TRPV1-FAAH-COX: The Couples Game in Pain Treatment

TRPV1-FAAH-COX in Pain Treatment: One for All and All for One

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Abstract: Pain is a complex sensation involving the perception and transduction of diverse environmental pain stimuli, and also cognitive and emotional processed by Central Nervous System (CNS). It can manifest as acute or chronic pain. Pain is controlled by a series of enzymes and receptors, implicated in a variety of interconnected mechanisms and pathways. In fact, several studies showed the Cannabinoid receptor 1 (CB1) and the Transient Receptor Potential Vanilloid Channel 1 (TRPV1) as new players in modulating the sophisticated pain transduction system, at the central level. At the peripheral one, the perception of pain involves the Cyclooxygenases (COXs) and the Fatty Acid Amide Hydrolase (FAAH), as recent studies demonstrate This minireview describes physiological aspects of receptors and enzymes mentioned above and focus on the consideration of dual mechanisms as new therapeutic approach in the treatment of pain.

1. Pain: enzymes and receptors

Pain is an unfriendly feeling that involves central and peripheral stimuli processed by Central Nervous System (CNS).[1] Pain is mainly classified as acute or chronic. Acute pain is due to a definite cause or trauma, often involving an inflammatory process, and it results in a short time with a proper drug treatment. Conversely, chronic pain does not have a determined origin, and in turn can be nociceptive (painful stimuli and tissue damages) and neuropathic (neuronal injury) pain, commonly associated with phenomena such as allodynia and hyperalgesia.[2] Directors of this scenario are Transient Receptor Potential Vanilloid Channel 1 (TRPV1) and Cannabinoid subtype 1 (CB1) receptors at the central level, while peripherally, Cyclooxygenase (COX) and Fatty Acid Amide Hydrolase (FAAH) enzymes, both acting on arachidonic-type molecules, are responsible for the consciousness of pain.

1.1 Transient Receptor Potential Vanilloid Channel 1 (TRPV1)

TRPV1 (Transient Receptor Potential Vanilloid subtype 1) receptor is a non-selective cation channel, mainly distributed in the peripheral and central terminals of sensory neurons. It is involved in both afferent (feeling pain) and efferent (neurotransmitters and neuro-peptides release) functions. In the peripheral nerve endings, TRPV1 can initiate a nociceptive signaling, thereby generating an action potential and increasing the membrane permeability to some cations, including Ca^{2+}. Its activation threshold is significantly lowered during inflammatory conditions resulting in sensitization of the receptor. In neuropathic pain, the TRPV1 expression increases; the retrograde transport of Nerve Growth Factor (NGF), released at the site of tissue injury, results in the activation of p38, a mitogen-activated protein kinase (MAPK). The thermal hypersensitivity, following the tissue damage, suggests the increased transduction and transport of TRPV1 protein, selectively to the peripheral terminals of sensory neurons. Instead, the PCK-mediated phosphorylation of TRPV1, expressed on the peripheral terminals, activates the receptors at the body temperature and leads an increased glutamatergic transmission. Capsaicin, the pungent active component of *Capsicum* specie fruits, is the major agonist of TRPV1 receptors, and shows an interesting analgesic activity, due to the desensitization of whole terminal nerve. Its therapeutic use suffers of evident limitations such as the irritating side effects. To overcome such aspect, an intense research activity is addressed to synthesize analogues less pungent. In addition, the discovery that TRPV1 knock-out mice are less sensitive to certain models of pain suggests TRPV1 receptors as promising pain relief drug target and molecules with both TRPV1 agonist and antagonist activity as new analgesic drugs.[3]

1.2 Cannabinoid Receptor 1 (CB1)

Identified in the 1990s, CB1 is one of the two main GPCR-coupled receptors responsible for the biological actions of the phytochemical THC (tetrahydrocannabinol). This psychoactive component of *Cannabis* was used for thousands years for the treatment of spasms and pain. Arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) are the two major endocannabinoids (ECs) binding both CB1 and CB2 receptors. Lately, further studies demonstrated that AEA can also activates TRPV1 receptors, although it has markedly less relative intrinsic activity at these receptors than capsaicin.[4] CB1 receptors are abundant on presynaptic nerve terminals and on peripheral sympathetic nerve terminals where they reduce neurotransmitter release and modulate adrenergic signaling, thus affecting the sensation of pain and supporting inflammation. In the nociceptive nerve fibers, CB1 and TRPV1 receptors are often co-expressed, becoming two interacting signalling systems in many physio/pathological conditions.[5]

1.3 Fatty Acid Amide Hydrolase (FAAH)

Fatty acid amide hydrolase is an enzyme of serine hydrolase family responsible for the degradation of anandamide, as well as of other acyl-ethanolamines, to arachidonic acid. It is constituted by the unusual catalytic triad Ser217 – Lys142 – Ser241. The catalytic cycle involves first the formation of an acyl intermediate, which is then hydrolysed, restoring the enzymatic function. The crystal structure of FAAH reveals the presence of three essential elements: the membrane access channel (MAC), the cytosolic access channel, and the acyl chain-binding pocket (ABP), important for its biological activity.[6] The catalytic triad was also able to hydrolyze the *N*-oleolylethanolamine and oleoylmethyl ester substrates, and this was evaluated by combined quantum mechanics/molecular mechanics (QM/MM) studies.[7]

1.4 Cyclooxygenases (COXs)

The cyclooxygenases (COXs) are oxidoreductases responsible for the conversion of arachidonic acid to prostaglandins and thromboxanes. The mechanism involves the formation of radicals: in the first step, during the oxidation reaction, hydroperoxy endoperoxide PGG_2 is formed and then reduced to the alcohol PGH_2; which is lastly converted to prostaglandins and thromboxane by isomerases. Thromboxane is responsible for platelet aggregation while prostaglandins are essential to the functioning of kidneys, stomach, intestines. Moreover, they are also involved in inflammation and pain.[8]
There are two isozymes of COXs: COX-1 and COX-2. Cyclooxygenase-1 is a constitutive enzyme and it is a housekeeper molecule, while Cyclooxygenase-2 is usually defined an inducible enzyme, which expression increases during inflammatory states.[9]
AEA and 2-AG are converted by COX in prostanoid-like derivatives.[10] While both ECs are poor substrates of COX-1, they are quickly transformed by COX-2 in oxygenate biologically active molecules not acting at conventional CB and prostanoid receptors.[11] During inflammatory states, this oxygenation process can lower ECs levels whereas its inhibition can contribute to increase the endocannabinoid tone.

2. To treat pain is just one way?

TRPV1 agonists, like capsaicin and the other vanilloids, can be used rationally for the treatment of the pain. They get excited and under constant activation desensitize the whole nociceptive neuron. This is due to the subsequent increase of intracellular Ca^{2+} levels and activation of calcineurin. This enzyme dephosphorylates TRPV1 and other proteins involved in the nociceptive stimulus transmission, including VAACs (Voltage-Activated Ca^{2+} Channels). The dephosphorylated TRPV1 is less sensitive to noxious stimuli and, simultaneously, the electrical activity of the nerve is reduced. This action strongly depends on the agonist concentration and its time of exposure. Therefore, this lasting refractory state is not only a feedback mechanism to protect the cell to a toxic overload of Ca^{2+}, but also contributes to observed analgesic effects.[12]
On the other hand, the treatment of neuropathic pain with intrathecal administration of URB597, a FAAH inhibitor, can increase the levels of AEA. AEA reduces neuropathic pain with a mechanism involving both CB1 and TRPV1. In rats the dose of 200 µg is able to increase the levels of PEA (*N*-palmitoylethanolamine), OEA and 2-AG and fully inhibits thermal and tactile nociception in a manner blocked uniquely by a TRPV1 antagonist. The complete inhibition of FAAH is a useful tool to unmask different metabolic pathways for AEA. Formation of 15-hydroxy-AEA, together with OEA and PEA, may contribute at producing TRPV1-mediated analgesia in rats with chronic constriction injury.[13]

The role of TRPV1 in neuropathic pain is highlighted by the fact that in patients with herpes zooster lesions there is a strong presence of this receptor, demonstrating that TRPV1 agonists/antagonists may reduce pain.[14] Other studies instead show as TRPV1 is involved in the genesis of neuropathic pain through its sensitization. In fact, the activation of different factors such as P38-MAPK, Camk2, PKA and PKC, modulates the phosphorylation state of the receptor, while the increase of proinflammatory cytokines, such as TNF-α, IL-1β and IL-6 lowers temperature threshold.[15] Also other molecules, not necessarily selective ligands for the TRPV1, can modulate its activity showing analgesic effect, as in the case of the sodium hyaluronate, which reduces the pain probably through its binding with the channel protein. In this context, TRPA1 and TRPM8 are not affected by sodium hyaluronate.[16]
TRPV1 receptor is involved in the analgesic mechanism of the acetaminophen, because its metabolite *N*-arachidonoylaminophenol (AM404) is a TRPV1 ligand endowed with agonist profile and, centrally, contributes to the antinociceptive effect. This also occurs with substances such as 4-aminophenol and 4-hydroxy-3-methoxybenzylamine (HMBA) which can be converted by FAAH, furnishing AM404 and other two powerful TRPV1 activators, i.e. arvanil and olvanil. Indeed, in FAAH null mice the antinociceptive effect of these metabolites is absent. Similarly to paracetamol, FAAH inhibition, TRPV1/ CB1 and spinal serotoninergic receptor blockade prevent the antinociceptive action of 4-aminophenol.[17]

The functional promiscuity of these two systems, namely endocannabinoid and endovanilloid, also reflects some chemical similarity among the numerous ligands of their receptors and enzymes.[18] In the literature, there are several examples of new molecules selectively designed for one of the two aforementioned targets and discovered able to interact also with the other.
One of these studies, recently, reports that a series of alkyl-resorcinol derivatives are good ligands of cannabinoid receptors. The most potent compound is the *N*-allyl-8-[3-hydroxy-5-(2-methyloctan-2-yl)phenoxy]octanamide, 11, showed nanomolar affinity values at both CB receptors (K_i CB1 7.22 nM and K_i CB2 7.36 nM). However, this compound caused a moderate reduction of the second phase of nociceptive behaviour only at the highest doses used (3 and 4 mg/Kg, i.p.) and showed a typical capsaicin-mediated abdominal writhing. Actually, compound 11 was found to interact with TRP channels, behaving a modest agonist at TRPV1 but an agonist as potent as allyl isothiocyanate at TRPA1.[19]

A different approach to pain management is represented by a novel ligand with analgesic properties, the 6-methyl-3-(2-nitro-1-(thiophen-2-yl)ethyl)-2-phenyl-1*H*-indole (ZCZ011), a positive allosteric modulator (PAM) of CB1 receptors, which is more potent than the CB1 orthosteric agonist, CP55,940. As proof of this, it

enhanced CB1 receptor-mediated antinociceptive effects in the chronic constriction nerve injury model (CCI) of neuropathic pain and in the carrageenan model of inflammatory pain. [20]
In numerous studies is reported that the activation of both CB1 and CB2 receptors by several FAAH inhibitors, reduced the nociceptive processing in animal models of neuropathic pain.[21,22]

The role of endocannabinoids has been also evaluated in a rheumatoid arthritis experimental model, where acyl-amides related to AEA, i.e. OEA and PEA, can reduce the inflammation event through TRPV1 desensitization. Although their own are not able to moderate hyperalgesia and inflammation induced by cytokines and matrix metallopeptidase 3 (MMP-3), PEA and OEA can efficiently potentiate the action of nimesulide (COX-2 inhibitor), resulting in a decreased production of the proinflammatory mediators.[23]

It is a fact that direct-acting cannabinoid receptor agonists, as endocannabinoids, as well as inhibitors of Fatty Acid Amide Hydrolase show a detectable analgesic effect in models of acute and inflammatory pain. In a very interesting study, Naidu and co-workers have investigated how the inhibition of FAAH enzyme is able to produce antinociception in a model of visceral nociception (acid abdominal stretching induced by acetic acid). The involvement of fatty acid amides emerged clearly in experiments carried out with transgenic FAAH (-/-) mice. These mice displayed the antinociceptive phenotype because express large amounts of fatty acid amides as PEA and *N*-acyl taurines in the brain. In addition, anandamide levels are improved, contributing to the described antihyperalgesic phenotype. Nevertheless, AEA is the only FAAH substrate that binds directly cannabinoid receptors. The study highlights how only CB1 receptors mediate the reduction of pain behavior in FAAH (-/-) mice because Rimonabant, a CB1 inverse agonist, but not SR144528, a CB2 antagonist, is able to block the antihyperalgesic phenotype of FAAH (-/-) or FAAH-inhibitor treated mice. While Chang and co-workers, in a previous study, reported that Naloxone was capable to block the pain-relieving effects of OL-135, a reversible FAAH inhibitor, Naidu and co-workers reported that opioid receptors are not involved in the antinociceptive effects showed by two blockers of FAAH enzyme (URB597 and OL-135). Moreover, the co-administration of URB597 and diclofenac (NSAID) produced synergic analgesic effect in the acetic acid abdominal stretching assay. This due to in part blocking the production of prostaglandins and activating CB1-mediated pathways, in part involving other mechanisms.[24] The synergic activity of FAAH and COX inhibitors has been confirmed also by Grim and collaborators in models of neuropathic (CCI) and inflammatory (intraplantar carrageenan) pain. Co-administration of subthreshold doses of a highly selective FAAH inhibitor, PF-3845, and Diclofenac sodium reduced pain behavior in both models. The heightened levels of anandamide, but not of 2-AG, cause an enhanced anti-allodynic effect through both CB1 and CB2 activation while increased levels of PEA and OEA, other FAAH substrates, may contribute through non-cannabinoid receptor pathways. Instead, diclofenac is not able to bind cannabinoid receptors and its anti-allodynic action is exclusively due to the inhibition of prostaglandin synthesis.[25]

3. The future is dual

Treatment of many features of pain is still an unreached goal because common analgesic options such as opioids, non-opioid drugs, NSAIDs, acetaminophen and other types of drugs (anticonvulsants and antidepressants) are not completely effective and show frequently serious side effects. Despite their positive properties, the chronic use of cannabinoids is usually associated with psychoactive consequences. As mentioned above, medicinal chemists are spurred to investigate about innovative strategies for pain management. In the literature, there are many examples of new potential analgesic molecules, which can selectively interact with known or new targets. Most of them are COX-inhibitors[26] or TRPV1 antagonists[27] or even FAAH inhibitors.[28] Nevertheless, the usual approach “one target-one drug” seems to be exceeded, opening the way to the concept that molecules interacting with more than one target, possibly relevant for the same disease, may show higher therapeutic effects and a safer biological profile.[29]

3.1 Dual TRPV1-FAAH blockers

Inhibition of FAAH, increasing the endocannabinoid tone through higher levels of AEA and analogous lipid modulators, produces analgesic effects without causing the typical side symptoms related to a direct CB activation.
However, compound PF-04457845, the first selective FAAH inhibitor that reached the phase II clinical trial, lacked of efficacy in patients affecting by osteoarthritis pain.[30] One of the possible explanation, in a very complex context, is that AEA, activating also TRPV1 receptors, may cause a concomitant pro-nociceptive signaling. The idea of synthesizing a dual inhibitor of FAAH-TRPV1 systems arises from the possibility of counteract this second behavior. Interestingly, an endogenous lipid amide, the *N*-arachidonoyl-5-hydroxytryptamine (AA-5-HT), inhibits FAAH enzyme by increasing the cannabinergic tone without enhancing TRPV1-mediated effects. Indeed, Maione and co-workers have shown that AA-5-HT behaves also as an antagonist at TRPV1 receptor (IC_{50} 37-40 nM against 100 nM of capsaicin), its action being reversed by either capsazepin or I-RTX (5’-iodo-resiniferatoxin). However, like other FAAH inhibitors, AA-5-HT appears to be less effective in antagonizing TRPV1 receptors when tissue pH is reduced, suggesting its lower effectiveness during chronic inflammatory conditions. In the same study, AA-5-HT caused anti-hyperalgesic effects *in vivo* depending on local or systemic administration and animal species used.[31]
Anyhow, the efficacy of this compound is greater than selective FAAH or TRPV1 inhibitors, so it is right to think that this molecule may represent a prototype of a dual inhibitor FAAH-TRPV1.

Rose and collaborators designed new serotonin-amide derivatives replacing arachidonic acid with various NSAID-scaffold acids. Among synthetized derivatives, fenoprofen-5-HT and naproxen-5-HT are TRPV1 blockers and inhibitors of COX-2 as potent as AA-5-HT, but they do not inhibit FAAH, even to the highest concentration (50 µM). Conversely, two 2-arylproponic acid derivatives ibuprofen-5-HT, and flurbiprofen-5-HT seem endowed with inhibitory activity to all three enzymes (for IC_{50} values see table 1).[32]

In the field of natural compounds, some products structurally similar to serotonin but conformationally restricted, showed vanilloid activity. Evodiamine[33] and rutaecarpine[34] for example, are TRPV1 agonists, although with a potency lower than

capsaicin. Both of them, relax vascular smooth muscle determining a marked antihypertensive effect and this action seems to involve also TRPV1 receptors. Besides, fatty acid amides of endogenous tetrahydroisoquinolines such as salsolinol and isosalsoline, conceptually related to TRPV1 antagonist capsazepine, showed a weak partial agonism at capsaicin receptor.[35] The previously mentioned molecules hold within their structure the pharmacophoric features to interact with TRPV1 receptors and they could represent excellent scaffolds for the synthesis of new dual FAAH-TRPV1 ligands. Keeping in mind this concept, Ortar and co-workers designed and synthesized a series of new tetrahydro-β-carboline amides, ureas and carbamates evaluating their ability to interact with FAAH and both TRPV1 and TRPA1 channels. While amides were the less interesting compounds, being inactive in FAAH inhibition and showing modest effect on both TRP receptors, among carbamates emerged some attractive compounds, such as 5a, 5b, 5c**,** and 5e, and 9b (see Table 1). All targeted compounds interact with the enzyme and both TRP channels. Compounds 5e and 9b, that bearing a biphenyl substituent in their structure, resulted as the most potent FAAH inhibitors and between them removal of 6-OH phenolic group (9b) caused a six fold increase in potency against the enzyme and both TRP receptors. Some urea derivatives, instead, are good TRPV1 ligands (submicromolar EC_{50} and IC_{50} values) endowing with an agonist profile, but do not interact with FAAH.[36]

Changing the nature of the linker between the two moieties of the molecule (i.e. amide vs urea vs carbamate group) and modulating lipophilic properties are well documented strategies to obtain hybrid TRPV1/FAAH blockers. Following this approach, Morera and co-workers designed a series of piperazinyl carbamates and ureas, potentially acting on FAAH and TRPV1. Beside some derivatives showed a good inhibitory activity against FAAH (submicromolar IC_{50} values) but completely ineffective at TRP receptors, the best carbamates with dual activity were compound 4, (4-(trifluoromethyl)phenyl4-(3-chloropyridin-2-yl)piperazine-1-carboxylate), compound 10, (3-(trifluoromethyl)phenyl4-(3-(trifluoromethyl)pyridin-2-yl)piperazine-1-carboxylate), and compound 12, (3-chlorophenyl 4-(3-(trifluoromethyl)pyridin-2-yl)piperazine-1-carboxylate) (see table 1).[37]

Other piperazinyl carbamates acting as TRP-FAAH modulators, named OMDM-198 and OMDM-202, are identified and tested *in vivo* for their analgesic properties. The two compounds inhibit the second phase of formalin-induced nocifensive behavior in mice. Furthermore, OMDM-198 inhibits carrageenan-induced paw thermal hyperalgesia and oedema in mice. Interestingly, the functional profile of the two derivatives is different; in fact, while OMDM-198 is a FAAH inhibitor and a TRPV1 blocker, OMDM-202 is about 10-fold more potent in inhibiting FAAH but it does not antagonize TRPV1 receptors. Its analgesic effect is rather due to the simultaneous activation/desensitization of TRPA1 channel. [38] Antihyperalgesic effects of OMDM-198 were also evaluated in a rat model of osteoarthritis. Its efficacy was found similar to those of the FAAH selective inhibitor URB-597 and the TRPV1 antagonist SB366,791. Analgesic activity of OMDM-198 was attenuated by a non-pungent TRPV1 agonist as palvanil but also by the selective CB1 antagonist AM-251 (*per se* inactive dose),[39] confirming the increase of endocannabinoid levels and the indirect-activation of their receptors.

Dual TRPV1-FAAH inhibitors have been recently obtained by Morera and co-workers combining a boronic acid group, as FAAH blocking moiety, with the pharmacophore backbone of several known TRPV1 antagonists.[40] A quite large family of benzyl/aryl amides, reverse-amide analogues and urea derivatives has been synthesized. Among most interesting compounds, four derivatives act as true TRPV1 antagonists and as good FAAH blockers paving the way for a new promising strategy.

3.2 Dual COX-FAAH inhibitors

Experimental data highlight as tandem COX and FAAH inhibition produces greater reduction of painful sensation in mechanical allodynia in the CCI and carrageenan pain models. Accordingly, a growing attention is directed toward investigating the dual FAAH-COX analgesic mechanism and identifying new combined blockers endowed with lower side gastric effects.

Trough X-ray crystallography studies on fatty acid amide hydrolase (FAAH) complexed with the NSAID carprofen emerge clearly as the compound interacts with the enzyme: in fact, carprofen, chemically the 2-(6-chloro-9*H*-carbazol-2-yl)propanoic acid, occupies a pocket at the entrance of the membrane channel of FAAH with the carboxylate moiety partially at the exterior of the enzyme. Additionaly, known FAAH inhibitors usually occupy the core of binding cavity.[41] Just a year earlier, Favia and co-workers have carried out a study of molecular docking of many NSAIDs to find the best FAAH ligand. Among these, carprofen was chosen as starting scaffold to develop new derivatives because *in vitro* pharmacological experiments evidenced that carprofen inhibits FAAH (IC_{50} 78.6±19.7 μM), COX-1 (IC_{50} 22.3±6.6 μM) and COX-2 (IC_{50} 3.9±1.0 μM). Structure-activity relationships highlighted how removing the chlorine atom from the carbazole nucleus or converting the acid functionality in the corresponding ester/amide dramatically reduced the inhibitory COX activity. Instead, the functionalization of the carbazole –NH- group furnished inhibitors of FAAH and COX-1/2 with different IC_{50} values. In particular, the best dual ligand appeared to be the compound 15c, bearing a 4-chlorobenzoyl group and showing satisfactory IC_{50} values against all three enzymes. Using the compound 15c as a model by additional SAR studies, other derivatives were further synthesized identifying the compound 15i (2-(6-chloro-9-(oxazole-4-carbonyl)-9*H*-carbazol-2-yl)propanoic acid) as the best inhibitor with IC_{50} values even lower than its precursor (see Table 1).[42]

N-(3-methylpyridin-2-yl) amide derivatives of flurbiprofen and Ibuprofen behave dual inhibitors of FAAH and COX more potent than the parent compounds with IC_{50} values in the submicromolar range. These molecules are able to interact with a region delimited by the acyl chain binding (ACB) and the membrane-access (MA) channels of FAAH. Both (*R*) and (*S*) isomers of 2-(2-fluorobiphenyl-4-yl)–*N*-(3-methylpyridin-2-yl)propanamide (Flu-AM1) inhibit FAAH with similar IC_{50} values (0.74 and 0.99 μM, respectively), while the (*S*)-isomer of 2-(4-isobutylphenyl)-*N*-(3-methylpyridin-2-yl)propanamide (Ibu-AM5) is more potent than the (*R*)-isomer (IC_{50} 0.59 μM and IC_{50} 5.7 μM, respectively).[43] Following the same aim, finalized to synthesize small molecules as potential FAAH-COX inhibitors starting from NSAID structure, previously, Cipriano and co-workers synthesized the racemic *N*-(3-methylpyridin-2-yl) amides of naproxen, flurbiprofen and 2-(2-(2-fluorobiphen-4-yl)propanamido)acetic acid. In this study, flurbiprofen inhibited rat brain [^{3}H]AEA hydrolysis with an IC_{50} value of 29 μM, while its *N*-(3-methylpyridin-2-yl) amide (Flu-AM1) was 60-fold more potent (IC_{50} 0.44 μM). The analogues

derivative 2-(2-fluorobiphenyl-4-yl)-*N*-(2-(3-methylpyridin-2-ylamino)-2-oxoethyl)propanamide (Flu-AM2) lost the potency (IC_{50} 17 µM) because an additional three atom linker (-NH-CH_2-CO-) between the carbonyl of the parent flurbiprofen and the pyridylamino group was added. Naproxen is a less potent inhibitor with low potency (IC_{50} 100 µM) and limited efficacy while its *N*-(3-methylpyridin-2-yl) amide (Nap-AM1) showed greater potency (0.74 µM) without exceeding Flu-AM1.[44]

It is noteworthy that *R*-isomers of arylpropionic acids (*R*-profens) such as ibuprofen, flurbiprofen, and naproxen inhibit COX-2 mediated endocannabinoids oxygenation but not the arachidonic acid's one (AA), thus showing "substrate-selectivity". Both, site-directed mutagenesis and molecular modeling studies, confirmed the crucial role of the ion-pairing interactions between the NSAID carboxyl group and the Arg-120 residue.[45] As previously reported,[46] the stable binding of *R*-arylpropionic acids within the active site of COX-2 was hindered by a steric interaction between the phenolic ring of Tyr355 and the α-methyl group of these molecules. Duggan and co-workers[47] showed that the α-methyl group is located adjacent to the Tyr355 residue. The substrate-selectivity is achieved through a negative cooperativity between the two monomers of COX-2 homodimer when only a molecule of *R*-profens (a weak and reversible inhibitor) binds the enzyme.

Inside the chemical diversity of FAAH inhibitors, the carbamate-based class furnished very selective and potent blockers, such as URB597.[48] By modifying the substituents on the aryl/alkyl group linked to the carbamate O- and N- atoms, it is possible to modulate FAAH inhibitory activity. In fact, it seems to be optimal a lipophilic *N*-alkyl and a bent *O*-aryl moieties.
With the aim to design new ligands able to inhibit both FAAH and COX 1/2, Sasso and co-workers tried to join pharmacophore and similar elements present in both carbamate-biphenyl FAAH inhibitor class and COX inhibitor, flurbiprofen. The new molecules are finally characterized by a common biphenyl core connecting the propionic acid framework, required for COX inhibition, and the *N*-alkyl carbamate functionality, essential to FAAH inhibition. The first synthetized compound 3, bringing at 3' position of distal phenyl ring the *N*-cyclohexylcarbamate moiety of URB597, showed modest inhibitory activity of FAAH (IC_{50} 8.2±2.4 µM) and COX-1 (IC_{50} 7.9±2.5 µM) and no activity against COX-2 (IC_{50}>100 µM). By modifying the *N*-alkyl substituent of the carbamate group, the potency toward the three targets progressively increased; in particular, when the cyclic aliphatic group was replaced by a linear alkyl chain of five carbon atoms, the obtained molecule, ARN2508, behaved a multiple ligand and inhibited FAAH, COX-1 and COX-2 with similar potencies (see Table 1). Removing the carbamate functionality, as in compound 5, the FAAH inhibition fully disappeared, while COX enzymes were still blocked. When the carbamate group was replaced with a urea fragment (see compound 6) or it was reversed (see compound 7) a weighty reduction of FAAH inhibition was anyway exhibited. As already pointed out, the presence of free carboxylate moiety is crucial for COX inhibitory effect, in fact, removing that group, it is possible to detect a decreased COX inhibition, without affecting FAAH interaction.[49] The interesting IC_{50} values of ARN2508 have prompted researchers to carry out the docking experiments of this molecule on several enzymes. In the active site of COX-1, the carboxyl group of ARN2508 forms stable hydrogen bonds with Tyr355 and Arg120, interaction similar to that exerted by the arylpropionic acid NSAIDs. Interestingly, the hybrid ARN2508 showed hydrophobic interaction pattern such as both flurbiprofen and AA: in fact, biphenyl system establishes Van der Waals interactions within the same region of COX-1 active site as flurbiprofen and the C_2-C_{11} portion of AA acyl chain, while *N*-pentyl linear tail leads a further hydrophobic contact (C_{14}-C_{20} region) within the enzyme cavity mimicking the arachidonoyl end. Concerning the carbamate-COX interaction, data suggest that the carbonyl group of ARN2508 and the Ser530, residue acetylated by aspirin, form H-hydrogen bonds without covalent binding. Conversely, the carbamate functionality causes the covalent inhibition of FAAH undergoing a nucleophilic attack by Ser241, according to carbamate-based FAAH blockers.[50]

Table 1. Compounds that interact as dual ligand

Name	**Activity**			
AA-5-HT[31]	TRPV1 [a] (IC_{50} nM) 36.8-39.9	FAAH (IC_{50} µM) 1-12	CB_1 (K_i) >50µM	CB_2 (K_i) >10µM
Fenoprofen-5-HT[32]	TRPV1 [b] (IC_{50} µM) 8 (6.5–9.6)	FAAH[c] 105% (9) [50 µM]	COX2 [d] 49% (2) 7 µM (2–25 µM)	
Naproxen-5-HT[32]	TRPV1[b] (IC_{50} µM) 13 (10.4–16.7)	FAAH[c] 73% (6) [50 µM]	COX2 [d] 48% (3) 18 µM (12–25 µM)	
Ibuprofen-5-HT[32]	TRPV1[b] (IC_{50} µM) 6 (5.2–7.6)	FAAH[c] 75% (9) 5 µM (3–8)	COX2 [d] 42% (2) 10 µM (8–13)	
Flurbiprofen-5-HT[32]	TRPV1[b] (IC_{50} µM) 9 (7.8–10.5)	FAAH[c] 85% (15) 15 µM (11–20)	COX2 [d] 38% (1) 8 µM (6–9)	
5a[36]	FAAH (IC_{50} µM) 3.69 ± 0.84	TRPV1[e] (efficacy %) 52.3 ± 0.5	TRPV1 (EC_{50} µM) 5.1 ± 2.3	TRPV1[f] (IC_{50} µM) 6.20 ± 0.10
5b[36]	FAAH (IC_{50} µM) 6.77 ± 0.85	TRPV1[e] (efficacy %) <10	TRPV1 (EC_{50} µM) ND	TRPV1[f] (IC_{50}) 6.40 ± 0.10 µM
5c[36]	FAAH (IC_{50} µM) 6.25 ± 0.82	TRPV1[e] (efficacy %) <10	TRPV1 (EC_{50} µM) ND	TRPV1[f] (IC_{50} µM) 5.10 ± 0.10
5e[36]	FAAH (IC_{50} µM) 1.74 ± 0.03	TRPV1[e] (efficacy %) 12.5 ± 0.1	TRPV1 (EC_{50} µM) 5.2 ± 0.1	TRPV1[f] (IC_{50} µM) 9.60 ± 0.20
9b[36]	FAAH (IC_{50} µM) 0.275 ± 0.04	TRPV1[e] (efficacy %) 20.6 ± 1.8	TRPV1 (EC_{50} µM) 2.40 ± 0.94	TRPV1[f] (IC_{50} µM) 7.01 ± 0.29
4[37]	TRPV1 (IC_{50}, µM) 3.9	AEA hydrolysis (IC_{50}, µM) 6.56	% Inhibition (c = 50 µM) 98.3	
10[37]	TRPV1 (IC_{50}, µM)	AEA hydrolysis (IC_{50}, µM)	% Inhibition	

	1.0	3.36	(c = 50 μM) 69.4
12[37]	TRPV1 (IC_{50}, μM) 2.19	AEA hydrolysis (IC_{50}, μM) 4.6	% Inhibition (c = 50 μM) 97.5
OMDM-198[38]	Human TRPV1 (IC_{50}, μM) 1	Rat brain FAAH (IC_{50}, μM) 3.36	
OMDM-202[38]	Human TRPV1 (IC_{50}, μM) > 10	Rat brain FAAH (IC_{50}, μM) 0.38	
Flu-AM1[44]	Human recombinant COX2 [h] (IC_{50}, μM) 42 (34-53)	Rat brain FAAH[g] (IC_{50}, μM) 0.44 (0.40-0.47)	Ovine COX1 [h] (IC_{50}, μM) 6.6 (4.4-9.8)
Nap-AM1[44]	Human recombinant COX2 [h] (IC_{50}, μM) >100	Rat brain FAAH[g] (IC_{50}, μM) 0.74 (0.62-0.90)	Ovine COX1 [h] (IC_{50}, μM) 56 (1.5-213)
15c[42]	COX2[i] (IC_{50},μM) 72.3±28.0	FAAH[i] (IC_{50}, μM) 22.0±4.2	COX1[i] (IC_{50}, μM) 74.3±28.0
15i[42]	COX2[i] (IC_{50}, μM) 27.8±9.7	FAAH[i] (IC_{50},μM) 84.8±10.6	COX1[i] (IC_{50}, μM) 30.0±12.1
ARN2508[49]	Human COX2 (IC_{50}, μM) 0.43±0.025	Rat FAAH (IC_{50},μM) 0.031±0.002	Ovine COX1 (IC_{50}, μM) 0.012±0.002

[a] No agonist activity up to 10 μM Antagonist activity (IC50 36.8–39.9 nM, against capsaicin, 100 nM). **[b]** (95% CI), DMSO. **[c]** % activity (SD), DMSO % activity (SD), EtOH ,IC50 (95% CI), EtOH. **[d]** % activity (SD), EtOH, IC50 (95% CI), EtOH. **[e]** As percent of ionomycin (4 μM). **[f]** Determined against the effect of capsaicin (0.1 μM). **[g]** 0.5 μM AEA. **[h]** Arachidonic acid 10 μM. **[i]** IC_{50} μM ±SD.
ND not determined when efficacy is lower than 10%.

Taking into consideration that the mode of pain treatment is strictly related to its inflammatory or neuropathic aspect, we can assert that is possible and hopefully reliable, to manage the whole process by using a single drug endowed with a dual action. In fact in the nociceptive nerve fibers, CB1 and TRPV1 receptors are often co-expressed, becoming two interacting signalling systems in many physio/pathological conditions.
While, due to the assumption that Cyclooxygenase-2 is usually defined an inducible enzyme, which expression increases during inflammatory states, in the inflammatory pain, the application of NSADs selective COX-2 inhibitor together with a FAAH inhibitor, can dramatically reduce the proinflammatory cascade and then generate pain relieve. On the other hand, the treatment of neuropathic pain with intrathecal administration of URB597, a FAAH inhibitor, can increase the levels of AEA. AEA reduces neuropathic pain with a mechanism involving both CB1 and TRPV1. The complete inhibition of FAAH is a useful tool to unmask different metabolic pathways for AEA. Formation of 15-hydroxy-AEA, together with OEA and PEA, may contribute at producing TRPV1-mediated analgesia. Nevertheless, the usual approach "one target-one drug" seems to be exceeded, opening the way to the concept that molecules that interact with more than one target, possibly relevant for the same disease, may show higher therapeutic effect and a safer biological profile.

In conclusion, in this minireview we have described the very complex and promiscuous network involved in pain perception and transmission, pointing out especially its inflammatory and neuropathic aspect.
Several sophisticated and interconnected systems of receptors (CB1/2 and TRPV1) and enzymes (FAAH and COX1/2) control this process, and experimental data demonstrate that the selective action of a molecule at only one of these targets dysregulates the other signaling systems, causing adverse side effects. Pain treatment with the combination of multiple drugs is currently the unique therapeutic option but, although yet usefulness, it is hopefully reliable to manage the whole pain process by using a single drug endowed with a dual action.
As alternative to a direct activation of cannabinoid receptors, primarily CB1, selective FAAH inhibition was considered an effective approach in order to elicit the cannabinoid desirable effects without psychotropic outcome. However, in the nociceptive nerve fibers, CB1 and TRPV1 are often co-expressed and heightened levels of AEA can activate, but not desensitize, the TRPV1 hyperalgesic pathway. In this context, the development of combined FAAH/TRPV1 blockers might have a more powerful therapeutic application, elevating contemporary endocannabinoid tone and the levels of other lipid mediators, and at the meantime silencing TRPV1 signaling.
Looking from a different perspective, by blocking exclusively FAAH metabolism, cyclooxygenases become the main enzymes responsible for EC degradation. In particular, the inducible COX-2, which expression increases during inflammatory states, could produce oxygenate metabolites contributing to the inflammatory process. Thus, dual FAAH/COX-2 inhibitors might dramatically reduce the proinflammatory cascade and then generate pain relief, contrasting at the same time typical gastric outcomes of selective COX drugs.
Albeit the usual approach "one target-one drug" seems to be exceeded, paving the way to the concept that molecules interacting with more than one target, possibly relevant for the same disease, may show higher therapeutic effect and a safer biological profile, this idea has not yet a therapeutic value because of the lack of appropriate tools. In fact, further studies will have to be performed with the aim to solve significant issues, as the improvement of pharmacologic/pharmacokinetic properties, and focused to balance the combined actions in these multi-target compounds.
From the medicinal chemistry point of view, it is a challenging and exciting goal to pursue the design and synthesis of hybrid functional molecules and, as herein described, inspirations may come from the field of natural compounds as well as combining pharmacophore features of known drugs, crucial for interaction with different targets.

Keywords: pain treatment • TRPV1 agonists/antagonists • COXs inhibitors • FAAH inhibitors • dual inhibitors

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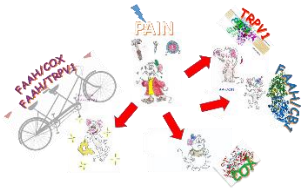

The pain management still represents an intriguing research topic, and despite the innovative analgesic available drugs, very few of them are therapeutically efficient without serious side effects. In particular, seems to be a useful action the contemporary modulation of two key targets in the pain transduction event, by a single molecule. This strategy has a double aim to reduce both the doses and the side effects of the single drugs.