Exploring the fatty acid amide hydrolase and cyclooxygenase inhibitory properties of novel amide derivatives of ibuprofen

Alessandro Deplanoa, †, Jessica Karlssonb, †, Mona Svenssonb, †, Federica Moracc, †, Bruno Catalanottic, †, Christopher J. Fowlerb, † and Valentina Onnic

aUnit of Pharmaceutical, Pharmacochemical and Nutraceutical Sciences, Department of Life and Environmental Sciences, University of Cagliari, Cagliari, Italy; bDepartment of Integrative Medical Biology, Umeå University, Umeå, Sweden; cDepartment of Pharmacy, University of Napoli Federico II, Napoli, Italy

ABSTRACT

Inhibition of fatty acid amide hydrolase (FAAH) reduces the gastrointestinal damage produced by non-steroidal anti-inflammatory agents such as sulindac and indomethacin in experimental animals, suggesting that a dual-action FAAH-cyclooxygenase (COX) inhibitor could have useful therapeutic properties. Here, we have investigated 12 novel amide analogues of ibuprofen as potential dual-action FAAH/COX inhibitors. N-(3-Bromopyridin-2-yl)–2-(4-isobutylphenyl)propanamide (Ibu-AM68) was found to inhibit the hydrolysis of [3H]anandamide by rat brain homogenates by a reversible, mixed-type mechanism of inhibition with a Ki value of 0.26 µM and an n value of 4.9. At a concentration of 10 µM, the compound did not inhibit the cyclooxygenation of arachidonic acid by either ovine COX-1 or human recombinant COX-2. However, this concentration of Ibu-AM68 greatly reduced the ability of the COX-2 to catalyse the cyclooxygenation of the endocannabinoid 2-arachidonoylglycerol. It is concluded that Ibu-AM68 is a dual-acting FAAH/substrate-selective COX inhibitor.

Introduction

The non-steroidal anti-inflammatory agents (NSAIDs) such as ibuprofen, naproxen and diclofenac have widespread usage around the world, but their use is hampered by the incidence of severe gastrointestinal side effects. The elderly have a high consumption of NSAIDs, and this has resulted in a high incidence of NSAID-related hospitalisations and deaths3. There is thus much to be gained by the discovery and development of compounds that are as efficacious as the NSAIDs, but which lack these deleterious gastrointestinal effects.

In a key study from 2009, Naidu, Lichtman and colleagues2 reported that the ulcerogenic potency of the NSAID diclofenac was lower in mice lacking the enzyme fatty acid amide hydrolase (FAAH) than in the corresponding wild-type mice. A similar result was found in wild-type mice pre-treated with the irreversible FAAH inhibitor URB597 (3-(3′-(aminocarbonyl)[1,1′-biphenyl]–3-yl)cyclohexylcarbamate). Further, URB597 and diclofenac acted synergistically in reducing acetic acid-induced visceral nociception2. FAAH catalyses the hydrolysis of the endogenous cannabinoid (endocannabinoid) ligand anandamide (AEA, arachidonoylthanolamide)3 and the effects of FAAH inhibition upon diclofenac-induced ulceration were not seen in mice lacking cannabinoid-1 receptors2. The ability of FAAH inhibition to reduce the ulcerogenic potency of NSAIDs has also been seen with a peripherally-restricted FAAH inhibitor, URB937 (N-cyclohexyl-carbamic acid, 3′-(aminocarbonyl)–6-hydroxy[1,1′-biphenyl]–3-yl ester) and with indomethacin as NSAID4. A second endocannabinoid, 2-arachidonoylglycerol (2-AG) is primarily hydrolysed by monoacylglycerol lipase, and inhibition of that enzyme also reduces the ulcerogenic potency of diclofenac5,6.

Taken together, the studies above suggest that a compound with dual-action effects towards both cyclooxygenase (COX, the primary target of NSAIDs) and FAAH (or monoacylglycerol lipase) may be a potentially useful anti-inflammatory agent lacking the problematic gastrointestinal unwanted effects associated with NSAIDs. In 2015, the Piomelli group reported the synthesis and pharmacological properties of ARN2508 ((±)-2-[3-fluoro-4-[3-(hexylcarbamoyloxy)phenyl]phenyl]propanoic acid), a compound combining the structural elements of URB597 and the NSAID flurbiprofen7,8. The compound inhibited FAAH, COX-1 and COX-2 with IC50 values of 31, 12 and 420 nM, respectively, and produced anti-inflammatory effects in vivo without causing gastric irritation7. The carbamate group in the molecule was required for (presumably irreversible) FAAH inhibition, but not for COX-inhibition7. Similar to the profens9, the compound shows substrate-selective inhibition of COX-2, being more potent when 2-AG is used as substrate than when arachidonic acid (AA) is used10.

An alternative approach has been to design compounds based on ibuprofen, which has modest FAAH-inhibitory activity11, and to optimise the FAAH-inhibitory properties while retaining the COX-inhibitory properties of the parent compound. The first such
compound, a heterocyclic amide ibuprofen analogue, *Ibu-AMS* (2-(4-isobutylphenyl)-N-(3-methylpyridin-2-yl)propanamide, Figure 1) had been shown previously by one of us in 2003 to have analgesic activity with respect to acetic acid-induced visceral nociception in the mouse, without appreciable ulcerogenic potency\(^1\), and successively further described in 2007 for its FAAH inhibitory activity\(^\text{12}\). Further studies by us have shown that the compound inhibits FAAH in a mixed-type manner in sub-micromolar concentrations (i.e. 2-3 orders of magnitude more potent than ibuprofen itself) while retaining the substrate-selective inhibition of COX-2 seen with ibuprofen\(^\text{14,15}\).

While *Ibu-AMS* is a potentially useful compound, it would be useful to explore its structure to determine whether more potent FAAH/COX dual inhibitors can be identified. SAR studies so far reported by us have\(^\text{14,16,17}\) however, been unsuccessful in that the most potent FAAH-inhibitory compound so far described, *N*-((3-chloropyridin-2-yl)-2-((2-(trifluoromethyl)pyridin-4-yl)amino)phenyl)propenamide, **TPA-14** (*N*-((3-chloropyridin-2-yl)-2-((2-(trifluoromethyl)pyridin-4-yl)amino)phenyl)propenamide, Figure 1), had a similar potency to that of **Ibu-AM5**, but lacked COX-inhibitory potency\(^\text{16}\). In the present study, we report the identification of a novel *Ibu-AMS* analogue that is more potent than *Ibu-AMS* but which retains its substrate-selective inhibition of COX-2.

**Experimental**

**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). Non-radioactive AEA, ovine COX-1 (cat. no. 60100), human recombinant COX-2 (cat. no. 60122), arachidonic acid (AA) and 2-AG were purchased from the Cayman Chemical Co. (Ann Arbor, MI, USA). All commercially available solvents and reagents were used without further purification and were purchased from Sigma-Aldrich (Milan, Italy).

**Chemistry**

NMR spectra were recorded on an Inova 500 spectrometer (Varian, Palo Alto, CA). The chemical shifts (\(\delta\)) are reported in part per million downfield from tetramethylsilane (TMS), which was used as internal standard, and the spectra were recorded in hexadeuterio-dimethylsulphoxide (DMSO-d$_6$). Infra-red 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 *M*AT 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-S CHN recorder elemental analyser (Yanagimoto, Kyoto, Japan) and the values found were within 0.4% of theoretical values. Ibuprofen amides **Ibu-AM38-73** were synthesised according to Schemes 1 and 2.

**Methyl 2-(4-isobutylphenyl)acetate (2)**

A solution of ibufenac 1 (1.92 g, 10 mmol) in CH$_2$OH (10 ml) was added at room temperature (r.t.) with 37% HCl (0.5 ml) and refluxed for 4 h. The solvent was removed under vacuum and the crude methyl ester was used without purification in the further step. Yield 85%. Oil. \(^{1}\)H NMR (DMSO-d$_6$) \(\delta\) 0.98 (d, \(J = 7.0\) Hz, 6H, CH$_3$), 1.93 (m, 1H, CH), 2.42 (d, \(J = 7.0\) Hz, 2H, CH$_2$), 3.65 (s, 2H, CH$_2$), 3.78 (s, 3H, CH$_3$), 7.15 (m, 2H, Ar), 7.19 (m, Hz, 2H, Ar). Elemental analysis: calculated for C$_{14}$H$_{21}$O$_2$ (206.29)% C 75.69; H 8.80; found % C 75.75; H 8.77. Physical and spectral data were in accordance with literature values\(^\text{18}\).

**General procedure for the synthesis of esters 3 and 4**

Lithium bis-(trimethylsilyl)amide (4.00 g, 24 mmol) was added to a solution of ester 2 (2.00 g, 9.7 mmol) in dry THF (40 ml) under argon at \(-78^\circ\) C, the mixture was stirred at this temperature for 45 min. Then methyl iodide (3.40 g, 24 mmol) or 1,2-dibromo-ethane (4.51 g, 12 mmol) was added dropwise to the stirred solution for an additional 1 h. The mixture was poured in water and the desired product was extracted with diethyl ether (2 \(\times\) 30 ml). The solvent was dried over Na$_2$SO$_4$, then it was evaporated under reduced pressure. The crude residue was purified via silica gel (200-400 mesh silica gel Merk KGaA) chromatography using petrol ether 40–60 °C and AcOEt 20:1.

**Methyl 2-(4-isobutylphenyl)-2-methylpropanoate (3)**

Yield 80%. Oil. \(^{1}\)H NMR (DMSO-d$_6$) \(\delta\) 0.95 (d, \(J = 7.0\) Hz, 6H, CH$_3$), 1.63 (s, 6H, CH$_3$), 1.82 (m, 1H, CH), 2.42 (d, \(J = 7.0\) Hz, 2H, CH$_2$), 3.66 (s, 3H, CH$_3$), 7.07 (d, \(J = 7.5\) Hz, 2H, Ar), 7.25 (d, \(J = 7.5\) Hz, 2H, Ar). Elemental analysis: calculated for C$_{15}$H$_{22}$O$_2$ (234.16)% C 76.88; H 9.46; found % C 76.92; H 9.44. Physical and spectral data were in accordance with literature values\(^\text{19}\).

**Methyl 1-(4-isobutylphenyl)cyclopropane-1-carboxylate (4)**

Yield 60%. Oil. \(^{1}\)H NMR (DMSO-d$_6$) \(\delta\) 0.91 (d, \(J = 7.0\) Hz, 6H, CH$_3$), 1.25 (m, 2H, CH$_2$), 1.58 (m, 2H, CH$_2$), 1.84 (m, 1H, CH), 2.43 (d, \(J = 7.0\) Hz, 2H, CH$_2$), 3.69 (s, 3H, CH$_3$), 7.15 (d, \(J = 7.5\) Hz, 2H, Ar), 7.23 (d, \(J = 7.5\) Hz, 2H, Ar). Elemental analysis: calculated for C$_{14}$H$_{21}$O$_2$ (232.32)% C 77.55; H 8.68; found % C 77.62; H 8.72. Physical and spectral data were in accordance with literature values\(^\text{19}\).

**General procedure for the synthesis of acids 5 and 6**

To a solution of ester 3 or 4 (1 mmol) in EtOH (10 ml) 5 N solution of NaOH (2 ml) and water (2 ml) were added. The resulting mixture was stirred at r.t. for 24 h. After removing EtOH under vacuum to the resulting solution ice was added and then acidified with...
aqueous 20% HCl solution until pH 3–4. The formed precipitate was filtrated, washed with water and re-crystallized from n-hexane.

2-(4-Isobutylphenyl)-2-methylpropanoic acid (5)

Obtained following the general procedure starting by ester 4. Yield 90%. m.p. 70-72 °C. 1H NMR (DMSO-d6) δ 0.90 (d, J = 7.0 Hz, 6H, CH₃), 1.64 (s, 6H, CH₃), 1.90 (m, 1H, CH), 2.55 (d, J = 7.0 Hz, 2H, CH₂), 7.09 (d, J = 7.5 Hz, 2H, Ar), 7.33 (d, J = 7.5 Hz, 2H, Ar). Elemental analysis: calculated for C₁₄H₂₀O₂ (220.15)% C 76.33; H 9.15; found % C 76.33; H 9.03. Physical and spectral data were in accordance with literature values19.

1-(4-Isobutylphenyl)cyclopropane-1-carboxylic acid (6)

Obtained following the general procedure starting by ester 5. Yield 90%. m.p. 74-76 °C. 1H NMR (DMSO-d6) δ 0.87 (d, J = 7.0 Hz, 6H, CH₃), 1.08 (m, 2H, CH₂), 1.41 (m, 2H, CH₂), 1.80 (m, 1H, CH), 2.42 (d, J = 7.0 Hz, 2H, CH₂), 7.06 (d, J = 7.5 Hz, 2H, Ar), 7.21 (d, J = 7.5 Hz, 2H, Ar). Elemental analysis: calculated for C₁₄H₁₈O₂ (218.30)% C 77.03; H 8.31; found % C 77.10; H 8.27. Physical and spectral data were in accordance with literature values19.

N-(3-Chloropyridin-2-yl)-2-(4-isobutylphenyl)propanamide (Ibu-AM58)

Obtained following the general procedure by the condensation between ibuprofen and 2-amino-3-chloropyridine. Yield 39%. Oil. 1H NMR (DMSO-d6) δ 0.85 (d, J = 7.0 Hz, 6H, CH₃), 1.41 (d, J = 7.0 Hz, 3H, CH₃), 1.83 (q, J = 7.0 Hz 1H, CH), 2.04 (s, 3H, CH₃), 2.42 (q, J = 7.0 Hz, 2H, CH₂), 3.88 (q, J = 7.0 Hz, 1H, CH), 7.04–7.10 (m, 6H, Ar), 7.31 (m, 2H, Ar), 9.30 (s, 1H, NH). 13C NMR (DMSO-d6) δ 21.6, 25.4 (2 C), 32.7, 47.3, 116.9, 120.2, 130.1 (2 C), 132.0 (2 C), 142.6, 143.2, 147.8, 150.1, 160.2, 178.7 IR (Film) 1660, 1512 cm⁻¹. Elemental analysis: calculated for C₂₀H₁₇ClN₂O (316.83)% C 68.24; H 6.68; N 8.84; found % C 68.30; H 6.65; N 8.81.

Scheme 1. Synthetic pathway for Ibu-AM72 and 73. Reagents and conditions: (i) HCl 37%, MeOH, reflux 4 h; (ii) Lithium bis-(trimethylsilyl)amide, THF, –78 °C, 45 min, then Mel or 1,2-dibromoethane, 1 h; (iii) 5N NaOH, H₂O, EtOH, r.t., 24 h; (iv) EDC, HOBt, MeCN, r.t. 36 h.

Scheme 2. Synthetic pathway for ibuprofen aryl- and pyridyl-amides. Reagents and conditions i) EDC, HOBt; MeCN, r.t. 36 h.

General procedure for the synthesis of amides Ibu-AM38-73

The solution of the appropriate acid 2, 5 or 6 (1 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (0.19 g, 1.1 mmol) and 1-hydroxybenzotriazole (HOBt) (0.13 g, 1 mmol) in anhydrous MeCN (10 ml) was stirred at r.t., after 30 min the opportune amine (1 mmol) was added and the mixture was stirred at r.t. for 36 h. After the solvent was removed under vacuum. The residue was dissolved in AcOEt (20 ml) and washed sequentially with brine (2 × 5 ml), 10% citric acid (2 × 5 ml), saturated NaHCO₃ aqueous solution (2 × 5 ml) and water (2 × 5 ml). The organic layer was dried over anhydrous Na₂SO₄ and evaporated under vacuum to give the title amides.
2-(4-Isobutylphenyl)-N-(3-(trifluoromethyl)pyridin-2-yl)propanamide (Ibu-AM60)

Obtained following the general procedure by the condensation between ibuprofen and 2-aminomethylpyridin. Yield 59%. m.p. 112–114 °C. 1H NMR (DMSO-d6) δ 0.85 (d, J = 6.0 Hz, 6H, CH3), 1.39 (d, J = 7.0 Hz, 3H, CH3), 1.81 (q, J = 6.5 Hz, 1H, CH), 2.42 (d, J = 7.0 Hz, 2H, CH2), 3.87 (q, J = 7.0 Hz, 1H, CH), 7.10–8.71 (m, 7H, Ar), 10.25 (s, 1H, NH). IR (Nujol) 3253, 1670, 1583 cm⁻¹. Elemental analysis: calculated for C19H23NO2 (350.16)% C 76.74; H 7.80; N 4.71. Obtained from the condensation between ibuprofen and 2-amino-3-(trifluoromethyl)pyridine. Yield 53%. Oil.

N-(3-Hydroxypyridin-2-yl)-2-(4-isobutylphenyl)propenamide (Ibu-AM69)

Obtained following the general procedure by the condensation between ibuprofen and 2-amino-3-hydroxypyridine. Yield 38%. Oil. 1H NMR (DMSO-d6) δ 0.85 (d, J = 7.0 Hz, 6H, CH3), 1.42 (d, J = 7.0 Hz, 3H, CH3), 1.80 (hept, J = 7.0 Hz, 1H, CH), 2.14 (m, 2H, CH2), 3.84 (q, J = 7.0 Hz, 1H, CH), 7.02–8.40 (m, 7H, Ar), 10.18 (s, 1H, NH). IR (Film) 3233, 1675, 1574 cm⁻¹. Elemental analysis: calculated for C19H19N3O (408.28)% C 52.95; H 5.18; N 6.86; found % C 53.01; H 5.16; N 6.90.

N-(3-Hydroxy-2-(4-isobutylphenyl)-aminopyridin-2-yl)-2-(4-isobutylphenyl)propenamide (Ibu-AM70)

Obtained following the general procedure by the condensation between ibuprofen and amino-2-hydroxypyridine. Yield 38%. Oil. 1H NMR (DMSO-d6) δ 0.85 (d, J = 7.0 Hz, 6H, CH3), 1.42 (d, J = 7.0 Hz, 3H, CH3), 1.79 (hept, J = 7.0 Hz, 1H, CH), 2.40 (d, J = 7.0 Hz, 2H, CH2), 3.62 (q, J = 7.0 Hz, 1H, CH), 7.09–7.88 (m, 7H, Ar), 10.24 (s, 1H, NH), 10.75 (s, 1H, OH). IR (Film) 1662, 1512 cm⁻¹. Elemental analysis: calculated for C39H24N2O2 (583.39)% C 72.46; H 7.43; N 9.39; found % C 72.54; H 7.45; N 9.42.

3-Methylpyridin-2-yl-1-(4-isobutylphenyl)cyclopropane-1-carboxylate (Ibu-AM73)

Obtained following the general procedure by the condensation between 2-(4-isobutylphenyl)-2-methylpropanoic acid (5) and 2-amino-3-methylpyridine. Yield 70%. Oil. 1H NMR (DMSO-d6) δ 0.86 (d, J = 7.0 Hz, 6H, CH3), 1.57 (s, 6H, CH3), 2.02 (m, 1H, CH), 2.03 (s, 3H, CH3), 2.44 (d, J = 7.0 Hz, 2H, CH2), 7.12–7.20 (m, 3H, Ar), 7.30–7.35 (m, 2H, Ar), 7.62 (m, 1H, Ar), 8.22 (m, 1H, Ar), 9.27 (s, 1H, NH). IR (Film) 3167, 2956, 1686, 1583 cm⁻¹. Elemental analysis: calculated for C35H29NO2 (530.44)% C 77.38; H 8.44; N 9.02; found % C 77.31; H 8.47; N 9.06.

2-(4-Isobutylphenyl)-N-(2-methoxy-2-methylpropyl)propenamide (Ibu-AM67)

Obtained following the general procedure by the condensation between ibuprofen and 4-methoxy-2-methylpropenyl. Yield 49%. m.p. 100–102 °C. 1H NMR (DMSO-d6) δ 0.85 (d, J = 7.0 Hz, 6H, CH3), 1.40 (d, J = 7.0 Hz, 3H, CH3), 1.81 (hept, J = 7.0 Hz, 1H, CH), 1.99 (s, 3H, CH3), 2.42 (d, J = 7.0 Hz, 2H, CH2), 3.70 (s, 3H, CH3), 3.81 (d, J = 7.0 Hz, 1H, CH), 6.69–7.31 (m, 7H, Ar), 9.21 (s, 1H, NH). IR (Nujol) 3298, 1655, 1613 cm⁻¹. Elemental analysis: calculated for C35H29NO2 (530.44)% C 77.38; H 8.44; N 9.02; found % C 77.31; H 8.47; N 9.06.

2-(4-Isobutylphenyl)-N-(3-hydroxy-2-(4-isobutylphenyl)aminopyridin-2-yl)propanamide (Ibu-AM66)

Obtained following the general procedure by the condensation between ibuprofen and 4-hydroxy-2-methylaminopyridine. Yield 63%. m.p. 133–135 °C. 1H NMR (DMSO-d6) δ 0.83 (d, J = 7.0 Hz, 6H, CH3), 1.35 (d, J = 7.0 Hz, 3H, CH3), 1.77 (hept, J = 7.0 Hz, 1H, CH), 1.90 (s, 3H, CH3), 2.40 (d, J = 7.0 Hz, 2H, CH2), 3.75 (q, J = 7.0 Hz, 1H, CH), 6.48–7.28 (m, 7H, Ar), 9.09 (s, 1H, OH). IR (Nujol) 3398, 3292, 1656, 1610 cm⁻¹. Calculated for C35H30O2N (531.43)% C 77.14; H 8.09; N 4.50; found % C 77.06; H 8.11; N 4.52.
7.07–7.25 (m, 4H, Ar), 7.37 (m, 1H, Ar), 7.62 (m, 1H, Ar), 8.19 (m, 1H, Ar), 8.59 (s, 1H, NH). IR (Film) 3397, 1687, 1582 cm$^{-1}$. Elemental analysis: calculated for C$_{20}$H$_{24}$N$_{2}$O (308.43)% C 77.89; H 6.53; N 10.01; O 5.63; found % C 77.79; H 8.11; N 10.04.

**Pharmacology**

**FAAH assay**

Frozen (−80 °C) brains (minus cerebellum) from adult Wistar or Sprague-Dawley rats were thawed and homogenised in 20 mM HEPES, 1 mM MgCl$_2$, pH 7.0. The homogenates were then centrifuged at ~3500 × 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. After 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. For the FAAH assay, test compounds, homogenates and [3H]AEA (diluted with non-radioactive AEA to give a substrate concentration of 0.5 μM) in 10 mM Tris-HCl buffer, pH 7.4, containing 1% w/v fatty acid-free bovine serum albumin were incubated for 10 min at 37 °C. Activated charcoal in 0.5 M HCl was added to adsorb the unmetabolized [3H]AEA and the samples were mixed and left at r.t. for ~30 min. Following centrifugation at 2000 g for 10 min, aliquots of the supernatants, containing the [3H]ethanolamine produced by hydrolysis of [3H]AEA, were analysed for tritium content by liquid scintillation spectrometry.

**COX-1 and 2 assay**

The assay was performed essentially according to the method of Meade et al. An oxygen electrode chamber with integral stirring (Oxygraph System, Hansatech Instruments, King’s Lynn, U.K.) was calibrated daily to 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 or 2-AG) in a final assay volume of 2 ml. After addition of test compound, a baseline was established for 5 min before initiation of reaction by addition of 200 units ovine COX-1 or human recombinant COX-2. The change in oxygen consumption as a measurement of enzyme activity was monitored for approximately 5 min.

**Computational studies**

**FAAH receptor and ligand preparation**

The crystal structure of the rat fatty acid amide hydrolase (fFAAH) (PDB ID: 3QKS) was downloaded from the Protein Data Bank website. Both monomers A and B were treated with the Protein Preparation Wizard tool implemented in Maestro ver. 11.12, in order to add all the hydrogen atoms and assign the correct bond orders. Subsequently, both the co-crystallized ligands and water molecules were removed. Residue Lys142 was considered in its deprotonated form, according to the proposed catalytic mechanism of FAAH. The 3D structure of Ibu-AM68 was built using the Graphical User Interface (GUI) of Maestro ver. 11.12. The protonation state of Ibu-AM68 at pH 7.4 in water has been calculated using the Epik module. Finally, Ibu-AM68 was then minimised using a protocol already adopted for Ibu-AM5. The molecular docking of Ibu-AM68 was performed only on the monomer A of the rat FAAH (fFAAH) receptor. Docking procedure was carried out with the Glide software package, using the Standard Precision (SP) algorithm of the GlideScore function and the OPLS 2005 force field. A grid box of 29 × 29 × 29 Å centered on the ligand binding cavity was created. A total amount of 200 poses was generated and the conformational sampling of the ligand was enhanced by two times, as reported by the default setting of Glide. Docking conformations of Ibu-AM68 were then clustered based on their RMSD cut-off of 2 Å. Globally, ten clusters were obtained and, among them, only the conformation included in the most populated cluster owing both the Glide Emodel and GlideScore lowest-energy value was considered. Such conformation was, finally, submitted to a further minimisation protocol using the OPLS 2005 force field, 20,000 minimisation steps and the Polak-Ribiere Conjugate Gradient (PRCG) algorithm.

**Results and discussion**

The potency of Ibu-AM5 towards AEA hydrolysis has been measured by different groups with different assay methodologies, FAAH preparations (rat brain, mouse brain, recombinant human FAAH) and substrate concentrations (0.5–2 μM). The IC$_{50}$ value for Ibu-AM5 from different studies in our laboratory using the same assay as here (of importance given the mixed-type nature of its inhibition of FAAH) ranges from 0.52–1.2 μM and we therefore have used the most potent value for comparative purposes, since the aim is to identify more potent compounds.

**FAAH inhibition**

Three series of Ibu-AM5 analogues were synthesised according to Schemes 1 and 2 and tested towards rat brain FAAH-catalysed hydrolysis of AEA. The first series of two compounds was motivated by the finding that for Ibu-AM5 removal of the methyl group at the C-2 carbon atom (“Ibufenac-AM1”) reduced the potency roughly 60-fold. In order to explore whether or not the methyl group at that position was optimal, two compounds were synthesised, with a dimethyl (Ibu-AM72) and cyclopropyl (Ibu-AM73) groups instead of the methyl group at the C-2 carbon atom. The amides were obtained starting from Ibufenac (1) that was converted into its methyl ester 2 and then alkylated at C-2 position to give intermediates 3 and 4 that were hydrolysed to the corresponding acid 5 and 6. These last were coupled with 2-amino-3-methylpyridine by EDC method to afford the target amides Ibu-AM72 and Ibu-AM73. The compounds inhibited FAAH with IC$_{50}$ values of 1.0 and 4.1 μM for Ibu-AM72 and Ibu-AM73, respectively (Figure 2A, Table 1). Although this is a very limited series, it would suggest that there is little to be gained by adjusting the methyl group at the C-2 carbon atom, and so we moved on to the amido moiety of Ibu-AM5 3-substituent on the pyridine ring of Ibu-AM5.
In our initial study, we reported that the potency of the pyridinamides of ibuprofen towards FAAH is related to the presence of both methyl and pyridine nitrogen in ortho positions to the amide nitrogen as in Ibu-AM5, the methyl absence or its moving in a position different from ortho to the amide nitrogen results in activity decrease. On this basis to evaluate the influence on the activity of the pyridine nitrogen, we changed pyridine ring with a chlorine and a bromine group. In Figure 2(B) and summarised in Table 1. The observed potencies of the substituents were -I (Ibu-AM69) and -Br (Ibu-AM68) > -CF₃ (Ibu-AM60) > -Cl (Ibu-AM58) > -OH (Ibu-AM70). The 95% confidence intervals for the mean IC₅₀ values for Ibu-AM69 (0.078–0.19 μM) and Ibu-AM68 (0.038–0.15) overlap, so we regard the two compounds as equipotent but more potent than Ibu-AM5.

The inhibition of FAAH by Ibu-AM68 was investigated in more detail. Preincubation of Ibu-AM68 with the homogenates for up to 60 min prior to addition of substrate did not increase the observed inhibition, indicating that there is no time-dependence of the inhibition (Figure 2(D)). For a fully reversible inhibitor, preincubation for 60 min with a concentration "x" of compound followed by a 20-fold dilution prior to addition of substrate should
produce the same observed inhibition as seen with a concentration of \( \frac{x}{20} \) of the compound added together with the substrate, and this was found to be the case for Ibu-AM68 (Figure 2(E)). Finally, kinetic experiments indicated a simple mixed-model inhibition of FAAH with a \( K_i \) value of 0.26 \( \mu \)M and an \( a \) value (the ratio of the \( K_i \) intercept: \( K_i \) slope values; for pure competitive inhibition, \( a \approx 1 \)) of 4.9 (Figure 2(F)). Thus, Ibu-AM68 is a reversible mixed inhibitor of rat brain FAAH with a greater potency than Ibu-AM5.

Inhibition of COX isoenzymes by Ibu-AM68

The ability of Ibu-AM68 to inhibit the cyclooxygenation of AA and 2-AG by COX-1 and COX-2 was investigated (Figure 3). In our hands under the assay conditions used, 30 \( \mu \)M ibuprofen itself produces approximately 50% inhibition of the cyclooxygenation of AA by COX-1 with at best minor inhibition of COX-2 at this concentration. However, \( \geq 10 \) and 30 \( \mu \)M ibuprofen produce a marked inhibition of 2-AG and AEA cyclooxygenation by COX-2 (neither endocannabinoid is a substrate for COX-1)\(^{14,35}\). Ibu-AM5 also shows substrate selective inhibition, reducing the rate of cyclooxygenation to about half at concentrations of 50 \( \mu \)M (COX-1, AA as substrate) and 3 \( \mu \)M (COX-2, AEA as substrate) whilst 100 \( \mu \)M Ibu-AM5 is without effect upon COX-2 with AA as substrate.\(^{14}\) At a concentration of 10 \( \mu \)M, a modest inhibition of AA cyclooxygenation by Ibu-AM68 was seen with COX-1 whereas the cyclooxygenation of 2-AG by COX-2 was almost completely inhibited. Higher concentrations of Ibu-AM68 (50 and 100 \( \mu \)M) produced a complete inhibition of COX-1 but did not inhibit AA cyclooxygenation by COX-2. This suggests that the substrate-selective inhibition of COX-2 reported first for the R-profens by Marnett and colleagues\(^{15}\) is also seen with Ibu-AM68. The mechanism of this inhibition has not been investigated, but Marnett et al.\(^{9,36}\) have suggested that it may be related to COX-2 (which has a homodimeric structure) acting as functional heterodimers, whereby the binding of the R-profen to one site acts allosterically to block 2-AG but not AA cyclooxygenation. It is possible that such a mechanism can explain the actions of Ibu-AM5 and Ibu-AM68.

FAAH docking on Ibu-AM68

Molecular docking calculations on (S)-Ibu-AM68 were performed with the Glide software\(^{29-31}\) in the crystal structure of rat FAAH (PDB ID: 3QK5)\(^{37}\). The software Glide was chosen since it showed to be able to well reproduce the binding poses of (R)- and (S)-Ibu-AM5 resulting by molecular dynamics and free energy calculations 0.17 The results were clustered and successively ranked according to the Glide Emodel and the Glide Score. The best pose showed the isobutyl moiety pointing to the catalytic triad and the pyridine moiety entering the membrane access channel (MAC) channel (Figure 4). In particular, the substituted pyridine ring established hydrophobic contacts with Leu404, Ile407, a T-shaped \( \pi-\pi \) interaction with Trp531 and a H-bond interaction with the hydroxyl group of Thr488. Moreover, polar contacts between the bromine atom and the backbone hydrogens of residues Asp403 and Leu404 were observed. An additional H-bond was established between the NH group of the ligand with the carbonyl of the Gly485. The Ibu-AM68 hydrophobic isobutyl-phenyl moiety resulted embedded in a hydrophobic region in the Acyl Chain Binding channel (ACB), and established hydrophobic contacts with residues Leu192 Phe244, Leu380, Thr488 and Ile491. The comparison with the binding mode of Ibu-AM5 showed high similarity in the isobutyl-phenyl moiety, but a different conformation of the pyridine ring with respect to the amide moiety. This different conformational behaviour maybe be likely due to different dipole alignment, being the slightly negative bromine atom better aligned with the NH group of the amide bond, while the methyl
substituent preferred an orientation orthogonal to the carbonyl group.

In conclusion, the present study has characterised in vitro an Ibu-AM5 analogue that is slightly more potent than Ibu-AM5 itself as FAAH inhibitor and which retains its COX-2 substrate-selectivity. Further studies are necessary to determine whether this compound behaves like the dual action FAAH-COX inhibitor ARN2508 in producing potentially beneficial effects in models of inflammatory pain without the ulcerogenic effects that are an issue with current NSAIDs.

Acknowledgements

The authors are grateful to Dr. Emmelie Björklund for running the FAAH assay for Ibu-AM38.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

C.F. would like to thank the Research Funds of the Medical Faculty, Umeå University, for financial support. V.O would like to thank the Region Autonoma della Sardegna Project L.R. 7/2007 under grant no. 2012_CRP-59473 and the University of Cagliari (grant FIR 2018–19). B.C. would like to thank Regione Campania under grant B61C17000070007–SATIN (POR Campania FESR 2014/2020). This work was supported by the Open Access Publishing Fund of the University of Cagliari, with the funding of the Region Autonoma della Sardegna – L.R. n. 7/2007.

ORCID

Alessandro Deplano http://orcid.org/0000-0002-8451-5831  Jessica Karlsson http://orcid.org/0000-0001-8572-5841  Federica Moraca http://orcid.org/0000-0002-1077-1971  Bruno Catalanotti http://orcid.org/0000-0002-7532-6959  Christopher J. Fowler http://orcid.org/0000-0002-6658-7874  Valentina Onnis http://orcid.org/0000-0002-2438-725X

References

1. Griffin M. Epidemiology of nonsteroidal anti-inflammatory drug-associated gastrointestinal injury. Am J Med 1998;104: 23S–9S.
2. Naidu P, Booker L, Cravatt B, Lichtman A. Synergy between enzyme inhibitors of fatty acid amide hydrolase and cyclooxygenase in visceral nociception. J Pharmacol Exp Ther 2009;329:48–56.
3. Deutsch DG, Chin SA. Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. Biochem Pharmacol 1993;46:791–6.
4. Sasso O, Bertorelli R, Bandiera T, et al. Peripheral FAAH inhibition causes profound antinociception and protects against indomethacin-induced gastric lesions. Pharmacol Res 2012;65:553–63.
5. Kinsey SG, Nomura DK, O’Neal ST, et al. Inhibition of monoa-cylglycerol lipase attenuates nonsteroidal anti-inflammatory drug-induced gastric hemorrhages in mice. J Pharmacol Exp Ther 2011;338:795–802.
6. Crowe MS, Kinsey SG. MAGL inhibition modulates gastric secretion and motility following nsaid exposure in mice. Eur J Pharmacol 2017;807:198–204.
7. Sasso O, Migliore M, Habrant D, et al. Multitarget fatty acid amide hydrolase/cyclooxygenase blockade suppresses intestinal inflammation and protects against nonsteroidal anti-inflammatory drug-dependent gastrointestinal damage. Faseb J 2015;29:2616–27.
8. Migliore M, Habrant D, Sasso O, et al. Potent multitarget faah-cox inhibitors: design and structure-activity relationship studies. Eur J Med Chem 2016;109:216–37.
9. Hermanson DJ, Gamble-George JC, Marnett LJ, Patel S. Substrate-selective cox-2 inhibition as a novel strategy for therapeutic endocannabinoid augmentation. Trends Pharmacol Sci 2014;35:358–67.
10. Goodman MC, Xu S, Rouzer CA, et al. Dual cyclooxygenase-fatty acid amide hydrolase inhibitor exploits novel binding interactions in the cyclooxygenase active site. J Biol Chem 2018;293:3028–38.
11. Fowler CJ, Tiger G, Stenström A. Ibuprofen inhibits rat brain deamidation of anandamide at pharmacologically relevant
concentrations. Mode of inhibition and structure-activity relationship. J Pharmacol Exp Ther 1997;283:729–34.

12. Cocco M, Congiu C, Onnis V, et al. Synthesis of ibuprofen heterocyclic amides and investigation of their analgesic and toxicological properties. Eur J Med Chem 2003;38:513–8.

13. Holt S, Paylor B, Boldrup L, et al. Inhibition of fatty acid amide hydrolase, a key endocannabinoid metabolizing enzyme, by analogues of ibuprofen and indomethacin. Eur J Pharmocol 2007;565:26–36.

14. Fowler CJ, Björklund E, Lichtman AH, et al. Inhibition of fatty acid amide hydrolase, a key endocannabinoid metabolizing enzyme, by analogues of ibuprofen and indomethacin. Eur J Pharmocol 2007;565:26–36.

15. Karlsson J, Morgillo CM, Deplano A, et al. Interaction of the n-(3-methylpyridin-2-yl)amide derivatives of flurbiprofen and ibuprofen with FAAH: enantiomeric selectivity and binding mode. PLoS One 2015;10:e0142711.

16. Deplano A, Morgillo CM, Demurtas M, et al. Novel propanamides as fatty acid amide hydrolase inhibitors. Eur J Med Chem 2017;136:523–42.

17. Deplano A, Cipriano M, Moraca F, et al. Benzylamides and piperazinoarylamides of ibuprofen as fatty acid amide hydrolase inhibitors. J Enzyme Inhib Med Chem 2019;34:562–76.

18. Adams AD, Jones AB, Berger JP, et al. Preparation of 2-aryloxy-2-arylalkanoic acids for diabetes and lipid disorders, Patent WO2002064094A2, 2002.

19. Windsor MA, Hermanson DJ, Kingsley PJ, et al. Substrate-selective inhibition of cyclooxygenase-2: development and evaluation of achiral profen probes. ACS Med Chem Letts 2012;3:759–63.

20. Boldrup L, Wilson SJ, Barbier AJ, Fowler CJ. A simple stopped assay for fatty acid amide hydrolase avoiding the use of a chloroform extraction phase. J Biochem Biophys Methods 2004;60:171–7.

21. Meade EA, Smith WL, DeWitt DL. Differential inhibition of prostaglandin endoperoxide synthase (cyclooxygenase) isoforms by aspirin and other non-steroidal anti-inflammatory drugs. J Biol Chem 1993;268:6610–4.

22. Sastry GM, Adzhigirey M, Day T, et al. Protein and ligand preparation: parameters, protocols, and influence on virtual screening enrichments. J Comput Aid Mol Des 2013;27:221–34.

23. Schrödinger Release 2019-1: Maestro, New York, NY: Schrödinger, LLC, 2019.

24. Palermo G, Rothlisberger U, Cavalli A, De Vivo M. Computational insights into function and inhibition of fatty acid amide hydrolase. Eur J Med Chem 2015;91:15–26.

25. Bracey M, Hanson MA, Masuda KR, et al. Structural adaptations in a membrane enzyme that terminates endocannabinoid signaling. Science 2002;298:1793–6.

26. Lodola A, Castelli R, Mor M, Rivara S. Fatty acid amide hydrolase inhibitors: a patent review (2009–2014). Expert Opin Ther Pat 2015;25:1247–66.

27. Shelley JC, Cholleti A, Frye L, et al. Epik: a software program for pKa prediction and protonation state generation for drug-like molecules. J Comp Aided Mol Design 2007; 21: 681–91.

28. Grippo L, Lucidi S. A globally convergent version of the Polak-Ribière conjugate gradient method. Math Program 1997; 78:375–91.

29. Glide, version 7.1. New York, NY: Schrödinger, LLC, 2019.

30. Friesner RA, Banks JL, Murphy RB, et al. Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. J Med Chem 2004; 47: 1739–49.

31. Halgren TA, Murphy RB, Friesner RA, et al. Glide: a new approach for rapid, accurate docking and scoring. 2. Enrichment factors in database screening. J Med Chem 2004;47:1750–9.

32. Banks JL, Beard HS, Cao Y, et al. Integrated modeling program, applied chemical theory (IMPACT). J Comp Chem 2005;26:1752–80.

33. De Wael F, Muccioli GG, Lambert DM, et al. Chemistry around imidazopyrazine and ibuprofen: discovery of novel fatty acid amide hydrolase (FAAH) inhibitors. Eur J Med Chem 2010;45:3564–74.

34. Favia AD, Habrant D, Scarpelli R, et al. Identification and characterization of carprofen as a multitarget fatty acid amide hydrolase/cyclooxygenase inhibitor. J Med Chem 2012;55:8807–26.

35. Karlsson J, Fowler CJ. Inhibition of endocannabinoid metabolism by the metabolites of ibuprofen and flurbiprofen. PLoS One 2014;9:e103589.

36. Duggan KC, Hermanson DJ, Musee J, et al. (R)-profens are substrate-selective inhibitors of endocannabinoid oxygenation by COX-2. Nat Chem Biol 2011;7:803–9.

37. Gustin DJ, Ma Z, Min X, et al. Identification of potent, non-covalent fatty acid amide hydrolase (FAAH) inhibitors. Bioorg Med Chem Lett 2011;21:2492–6.