Supporting Information

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SI Methods

Reagents. Oleamide was purchased from Sigma. Nicotinamide adenine dinucleotide, reduced form (NADH) and glutamate dehydrogenase (GDH) were from Roche Diagnostics. Triton X-100 and EDTA were obtained from Calbiochem and Biochemika, respectively. ADP and α-ketoglutaric acid were purchased from Amresco. OL-135 was synthesized according to the published procedure (1). *N*-phenyl-4-(quinolin-3-ylmethyl)piperidine-1-carboxamide (PF-750) and *N*-phenyl-4-(quinolin-2-ylmethyl)piperazine-1-carboxamide (PF-622) were synthesized as described previously (2). Inhibitors were stored as dry powders at room temperature and dissolved in DMSO to prepare concentrated stock solutions. Polystyrene 96-well microplates were purchased from Rainin. All reagents used were the highest quality commercially available.

Generation of FAAH Expression Constructs. Constructs for all three species of FAAH (hFAAH, rFAAH, and h/rFAAH), were generated as the N-terminal transmembrane-deleted truncated forms (ΔTM-FAAH) with N-terminal His_6 tags. The N-terminal amino acid residues 1–29 constitute a predicted transmembrane domain, and for rFAAH, this deletion has been shown to enhance expression and purification greatly and to afford identical enzymatic properties to the wild-type rFAAH (3). For the hFAAH construct, the cDNA containing the *Escherichia coli* codon-optimized cDNA for hFAAH was custom-synthesized at Blue Heron Biotechnolgy and cloned into the pET28a(+) vector (Novagen) by using standard molecular biology techniques to generate an N-terminally His-tagged pET38a-ΔTM-hFAAH construct (amino acids 32–579). For the rFAAH construct, the rFAAH cDNA was amplified from the rat brain Quick-clone cDNA library (BD Biosciences Clontech). The resulting PCR product was subcloned into the pET28a(+) vector to generate an N-terminally His-tagged pET28a-ΔTM-rFAAH construct (amino acids 30–579). For the h/rFAAH construct, three rounds of mutagenesis were performed on the pET38a-ΔTM-rFAAH construct (amino acids 30–579) by using the QuikChange procedure (Stratagene). At each round, mutations on 2 amino acid residues were performed with the order of A377T/S435N, L192F/F194Y, and I491V/V495M mutations to generate the construct containing all six mutations, pET28a-ΔTM-h/rFAAH construct (amino acids 30–579). All constructs were confirmed by sequencing in both directions.

FAAH Expression and Purification. Human FAAH plasmid, pET28-ΔTM-hFAAH (amino acids 32–579), was transformed into the *E. coli* BL21 AI strain. Two-liter cultures of the freshly transformed expressing strains were grown in Superbroth medium in the presence of 30 μM kanamycin at 37°C. At A_{600} of ≈0.12, the cultures were transferred to 25°C and induced at A_{600} of 0.6–0.65 with final concentrations of IPTG and L-arabinose at 100 μM and 0.2%, respectively, for 20 h at 25°C. All operations below were at 4°C unless otherwise noted. The cells were then harvested by centrifugation at 5,000 × *g*, and the cell pellets were washed by resuspending in 700 ml of PBS and collected by centrifugation at 5,000 × *g*. The cell paste was frozen and stored at −80°C until needed. The cells were resuspended in 100 ml of buffer A [20 mM NaP_i (pH 7.8), 100 mM NaCl] with stirring. After adding 500 units of benzonase, the cell suspension was stirred for an additional 15 min before lysing the cells by passing through the microfluidizer processor (Microfluidics) according to the manufacturer's instructions. The cell debris was removed by centrifugation at 5,000 × *g* for 20 min, and the supernatant was centrifuged at 100,000 × *g* for 1 h. The resulting pellet was resuspended in 100 ml of buffer A and centrifuged again at 100,000 × *g* for 1 h. After repeating the last step two more times, the pellet was resuspended in 80 ml of buffer B [20 mM NaP_i (pH 7.8), 500 mM NaCl, 1% Triton X-100] and centrifuged at 100,000 × *g* for 1 h, and the resulting supernatant was loaded at 0.5–1 ml/min onto a 1 ml Ni HiTrap chelating HP column (GE Healthcare) that had been equilibrated with buffer B. The column was washed with 20 ml of buffer B at 0.2 ml/min. The column was further washed with 10 ml of buffer B containing 20 mM imidazole and then with 10 ml of buffer B containing 50 mM imidazole at 0.2 ml/min. Elution was with a 20-ml gradient (buffer B, 50–200 mM imidazole) at 0.2 ml/min. Fractions of 1 ml were collected. Peak fractions were dialyzed against buffer C [20 mM NaP_i (pH 7.8), 500 mM NaCl] overnight. Identical conditions (with an exception of typically larger Ni columns caused by higher expression) were used for expression and purification of ΔTM-rFAAH and ΔTM-h/rFAAH. This procedure generated all three forms of FAAH with greater than 95% purity based on SDS/PAGE visualized by Coomassie blue staining. Protein concentrations were determined by using the BCA protein assay kit (Pierce). Typically, 1–2, 20, and 10 mg of purified hFAAH, rFAAH, and h/rFAAH were obtained per liter culture.

For expression and purification of h/rFAAH for crystallization in complex with PF-750, a typically shorter induction time of 6 h was used, and a French press was used for lysis. The membrane fractions were resuspended in a buffer containing 1% *n*-dodecyl-β-D-maltoside (instead of Triton X-100) and 50 μM PF-750. After the Ni chelating column, an additional HiTrap heparin HP column was used for purification, during which the NaP_i buffer was exchanged with Hepes-NaOH (pH 7.0), 0.2% *n*-decyl-β-D-maltoside, 2 mM DTT. The last step of the purification before crystallization consisted of a size exclusion chromatography using Superdex 200 medium (GE Healthcare). Protein concentration was performed by pressure filtration (10-ml cell; Amicon) or by 2.5-ml Vivaspin microconcentrators (Vivascience) using 100-kDa cutoff membranes. PF-750 at 50 μM was added throughout the purification up to the size exclusion column.

Inhibitor Synthesis. ***Synthesis of 4-(naphthalen-2-ylmethyl)-N-(pyridin-3-yl)piperazine-1-carboxamide (compound 3).*** To a solution of 3-isocyanatopyridine (6.0 g, 50.0 mmol) in toluene (100 ml) and CH_2Cl_2 (100 ml) was added *t*-butyl piperazine-1-carboxylate (10.2 g, 55.0 mmol). The reaction mixture was stirred overnight at RT and a white precipitate formed. The precipitate was collected by filtration, washed with toluene and dried in a vacuum oven to give *t*-butyl 4-(pyridin-3-ylcarbamoyl)piperazine-1-carboxylate as a white solid (13.2 g, 86%).

t-Butyl 4-(pyridin-3-ylcarbamoyl)piperazine-1-carboxylate (11.3 g, 36.9 mmol) was dissolved in CH_2Cl_2:MeOH (1:1, 110 ml), treated with 4 M HCl in dioxane (55.3 ml, 221 mmol), and stirred at room temperature overnight under a nitrogen atmosphere. The resulting white solid was collected by filtration, washed with a small amount of CH_2Cl_2, and dried in a vacuum oven at 40°C to give *N*-(pyridin-3-yl)piperazine-1-carboxamide dihydrochloride (10.10 g, 98%).

To a solution of *N*-(pyridin-3-yl)piperazine-1-carboxamide dihydrochloride (0.36 g, 1.29 mmol) and 2-naphthaldehyde (0.20 g, 1.29 mmol) in CH_2Cl_2 (6 ml) was added triethylamine (0.72 ml,

5.16 mmol). The mixture was stirred for 10 min, and sodium triacetoxyborohydride (0.38 g, 1.8 mmol) was added. The reaction was allowed to stir overnight at room temperature. The mixture was quenched with aqueous sodium bicarbonate and extracted with CH_2Cl_2. The mixture was concentrated, and the residue was purified by SiO_2 column chromatography (0–5% MeOH/CH_2Cl_2) to give the title compound as a foam (0.318 g, 71%). MS: M + 1 = 347.2 (APCI).

Synthesis of* N-*phenyl-4-(quinoline-3-carbonyl)piperidine-1-carboxamide (compound 4). To a cooled (−0°C) solution of *t*-butyl 4-[methoxy(methyl)carbamoyl]piperidine-1-carboxylate (29.45 g, 0.108 mol) and 3-bromoquinoline (45 g, 0.216 mol) in THF (300 ml) was added *n*-BuLi (84.85 ml, 0.119 mol) in hexane such that the temperature was less than −90°C. The reaction mixture was stirred for 1.5 h and then warmed to −5°C for 1 h. The reaction mixture was quenched with saturated aqueous ammonium chloride (500 ml) and stirred for 18 h. The reaction mixture was partitioned between ethyl acetate and saturated ammonium chloride. The organic layer was dried over Na_2SO_4 and concentrated. Purification by SiO_2 column chromatography (15% ethyl acetate/hexane) followed by slurrying the compound in 10% ether/hexane provided *t*-butyl 4-(quinoline-3-carbonyl)piperidine-1-carboxylate as a pale yellow solid (20.0 g, 54%). mp 88°C.

To a solution of *t*-butyl 4-(quinoline-3-carbonyl)piperidine-1-carboxylate (0.75 g, 2.20 mmol) in CH_2Cl_2 (25 ml) was added trifluoroacetic acid (5 ml), and the reaction was stirred for 3 h. The mixture was concentrated, and the residue was partitioned between CH_2Cl_2 and saturated $NaHCO_3$. The organic layer was dried over Na_2SO_4 and concentrated to give piperidin-4-yl(quinolin-3-yl)methanone (0.31 g). Additional product was in the aqueous layer so NaCl was added until the aqueous layer was saturated. The aqueous layer was extracted with $CHCl_3$, which provided another 0.21 g of product (total: 0.52 g, 98%).

To a solution of piperidin-4-yl(quinolin-3-yl)methanone (0.240 g, 1.0 mmol) in CH_2Cl_2 (20 ml) was added phenyl isocyanate (0.11 ml, 1.0 mmol). The reaction mixture was stirred at room temperature for 5 h and then quenched with saturated $NaHCO_3$. The mixture was extracted with CH_2Cl_2, and the organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by SiO_2 column chromatography (20:1 CH_2Cl_2:1 N NH_3 in MeOH) to give the title compound as a white solid after trituration with diethyl ether (0.300 g, 84%). MS: M + 1 = 360.1 (APCI).

Synthesis of 4-(3-phenylpropyl)-*N*-(pyrimidin-5-yl)piperidine-1-carboxamide (compound 5). Ethyl pyrimidin-5-ylcarbamate (0.250 g, 1.496 mmol) was combined with 4-(3-phenylpropyl)-piperidine (0.456 g, 2.24 mmol) in anhydrous acetonitrile (2.5 ml) in a microwave reaction vial. The vial was capped and the mixture was microwaved to 180°C for 15 min. The homogenous reaction mixture was concentrated, and the residue was purified by SiO_2 column chromatography (0–50% acetone-hexanes, CombiFlash gradient elution) to give the title compound as a flaky white solid (0.267 g, 55%). MS: M + 1 = 325.2 (APCI); ^{1}H NMR (400 MHz, $CDCl_3$) ppm 1.18 (m, 2H), 1.32 (m, 2H), 1.49 (m, 1H), 1.66 (m, 2H), 1.78 (m, 2H), 2.61 (t, J = 7.70 Hz, 2H), 2.90 (m, 2H), 4.05 (d, J = 13.26 Hz, 2H), 6.48 (s, 1H), 7.18 (m, 3H), 7.28 (m, 2H), 8.81 (s, 2H), 8.87 (s, 1H).

1. Boger DL, *et al*. (2005) Discovery of a potent, selective, and efficacious class of reversible α-ketoheterocycle inhibitors of fatty acid amide hydrolase effective as analgesics. *J Med Chem* 48:1849–1856.
2. Ahn K, *et al*. (2007) Novel mechanistic class of fatty acid amide hydrolase inhibitors with remarkable selectivity. *Biochemistry* 46:13019–13030.
3. Patricelli MP, Lashuel HA, Giang DK, Kelly JW, Cravatt BF (1998) Comparative characterization of a wild-type and transmembrane domain-deleted fatty acid amide hydrolase: Identification of the transmembrane domain as a site for oligomerization. *Biochemistry* 37:15177–15187.

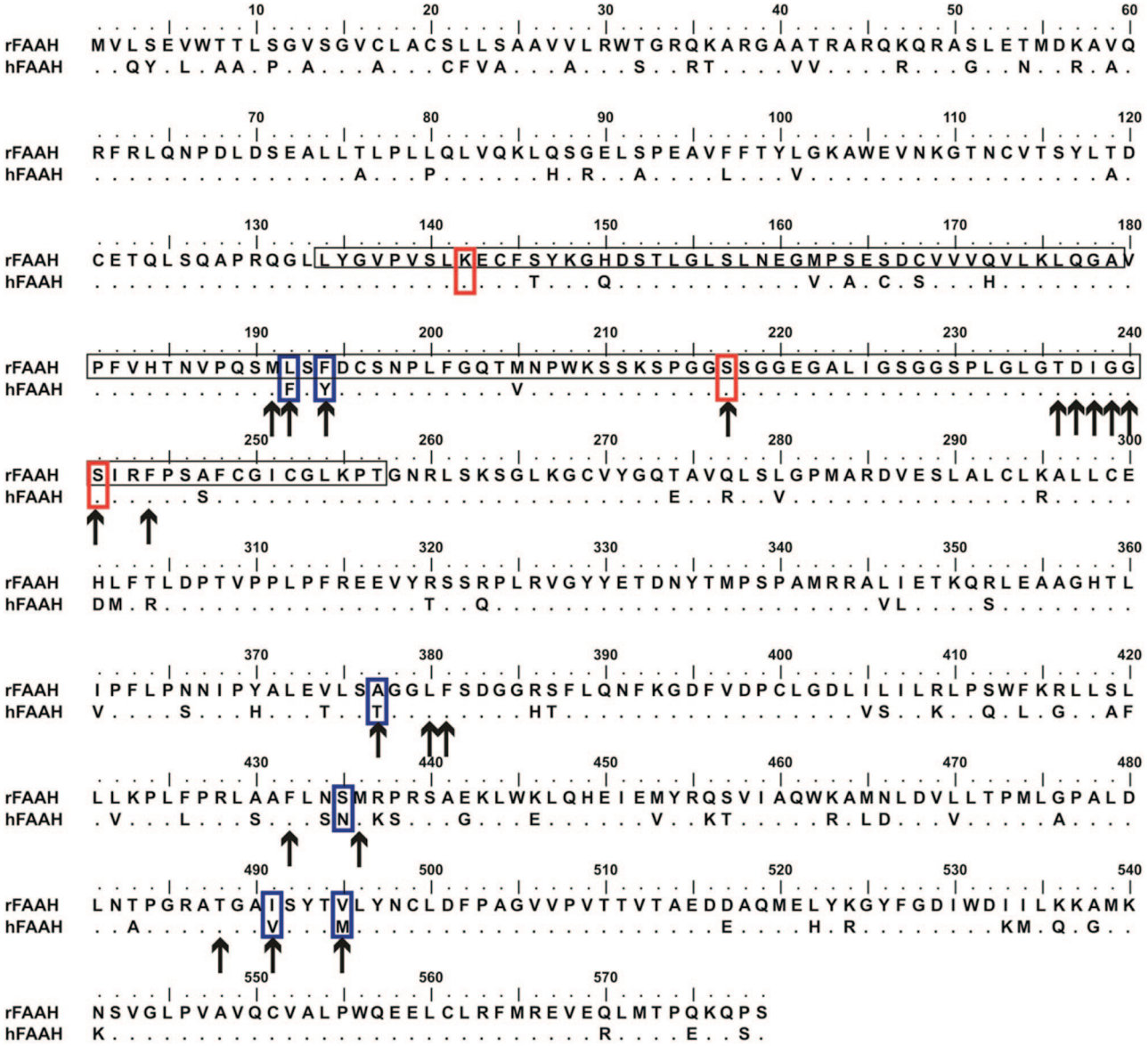

Fig. S1. Sequence alignment of rFAAH and hFAAH (82% identity). The box surrounding amino acids 134–257 denotes the AS signature sequence. Humanized residues are highlighted with violet rectangles. Residues that form the serine–serine–lysine catalytic triad are highlighted with red rectangles. The small arrows indicate residues that are within 5 Å distance from the PF-750 inhibitor.

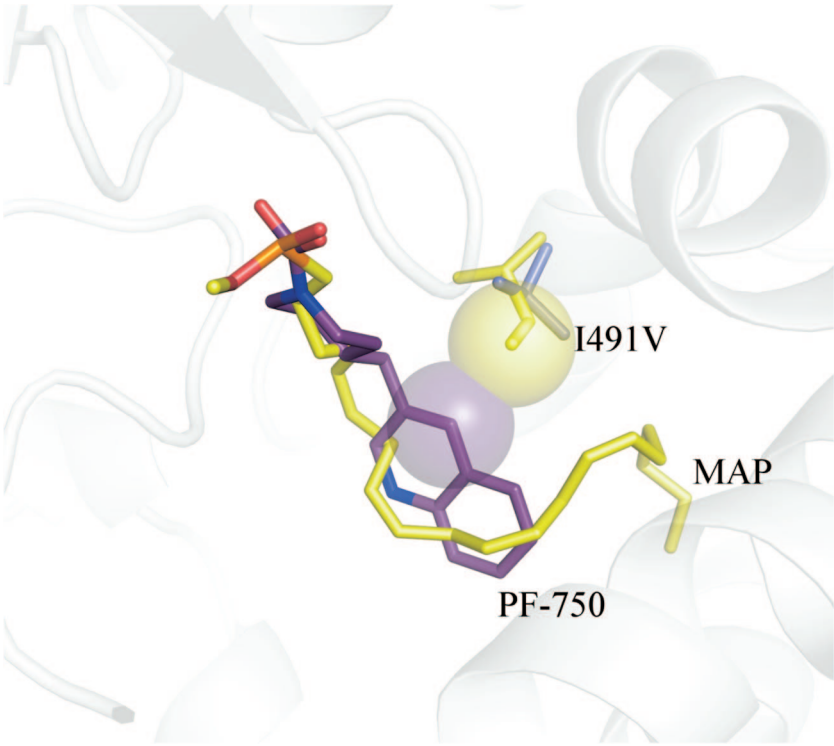

Fig. S2. Potential steric hindrance between I491 (yellow sticks) from the rFAAH structure (PDB code 1MT5) and the inhibitor PF-750 (purple sticks) from the h/rFAAH structure. The residue V491 from the h/rFAAH structure is shown in violet sticks. The spheres indicate van der Waals radii of carbon in position 4 (purple) and distal carbon of the I491 side chain (yellow). The MAP adduct (yellow sticks) is shown to indicate its different arrangement compared with the PF-750 adduct. The monomers of the two structures have been superposed by using the program Coot.

Table S1. Data collection and refinement statistics (molecular replacement)

Parameter	PF-750-ΔTM-h/rFAAH
Data collection	
Space group	$P3_221$
Cell dimensions	
a, *b*, *c* , Å	103.69, 103.69, 253.87
α, β, γ, °	90.0, 90.0, 120.0
Resolution, Å	40–2.75 (2.85–2.75)
R_{sym}	14.3 (56.2)
I / σI	7.0 (2.1)
Completeness, %	95.6 (98.6)
Redundancy	3.0 (3.0)
Refinement	
Resolution, Å	2.75
No. reflections	38,162
R_{work}/R_{free}	18.8 (28.5)/23.9 (33.5)
No. atoms	
Protein	8,365
Ligand/ion	39
Water	84
B factors	
Protein	37.8
Ligand/ion	30.6
Water	30.2
rms deviations	
Bond lengths, Å	0.012
Bond angles, °	1.585

The values in parentheses are the highest resolution shell.