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The dual fatty acid amide hydrolase/TRPV1 blocker, *N*-arachidonoyl-serotonin, relieves carrageenan-induced inflammation and hyperalgesia in mice

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ABSTRACT

Given that the pharmacological or genetic inactivation of fatty acid amide hydrolase (FAAH) counteracts pain and inflammation, and on the basis of the established involvement of transient receptor potential vanilloid type-1 (TRPV1) channels in inflammatory pain, we tested the capability of a dual FAAH/TRPV1 blocker, *N*-arachidonoyl-serotonin (AA-5-HT), to relieve oedema and pain in a model of acute inflammation, and compared its efficacy with that of a single FAAH inhibitor (URB597) or TRPV1 antagonist (capsazepine). Acute inflammation was induced by intraplantar injection of λ-carrageenan into mice and the anti-inflammatory and anti-nociceptive actions of AA-5-HT were assessed at different doses, time points and treatment schedule. In addition, endocannabinoid levels were measured in paw skin and spinal cord. Systemic administration of AA-5-HT elicited dose-dependent anti-oedemigen and anti-nociceptive effects, whereas it was devoid of efficacy when given locally. When tested in a therapeutic regimen, the compound retained comparable anti-inflammatory effects. TRPV1 receptor mediated the anti-inflammatory property of AA-5-HT, whereas both CB_1 and TRPV1 receptors were involved in its anti-hyperalgesic activity. These effects were accompanied by an increase of the levels of the endocannabinoid anandamide (AEA) in both inflamed paw and spinal cord. AA-5-HT was more potent than capsazepine as anti-oedemigen and anti-hyperalgesic drug, whereas it shows an anti-oedemigen property similar to URB597, which was, however, devoid of the anti-nociceptive effect. AA-5-HT did not induce unwanted effects on locomotion and body temperature. In conclusion AA-5-HT has both anti-inflammatory and anti-hyperalgesic properties and its employment offers advantages, in terms of efficacy and lack of adverse effects, deriving from its dual activity.

1. Introduction

N-arachidonoyl-serotonin (AA-5-HT) is a synthetic derivative of arachidonic acid acting as inhibitor of fatty acid amide hydrolase (FAAH) [1], the enzyme responsible for the inactivation of the endocannabinoid anandamide (AEA). This FAAH inhibitor (IC_{50} = 1–12 μM in various preparations [1,2]) lacks agonistic activity at the cannabinoid CB_1 and CB_2 receptors, is devoid of activity on serotonin receptor, does not inhibit AEA cellular uptake and does not significantly interfere with cytosolic phospholipase A_2 [1]. Furthermore, with respect to other FAAH inhibitors developed so far (e.g. URB597, OL-135), AA-5-HT does not inhibit other carboxylesterases at concentrations lower than 10 μM. Importantly, AA-5-HT is not hydrolysed by FAAH, and, hence, does not generate its parent bioactive compounds, arachidonic acid and serotonin [1]. Accordingly, i.p. administration of AA-5-HT to rats does not elevate serotonin levels in the brain [3]. This latter observation, together with the demonstration that AA-5-HT forms a rather stable even though not covalent, adduct with the enzyme, implies that this compound can be a highly suitable FAAH inhibitor to be employed *in vivo*. The ability of AA-5-HT to elevate AEA levels *in vivo* following various types of administration protocols [3,4] is of significance, too. Owing to its inhibitory activity on FAAH, AA-5-HT has been tested in some animal models of diseases associated with alterations in the endocannabinoid tone and for which an increase in AEA level could be therapeutically useful. Particularly, AA-5-HT was effective in an animal model of inflammatory bowel disease [5], potentiated stress-induced analgesia [4], inhibited cancer growth both *in vitro* [6] and *in vivo* [7], and, more recently, its ability to counteract both acute peripheral and chronic neuropathic pain was established [8]. Interestingly, AA-5-HT is also capable to antagonize human and rat recombinant vanilloid TRPV1 receptors, with potency slightly higher than that of the widely used

Abbreviations: AA-5-HT, *N*-arachidonoyl-serotonin; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; FAAH, fatty acid amide hydrolase; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; TRPV1, transient receptor potential vanilloid type-1.

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TRPV1 antagonist, capsazepine [8]. Thus, AA-5-HT is now defined as a unique dual FAAH/TRPV1 blocker [8]. Given that the pharmacological or genetic inactivation of FAAH usually counteracts pain and inflammation [9–12], and on the basis of the well established involvement of TRPV1 receptors in driving inflammatory nociceptive response [13], the capability of AA-5-HT to modulate simultaneously these two distinct targets might be potentially very useful. These observations prompted us to test this compound in a murine model of inflammation/inflammatory pain such as that evoked by carrageenan. A further aim of this study was to compare AA-5-HT efficacy with that of a FAAH inhibitor, such as URB597, and of a TRPV1 antagonist, i.e. capsazepine. We report that AA-5-HT possesses both anti-inflammatory and anti-hyperalgesic properties and that vanilloid and cannabinoid receptors are differently involved in these effects. Finally, we provide evidence that the use of AA-5-HT offers some advantages deriving from its dual activity as compared to selective inhibitors.

2. Materials and methods

2.1. Carrageenan-induced inflammation

All experiments performed were in accordance with Italian and European regulations governing the care and treatment of laboratory animals (Permission No. 41/2007B) and conformed to the guidelines for the study of pain in awake animals established by the International Association for the Study of Pain [14]. Male C57BL/6J mice (9 weeks old, Harlan, Italy) were housed under controlled illumination (12 h light/12 h dark cycle) and standard environmental conditions (room temperature $22 \pm 1\,^{\circ}C$, humidity $60 \pm 10\%$) and allowed to acclimatize for at least one week before experimental use. Standard food and water were available *ad libitum*. All efforts were made to reduce both animal numbers and suffering during the experiments, in agreement with Ethical Guidelines of the IASP. All behavioural evaluations were performed by experimenters blind to treatments. Mice were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.). Inflammation was induced by intraplantar injection of 20 μl of λ-carrageenan (2% in saline) into the right hind paw.

2.2. Experimental design

AA-5-HT (0.1, 1 and 5 mg/kg, i.p.), URB597 (0.1, 1 and 3 mg/kg, i.p.) or capsazepine (2.5, 5, 10 and 20 mg/kg, i.p.) or their vehicle were administered 30 min before carrageenan injection. AA-5-HT, URB597 and capsazepine were dissolved in 10% dimethylsulphoxide and 90% saline. Capsazepine and URB597 were also i.p. co-administered at submaximal doses (5 mg/kg capsazepine plus 1 mg/kg URB597) 30 min before carrageenan. To investigate the receptors involved in the effect of AA-5-HT (5 mg/kg), mice were pre-treated (15 min before AA-5-HT) with the CB_1 receptor antagonist, rimonabant (0.5 mg/kg i.p), or with the selective CB_2 receptor antagonist SR144528 (1 mg/kg i.p.), or with olvanil (1 mg/kg i.p.), a selective TRPV1 receptor agonist. Rimonabant and SR144528 were dissolved in a mixture of Tween80:DMSO:distilled water (1:2:7); olvanil was dissolved in a mixture of ethanol:Tween80:saline (1:1:8). In order to evaluate the contribution of a local effect versus systemic one, in one set of experiments, AA-5-HT was administered subcutaneously into the hindpaw (10, 250 and 1000 μg each mouse), 5 min prior to carrageenan. Finally, to determine the ability of AA-5-HT to improve the established disease, in a series of experiments AA-5-HT was administered at 5 mg/kg, i.p to mice in a therapeutic regimen: 24 h after the intraplantar injection of carrageenan.

2.3. Evaluation of paw oedema and thermal and mechanical hyperalgesia

The volume of the injected, as well as the contralateral paw, was assessed with a plethysmometer (Ugo Basile, Varese, Italy) 2, 4 and 24 h after carrageenan injection. Data are expressed as oedema (difference in volume between the injected and non injected paws). Thermal hyperalgesia was evaluated on the same animals used to determine oedema, employing the radiant heat method [15]. After recording of baseline withdrawal latencies (s), latency of the withdrawal of both hind paws was estimated 3, 6 and 24 h after carrageenan injection. Since at the 2 h time point some animals had not still recovered from anaesthesia, the test was performed when they had completely recovered, that is 3 h after carrageenan. Briefly, animals were placed in a clear plexiglass box and allowed to acclimatize. A constant intensity, radiant heat source was aimed at the midplantar area of the hind paw. The time, in seconds, from initial heat source activation until paw withdrawal, was recorded. To determine whether AA-5-HT was also effective at counteracting mechanical hyperalgesia, mice treated with vehicle or with the highest dose of AA-5-HT (5 mg/kg) were submitted to the Randall-Selitto apparatus (Ugo Basile, Varese, Italy) evaluating the withdrawal threshold following a mechanical stimulus.

2.4. Endocannabinoid extraction

A group of non pathological mice, as well as mice injected with carrageenan and treated with AA-5-HT (5 mg/kg, i.p.) or with vehicle, were sacrificed 2 h after the phlogogen. Ipsilateral and contralateral paw skin samples as well as spinal cords (L4-L6 tract) were removed and homogenized in 5 vol of chloroform:methanol:Tris-HCl 50 mM (2:1:1) containing 50 pmol of d_8-AEA, d_4-palmitoylethanolamide (PEA), d_5–2-arachidonoylglycerol (2-AG) and d_4–oleoylethanolamide (OEA). Homogenates were centrifuged at $13{,}000 \times g$ for 16 min (4 °C), the aqueous phase plus debris were collected and extracted again twice with 1 vol of chloroform. The organic phases from the three extractions were pooled and the organic solvents evaporated in a rotating evaporator.

2.5. Evaluation of endocannabinoid content

Lyophilized extracts were resuspended in chloroform:methanol (99:1). The solutions were then purified by open bed chromatography on silica as previously described [16]. Fractions eluted with chloroform:methanol (9:1) and containing AEA, PEA, 2-AG and OEA, were collected and the excess solvent evaporated with a rotating evaporator, and aliquots analysed by isotope dilution-liquid chromatography/atmospheric pressure chemical ionization/mass spectrometry (MS) carried out under conditions described previously [16] and allowing the separations of 2-AG, PEA, AEA and OEA. Results are expressed as nmol per g of wet tissue, or pmol per g in the case of AEA.

2.6. Evaluation of central unwanted effects

A series of four consecutive observations were conducted on each carrageenan-injected mice, following a standard procedure employed to evaluate psychoactive cannabinoid-induced effects [17]. Briefly, mice were tested for body temperature, nociceptive threshold, spontaneous locomotor activity and ring immobility 30, 60, 150, 210 and 240 min after drug or vehicle administration. Body temperature was measured with a rectal thermistor probe (Ellab, Roedrove, Denmark) inserted to a constant depth of 2 cm. To determine thermal nociception, mice were tested for responsiveness to radiant heat applied to the non injected paw with a plantar test (Ugo

Basile, Varese, Italy). Spontaneous locomotor activity was counted for 5 min and performed with an activity cage (Ugo Basile, Varese, Italy), which recorded the number of horizontal animal movements through infrared sensors. Immobility time was measured by the ring test described by Pertwee [18]. The apparatus consisted of a wire ring, 6 cm in diameter, fixed horizontally to a ring stand at a point 22 cm above the desk. The experimenter placed the mouse across the ring, so that it was supported only by its front and rear paws. Its forepaws were placed on diametrically opposite points on the ring. The number of seconds the mouse remained motionless on the ring (except for breathing movements) was recorded over a period of 5 min. Data are expressed as percentage of immobility.

2.7. Drugs and reagents

AA-5-HT was synthesized in our laboratory as previously described [1]. Rimonabant and SR144528 were kindly supplied by Sanofi-Aventis (Montpellier, France). URB597 was purchased from Cayman Chemical (Ann Arbor, MI, USA) whereas olvanil, capsazepine and carrageenan were purchased from Sigma–Aldrich (Milano, Italy). Deuterated compounds were either synthesised in house or purchased from Cayman Chemical (Ann Arbor, MI, USA).

2.8. Statistical analysis

The results are expressed as the mean ± S.E.M. Data were analyzed by ANOVA followed by Tukey's *post hoc* test for multiple comparisons. Significance was set at $P < 0.05$. All statistical analyses, including linear regression analysis and the calculation of the mean effective dose (ED_{50}), were done using the statistical GraphPad Software package (San Diego, CA, U.S.A.).

3. Results

3.1. Anti-inflammatory and anti-hyperalgesic effect of AA-5-HT

Intraplantar injection of carrageenan led to an increase in ipsilateral hindpaw volume, expressed as oedema, that is the difference between the ipsilateral paw and the non-injected contralateral paw (Fig. 1A). At 2 h after carrageenan, the oedema was significantly reduced by the pre-administration of AA-5-HT in a dose-dependent manner with a maximum effect elicited by 5 mg/kg ($r^2 = 0.9950$, $F = 200.6$, $P < 0.05$) (Fig. 1A). Carrageenan injection also induced a marked thermal hyperalgesia, as shown in Fig. 1B, assessed as decreased paw withdrawal latency in response to a thermal stimulus. AA-5-HT administration resulted in a dose-related reduction of thermal hypersensitivity ($r^2 = 0.9310$, $F = 94.42$, $P < 0.001$) (Fig. 1B). At no dose did AA-5-HT affect the withdrawal latency of the contralateral paws (9.05 ± 0.426, 9.60 ± 0.569, 9.20 ± 0.153 s for AA-5-HT 5, 1 and 0.1 mg/kg, respectively, versus 9.12 ± 0.471 s of control/inflamed mice). The carrageenan-induced oedema remained high throughout the observation period of 24 h (Fig. 2A). At this time, the animals treated with the highest dose of AA-5-HT (5 mg/kg) were significantly different from both non-inflamed and inflamed mice, suggesting that the anti-inflammatory effect was reduced but still present (Fig. 2A). Thermal hyperalgesia evoked by carrageenan remained unaltered at 24 h (Fig. 2B). The anti-hyperalgesic effect of AA-5-HT (5 mg/kg) diminished over the observation period and disappeared at 24 h (Fig. 2B). Interestingly, AA-5-HT failed to evoke significant anti-oedemigen and anti-hyperalgesic effects when administered locally before carrageenan up to 1 mg/paw (Fig. 3A and B). The highest dose of AA-5-HT exerted also a significant relief of carrageenan-induced mechanical hyperalgesia. Particularly, carrageenan injection reduced the withdrawal threshold following a mechanical stimulus from 135 ± 5 g to 50 ± 2.04 g; AA-5-HT pre-administration (5 mg/kg, i.p.) significantly increased the nociceptive threshold to 108.3 ± 1.67 g. Collectively these findings highlight the anti-inflammatory and anti-hyperalgesic efficacy of AA-5-HT when systemically administered in a preventive regimen.

3.2. Potency of AA-5-HT in comparison with URB597 and capsazepine

Since AA-5-HT acts as both FAAH inhibitor and TRPV1 receptor antagonist, we examined the advantages deriving from its use with respect to a FAAH inhibitor and to a TRPV1 receptor antagonist, by comparing the efficacy of AA-5-HT as anti-oedemigen and anti-hyperalgesic with that of URB597 (FAAH inhibitor) and capsazepine (TRPV1 receptor antagonist), in the same animal model. Fig. 1A and B shows the effect of different doses of URB597 and capsazepine on carrageenan-induced oedema and thermal hyperalgesia, respectively, in comparison with AA-5-HT. Both compounds elicited a dose-dependent anti-oedemigen effect when administered i.p. before carrageenan. For AA-5-HT, URB597 and capsazepine, a dose–response curve was obtained correlating the anti-oedemigen effect (expressed as percentage of oedema inhibition) and the log dose of compound, in order to calculate the ED_{50}. Table 1 shows the ED_{50} values for the three compounds tested with the relative 95% confidence intervals. URB597 is the most active compound in suppressing carrageenan-induced oedema: its potency is about 2-fold greater than that of AA-5-HT and 29-fold

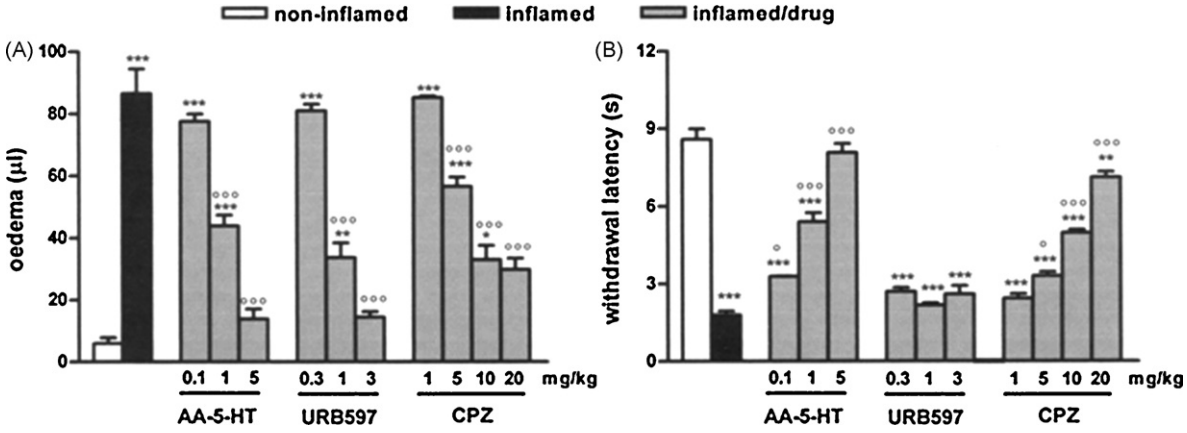

Fig. 1. Dose-related effect of AA-5-HT, URB597 and capsazepine (CPZ), administered in a preventive regimen, on oedema (A) evaluated 2 h after carrageenan, and on thermal hypersensitivity (B) evaluated 3 h after carrageenan. Data represent means ± S.E.M. ($N = 8$ for all groups). $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ versus non-inflamed; $^{\circ\circ\circ}P < 0.001$, $^{\circ}P < 0.05$ versus inflamed.

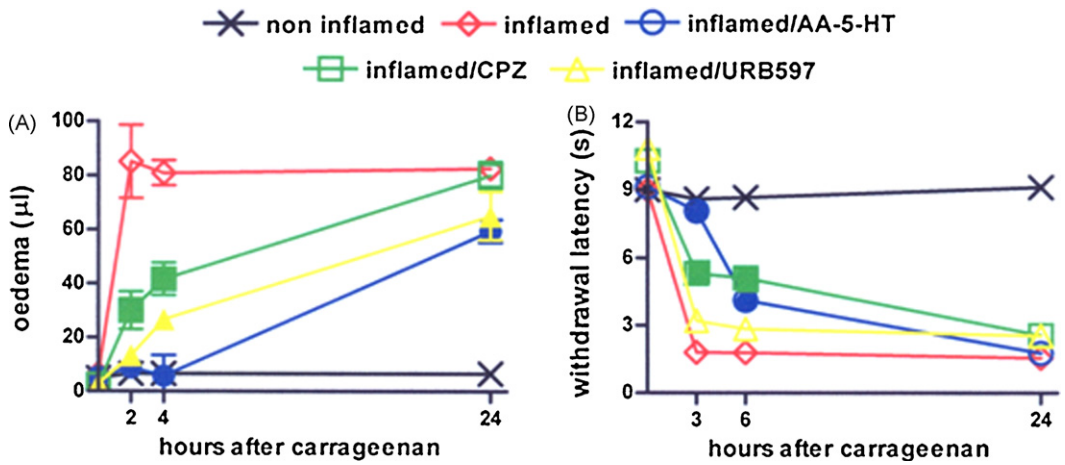

Fig. 2. Effect of AA-5-HT (5 mg/kg i.p.), capsazepine (CPZ, 10 mg/kg i.p.) and URB597 (3 mg/kg i.p.) on oedema (A) and thermal hypersensitivity (B) at different time points after carrageenan. Data represent means ± S.E.M. ($N = 5$ for all groups). Significant differences from the control (inflamed) group are indicated by filled symbols. $P < 0.05$ was considered statistically significant.

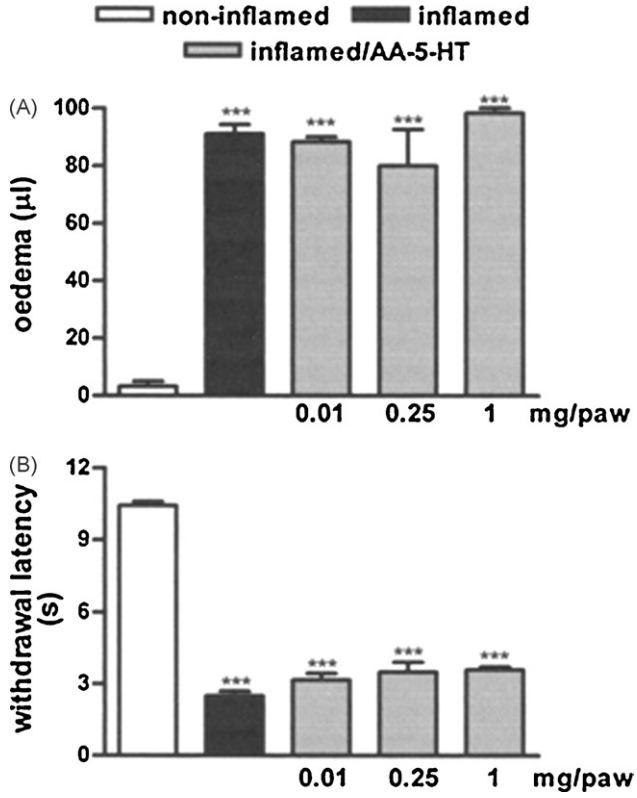

Fig. 3. Effect of AA-5-HT locally administered 5 min before carrageenan on oedema (A) and on thermal hypersensitivity (B). Data represent means ± S.E.M. ($N = 3$ for all groups). $^{***}P < 0.001$ versus non-inflamed.

greater than that of capsazepine (Table 1). When tested as an anti-hyperalgesic compound, capsazepine reduced thermal hyperalgesia in a dose-dependent manner, with an ED_{50}, obtained as previously described for anti-oedema effect, of 11 mg/kg (Table 1). On the contrary URB597 did not affect thermal hyperalgesia in carrageenan-treated mice even at the dose able to completely prevent oedema (3 mg/kg). The comparison between the compounds indicates that AA-5-HT is 4-fold more potent than capsazepine in relieving pain hypersensitivity (Table 1). These data suggest that AA-5-HT is more potent than capsazepine as anti-inflammatory and anti-hyperalgesic drug; AA-5-HT shows a similar anti-oedemigen property to URB597, but the latter is devoid of anti-nociceptive effect. Fig. 2A and B shows the time course of the effects of capsazepine and URB597 in carrageenan-injected mice at the maximal effective dose found during the experiments described in Fig. 1, in comparison with AA-5-HT. Both URB597 and capsazepine evoked an anti-oedemigen effect that peaked 2 h after carrageenan and decreased during the subsequent time points (Fig. 2A). At no time point did URB597 induce relief of thermal hyperalgesia, whereas capsazepine evoked a similar partial effect both at the 3 h and 6 h time points (Fig. 2B). As for AA-5-HT such an effect disappeared 24 h after carrageenan (Fig. 2B). To further exploit the advantage of AA-5-HT, we compared its effect with that evoked by the co-administration of URB597 and capsazepine (Fig. 4). A significant reduction of oedema development was reached with URB597 and capsazepine together, even if no improvement was shown in comparison with both the compounds alone and AA-5-HT (Fig. 4A). Conversely, the simultaneous administration of URB597 and capsazepine elicited an anti-hyperalgesic effect stronger than that evoked by the compounds given alone, but weaker than that induced by AA-5-HT (Fig. 4B).

3.3. CB_1, CB_2 and TRPV1 receptor involvement in AA-5-HT-induced anti-inflammatory and anti-hyperalgesic effects

The ability of rimonabant, a selective antagonist of the CB_1 receptor, SR144528, a selective antagonist of the CB_2 receptor, and olvanil, a selective TRPV1 receptor agonist, to reverse the effect elicited by AA-5-HT, was tested on both oedema and thermal hypersensitivity. The mentioned compounds were administered 15 min before AA-5-HT 5 mg/kg and oedema and thermal hyperalgesia evaluations were performed 2 h and 3 h after carrageenan injection,

Table 1
ED_{50} values of tested compounds as anti-inflammatory[a] and anti-hyperalgesic[b] compounds.

Compound	[a]ED_{50} (mg/kg, i.p.)	95% confidence intervals	[b]ED_{50} (mg/kg, i.p.)	95% confidence intervals
AA-5-HT	1.00	0.48–2.07	2.46	1.24–4.89
URB597	0.54	0.21–1.35	No effect	–
Capsazepine	15.6	3.43–71.1	11.00	5.12–23.6

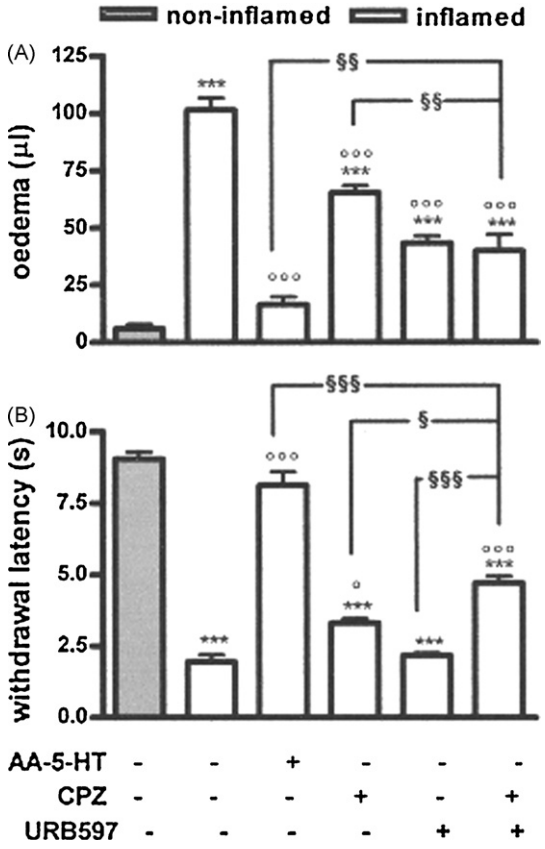

Fig. 4. Effect of capsazepine (CPZ, 5 mg/kg i.p.) and URB597 (1 mg/kg i.p.) administered alone or in combination on oedema (A), evaluated 2 h after carrageenan, and on thermal hypersensitivity (B), evaluated 3 h after carrageenan, in comparison with AA-5-HT (5 mg/kg i.p.). Data represent means ± S.E.M. ($N = 5$ for all groups). $^{***}P < 0.001$ versus non-inflamed; $^{\circ\circ\circ}P < 0.001$, $^{\circ}P < 0.05$ versus inflamed; §§§ $P < 0.001$, §§ $P < 0.01$, § $P < 0.05$.

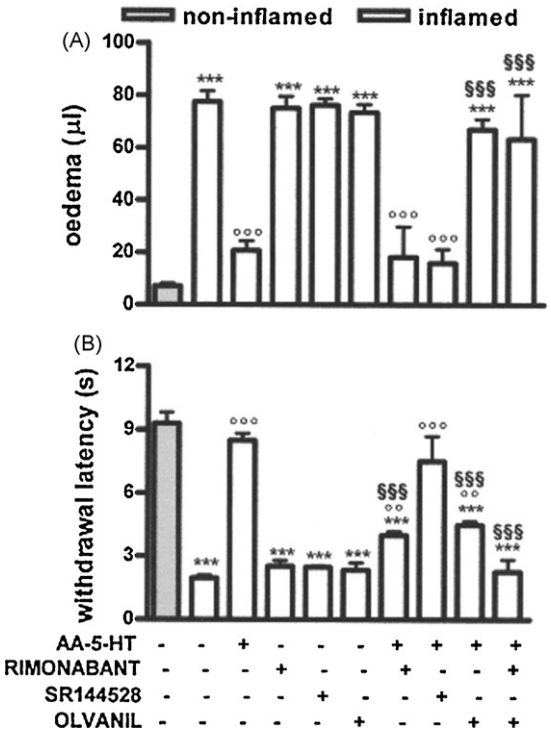

Fig. 5. Effect of rimonabant (0.5 mg/kg i.p), SR144528 (1 mg/kg i.p.) and olvanil (1 mg/kg i.p.) on anti-oedemigen (A) and anti-hyperalgesic (B) effect evoked by AA-5-HT (5 mg/kg i.p.). The compounds were administered 15 min before the drug and behavioural evaluations were assessed 2 h (oedema) and 3 h (thermal hyperalgesia) after carrageenan. Data represent means ± S.E.M. ($N = 6$ for non inflamed, inflamed/vehicle and inflamed/antagonists alone groups; $N = 8$ for inflamed/AA-5-HT and for inflamed/AA-5-HT/antagonist groups). $^{***}P < 0.001$ versus non-inflamed; $^{\circ\circ\circ}P < 0.001$, $^{\circ\circ}P < 0.01$ versus inflamed; §§§ $P < 0.001$ versus AA-5-HT.

respectively. Fig. 5A shows that only olvanil was able to reverse the anti-oedemigen effect evoked by AA-5-HT, suggesting TRPV1 receptor as the unique target for the anti-inflammatory activity of the compound. Olvanil was also able to partially antagonized the AA-5-HT-induced anti-hyperalgesia; moreover, this effect was partially reversed by rimonabant (Fig. 5B). The combination of both drugs completely reversed the anti-hyperalgesic property of AA-5-HT, thus suggesting that this effect was mediated by both CB_1 and TRPV1 receptors (Fig. 5B). Rimonabant, SR144528 and olvanil given alone at the doses employed in the antagonism studies, did not affect the paw oedema and the nociceptive response of animals (Fig. 5A and B). The doses of rimonabant and SR144528 employed herein were previously shown to be able to highlight the involvement of CB_1 or CB_2 receptors, respectively, by us [10,19] and others [20]. To ascertain the capability of the employed dose of olvanil to counteract an effect induced by a TRPV1 antagonist, we pre-administered to inflamed mice the same dose of olvanil before capsazepine (10 mg/kg), and showed that olvanil antagonized also the partial anti-oedemigen and anti-hyperalgesic effect of capsazepine (data not shown).

3.4. Endocannabinoid levels following AA-5-HT administration in inflamed mice

The evaluation of the levels of the two major endocannabinoids (AEA and 2-AG), as well as of PEA and OEA, in the ipsilateral paw skin from non-inflamed and inflamed mice demonstrated a significant decrease of AEA (41%), PEA (52%) and OEA (59%), whereas the levels of 2-AG remained unchanged (Fig. 6A). The single administration of AA-5-HT (5 mg/kg) to inflamed mice significantly prevented AEA decrease without affecting the reduced levels of either PEA or OEA (Fig. 6A). The tissue concentrations of these endogenous substances in the contralateral paw were unaffected by carrageenan (data not shown). Interestingly, AA-5-HT administration did not alter AEA levels in the contralateral paw (data not shown). These data highlight the ability of AA-5-HT to prevent the changes of the endocannabinoid AEA in the damaged site. The measurement of the same endogenous compounds in the spinal cord revealed only a decrease (31%) in AEA content that, however, did not reach the statistical significance, and a general tendency towards a decrease for the others (Fig. 6B). Opposite to that observed in the paw skin, the single administration of AA-5-HT (5 mg/kg) to carrageenan-treated mice not only prevented AEA decrease in the spinal cord, but it brought AEA content markedly over the physiological one (Fig. 6B), without affecting the level of 2-AG, PEA and OEA (Fig. 6B).

3.5. Effects on motor activity, body temperature and general CNS function

Because of the involvement of CB_1 and TRPV1 receptors in the pharmacological effect of AA-5-HT, we evaluated the potential unwanted effects associated with the activation of CB_1 receptor (psychoactivity and hypolocomotion) and the blockade of TRPV1 (hyperthermia). At 30, 60, 150, 210 and 240 min after a single i.p. administration of AA-5-HT (5 mg/kg), the body temperature, nociceptive threshold, locomotor activity and ring immobility of each mouse were consecutively measured. The data, shown in Fig. 7, indicate this dose did not alter body temperature and locomotor activity and did not induce catalepsy or analgesia at any time

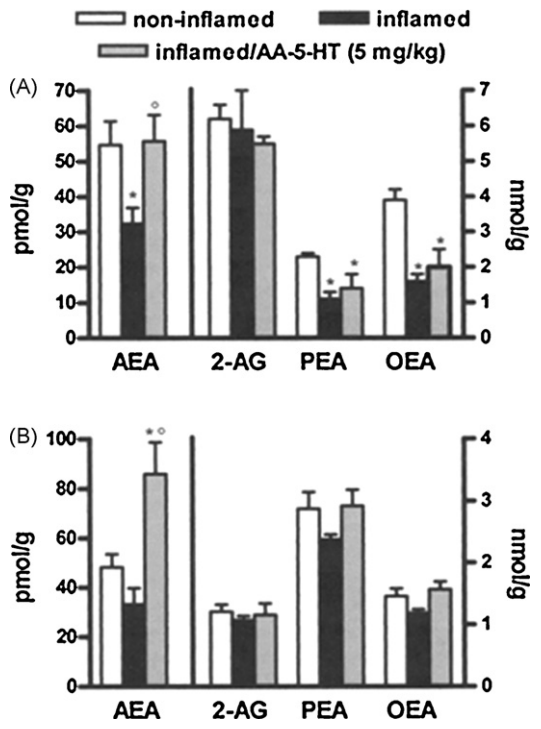

Fig. 6. Levels of the two endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) and of the two congeners palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) in the ipsilateral paw skin (A) and in the spinal cord (L4-L6 tract) (B) collected 2 h after carrageenan. Data are normalized on g of fresh tissue. Data represent means ± S.E.M. ($N = 4$ for non inflamed and inflamed group; $N = 5$ for AA-5-HT group). $^{*}P < 0.05$ versus non-inflamed; $^{\circ}P < 0.05$ versus inflamed.

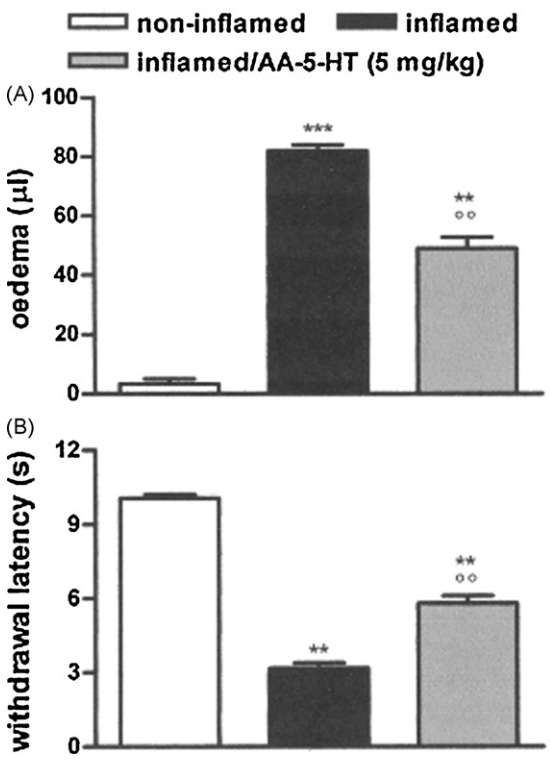

Fig. 8. Effect of AA-5-HT i.p administered in a therapeutic regimen (24 h after carrageenan), on oedema (A), evaluated 150 min after drug, and on thermal hypersensitivity (B), evaluated 210 min after drug. Data represent means ± S.E.M. ($N = 5$ for all groups). $^{***}P < 0.001$, $^{**}P < 0.01$ versus non-inflamed; $^{\circ\circ}P < 0.01$ versus inflamed.

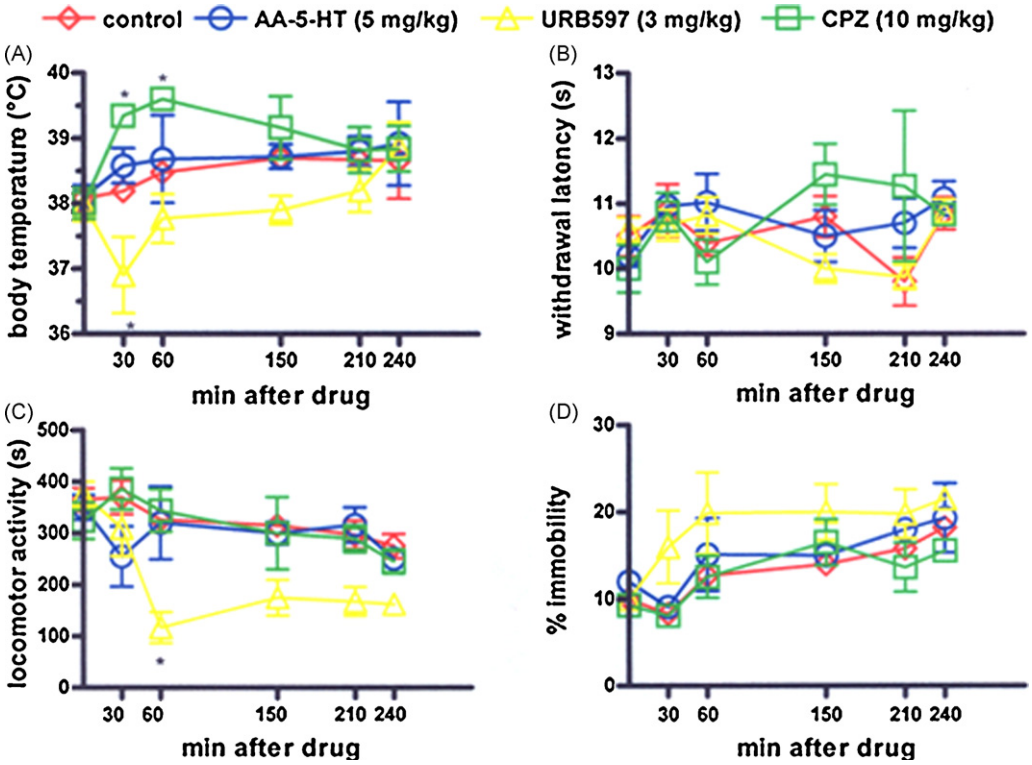

Fig. 7. Effect of AA-5-HT, URB597 and capsazepine (CPZ), 30, 60, 150, 210 and 240 min after their i.p. administration on body temperature (A), nociceptive threshold (B), spontaneous locomotor activity (C), immobility on a ring (D). Data represent means ± S.E.M. ($N = 4$ for control group; $N = 5$ for drug-treated groups). $^{*}P < 0.05$ versus control.

points, suggesting that AA-5-HT is unable to induce the typical unwanted effects of CB_1 agonists or TRPV1 antagonists in mice. In the same figure (Fig. 7) we reported the effect of URB597 and capsazepine i.p. administration in the same tetrad of assays. Despite the fact that high doses of this FAAH inhibitor (10 mg/kg) are needed to exert in mice the typical signs of cannabimimetic effects (data not shown), following the administration of a dose active on carrageenan-induced oedema (3 mg/kg), mice exhibited slight although significant hypothermia and hypomotility at 30–60 time point (Fig. 7). For a comparison, we evaluated, in the same model, the effect of capsazepine, and showed that 10 mg/kg i.p. of capsazepine evoked a significant increase in mice body temperature (+1.3 °C) already 30 min following the administration and much more after 60 min (+1.6 °C); body temperature then decreased during the subsequent time points to values no more different from controls (Fig. 7).

3.6. Anti-inflammatory and anti-nociceptive therapeutic effect of AA-5-HT

To test the ability of AA-5-HT to counteract an established inflammatory process, the maximal dose of the drug (5 mg/kg) was administered in a therapeutic regimen, that is 24 h after carrageenan injection. Fig. 8A shows that AA-5-HT counteracted oedema (about 40%) evoked by carrageenan. With this regimen, AA-5-HT also significantly reduced carrageenan-induced thermal hyperalgesia (Fig. 8B).

4. Discussion

Data from FAAH knockout mice indicate that there are significantly reduced inflammatory responses following genetic ablation of FAAH in both the formalin and carrageenan models [9]. Accordingly, the pharmacological inhibition of this enzyme by URB597 resulted in a significant reduction of oedema development in a murine model of carrageenan-induced acute inflammation [10]. The development of selective FAAH inhibitors opens an important therapeutic scenario based on the assumption that this approach leads to the enhancement of endocannabinoid level mainly in those tissues in which there is an ongoing production of otherwise silent endocannabinoids, thus avoiding the unwanted side effects due to the use of direct cannabinoid receptor agonists [21]. However, FAAH inhibitors are likely to prolong the lifespan also of other substrates of this enzyme, such as OEA and PEA, which stimulate targets different from cannabinoid receptors. Further problems with FAAH inhibitors are represented by the redundancy of pathways inactivating endocannabinoids: both AEA and 2-AG might become substrates for COX-2 and cytochrome P450, thereby potentially giving rise to other active metabolites [22]. Particularly, AEA and 2-AG can be converted by COX-2 to prostamides and prostaglandin glyceryl esters, respectively, whereas cytochrome P450 metabolizes AEA and 2-AG into hydroxyeicosatetraenoic acids [23 for review]. Although the biological effects of such metabolites await further investigation, it has been recently reported that some COX-2 metabolites of 2-AG acted as pro-nociceptive ligands [24] and that oxidative metabolites of endocannabinoids also behave as PPARα ligands [23]. In addition, the increased levels of AEA following FAAH inhibition, might lead to the stimulation of TRPV1 receptors, known to trigger intracellular events that are often opposite to those produced by cannabinoid receptor activation, and recently shown to inhibit the biosynthesis of the other endocannabinoid, 2-AG, in some brain areas [25,26]. TRPV1 receptors are non-selective cation channels activated by noxious heat, low pH and vanilloids and abundantly expressed in sensory neurons, and are required for inflammatory sensitization to noxious thermal stimuli [13]. The analgesic potential of TRPV1 receptor blockade has been demonstrated by various approaches including gene disruption [27,13], neutralizing antibodies [28] or receptor antagonism [29]. In spite of the beneficial effects of TRPV1 antagonists against painful diseases, their employment in both preclinical and clinical studies revealed some potential side effects including gastric ulcer formation, hypertension and hyperthermia. This latter symptom is the most relevant unwanted effect observed during recent clinical trials [30]. Here we tested the hypothesis that one possible strategy to retain/ameliorate the beneficial properties of FAAH inhibitors and TRPV1 antagonists with a simultaneous decrease in the potential unwanted effects may be to concentrate in one molecule the capability to inhibit FAAH and to antagonize TRPV1 receptors, such as in AA-5-HT.

Consistent with the efficacy of this approach, we report for the first time here that the systemic administration of AA-5-HT prevents the development of carrageenan-induced oedema and hyperalgesia. A dose-dependent relationship was found for both effects, with a dose of 5 mg/kg decreasing oedema and thermal hyperalgesia to levels undistinguishable from non-inflamed mice. The anti-oedemigen effect was still present at 24 h, indicating a lasting efficacy of this compound, whereas the relief of pain hypersensitivity decreased between 6 and 24 h. Previous studies highlighted the metabolic stability of AA-5-HT and, particularly, experiments performed *in vivo* after systemic administration of AA-5-HT failed to detect serotonin or its metabolite 5-IHAA [3]. These data support our finding of a long-lasting activity of AA-5-HT, although the hydrolysis of this compound by enzymes different from FAAH cannot be ruled out. Consistent with a selective anti-hyperalgesic, rather than analgesic, effect, AA-5-HT failed to increase the thermal withdrawal latency in the paw contralateral to the inflammatory injury. Although a recent report highlighted the anti-nociceptive property of AA-5-HT in different models of chronic pain [8], this is the first report showing the potent effect of this compound against inflammatory pain. We also report here the ability of the compound to counteract both oedema and hyperalgesia after the inflammation has developed, in a situation that more closely reproduces the clinical one, thus showing that this substance might be therapeutically useful.

In order to examine the potential advantages deriving from AA-5-HT employment, we compared its efficacy with that of URB597, a FAAH inhibitor, and with capsazepine, a TRPV1 antagonist. As expected, all compounds exhibited anti-oedemigen activity in the carrageenan model. However, the comparison of ED_{50} values demonstrated that AA-5-HT has a potency not statistically different from URB597, whereas it is about 15 fold more potent than capsazepine. Capsazepine was tested against carrageenan-induced oedema previously [31] and failed to reduce the increase in the paw perimeter when injected locally, supporting the high ED_{50} observed here. However, more recently, the anti-oedemigen activity in the carrageenan model of a new non-competitive TRPV1 receptor antagonist was reported [32], suggesting that TRPV1 antagonists are good candidates for anti-inflammatory drug development beyond their well established analgesic properties. The profile of the anti-oedemigen activity of URB597 is in agreement with our previous report [10]. When tested as an anti-hyperalgesic compound, URB597 failed to relieve thermal hyperalgesia even at the maximum anti-oedemigen dose. A very recent study showed that local administration of URB597 partially attenuated carrageenan-induced hyperalgesia even though only at 25 μg/paw, without exerting this slight effect at a higher dose [33]. The reasons for these differences are unclear, and may have been due to a number of factors. The local versus systemic route of administration could account for the mild anti-hyperalgesic effect observed by Jhaveri et al. [33], also because a previous study showed that

peripheral, but not systemic, administration of anandamide inhibited the induction of hyperalgesia in carrageenan-injected animals [34]. Furthermore, the experimental method employed by Jhaveri et al. [33] to evaluate hyperalgesia did not involve the application of a thermal stimulus, but it was a non-pain inducing method that evaluated spontaneous pain and was linked to a mechanical input, involving fibers different from those involved in thermal perception. Finally, since Jhaveri et al. [33] found URB597 effective only at a very specific dose, it is also possible that following the dosage employed by us, URB597 cannot reach the same concentration in the periphery. Although another study found URB597 effective against thermal hyperalgesia in the CFA model of inflammatory pain after systemic administration [11], it is conceivable that the differences between the two models may reflect differences also in endocannabinoid adaptations in pain transmission and modulation. The lack of anti-hyperalgesic effect observed by us might be also due to the capability of URB597 to activate other targets, such as TRPA1 channels which are also involved in pain transduction [35], thereby affecting its potential analgesic properties. However, we believe that the lack of anti-hyperalgesic activity by URB597 might be due to the activation of pro-nociceptive TRPV1 by the elevated levels of AEA induced by this FAAH inhibitor. In this context, the dual activity of AA-5-HT allows AEA elevation and concomitant blockade of TRPV1, which should minimize the potential proalgesic activity of FAAH inhibition. As expected from a large body of literature (see [36] for review), capsazepine reversed inflammatory pain, although with a potency 5 fold lesser than AA-5-HT. When analysing the previous studies employing capsazepine in models of carrageenan-induced inflammatory hyperalgesia, conflicting results are highlighted. In fact, peripherally applied capsazepine reduced thermal hyperalgesia in carrageenan-treated rats [31], whereas systemic administration of this compound was ineffective in this model in rats and effective in guinea pigs [29], suggesting species-dependent effects. Further evidence supporting the pharmacological advantages associated with the dual activity of AA-5-HT derives from the data obtained employing the co-administration of the single FAAH inhibitor and TRPV1 antagonist. The potentiated anti-hyperalgesic effect shown following the combined drugs versus the single administrations is an agreement with the aforementioned hypothesis that the inhibition of TRPV1 is necessary to obtain relief of pain by a FAAH inhibitor. However, the combined drugs, as compared to AA-5-HT, evoked a weaker anti-hyperalgesic effect than AA-5-HT, which could not be improved by administering higher doses of the compounds since these led to the appearance of side effects. Unlike the anti-hyperalgesic effect, the antioedemigen effect of combined URB597 and capsazepine co-administration did not ameliorate the anti-inflammatory effect evoked by the drugs when administered separately, although it was again weaker than that exerted by AA-5-HT. It is possible that the co-administration of the two drugs does not result in them reaching their targets at the same time.

Interestingly, unlike what previously observed with URB597 and capsazepine [31,33], AA-5-HT was found here to be inactive when administered locally up to a dose 1 mg/mouse similar to that previously found to be efficacious against the second phase of the formalin-induced nocifensive behaviour in rats [8]. This might suggest that, although the experimental models used in the two studies are different, also for AA-5-HT species differences might influence its efficacy depending on the route of administration. It is possible that, in the mouse, AA-5-HT, when injected directly in the skin, is more rapidly degraded, or has less favourable pharmacodynamics, possibly due to the lower local pH occurring during inflammation. Indeed, low pH was previously reported to reduce the potency of AA-5-HT as a FAAH inhibitor [37], and to elevate AEA activity at TRPV1 [38], which might reduce the efficacy of AA-5-HT at antagonizing these channels. Finally, the varying effect depending on the route of administration may also reflect subtle tissue-specific differences in FAAH activity, endocannabinoid levels, TRPV1 and cannabinoid receptor expression.

Next, we tested whether the pharmacological effects induced by AA-5-HT were accompanied by the most likely adverse effects due to CB_1 receptor activation or TRPV1 antagonism. The results reported here, in agreement with data obtained in healthy mice [1], suggest that AA-5-HT is totally devoid of cannabimimetic activity and that its employment *in vivo* does not induce the central unwanted effects that limit the therapeutic use of direct cannabinoid receptor agonists. In addition, our data demonstrate that no changes in body temperature occur following systemic AA-5-HT, indicating that the most frequent side effect evoked by TRPV1 antagonists (hyperthermia) is unlikely after AA-5-HT administration. It is possible to exclude that this lack of central effects can be due to the inability of AA-5-HT to cross the blood brain barrier, since a previous work [3] provides clear evidence that the systemic (i.p.) administration of AA-5-HT at the same dose (5 mg/kg) and at the same time points (from 1 to 12 h after administration) as in this work, significantly elevated endocannabinoid level (especially AEA) in the brain. For comparison, we tested, in the same experimental conditions, URB597 in the tetrad assay, and capsazepine, on body temperature. Whilst URB597 evoked a slight and short-lasting hypothermia and hypolocomotion within 1 h from administration, capsazepine evoked hyperthermia (+1.6 °C). In summary, we show here that AA-5-HT exerts anti-inflammatory and anti-hyperalgesic effects that are overall more efficacious than those obtained employing a single FAAH inhibitor or TRPV1 antagonist, thus suggesting that the use of AA-5-HT against acute inflammatory diseases might offer advantages in terms of both efficacy and lack of adverse effects.

Finally, we wanted to make sure that the mechanism of action of AA-5-HT postulated here does include blockade of TRPV1 receptors and indirect activation of cannabinoid receptors. Owing to the dual targets of AA-5-HT, we studied the relative contribution of cannabinoid CB_1 and CB_2 as well as TRPV1 receptors in its anti-oedemigen and anti-hyperalgesic effects. The data demonstrate that AA-5-HT–induced anti-oedemigen effect can be prevented by a TRPV1 agonist but not by CB_1 and CB_2 antagonists, suggesting that TRPV1 is the only receptor responsible for the anti-inflammatory properties of AA-5-HT, and possibly explaining why this compound was not found here to be more efficacious at reducing oedema than URB597 in carrageenan-treated mice. We previously showed that the anti-oedemigen effect of URB597 was mediated exclusively by CB_2 receptors [10]; this apparent discrepancy may be ascribed to the capability of URB597 to increase the local levels of 2-AG (a potent ligand of CB_2 receptor) as well as those of AEA [33], whereas AA-5-HT, at a pharmacologically active dose, elevated AEA, but not 2-AG, levels in the carrageenaan-treated paw. Conversely, both TRPV1 agonism and CB_1 antagonism partially reversed the anti-hyperalgesic efficacy of AA-5-HT, clearly indicating an involvement of both receptor types in this effect, and possibly explaining why this compound was more efficacious than both capsazepine and URB597 at inhibiting carrageenan-induced hyperalgesia. These data again suggest that, despite the failure of URB597 to reduce hyperalgesia in this model, FAAH inhibition also reduces this consequence of carrageenan-induced inflammation, provided that also TRPV1 is blocked at the same time. It is possible that strongly elevated AEA levels, caused by a very potent FAAH inhibitor like URB597, activate not only cannabinoid but also TRPV1 receptors, which are sensitised during inflammation, thus counteracting the anti-hyperalgesic effect of the former receptors. Indeed, also in other contexts, URB597-induced inhibition of FAAH was shown to lead to TRPV1 activation, thus counteracting in part the effects of CB_1 activation [16,39,40].

Table 2
Summary of the current knowledge on the anti-nociceptive effects of AA-5-HT.

Animal model	Test	Dose (administration route)	Effect	Receptor involved	Ref.
SIA	Thermal nociception	20 nmol (intra PAG or RVM)	Enhancement of SIA	CB_1	[4]
Formalin	Nocifensive behaviour	5 mg/kg (i.p.)	Reversal of the second phase of nociception	CB_1 and TRPV1	[8,50]
		1 mg (intra paw)			
CCI	Thermal hyperalgesia and mechanical allodynia	5 mg/kg (s.c.)	Anti-hyperalgesic and anti-allodynic	CB_1 (anti-hyperalgesia)	[8]
				CB_1 and TRPV1 (anti-allodynia)	
Formalin	Nocifensive behaviour	0.5 nmol (intra PAG)	Reversal of the second phase of nociception	CB_1 and TRPV1	[51]
Acute thermal nociception	Thermal nociception	0.2 and 0.5 nmol (intra PAG)	Increase in latency	CB_1 and TRPV1	[51]
Carrageenan	Thermal and mechanical hyperalgesia	1–5 mg/kg (i.p.)	Anti-hyperalgesic	CB_1 and TRPV1	Present study

SIA, stress-induced analgesia; CCI, chronic constriction injury; PAG, periaqueductal grey; RVM, rostral ventromedial medulla; s.c., subcutaneous; i.p., intraperitoneal.

On the basis of our findings, we hypothesize that the anti-hyperalgesic effect elicited by AA-5-HT is due to inhibition of sensory pro-nociceptive neurons mediating thermal pain transmission, through both direct antagonism of TRPV1 receptors and CB_1 stimulation caused by increased AEA levels (which can only act at CB_1 receptors since TRPV1 is concomitantly blocked by the compound). By contrast, only TRPV1 is involved in the anti-oedemigen effect of AA-5-HT. This is not completely surprising given the aforementioned lack of capability of this compound to elevate the levels of 2-AG (which does not activate TRPV1) in the inflamed paw, and the pivotal role of TRPV1, in sensory neurons, epithelial cells and keratinocytes, at mediating inflammation also via neurogenic peptide-, prostaglandin E_2-, ATP-, bradykinin- and nerve growth factor-mediated, and non-endovanilloid-mediated, effects [41–46].

We reported here that the levels of endocannabinoids AEA and OEA, and of the related endogenous substance (PEA), are significantly lower in the mouse paw skin following carrageenan injection, as recently demonstrated in rats [33], and that, however, pre-administration of AA-5-HT prevented only the decrease in AEA levels. This finding is unlikely due uniquely to the lower efficacy of the compound on FAAH, since it was previously shown that AA-5-HT does elevate both AEA and 2-AG levels in the paw of mice treated with formalin [8]. We hypothesize that, during carrageenan-induced inflammatory conditions, other enzymes that are not inhibited by AA-5-HT, for example the *N*-acylethanolamine acid amidohydrolase (NAAA) or ceramidase [47], participate in the degradation of PEA and OEA. This hypothesis is in agreement with the finding that macrophages express functionally active NAAA and FAAH, both of which appeared to cooperatively degrade various *N*-acylethanolamines [48]. The inhibition of FAAH exerted by AA-5-HT fully restored the physiological levels of AEA, thus arguing against the possibility that other enzymes, e.g. COX-2, which is not likely inhibited by AA-5-HT, are up-regulated and participate in AEA degradation during inflammation [49]. We found that, beside the paw skin, AA-5-HT elevated AEA levels also in the spinal cord after systemic administration. We report here for the first time that, in addition to altering the levels of endocannabinoids at the site of injury, carrageenan-evoked inflammation decreased endocannabinoids at other targets such as the spinal cord, so contributing to hypersensitivity to pain. The ability of AA-5-HT to increase AEA in the spinal cord over the physiological levels strongly suggests that the compound acts as anti-hyperalgesic and anti-inflammatory through both central and peripheral mechanisms.

In conclusion, the data presented herein extended the current knowledge about the anti-nociceptive properties of the dual FAAH/TRPV1 blocker, AA-5-HT (Table 2). We showed here that, by combining the features of FAAH inhibitors and TRPV1 channel antagonists, AA-5-HT exhibits notable anti-inflammatory and anti-hyperalgesic activity in a pre-clinical model of acute inflammation. This compound seems to be devoid of the main side effects of CB_1 agonists and TRPV1 antagonists, and could be used as a template from which new drugs [50,51] might evolve in the future.

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