Research paper

Paracetamol analogues conjugated by FAAH induce TRPV1-mediated antinociception without causing acute liver toxicity


1. Introduction

The pharmacological options for analgesic therapy are limited with only a few distinct drug classes on the market. For many chronic pain conditions the analgesic treatments do not offer adequate pain relief and are often associated with adverse effects that limit a long-term use. Today, this is highlighted by the opioid crisis urging for the discovery of new analgesics. Paracetamol (acetaminophen) is one of the most used fever and pain-relieving drugs worldwide and its consumption increases [1,2]. It is currently recommended as first-line pharmacological therapy for many pain conditions, although some observations show that paracetamol has a limited efficacy, particularly in several chronic painful conditions including osteoarthritis [3–6] and low back pain [3,7]. Variation in human genotype may also contribute to the variable effectiveness of paracetamol as an analgesic [8]. Furthermore, paracetamol is converted into the liver-toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI) already at therapeutic doses, urging for safer paracetamol analogues. Primary amine analogues with chemical structures similar to paracetamol were evaluated for their propensity to undergo FAAH-dependent biotransformation into N-arachidonoylphenolamine (AM404), which mediates TRPV1-dependent antinociception in the brain of rodents. However, paracetamol is also converted to the liver-toxic metabolite N-acetyl-p-benzoquinone imine already at therapeutic doses, urging for safer paracetamol analogues. Primary amine analogues with chemical structures similar to paracetamol were evaluated for their propensity to undergo FAAH-dependent N-arachidonoyl conjugation into TRPV1 activators both in vitro and in vivo in rodents. The antinociceptive and antipyretic activity of paracetamol and primary amine analogues was examined with regard to FAAH and TRPV1 as well as if these analogues produced acute liver toxicity. 5-Amino-2-methoxyphenol (2) and 5-aminooindazole (3) displayed efficient target protein interactions with a dose-dependent antinociceptive effect in the mice formalin test, which in the second phase was dependent on FAAH and TRPV1. No hepatotoxicity of the FAAH substrates transformed into TRPV1 activators was observed. While paracetamol attenuates pyrexia via inhibition of brain cyclooxygenase, its antinociceptive FAAH substrate 4-AP was not antipyretic, suggesting separate mechanisms for the antipyretic and antinociceptive effect of paracetamol. Furthermore, compound 3 reduced fever without a brain cyclooxygenase inhibitory action. The data support our view that analgesics and antipyretics without liver toxicity can be derived from paracetamol. Thus, research into the molecular actions of paracetamol could pave the way for the discovery of analgesics and antipyretics with a better benefit-risk ratio.

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causing hepatotoxicity, a well-known adverse effect of the drug [9–11] making paracetamol the principal cause of acute liver failure in the Western world [12–15].

In a series of publications, we have developed a conceptual framework for the antinociceptive action of paracetamol, involving fatty acid amide hydrolase (FAAH) and TRPV1 [16–20]. Accordingly, paracetamol as a prodrug undergoes hepatic deacetylation into 4-aminophenol (4-AP) followed by conjugation of 4-AP with arachidonic acid through a FAAH-dependent mechanism in the brain yielding N-arachidonoylphenolamine (AM404). While the endogenous role of FAAH mainly is to hydrolyse anandamide and related N-acylethanolamines [21], it can also synthesize N-arachidonoyl conjugates, including AM404 and arvanil [16–18]. Activation of TRPV1 in the brain by AM404 and arvanil induces antinociception, and previous reports have demonstrated that modulation of supraspinal FAAH and TRPV1 activity affects nociceptive signaling as well as that FAAH and TRPV1 are critical for the antinociceptive efficacy of paracetamol in mice [17–20,22–26]. It has also been shown that AM404 inhibits cyclooxygenase (COX)-2 [16,27], which could explain the antipyretic effect of paracetamol. Noteworthy, AM404 has not only been identified in the brain of rodents, but also in human cerebrospinal fluid after paracetamol administration [28]. Furthermore, there is genetic evidence that TRPV1 is involved in the analgesic action of paracetamol in humans [8].

The objective of this study was to understand the structure-activity relationship of paracetamol and primary amine analogues that undergo a FAAH-dependent biotransformation into N-arachidonoyl conjugates (Fig. 1) mediating a TRPV1-induced antinociception, and to examine if these analogues could produce antinociception without causing acute liver toxicity. The antipyretic effect of paracetamol and analogues was also examined.

2. Methods

2.1. Drugs

4-Aminophenol, 4-(aminomethyl)-2-methoxyphenol (HMBA), 4-amino-2-methoxyphenol (1), 5-amino-2-methoxyphenol (2), 5-aminoindazole (3), 5-aminomethyl indazole (4), paracetamol, capsazepine, ibuprofen and Nω-nitro-ω-arginine and phenylephrine were purchased from Sigma-Aldrich (St Louis, MO, USA). Indomethacin (Confortide®) was obtained from Dumex (Copenhagen, Denmark). N-arachidonoyl conjugates: N-(4-hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (AM404), N-(4-hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide-d4 (AM404-d4), N-(4-hydroxy-3-methoxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (arvanil), methyl arachidonyl fluorophosphonate (MAFP) and 3′-carbamoyl-6-hydroxy-[11′′-biphenyl]-3-ylcyclohexylcarbamate (URB937) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). N-(4-hydroxy-3-methoxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (1aa); conjugate of 4-amino-2-methoxyphenol and N-(3-hydroxy-4-methoxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (2aa; conjugate of 5-amino-2-methoxyphenol) were purchased from Bioduro (San Diego, CA, USA). N-(1H-indazol-5-yl) 5Z,8Z,11Z,14Z-eicosatetraenamide (3aa; conjugate of 5-aminooindazole) and N-(1H-
2.3. Animals

All experiments were approved by the Linköping and Lund Animal Ethic Committees (ethical permission No. 44/13 (ID1854), M 48–13, M 49–134818/2017, 44–13; ID 1854) as well as the local French ethical committees (CEEMA Auvergne, France, ethical permission No. 02175.02). Experiments were performed according to European legislation (Directive 2010/63/EU) on the protection of animals used for scientific purposes, and complied with the recommendations of the International Association for the Study of Pain [31]. The design, analysis and reporting of the research complied with the ARRIVE guidelines [32]. All efforts were made to minimize discomfort and use as few animals as possible. Male Wistar rats (200–250 g) were purchased from Charles River Laboratories (RRID:RGD:10395233, Sulzfeld, Germany). FAAH−/− and FAAH+/− mice, originally generated by Benjamin Cravatt [33] and back-crossed with C57BL/6 mice for at least ten generations in our laboratory, were obtained from our own homozygous breeding for 2–3 generations. To minimize the number of animals used for the in vitro and in vivo FAAH metabolic studies, both male (25–35 g) and female (18–25 g) mice were used as previous studies have not revealed any difference between sex with regard to such studies [16–18]. For behavioural and temperature recording studies, eight-week-old male C57BL/6 mice were obtained from Janvier Laboratories and (RRID:MGJ:5657942, Le Genest-Saint-Ise, France). All mice were kept 4–6 per cage at specific pathogen free facilities and fed ad libitum under controlled conditions with a 12 h light-dark cycle. At the end of the experiment animals were euthanized by CO2 inhalation. For liver toxicity studies, eight-week-old male C57BL/6 mice were obtained from Charles River Laboratories (RRID:RGD:10395233, Sulzfeld, Germany).

2.4. TRPV1-mediated vasorelaxation

The TRPV1 potency of N-arachidonoyl conjugates was assessed using mesenteric arteries from rats by recording the nerve-mediated vasorelaxation [16,18,34]. In short, arterial segments were dissected and mounted in organ baths between two force sensitive wires at a tension corresponding to a blood pressure of 90 mm Hg. The surrounding buffer was composed of a physiological salt solution (NaCl 119, KCl 4.6, CaCl2 1.5, MgCl2 1.2, NaHCO3 15, NaH2PO4 1.2 and D-glucose 6 (mM); pH 7.4) which was temperature-controlled (37 °C) and aerated with 95% O2 and 5% CO2. To create an environment in which phenylephrine (dissolved in saline) induces stable and long-lasting contractions all experiments were performed in the presence of indomethacin (10 µM) and N0-nitro-l-arginine (100 µM), both dissolved in saline, to diminish the production of COX- and nitric oxide synthase-derived vasodilators, respectively. After submaximal vasconstriction mediated by phenylephrine (3 µM), the arteries were exposed to N-arachidonoyl conjugates (dissolved in ethanol) to estimate their sensory-nerve mediated vasodilator properties in the absence and presence of the TRPV1 antagonist capsazepine (3 µM) dissolved in ethanol. The final concentration of ethanol reached 1% and does not affect blood vessel contractile properties [16,18,34]. Changes in the tension of vessels were registered by the force-displacement transducer model FT03C (Grass Instruments; Rhode Island, USA).

2.5. Evaluation of FAAH-mediated conjugation in vitro

Brains from FAAH−/− and FAAH+/− mice were removed and frozen in liquid nitrogen and homogenized in 4 ml ice-cold 10 mM Tris buffer supplemented with 0.2 mM EDTA and 0.3 mM ascorbic acid. The homogenate was divided into aliquots of 200 µl and preheated to 37 °C prior to addition of primary amines (0.1 mM). The samples were incubated at 37 °C under stirring for 20 min before the reaction was stopped by adding 1 ml of ice-cold acetone containing an internal standard (AM404-d4). The conjugate content of samples was determined using the LC-MS/MS technique (see below). For details on methodology, see Refs. [16–18].

2.6. Evaluation of FAAH-mediated conjugation in vivo

FAAH−/− and FAAH+/− mice were injected intraperitoneally (i.p.) with the primary amines (100 or 300 µl kg−1) dissolved in saline (occasionally supplemented with 5% DMSO). The animals were anesthetized with isoflurane and decapitated 20 min after the injection. The brains were removed and promptly frozen in liquid nitrogen, and stored at −80 °C. They were homogenized in 2 ml ice-cold 10 mM Tris buffer, which had been supplemented with 1 mM EDTA and 0.3 mM ascorbic acid and 10 mM of the FAAH inhibitor MAPF to prevent N-arachidonoyl formation and degradation post mortem. To determine N-arachidonoyl conjugate levels, aliquots of 200 µl homogenate were precipitated with 1 ml ice-cold acetone containing 0.3 mM ascorbic acid, 5 mM AM404-d4 (Cayman Chemicals) and 10 mM PGE1-d4 (Cayman Chemicals), as internal standards, and analyzed by the LC-MS/MS technique (see below). For details on methodology, see Refs. [16–18].

2.7. Lipopolysaccharide-induced fever and prostanoid levels in vivo

Lipopolysaccharide (LPS), 100 µg kg−1 i.p. from Escherichia coli (O111:B4; Sigma-Aldrich, St. Louis, MO), or saline was administered 3 h prior to i.p. injection of paracetamol (100 and 150 mg kg−1), 4-AP (30 mg kg−1), 3 (25 mg kg−1), or their vehicle (Ringer’s acetate solution, Fresenius Kabi AB, Uppsala, Sweden (NaCl 131 mM, Cl− 112 mM, acetate 30 mM, K+ 4 mM, Ca2+ 2 mM, Mg2+ 1 mM)). Forty minutes after the injection, the animals were anesthetized with isoflurane and decapitated. Blood was collected from carotid arteries into heparin tubes containing sodium citrate and the COX-inhibitor ibuprofen. The brain was removed and the diencéphalon, including the hypothalamus (considered to be the site of the
antipyretic effect of paracetamol through COX inhibition [35]) was promptly dissected, snap frozen in liquid nitrogen, and stored at −80 °C. The dienccephalon was homogenized in 1 ml ice-cold 10 mM Tris buffer, to which had been added 1 mM EDTA, 300 μM ascorbic acid, 10 μM MAPF and 100 μM ibuprofen, the latter to prevent in vitro prostanoid formation and degradation. In order to determine prostanoid levels, aliquots (200 μl) of blood and homogenates of the dienccephalon were precipitated with 1 ml ice-cold acetone, containing 300 μM ascorbic acid and 10 mM PGF1−
/d4 (Cayman Chemicals, Co., Ann Arbor, USA) as internal standard, and analyzed by the LC-MS/MS technique (see below).

For details on telemetric temperature recordings, see Refs. [36,37]. In short, under anesthesia (1% isoflurane, Abbot Scandinavia, Solna, Sweden), a temperature-recording probe was implanted intraperitoneally (TA11TAF10; Data Sciences International, St. Paul, MN, USA) and postoperative analgesia was given i.m. at a dose of 5 mg kg−1 in 0.1 ml saline i.p.; RB Pharmaceuticals, Slough, Berkshire, United Kingdom). To provide near-thermoneutral conditions the mice were transferred to a room in which the ambient temperature was set to 29 °C. The animals were left to recover for at least 1 week before start of experiments.

2.8. Quantitative LC-MS/MS analysis

All precipitated samples were centrifuged at 17960 g for 10 min at 4 °C. The supernatants were vacuum evaporated and each extraction residual was subsequently reconstituted in 200 μl of a mixture of 99.5% methanol and 0.5% acetic acid (for N-arachidonoyl conjugates) or 80% methanol, 19.5% H2O and 0.5% acetic acid (for prostanoids). The protein content of the pellet was determined by Coomassie protein assay (Pierce, IL, USA), using bovine serum albumin as standard reference.

All samples were analyzed on a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) coupled to a Sciex 5500 tandem mass spectrometer (Applied Biosystems/MD Sciex, Darmstadt, Germany). Sample aliquots of 5 μl were injected onto a Waters CSH C18 column (50 × 2 mm, 3 μm (Waters, Milford, MA, USA)) held at 50 °C. All mobile phases were water-acetonitrile gradients, containing 0.1% formic acid and with a flow rate of 500 μl min−1. The acetonitrile content was initially 20%, then increased linearly to 100% over 3 min and kept at this level for 3 min 15 s, followed by an equilibration at 20% for 45 s.

All N-arachidonoyl conjugates were quantified by LC-MS/MS with the electrospray interface operating in a positive ion mode at 550 °C with the ion spray voltage set to 5000 V. The prostanoids PGF2α (Sigma-Aldrich, Schnelldorf, Germany), PGE2 (Sigma-Aldrich, Schnelldorf, Germany), 6-keto-PGF1α (Cayman Chemicals) and TXB2 (Biomol GmbH, Hamburg, Germany) were quantified by LC-MS/MS with the electrospray interface operating in a negative ion mode at 550 °C with the ion spray voltage set to 4500 V. The mass transitions (m/z) for N-arachidonoyl conjugates and prostanoids were as follows: 396.2/110.0 (AM404); 400.2/287.4 (AM404-d4); 440.3/137.1 (arvanil); 426.3/140.1 (1aa and 2aa); 420.3/134.1 (3aa); 434.0/131.2 (4aa); 351.2/315.3 (PGF2α); 357.2/321.2 (PGE2-d4); 369.2/169.1 (TXB2); 353.4/309.3 (PGE2α) and 369.4/163.1 (6-keto-PGF1α).

N-arachidonoyl conjugates and prostanoids were analyzed using AM404-d4 and PGE2-d4, respectively, as internal standard. The detection limits were calculated as the concentrations corresponding to three times the standard deviation of the blanks. When levels were below detection limit for N-arachidonoyl conjugates (AM404, 3.58 pmol g−1 protein; arvanil, 14.34 pmol·g−1 protein; 1aa, 14.34 pmol·g−1 protein; 2aa, 3.58 pmol·g−1 protein; 4aa, 14.34 pmol·g−1 protein) and prostanoids (PGE2, 493.26 pmol·g−1 protein; TXB2, 162.88 pmol·g−1 protein; 6-

keto-PGF1α, 264.30 pmol g−1 protein; PGF2α, 1874.68 pmol g−1 protein), numerical values of 1/4 of the detection limit were used in the calculations.

2.9. Animal behavioural tests

Animals were randomly divided into 5–10 per group. Randomized treatment administrations were performed according to the method of equal blocks to assess the effect of the different treatments at the same time interval to avoid unverifiable and time-variable environmental influences. All experiments were performed in a quiet room by the same blinded experimenter. To ensure the methodological quality of this study, we followed recommendations from Rice et al. [38]. The two compounds, 2 and 3, were injected i.p., 10 min before locomotor or nociceptive tests. For intracerebroventricular (i.c.v.) administrations, URB937 was injected at a dose of 5 μg in 5 μl and capsazepine at a dose of 10 μg in 5 μl, 5 min before injection of compounds 2 or 3.

Locomotor activity: Motor impairment was measured on a rotarod apparatus (Bioseb, Chaville, France). Before the test, animals were trained under continuous rotation (4 rpm) in 1-minute sessions. For the test, mice were placed individually on the revolving drum. Once mice were balanced, the drum was accelerated from 4 to 40 revolutions per minute over the course of 5 min. The time point at which each mouse fell off the rod was recorded.

Formalin test: The animals received an intraplantar injection of a 2.5% formalin solution (25 μl/mouse) into a hind paw. The time spent biting and licking of the injected paw was monitored during the two typical phases of the nociceptive response (phase I: 0–5 min; phase II: 15–40 min).

The i.c.v. administrations were performed following the method previously described [25]. In short, all animals were briefly anesthetized with isoflurane (1–2%). The site of injection was 2 mm from either side of the median on a line drawn through the anterior base of the ears. Animals were injected with a volume of 5 μl using a 10 μl Hamilton syringe fitted with a 26-gauge needle with the tip adjusted to be inserted 2 mm deep.

2.10. Liver histology and aminotransferases activity

Mice were randomly allocated into one of the treatment groups. The food was withdrawn 12–16 h prior to the administration of substances. All substances were dissolved in saline (using minor heating) and administered at a dose of 300 mg kg−1 (i.p.), a dose often used to study paracetamol-induced liver injury in mice [39]. After 12 h the animals were briefly anesthetized with isoflurane and decapitated. The blood was collected in heparin tubes and the left medial hepatic lobe was removed for analysis.

Blood-containing heparin tubes were centrifuged at 2000 g for 10 min. The serum was collected and the aminotransferases activity was measured within 12 h using Cobas 6000/8000 analyzer series (Roche Diagnostics, Rotkreuz, Switzerland).

The excised liver tissue was fixed in 4% cold formaldehyde dissolved in PBS for 90 min and subsequently rinsed in PBS supplemented with 15% sucrose at 4 °C. Samples were then frozen in isopentane before being cut serially at 10 μm, using a cryostat (Leica CM3050, Leica microsystems, Wetzlar, Germany). All sections were stained with haematoxylin & eosin (Sigma, St Louis, MO). A Nikon Eclipse TE2000-S bright-field microscope (Nikon, Tokyo, Japan) was used to evaluate the morphology.

2.11. Calculations and statistical analysis

GraphPad Prism 8 (GraphPad Software, La Jolla, CA) was used for statistical analysis and to perform curve fitting (non-linear}
regressions) and calculations of pEC<sub>50</sub> values (the negative log concentration that elicited half-maximal vasorelaxation). The level of statistical significance was set at P < 0.05. Comparisons between two groups of data were performed using Student’s t-test. Comparisons of more than two groups of data were performed using 1-way ANOVA or repeated measures ANOVA followed by either Dunnett’s, Tukey’s or Sidak’s multiple comparisons test, Welch’s ANOVA test or 2-way ANOVA followed by Sidak’s multiple comparisons test. Data are presented as the mean ± SEM; n indicates the number of animals examined.

3. Results

3.1. In vitro FAAH-mediated fatty acid conjugation of primary amines and evaluation of TRPV1 agonistic properties of the corresponding N-arachidonyl conjugates

The paracetamol metabolite 4-AP as well as the primary amine 4-(aminomethyl)-2-methoxyphenol (HMBA) undergo FAAH-mediated fatty acid conjugation into the N-arachidonyl conjugate AM404 and arvanil, respectively, both of which activate TRPV1 in vitro (Fig. 2, Table 1) [16–18]. Here, we have explored additional primary amino analogues in a LC-MS/MS based assay for evaluation of their predisposition to undergo FAAH-mediated fatty acid conjugation in vitro into their corresponding N-arachidonyl conjugates denoted with N (Fig. 2A, Table 1). The vanillinid structure 4-amino-2-methoxyphenol (1) was found to be a poor FAAH substrate, whereas the regiosomer 5-amino-2-methoxyphenol (2) was conjugated by FAAH in greater amounts, albeit less efficiently than 4-AP (Fig. 2A, Table 1). 5-Aminooindazole (3), a bioisostere of 4-AP, underwent a more efficient FAAH-mediated conjugation than 4-AP and 5-aminomethyl indazole (4) (Fig. 2A, Table 1). The aliphatic amines (4) and HMBA yielded similar amounts of their corresponding N-arachidonyl conjugates, but less than the amount of AM404 produced by 4-AP (Fig. 2A, Table 1). The FAAH-dependent structural requirements of the conjugation of the primary amines was apparent by the reduced formation of their corresponding conjugates in FAAH−/− preparations (Table 1).

The TRPV1 agonistic properties of the N-arachidonyl conjugates of compounds 1–4 were determined by their ability to induce a TRPV1-dependent primary sensory nerve-mediated vaso-relaxation of pre-contracted rat isolated mesenteric arterial segments, an in vitro assay that has been extensively used in pharmacological studies of native TRPV1 [40]. In this assay, all N-arachidonyl conjugates produced complete relaxations and were more potent than the TRPV1 active paracetamol metabolite AM404 (Fig. 2B, Table 2). The TRPV1 antagonist capsazepine significantly reduces the potency of each N-arachidonyl conjugate similar to what has been shown previously for the TRPV1 agonist capsaicin, AM404 and arvanil [16,18,34] (Table 2).

3.2. In silico studies of the FAAH-mediated production of N-arachidonyl conjugates and their interactions with TRPV1

To understand the interaction of the N-arachidonyl conjugates with both target proteins, FAAH and TRPV1, the corresponding N-arachidonyl conjugates of 4-AP, 2 and 3 were chosen as key compounds from the *in vitro* studies for modelling with FAAH (PDB: 3PR0, 2.2 Å, X-ray) and TRPV1 (PDB: 5IRZ, 3.28 Å, cryo-EM) [29,30]. The two proteins were prepared using the Protein Preparation Wizard Script within Maestro (Schrödinger Inc.) before manual introduction of the N-arachidonyl conjugates of 4-AP, 2 and 3.

The interaction of AM404 with FAAH was first studied (Fig. 3A). Upon FAAH-mediated conjugation of 4-AP, the tetraheiral intermediate of AM404 is covalently attached to Ser241 via its carbonyl carbon [41], whereas the amide hydrogen and nitrogen of AM404 are interacting with backbone Met191 and the hydroxyl group of

### Table 1

In vitro fatty acid conjugation of primary amines by FAAH into N-arachidonyl conjugates.

<table>
<thead>
<tr>
<th>Compound</th>
<th>N-arachidonyl conjugates (nmol g⁻¹ protein)</th>
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<tbody>
<tr>
<td></td>
<td>FAAH&lt;sup&gt;+/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>4-AP</td>
<td>8.8 ± 0.7</td>
</tr>
<tr>
<td>HMBA</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>1</td>
<td>0.54 ± 0.09</td>
</tr>
<tr>
<td>2</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>12 ± 0.6</td>
</tr>
<tr>
<td>4</td>
<td>2.5 ± 0.2</td>
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The fatty acid amide hydroxase (FAAH) in vitro activity was determined by measuring the content of N-arachidonyl conjugates after incubation of brain homogenates from FAAH<sup>+/−</sup> or FAAH<sup>−/−</sup> mice with 0.1 mM of primary amines (Compound). Compared to AM404, the content of each other N-arachidonyl conjugate was different in FAAH<sup>−/−</sup> mice (see also Fig. 2A). Data are presented as mean ± SEM, and n indicates the number of animals.

![Fig. 2](image-url). *In vitro* fatty acid conjugation of primary amines by FAAH into corresponding N-arachidonyl conjugates activating TRPV1. (A) *In vitro* FAAH activity was evaluated by the content of N-arachidonyl conjugates after addition of primary amines (0.1 mM) to brain homogenate from FAAH<sup>+/−</sup> mice. The production of all N-arachidonyl conjugates were FAAH-dependent (Table 1). Compared to AM404, the content of each other N-arachidonyl conjugate was different in FAAH<sup>−/−</sup> mice (P < 0.0001, 1-way ANOVA followed by Sidak’s multiple comparisons test, n = 5–7). Also, using the same test, differences were observed between the N-arachidonyl conjugates of the phenolic regioisomers 1 and 2 (P < 0.0001), and the indazoles 3 and 4 (P < 0.0001) (n = 5–6), (B) Concentration-response curves for TRPV1-mediated vasorelaxation of rat isolated mesenteric arteries evoked by the N-arachidonyl conjugates (n = 6–10). The potency (pEC<sub>50</sub>) of N-arachidonyl conjugates are listed in Table 2. The TRPV1 antagonist capsazepine (3 μM) caused a rightward shift of the concentration-response curves without affecting the maximum relaxation (Table 2). Data are presented as mean ± SEM.
Ser217, respectively (Fig. 3A and B). Interestingly, two energy minima were observed, as the amide nitrogen of the covalently bound AM404 intermediate could assume either a pyramidal (sp3) or a planar (sp2) geometry. Similar to AM404, the N-arachidonoyl conjugates of 2 and 3 are displaying planar sp2 and pyramidal sp3 geometries, respectively, in their energy minima when bound to FAAH (Fig. 3C and D). Compared to 4-AP and 2, the N-arachidonoyl conjugate of 3 assumes a planar amide nitrogen geometry at a lower energy state (3E: ΔE_pyramidal-planar = 14.4 kJ mol⁻¹ versus AM404: ΔE_pyramidal-planar = −3.1 kJ mol⁻¹ and 2E: ΔE_pyramidal-planar = −23.2 kJ mol⁻¹).

Next, the interaction of AM404 with TRPV1 was studied (Fig. 4). In TRPV1, AM404 was introduced to the capsaicin-binding site (Fig. 4A). The arachidonic tail of AM404 facilitates non-specific, hydrophobic interactions, similar to the hydrophobic moieties of the ultrapotent TRPV1 activator resiniferatoxin [42]. Furthermore, two polar interactions fixate the amide linker region of AM404 towards Tyr511 and Thr550 entrapping AM404 in a VRdown binding pose [30,43]. This geometry also allows for π–π interactions towards Tyr511. Additional polar interactions are formed between the hydroxyl group and Arg557/Glu570 in the TRPV1 S4–S5 linker region. In vitro data and insights from the computational studies regarding AM404 encouraged studies of the N-arachidonoyl conjugate of 3, having an indazole moiety instead of a phenolic/vanilloid moiety as in AM404 and 2 (Fig. 4B). The conjugate with 3 assumed a binding mode within TRPV1 similar to AM404, only lacking an apparent π–π interaction towards Tyr511, but with closer contact to Arg557 and Glu570 through hydrogen accepting and donating atoms, respectively.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>TRPV1 potency of N-arachidonoyl conjugates</th>
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<tbody>
<tr>
<td>Compound</td>
<td>TRPV1 potency (pEC50)</td>
</tr>
<tr>
<td>AM404</td>
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<tr>
<td>Arvanil</td>
<td></td>
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<tr>
<td>1aa</td>
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<td>2aa</td>
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<td>4aa</td>
<td></td>
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<tr>
<td>Control</td>
<td>n</td>
</tr>
<tr>
<td>AM404</td>
<td>****</td>
</tr>
<tr>
<td>Arvanil</td>
<td>****</td>
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<tr>
<td>1aa</td>
<td>ns</td>
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<tr>
<td>2aa</td>
<td>ns</td>
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<tr>
<td>3aa</td>
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<td>4aa</td>
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N-arachidonoyl conjugates of primary amines (Compound) were assessed for their ability to induce TRPV1-mediated relaxation of rat isolated mesenteric arteries. The negative log molar concentration of the conjugate that evoked half-maximal response (pEC50) was calculated in the absence and presence of the TRPV1 antagonist capsazepine (CZ, 3 μM). Capsazepine significantly reduced the potency of each N-arachidonoyl conjugate (P < 0.001 for 1, P < 0.0001 for 2, P < 0.01 for 3 and P < 0.0081 for 4 using the Student’s unpaired t-test). The potencies of N-arachidonoyl conjugates were compared by 1-way ANOVA followed by Tukey’s multiple comparisons test (****P < 0.001, ***P < 0.001, **P < 0.01, *P < 0.05; P > 0.05 is not significant = ns). Data are presented as mean ± SEM, and n denotes the number of animals. n.d. = not determined.

![Fig. 3](image-url)

Fig. 3. Representative docking poses for the paracetamol metabolite AM404, 2 and 3 within FAAH. (A) FAAH is a ubiquitous, dimeric, membrane bound enzyme characterized by a distinctive catalytic triad (Ser241, Ser217, Lys142) and an oxyanion hole which stabilizes the carbonyl oxygen in the tetrahedral transition state of AM404, a geometry in the FAAH-mediated conjugation process that has been proposed as a stable intermediate [41,56]. (B) A planar (blue) and a pyramidal (green) geometry of the amide nitrogen of AM404 were observed (ΔE_pyramidal-planar = −3.1 kJ mol⁻¹), which have different bond lengths towards Ser217 (red and light blue dots). (C,D) Like AM404 the N-arachidonoyl conjugates of 2 (C) and 3 (D) display planar (2, orange; 3, magenta) and pyramidal (2 and 3, green) energy minima within FAAH (ΔE_pyramidal-planar = −23.2 kJ mol⁻¹, and ΔE_pyramidal-planar = 14.4 kJ mol⁻¹, respectively). Yellow dots show polar bonds. Only polar hydrogens are shown for clarity. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
3.3. In vivo evaluation of N-arachidonoyl conjugate production in the brain and assessment of the antinoceptive action of selected primary amines

The primary amines (Fig. 1) were administered intraperitoneally (100 mg kg⁻¹ or 300 mg kg⁻¹) to mice to examine their capacity to reach the brain and undergo FAAH-dependent fatty acid conjugation in vivo (Fig. 5A). The conjugations of 2, 3, 4-AP and HMBA, but not 1 and 4, were FAAH-dependent, and the content of each N-arachidonoyl conjugate was different from that of AM404 in FAAH⁻/⁻ mice (Fig. 5A, Table 3). The biotransformation of HMBA and 4 into their corresponding N-arachidonoyl conjugates was near the detection limit, as also previously reported for HMBA [18]. Therefore, compounds 2 and 3, displaying compatible interactions with both FAAH and TRPV1 in vitro/in vivo, were evaluated for their antinoceptive properties. Compounds 2 (Fig. 5B and C) and 3 (Fig. 5D and E) displayed dose-dependent antinoceptive properties in the formalin test, and at doses that were without effect on locomotor activity (Fig. 5F). Furthermore, the blood-brain-barrier impermeable FAAH inhibitor, URB937, and the TRPV1 antagonist capsazepine were injected intracerebroventricularly to investigate if the antinoceptive effect of compounds 2 and 3 was dependent on brain FAAH and TRPV1 (Fig. 6). This restrictive pharmacologic intervention inhibited the antinoceptive effects of 2 and 3 during the second (Fig. 6B,D and F,H), but not the first (Fig. 6A,C and E,G), phase of the formalin test.

3.4. Antipyretic effects of paracetamol and its FAAH substrate analogues

To reveal any antipyretic effect of the antinoceptive FAAH substrate analogues of paracetamol, we compared the ability of 4-AP and the most efficient antinoceptive analogue 3 with that of paracetamol to reduce fever. Mice were pretreated with either lipopolysaccharide (LPS; 100 μg kg⁻¹ i.p.) to induce fever or injected with saline (controls) and then exposed to drugs or vehicle in an in vivo model for telemetric temperature recordings to monitor the body temperature (Fig. 7). While paracetamol suppressed the fever at a dose of 100 mg kg⁻¹ (Fig. 7A,A1), which is not antinoceptive in the mouse formalin test [17], the antinoceptive dose of 4-AP (30 mg kg⁻¹) was unable to attenuate the LPS-induced fever (Fig. 7B1). Febrile animals exposed to 3 at an antinoceptive dose (25 mg kg⁻¹) displayed a rapid decrease in core body temperature (Fig. 7C,C1).

3.5. Evaluation of liver-toxic effects

High paracetamol doses deplete the hepatic stores of protective glutathione which allows NAPQI to bind liver proteins and ultimately cause liver necrosis. We therefore tested 4-AP, HMBA, 2 and 3 in a mouse model for paracetamol-induced liver injury to evaluate the potential liver toxicity of these primary amines. Paracetamol (300 mg kg⁻¹, i.p.) created prominent centrilobular necrosis (Fig. 8A). These histological characteristics were not observed in livers from animals treated with an intraperitoneal injection of either 4-AP, HMBA, 2 or 3 at 300 mg kg⁻¹ (Fig. 8B–E). The levels of hepatic alanine aminotransferase (ALT) and aspartate aminotransferase (AST), as well as the ratio between these aminotransferases, were also evaluated in the same animals to disclose potential...
The fatty acid amide hydrolase (FAAH) in vivo activity was determined in brains obtained from FAAH knockout or FAAH wild-type mice 20 min after intraperitoneal injection (i.p.) of the primary amine (Compound). Except for HMBA, of which the corresponding N- arachidonoyl conjugate arvanil was only detected at 300 mg kg$^{-1}$, the other primary amines were administered at a dose of 100 mg kg$^{-1}$. The primary amine 2 was further assessed for antinociceptive properties in the formalin test. Both 2 and 3 displayed antinociceptive properties during the first and second phase, respectively, of the formalin test (${P < 0.05}$, ${** P < 0.01}$, ${**** P < 0.0001}$ determined by 1-way ANOVA followed by Dunnett’s multiple comparisons test). The primary amine 4 displayed antinociceptive properties during the first and second phase, respectively, of the formalin test ($P < 0.05$ and $** P < 0.01$ for vehicle vs 2 (75 mg kg$^{-1}$) [Repeated measures two-way ANOVA followed by Dunnett’s multiple comparisons test, $n = 8$]. Data are presented as mean $\pm$ SEM.

The fatty acid amide hydrolase (FAAH) in vivo activity was determined in brains obtained from FAAH knockout or FAAH wild-type mice 20 min after intraperitoneal injection (i.p.) of the primary amine (Compound). Except for HMBA, of which the corresponding N-arachidonoyl conjugate arvanil was only detected at a higher dose of 300 mg kg$^{-1}$, each other primary amine was administered at a dose of 100 mg kg$^{-1}$. The content of N-arachidonoyl conjugates in FAAH knockout vs FAAH wild-type mice was compared with significant differences obtained for 4-AP ($P < 0.01$, 1-way ANOVA followed by Sidak’s multiple comparisons test), 2 and 3 ($P < 0.01$) but not 1 and 4 using the Student’s unpaired t-test. Compared to AM404, the content of each other N-arachidonoyl conjugate was different in FAAH knockout mice ($P < 0.01$ for arvanil, 4-AP, HMBA vs FAAH knockout) but not for arvanil, 4-AP vs FAAH knockout.

The fatty acid amide hydrolase (FAAH) was determined in brain tissue of male and female mice treated with paracetamol and subsequent primary amine (Fig. 8B). Statistical analysis revealed significant differences in ALT levels between paracetamol and either of 4-AP, HMBA, 2 or 3 (Fig. 8F). Although no histological changes were noted for 4-AP (Fig. 8B), it increased the ALT/AST ratio close to 1 (Fig. 8G).

4. Discussion

The objective of this study was to explore the structure-activity relationship of paracetamol and primary amine analogues with regard to their ability to undergo a FAAH-dependent biotransformation into N-arachidonoyl conjugates mediating TRPV1-induced antinociception. To exploit differences in the FAAH-mediated conjugation of primary amines and the TRPV1 efficacy of their corresponding N-arachidonoyl conjugates, a computational analysis was conducted. The outcome from our initial in vitro functional studies of FAAH and TRPV1 activity was pertinent. Thus, we performed a computational study of the interaction between FAAH and the N-arachidonoyl conjugates of compounds 2 and 3 compared with the paracetamol metabolite AM404. The covalently linked tetrahedral FAAH-intermediate of the corresponding N-arachidonoyl conjugate of 3 had a low-energy conformation with a planar sp2-hybridised amide nitrogen in FAAH, while the corresponding covalently linked FAAH-intermediate of AM404 and 2 had pyramidal amide nitrogens in the energy-minimized complexes. These observations, together with the in vitro findings of FAAH-mediated conjugation of compounds 2, 3 and 4-AP, suggest that the amount of N-arachidonoyl conjugate produced may be related to the geometry of its amide nitrogen in the covalently linked

hepatoxicity of the primary amines (Fig. 8F and G). Statistical analysis revealed significant differences in ALT levels between paracetamol and either of 4-AP, HMBA, 2 or 3 (Fig. 8F). Although no
F AAH-intermediate. However, further experiments are needed to consolidate a causal relationship for this observation. The computational studies of AM404 and TRPV1 disclosed that the hydroxyl group enables key interactions with Arg557 and Glu570, which is in line with recent findings [44] and points to a putative link towards the TRP-domain (represented by Gln700), a structure important for the potency and specificity of vanilloid ligands [42,45-47]. Interestingly, when the distance between the hydrogen donating and accepting atom of this hydroxyl group was increased/separated by the presence of an extra heteroatom in 2 and 3, i.e. the hydroxy and methoxy groups in 2 and the two nitrogens of the indazole moiety in 3, the TRPV1 potency of the corresponding N-arachidonoyl analogues was increased. Hypothetically, these moieties better resemble the vanilloid head group of the potent TRPV1 activators capsaicin and resiniferatoxin and possess more optimized interactions towards Arg557 and Glu570. Our findings indicate that aliphatic amines form N-arachidonoyl conjugates with higher TRPV1 potency than aromatic amines, but are poorly biotransformed by FAAH yielding low amounts of their corresponding N-arachidonoyl conjugates. However, the low yield of such N-arachidonoyl conjugates are not necessarily caused by steric hindrance, since FAAH is able to accommodate larger and more complex structures. Thus, additional computational studies are needed in order to develop new analogues with better FAAH interface. In this context, structural variations of compounds 2 and 3 that do not lead to major steric or geometric alteration may be interesting further developments of these two compounds.

Even though the N-arachidonoyl conjugates of 1 and 4 were full agonists and more potent than AM404 as activators of TRPV1, their in vivo transformation into corresponding N-arachidonoyl conjugates was independent of FAAH, and we therefore chose to investigate the antinociceptive properties of 2 and 3 with regard to supraspinal FAAH and TRPV1 dependence. Compounds 2 and 3 demonstrated dose-dependent antinociceptive properties in the two phases of the formalin test, suggesting that their antinociceptive profile resembles that of paracetamol and is different from that of commonly used NSAIDs [17,48]. Interestingly, compound 3 showed an antinociceptive efficacy at least twice as high as compound 2, most likely because 3 is a better substrate for FAAH. Furthermore, compounds 2 and 3 showed an antinociceptive profile that was associated with functional FAAH- and TRPV1-mediated actions in the brain. These effects were, however, restricted to the second phase of the formalin test as FAAH and TRPV1 inhibition by the i.c.v. administration of URB937 and capsazepine, respectively, did not prevent the inhibition of the nociceptive response during first phase of the formalin test in contrast to what has been reported when animals are given paracetamol, 4-AP or HMBA [17,18]. Since URB937 and capsazepine were administered at well-established doses these differences may be best explained by additional FAAH and TRPV1-independent antinociceptive mechanisms of compounds 2 and 3.

The antipyretic action of paracetamol has been attributed to inhibition of central COX [36,49], and the N-arachidonoyl conjugate AM404, which is produced by FAAH from paracetamol-derived 4-AP, has been shown to inhibit COX-mediated production of the pyrogenic mediator PGE2 in vitro [16,27]. We therefore compared the antipyretic effects of compound 3, which was the most efficient FAAH-dependent antinociceptive antinociceptive analogue, and 4-AP with the antipyretic effect of paracetamol. Interestingly, when given at doses that produced antinociception, compound 3 reversed the febrile response in mice, whereas 4-AP had no effect. To better understand the temperature regulating effects of 3 with
regard to pyrexia, we performed additional experiments determining brain prostanoid levels in febrile mice. The effects of the antipyretic paracetamol and the non-antipyretic 4-AP were also studied. In line with our recent findings [37], paracetamol decreased the prostanoid levels in the diencephalon and attenuated the pyrexia in immune challenged mice at a dose that is not antinociceptive (100 mg kg\(^{-1}\)). Interestingly, an antinociceptive dose of 4-AP (30 mg kg\(^{-1}\)) partially reduced the LPS-induced elevation of prostanoids in the diencephalon, but, as reported above, it did not affect the febrile response. Furthermore,
compound 3 did not suppress the LPS-induced elevation of prostanoid levels in the diencephalon of febrile mice, and thus the rapid decrease in core body temperature mediated by 3 is unrelated to inhibition of supraspinal COX synthesis of prostanoids. An acetylated analogue of 3 has been shown to selectively inhibit COX-2 in certain experiments indicating that minor modifications of amino-indazole compounds may counteract febrile responses by multiple mechanisms [56]. The findings that compound 3 in the present study and 4-AP [18] at antinociceptive doses did not cause hypothermia in non-febrile animals and that they did not affect locomotor activity in mice are also important observations as hypothermia is known to affect the antinociceptive readout in animal behavioral studies. Finally, paracetamol was able to equally prevent LPS-induced prostanoid synthesis in the diencephalon of wild-type and FAAH deficient mice, demonstrating that the supraspinal COX inhibition mediated by paracetamol occurs independent of FAAH. Thus, the antipyretic effect of paracetamol, in contrast to its antinociceptive effect [17,25], is not mediated by AM404. Consequently, future therapies that utilize the potential dichotomy between the antipyrctic and antinociceptive effects of paracetamol may be of value as fever is presumably beneficial by enhancing the capacity of the immune system while impairing pathogens [51].

One of the major drawbacks with paracetamol is its degradation into the liver-toxic metabolite NAPQI. Although our analogues 2 and 3 may be less prone to form such electrophilic species, the full molecular mechanism of acetaminophen-induced hepatotoxicity is still unclear. While pharmacological inhibition of TRPV1 did not alleviate acetaminophen-induced hepatotoxicity [52], a recent publication showed that TRPV4 inhibitors are able to reduce paracetamol-induced necrosis in primary human hepatocytes [53]. To evaluate liver toxicity, we performed biochemical and histological liver examinations of animals exposed to paracetamol, 4-AP, HMBA, 2 or 3. We found that 4-AP, HMBA, 2 and 3 did not induce a paracetamol-like hepatotoxicity at a dose at which paracetamol produces a robust injury. 4-AP caused a borderline risk ALT/AST ratio >1, perhaps by conversion into its p-benzoquinone metabolite or by acetylation into paracetamol [54,55].

5. Conclusions

In this study, we have combined in vitro/in vivo studies of FAAH and TRPV1 activity with computational studies to understand the

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**Table 4**

Effect of paracetamol, 4-AP and 3 on LPS-induced prostanoid levels in mouse diencephalon.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PGE₂</th>
<th>PGE₃</th>
<th>6-keto-PGF₁₂</th>
<th>TXB₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paracetamol (100 mg kg⁻¹)</td>
<td>13 ± 5</td>
<td>18 ± 1</td>
<td>17 ± 5</td>
<td>36 ± 5</td>
</tr>
<tr>
<td>4-AP (30 mg kg⁻¹)</td>
<td>44 ± 9</td>
<td>81 ± 8</td>
<td>60 ± 10</td>
<td>60 ± 23</td>
</tr>
<tr>
<td>3 (25 mg kg⁻¹)</td>
<td>96 ± 7</td>
<td>100 ± 6</td>
<td>99 ± 4</td>
<td>91 ± 5</td>
</tr>
<tr>
<td>Paracetamol (150 mg kg⁻¹)</td>
<td>8 ± 2</td>
<td>19 ± 4</td>
<td>15 ± 3</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>FAAH⁻⁻⁻⁻⁻</td>
<td>9 ± 3</td>
<td>10 ± 3</td>
<td>12 ± 2</td>
<td>18 ± 6</td>
</tr>
</tbody>
</table>

The inhibition of COX was evaluated by analysing the content of prostanoids in diencephalon from mice immune challenged with LPS (100 mg kg⁻¹) or saline, administered intraperitoneally (i.p.) 3 h prior to treatment for 40 min with paracetamol, 4-AP, 3 or their vehicle (n = 6). P < 0.0001 (PGE₂, PGE₃ and 6-keto-PGF₁₂). P < 0.01 (6-keto-PGF₁₂). P < 0.001 (PGE₂, P < 0.05 (PGF₃α). P < 0.001 (6-keto-PGF₁₂) for 4-AP vs vehicle (control). No difference in prostanoid contents was observed after treatment with 3. Statistical significance was accepted when P < 0.05 (1-way ANOVA followed by Dunnett’s multiple comparisons test). The prostanoid assay was also used in a separate set of experiments to evaluate the ability of paracetamol (150 mg kg⁻¹ i.p.) to inhibit COX in FAAH⁻⁻⁻⁻⁻ (n = 10–11) and FAAH⁻⁻⁻⁻⁻ (n = 5) mice. No difference was observed in the prostanoid content between FAAH genotypes (P = 0.1, Student’s unpaired t-test). Values are calculated as a percentage of those obtained in febrile animals treated with compound vehicle. Data are presented as mean ± SEM.

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**Fig. 8**

Evaluation of liver injury. Male C57/BL6 mice were fasted for 12–16 h before receiving an intraperitoneal injection of 300 mg kg⁻¹ paracetamol, 4-AP, HMBA, 2 or 3. Serum samples and liver tissues were collected 12 h after administration. (A–E) Micrographs of liver samples from paracetamol (A), 4-AP (B), HMBA (C), 2 (D) and 3 (E) treated animals, stained with haematoxylin and eosin. Necrotic areas are visualized as lighter zones surrounding hepatic vessels (arrows). Scale bar = 200 μm. (F) The liver damage caused by paracetamol is associated with increased hepatic alanine aminotransferase (ALT) levels. The ALT levels were significantly higher after exposure to paracetamol compared to those seen after exposure to 4-AP (*P = 0.0139), HMBA (*P = 0.0122), 2 (*P = 0.0121) and 3 (*P = 0.0124) as determined by the Welch’s ANOVA test. (G) A ratio between ALT and aspartate aminotransferase (AST) >1 is a clinical marker for hepatic injury. Paracetamol and 4-AP increased this ratio close to 1 (4-AP) and above (paracetamol, *P = 0.0191), whereas the ratios for HMBA, 2 and 3 were far below 1 (**P < 0.0001); one sample t-test compared to the ratio of 1 (ns = not significant). Data are presented as mean ± SEM (n = 6–7).
structure-activity relationship of paracetamol and primary amine analogues thereof at FAAH and TRPV1, with the aim to find paracetamol analogues that are antinociceptive at lower doses and without acute liver toxicity. We found that the N-arachidonoyl conjugates of 5-amino-2-methoxyphenol (2) and 5-aminooindazole (3) displayed efficient target protein interactions with FAAH and TRPV1, and possessed a dose-dependent antinociception in both phases of the formalin test that during the second phase was mediated by FAAH and TRPV1 in the brain of mice. Importantly, none of the compounds demonstrated hepatotoxicity. We also demonstrate that the antipyrine COX inhibitory action of paracetamol, in contrast to its antinociceptive mechanism, does not involve FAAH-dependent biotransformation of paracetamol into its N-arachidonoyl conjugate AM404. Furthermore, compound 3 reduced fever without inhibiting prostanoid formation in the brain, suggesting a COX-independent mechanism. Our study shows that analogues and antipyrines without liver toxicity can be derived from paracetamol. Thus, further research into the molecular actions of paracetamol could pave the way for the discovery of analgesics and antipyrines with a better benefit-to-risk ratio.

Author contributions


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