

Reversible Inhibitors of Fatty Acid Amide Hydrolase That Promote Analgesia: Evidence for an Unprecedented Combination of Potency and Selectivity

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ABSTRACT

Fatty acid amide hydrolase (FAAH) is the primary catabolic regulator of several bioactive lipid amides in vivo, including the endogenous cannabinoid anandamide and the sleep-inducing substance oleamide. Inhibitors of FAAH are considered a potential therapeutic approach for the treatment of several nervous system disorders, including pain, anxiety, and insomnia. However, for FAAH inhibitors to achieve clinical utility, they must not only display efficacy in vivo but also selectivity for this enzyme relative to the numerous other serine hydrolases present in mammalian proteomes. Here, we report a general strategy for evaluating the pharmacological activity and target specificity of FAAH inhibitors and its implementation to develop the first class of selective reversible inhibitors of this enzyme

that are highly efficacious in vivo. Using a series of functional proteomics, analytical chemistry, and behavioral pharmacology assays, we have identified a class of α -keto-heterocycles that show unprecedented selectivity for FAAH relative to other mammalian hydrolases, and, when administered to rodents, raise central nervous system levels of anandamide and promote cannabinoid receptor 1-dependent analgesia in several assays of pain sensation. These studies provide further evidence that FAAH may represent an attractive therapeutic target and describe a general route by which inhibitors of this enzyme can be optimized to achieve exceptional potency, selectivity, and efficacy in vivo.

Fatty acid amides represent an emerging class of signaling lipids that include the endogenous cannabinoid (endocannabinoid) anandamide (Devane et al., 1992), the sleep-inducing substance oleamide (Cravatt et al., 1995), the anti-inflammatory compound *N*-palmitoylethanolamine (PEA) (Lambert et al., 2002), and the appetite-suppressing agent *N*-oleoylethanolamine (OEA) (Rodriguez de Fonseca et al.,

2001). The signaling function of fatty acid amides is tightly controlled by the integral membrane enzyme fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996), which hydrolyzes these compounds to their corresponding acids (Deutsch and Chin, 1993; Desarnaud et al., 1995; Maurelli et al., 1995; Ueda et al., 1995; Cravatt et al., 1996). Mice lacking FAAH possess highly elevated endogenous levels of fatty acid amides in several brain regions (Clement et al., 2003) and display cannabinoid receptor (CB1)-dependent analgesia (Cravatt et al., 2001; Lichtman et al., 2004). These data confirm the central role that FAAH plays in regulating fatty acid amide signaling in vivo and suggest that this enzyme may represent an attractive target for the treatment of pain, as well as other nervous system disorders. Consistent with this

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ABBREVIATIONS: PEA, *N*-palmitoylethanolamine; OEA, *N*-oleoylethanolamine; FAAH, fatty acid amide hydrolase; CB1, central cannabinoid receptor; DMSO, dimethyl sulfoxide; FP, fluorophosphonate; PAGE, polyacrylamide gel electrophoresis; %MPE, percent maximum possible effect; TGH, triacylglycerol hydrolase; AAD, arylacetamide deacetylase; CE-1, carboxylesterase 1; LPL, lipoprotein lipase; MAGL, monoacylglycerol lipase; SR141716, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-di-chlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride; SR144528, *N*-[1*S*]-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; OL-135, 1-oxo-1[5-(2-pyridyl)-2-yl]-7-phenylheptane; OL-92, 1-(oxazolo[4,5-*b*]pyridin-2-yl)-1-oxo-7-phenylheptane; CP 55,940, (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; URB532, *n*-butylcarbamic acid 4-benzoyloxyphenyl ester; URB597, cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-yl ester; BMS-1, [6-(2-methyl-4,5-diphenyl-imidazol-1-yl)-hexyl]-carbamic acid 2-fluoro-phenyl ester.

notion, carbamate FAAH inhibitors have recently been shown to produce both analgesic and anxiolytic effects in rodents (Kathuria et al., 2003). Nonetheless, these agents, like nearly all FAAH inhibitors described to date (Deutsch et al., 1997; Boger et al., 1999, 2000), possess electrophilic groups that either reversibly or irreversibly inactivate this enzyme by engaging its conserved serine nucleophile. Although this "electrophile-based" strategy for FAAH inhibition has engendered highly potent compounds, the selectivity of these agents is less clear. Indeed, testing the specificity of FAAH inhibitors is a predictably daunting challenge given the immense size of the serine hydrolase superfamily to which it belongs (e.g., greater than 200 serine hydrolases are encoded by the human genome; Lander et al., 2001).

Recently, we reported a chemical strategy for evaluating the potency and selectivity of serine hydrolase inhibitors in whole proteomes (Leung et al., 2003). We have since used this functional proteomic method, in combination with chemical synthesis and enzymatic assays, to identify a class of highly potent and selective reversible FAAH inhibitors (Boger et al., 2004). Here we demonstrate that these agents 1) exhibit an unprecedented selectivity for FAAH compared with previously described inhibitors, 2) augment the endogenous levels of fatty acid amides in the central nervous system, and 3) produce CB1-dependent analgesic effects in both thermal and chemical pain models. Collectively, these studies provide additional evidence that FAAH is an attractive potential drug target and describe a general route by which inhibitors of this enzyme can be optimized to achieve exceptional potency, selectivity, and efficacy *in vivo*.

Materials and Methods

Experimental Animals. ICR mice (Harlan, Indianapolis, IN) were used for the initial *in vivo* screening of FAAH inhibitors and C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were used for the evaluation of OL-135 in thermal and formalin pain models. FAAH(−/−) mice used in this study were sixth generation offspring

backcrossed onto a C57BL/6 background (Clement et al., 2003). All subjects were male, weighed between 20 and 30 g, and were housed five animals per cage in a temperature-controlled (20–22°C) facility. The Institutional Animal Care and Use Committee at Virginia Commonwealth University approved all experiments. Mice were given unlimited access to food and water and were maintained on a 12:12-h light/dark cycle.

Drugs. The structures of the reversible FAAH inhibitors OL-92 and OL-135, the synthesis of which will be described elsewhere (Boger et al., 2004), are presented in Table 1. National Institute on Drug Abuse (Bethesda, MD) provided SR141716 and SR144528, selective receptor antagonists for the CB1 (Rinaldi-Carmona et al., 1994) and CB2 (Rinaldi-Carmona et al., 1998) receptors, respectively. All drugs were administered via either i.p. or i.v. routes of administration in a mixture of 1:1:18 ethanol/alkamuls-620 (Rhône-Poulenc, Princeton, NJ)/saline using an injection volume of 100 μ l/10 g body weight.

Proteomic Analysis of Inhibitor Selectivity. Inhibitor analysis using activity-based proteomic methods was carried out as described previously (Leung et al., 2003). Briefly, mouse tissues were Dounce-homogenized in Tris buffer (50 mM Tris-HCl buffer, pH 8.0) with 320 mM sucrose and separated by high-speed centrifugation at 4°C. Sequential spins of 1100g for 5 min and 22,000g for 30 min yielded the membrane fraction, which was washed and resuspended in Tris buffer. Supernatant from the second spin yielded the soluble fraction. Proteome samples (1 mg/ml) were preincubated with inhibitors over a concentration range of 100 pM to 100 μ M for 10 min and then treated with fluorophosphonate (FP)-rhodamine (100 nM) (Patricelli et al., 2001) at room temperature for 10 min. Both FAAH inhibitors and FP-rhodamine were added from concentrated DMSO stocks to give a final DMSO concentration of 2%. Reactions were quenched by the addition of 1 volume of 2 \times standard SDS-PAGE loading buffer (reducing), run on SDS-PAGE, and visualized in-gel using a Hitachi FMBio IIe flatbed fluorescence scanner (MiraBio, Alameda, CA). Labeled proteins were quantified by measuring integrated band intensities (normalized for volume). The band intensities of proteome samples treated with DMSO alone were considered 100% activity, and band intensities of proteins inhibited by compound library members were expressed as a percentage of remaining activity. Potent inhibitors (IC₅₀ values <10 nM) also were tested at 0.5 to 50 nM with proteome samples adjusted to 0.1 mg/ml. IC₅₀

TABLE 1

IC₅₀ values [nanomolar concentration (95% confidence limits)] determined by activity-based protein profiling for serine hydrolases sensitive to FAAH inhibitors

| Inhibitor | FAAH | TGH | AAD | CE-1 | LPL | MAGL | KIAA1363 |
|-----------|---------------------|------------------------|-------------------|------------------------|------------------------|---------------------|-----------------------|
| OL-135 | 2.1 (1.5-3.0) | 620 (490-870) | > 100000 | > 100000 | > 100000 | > 100000 | > 100000 |
| OL-92 | 0.28 (0.15-0.52) | 2.9 (2.2-3.7) | > 100000 | > 100000 | > 100000 | > 100000 | 17000 (9500-28000) |
| URB-532 | 610 (360-630) | 66 (36-99) | 186 (88-320) | 28000 (13000-59000) | 23000 (12000-44000) | > 100000 | > 100000 |
| URB-597 | 113 (79-170) | 192 (96-320) | > 100000 | > 100000 | > 100000 | > 100000 | > 100000 |
| BMS-1 | 200 (150-270) | 14000 (11000-17000) | 440 (140-1400) | 170 (94-300) | 1300 (640-2400) | 3300 (2800-4700) | 4100 (1800-7900) |

values were determined from dose-response curves from three trials at each inhibitor concentration using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Enzyme targets were affinity isolated and identified using biotinylated FP probes (Liu et al., 1999) and avidin chromatography-mass spectrometry procedures, as described previously (Kidd et al., 2001).

Ligand Binding. OL-135 binding to CB1 or CB2 receptors was assessed in [³H]CP 55,940 binding competition assays using membranes from either mouse whole brain or from Chinese hamster ovary cells transfected with the human CB2 receptor cDNA (Showalter et al., 1996), respectively. Details of the binding assays were described previously (Aung et al., 2000).

Behavioral Studies. Subjects were assessed for basal responses in the tail immersion and hot plate assays, with the intensity set at $56.0 \pm 0.5^\circ\text{C}$. In the tail immersion test, each mouse was hand-held, with approximately 1 cm of the tip of the tail immersed into a water bath and the latency for the animal to withdraw its tail from the water within 10 s was scored (Cravatt et al., 2001). In the *in vivo* screening experiment (Fig. 2A), the tail withdrawal data were depicted as percent maximum possible effect (MPE), in which $\% \text{MPE} = 100 \times (\text{postinjection latency} - \text{preinjection latency}) / (10\text{-s ceiling} - \text{preinjection latency})$. In the hot-plate test, the latency to jump or lick/shake a hind paw within a 60-s observation period was scored (Cravatt et al., 2001).

In the formalin assay, subjects were given an intraplantar injection containing 20 μl of a 2.5% formalin solution into the right hind paw (Cravatt et al., 2001). The total amount of time spent licking or lifting the afflicted paw was recorded for both the early phase (i.e., 0–5 min) and the late phase (i.e., 10–25 min). Peak pain behavior was observed during each respective time period in control mice.

Rectal temperature was determined by inserting a thermocouple probe 2.0 cm into the rectum, and temperature was obtained from a telethermometer. Baseline temperatures were obtained before injections ($36.5 \pm 0.1^\circ\text{C}$, mean \pm S.E.) and again 60 min after anandamide or vehicle injection.

Locomotor activity was assessed by placing individual mice into photocell activity cages (6.5×11 in.), and interruptions of the photocell beams were recorded for 10 min using a Digiscan animal activity monitor (Omnitech Electronics Inc., Columbus, OH). Motor incoordination was evaluated in the inverted-screen test in which mice were placed on a wire screen that was immediately inverted, and the latency to climb onto the top within 60 s was recorded.

Determination of Tissue Levels of Fatty Acid Amides. The levels of three representative fatty acid amides, anandamide, OEA, and PEA, in mouse tissues were measured by isotope-dilution liquid chromatography-mass spectrometry as described previously (Di Marzo et al., 2000; Cravatt et al., 2001) using an Agilent 1100 series high-performance liquid chromatograph coupled to an Agilent MSD mass spectrometer in the selected-ion monitoring mode.

Data Analysis. Analysis of variance was used to analyze the data. Dunn's test was used for post hoc analysis. Differences were considered significant at the $p < 0.05$ level. The ED_{50} values with 95% confidence intervals of OL-135 were determined by least-squares linear regression analysis on the logs of the doses using Microsoft Excel (Microsoft, Redmond, WA).

Results

A Comparative Proteomic Analysis of the Selectivity of FAAH Inhibitors. Several FAAH inhibitors have recently been described that produce intriguing behavioral effects in rodents. These include URB532 and URB597, which produce analgesic and anxiolytic effects (Kathuria et al., 2003), and BMS-1, which exhibits analgesic properties in several acute and chronic pain models (Sit and Xie, 2002) (Table 1). Each of these inhibitors possesses a carbamate group that presumably causes the irreversible inactivation of FAAH

through modification of its serine nucleophile. Thus, these carbamate inhibitors likely achieve their potency for FAAH, at least in part, by exploiting a feature of the enzyme's active site that is conserved among all members of the serine hydrolase superfamily, raising potential concerns about the selectivity that these agents will display for FAAH relative to other hydrolytic enzymes. We therefore screened these carbamate inhibitors for activity against numerous serine hydrolases in parallel using a recently described functional proteomics assay (Leung et al., 2003) and compared the resulting global serine hydrolase inhibition profiles to those of OL-135 and OL-92, two recently identified potent and selective reversible FAAH inhibitors (Boger et al., 2004).

In this functional proteomic screen, inhibitors are evaluated for their ability to compete the labeling of serine hydrolases by an active site-directed FP-rhodamine probe (Leung et al., 2003). Inhibitors were tested over a range of concentrations (100 pM–100 μM) against the soluble and membrane fractions of three mouse tissue proteomes (brain, heart, and kidney), and from these data, IC_{50} values were determined for hydrolases that showed sensitivity to one or more of the inhibitors. Inhibitor-sensitive hydrolases were then identified using biotinylated FP probes (Liu et al., 1999) and avidin chromatography-mass spectrometry procedures, as described previously (Kidd et al., 2001). A representative gel comparing the FP-rhodamine labeling of a series of serine hydrolases in the presence of 1 μM of each FAAH inhibitor is shown in Fig. 1A. Each carbamate inhibitor of FAAH was found to show significant activity against other serine hydrolases. For example, both URB532 and URB597 inhibited the heart enzyme triacylglycerol hydrolase (TGH) with potencies equivalent to or greater than their IC_{50} values for FAAH (Table 1). Interestingly, BMS-1 only weakly inhibited TGH (IC_{50} value of 14 μM), but instead showed broad activity against several other serine hydrolases, including arylacetamide deacetylase (AAD; IC_{50} value of 0.44 μM), carboxylesterase 1 (CE-1; IC_{50} value of 0.17 μM), lipoprotein lipase (LPL; IC_{50} value of 1.3 μM), and monoacylglycerol lipase (MAGL; IC_{50} value of 3.3 μM). In contrast to these carbamate inhibitors, the reversible inhibitor OL-135 exhibited exceptional selectivity for FAAH, displaying an IC_{50} value for this enzyme that was more than 300-fold lower than its IC_{50} value for any of the other hydrolases examined (Table 1). An analog of OL-135, OL-92, which bears a fused pyridyl oxazole heterocycle, also showed excellent potency and selectivity for FAAH (Table 1). The remarkable selectivity of OL-135 and OL-92 is graphically summarized in Fig. 1B, which compares the relative activity of these inhibitors and the carbamate inhibitors for FAAH versus other hydrolases in the proteome. Thus, once having discovered that OL-135 and OL-92 showed an unprecedented level of selectivity for FAAH, we next examined whether these reversible inhibitors were efficacious *in vivo*.

The FAAH Inhibitor OL-135 Augments the Pharmacological Activity of Anandamide *In Vivo*. OL-135 and OL-92 were first screened for their ability to augment anandamide-induced analgesia, as assessed in tests for pain sensitivity and thermoregulation. FAAH inhibitors (10 mg/kg *i.v.*) or vehicle was administered to mice 10 min before treatment with anandamide (50 mg/kg *i.p.*) or vehicle, and, after 20 min, animals were evaluated in the tail immersion test for thermal pain sensation. As reported previously (Cravatt et al., 2001), anandamide failed to significantly alter the pain

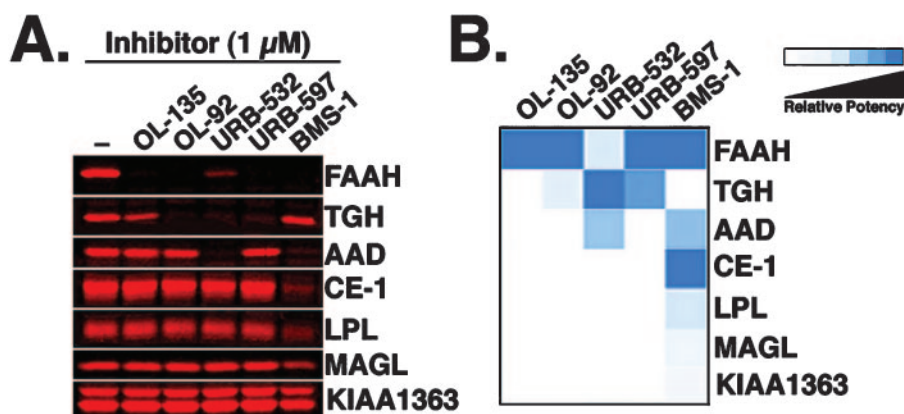


Fig. 1. Proteomic analysis of the selectivity of FAAH inhibitors. A, representative brain, heart, and kidney serine hydrolases exhibiting sensitivity to one or more FAAH inhibitors. Shown are fluorescent gel images of competition reactions in which FAAH inhibitors ($1\ \mu\text{M}$) and the activity-based probe FP-rhodamine ($100\ \text{nM}$) were incubated with mouse tissue proteomes ($1\ \text{mg protein/ml}$) for $10\ \text{min}$. Enzymes were identified from tissue proteomes using biotinylated FP probes and avidin chromatography-mass spectrometry procedures (Kidd et al., 2001). B, comparison of the relative selectivities of FAAH inhibitors. The IC_{50} values of each FAAH inhibitor for the indicated enzymes were determined from activity-based proteomic reactions containing different inhibitor concentrations ($100\ \text{pM}$ – $100\ \mu\text{M}$) and were normalized to the most potently inhibited enzyme.

responses of wild-type mice. In contrast, wild type mice pretreated with the FAAH inhibitor OL-135 before anandamide exhibited robust antinociceptive responses that were equivalent in magnitude to FAAH(–/–) mice treated with this endocannabinoid (Fig. 2A). OL-135 was also found to dramatically augment the hypothermic effects of anandamide in wild-type mice (Fig. 2B). The CB1 receptor antagonist SR141716 ($3\ \text{mg/kg i.p.}$) blocked the analgesic and hypothermic effects of anandamide in OL-135-treated mice, indicating that these pharmacological activities are mediated by CB1 receptors. Finally, a dose-response profile of OL-135 (1 – $10\ \text{mg/kg i.p.}$) revealed that this agent increased the analgesic (Fig. 2C) and hypothermic (Fig. 2D) activity of anandamide with ED_{50} values (95% confidence limits) of 1.9 (1.0 – 3.7) and 1.7 (1.0 – 2.6) mg/kg , respectively. In contrast to these findings, the fused pyridyl oxazole inhibitor OL-92 failed to augment the pharmacological activity of anandamide (Fig. 2, A and B), indicating that this agent was not active *in vivo*.

The FAAH Inhibitor OL-135 Produces CB1-Dependent Analgesia and Elevates Endocannabinoid Levels *in Vivo*. Previous studies have shown that FAAH(–/–) mice possess dramatically elevated brain levels of anandamide and related fatty acid amides (Cravatt et al., 2001) that correlate with a CB1-dependent analgesic phenotype in thermal and chemical models of pain (Cravatt et al., 2001; Lichtman et al., 2004). Therefore, we evaluated whether the FAAH inhibitor OL-135 could produce similar neurochemical and behavioral effects in wild-type mice. As shown in Fig. 3, OL-135 ($10\ \text{mg/kg i.p.}$) produced significant antinociception in both the tail immersion (Fig. 3A) and hot-plate (Fig. 3B) tests measured $60\ \text{min}$ postinjection. Time-course experiments revealed that the analgesic activity of OL-135 persisted from 15 to $240\ \text{min}$ postinjection (Fig. 3, C and F). OL-135 was also found to reduce pain behavior in the formalin test of noxious chemical pain (Fig. 4). An *i.p.* injection of OL-135 given $60\ \text{min}$ before formalin elicited a dose-dependent decrease in pain behavior in both the first (Fig. 4A) and second (Fig. 4B) phases of this test. A maximal analgesic effect of approximately 50% was found, which is similar in magnitude to that observed in FAAH(–/–) mice (Cravatt et al., 2001; Lichtman et al., 2004). The ED_{50} (95% confidence intervals) values for the analgesic effects of OL-135 in phase

1 and phase 2 of the formalin test were 7.9 (6.6 – 9.4) and 7.5 (6.2 – 9.0) mg/kg , respectively.

Pretreatment with the CB1 receptor antagonist SR141716 ($3\ \text{mg/kg i.p.}$) completely blocked the analgesic effects of OL-135 in the tail immersion (Fig. 3B), hot-plate (Fig. 3E), and formalin (Fig. 4, C and D) tests, indicating a CB1-mediated mechanism of action for this inhibitor in each of these assays. Consistent with this premise, the endogenous levels of the endocannabinoid anandamide (and other *N*-acyl ethanolamines; e.g., OEA and PEA) were significantly elevated in the brains and spinal cords of OL-135-treated mice (measured $60\ \text{min}$ postinjection) (Fig. 5). To determine whether OL-135 had direct effects at cannabinoid receptors, we evaluated whether it would displace [^3H]CP 55,940 from CB1 (rat whole brain homogenate) or CB2 (transfected Chinese hamster ovary cells) receptors. OL-135 failed to bind to either CB1 (22% maximal displacement at $10\ \mu\text{M}$) or CB2 (30% maximal displacement at $10\ \mu\text{M}$) receptors, indicating that its pharmacological effects are not the result of a direct activation of cannabinoid receptors. Finally, to confirm that OL-135 did not produce motor deficits that might confound the interpretation of pain responses in the formalin and hot-plate tests, mice were given an *i.p.* injection of this inhibitor and $60\ \text{min}$ later evaluated for gross locomotor activity (Compton et al., 1993) and in the inverted screen test of motor coordination. At doses of 10 and $30\ \text{mg/kg i.p.}$, OL-135 failed to affect motility or cause motor impairment compared with vehicle controls (data not shown).

Discussion

The fatty acid amide signaling system has been implicated in a number of (patho)physiological processes, including pain (Calignano et al., 1998; Jaggar et al., 1998; Walker et al., 1999), sleep (Cravatt et al., 1995), feeding (Di Marzo et al., 2001; Rodriguez de Fonseca et al., 2001), blood pressure (Wagner et al., 1997), and inflammation (Franklin et al., 2003). Representative fatty acid amides include the endocannabinoid anandamide (Devane et al., 1992), the sleep-inducing substance oleamide (Cravatt et al., 1995), the anti-inflammatory compound PEA (Lambert et al., 2002), and the appetite-suppressing agent OEA (Rodriguez de Fonseca et

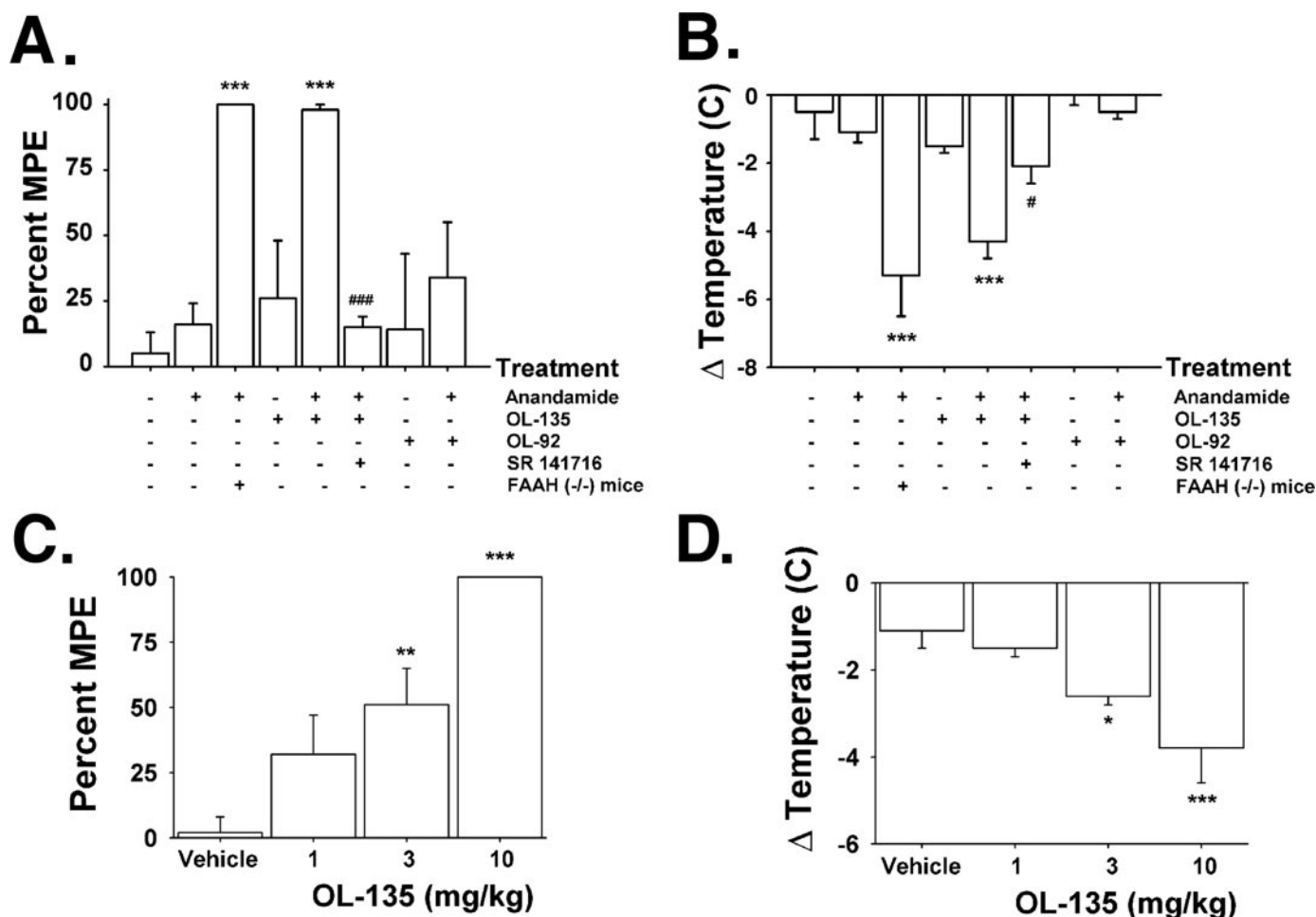


Fig. 2. Rapid pharmacological screen to evaluate the activity of FAAH inhibitors in vivo. The reversible FAAH inhibitors OL-135 and OL-92 were tested for their ability to augment the behavioral effects of anandamide (50 mg/kg i.p.) in wild-type mice. Anandamide given alone failed to produce either analgesia in the tail withdrawal test (A) or hypothermia (B), but it elicited significant effects in both assays when administered to FAAH (-/-) mice (positive control group) or mice pretreated with OL-135 (10 mg/kg i.v., administered 10 min before anandamide). SR141716 (3 mg/kg i.p., administered 60 min before OL-135) completely blocked the antinociceptive and hypothermic effects in mice treated with both anandamide and OL-135. The fused pyridyl oxazole agent OL-92 (10 mg/kg i.v.) failed to augment the pharmacological effects of anandamide or produce any overt effects on its own. Antinociception and hypothermia were measured 20 and 60 min postadministration of anandamide, respectively. ***, $p < 0.001$ for each respective group versus either the vehicle control group or the anandamide alone group. #, $p < 0.05$ and ###, $p < 0.001$ for mice treated with SR141716 before the OL-135 and anandamide combination compared with mice treated with the combination of OL-135 and anandamide alone. C and D, OL-135 dose-dependently (1–10 mg/kg i.v.) augmented the analgesic (C) and hypothermic (D) effects of anandamide (50 mg/kg). *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ for each respective group versus the vehicle control group. Results shown as means \pm S.E., $n = 6$ –12 mice/group.

al., 2001). All of these signaling lipids seem to share a common route for catabolism in vivo, principally mediated by the integral membrane protein FAAH (Cravatt et al., 1996). Accordingly, inhibitors of FAAH have been suggested as a novel therapeutic strategy for the treatment of several diseases, including pain, anxiety, and inflammation (Cravatt and Lichtman, 2003; Gaetani et al., 2004). Nonetheless, to date, the only FAAH inhibitors that have been shown to possess in vivo efficacy are nucleophile-directed, irreversible agents (e.g., carbamates), thus raising concerns about their selectivity for FAAH relative to the more than 200 additional serine hydrolases present in mammalian proteomes.

Here we have presented a general platform for the discovery and in vivo characterization of FAAH inhibitors that incorporates several unique features. First, we have applied an activity-based proteomic screen (Leung et al., 2003) to assess the global selectivity of FAAH inhibitors against numerous serine hydrolases in parallel. These studies have identified several enzymes that represent “hot spots” in the

proteome for off-target activity of FAAH inhibitors. Notably, many of these enzymes, including TGH (Alam et al., 2002), LPL (Stein and Stein, 2003), and AAD (Trickett et al., 2001), regulate the mobilization/storage of triacylglycerides in the periphery, and therefore their inhibition may produce untoward effects on the ratio of high-density and low-density lipoprotein particles. Each of the previously described carbamate inhibitors of FAAH showed equal or greater potency on one or more of these peripheral lipases. In contrast, the reversible α -ketoheterocycles OL-92 and OL-135 were very selective for FAAH, with the latter compound displaying a remarkable ~ 300 -fold greater potency for FAAH than any other serine hydrolase examined in brain, heart, or kidney proteomes. In addition, OL-135 did not bind to CB1 or CB2 receptors. Finally, it is noteworthy that none of the additional hydrolases found to be sensitive to FAAH inhibitors share any sequence homology with FAAH, highlighting that widely divergent enzymes can possess similar active site structures. In this regard, the selectivity profile of the BMS-1

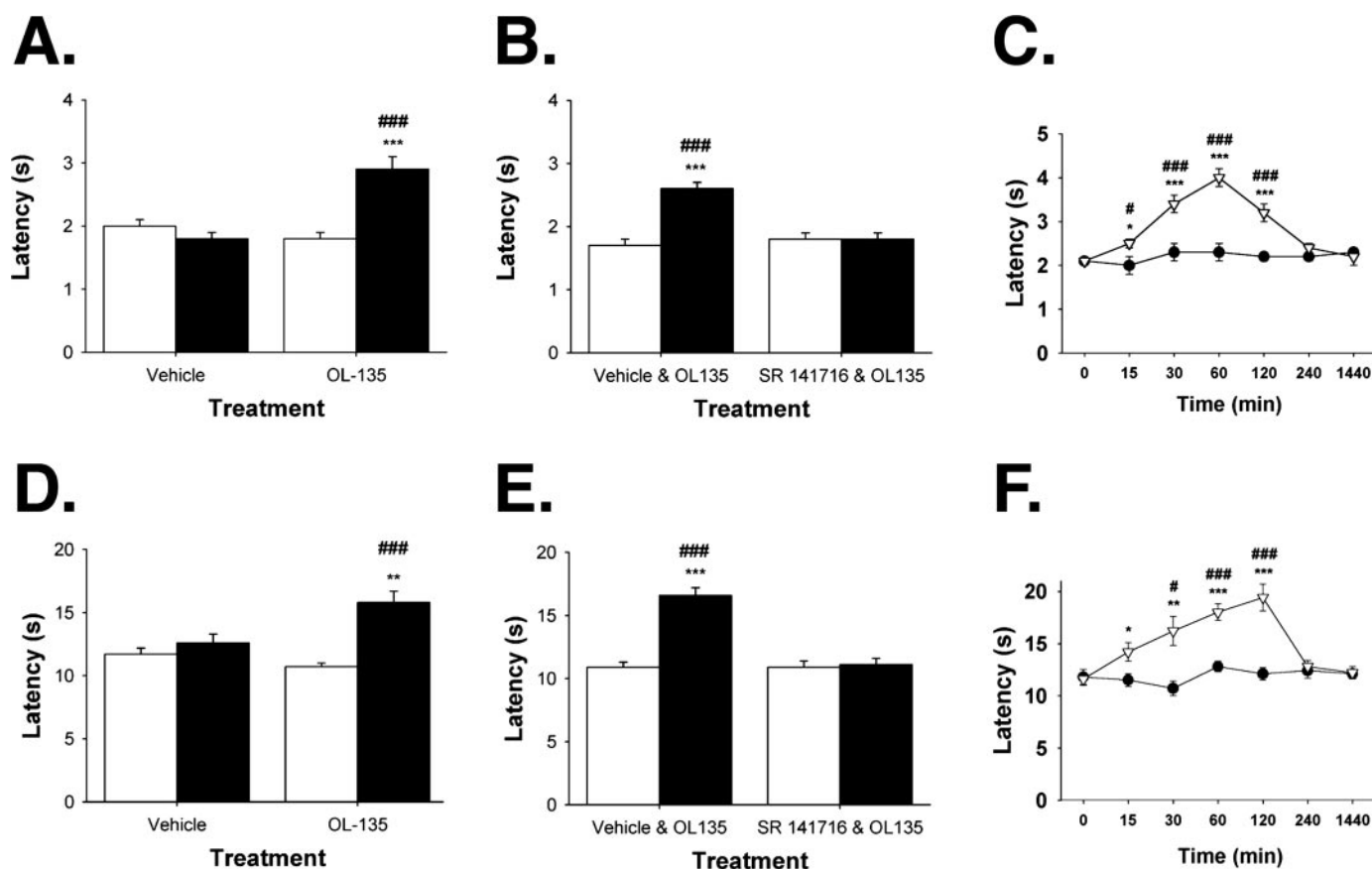


Fig. 3. OL-135 promotes analgesia in thermal pain assays in rodents. OL-135 (10 mg/kg i.p.) produced significant increases in response latencies in both the tail immersion (A) and hot plate (D) tests (tested 60 min postadministration). SR141716 (3 mg/kg) completely blocked the hypoalgesic effects of OL-135 (10 mg/kg i.p.) in both tests (B and E, respectively). White bars reflect preinjection latencies; black bars represent latencies 60 min postinjection. A time-course analysis of the hypoalgesic effects of OL-135 in the tail withdrawal (C) and hot-plate (F) tests shows that significant differences occurred within 15 min of administration and were completely reversed by 240 min. *, $p < 0.05$ and ***, $p < 0.001$ for OL-135 treatment versus vehicle treatment at each respective time point. #, $p < 0.05$ and ###, $p < 0.001$ for latencies 60 min after OL-135 treatment compared with preinjection latencies. Results shown as means \pm S.E., $n = 8$ –12 mice/group.

inhibitor is particularly striking, as this agent blocked FAAH and AAD with similar potencies, but showed much weaker activity against the uncharacterized brain hydrolase KIAA1363, which shares ~50% sequence identity with AAD. These data indicate the inhibitor sensitivity profiles of enzymes cannot easily be predicted from their primary structures and thus highlight the importance of global screening strategies such as the activity-based proteomics method described herein for evaluating the selectivity of inhibitors against numerous enzymes in parallel.

Although our functional proteomics screen enabled the discovery of highly selective FAAH inhibitors, this assay did not directly address whether these agents would be active in vivo. To evaluate the in vivo activity of FAAH inhibitors, we developed a rapid pharmacological assay in which the ability of these agents to augment the behavioral effects of anandamide was compared with the effects of this endocannabinoid in FAAH(–/–) mice. In this in vivo screen, OL-135 was fully efficacious at promoting the analgesic and hypothermic effects of anandamide in wild-type mice. In contrast, the fused heterocycle OL-92, despite displaying greater potency than OL-135 in vitro, failed to elevate the pharmacological effects of anandamide. These data indicate that the in vitro potency and in vivo efficacy of FAAH inhibitors do not necessarily correlate, even for structurally related agents, and empha-

size the importance of integrating an in vivo pharmacological screen at an early stage during the development of these agents.

The in vivo screening procedure used here detected functional consequences of preventing anandamide catabolism in the central nervous system and therefore did not address whether FAAH inhibitors that were negative in these assays (e.g., OL-92) might show activity in the periphery. This issue is important to address in future studies, as we have recently demonstrated a disassociation between central and peripheral actions of fatty acid amides using a transgenic mouse model in which FAAH expression is restricted to the nervous system (Cravatt et al., 2004). These transgenic mice express wild-type levels of fatty acid amides in the central nervous system, but they have significantly increased levels of these lipids in the periphery. This anatomically restricted elevation in fatty acid amides correlates with a reversion of the reduced pain sensitivity of FAAH(–/–) mice, but maintenance of an anti-inflammatory phenotype, thus indicating that the latter effect is mediated by the peripheral fatty acid amide signaling system.

In addition to augmenting the behavioral effects of exogenously administered anandamide, OL-135 was also found to elevate the activity of endogenously produced fatty acid amides. Indeed, within 30 min after administration, OL-135

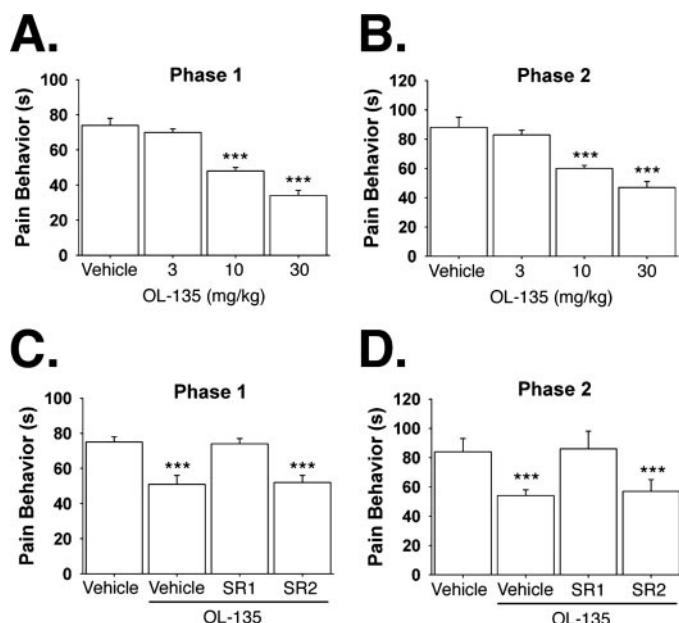


Fig. 4. OL-135 promotes analgesia in the formalin test of noxious pain in rodents. OL-135 produced a dose-dependent decrease in pain behavior during both phase 1 (A) and phase 2 (B) of the formalin test. ***, $p < 0.001$ for OL-135 treatment versus vehicle treatment. The analgesic effects of OL-135 (10 mg/kg i.p.) during both phase 1 (C) and phase 2 (D) were completely blocked by the CB1 receptor antagonist SR141716 (SR1), but not by the CB2 receptor antagonist SR144528 (SR2). ***, $p < 0.001$ for each respective group versus either the control group given two injections of vehicle or the group pretreated with SR144528 prior to OL-135. $n = 6$ mice/group. Results shown as means \pm S.E.

produced significant antinociception in the tail immersion and hot-plate tests for thermal pain sensation. In addition to reducing thermal pain sensitivity, OL-135 also promoted analgesia in the first and second phases of the formalin test of noxious chemical pain. In each of these cases, the analgesic effects of OL-135, which seemed to last at least 2 h, were blocked by the CB1 receptor antagonist SR141716, suggesting that FAAH inhibition augments endocannabinoid signaling in vivo. Consistent with this premise, OL-135 was also found to elevate brain and spinal cord levels of anandamide (and other fatty acid amides). Although OL-135 produced a CB1 receptor-mediated effect in all three pain assays used, there is growing evidence indicating that stimulation of peripheral CB2 receptors can suppress pain in a variety of pain

models (Clayton et al., 2002; Ibrahim et al., 2003; Hohmann et al., 2004). Thus, it will be important to assess OL-135 and other FAAH inhibitors in a variety of inflammatory and neuropathic pain models that have been shown to have a CB2 component. Finally, it is noteworthy that the antinociceptive properties of OL-135 occurred in the absence of any significant effects on motility or motor coordination that typically accompany the global activation of CB1 receptors by direct agonists (Compton et al., 1993). These data provide further evidence that augmentation of endocannabinoid signaling via the inactivation of FAAH produces a provocative subset of the behavioral effects observed with CB1 agonists (Cravatt et al., 2001; Kathuria et al., 2003) and suggest a key role for the FAAH-endocannabinoid system in modulating multiple pain pathways.

In summary, we describe in this manuscript a general platform for identification and pharmacological characterization of inhibitors of FAAH, resulting in the discovery of a highly potent and selective agent OL-135 that augments endocannabinoid signaling and produces analgesia in vivo. Based on the FAAH crystal structure (Bracey et al., 2002), it is interesting to speculate that OL-135 may achieve its remarkable selectivity for FAAH by interacting with both the acyl chain-binding and cytoplasmic tunnels of the enzyme's unusual bifurcated active site. Indeed, the unfused heterocycle group of OL-135, which would be predicted to extend into the cytoplasmic tunnel of FAAH (assuming that the ketone of OL-135 binds to the enzyme's serine nucleophile), seems to be a key specificity factor, because conversion of this moiety to a fused heterocycle or trifluoromethyl group reduces (Table 1) and abolishes (Leung et al., 2003) selectivity for FAAH, respectively. Thus, these studies have offered an intriguing initial structure-activity relationship for FAAH inhibitors, which should help to guide the design of future agents that display increasing potency and selectivity for this enzyme. Finally, we suggest that the functional proteomics strategy described herein, which does not require that enzymes be recombinantly expressed or purified before analysis, should prove of general use for the development of inhibitors for many members of the serine hydrolase superfamily (as well as other enzyme classes; Adam et al., 2002; Greenbaum et al., 2002), thereby enabling the concurrent optimization of the potency and selectivity of inhibitors for a large number of biomedically important enzymes.

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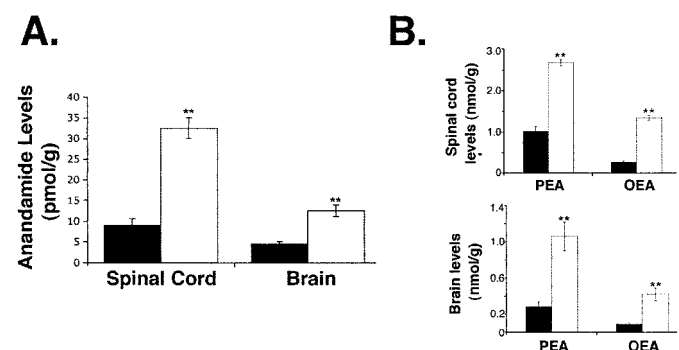


Fig. 5. OL-135 significantly elevates the endogenous levels of anandamide (A) and other fatty acid amides (B) in the central nervous system of wild-type mice. Fatty acid amide levels were measured 60 min after i.p. administration of either vehicle (black columns) or OL-135 (30 mg/kg; white columns). **, $p < 0.01$ for OL-135 treatment versus vehicle treatment. Results shown as means \pm S.E., $n = 8$ mice/group.

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