shown are means \pm s.e.m., $n=3$. Panel D. Reversibility of inhibition of rat brain [^3H]AEA hydrolysis by **9**. Samples were preincubated for 60 min with either vehicle or the concentration shown of **9** and then diluted twenty-fold (shown as 12 \rightarrow 0.6 in the Figure), after which substrate was added and the samples were incubated for 10 min. Following the preincubation phase, vehicle samples were either treated with vehicle or the concentrations of inhibitor shown. The values are expressed as % of the vehicle control treated in the same way. Shown are means and s.e.m., $n=3$.

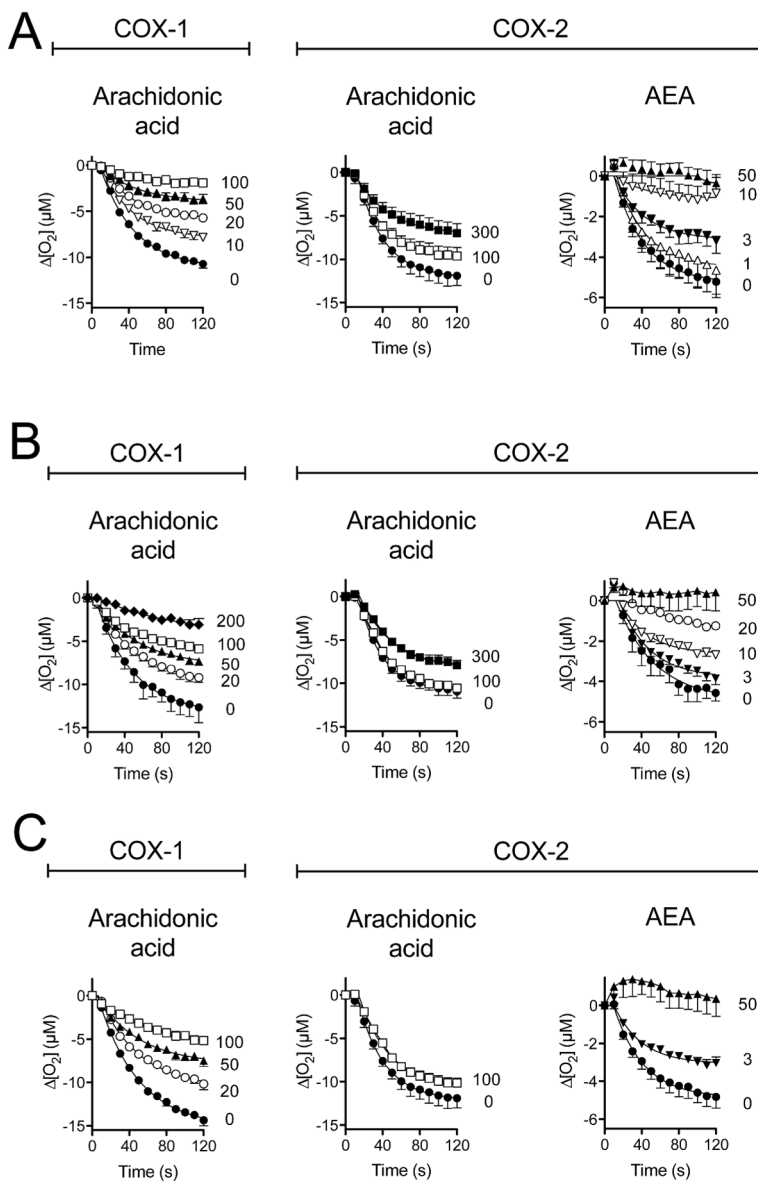


Figure 3. Inhibition of ovine COX-1 and human recombinant COX-1 by AEA, **1**, **2** and **9**. The substrates (10 μM) are shown in the figure, as are the concentrations (in μM) of the test compounds. The vehicle used was ethanol. Shown are means and s.e.m. (when not enclosed by the symbols), $n=3$.

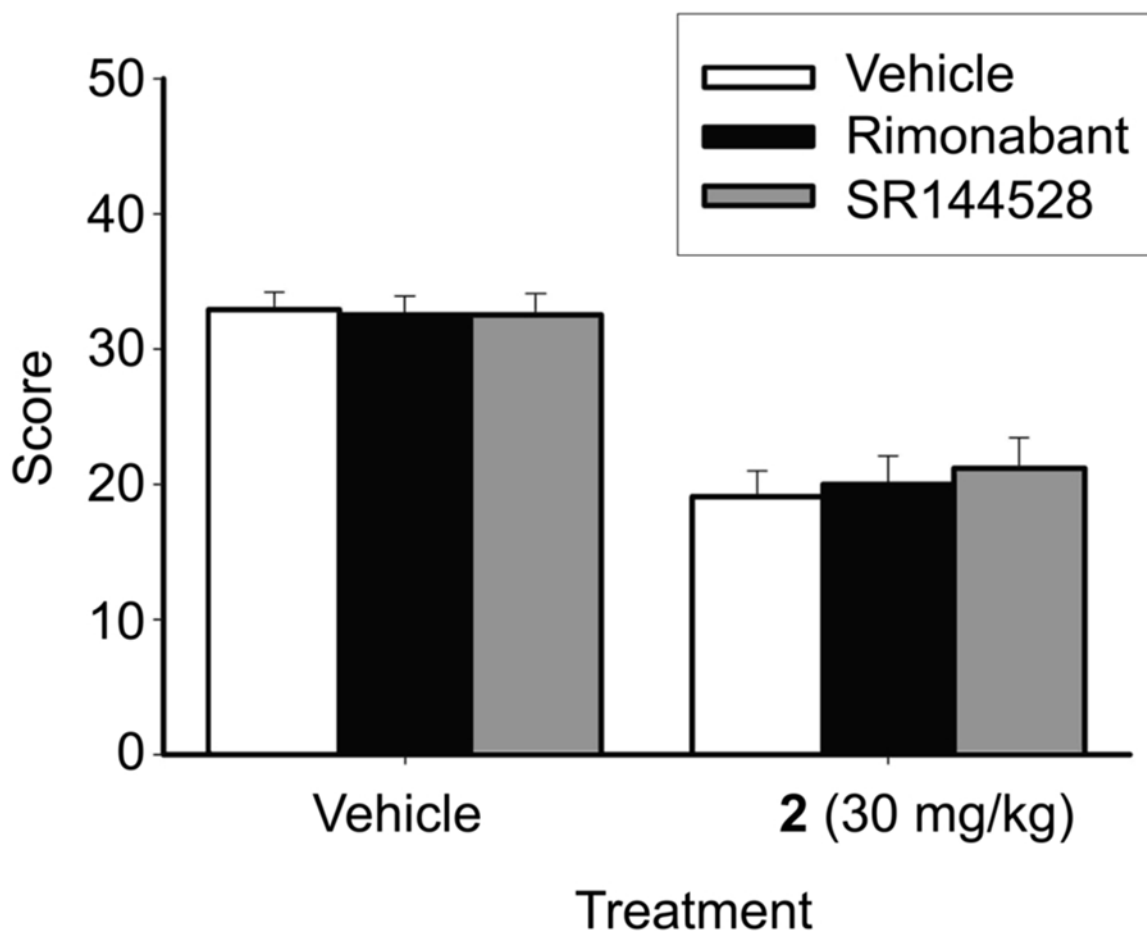
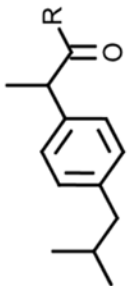
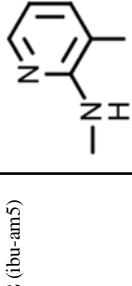
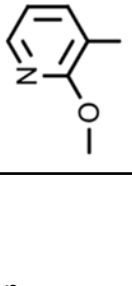
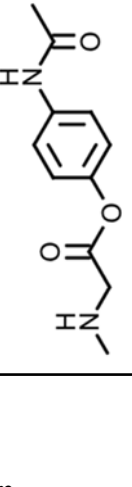
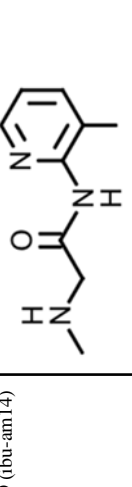


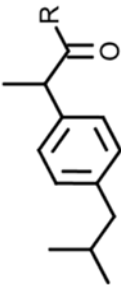
Figure 4. Effects of the CB₁ receptor antagonist rimonabant and the CB₂ receptor antagonist SR144528 upon the efficacy of **2** on the number of abdominal stretches elicited by acetic acid. Vehicle or receptor antagonist (3 mg/kg s.c.) were given 10 min before either vehicle or **2** (s.c.) and acetic acid was injected 30 min later. Data are depicted as means \pm s.e.m., n= 8 mice/group.

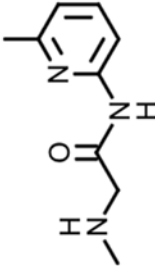
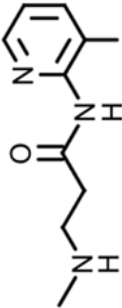
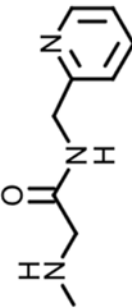
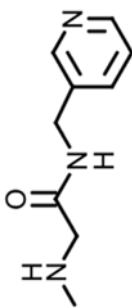
Table 1

Inhibition of FAAH by compounds **1–3**, **8–13** (legend overpage)

| Compound | Substituent (R) | Solvent | pI ₅₀ ^a | IC ₅₀ (μM) | Max inhibition % |
|----------------------|---|---------|-------------------------------|-----------------------|------------------|
| 1 (ibuprofen) |  | EtOH | 3.87±0.06 | 134 | 79±5 |
| | | DMSO | ~3.79±0.01 | ~163 | ~86±2 |
| 2 (ibu-am5) |  | EtOH | 6.28±0.01 | 0.52 | 100 |
| | | DMSO | 6.19±0.02 | 0.65 | 100 |
| 3 |  | EtOH | 3.94±0.03 | 115 | 100 |
| 8 |  | EtOH | 5.83±0.05 | 1.5 | 84±3 |
| 9 (ibu-am14) |  | EtOH | 5.44±0.03 | 3.6 | 100 |

Basic structure:



| Compound | Substituent (R) | Solvent | pI ₅₀ ^a | IC ₅₀ (μM) | Max inhibition % |
|----------|--|---------|-------------------------------|-----------------------|------------------|
| 10 |  | EtOH | 3.82±0.07 | 150 | 100 |
| 11 |  | DMSO | 5.03±0.03 | 9.3 | 90±3 |
| 12 |  | DMSO | 4.05±0.02 | 90 | 100 |
| 13 |  | DMSO | 4.43±0.09 | 37 | 81±8 |

^aThe inhibitor concentrations used to construct concentration-response curves and thereby the I₅₀ and hence IC₅₀ values in all cases straddled the IC₅₀ values and were in half-log increments (i.e. 1, 3, 10, 30 μM etc). The number of concentrations used were 6 for all compounds except **1** (EtOH) (8 concentrations), **2** (8 concentrations for both EtOH and DMSO), **9** (8 concentrations), **10** (5 concentrations), and **1** (DMSO) (4 concentrations, which is the reason why the values are given as approximate in the table). Combined data from 3–9 experiments were used. Abbreviation: EtOH, ethanol, DMSO, dimethylsulfoxide.

Table 2

Inhibition of cyclooxygenase isoforms by compounds 1, 2 and 9

| Compound/Concentration (μ M) | Initial activity (% of vehicle control) | | | | | | | |
|-----------------------------------|---|------------------|------------|------|----------------|----------------|------------------|------------------|
| | COX-1 (AEA) | | COX-2 (AA) | | COX-2 (AEA) | | DMSO | |
| Vehicle: | EtOH | DMSO | EtOH | DMSO | EtOH | DMSO | EtOH | DMSO |
| Ibuprofen (1) | | | | | | | | |
| 1 | | | | | | | 90 \pm 14 (3) | 103 \pm 16 (3) |
| 3 | | | | | | | 71 \pm 6 (6) | 104 \pm 8 (8) |
| 10 | 81 \pm 7 (3) | | | | | | 36 \pm 17 (3) | 31 \pm 4 (3) |
| 20 | 55 \pm 6 (3) | 78 (2) | | | | | | |
| 50 | 35 \pm 9 (6) | 59 \pm 3 (3) | | | | | 0.8 \pm 4 (4) | -0.6 \pm 4 (3) |
| 100 | 25 \pm 9 (6) | 44 \pm 6 (4) | | | 88 \pm 4 (4) | 87 \pm 1 (3) | | |
| 200 | 16 \pm 18 (3) | 30 \pm 5 (4) | | | 64 \pm 8 (4) | 70 \pm 5 (3) | | |
| 300 | | | | | | | | |
| 500 | | 15 \pm 3 (7) | | | | | | |
| Ibu-ams (2) | | | | | | | | |
| 3 | | | | | | | 111 \pm 15 (6) | |
| 10 | | | | | | | 77 \pm 10 (3) | |
| 20 | 70 \pm 6 (3) | 106 \pm 15 (3) | | | | | 51 \pm 17 (3) | |
| 50 | 57 \pm 0.6 (3) | 82 \pm 4 (6) | | | | | 0.7 \pm 9 (4) | |
| 100 | 45 \pm 8 (3) | 63 \pm 6 (6) | | | 88 \pm 8 (3) | | | |
| 200 | 14 \pm 4 (3) | 59 \pm 8 (6) | | | 59 \pm 4 (4) | | | |
| 300 | | | | | | | | |
| 400 | | 36 \pm 0.9 (3) | | | | | | |
| Ibu-am14 (9) | | | | | | | | |
| 3 | | | | | | | 83 \pm 8 (3) | |
| 20 | 76 \pm 8 (3) | | | | | | | |
| 50 | 56 \pm 4 (3) | | | | | | | |
| 100 | 23 \pm 4 (3) | | | | 82 \pm 5 (4) | | | |
| | | | | | | | | -11 \pm 4 (3) |

The substrate used (assay concentration 10 μM) is indicated for each COX isoform. The initial activity (i.e. the change in O_2 concentration between 10 and 30 s after addition of enzyme to start the reaction) was determined and the values calculated as % of vehicle controls. Data are shown as means \pm s.e.m. with the number of experiments given in brackets.