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Peripheral FAAH inhibition causes profound antinociception and protects against indomethacin-induced gastric lesions

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Abstract

Fatty-acid amide hydrolase (FAAH) catalyzes the intracellular hydrolysis of the endocannabinoid anandamide and other bioactive lipid amides. In the present study, we conducted a comparative characterization of the effects of the newly identified brain-impermeant FAAH inhibitor, URB937 ([3-(3-carbamoylphenyl)-4-hydroxy-phenyl] N-cyclohexylcarbamate), in various rodent models of acute and persistent pain. When administered by the oral route in mice, URB937 was highly active (median effective dose, ED₅₀, to inhibit liver FAAH activity: 0.3 mg·kg⁻¹) and had a bioavailability of 5.3%. The antinociceptive effects of oral URB937 were investigated in mouse models of acute inflammation (carrageenan), peripheral nerve injury (chronic sciatic nerve ligation) and arthritis (complete Freund's adjuvant). In all models, URB937 was as effective or more effective than standard analgesic and anti-inflammatory drugs (indomethacin, gabapentin, dexamethasone) and reversed pain-related responses (mechanical hyperalgesia, thermal hyperalgesia, and mechanical allodynia) in a dose-dependent manner. ED₅₀ values ranged from 0.2 to 10 mg·kg⁻¹, depending on model and readout. Importantly, URB937 was significantly more effective than two global FAAH inhibitors, URB597 and PF-04457845, in the complete Freund's adjuvant model. The effects of a combination of URB937 with the non-steroidal anti-inflammatory agent, indomethacin, were examined in the carrageenan and chronic sciatic nerve ligation models. Isobolographic analyses showed that the two compounds interacted synergistically to attenuate pain-related behaviors. Furthermore, URB937 reduced the number and severity of gastric lesions produced by indomethacin, while exerting no ulcerogenic effect when administered alone. The results indicate that the peripheral FAAH inhibitor URB937 is more effective than globally active FAAH inhibitors at inhibiting inflammatory pain. Our findings further suggest that FAAH and cyclooxygenase inhibitors interact functionally in peripheral tissues, to either enhance or hinder each other's actions.

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Conflict of interest

The authors declare the following conflict of interest: T.B., G.M.S., and D.P. are inventors in patent applications that describe peripheral FAAH inhibitors.

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Keywords

inflammation; anandamide; neuropathic pain; gastric lesions; cannabinoid receptors

1. Introduction

Fatty-acid amide hydrolase (FAAH) [1] is an intracellular serine hydrolase that catalyzes the hydrolysis of endogenous lipid amides, which include agonists of cannabinoid receptors, such as anandamide [2, 3], and agonists of type- α peroxisome proliferator-activated receptors (PPAR- α), such as oleoylethanolamide and palmitoylethanolamide [4, 5]. These lipid-derived messengers are thought to serve important physiological functions both in the central nervous system (CNS) and in peripheral tissues, and deficits in their intrinsic activity have been implicated in a variety of disease conditions [6, 7]. Inhibition of intracellular FAAH activity has been the focus of substantial drug discovery efforts, and several chemically distinct classes of FAAH inhibitors have been identified [8]. Among them, two well-characterized examples are the *O*-aryl carbamates, such as URB597 [cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-yl ester] [9–11], and the piperidine/piperazine ureas, such as PF-04457845 [benzylidenepiperidine pyridazine urea] [12]. Preclinical studies have shown that URB597 readily enters the CNS and exerts a combination of anxiolytic, antidepressant and analgesic effects [9, 13, 14]. Another recently disclosed *O*-aryl carbamate derivative, the compound URB937, is potent at inhibiting FAAH activity in peripheral tissues, but is extruded from the CNS by the activity of the membrane transporter ATP-binding cassette G2 (ABCG2, also called breast cancer resistance protein) [15, 16]. Initial pharmacological studies have shown that URB937 reduces nociceptive responses in rodent models of acute and persistent pain through a mechanism that requires elevation of anandamide levels and consequent activation of peripheral CB₁-type cannabinoid receptors [15]. These findings suggest that peripheral blockade of FAAH activity may enhance an intrinsic analgesic mechanism, mediated by anandamide, which is involved in controlling the transmission of pain signals from peripheral tissues to the spinal cord and the brain. To further evaluate the potential therapeutic usefulness of URB937, and compare it to that of globally active FAAH inhibitors such as URB597, in the present study we characterized the effects of oral administration of this compound in mouse models of acute inflammation (carrageenan), arthritis (complete Freund's adjuvant, CFA) and peripheral nerve injury (sciatic nerve ligation). In addition, we evaluated the occurrence of possible functional interactions *in vivo* between URB937 and the non-steroidal anti-inflammatory drug (NSAID), indomethacin.

2. Materials and methods

2.1. Synthesis of FAAH inhibitors

All reagents were purchased from Sigma-Aldrich (Milan, Italy) in the highest quality commercially available. Solvents were RP grade unless otherwise indicated. Purification of the crude products was performed by flash column chromatography on silica gel (Kieselgel 60, 0.040–0.063 mm, Merck). TLC analyses were performed on precoated silica gel on aluminum sheets (Kieselgel 60 F254, Merck). Melting points were determined on a Büchi SMP-510 capillary melting point apparatus and are uncorrected. The structures of the unknown compounds were unambiguously assessed by MS and ^1H NMR. EI-MS spectra (70 eV) were recorded with a Fisons Trio 1000 spectrometer. ^1H NMR spectra were recorded at 300K on a Bruker AC 200 spectrometer and on a Bruker Avance III 400 system equipped with a BBI inverse probe and Z-gradients. ^1H chemical shifts are reported in δ values in parts per million (ppm) downfield with the deuterated solvent as the internal standard.

Synthesis of [3-(3-carbamoylphenyl)-4-hydroxy-phenyl] N-cyclohexylcarbamate (URB937)—

URB937 was synthesized in a 5-step procedure starting from the commercially available 3-bromo-4-hydroxybenzaldehyde. This compound was converted into 4-benzyloxy-3-bromophenol, as previously described [17,18], by benzylation (BzCl , Cs_2CO_3 , DMF, rt, 3 h, 78%) [17] followed by oxidation and hydrolysis (m -CPBA, CH_2Cl_2 , rt, 4h; then, 1M NaOH, MeOH, rt, 18h, 46%) [18]. Elaboration of 4-benzyloxy-3-bromophenol to URB937 was accomplished as follows.

3-(2-benzyloxy-5-hydroxy-phenyl)benzamide—To a stirred solution of 4-benzyloxy-3-bromophenol (6.1 g, 21.7 mmol) in toluene (100 mL) were added $\text{d}(\text{PPh}_3)_4$ (1.3 g, 1.1 mmol), a solution of Na_2CO_3 (11.5 g, 108.7 mmol) in H_2O (100 mL) and 3-carbamoylbenzeneboronic acid (4.3 g, 26.1 mmol). The mixture was refluxed under argon atmosphere for 18h. After cooling at room temperature, 2M HCl (200 mL) was added and the mixture was extracted with ethyl acetate (3×200 mL). The combined organic layers were dried over MgSO_4 , filtered and concentrated. The residue was purified by flash column chromatography on silica gel, using ethyl acetate/cyclohexane (1:1) as eluent to give 3-(2-benzyloxy-5-hydroxy-phenyl)benzamide (3.3 g, 47%) as a light brown solid. Mp: 148–150 °C (dichloromethane/*n*-hexane). MS (ESI) $\text{C}_{20}\text{H}_{17}\text{NO}_3$ requires m/z 319, found 320 ($\text{M} + \text{H}^+$). ^1H NMR (200 MHz, DMSO): δ 9.15 (s, 1H), 8.02–8.04 (m, 2H), 7.78–7.82 (m, 1H), 7.63–7.67 (m, 1H), 7.27–7.49 (m, 7H), 7.00–7.05 (m, 1H), 6.70–6.78 (m, 2H), 4.98 (s, 2H).

[4-benzyloxy-3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate—To a stirred solution of 3-(2-benzyloxy-5-hydroxy-phenyl)benzamide (2.7 g, 8.5 mmol) in ethanol (25 mL) and acetonitrile (25 mL) were added cyclohexylisocyanate (1.2 mL, 9.3 mmol) and triethylamine (0.2 mL, 1.3 mmol). The mixture was refluxed for 4h, then cooled and concentrated. The residue was purified by flash column chromatography on silica gel, using ethyl acetate/cyclohexane (7:3) as eluent to give [4-benzyloxy-3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate (2.1 g, 56%) as a white solid. Mp: 180–

182 °C (ethanol). MS (ESI) $C_{27}H_{28}N_2O_4$ requires m/z 444, found 445 ($M+H$)⁺. 1H NMR (200 MHz, DMSO) δ 8.02–8.04 (m, 2H), 7.78–7.82 (m, 1H), 7.68–7.71 (m, 2H), 7.03–7.49 (m, 10H), 5.11 (s, 2H), 3.32–3.34 (m, 1H), 1.19–1.82 (m, 10H).

[3-(3-carbamoylphenyl)-4-hydroxy-phenyl] *N*-cyclohexylcarbamate (URB937) was prepared from [4-benzyloxy-3-(3-carbamoylphenyl)phenyl] *N*-cyclohexylcarbamate (2.1 g, 4.8 mmol) following a previously reported procedure [15]. White solid (0.9 g, 54%). Mp: 128–130 °C (dichloromethane/*n*-hexane). MS (ESI) $C_{20}H_{22}N_2O_4$ requires m/z 354, found 355 ($M+H$)⁺. 1H NMR (200 MHz, CDCl₃) δ 7.68–7.75 (m, 2H), 7.56 (m, 1H), 7.34–7.41 (m, 1H), 7.07 (s, 1H), 6.74–6.95 (m, 3H), 6.59 (br s, 1H), 5.85 (br s, 1H), 5.13 (br d, 1H), 3.55 (m, 1H), 1.13–2.02 (m, 10H).

N-pyridazin-3-yl-4-[[3-[[5-(trifluoromethyl)-2-pyridyl]oxy]phenyl]methylene]piperidine-1-carboxamide (PF-04457845) was synthesized starting from 2-(3-piperidin-4-ylidenemethylphenoxy)-5-trifluoromethyl-pyridine hydrochloride (5.0 g, 13.0 mmol) and phenyl pyridazin-3-ylcarbamate (2.8 g, 13.0 mmol) following a reported procedure [19, 20]. White solid (3.8 g, 60%).

MS (ESI) $C_{23}H_{20}F_3N_5O_2$ requires m/z 455, found 456 ($M+H$)⁺. 1H NMR (400 MHz, DMSO) δ 9.86 (s, 1H), 8.84 (dd, $J = 4.7, 1.5$ Hz, 1H), 8.58 (s, 1H), 8.23 (dd, $J = 8.7, 2.6$ Hz, 1H), 8.01 (dd, $J = 9.1, 1.5$ Hz, 1H), 7.56 (dd, $J = 9.1, 4.6$ Hz, 1H), 7.43 (m, 1H), 7.24 (d, $J = 8.7$ Hz, 1H), 7.16 (d, $J = 7.7$ Hz, 1H), 7.07 (m, 2H), 6.42 (s, 1H), 3.62 (t, $J = 5.8$ Hz, 2H), 3.54 (t, $J = 5.8$ Hz, 2H), 2.48 (m, 2H, overlapped with DMSO signal), 2.38 (t, $J = 5.6$ Hz, 2H).

2.2. Animals

Male CD1 mice, weighing 25–30 g, (Charles River, Calco, Italy) were used. Procedures were in accordance with the Ethical Guidelines of the International Association for the Study of Pain and were approved by Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192) as well as with European Economic Community regulations (O.J. of E.C. L 358/1 12/18/1986). Mice were housed in groups of 5 in ventilated cages containing autoclaved cellulose paper as nesting material, with free access to food and water. They were maintained under a 12 h light/dark cycle (lights on at 08:00 a.m.), at controlled temperature (21 ± 1 °C) and relative humidity ($55 \pm 10\%$). The animals are randomly divided in groups of 6 mice. Behavioral testing was performed between 9:00 a.m. and 5:00 p.m. Scientists running the experiments were not aware of the treatment protocol at the time of the test (blind procedure).

2.3. Chemicals

Carboxymethylcellulose (CMC), λ -carrageenan, chloral hydrate, CFA, heparin sodium salt, PEG 400, TWEEN 80, gabapentin and indomethacin were purchased from Sigma-Aldrich (Milan, Italy). Drug solutions were prepared immediately before use in a vehicle of 0.5% CMC and orally administered in a volume of $2.5 \text{ mL} \cdot \text{kg}^{-1}$.

2.4. Pharmacokinetic Analyses

For oral treatment, URB937 was dissolved in 0.5% CMC at the dose of 10 mg·kg⁻¹, while for intravenous studies URB937 was dissolved in 10% PEG 400 /10% Tween /80% saline at the dose of 10 mg·kg⁻¹. Blood samples were collected from the site where the animal was decapitated with prior administration of CO₂ at 5, 15, and 30 min, and 1, 2, 4, 6 and 24 h after intravenous administration, and at 30 min, and 1, 2, 4, 6, 8, and 24 h after oral administration. All blood samples (200 µL) were collected into tubes containing heparin sodium salt (20 µL, 5000 U·ml⁻¹) as anticoagulant. The plasma fraction was immediately separated by centrifugation (3000×g, 10 min, 4°C), frozen on dry ice, and stored at -20°C until analyses. Plasma levels of URB937 were monitored using a Xevo TQ UPLC-MS/MS system (Waters, Milford, CT), using a calibration curve and URB597 as an internal standard (I.S.). Details of the analytical method were as follow: chromatography was performed on an Acquity BEH C18 column (2.1×50mm, 1.7 µm particle size; Waters). Flow rate was at 0.5 mL·min⁻¹ with a gradient from 10% solvent B to 98% solvent B in 2 min, 30 s at 10% and 30 s at 98% before and after the gradient. Solvent A = water plus formic acid 0.1%; solvent B = acetonitrile plus formic acid 0.1%. Mass spectrometry parameters: capillary voltage 3KV, cone voltage 20V, source temperature 150°C, cone gas 20 L·h⁻¹, desolvation gas 500 L·h⁻¹, desolvation temperature 500°C. The following multiple reaction monitoring transitions were followed: 355>230, 15V for URB937 and 339>197, 20V for I.S.. Sample preparation: after a short centrifugation, mouse plasma samples (50µL) were transferred into Eppendorf tubes and diluted with 150µL of acetonitrile spiked with I.S. to a final 500nM concentration. After vortexing (30 s) samples were centrifuged at 14000×g for 10 min at 4°C. 5 µL of supernatant were loaded onto the LC column. Calibration curve and quality controls: URB937 was spiked in phosphate-buffered saline (PBS) solution (pH 7.4) and a calibration curve over the 1nM – 10µM range was prepared. To check for recovery, 3 quality control samples were also prepared by spiking mouse plasma with URB937 to final concentrations of 20 nM, 200 nM and 2000 nM. Calibrators and quality controls were crashed with acetonitrile spiked with I.S. as described for plasma samples. Calibrators, quality controls and samples were analyzed in triplicate. The linear regression of the calibration curve yielded an R² value of 0,995. The recovery of the quality controls (back-calculated from the regression curve) ranged from 85 to 95%. Pharmacokinetic Data calculation: PK data were calculated on the basis of the time versus plasma concentration profiles of URB937 using the PK Solutions 2.0 software (Summit Research Services, USA) for non-compartmental PK data analysis. Experimental points were fitted using three exponentials terms for both the PO and IV arms (three phases fitting: elimination, distribution and adsorption).

2.5. Surgeries and Treatments

We injected the pro-inflammatory agent a sulfated polysaccharides that are extracted from red seaweeds, λ-carrageenan (1% weight-vol⁻¹ in sterile water, 20 µL) or CFA (20 µL, 1mg·ml⁻¹) into the left hind paw of slightly restrained mice, and measured paw volume with a plethysmometer (Ugo Basile, Comerio, Italy). Sciatic nerve ligations were performed according to Bennett and Xie [21]. Mice were anesthetized with 2–3% isoflurane, and the left sciatic nerve was exposed at mid-thigh level through a small incision and tied at two distinct sites (spaced at a 2-mm interval) with a silk thread. The wound was closed with a single muscle suture and skin clips, and dusted with streptomycin. In sham-operated animals, the nerve was exposed but not tied.

2.6. Behavioral Assays

We evaluated mechanical hyperalgesia by measuring the latency (in s) to withdraw the paw from a constant mechanical pressure exerted onto the dorsal surface. A 15-g calibrated glass cylinder (diameter = 10 mm) chambered to a conical point (diameter = 3 mm) was used to

exert the mechanical force. The weight was suspended vertically between two rings attached to a stand and was free to move vertically. A cutoff time of 180 s was used. Withdrawal thresholds were measured on both ipsilateral (experimental) and contralateral (control) paws at various times after drug administration. We assessed thermal hyperalgesia by the method of Hargreaves et al. [22], measuring the latency to withdraw the hind paw from a focused beam of radiant heat (thermal intensity: infrared 3.0) in a plantar test apparatus (Ugo Basile, Comerio, Italy). Cutoff time was set at 30 s. Withdrawal latencies were measured on both ipsilateral and contralateral paws at various time points after drug administration. We evaluated mechanical allodynia using a device for automated von Frey hair assessment (Dynamic Plantar Aesthesiometer, Ugo Basile, Comerio, Italy). Mice were placed individually in a small testing arena (20×18.5×13 cm) with a wire mesh floor. The device was positioned beneath the animal, so that the filament was placed directly under the surface of the test paw. The device raised the filament to touch the foot and progressively increased force until the animal withdrew its paw, or until it reached a maximum of 5 g of force. The device automatically recorded the force at which the foot was withdrawn and the withdrawal latency. Each test was conducted in duplicate and the mice are fully awake at every time points.

2.7. Indomethacin-Induced Gastric Lesions

We followed the protocol of Chan et al. [23]. Food-deprived (18–24 h) mice, using an oral gavage, received a single dose of indomethacin (3 or 10 mg·kg⁻¹) preceded by oral URB937 (0.3 and 1 mg·kg⁻¹) or its vehicle. After 4 h, the mice were killed by CO₂ asphyxiation. The stomachs were removed, opened, rinsed in PBS, and the stomach lining was photographed. Scoring was done by observers blinded to experimental conditions, as previously reported [24]: red coloration = 0.5; spot ulcers = 1; hemorrhagic streaks = 1.5. If 3 or 4 ulcers were observed, a value of 2 was added to the score. If 5 or more ulcers were observed, a value of 3 was added to the score. The overall ulceration index was the sum of these scores, with a maximal value of 6.

2.8. Isobolographic Analyses

Analyses were performed as described by Tallarida [25]. Median effective dose (ED₅₀) values for each drug combination were plotted on the x- and y-axes. The line joining the two axes corresponded to the theoretical additive line (isobole). In such a plot, drug combinations producing pharmacological synergism with potentiation generate experimental points that fall below the isobole. Conversely, drug combinations producing antagonism generate experimental points that fall above the isobole.

2.9. Statistical Analyses

Results are expressed as the mean±S.E.M., or 95% confidence limits (95% CL). Effective doses were determined by linear regression analysis of dose–response curves. Individual slopes of the dose–response curves were compared by Student's *t* test, according to the test of parallelism, and isobolographic analyses were performed using the Prism software (GraphPad Software, San Diego, CA). The data from mechanical and thermal hyperalgesia and mechanical allodynia were compared using two-way analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons. For gastric toxicity, the significance of differences between groups was determined by one-way ANOVA followed by a Bonferroni's test for multiple comparisons.

3. Results

3.1. Pharmacokinetic profile

We dosed URB937 intravenously or orally in mice, and monitored plasma concentrations for 24 h following administration (Fig. 1). After intravenous dosing ($10 \text{ mg}\cdot\text{kg}^{-1}$), we measured a maximal plasma concentration of $12,828 \text{ ng}\cdot\text{mL}^{-1}$ (Fig. 1A). The half-life of the compound for the elimination phase was 1.6 h. URB937 showed a volume of distribution of $4.6 \text{ L}\cdot\text{kg}^{-1}$ and disappeared from the systemic circulation with a clearance of $2 \text{ L}\cdot\text{h}\cdot\text{kg}^{-1}$ (Table 1). After oral administration at $10 \text{ mg}\cdot\text{kg}^{-1}$ (Fig. 1B), the maximal plasma concentration of URB937 was observed 30 min after dosing ($123 \text{ ng}\cdot\text{mL}^{-1}$). Plasma concentration after 24 h was $1.1 \text{ ng}\cdot\text{mL}^{-1}$. The oral bioavailability of URB937, calculated from the area under the curve (AUC) following oral and intravenous exposure, was 5.3% (Table 1). In a separate experiment, we tested the oral activity of URB937 in mice. Orally administered URB937 inhibited FAAH activity in liver tissue with a median effective dose (ED_{50}) of $0.3 \text{ mg}\cdot\text{kg}^{-1}$ without affecting FAAH activity in brain tissue (Supplementary Fig. 1).

3.2. Antihyperalgesic effects in a model of acute inflammation

Previous studies have shown that intraperitoneal administration of a $1 \text{ mg}\cdot\text{kg}^{-1}$ dose of URB937 exerts a marked analgesic effect in the carrageenan model of acute inflammation [15]. This effect is likely mediated by activation of CB_1 receptors because it is blocked by the CB_1 antagonist AM251, but not by the CB_2 antagonist AM630 [15]. Consistent with those results, oral administration of URB937 (0.1 , 0.3 , 1 and $3 \text{ mg}\cdot\text{kg}^{-1}$) produced a dose-dependent and persistent suppression of carrageenan-induced edema (Fig. 2A), mechanical hyperalgesia (Fig. 2B), and thermal hyperalgesia (Fig. 2C). ED_{50} values for URB937 were $0.5 \text{ mg}\cdot\text{kg}^{-1}$ on edema (CL 95% = 0.038 – $0.47 \text{ mg}\cdot\text{kg}^{-1}$); $0.8 \text{ mg}\cdot\text{kg}^{-1}$ on mechanical hyperalgesia (CL 95% = 0.021 – $0.43 \text{ mg}\cdot\text{kg}^{-1}$); and $0.2 \text{ mg}\cdot\text{kg}^{-1}$ on thermal hyperalgesia (CL 95% = 0.058 – $0.46 \text{ mg}\cdot\text{kg}^{-1}$). Notably, oral URB937 was more potent ($p < 0.001$ for $3 \text{ mg}\cdot\text{kg}^{-1}$, $F = 16.77$) than oral indomethacin at inhibiting the responses induced by carrageenan. ED_{50} values for indomethacin were $20 \text{ mg}\cdot\text{kg}^{-1}$ on edema (CL 95% = 5.82 – $8.02 \text{ mg}\cdot\text{kg}^{-1}$); $16 \text{ mg}\cdot\text{kg}^{-1}$ on mechanical hyperalgesia (CL 95% = 5.13 – $9.52 \text{ mg}\cdot\text{kg}^{-1}$); and $3.3 \text{ mg}\cdot\text{kg}^{-1}$ on thermal hyperalgesia (CL 95% = 1.95 – $2.3 \text{ mg}\cdot\text{kg}^{-1}$) (Supplementary Fig. 2 A, B and C).

3.3. Antihyperalgesic effects in a model of peripheral neuropathy

The results reported above suggest that URB937 prevents the development of acute pain responses induced by carrageenan in mice. To test the ability of URB937 to alleviate established chronic pain, we determined whether the compound influences the persistent hyperalgesia associated with nerve injury. We produced a peripheral neuropathy in mice by loosely tying the left sciatic nerve, a surgical procedure that results in the development of mechanical and thermal hyperalgesia (Fig. 3A, B), and mechanical allodynia (Fig. 3C) in the operated limb. We administered oral URB937 (0.3 – $3 \text{ mg}\cdot\text{kg}^{-1}$) once on day 3, day 7 or day 14 following the ligation, and measured pain behavior 2 h after dosing. As shown in Figure 3, a single administration of URB937 was sufficient to cause a rapid reversal of established hyperalgesia and allodynia ($p < 0.01$ for $1 \text{ mg}\cdot\text{kg}^{-1}$ and $p < 0.001$ for $3 \text{ mg}\cdot\text{kg}^{-1}$). ED_{50} values were $1.6 \text{ mg}\cdot\text{kg}^{-1}$ on mechanical hyperalgesia (CL 95% = 0.33 – $0.41 \text{ mg}\cdot\text{kg}^{-1}$); $1.0 \text{ mg}\cdot\text{kg}^{-1}$ on thermal hyperalgesia (CL 95% = 0.19 – $0.27 \text{ mg}\cdot\text{kg}^{-1}$); and $1.3 \text{ mg}\cdot\text{kg}^{-1}$ on mechanical allodynia (CL 95% = 0.24 – $0.36 \text{ mg}\cdot\text{kg}^{-1}$). The maximal dose of URB937 tested ($3 \text{ mg}\cdot\text{kg}^{-1}$) was as effective at alleviating pain responses ($p < 0.001$, $F = 16.41$) as an oral $50 \text{ mg}\cdot\text{kg}^{-1}$ dose of the centrally active analgesic, gabapentin (Fig. 3). Moreover, URB937 was substantially more potent and effective ($p < 0.001$, $F = 15.89$) than indomethacin (Supplementary Fig. 3), which gave ED_{50} values of $40 \text{ mg}\cdot\text{kg}^{-1}$ on mechanical hyperalgesia

(CL 95% = 2.00–2.05 mg·kg⁻¹), 28 mg·kg⁻¹ on thermal hyperalgesia (CL 95% = 1.71–1.90 mg·kg⁻¹), and 13 mg·kg⁻¹ on mechanical allodynia (CL 95% = 1.690–1.91 mg·kg⁻¹).

3.4. Antihyperalgesic effects in an experimental model of arthritis

To examine whether the compound might exert a therapeutic effect on established inflammatory pain, we tested its activity in the CFA model of arthritis. We administered URB937 (1–30 mg·kg⁻¹) orally once on day 3, day 7 or day 14 following intraplantar CFA injection, and measured pain behavior 2 h after dosing. Irrespective of the day on which it was administered, the FAAH inhibitor produced a marked suppression of mechanical and thermal hyperalgesia ($p < 0.01$ for 3 mg·kg⁻¹ and $p < 0.001$ for 10 and 30 mg·kg⁻¹) (Fig. 4A, B). ED₅₀ values were 10 mg·kg⁻¹ on mechanical hyperalgesia (CL 95% = 0.62–3.39 mg·kg⁻¹) and 3.1 mg·kg⁻¹ on thermal hyperalgesia (CL 95% = 0.30–1.69 mg·kg⁻¹). At the highest dose tested (30 mg·kg⁻¹), URB937 was more effective ($p < 0.001$ for 30 mg·kg⁻¹, $F = 15.53$) than a 100 mg·kg⁻¹ oral dose of the potent steroidal antiinflammatory agent, dexamethasone (Fig. 4; Supplementary Fig. 4). In this model, URB937 was also significantly more effective ($p < 0.001$ for 30 mg·kg⁻¹, $F = 17.01$) than the globally active FAAH inhibitors, URB597 and PF-04457845 (Fig. 4).

3.5. Synergistic interactions between URB937 and indomethacin

Previous studies have shown that URB597, a globally active FAAH inhibitor, acts synergistically with the NSAID diclofenac to reduce pain behavior in mice [26]. To determine whether this synergistic interaction occurs at central or peripheral sites, we investigated the effects of a combination of URB937 plus indomethacin on carrageenan-induced edema and hyperalgesia. Co-administration of the two drugs, at doses that were ineffective when administered separately (0.1 mg·kg⁻¹ URB937 and 1 mg·kg⁻¹ indomethacin) (Fig. 5; Supplementary Fig. 2), exerted profound antiinflammatory (Fig. 5A) and antihyperalgesic effects (Fig. 5B, C). An isobolographic analysis of the data confirmed that URB937 and indomethacin acted synergistically to reduce pain responses (Fig. 5). Similar results were obtained when URB937 and indomethacin were tested alone or in combination in the sciatic nerve ligation model (Fig. 6).

3.7. Effects of URB937 on Indomethacin-induced gastric ulcers

Irritation of the gastric mucosa is a common adverse event associated with the use of indomethacin and other NSAIDs [27]. Prior work has suggested that the globally active FAAH inhibitor URB597 attenuates indomethacin-induced gastric ulcer formation [26]. We evaluated therefore whether peripheral FAAH blockade with URB937 might result in a similar protective effect. Oral administration of indomethacin (3 and 10 mg·kg⁻¹) to food-deprived mice was accompanied by substantial gastric irritation and appearance of gastric ulcers (Fig. 7). Co-administration of URB937 (0.3 and 1 mg·kg⁻¹), administered orally at the same time as indomethacin, markedly decreased the severity of these effects ($p < 0.01$ for 0.3 mg·kg⁻¹ and $p < 0.001$ for 1 mg·kg⁻¹) (Fig. 7). Strikingly, when combined with the lowest dose of URB937 (0.3 mg·kg⁻¹), the highest dose of indomethacin (10 mg·kg⁻¹) caused irritation but no ulceration (Fig. 7). Administration of URB937 alone had no ulcerogenic effect (Fig. 7).

4. Discussion

Pain perception is controlled by neurotransmitter systems that are localized both inside and outside the CNS. In the periphery of the body, the intrinsic regulation of pain transmission is thought to occur primarily at terminals of afferent nerve fibers. One well-known example of this regulation is provided by endogenous opioid peptides, which are secreted from immune cells during inflammation and inhibit pain initiation by interacting with opioid receptors

present on sensory nerve endings [28]. Several lines of evidence suggest that endocannabinoid lipid messengers such as anandamide may serve an analogous function to that of the opioids. Pharmacological studies have shown that activation of peripheral CB₁ and CB₂ receptors by local administration of cannabinoid agonists reduces nociceptive responses in rodents [29–31]. Moreover, genetic disruption of CB₁ receptor expression in mouse primary nociceptive neurons markedly exacerbates nociception, suggesting that an intrinsic endocannabinoid tone modulates the activity of peripheral sensory fibers [32]. Pointing to a possible clinical significance of these findings, human studies have shown that painful conditions such as complex regional pain syndrome and arthritis are accompanied by elevations in the levels of anandamide in peripheral tissues [33, 34].

We have recently described a peripherally restricted FAAH inhibitor, the compound URB937, which is extruded from the brain and spinal cord by the xenobiotic transporter ABCG2 and therefore selectively interrupts anandamide deactivation in peripheral tissues [15]. The ability of this compound to reduce nociception in rodent models of acute and persistent pain suggests that peripheral FAAH inhibition may heighten the activity of an endogenous analgesic mechanism, mediated by anandamide, which modulates the traffic of emerging pain signals from the periphery toward the spinal cord and the brain. To test this hypothesis further, in the present study we investigated the effects of oral administration of URB937 in various mouse models of acute and persistent pain. Our results show that URB937 is orally available in mice, and reduces the hyperalgesia and allodynia resulting from carrageenan injection, surgical ligation of the sciatic nerve, and intraplantar injection of CFA. The remarkable efficacy demonstrated by URB937 in these models – relative to the efficacies displayed by standard analgesics, such as indomethacin and gabapentin – supports the hypothesis that peripheral anandamide signaling plays a key role in regulating the intensity of pain stimuli as they arise in damaged tissues. This discovery may have important therapeutic implications. Indeed, even though globally active FAAH inhibitors exert few side effects in animals, human genetic studies suggest that long-term impairment of brain FAAH activity may result in increased vulnerability to substance abuse [35, 36]. Peripherally restricted FAAH inhibitors such as URB937 would not be subject to this potential risk.

We found URB937 to be substantially more effective at than two globally active FAAH inhibitors – the *O*-aryl carbamate URB597 and the piperidine urea PF-04457845 – in the CFA model of arthritis pain. This surprising result cannot be attributed to differences in inhibitory potency toward FAAH, because URB597 and PF-04457845 are more potent than URB937 at inhibiting FAAH activity in vitro [11, 12]. An alternative explanation, which will require experimental test, is that the greater efficacy of URB937 may be a consequence of the peripheral distribution of this compound. One possibility is that URB937 may be able to access damaged tissues more effectively than other FAAH inhibitors do. Another possibility is that the peripheral distribution of URB937 may limit its ability to activate central hyperalgesic processes that might functionally counteract its peripheral anti-hyperalgesic activity. Consistent with this idea, it has been shown that spinal anandamide can act as a mediator of heterosynaptic pain sensitization and that intrathecal administration of URB597 prolongs the secondary hyperalgesia caused in mice by administration of capsaicin [37].

Taking advantage of the restricted peripheral distribution of URB937, we utilized this compound to examine whether blocking FAAH activity outside the CNS might influence the pharmacological effects of the potent cyclooxygenase-1/cyclooxygenase-2 inhibitor, indomethacin. We found that URB937 enhances the analgesic effects of indomethacin, but strongly reduces the ulcerogenic activity of this NSAID. These results are consistent with those previously reported for the combination of the brain-permeant FAAH inhibitor

URB597 and the NSAID diclofenac [26], and raise the question of how anandamide and cyclooxygenase-derived eicosanoids might interact to influence such diverse functions as pain processing and gastric mucosa renewal. The available data suggest that anandamide may act as a diffuse paracrine signal in peripheral tissues, a role similar to that ascribed to the eicosanoids [38]. It is possible therefore that these two lipid-derived signaling systems operate either synergistically or antagonistically, depending on the locale in which they are generated. Two additional findings further underscore this possibility: first, that CB₁ receptor antagonists block the analgesic effects of various cyclooxygenase inhibitors [39–41] and, second, that anandamide protects gastric mucosal cells from stress-induced damage through a CB₁ receptor-dependent effect [42].

In conclusion, the present findings provide evidence that blockade of peripheral FAAH activity is a highly effective strategy to modulate nociception in animal models of human pain. The results also show that inhibitors of FAAH and cyclooxygenase activities interact functionally in peripheral tissues, to either enhance or hinder each other's action. The marked pharmacological efficacy demonstrated by URB937, superior to that of globally active analgesics, encourages the further evaluation of peripheral FAAH inhibitors as analgesic medicines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AUC	area under the curve
CB	cannabinoid receptor
CFA	complete Freund's adjuvant
CL	confidence limits
CMC	carboxymethylcellulose
CNS	central nervous system
ED₅₀	median effective dose
FAAH	fatty-acid amide hydrolase
IS	internal standard
NSAID	non-steroidal anti-inflammatory drug
PBS	phosphate buffered saline
PPARα	α peroxisome proliferator-activated receptor
V_d	volume of distribution

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Fig. 1. Pharmacokinetic profile of URB937 in mice. (A) URB937 concentrations in plasma after intravenous administration of a 10 mg·kg⁻¹ dose. (B) URB937 concentrations in plasma after oral administration of a 10 mg·kg⁻¹ dose.

Fig. 2.

Antihyperalgesic effects of URB937 in the carrageenan model of acute inflammation. URB937 (0.1–3 mg·kg⁻¹, per os) produced a reduction in paw edema (A), mechanical hyperalgesia (B) and thermal hyperalgesia (C). The drug was dissolved in 0.5% CMC and administered orally before intraplantar injection of carrageenan. Paw edema (A), mechanical hyperalgesia (B) and thermal hyperalgesia (C) were measured before (0 h) or 2, 4, 6, 24, 48, and 72 h after URB937 injection and were significantly different for URB937 (0.3–3 mg·kg⁻¹), compared to vehicle-treated groups. Results are expressed as mean ± SEM (n=6, each group). The data were compared using two-way analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons. * p<0.05, ** p<0.01 and *** p<0.001 vs. vehicle.

Fig. 3.

Antihyperalgesic effects of URB937 in the sciatic nerve constriction model of peripheral neuropathy. URB937 (0.3–3 mg·kg⁻¹, per os) reduced mechanical hyperalgesia (A), thermal hyperalgesia (B), and mechanical allodynia (C). The effects of gabapentin (50 mg·kg⁻¹, per os) is shown for comparison. URB937 was dissolved in 0.5% CMC and administered orally once on day 3, day 7, and day 14 following the sciatic nerve ligation. Mechanical hyperalgesia (A), thermal hyperalgesia (B) and mechanical allodynia (C) were measured 2 h after drug treatment and were significantly different for URB937 (0.3–3 mg·kg⁻¹) and gabapentin, compared to vehicle-treated groups. Results are expressed as mean ± SEM (n=6, each group). The data were compared using two-way analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons. ** p<0.01 and *** p<0.001 vs. vehicle; ###p<0.001 vs. sham-operated mice.

Fig. 4.

Antihyperalgesic effects of FAAH inhibitors URB597, URB937, and PF-04457845 in the CFA model of chronic inflammatory pain. FAAH inhibitors (1–30 mg·kg⁻¹, per os) reduced mechanical hyperalgesia (A) and thermal (C) hyperalgesia. The effect of dexamethasone (100 mg·kg⁻¹, per os) is shown for comparison. FAAH inhibitors were dissolved in 0.5% CMC and administered orally once on day 3, day 7, and day 14 following intraplantar CFA injection. Mechanical hyperalgesia (A) and thermal hyperalgesia (B) were measured 2 h after drug treatment and were significantly different for URB597 (10–30 mg·kg⁻¹), URB937 (1–30 mg·kg⁻¹), PF-04457845 (3–30 mg·kg⁻¹) and dexamethasone, compared to vehicle-treated groups. Results are expressed as mean ± SEM (n=6, each group). The data were compared using two-way analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons. ** p<0.01 and *** p<0.001 vs. vehicle; ###p<0.001 vs. sham-operated mice.

Fig. 5.

Synergistic interaction between URB937 and indomethacin in the carrageenan model.

URB937 and indomethacin were dissolved in 0.5% CMC and administered orally just before intraplantar injection of carrageenan. Paw edema (A) as well as mechanical (B) and thermal hyperalgesia (C) were measured immediately before (0 h) or 2, 4, 6, 24, 48, and 72 h after drug treatments. Co-administrations of URB937 and indomethacin (0.1 mg·kg⁻¹ of URB937 + 1 mg·kg⁻¹ of indomethacin, and 0.3 mg·kg⁻¹ of URB937 + 3 mg·kg⁻¹ of indomethacin, respectively) exerted profound anti-inflammatory (A) and antihyperalgesic effects (B and C). Isobolographic analysis of the data are shown in the right panels for each test. Filled circles represent the theoretical ED₅₀ with 95 % confidence limits (CL). Open circles the experimental ED₅₀ with 95 % CL. Results are expressed as mean ± SEM (n=6, each group). Control animals received vehicle. The data were compared using two-way analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons. * p<0.05, ** p<0.01 and *** p<0.001 vs. vehicle.

Fig. 6.

Synergistic interaction between URB937 and indomethacin in the nerve injury model. URB937 and indomethacin were dissolved 0.5% CMC and administered orally once on day 7 following the ligation. Mechanical hyperalgesia (A) and thermal hyperalgesia (B) as well as mechanical allodynia (C) were measured 2 h after drug treatments. Coadministration of URB937 and indomethacin ($0.3 mg \cdot kg^{-1}$ of URB937 + $3 mg \cdot kg^{-1}$ of indomethacin, and $1 mg \cdot kg^{-1}$ of URB937 + $10 mg \cdot kg^{-1}$ of indomethacin, respectively) exerted profound antihyperalgesic (A and B) and antiallodynic effects (C). Isobolographic analysis of the data are shown in the right panels for each test. Filled circles represent the theoretical ED_{50} with 95 % confidence limits (CL). Open circles the experimental ED_{50} with 95 % CL. Results ($n=6$, each group) are expressed as mean $\pm$ SEM. The data were compared using two-way analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons. ** $p < 0.01$ and *** $p < 0.001$ vs. vehicle; ### $p < 0.001$ vs. sham-operated mice.

Fig. 7.

Gastroprotective effects of URB937 on ulcerogenic activity of indomethacin. URB937 and indomethacin were dissolved in 0.5% CMC and administered orally to food-deprived mice. Gastric irritation score was measured 4h after drug treatments. URB937 displayed no ulcerogenic activity when applied alone (closed triangle). Coadministration of URB937 (0.3 and 1 mg·kg⁻¹, p.o.) and indomethacin (3 p.o., gray circles; or 10 mg·kg⁻¹, closed squares) markedly decreased gastric irritation produced by indomethacin injection alone. Results are expressed as mean ± SEM. The significance of differences between groups was determined by one-way ANOVA followed by Bonferroni's test for multiple comparisons. *p<0.05, **p<0.01 and ***p<0.001 vs. indomethacin.

Table 1

Plasma pharmacokinetic parameters of URB937 after a single intravenous or oral administration ($10 \text{ mg}\cdot\text{kg}^{-1}$) in mice.

Parameter		IV $10 \text{ mg}\cdot\text{kg}^{-1}$	PO $10 \text{ mg}\cdot\text{kg}^{-1}$
$C_{\max}$ (obs)	ng/mL	12828	123
$T_{\max}$ (obs)	h	0,08	0,5
AUC(0-t) (obs area)	ng-h/mL	4855	258
V_d (area) / kg	mL/kg	4613	312816
CL (area) / kg	mL/h/kg	2001	37302
Half-life (Elimination Phase)	h	1,6	5,8
F (oral bioavailability)			5,3%

$C_{\max}$ = Maximum observed concentration; AUC=Cumulative area under curve for experimental time points (0–24h); V_d =Distribution Volume; CL=Systemic clearance based on observed data points (0–24h); $t_{1/2}$ =Time for concentration to diminish by one-half for Elimination phase (E) and Distribution phase (D)