

The Fatty Acid Amide Hydrolase Inhibitor URB597 (Cyclohexylcarbamic Acid 3'-Carbamoylbiphenyl-3-yl Ester) Reduces Neuropathic Pain after Oral Administration in Mice

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ABSTRACT

Fatty acid amide hydrolase (FAAH) is an intracellular serine hydrolase that catalyzes the cleavage of bioactive fatty acid ethanolamides, such as the endogenous cannabinoid agonist anandamide. Genetic deletion of the *faah* gene in mice elevates brain anandamide levels and amplifies the antinociceptive effects of this compound. Likewise, pharmacological blockade of FAAH activity reduces nocifensive behavior in animal models of acute and inflammatory pain. In the present study, we investigated the effects of the selective FAAH inhibitor URB597 (KDS-4103, cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-yl ester) in the mouse chronic constriction injury (CCI) model of neuropathic pain. Oral administration of URB597 (1–50 mg/kg, once daily) for 4 days produced a dose-dependent reduction in

nocifensive responses to thermal and mechanical stimuli, which was prevented by a single i.p. administration of the cannabinoid CB₁ receptor antagonist rimonabant (1 mg/kg). The antihyperalgesic effects of URB597 were accompanied by a reduction in plasma extravasation induced by CCI, which was prevented by rimonabant (1 mg/kg i.p.) and attenuated by the CB₂ antagonist SR144528 (1 mg/kg i.p.). Oral dosing with URB597 achieved significant, albeit transient, drug levels in plasma, inhibited brain FAAH activity, and elevated spinal cord anandamide content. The results provide new evidence for a role of the endocannabinoid system in pain modulation and reinforce the proposed role of FAAH as a target for analgesic drug development.

The endogenous cannabinoid ligand anandamide (Devane et al., 1992; Di Marzo et al., 1994) and the analgesic and anti-inflammatory factor palmitoylethanolamide (PEA) (Calignano et al., 1998) are members of the fatty acid ethanolamide (FAE) family of lipid mediators. These compounds are found in most mammalian tissues, where they are thought to be stored as the phospholipid precursor *N*-acylphosphatidylethanolamine and

to be produced in a stimulus-dependent manner by activation of phospholipase D or phospholipase C activities (Okamoto et al., 2004; Liu et al., 2006).

After release from cells, anandamide may be eliminated via a two-step process consisting of high-affinity transport into cells (Di Marzo et al., 1994; Beltramo et al., 1997) followed by intracellular degradation, catalyzed by fatty acid amide hydrolase (FAAH) (McKinney and Cravatt, 2005). On the other hand, saturated and monounsaturated FAEs, such as PEA, are poor substrates for the anandamide transport system, and their deactivation may be primarily mediated by intracellular hydrolysis catalyzed by FAAH and/or a distinct *N*-acylethanolamine acid amidase (Sun et al., 2005).

Mutant mice lacking the gene encoding for FAAH (*faah*) display reduced FAE hydrolysis and elevated brain levels of

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ABBREVIATIONS: PEA, palmitoylethanolamide; FAE, fatty acid ethanolamide; FAAH, fatty-acid amide hydrolase; CB₁, cannabinoid receptor type I; URB597 (KDS-4103), cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-yl ester; CCI, chronic constriction injury; 2-AG, 2-arachidonoylglycerol; SR144528, *N*-[(1*S*)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide; SR141716, rimonabant, 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide; LC, liquid chromatography; MS, mass spectrometry; OEA, oleoylethanolamide; AUC, area under the curve; CB₂, cannabinoid receptor type II.

these lipid amides (McKinney and Cravatt, 2005). Furthermore, FAAH-null mice show signs of enhanced anandamide signaling at cannabinoid CB₁ receptors (e.g., decreased pain sensation) and increased sensitivity to exogenous anandamide, although their overall behavioral phenotype is similar to that of wild-type mice (McKinney and Cravatt, 2005). This finding suggests that drugs targeting FAAH might heighten the tonic actions of anandamide while avoiding unwanted psychotropic effects due to direct activation of CB₁ receptors.

We have recently identified a class of highly selective *O*-arylcabamate inhibitors of FAAH activity (Kathuria et al., 2003; Mor et al., 2004). Systemic administration of a lead compound in this class, URB597 (KDS-4103, cyclohexylcarbamate acid 3'-carbamoylebiphenyl-3-yl ester), produces profound inhibition of brain FAE hydrolysis in rats and mice, which is accompanied by elevations of brain FAE content and potentiation of the actions of anandamide (Kathuria et al., 2003; Fegley et al., 2005). Furthermore, URB597 exerts anxiolytic-like (Kathuria et al., 2003; Patel and Hillard, 2006), antidepressant-like (Gobbi et al., 2005), antihypertensive (Bátkai et al., 2001), and analgesic (Jayamanne et al., 2006; Jhaveri et al., 2006) effects in rodents. In particular, URB597 reduces pain behaviors in the hot-plate model of thermal nociception (Kathuria et al., 2003) and the adjuvant model of inflammatory pain (Jayamanne et al., 2006). However, the effects of URB597 in neuropathic pain, a condition that affects more than 2 million patients in the United States alone, have not been established. In a recent study, intrathecal administration of URB597 reduced the responses of spinal wide dynamic range neurons in spinal nerve-ligated neuropathic rats (Jhaveri et al., 2006). In contrast, a single systemic injection of URB597 did not reduce mechanical allodynia in neuropathic rats with partially ligated sciatic nerves (Jayamanne et al., 2006). These contradictory findings prompted us to ask whether repeated treatment with URB597 could effectively reduce pain behaviors in chronic nerve constriction-injured (CCI) mice, a widely used model of neuropathic pain (Bennett and Xie, 1988).

Materials and Methods

Chemicals. URB597 (KDS-4103) was provided by Kadmus Pharmaceuticals, Inc. (Irvine, California) and the Institute of Medicinal Chemistry, University of Urbino "Carlo Bo" (Urbino, Italy). We purchased fatty acid chlorides from Nu-Chek Prep (Elysian, MN), [²H₄]ethanolamine from Cambridge Isotope Laboratories (Andover, MA), and [²H₈]2-arachidonoylglycerol (2-AG) from Cayman Chemical (Ann Arbor, MI). SR144528 and rimonabant (SR141716) were provided by RBI (Natick, MA) as part of the Chemical Synthesis Program of the National Institutes of Mental Health. Standard ²H₄-labeled FAEs were synthesized by the reaction of the corresponding fatty acid chlorides with ²H₄-labeled ethanolamine. Fatty acyl chlorides were dissolved in dichloromethane (10 mg/ml) and allowed to react with 1 Eq of ²H₄-labeled ethanolamine for 15 min at 0 to 4°C. The reaction was stopped by adding purified water. After vigorous stirring and phase separation, the upper aqueous phase was discarded, and the organic phase was washed twice with water to remove unreacted ethanolamine. The reaction results in quantitative formation of ²H₄-labeled FAEs, which were concentrated at dryness under a stream of N₂ and reconstituted in chloroform at a concentration of 20 mM. FAE solutions were stored at -20°C until use. Identity and chemical purity (>99.9%) of the synthesized FAEs were determined by thin-layer chromatography and liquid chromatography (LC) coupled to mass spectrometry (MS). All other chemi-

cals were from Sigma-Aldrich (St. Louis, MO). Fresh drug solutions were prepared immediately before use: KDS-4103 and gabapentin were prepared in a vehicle of 0.5% sodium carboxymethyl cellulose and 0.4% polysorbate 80 in water (w/w/w). Rimonabant and SR144528 were prepared in a vehicle of 90% saline-5% polysorbate-80-5% polyethylene glycol (PEG-400) for i.p. administrations (12 ml/kg).

Animals. All procedures met the National Institutes of Health *Guidelines for the Care and Use of laboratory Animals* and those of the Italian Ministry of Health (Decreto Legge 116/92). Male Swiss mice (20–25g) were obtained from Charles River Laboratories, (Wilmington, MA). All animals were maintained on a 12-h/12-h light/dark cycle with free access to water and chow (RMH 2500; Prolab, Framingham, MA) and were habituated to their surroundings for 2 h before experimentation.

CCI Model. Sciatic nerve ligation was performed following the method of Bennett and Xie (1988). Mice were first anesthetized with xylazine (10 mg/kg i.p.) and ketamine (100 mg/kg i.p.), the left thigh was shaved and scrubbed with Betadine, and a small incision (2 cm in length) was made in the middle of the left thigh to expose the sciatic nerve. The nerve was loosely ligated at two distinct sites (spaced at a 2-mm interval) around the entire diameter of the nerve using silk sutures (7-0). The surgical area was dusted with streptomycin powder and closed with a single muscle suture and two skin clips and finally scrubbed with Betadine. In sham-operated animals, the nerve was exposed but not ligated. The animals were placed under a heat lamp until they awakened.

Behavioral Tests. Pain withdrawal thresholds to mechanical or thermal stimuli were measured on both the ipsilateral paw (ligated) and contralateral paw (nonligated) 2 h after drug administration for acute administration experiments or 2 h after the last drug administration for chronic administration experiments. Cannabinoid antagonists were administered 30 min before behavioral testing. *Mechanical hyperalgesia* was assessed by measuring the latency in seconds to withdraw the paw away from a constant mechanical pressure exerted onto its dorsal surface. A 15-g calibrated glass cylindrical rod (diameter = 10 mm) chamfered 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. *Thermal hyperalgesia* was assessed by the method of Hargreaves et al. (1988) by measuring the latency to withdraw the hind paw from a focused beam of radiant heat (thermal intensity: infrared 3.0) applied to the plantar surface, using a commercial apparatus (Ugo Basile, Varese, Italy). The cutoff time was set at 30 s. *Mechanical allodynia* was assessed using a Dynamic Plantar Anesthesiometer (Ugo Basile, Italy) by measuring the latency to withdraw the hind paw from a graded force applied to the plantar surface of the paws using a Von Frey filament. The cutoff force was set at 50 g. *Locomotor activity* was assessed using a fully automated system (Technical and Scientific Equipment, Bad Homburg, Germany). The motility system consists of 2 × 6 infrared light-barriers per cage disposed at right angles on the *x-y* axes to determine the animal's center of gravity and its displacement over time. Animals were habituated to test cages for 3 days before trials. Animals were monitored for 96 h immediately after the first drug administration on day 3.

Plasma extravasation was assessed by the method of Joris et al. (1990). In brief, on day 7 after CCI, Evans' blue dye was injected i.v. (75 mg/kg), and 30 min later, the mice were sacrificed, and the paws were excised. Plantar skin biopsies (6-mm-diameter punches) were taken from the hind paws, and the dye was extracted with 1 ml of formamide for 72 h. Evans' blue dye concentrations were determined by measuring optical density at λ = 550 nm.

FAAH activity was measured in homogenates under conditions that were linear with respect to protein concentration and time as described previously (Fegley et al., 2005). In brief, homogenates were incubated with anandamide[ethanolamine-³H] (60 Ci/mmol; Ameri-

can Radiolabeled Chemicals, St. Louis, MO) at 37°C for 30 min in 0.5 ml of Tris buffer (50 mM, pH 7.5) containing fatty acid-free bovine serum albumin (0.05%). Radioactivity was measured in the aqueous phase after chloroform extraction.

Lipid Extractions. Frozen tissue samples were weighed and homogenized. Tissue homogenates were spiked with [$^2\text{H}_4$]oleoyl-ethanolamide (OEA), [$^2\text{H}_4$]PEA, [$^2\text{H}_4$]anandamide, and [$^2\text{H}_8$]2-AG and were subjected to methanol-chloroform (1:2, v/v) extraction. After centrifugation, the organic layer was carefully removed and transferred to another vial. The aqueous layer was reextracted with additional methanol-chloroform-water (1:2:1, v/v/v), and the organic layers from the first and second extractions were combined and concentrated under N_2 and fractionated by open-bed Silica Gel G column chromatography. In brief, the lipid extracts were reconstituted in chloroform and loaded onto small glass columns packed with Silica Gel G (60-Å 230–400 mesh ASTM; Whatman, Clifton, NJ). Analytes were eluted with 9:1 (v/v) chloroform-methanol. Eluates were dried under N_2 and reconstituted in 0.1 ml of chloroform-methanol (1:4, v/v) for LC/MS analyses. LC/MS analysis of FAE and 2-AG was performed using an 1100-LC system coupled to a 1946A-MS detector (Agilent Technologies, Palo Alto, CA) equipped with an electrospray ionization interface. An XDB Eclipse C_{18} column (50 × 4.6 mm i.d., 1.8 μm , Zorbax; Agilent Technologies) was eluted with a gradient of methanol in water (from 85 to 90% methanol in 2.5 min) at a flow rate of 1.5 ml/min. Column temperature was kept at 40°C. Mass spectrometric detection was in the positive ionization mode, capillary voltage was set at 3 kV, and fragmentor voltage was varied from 120 to 140 V. N_2 was used as drying gas at a flow rate of 13 liters/min and a temperature of 350°C. Nebulizer pressure was set at 60 psi. We monitored Na^+ adducts of the molecular ions $[\text{M}+\text{Na}]^+$ in the selected ion-monitoring mode.

Analysis of URB597 in Plasma. Plasma samples (0.1 ml) were subjected to protein precipitation with acetonitrile (0.25 ml) containing an internal standard (KDS-0017). KDS-0017 is a sulfonamide derivative of URB597, in which the carbamoyl (CONH_2) moiety of URB597 is substituted with a sulfamoyl (SO_2NH_2) (Mor et al., 2004). Samples were immediately vortexed and centrifuged (21,000g) for 5 min, and supernatants were transferred to 96-well plates for LC-MS/MS analysis. LC separations were performed on a Waters 2790 Alliance system (Milford, MA). Separations were carried out using a Luna C_{18} column (2 × 50 mm, 5 μm ; Phenomenex, Torrance, CA) and a gradient consisting of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The flow rate was 0.3 mm/min, column temperature was 45°C, and run times were 7 min. The LC system was interfaced with a Micromass Quattro Ultima tandem mass spectrometer (Micromass, Beverly, MA). The samples were analyzed using electrospray in the positive ionization mode with the cone voltage set at 40 V and capillary voltage of 3.2 kV. The source and desolvation temperature settings were 130 and 500°C, respectively. The voltage of the collision-induced dissociation chamber was set at -15 eV. Multiple reaction monitoring was used for the detection of URB597 as $[\text{M}+\text{H}]^+$ (m/z 339 > 214) and KDS-0017 as $[\text{M}+\text{H}]^+$ (m/z 375 > 250).

Statistical Analyses. Results are expressed as the mean \pm S.E.M. of n experiments. Analyses of data were conducted using GraphPad Prism software (GraphPad Software, San Diego, CA). The significance of differences between groups was determined by one-way analysis of variance followed by a Dunnett's or Tukey's test for multiple comparisons where appropriate. Within group analysis was conducted with a Student's t test. A value of $P < 0.05$ was considered significant.

Results

URB597 Is Systemically Absorbed after Oral Administration. To determine whether URB597 is absorbed after oral administration, we measured the drug in plasma at

various time points after single p.o. dosing in mice (10 or 50 mg/kg). LC/MS/MS analyses of plasma samples taken 15 min after administration revealed that URB597 reached maximal concentrations (C_{max}) of 16 ng/ml at the 10 mg/kg dose and 90 ng/ml at the 50 mg/kg dose (Fig. 1A; Table 1). The drug was cleared from circulation within 1 h of administration at the 10 mg/kg dose and within 12 h at the 50 mg/kg dose (Fig. 1A; Table 1). To assess the pharmacodynamic consequences of oral URB597 treatment, in the same set of experiments we determined the ability of this agent to inhibit brain FAAH activity. Ex vivo measurements in brain homogenates showed that URB597 produced a long-lasting inhibition of FAAH activity (Fig. 1B). It is noteworthy that the lower dose of URB597 (10 mg/kg) was cleared more rapidly and produced lower plasma exposure levels ($\text{AUC}_{(0-T)}$: 26 ng*h/ml) than did the higher dose (50 mg/kg) ($\text{AUC}_{(0-T)}$: 170 ng*h/ml) (Fig. 1A; Table 1). Both doses caused maximal inhibition of FAAH activity (Fig. 1B), albeit with different time courses. The results indicate that URB597 is systemically absorbed and inhibits brain FAAH activity after oral administration.

Oral URB597 Reduces Mechanical Hyperalgesia. We next asked whether oral URB597 inhibits pain behavior in neuropathic mice. We produced peripheral neuropathy by loosely ligating the left sciatic nerve, a surgical procedure that results in the development of mechanical and thermal hyperalgesia (Bennett and Xie, 1988), as well as plasma extravasation in the operated limb. Three days after surgery,

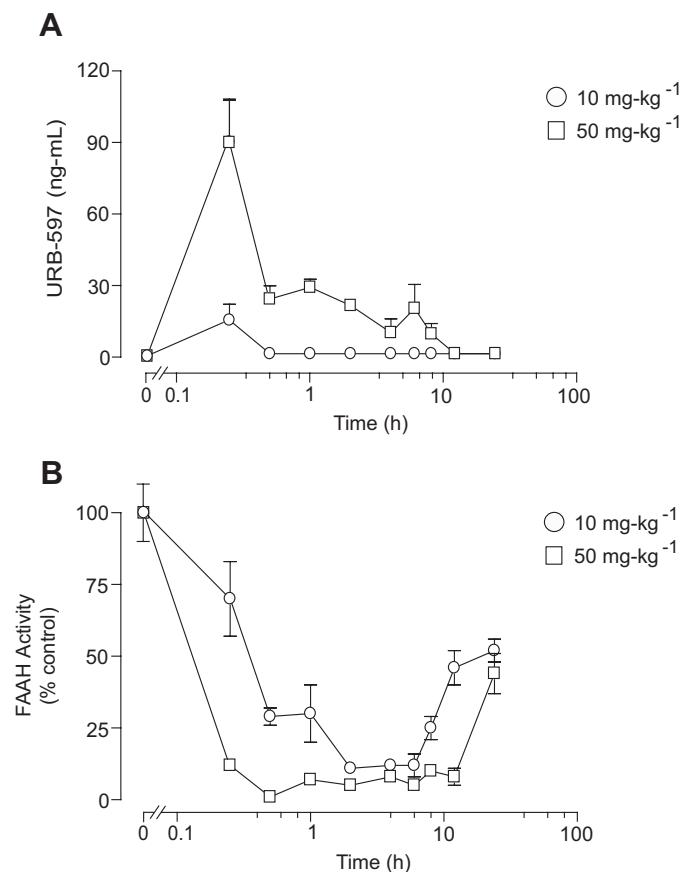


Fig. 1. URB597 is systemically absorbed and inhibits FAAH after oral administration. Plasma URB597 levels (A) and brain FAAH activity (B) after a single dose of URB597 (10 or 50 mg/kg p.o.) in mice ($n = 3-4$). Basal FAAH activity levels for the 10 and 50 mg/kg treatment groups were 422 ± 36 and 524 ± 46 pmol/min/mg protein, respectively.

TABLE 1

Pharmacokinetic profile of URB597 after oral administration in mice ($n = 3-4$)

Dose	AUC	C_{max}	T_{max}
mg/kg	ng*h/ml	ng/ml	min
10	26	16	15
50	170	90	15

when pain behavior is maximal in mice [day 0, presurgery: 54.5 ± 2.1 , day 3: 23.9 ± 3.1 , day 7: 21.3 ± 1.4 ; results are paw withdrawal latencies(s) for a mechanical stimulus], we initiated a 4-day treatment regimen with either vehicle or URB597 (10 mg/kg p.o.) administered once daily. On the fourth day of treatment, 7 days after surgery, paw withdrawal latencies were significantly decreased in ligated mice (Fig. 2A), but not in sham-operated animals (Fig. 2B). Administration of URB597 (10 mg/kg p.o.) significantly reduced mechanical hyperalgesia in the operated paw (Fig. 2A) without affecting withdrawal latencies in the nonoperated (contralateral) limb (Fig. 2A). Administration of a single acute dose of URB597 (10 mg/kg p.o.), 7 days after surgery and 2 h before pain testing, produced only a limited effect (Fig. 2C). The antihyperalgesic effects of repeated URB597 dosing were dose-dependent (Fig. 3A) and comparable in magnitude with those elicited by the clinically used analgesic gabapentin (50 mg/kg p.o., once daily for 4 days) (Fig. 3B). Moreover, these effects were not accompanied by any significant change in locomotor activity when measured for 24 h after the last dose on day 4 (vehicle, $18,256 \pm 1095$; URB, $17,241 \pm 1705$; results are expressed as total beam breaks).

To explore the contribution of cannabinoid receptors to URB597-mediated antihyperalgesia, on day 7 after nerve ligation, we administered the CB_1 -selective antagonist rimonabant (SR141716) or the CB_2 -selective antagonist SR144528 to CCI mice 30 min before pain assessment. Confirming a role for CB_1 receptors, rimonabant (1 mg/kg i.p.) (Fig. 4A) completely prevented the antihyperalgesic actions of URB597 (10 mg/kg p.o.), whereas SR144528 (1 mg/kg i.p.) had no such effect (Fig. 4B). These findings suggest that multiple oral dosing with URB597 reduces mechanical hyperalgesia in neuropathic mice through a CB_1 -dependent mechanism.

Oral URB597 Reduces Thermal Hyperalgesia and Mechanical Allodynia. Treatment with URB597 (10 mg/kg p.o., once daily for 4 days) reduced thermal hyperalgesia (Fig. 5A) and mechanical allodynia in CCI mice (Fig. 5B). In both tests, the analgesic effects of URB597 were prevented by rimonabant (1 mg/kg i.p., 30 min before pain assessment) (Fig. 5, A and B) and attenuated by SR144528 (1 mg/kg i.p.,

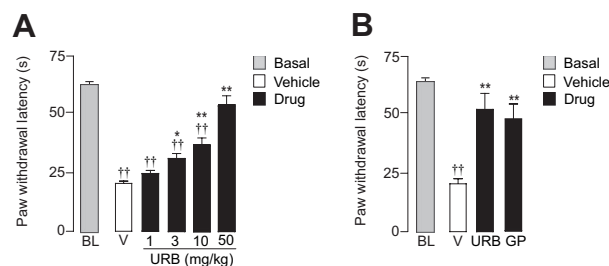


Fig. 3. Oral URB597 dose dependently reduces mechanical hyperalgesia with an efficacy comparable with that of gabapentin. A, effects of vehicle (V, □) or URB597 (URB, ■, 1–50 mg/kg p.o.) on mechanical paw withdrawal latencies on day 0 before surgery (BL, baseline, ▨) or after 1 week (□ and ■) of CCI in mice. B, effects of vehicle (V, □), URB597 (URB, 50 mg/kg p.o.), or gabapentin (GP, 50 mg/kg p.o.) on mechanical paw withdrawal latencies before surgery (BL, basal, ▨) or after 1 week (□ and ■) of CCI in mice ($n = 12$). *, $P < 0.05$; **, $P < 0.01$ versus CCI vehicle; ††, $P < 0.01$ versus BL.

30 min before pain assessment) (Fig. 5, A and B). In agreement with our previous findings (Fig. 2B), URB597 did not change nocifensive responses to thermal stimuli (Fig. 5A) or mechanical pressure (Fig. 5B) applied to nonoperated paws.

Oral URB597 Reduces Plasma Extravasation. The predominant mechanism by which CB_1 receptor activation produces analgesia involves the suppression of nociceptive neuron activity (Walker and Hohmann, 2005). However, CB_1 agonists may also exert local anti-inflammatory effects that might reduce pain sensation (Marchalant et al., 2007). To investigate whether URB597 affects the neurogenic inflammatory response produced by sciatic nerve ligation, we examined whether this drug influences plasma extravasation in the paws of CCI mice. On day 7 after surgery, vehicle-treated mice (once daily for 4 days p.o.) displayed a significant increase in Evans' blue dye permeability in paw tissue compared with control, nonligated animals (Fig. 6). Oral administration of URB597 (10 mg/kg) for 4 days markedly reduced this response (Fig. 6), without changing Evans' blue dye permeability in nonligated paws (Fig. 6). These anti-inflammatory effects of URB597 were completely prevented by rimonabant (1 mg/kg i.p., 30 min before pain assessment) and significantly reduced by SR144528 (1 mg/kg i.p.).

Oral URB597 Increases Spinal Cord FAE Levels. Inhibition of FAAH by URB597 has been shown to increase the levels of anandamide and other noncannabinoid analgesic FAEs, such as PEA, in regions of the brain that process nociceptive stimuli (Fegley et al., 2005; Gobbi et al., 2005; Bortolato et al., 2007). To examine whether similar changes occur in the spinal cord, we quantified FAE levels in lumbar spinal cord segments (L1–L5) of CCI mice treated with either

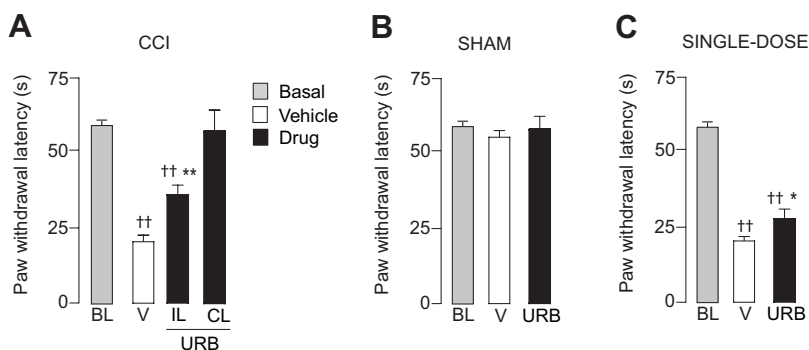


Fig. 2. Oral URB597 reduces mechanical hyperalgesia. A and B, effects of vehicle (V, □) or URB597 (URB, ■, 10 mg/kg p.o.) on mechanical paw withdrawal latencies before surgery (BL, baseline, ▨) after (B) repeated dosing or (C) a single drug administration or after 1 week (□ and ■) of CCI in the (A) ligated (IL, ipsilateral) or nonligated (CL, contralateral) paws of CCI mice ($n = 12$) or (B) sham-operated mice after repeated dosing. *, $P < 0.05$; **, $P < 0.01$ versus CCI vehicle; ††, $P < 0.01$ versus BL.

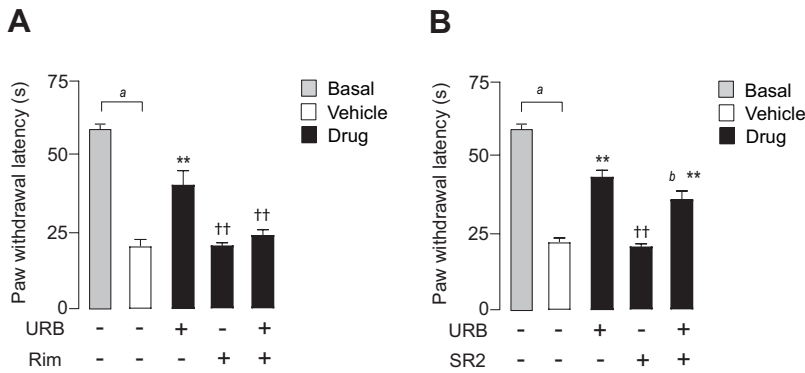


Fig. 4. URB597 analgesia is prevented by the CB₁ antagonist rimonabant. A and B, effects of URB597 (URB, 10 mg/kg p.o.) in the presence or absence of vehicle (V), (A) rimonabant (Rim, 1 mg/kg i.p.) or (B) SR144528 (SR2, 1 mg/kg i.p.), each administered 30 min before pain assessment, on mechanical paw withdrawal latencies before surgery (BL, baseline, □) or after 1 week (□ and ■) of CCI in mice (*n* = 12). **, *P* < 0.01 versus CCI vehicle, *a*, *P* < 0.01 versus BL; ††, *P* < 0.01 versus URB alone; *b*, *P* < 0.01 versus SR2 alone.

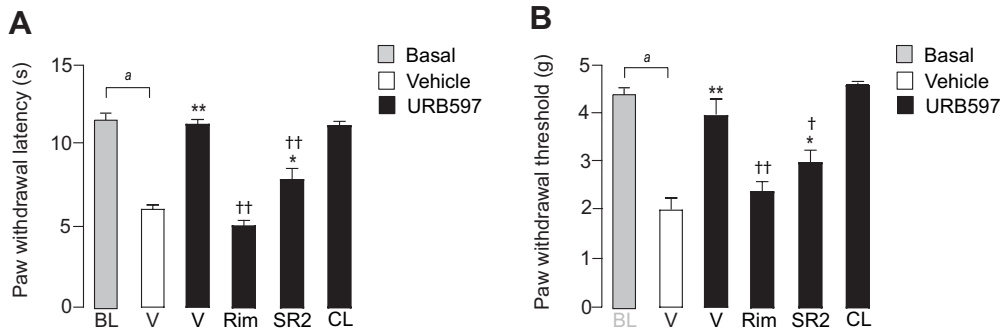


Fig. 5. URB597 reduces thermal hyperalgesia and mechanical allodynia. A and B, effects of vehicle (□), URB597 (■, 10 mg/kg p.o.) in the presence or absence of vehicle (V), rimonabant (Rim, 1 mg/kg i.p.), or SR144528 (SR2, 1 mg/kg i.p.), each administered 30 min before pain assessment, on thermal paw withdrawal latencies (A) or paw withdrawal thresholds (B) before surgery (BL, basal, □) or after 1 week (□ and ■) of CCI in mice. Control, nonligated (CL, contralateral) values are shown (*n* = 12). *, *P* < 0.05; **, *P* < 0.01 versus CCI vehicle (□); *a*, *P* < 0.01 versus BL; †, *P* < 0.05; ††, *P* < 0.01 versus V/URB.

vehicle or URB597 (10 mg/kg, once daily for 4 days). As anticipated, URB597 selectively increased spinal levels of anandamide (Fig. 7A), PEA (Fig. 7B), and OEA (Fig. 7C),

without affecting levels of 2-AG (Fig. 7D), an endocannabinoid lipid that is not a substrate for FAAH.

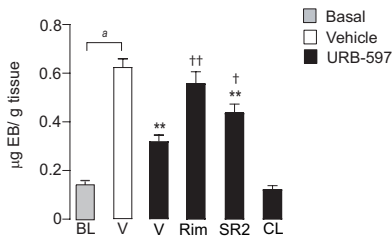


Fig. 6. URB597 inhibits plasma extravasation. Effects of vehicle (□), URB597 (■, 10 mg/kg p.o.) in the presence or absence of vehicle (V), rimonabant (Rim, 1 mg/kg i.p.), or SR144528 (SR2, 1 mg/kg i.p.), each administered 30 min before pain assessment, on plasma extravasation before surgery (BL, baseline, □) or after 1 week (□ and ■) of CCI in mice. Control, nonligated (CL, contralateral) values are shown here (*n* = 12). **, *P* < 0.01 versus CCI vehicle (□); *a*, *P* < 0.01 versus BL; †, *P* < 0.05; ††, *P* < 0.01 versus vehicle/URB.

Discussion

The main finding of the present study is that repeated oral administration of URB597 produces significant antihyperalgesic and antiallodynic effects in the mouse CCI model of neuropathic pain (Bennett and Xie, 1988). These effects are accompanied by an increase in spinal cord anandamide levels, are prevented by the CB₁ antagonist rimonabant, and are reduced by the CB₂ antagonist SR144528 when the stimuli are thermal or tactile, suggesting that they are caused by anandamide-mediated activation of both CB₁ and CB₂ receptors. It is noteworthy that the analgesic actions of URB597 are associated with a marked reduction in plasma extravasation, a finding that supports a role for anandamide in the modulation of neurogenic inflammation (Richardson et al., 1998).

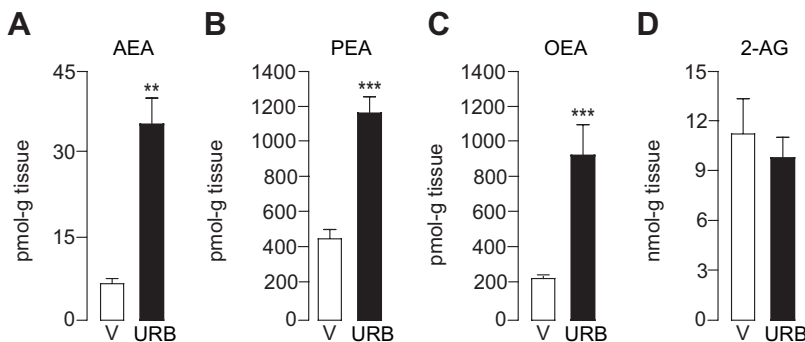


Fig. 7. URB597 increases spinal cord fatty acid ethanolamide levels. Effects of vehicle (V, □) or URB597 (URB, ■, 10 mg/kg p.o.) on spinal cord (A) anandamide (AEA), PEA, (C) OEA, or (D) 2-AG in CCI mice (*n* = 12). Spinal cords were removed 2 h after the fourth treatment with URB597 or vehicle, 7 days after CCI. **, *P* < 0.01 versus vehicle, ***, *P* < 0.001 versus vehicle.

A large body of evidence indicates that direct-acting cannabinoid agonists reduce nocifensive behaviors in animals and alleviate pain in humans. In animals, systemic or intracerebral administration of cannabinoid agonists exerts profound antinociceptive effects and suppresses activity of CB₁-expressing nociceptive neurons in the thalamus, midbrain, and brainstem (Walker and Hohmann, 2005). In addition to these central actions, cannabinoid agonists also prevent formalin-evoked pain responses in mice (Calignano et al., 1998; Jaggat et al., 1998) and capsaicin-evoked pain in monkeys and human volunteers (Ko and Woods, 1999; Rukwied et al., 2003) presumably by interacting with CB₁ or CB₂ receptors localized on peripheral sensory neuron terminals or resident non-neural cells (Hohmann et al., 1999; Ibrahim et al., 2003).

Three sets of results support the idea that the FAAH inhibitor URB597 produces its analgesic effects by blocking anandamide hydrolysis, thus magnifying the ability of this endocannabinoid ligand to activate CB₁ and CB₂ receptors. First, CB₁ receptor blockade enhances pain behaviors in various pain models (Calignano et al., 1998; Strangman et al., 1998) and abrogates nonopioid stress-induced analgesia, suggesting the existence of an analgesic endocannabinoid tone mediated by anandamide. Second, genetic deletion of the *faah* gene and pharmacological inhibition of FAAH activity, each of which elevates brain anandamide levels, reduce nocifensive behaviors in mice and rats (Kathuria et al., 2003; McKinney and Cravatt, 2005). Third, genetic linkage studies have identified FAAH haplotypes in humans, which are linked to variations in pain sensitivity (Kim et al., 2006).

In the present study, we show that URB597 reduces hyperalgesia and allodynia associated with CCI and increases spinal cord levels of anandamide. URB597 does not directly interact with cannabinoid receptors (Kathuria et al., 2003; Piomelli et al., 2006), yet its analgesic effects are blocked by the CB₁ antagonist rimonabant. This suggests that one mechanism by which URB597 produces analgesia is elevating anandamide levels at CB₁ receptors. In addition, recent studies in neuropathic rats have identified analgesic effects mediated by CB₂ receptors (Ibrahim et al., 2003; Scott et al., 2004; Whiteside et al., 2005), raising the possibility that URB597 might modulate pain through both cannabinoid receptor subtypes. Supporting this hypothesis, we found that the CB₂ antagonist SR144528 reduced URB597-mediated reductions in plasma extravasation, neuropathic thermal hyperalgesia, and allodynia. In contrast, SR144528 did not affect URB597-mediated analgesia when the pain stimulus was mechanical. These differences may be partly explained by the predominant localization of CB₁ receptors to neurons, which contrasts the more predominant expression of CB₂ receptors in immune cells and microglia, where they are thought to regulate neuroinflammatory processes (Cabral and Marciano-Cabral, 2005).

An additional possibility is that anandamide and PEA, a noncannabinoid FAE that produces broad spectrum analgesia by activating the nuclear receptor peroxisome proliferator-activated receptor- α (LoVerme et al., 2006), cooperate to reduce pain synergistically. Indeed, synergistic interactions between PEA and anandamide have been reported (Calignano et al., 1998; Russo et al., 2007).

The analgesic effects of URB597 reported here, when the drug is administered for 4 days, contrast those of a previous study reporting that a single dose of URB597 does not affect

mechanical allodynia in neuropathic rats (Jayamanne et al., 2006). Indeed, experiments in our laboratory have confirmed that acute URB597 administration (10 mg/kg p.o.) has only limited effects in CCI mice (day 0, presurgery: 54.5 ± 2.1 , day 3: 23.9 ± 3.1 , day 7: 21.3 ± 1.4 ; results are paw withdrawal latencies (seconds) from a mechanical stimuli). One plausible explanation for this result is that short-term dosing with URB597 might induce neuroplastic changes that are responsible for the enhanced efficacy of the drug. Similar enhancements in efficacy after repeated administrations have been observed with the ability of URB597 to increase serotonergic neuron firing in the dorsal raphe nucleus (Gobbi et al., 2005), as well as with the analgesic effects of cannabinoid agonists (Costa et al., 2004) and gabapentin (Fox et al., 2003). The alternate possibility that repeated dosing with URB597 causes incremental elevations in anandamide levels, for example, through alterations in cellular uptake (Kaczocha et al., 2006), is rendered less likely by our finding that single or repeated administration of URB597 elicits similar changes in spinal cord FAE levels (unpublished data).

The side effects and abuse potential of agonists that target CB₁ receptors are well documented, making these drugs less than ideal for clinical use. Previous experiments have shown that URB597 does not share the pharmacological profile of direct-acting cannabinoid agonists (Piomelli et al., 2006). In particular, FAAH inhibition does not cause hypothermia, catalepsy, or hyperphagia, three typical signs of CB₁ receptor activation (Kathuria et al., 2003). Moreover, URB597 does not produce rewarding effects in the rat conditioned place preference test and does not substitute for cannabinoid agonists in a rat drug discrimination test (Gobbi et al., 2005). This lack of overt cannabinoid effects has been attributed to the ability of URB597 to inhibit FAAH activity without directly activating CB₁ receptors (Kathuria et al., 2003). The favorable pharmacological properties of URB597 underscore the value of FAAH as a target for innovative analgesic drugs.

References

- Bátkai S, Járari Z, Wagner JA, Goparaju SK, Varga K, Liu J, Wang L, Mirshahi F, Khanolkar AD, Makriyannis A, et al. (2001) Endocannabinoids acting at vascular CB₂ receptors mediate the vasodilated state in advanced liver cirrhosis. *Nat Med* **7**:827–832.
- Beltramo M, Stella N, Calignano A, Lin SY, Makriyannis A, and Piomelli D (1997) Functional role of high-affinity anandamide transport, as revealed by selective inhibition. *Science* **277**:1094–1097.
- Bennett GJ and Xie YK (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* **33**:87–107.
- Bortolato M, Mangieri R, Fu J, Kim J, Arguello O, Duranti A, Tontini A, Mor M, Tarzia G, and Piomelli D (2007) Antidepressant-like activity of the fatty acid amide hydrolase inhibitor URB597 in a rat model of chronic mild stress. *Biol Psychiatry*, in press.
- Cabral GA and Marciano-Cabral F (2005) Cannabinoid receptors in microglia of the central nervous system: immune functional relevance. *J Leukoc Biol* **78**:1192–1197.
- Calignano A, La Rana G, Giuffrida A, and Piomelli D (1998) Control of pain initiation by endogenous cannabinoids. *Nature* **394**:277–281.
- Costa B, Colleoni M, Conti S, Trovato AE, Bianchi M, Sotgiu ML, and Gagnoni G (2004) Repeated treatment with the synthetic cannabinoid WIN 55,212-2 reduces both hyperalgesia and production of pronociceptive mediators in a rat model of neuropathic pain. *Br J Pharmacol* **141**:4–8.
- Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, and Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* **258**:1946–1949.
- Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz JC, and Piomelli D (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature* **372**:686–691.
- Fegley D, Gaetani S, Duranti A, Tontini A, Mor M, Tarzia G, and Piomelli D (2005) Characterization of the fatty-acid amide hydrolase inhibitor cyclohexyl carbamic acid 3'-carbamoyl-biphenyl-3-yl ester (URB597): effects on anandamide and oleylethanolamide deactivation. *J Pharmacol Exp Ther* **313**:352–358.
- Fox A, Gentry C, Patel S, Kesingland A, and Bevan S (2003) Comparative activity of the anti-convulsants oxcarbazepine, carbamazepine, lamotrigine and gabapentin in a model of neuropathic pain in the rat and guinea-pig. *Pain* **105**:355–362.

- Gobbi G, Bambico FR, Mangieri R, Bortolato M, Campolongo P, Solinas M, Cassano T, Morgese MG, Debonnel G, Duranti A, et al. (2005) Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. *Proc Natl Acad Sci U S A* **102**:18620–18625.
- Hargreaves K, Dubner R, Brown F, Flores C, and Joris J (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32**: 77–88.
- Hohmann AG, Briley EM, and Herkenham M (1999) Pre- and postsynaptic distribution of cannabinoid and μ opioid receptors in rat spinal cord. *Brain Res* **822**:17–25.
- Ibrahim MM, Deng H, Zvonok A, Cockayne DA, Kwan J, Mata HP, Vanderah TW, Lai J, Porreca F, Makriyannis A, and Malan TP Jr (2003) Activation of CB₂ cannabinoid receptors by AM1241 inhibits experimental neuropathic pain: pain inhibition by receptors not present in the CNS. *Proc Natl Acad Sci U S A* **100**: 10529–10533.
- Jaggar SI, Hasnie FS, Sellaturay S and Rice AS (1998) The anti-hyperalgesic actions of the cannabinoid anandamide and the putative CB₂ receptor agonist palmitoylethanolamide in visceral and somatic inflammatory pain. *Pain* **76**:189–199.
- Jayamanne A, Greenwood R, Mitchell VA, Aslan S, Piomelli D, and Vaughan CW (2006) Actions of the FAAH inhibitor URB597 in neuropathic and inflammatory chronic pain models. *Br J Pharmacol* **147**:281–288.
- Jhaveri MD, Richardson D, Kendall DA, Barrett DA, and Chapman V (2006) Analgesic effects of fatty acid amide hydrolase inhibition in a rat model of neuropathic pain. *J Neurosci* **26**:13318–13327.
- Joris J, Costello A, Dubner R, and Hargreaves KM (1990) Opiates suppress carrageenan-induced edema and hyperthermia at doses that inhibit hyperalgesia. *Pain* **43**:95–103.
- Kaczocha M, Hermann A, Glaser ST, Bojesen IN, and Deutsch DG (2006) Anandamide uptake is consistent with rate-limited diffusion and is regulated by the degree of its hydrolysis by fatty acid amide hydrolase. *J Biol Chem* **281**:9066–9075.
- Kathuria S, Gaetani S, Fegley D, Valino F, Duranti A, Tontini A, Mor M, Tarzia G, La Rana G, Calignano A, et al. (2003) Modulation of anxiety through blockade of anandamide hydrolysis. *Nat Med* **9**:76–81.
- Kim H, Mittal DP, Iadarola MJ, and Dionne RA (2006) Genetic predictors for acute experimental cold and heat pain sensitivity in humans. *J Med Genet* **43**:e40.
- Ko MC and Woods JH (1999) Local administration of Δ^9 -tetrahydrocannabinol attenuates capsaicin-induced thermal nociception in rhesus monkeys: a peripheral cannabinoid action. *Psychopharmacology* **143**:322–326.
- Liu J, Wang L, Harvey-White J, Osei-Hyiaman D, Razdan R, Gong Q, Chan AC, Zhou Z, Huang BX, Kim HY, et al. (2006) A biosynthetic pathway for anandamide. *Proc Natl Acad Sci U S A* **103**:13345–13350.
- LoVerme J, Russo R, La Rana G, Fu J, Farthing J, Mattace-Raso G, Meli R, Hohmann A, Calignano A, and Piomelli D (2006) Rapid broad-spectrum analgesia through activation of peroxisome proliferator-activated receptor- α . *J Pharmacol Exp Ther* **319**:1051–1061.
- Marchalant Y, Rosi S, and Wenk GL (2007) Anti-inflammatory property of the cannabinoid agonist WIN-55212-2 in a rodent model of chronic brain inflammation. *Neuroscience* **144**:1516–1522.
- McKinney MK and Cravatt BF (2005) Structure and function of fatty acid amide hydrolase. *Annu Rev Biochem* **74**:411–432.
- Mor M, Rivara S, Lodola A, Plazzi PV, Tarzia G, Duranti A, Tontini A, Piersanti G, Kathuria S, and Piomelli D (2004) Cyclohexylcarbamic acid 3'- or 4'-substituted biphenyl-3-yl esters as fatty acid amide hydrolase inhibitors: synthesis, quantitative structure-activity relationships, and molecular modeling studies. *J Med Chem* **47**:4998–5008.
- Okamoto Y, Morishita J, Tsuboi K, Tonai T, and Ueda N (2004) Molecular characterization of a phospholipase D generating anandamide and its congeners. *J Biol Chem* **279**:5298–5305.
- Patel S and Hillard CJ (2006) Pharmacological evaluation of cannabinoid receptor ligands in a mouse model of anxiety: further evidence for an anxiolytic role for endogenous cannabinoid signaling. *J Pharmacol Exp Ther* **318**:304–311.
- Piomelli D, Tarzia G, Duranti A, Tontini A, Mor M, Compton TR, Dasse O, Monaghan EP, Parrott JA, and Putman D (2006) Pharmacological profile of the selective FAAH inhibitor KDS-4103 (URB597). *CNS Drug Rev* **12**:21–38.
- Richardson JD, Kilo S, and Hargreaves KM (1998) Cannabinoids reduce hyperalgesia and inflammation via interaction with peripheral CB₁ receptors. *Pain* **75**:111–119.
- Rukwied R, Watkinson A, McGlone F, and Dvorak M (2003) Cannabinoid agonists attenuate capsaicin-induced responses in human skin. *Pain* **102**:283–288.
- Russo R, LoVerme J, La Rana G, D'Agnostino G, Sasso O, Calignano A, and Piomelli D (March 19, 2007) Synergistic antinociception by the cannabinoid agonist anandamide and the PPAR- α agonist GW7647. *Eur J Pharmacol* [Epub ahead of print].
- Scott DA, Wright CE, and Angus JA (2004) Evidence that CB-1 and CB-2 cannabinoid receptors mediate antinociception in neuropathic pain in the rat. *Pain* **109**: 124–131.
- Strangman NM, Patrick SL, Hohmann AG, Tsou K, and Walker JM (1998) Evidence for a role of endogenous cannabinoids in the modulation of acute and tonic pain sensitivity. *Brain Res* **813**:323–328.
- Sun YX, Tsuboi K, Zhao LY, Okamoto Y, Lambert DM, and Ueda N (2005) Involvement of *N*-acylethanolamine-hydrolyzing acid amidase in the degradation of anandamide and other *N*-acylethanolamines in macrophages. *Biochim Biophys Acta* **1736**:211–220.
- Walker JM and Hohmann AG (2005) Cannabinoid mechanisms of pain suppression. *Handb Exp Pharmacol* **168**:509–554.
- Whiteside GT, Gottshall SL, Boulet JM, Chaffer SM, Harrison JE, Pearson MS, Turchin PI, Mark L, Garrison AE, and Valenzano KJ (2005) A role for cannabinoid receptors, but not endogenous opioids, in the antinociceptive activity of the CB₂-selective agonist, GW405833. *Eur J Pharmacol* **528**:65–72.

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