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SPECIAL ISSUE

A *Double Whammy*: Targeting Both Fatty Acid Amide Hydrolase (FAAH) and Cyclooxygenase (COX) To Treat Pain and Inflammation

Rita Scarpelli,[a] Oscar Sasso,[a] and Daniele Piomelli*[a, b]

Pain states that arise from non-resolving inflammation, such as inflammatory bowel disease or arthritis, pose an unusually difficult challenge for therapy because of the complexity and heterogeneity of their underlying mechanisms. It has been suggested that key nodes linking interactive pathogenic pathways of non-resolving inflammation might offer novel targets for the treatment of inflammatory pain. Nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit the cyclooxygenase (COX)-mediated production of pain- and inflammation-inducing prostanoids, are a common first-line treatment for this condition, but their use is limited by mechanism-based side effects. The endogenous levels of anandamide, an endocannabinoid mediator with analgesic and tissue-protective functions, are regulated by fatty acid amide hydrolase (FAAH). This review outlines the pharmacological and chemical rationale for the simultaneous inhibition of COX and FAAH activities with designed multitarget agents. Preclinical studies indicate that such agents may combine superior anti-inflammatory efficacy with reduced toxicity.

Introduction

Despite substantial advances made in the last decades, the identification of safe and effective therapeutics for the management of acute and chronic pain remains a formidable challenge in drug development. The current standards of care—opioids, nonsteroidal anti-inflammatory drugs (NSAIDs), and gabapentinoids—help many patients, but it is estimated that only 25% of them receive adequate pain relief. Moreover, available analgesic drugs exert acute and long-term side effects and can have, as in the case of the opioids, significant potential to induce dependence and addiction. Pain conditions that arise from non-resolving inflammatory pathologies, such as inflammatory bowel disease and arthritis, are particularly challenging owing to the complexity and heterogeneity of their underlying pathogenesis. In such conditions, key control nodes that link interactive molecular pathways involved in promoting and maintaining pain, inflammation, and tissue damage might be exploited to achieve greater clinical benefits.[1] In this context, attention has focused on two important families of lipid-derived mediators: prostanoids and endocannabinoids. Accumulated evidence points to the presence of multiple biochemical and functional interactions between these signaling pathways, which might offer useful molecular targets for analgesic and anti-inflammatory therapy.[2]

Herein we outline the scientific rationale for simultaneously inhibiting two pivotal enzymes of endocannabinoid and prostanoid metabolism—fatty acid amide hydrolase (FAAH) and cyclooxygenase (COX)—and review medicinal chemistry approaches and structure–activity relationship (SAR) studies of the multitarget FAAH/COX inhibitors discovered to date.

Interacting Lipid Pathways

Fatty acid ethanolamides (FAEs) such as arachidonoylethanolamide (anandamide), palmitoylethanolamide (PEA), and oleoylethanolamide (OEA) (Figure 1) constitute a family of signaling lipids involved in the regulation of a wide range of physiologi-

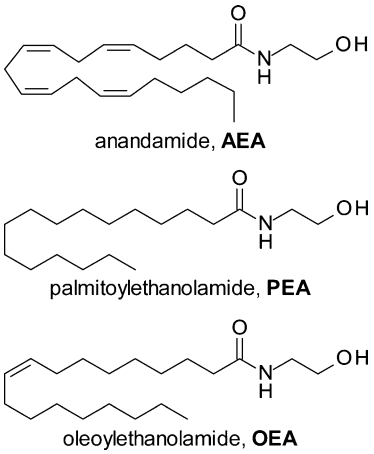

Figure 1. Chemical structures of representative FAEs.

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cal and pathological processes, including pain and inflammation.[3]

The FAEs exert their biological actions by binding to selective protein receptors in cells. Anandamide is an endogenous agonist of G-protein-coupled CB_1 and CB_2 cannabinoid receptors, which mediate the majority of its biological effects.[4] Other pharmacological targets for anandamide, such as transient receptor potential vanilloid type 1 (TRPV-1), have been identified, but their physiological significance remains unclear.[5]

OEA and PEA do not productively interact with cannabinoid receptors, but act by engaging nuclear peroxisome proliferator-activated receptor-α (PPAR-α) and, to a lesser extent, GPR119.[6]

The FAEs are produced from phospholipid precursors in membranes through either tonic or 'on-demand' mechanisms. Therefore, the signaling activity of these substances is strictly dependent on the enzymes responsible for their formation and degradation.[6d,7] FAAH is the main enzyme involved in the degradative hydrolysis of anandamide into arachidonic acid (AA) and ethanolamine (Figure 2).[8] It is a membrane-bound serine hydrolase localized to intracellular organelles such as microsomes and mitochondria, and is highly expressed in brain and liver, among other tissues.[9]

Soon after its discovery,[8a,10] FAAH attracted attention as a potential target for indirect-acting cannabinoid agents, that is, for compounds that stimulate cannabinoid receptors by modulating endogenous anandamide levels.[5b] There are multiple precedents that justify the adoption of this indirect strategy (e.g., neurotransmitter uptake inhibitors), which are further supported by the fact that direct cannabinoid receptor activation causes a variety of central and peripheral side effects.[11] Conversely, protecting anandamide from FAAH-mediated degradation might result in a more physiological activation of cannabinoid receptors—similar to that caused by endogenously released anandamide—and might thus exert a restricted, and hopefully favorable, set of pharmacological effects relative to exogenous direct-acting agonists.[12] One important piece of evidence in support of this theory comes from experiments with mice lacking the gene encoding for FAAH. These mutant mice display an analgesic and anti-inflammatory phenotype, yet do not show any of the typical signs of direct CB_1 receptor activation, such as decreased motor activity, lowered body temperature, and catalepsy.[13]

Another element in support of FAAH's value as a target for analgesic drugs is provided by the preclinical pharmacological profile of FAAH inhibitors. Several classes of such inhibitors, both reversible and irreversible, have been disclosed in the scientific and patent literature.[14] A few of the most investigated classes are exemplified in Figure 3.

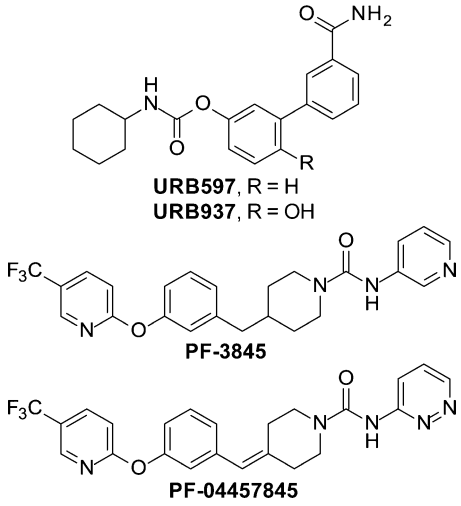

Figure 3. Chemical structures of representative FAAH inhibitors.

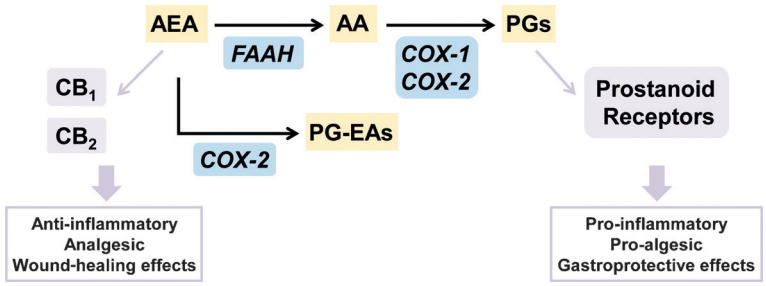

Figure 2. Simplified scheme of the endocannabinoid and prostanoid signaling pathways with some nodes of biological interactions.

The first selective and systemically active FAAH inhibitor to be disclosed, the *O*-arylcarbamate derivative URB597,[12c,15] exerts mild anti-hyperalgesic and anti-allodynic effects in animal models of acute and chronic pain.[12c,16] Interestingly, its peripherally restricted analogue, URB937, is markedly more effective in the same models.[17] This is consistent with the presence of a pro-algesic anandamide-dependent circuit in the dorsal spinal cord, which is potentiated by global FAAH blockade.[18]

The compound PF-3845 provides an example of a structurally different class of FAAH inhibitors, based on a urea scaffold, which effectively decreases nociceptive responses in a rat model of chronic inflammatory joint pain (complete Freund's adjuvant, CFA).[19] Improvements on PF-3845 yielded PF-04457845, which shows excellent pharmacokinetics and is also active in the rat CFA model.[20] PF-04457845 progressed into phase II clinical trials for the treatment of osteoarthritic pain, but was found to be ineffective.[21] While disappointing, this negative result is consistent with animal studies suggesting that FAAH inhibitors are only weakly active in pain conditions—such as osteoarthritic and neuropathic pain—that are characterized by profound neuroplastic changes in the central

nervous system.[22] Acute pain states, such as those occurring after surgery or during treatment with antineoplastic drugs, might provide better indications for these agents.[23]

In addition to anandamide, FAAH catalyzes the hydrolysis of PEA, OEA (Figure 1), and other bioactive FAEs.[24] Thus, FAAH inhibition might produce beneficial effects in models of pain and inflammation through the contribution of PEA- and OEA-mediated activation of PPAR-α.[13b,25] Moreover, other endogenous signaling molecules, such as *N*-acylglycines and *N*-acyltaurines, may also contribute.[26]

Anandamide not only modulates nociception, but can also attenuate neutrophil migration and immune-cell recruitment during inflammation, through the activation of CB_1 and CB_2 receptors.[27] This may be a factor in the ability of FAAH inhibitors to alleviate inflammatory symptoms in animal models of intestinal inflammation.[13c,28]

Innate immune and other host-defense cells produce a variety of pro-algesic and inflammatory eicosanoids through the action of the bifunctional membrane-bound enzyme, cyclooxygenase (COX) (Figure 2).[29] There are two COX isoforms, COX-1 and COX-2, which share ~60% sequence identity and are located on the luminal surfaces of the endoplasmic reticulum and the inner and outer membranes of the nuclear envelope. COX-1 is considered to be a constitutive enzyme and is expressed in most cells of the body. COX-2 is inducible in many tissues, but is also normally expressed in the brain, spinal cord, and kidney.[30] COX-2 induction occurs during tissue damage or inflammation in response to cytokines, mitogens, and growth factors.[31]

Both COX isoforms catalyze the first committed step in the pathway that converts membrane-derived AA into a large family of prostanoids that includes prostaglandin E_2 (PGE_2), prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) (Figure 4).[29] These signaling molecules play important roles in biology through the activation of selective G-protein-coupled receptors. Cardiovascular homeostasis, mucosal protection, inflammation, and cancer are among the diverse physiological and pathological processes modulated by these agents.[32]

Conventional NSAIDs such as ibuprofen, flurbiprofen, diclofenac, and indomethacin (Figure 5) are nonselective inhibitors of both COX isoforms.[33] Although they are widely prescribed to treat pain, inflammation, and fever, their chronic use can produce serious gastrointestinal (GI) and renal damage.[34] Inhibition of constitutive COX-1 activity in the gut mucosa is thought to underpin, at least in part, the toxic effects of NSAIDs on the GI tract.[34a] This is due to the fact that the tonic production of prostanoids (e.g., PGE_2) by epithelial cells in the GI mucosa protects these cells from injury, a property that is also shared by anandamide (Figure 6).[35]

To avoid COX-1-dependent GI toxicity, various COX-2-preferring inhibitors ('coxibs'; Figure 5) have been developed.[29] These compounds are highly effective at reducing pain and inflammation, but exert their own set of potentially severe ad-

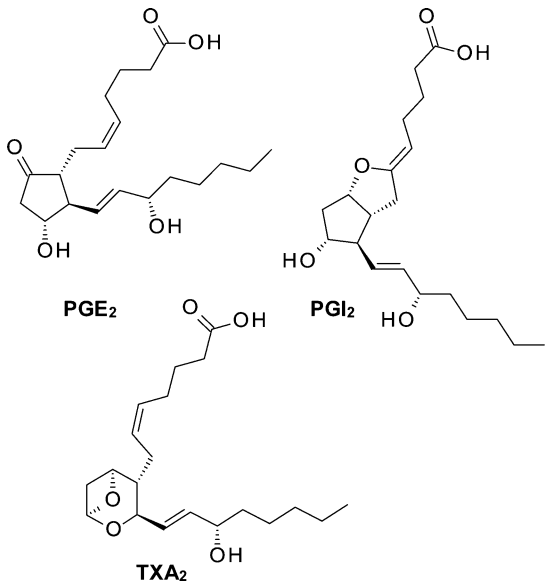

Figure 4. Chemical structures of representative prostanoids: prostaglandin E_2 (PGE_2), prostacyclin (PGI_2), and thromboxane (TXA_2).

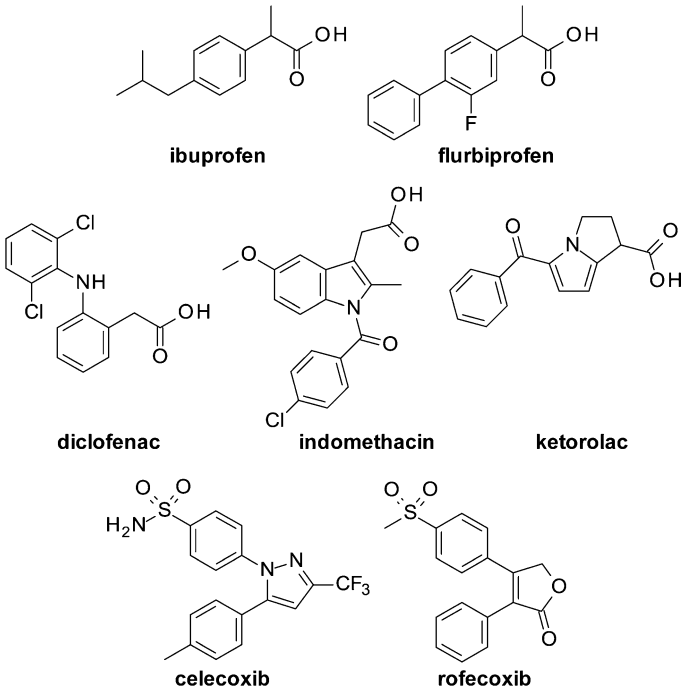

Figure 5. Chemical structures of representative COX inhibitors.

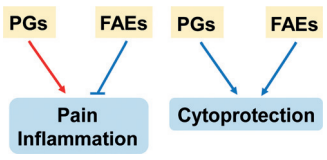

Figure 6. Prostanoids and FAEs, such as anandamide, exert antagonistic effects on pain and inflammation, but act synergistically to promote cytoprotection.

verse events, which are mostly localized to the renal and cardiovascular systems.[36]

COX-2, but not COX-1, converts anandamide into a family of biologically active prostaglandin ethanolamides ('prostamides').[37] These agents are pro-algesic and do not interact with cannabinoid or prostanoid receptors, but their mechanism of action is still under investigation.[38] Nevertheless, they represent an interesting point of intersection between endocannabinoid and prostanoid systems. The COX-2-mediated transformation of anandamide into pro-algesic prostamides has led to the hypothesis of the presence of deeper functional connections between the endocannabinoid and prostanoid systems. Confirming this idea, it was shown that genetic deletion or pharmacological blockade of CB_1 receptors prevents the antinociceptive effects produced by spinal administration of indomethacin in the mouse formalin test.[39] Similar results were obtained with another NSAID, flurbiprofen, given by intrathecal injection.[40]

Further supporting the existence of a tight link between endocannabinoid and prostanoid signaling, local co-administration of ibuprofen and anandamide in the rat hind paw was found to produce synergistic antinociceptive effects in the formalin test.[41] The CB_1 receptor antagonist AM251 prevented this synergy.[41] Another study in which anandamide was used along with the selective COX-2 inhibitor rofecoxib or the nonselective COX inhibitor ibuprofen showed that these combinations decrease mechanical and thermal hyperalgesia in the rat sciatic nerve ligation model.[42] In addition, co-administration of the NSAID ketorolac and the nonselective cannabinoid agonist WIN-555,212-2 exerted additive analgesic effects in the mouse acetic acid test of visceral pain.[43] Collectively, these findings strongly imply that the endocannabinoid and prostanoid systems interact at multiple levels to regulate nociception.

Additional evidence in support of this hypothesis comes from experiments in which various combinations of FAAH inhibitors and NSAIDs were tested in animal models. These experiments have consistently shown that simultaneous FAAH and COX blockade produces synergistic analgesic responses, along with a marked decrease in NSAID-induced GI damage. In one study, the FAAH inhibitor URB597 synergistically enhanced the effect of diclofenac in the acetic acid test.[44] In the same report, URB597 was also able to reduce gastric hemorrhages caused by the NSAID.[44] This protective effect was likely due to FAAH blockade, as it was reproduced by genetic FAAH deletion, and required CB_1 receptor activation, because it was absent in mice lacking CB_1, but not CB_2, receptors.[44] Like URB597, its peripherally restricted derivative URB937 decreased the number and severity of gastric lesions produced by indomethacin.[17b] In addition to this gastroprotective effect, URB937 synergistically enhanced the antinociceptive effects of indomethacin in mouse models of inflammatory (carrageenan) and neurogenic (sciatic nerve ligation) pain. Notably, the antinociceptive and gastroprotective effects of URB937 were superior to those of URB597.[44] Finally, co-administration of diclofenac and PF-3845 attenuated mechanical allodynia in models of inflammatory (carrageenan) and neurogenic pain (sciatic nerve ligation).[45] Blockade of either CB_1 or CB_2 receptors attenuated the effects produced by the combined administration of FAAH and COX inhibitors.

These findings have provided a rationale for the design of molecules that carry multitarget FAAH and COX inhibitory activities. Single molecules with such a profile are expected to have several advantages over combination therapies, including more a predictable pharmacokinetic profile and a decreased potential for drug–drug interactions.

Approaches to the Design of Multitarget FAAH/COX Inhibitors

From single to multiple target inhibitors

FAAH is a homodimer of 64 kDa subunits and belongs to the amidase signature family, which is characterized by an unusual Ser241-Ser217-Lys142 catalytic triad. The residues of the catalytic triad interact through a network of hydrogen bonds that facilitates proton exchange, activating the nucleophilic residue Ser241 (via deprotonation) and the leaving group of the substrate (via protonation). Adjacent to the nucleophilic serine side chain is a circle of four amide N–H bonds that form the oxyanion hole of FAAH. In addition to the catalytic core, FAAH is characterized by a series of channels and cavities, which include the membrane access channel (MAC) connecting the active site to an opening located at the membrane anchoring face of the enzyme, the cytosolic port (CP) that allows exit of hydrophilic products from the active site, and the acyl chain binding pocket (ABP): a narrower, longer, and more hydrophobic channel that interacts with the acyl chain of the substrate during the catalytic reaction.[14a,46]

COX-1 and COX-2 are homodimers of 70 and 72 kDa subunits, respectively. Dimerization is required for structural integrity and catalytic activity. Each subunit contains one cyclooxygenase and one peroxidase active site, which are located on opposite sides of the catalytic domain, with the heme prosthetic group positioned at the base of the peroxidase site.

Each monomer of COX consists of three structural domains: a short N-terminal epidermal growth factor domain, a membrane binding domain (MBD), and a large, globular C-terminal catalytic domain. The catalytic domain constitutes the majority of the COX monomer and is the site of substrate binding and NSAID action. The entrance to the COX active site occurs at the base of the MBD and leads to a long hydrophobic channel that extends deep into the interior of the catalytic domain to form a constriction composed of three residues (Arg120, Tyr355, and Glu524) that separate the entrance from the active site. Tyr385 is in the COX active site for both isoforms and is positioned at the end of the channel on the opposite side of Arg120. The peroxidase reaction converts Tyr385 into a tyrosyl radical species, which is necessary for activation of the COX reaction cycle. COX-1 and COX-2 active sites are very similar, but differ in the presence of a side pocket in COX-2 located above the Arg120/Tyr355/Glu524 region. This COX-2 side pocket contains Val523 (Ile523 in COX-1) and a conserved Arg513 (His513 in most COX-1) at the base of the pocket, and it has been pro-

posed to be essential for selective COX-2 binding recognition.[47]

Despite being evolutionarily unrelated, FAAH and COX have some commonalities, possibly linked to the structural closeness of their substrates, which might be exploited in the design of multitarget inhibitors. In addition, knowledge of the chemical structures of the many FAAH and COX inhibitors now available may be helpful in the rational design of potential binders with desired multitarget pharmacology and drug-like properties.[14a,29]

Various drug design approaches have been developed for the identification of multitarget inhibitors leading to hybrid, fused, or chimeric compounds.[48] The following section illustrates the most relevant strategies and representative examples reported in the scientific and patent literature for the identification of FAAH and COX inhibitors.

One strategy for the design of multitarget FAAH/COX inhibitors has been the identification of known single-target inhibitors (e.g., COX) that also show weak activity at the other target (e.g. FAAH), and are amenable to structural modifications aimed at enhancing activities on that target. Some NSAIDs directly bind to FAAH and inhibit its catalytic activity. In 1996, Paria et al. reported that indomethacin decreased anandamide hydrolysis in mouse uterus.[49] Subsequent in vitro studies showed that suprofen, ibuprofen, fenoprofen, naproxen, ketoprofen, diclofenac,[50] ketorolac, flurbiprofen,[51] and indomethacin[52] inhibit FAAH activity, albeit with weak potencies (median inhibitory concentrations (IC_{50} values) in the low to high micromolar range).[50a,53]

These observations stimulated interest in identifying NSAID-based compounds that also express FAAH inhibitory activity.[50a,51] In 2003, Cocco et al. described a series of heteroaromatic ibuprofen anilides bearing substituted pyridine or pyrimidine groups, which showed improved analgesic activity and reduced GI side effects relative to the parent acid.[54] This exploration identified compound **1** (ibu-am5) as the best analogue. This compound produced complete inhibition of acetic acid induced writhing after oral administration in rats, which was associated with very low ulcerogenic effects relative to its parent molecule, ibuprofen (Figure 7).

The reduced GI toxicity of **1** was initially attributed to the amidation of the free carboxylic acid group present in ibuprofen, and the consequent reduction of its local irritating action[55].Alternatively, weaker GI effects were ascribed to the potential inhibitory action of this set of amide derivatives on COX-2, as previously reported for ester and amide analogues of aryl acetic and fenamic acids.[56] Nevertheless, no additional data were reported in this study to support either of these hypotheses.

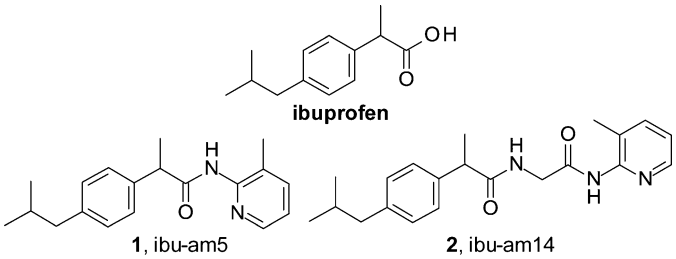

Figure 7. Ibuprofen and representative amide analogues, **1** and **2**.

A more complete biochemical evaluation of compound **1**, together with 13 additional heteroaromatic amides of ibuprofen and indomethacin, was reported[57] and later integrated with additional comparative data with novel analogues (Figure 7).[58] In the former report,[57] none of the six indomethacin amide analogues caused complete inhibition of FAAH activity at the concentration tested and, for this reason, were not further profiled in COX assays. Within the ibuprofen amide series, compound **1** was confirmed to be the most promising analogue, showing a more potent inhibitory activity against rat FAAH, but with a similar inhibitory profile against ovine COX-1 and COX-2 relative to ibuprofen. It was found that **1** inhibits FAAH activity in a noncompetitive manner with IC_{50} values of 4.7 and 2.5 μM at pH 6 and 8, respectively (for the FAAH assay conditions used, see ref. [59]). In comparison, the IC_{50} values of ibuprofen for FAAH were found to be 130 and 750 μM at pH 6 and 8, respectively. In intact C6 glioma cells, **1** inhibited FAAH with an IC_{50} value of 1.2 μM at pH 6 and 8 (for the FAAH assay conditions used, see ref. [60]). In addition, **1** showed a good selectivity profile against other hydrolases, such as *N*-acylethanolamine acid amidase and monoacylglycerol lipase. The effects of **1**, ibuprofen, and indomethacin on the specific binding of [^{3}H]CP55,940 to rat CB_1 and human CB_2 receptors were also investigated.[57] The compound displayed little or no ability to interact with cannabinoid receptors (IC_{50} = 41 and 24 μM for **1** toward CB_1 and CB_2, respectively).

In light of the overall in vitro pharmacological profile of **1**, a retrospective interpretation of its in vivo efficacy in the acetic acid model and its reduced ulcerogenic properties[54] was proposed. The lower GI toxicity of **1** relative to ibuprofen was ascribed more to differences in the physicochemical properties of the two compounds, rather than to their inhibitory potencies toward COX-1. On the other hand, the different analgesic effect between the two molecules was linked to the ability of **1** to inhibit both COX and FAAH.

In a subsequent study,[58] **1** was compared with ibuprofen and eight additional amide analogues of **1**. Compounds **1** and **2** were the most potent inhibitors of rat FAAH relative to ibuprofen (IC_{50} = 134 μM), showing IC_{50} values of 0.65 and 3.6 μM, respectively (Figure 7). Different from previous reports from the same group,[57] COX activity was measured using an oxygen electrode assay with commercially available ovine COX-1 and human recombinant COX-2 as enzyme sources (for the COX assay conditions used, see ref. [61]). Under those assay conditions, ibuprofen inhibited the activity of ovine COX-1 toward AA with an IC_{50} value of ~29 μM (using ethanol as a vehicle) and ~77 μM (using DMSO as a vehicle). Compound **1** was less potent than ibuprofen at inhibiting COX-1, with an IC_{50} value of ~60 μM (ethanol) and ~240 μM (DMSO). Compound **2** inhibited COX-1 with an IC_{50} value of ~50 μM (ethanol). Ibuprofen, **1**, and **2** also showed substrate-selective inhibition of COX-2, being poor inhibitors of the cyclooxygenation of AA by COX-2 and producing 36, 41, and 18% inhibition at

the highest concentrations tested (300, 300, and 100 μM, respectively). However, when anandamide was used as substrate, these compounds were relatively potent inhibitors of COX-2, with IC_{50} values of ~6 (ibuprofen), ~19 (**1**) and ~10 μM (**2**).

In addition, **1** was confirmed to be active at the dose of 30 mg kg^{-1} (subcutaneous) in a murine model of visceral nociception, but this effect was not blocked by either CB_1 or CB_2 receptor antagonists. Two possible explanations were proposed: 1) the contribution of analgesia due to the COX inhibitory properties of **1** dominates at the dose and in the model used, or 2) compound **1** is metabolized in vivo to a species that retains (or even improves) its COX inhibitory properties, but loses its FAAH inhibitory activity. The authors excluded ibuprofen being the metabolite, considering that in rats at the lower dose of 20 mg kg^{-1} (intraperitoneal) ibuprofen is significantly less effective than **1** in the acetic acid test.[54]

A small series of racemic flurbiprofen and naproxen amide derivatives were also investigated (Figure 8).[62] Within this limited set, **3** (flu-am1) and **4** (nap-am1) demonstrated increased inhibitory potency for FAAH, relative to the parent NSAIDs (flurbiprofen and **3**, FAAH IC_{50} values of 29 and 0.44 μM, respectively; naproxen and **4**, FAAH IC_{50} values of >100 and 0.74 μM, respectively) (for FAAH assay conditions, see ref. [59]).

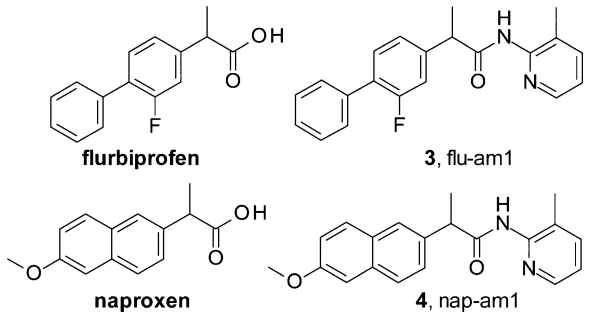

Figure 8. Flurbiprofen, naproxen, and their amide analogues, compounds **3** and **4**.

Compounds **3** and **4** were also investigated for their ability to inhibit COX-1 and COX-2, using the oxygen electrode assay and two different substrates: AA (for both isoforms) and 2-AG (for COX-2) (for COX assay conditions, see ref. [61]). The study confirmed that flurbiprofen inhibited the cyclooxygenation of AA catalyzed by ovine COX-1 (IC_{50} = 3.6 μM) and had little effect on the cyclooxygenation of AA catalyzed by human recombinant COX-2 (IC_{50} = 103 μM). Flurbiprofen also inhibited the cyclooxygenation of 2-AG catalyzed by human recombinant COX-2 (IC_{50} = 1.3 μM). A similar pattern of COX-1 and COX-2 inhibition was observed with **3**, although in general 2–3-fold higher concentrations were required to produce the same effect as flurbiprofen [COX-1 (AA) IC_{50} = 6.6 μM; COX-2 (AA) IC_{50} = 42 μM and COX-2 (2-AG) IC_{50} = 1.7 μM, respectively]. By contrast, **4** was a rather poor inhibitor of both COX isoforms [COX-1 (AA) IC_{50} = 56 μM; COX-2 (AA) IC_{50} >100 μM and COX-2 (2-AG) IC_{50} = 6.3 μM, respectively].

Compound **3**, which reversibly inhibits FAAH, was further investigated in vivo for its ability to inhibit the enzyme in the brain. The compound was administered by intraperitoneal injection in rats 30 min prior to the administration of [^{18}F]3-(4,5-dihydrooxazol-2-yl)phenyl (5-fluoropentyl)carbamate (^{18}F-DOPP), an irreversible and brain-penetrant ^{18}F-labeled FAAH inhibitor.[63] It was postulated that pretreatment with **3** would prevent the interaction of ^{18}F-DOPP with FAAH.[64] However, administration of **3** at two different dosages had no effect on brain labeling with ^{18}F-DOPP, which is suggestive of limited penetration.

Using a computational strategy,[65] Favia et al. identified the 2-arylpropionic acid, carprofen, as a multitarget FAAH, COX-1, and COX-2 inhibitor[66] (Figure 9). The compound was shown to inhibit rat FAAH with IC_{50} = 79 μM, ovine COX-1 with IC_{50} = 22 μM, and human COX-2 with IC_{50} = 4 μM (for assay conditions, see refs. [12c, 66]). Medicinal chemistry efforts focused on a diversified SAR exploration around different regions of the carprofen scaffold were also reported in that study. In particular, the functionalization at the nitrogen atom of the carbazole ring of carprofen led to the identification of novel analogues, compounds **5** and **6** (Figure 9), which showed an improved potency profile for both FAAH and COX (IC_{50} [μM]: compound **5**: FAAH = 22, COX-1 = 74, COX-2 = 72; compound **6**: FAAH = 85, ovine COX-1 = 30, human COX-2 = 28). In vitro profile studies showed that only the *S* enantiomer of carprofen retained multitarget FAAH and COX inhibition, whereas the enantiomers of **5** and **6** lost activity for one of the two targets.

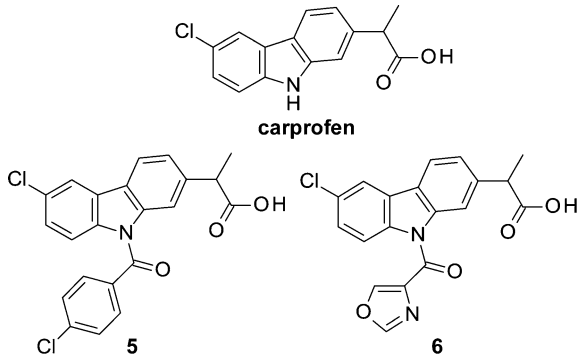

Figure 9. Carprofen and some representative N-functionalized analogues, **5** and **6**.

Docking analysis of the putative binding modes of carprofen on FAAH and COX-2 were also reported.[66] In COX-2, it was proposed that carprofen may preferentially interact, via hydrogen bonds formed by its carboxylic group, with Arg120 and Tyr238, while the carbazole scaffold may lie deep in the pocket, facing Tyr385. This putative binding mode is consistent with the experimentally observed modes of interaction of several NSAIDs in complex with either COX-1 or COX-2. In FAAH, carprofen was shown to fit the acyl chain binding channel and establish hydrogen bond interactions with the oxyanion hole through its carboxylic group, although other binding modes could not be excluded. In addition, accurate computational analysis of the structural similarities between FAAH and COX-2 were also reported, mapping hydrophobic (i.e., acyl chain

binding channel in FAAH; catalytic binding site in COX-2) and hydrophilic (i.e., oxyanion hole and cytosolic channel in FAAH; exit of catalytic binding site in COX-2) portions of the two enzymes, by the use of two known probes in complex with FAAH (methyl arachidonyl fluorophosphonate) and COX-2 (AA).

In 2013, Bertolacci et al. solved the X-ray crystal structure of rat FAAH in complex with carprofen at 2.25 Å resolution.[67] This was the first structural determination of the molecular interactions of an NSAID in complex with FAAH. Diffraction-quality crystals of the FAAH–carprofen complex were obtained by pre-incubating the enzyme with the *O*-arylcarbamate inhibitor, URB597.[15] The molecular details of the interactions were investigated using a combination of site-directed mutagenesis, WaterLOGSY and FABS NMR spectroscopy, with the goal of providing structural insight that might be useful to design novel FAAH/COX inhibitors.

A number of dual FAAH/COX inhibitors (mainly COX-2 inhibitors) with indomethacin-like structures are described in patent applications from Microbia Inc. (changed to Ironwood Pharmaceuticals on April 7, 2008). These compounds are exemplified by **7** and **8** (Figure 10).[68] However, a systematic SAR analysis for this class of molecules has not been reported.

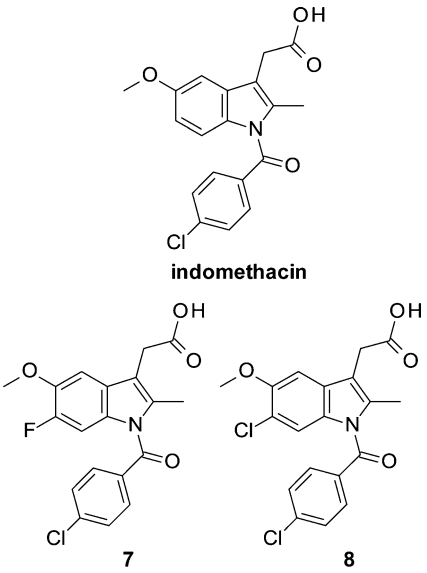

Figure 10. Representative dual FAAH/COX inhibitors, **7** and **8**, from Microbia Inc.

Rational drug design: merging strategy

An effective approach to the design of multitarget inhibitors is to exploit the presence of structural similarities present in selective binders.[48] Compounds with overlapping or integrated pharmacophores are likely to have lower molecular weights and potentially more drug-like physicochemical properties. The rational design of compound **9**, designed using this strategy, was recently reported.[69] This agent combines structural commonalities and pharmacophore groups of two classes of known FAAH and COX inhibitors—*O*-aryl carbamate FAAH inhibitors such as URB597,[15] and 2-aryl propionic acid NSAIDs such as flurbiprofen[47c,70]—which share a biphenyl core as common structural motif (Figure 11). Moreover, the previously reported SAR surrounding these scaffolds support the hypothesis of additional elements of structural overlapping, such as the oxygenated substituents at the 3′ position of the A phenyl ring, corresponding to the carbamate functionality of URB597[15,71] and the ether moieties of flurbiprofen analogues **10a** or **10b**, respectively.[70b]

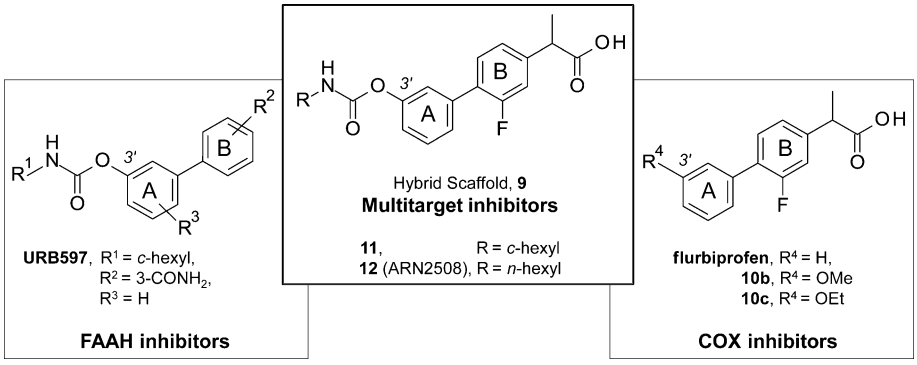

Figure 11. Rational design of the 'hybrid scaffold' **9** and initial SAR results.

An initial SAR exploration of the hybrid scaffold **9** around the alkyl substituent of the carbamate group, starting from the hit **11** (IC_{50} [μM]: FAAH = 8.2, COX-1 = 7.9, COX-2 > 100) led to the identification of compound **12** (ARN2508) as the first highly potent and selective multitarget FAAH/COX inhibitor (IC_{50}: FAAH = 31 nM, COX-1 = 12 nM, COX-2 = 430 nM). The pharmacological activity of this compound was evaluated in mouse models of inflammatory bowel disease, in which it is known that both FAAH and COX-2 are expressed at abnormally high levels.[72] After oral administration, **12** fully engaged its intended targets and elicited a profound anti-inflammatory response, without any overt signs of gastric toxicity. Pharmacological and genetic experiments provided evidence that such response was caused by dual FAAH/COX inhibition. The results suggest that multitarget FAAH/COX inhibitors such as **12** might achieve marked anti-inflammatory efficacy while protecting the GI tract from NSAID-induced damage.[69] The 'double whammy' these compounds produce by blocking both FAAH and COX activities might interrupt the pathological loop established in inflamed tissues by the concomitant upregulation of these enzymes (Figure 12).

Future Perspectives

Where do we go from here? It is reasonable to expect that multitarget ligands such as ARN2508 (**12**) will continue to be useful in probing the functional interactions between FAAH

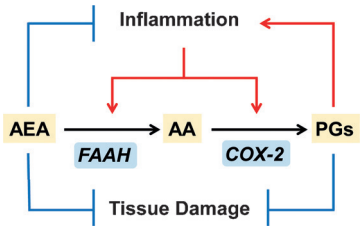

Figure 12. Concomitant upregulation of FAAH and COX may establish a pathological loop that exacerbates or prolongs inflammation by amplifying inflammatory COX-dependent signals at the expense of defensive anandamide (AEA)-dependent responses. This hypothesis predicts that drugs targeting both FAAH and COX should have substantial anti-inflammatory efficacy combined with decreased GI toxicity.

and COX, and as a means to explore the potential therapeutic value of FAAH/COX blockade. In addition to inflammatory bowel disease, where ARN2508 appears to be more effective than standards of therapy and single-target FAAH inhibitors,[69] multitarget FAAH/COX agents might prove valuable in other chronic inflammatory conditions in which FAAH and COX-2 (but also COX-1) are expressed at pathologically high levels. These include a variety of peripheral and central inflammatory states as well as certain forms of cancer.[23,73] The wide potential applicability of these agents provides a great therapeutic opportunity, but also poses interesting challenges to medicinal chemistry. In addition to balanced potency for FAAH and COX, future molecules should be able to engage these proteins in all compartments of the body, including the central nervous system, and to selectively target FAAH/COX-1 versus FAAH/COX-2.

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Keywords: drug design · enzymes · inflammation · multitarget inhibitors · pain · structure–activity relationships

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