A binding site for non-steroidal anti-inflammatory drugs in FAAH

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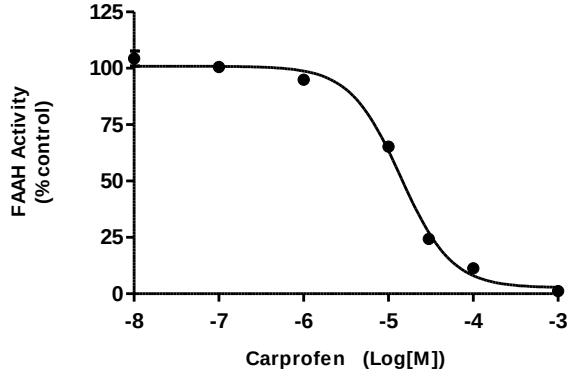
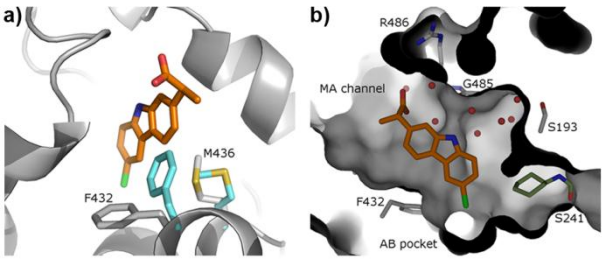
SUPPORTING INFORMATION

Supplementary Figures and Tables



Supplementary Figure 1. Inhibition of FAAH activity by carprofen at pH 6.0 (●). FAAH activity was assessed in rat brain membrane using [^{3}H]-anandamide as a substrate (Methods).



Supplementary Figure 2. Carprofen binding to the membrane access channel of FAAH. a) FAAH/carprofen interaction dynamics. The structure of the FAAH-carprofen complex is colored in gray (carprofen carbon atoms colored in orange). Residues F432 and M436 are superimposed with those of the FAAH/URB597 complex (PDB code: 3LJ7, carbon atoms in cyan) to show their different orientations. b) Position of carprofen (carbon atoms colored in orange) and O-carbamoylated (carbon atoms colored in dark green) catalytic S241. Water molecules of the membrane access channel (MA) are shown as red spheres (AB, acyl binding). Single-letter abbreviations of amino acids have been used for clarity.

Table 1. Crystallographic analysis.

	FAAH-Carprofen complex
Data collection	
Space group	$P2_12_12_1$
Cell dimensions	
a, b, c (Å)	103.47, 104.37, 147.62
α, β, γ (deg)	90, 90, 90
Resolution (Å) [a]	44.44 - 2.25 (2.37 - 2.25)
Rsym [a, b]	0.11 (0.58)
I / σI [a]	15.9 (4.1)
Completeness (%) [a]	99.8 (98.9)
Redundancy [a]	11.6 (10.8)
Refinement statistics	
Resolution (Å) [a]	44.44 - 2.25 (2.31-2.25)
No. reflections	68597
R_{work} / R_{free} [a, c]	0.164 (0.213) / 0.199 (0.271)
No. atoms	8986
Protein	8431
Ligand / ion	57
Water	533
B-factors	28.8
Protein	29.5
Ligand / ion	42.8
Water	36.1
rms deviations	
Bond lengths (Å)	0.02
Bond angles (deg)	1.83
Ramachandran plot	
preferred (%)	96.2
allowed (%)	3.7
outliers (%)	0.1

[a] In parentheses, values for the highest resolution shell data.

[b] R_{sym} is defined as: $\Sigma h \Sigma i(|Ii(h) - \langle I(h)\rangle|)/\Sigma h \Sigma i Ii(h)$, where Ii(h) is the ith integrated intensity of a given reflection and <I(h)> is the weighted mean of all measurements of I(h).

[c] R_{free} calculated on a subset of reflections excluded from all stages of refinement.

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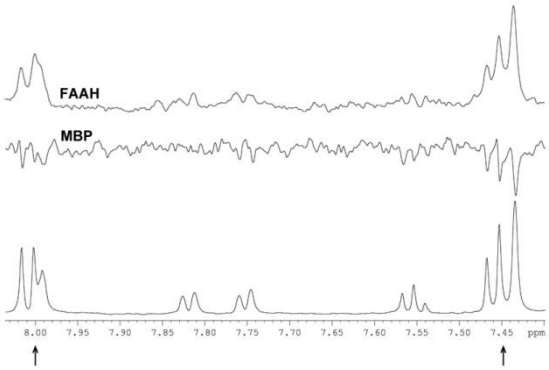
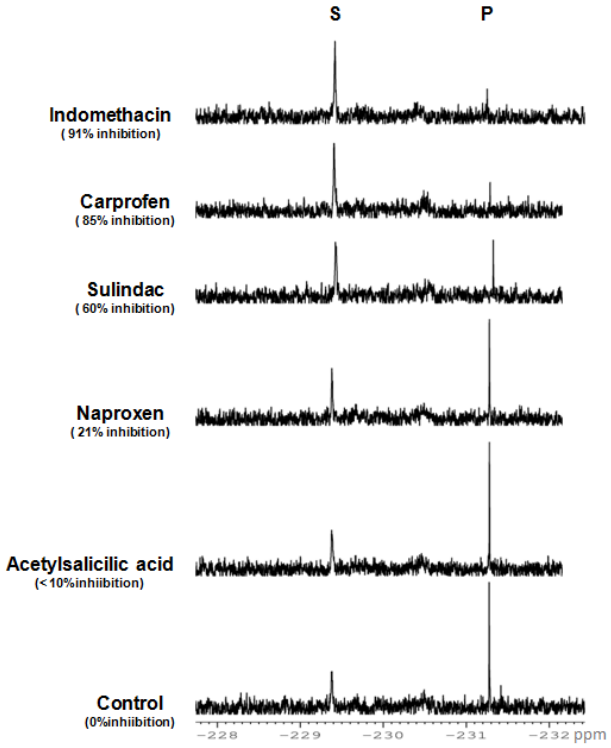


Supplementary Figure 3. WaterLOGSY binding experiments (BOX1). WaterLOGSY binding experiments recorded for FAAH (wild-type, maltose-binding protein (MBP)-fused FAAH) or MBP (0.7 μM) showing the interaction of carprofen (200 μM), in the presence of URB597 (100 μM). NMR spectra indicate the presence (positive LOGSY signals) and absence (negative LOGSY signals) of interaction with FAAH and MBP, respectively. Bottom, aromatic region of the 1D 1H spectrum of carprofen (arrows) and URB597.



Supplementary Figure 4. FABS-based inhibition profile (BOX1) of representative NSAIDs. ^{19}F NMR spectra show signals of the fluorinated anandamide analog ARN1302 (S) and its reaction product (P). The concentration of FAAH and ARN1302 was 15 nM and 30 μM, respectively. From top to bottom, ^{19}F NMR spectra were recorded in the presence or absence of select NSAIDs (200 μM). The amount of product for each sample was obtained integrating its ^{19}F NMR signal (Table 2). Two replicate measurements were carried out with a single point measurement.

Supplementary BOX 1

WaterLOGSY

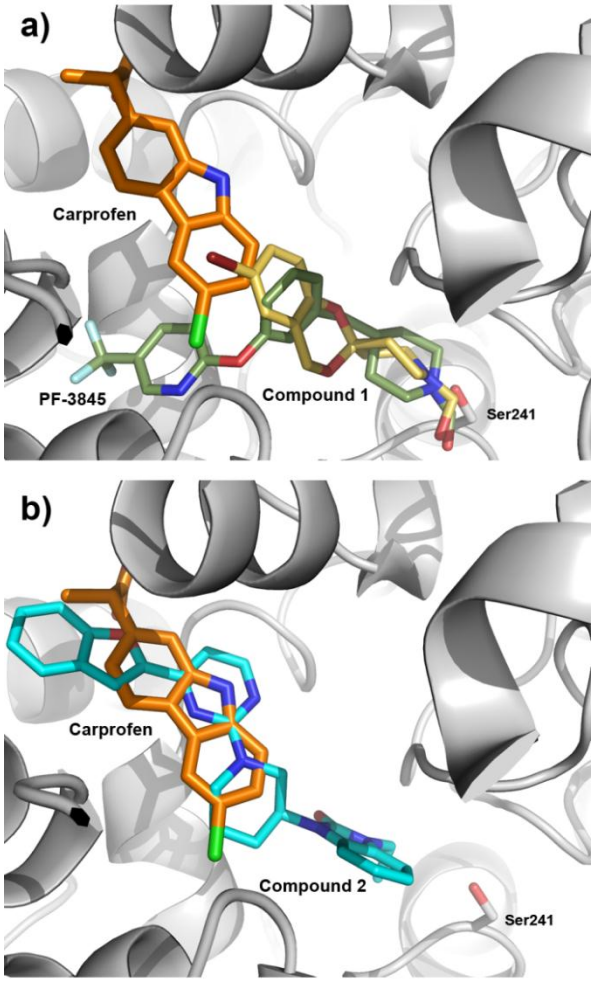
WaterLOGSY is a powerful NMR-based methodology for the identification of compounds interacting with protein targets. The magnetization of the bulk water is partially transferred via the protein-ligand complex to the free ligand in a selective manner. As a result, the resonances of binding compounds appear with opposite sign and tend to be stronger than those of the non-interacting ligands (Dalvit C et al. *J. Biomol. NMR*. 2001, 21, 349–359).

FABS

Fluorine Atoms for Biochemical Screening (FABS) requires the labeling of the substrate with a Fluorine-containing chemical moiety. FABS utilizes ^{19}F NMR spectroscopy for the detection of the starting substrate and the enzyme reaction product. The method allows for high-quality screening of large compound collections, and for measuring their IC50 values (Dalvit C et al. *J. Am. Chem. Soc.* **2003**, *125*, 14620–14625).

Table 2. FABS-based NMR spectroscopy.

NSAID	Chemical structure	Concentration (µM)	Area S [-231.26; -231.29] (ppm)	Area P [-231.26; -231.29] (ppm)	S (µM)	P (µM)	% Inhibition	% Mean inhibition	Standard deviation
Indomethacin		200	1	0,0294	29,1	0,9	94	**91**	**3,0**
				0,056	28,4	1,6	88		
Carprofen		200	1	0,0634	28,2	1,8	87	**85**	**2,2**
				0,0816	27,7	2,3	82		
Tenidap		200	1	0,2028	24,9	5,1	63	**66**	**3,7**
				0,1473	26,1	3,9	70		
Sulindac		200	1	0,2061	24,9	5,1	62	**60**	**2,3**
				0,2217	24,6	5,4	58		
Flufenamic Acid		200	1	0,3594	22,1	7,9	41	**42**	**0,8**
				0,3232	22,7	7,3	43		
Valdecoxib		200	1	0,351	22,2	7,8	42	**42**	**0,3**
				0,3309	22,5	7,5	42		
Flurbiprofen		200	1	0,3696	21,9	8,1	40	**40**	**0,2**
				0,3478	22,3	7,7	40		
Ibuprofen		200	1	0,4662	20,5	9,5	29	**31**	**1,5**
				0,4068	21,3	8,7	32		
Diclofenac		200	1	0,4768	20,3	9,7	28	**29**	**0,3**
				0,437	20,9	9,1	29		
Naproxen		200	1	0,5511	19,3	10,7	21	**21**	**0,3**
				0,5028	20,0	10,0	22		
Tenoxicam		200	1	0,5714	19,1	10,9	19	**20**	**0,7**
				0,5121	19,8	10,2	21		
Lumiracoxib		200	1	0,67	18,0	12,0	11	**20**	**8,9**
				0,4385	20,9	9,1	29		
Mefenamic Acid		200	1	0,5974	18,8	11,2	17	**17**	**0,2**
				0,5449	19,4	10,6	17		
Piroxicam		200	1	0,5918	18,8	11,2	17	**16**	**1,4**
				0,5741	19,1	10,9	15		
Sulfinpyrazone		200	1	0,6596	18,1	11,9	12	**12**	**0,3**
				0,5997	18,8	11,2	12		
Nabumetone		200	1	0,7759	16,9	13,1	3	**< 10**	
				0,6967	17,7	12,3	4		
Ketoprofen		200	1	0,8165	16,5	13,5	0	**< 10**	
				0,6806	17,9	12,1	5		
Etodolac		200	1	0,7632	17,0	13,0	4	**< 10**	
				0,6936	17,7	12,3	4		
Acetaminophen		200	1	0,8081	16,6	13,4	1	**< 10**	
				0,7087	17,6	12,4	3		
Acetylsalicilic acid		200	1	0,7842	16,8	13,2	2	**< 10**	
				0,6889	17,8	12,2	4		
control			1	0,8181	16,5	13,5			
				0,8194	16,5	13,5			
				0,7315	17,3	12,7			
				0,7595	17,1	12,9			



Supplementary Figure 5. Mode of binding of carprofen and representative FAAH inhibitors. a) Non-covalent binding of carprofen (carbon atoms colored in orange) to FAAH, and position of the covalent inhibitors PF-3845 (carbon atoms colored in dark green) and Compound 1 [6-bromo-1'H,4H-spiro[1,3-benzodioxine-2,4'- piperidin]-1'-yl)methanol] (carbon atoms colored in paleyellow) (Min X. et al. *Proc. Natl. Ac. Sci.* **2011**, 108, 7379–7384). The crystallographic bound conformation of these inhibitors (PDB ID: 2WAP and 3QKV) was superimposed on that of the FAAH/carprofen complex (PDB ID: 4DO3). The residue Ser241 is the enzyme nucleophile. b) Binding of carprofen and Compound 2 [1-{(3S)-1-[4-(1-benzofuran-2-yl)pyrimidin- 2-yl]piperidin-3-yl}-3-ethyl-1,3-dihydro- 2H-benzimidazol-2-one] (carbon atoms colored in cyan) (Min X. et al. *Proc. Natl. Ac. Sci.* **2011**, 108, 7379–7384). The crystallographic bound conformation of Compound 2 (PDB ID: 3QJ9) was superimposed on that of the FAAH/carprofen complex (PDB ID: 4DO3). Compound 2, which has no structural analogy with carprofen, occupies entirely the MA channel of FAAH, and was designed and synthesized starting from suicide urea inhibitors (Gusting D.J. et al. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 2492–2496).

Methods

Materials. Compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chemically competent DH5α and BL21(DE3) cells were from Novagen (Merck - Darmstadt, Germany). Vectors, restriction enzymes and T4 DNA ligase were from New England BioLabs (Ipswich, MA, USA). Centricons were from Sartorius (Goettingen, Germany) and dialysis cassettes from Pierce (Thermo Fisher Scientific Inc. - Rockford, IL, USA). NuPAGE Mini Gels were from Life technologies (Life Technologies - Paisley PA4 9RF, UK). Arachidonoyl ethanolamide (AEA) was purchased from Cayman chemical (item No. 90050, Ann Arbor, MI, USA); Arachidonyl-[1-3H] ethanolamine ([3H]-AEA) was from American radiolabel Chemicals Inc. (Item No. ART 0626, Saint Louis, MO, USA).

Construct generation and mutagenesis. The encoding sequence of rat (r)FAAHΔTM (97-1722bp) was amplified from the cDNA clone 7370226 (Open Biosystem) using the following primer pair: forward 5'-GGGAATTCCATATGGGGCGCCAGAAGGCCC-3' (NdeI site is underlined); reverse 5'-ATAGTTTAGCGGCCGC**TCAATGATGATGATGATGATG**AG GGGTCATCAGCTG-3' (NotI site is underlined). A (6x)Histidine tag was introduced in the reverse primer sequence (colored in bold). To obtain the construct MBP-rFAAH-6xHis pMALc5x, the amplified rFAAHΔTM was cloned in pMALc5x vector in frame with the N-terminal MBP. For the generation of the construct (6x)His-rFAAH-(6x)His pET28, NdeI and EcoRI restriction enzymes were used to move the rFAAH-(6x)His sequence from MBP-rFAAH-(6x)His pMALc5x construct to pET28, in frame with the vector N-terminal (6x)His tag. Mutagenesis was performed using QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) following manufacturer's instructions, the construct (6x)His-rFAAH-(6x)His pET28 was used as template. The following primers were designed to introduce the single point-mutation Thr488Ala in rFAAH: forward 5'-CACCGGGCAGAGCCGCAGGGGCTATCAGC-3' and reverse 5'-GCTGATAGCCCCTGCGGCTCTGCCCGGTG-3'.

Protein expression and purification. Expression of the MBP-rFAAH-6xHis protein was carried out in the E. coli strain Rosetta gami 2 (DE3)pLysS (Novagen). At an optical density (OD600) of 0.6, bacteria were induced by the addition of 0.25 mM IPTG for 16h at 25 ºC. Cells were then harvested by centrifugation, resuspended in buffer [sodium phosphate 50 mM, sodium chloride 0.3 M, imidazole 10 mM pH 7.4] and lysed by sonication and 1% Triton-X100 addition. The lysate was incubated for 1h with benzonase nuclease and centrifuged at 15,000xg for 30 min. The clarified supernatant was incubated for 2h with NiNTA Agarose (Qiagen, Germantown, MD, USA) and washed with buffer containing increasing concentrations of imidazole. Elution was performed with buffer containing 0.25 M Imidazole. BL21 codon plus (DE3)-RIPL cells (Agilent Technologies, Santa Clara, CA, USA) were used to express the 6xHis-rFAAH-6xHis protein, both wild

type and mutant. At an OD600 of 0.6, cells were induced by the addition of 0.1 mM IPTG for 16h at 25 ºC. Cells were then harvested, resuspended in Buffer A [sodium phosphate 20 mM, sodium chloride 0.1 M, pH 7.8], and disrupted by lysozyme-induced lysis and sonication. Buffer A was supplemented with 50 μM URB597 for the purification of rFAAH to be used for crystallization. The cell lysate was centrifuged at 5000xg, and the collected supernatant was centrifuged at 100,000xg for 1h. Pellets were resuspended in Buffer A and centrifuged again at 100,000xg for 1h. This last step was repeated 3 times. Finally the membrane pellet was solubilized with Buffer B [sodium phosphate 20 mM, sodium chloride 0.5 M, 1% Triton X100 pH 7.8] and centrifuged at 100,000xg for 1h. The supernatant was incubated with NiNTA affinity resin overnight at 4 ºC. The resin was washed using Buffer B containing increasing concentrations of imidazole. Protein was eluted with a buffer containing [20mM Hepes pH 7.0, NaCl 0.1 M, 0.02% n-undecyl-β-D-maltoside, and 0.5 M Imidazole]. Finally, the eluted sample was supplemented with 2mM dithiothreitol and 2mM ethylenediaminetetraacetic acid. For purification in the absence of URB597 (rFAAH apoprotein wt and T488A mutant), 10% glycerol was added to the elution buffer.

General experimental details for synthesis. All the commercial available reagents and solvents were used as purchased from vendors without further purification. Abbreviations for solvents and reagents are: acetic acid (AcOH), acetonitrile (MeCN), cyclohexane (Cy), dichloromethane (DCM), dimethylsulfoxide (DMSO), ethylacetate (EtOAc), *N,N*-dimethylformamide (DMF) and triethylamine (Et_3N). Automated column chromatography purifications were run using a Teledyne ISCO apparatus (CombiFlash® Rf) with prepacked silica gel columns of different sizes (from 4 g up to 24 g). NMR experiments were run on a Bruker Avance III 400 system (400.13 MHz for ^1H, and 100.62 MHz for ^{13}C), equipped with a BBI probe and Z-gradients. Spectra were acquired at 300 K, using deuterated dimethylsulfoxide (DMSO-d_6) or deuterated chloroform (chloroform-*d*) as solvents. Chemical shifts (δ) for ^1H and ^{13}C spectra are reported in parts per million (ppm) using the residual non-deuterated solvent resonance as the internal standard (for chloroform-*d*: 7.26 ppm, ^1H and 77.16 ppm, ^{13}C; for DMSO-d_6: 2.50 ppm, ^1H; 39.52 ppm, ^{13}C). Data are reported as follows: chemical shift (sorted in ascending order), multiplicity (indicated as: s, singlet; d, doublet; t, triplet; q, quartet; p, pentet; m, multiplet and combinations thereof), coupling constants (*J*) in Hertz (Hz) and integration. UPLC-MS analyses were run on a Waters ACQUITY UPLC-MS system consisting of a SQD (Single Quadrupole Detector) Mass Spectrometer (MS) equipped with an Electrospray Ionization (ESI) interface and a Photodiode Array (PDA) Detector. PDA range was 210–400 nm. Analyses were performed on an ACQUITY UPLC HSS T3 C_{18} column (50 × 2.1 mm ID, particle size 1.8 μm) with a VanGuard HSS T3 C_{18} pre-column (5 × 2.1 mm ID, particle size 1.8 μm). Mobile phase was (A) 10 mM NH_4OAc in H2O at pH 5 and (B) 10 mM NH_4OAc in MeCN/H_2O (95:5) at pH 5, gradient either 5% to 95% B or 50% to 100% B over 3 min, flow rate 0.5 mL/min, temperature 40°C. Electrospray ionization in positive and negative mode was applied. Purifications by preparative HPLC-MS were run on a Waters Autopurification system consisting of a 3100 Single Quadrupole Mass Spectrometer equipped with an Electrospray Ionization interface and a 2998 Photodiode Array Detector. HPLC system included a 2747 Sample Manager, 2545 Binary Gradient Module, System Fluidic Organizer and 515 HPLC Pump. PDA range was 210–400 nm. Purifications were performed on a XBridge™ Prep C_{18} OBD column (100 × 19 mm ID, particle size 5 μm) with a XBridge™ Prep C_{18} (10 × 19 mm ID, particle size 5 μm) Guard Cartridge. Mobile phase was (A) H_2O and (B) MeCN, gradient 80% to 90% B over 7 min with a flow rate of 20 mL/min, temperature 25°C. Electrospray ionization in positive and negative mode was used.

Synthesis of (5Z, 8Z, 11Z, 14Z)-N-(3-fluoro-2-hydroxypropyl)icosa-5,8,11,14-tetraenamide (ARN1203). **Step1:** To a suspension of potassium phthalimide (300 mg, 1.60 mmol) in dry DMF (3.2 mL) was added 1-chloro-3-fluoro-propan-2-ol (150 mg, 1.33 mmol) at room temperature. After stirring at 80 °C for 4 h, the reaction was cooled to room temperature and H_2O (5 mL) was added. The reaction mixture was extracted with EtOAc (5 × 15 mL), and the organic layer was washed with brine and dried over Na_2SO_4. After evaporation of the solvent, the crude was purified by automated column chromatography eluting with Cy/EtOAc (from 100:0 to 75:25) to afford 2-(3-fluoro-2-hydroxy-propyl)isoindoline-1,3-dione (180 mg, 61%) as white solid. ^1H NMR (400 MHz, chloroform-*d*) δ 2.71 (s, 1H), 3.90–3.95 (m, 2H), 4.16 (dp, *J* = 20.0, 4.9 Hz, 1H), 4.47 (ddd, *J* = 47.0, 9.8, 4.7 Hz, 1H), 4.51 (ddd, *J* = 47.2, 9.8, 4.1 Hz, 1H), 7.75 (dd, J = 5.5, 3.1 Hz, 2H), 7.88 (dd, *J* = 5.5, 3.1 Hz, 2H); MS (ESI) *m/z*: $[M+H]^+$ calcd for $C_{11}H_{11}FNO_3$ 224.07; found 224.1. **Step2:** A suspension of 2-(3-fluoro-2-hydroxy-propyl)isoindoline-1,3-dione (125 mg, 0.56 mmol) in 13% HCl solution (1 mL) was stirred at 100 °C overnight. The reaction was then cooled to room temperature and H_2O was added. The mixture was washed with EtOAc (3 × 15 mL) and the aqueous phase was evaporated under reduced pressure to dryness to afford 1-amino-3-fluoro-propan-2-ol hydrochloride (66 mg, 92%) as white solid. ^1H NMR (400 MHz, DMSO-d_6) δ 2.68–3.01 (m, 2H), 3.88–4.01 (m, 1H), 4.37 (ddd, *J* = 47.2, 9.7, 4.8 Hz, 1H), 4.41 (ddd, *J* = 47.2, 9.6, 4.3 Hz, 1H), 5.80 (s, 1H), 7.99 (s, 3H); MS (ESI) *m/z*: $[M+H]^+$ calcd for C_3H_9FNO 94.07; found 93.9. **Step3:** To a solution of arachidonic acid (100 mg, 0.33 mmol) in dry DCM (2 mL) DMF (1 μL, 0.01 mmol) and oxalyl chloride (55 μL, 0.66 mmol) were added at 0 °C under N_2 atmosphere. After stirring for 3 h, the solvent was removed under reduced pressure and the crude was washed with dry DCM (3 × 2 mL, evaporation after each step). The residue was then dissolved in dry DCM (2 mL) under N_2 atmosphere and added via cannula to a solution of dry Et_3N (120 μL, 0.82 mmol) and 1-amino-3-fluoro-propan-2-ol hydrochloride (55 mg, 0.43 mmol) in dry DCM (2.5 mL) at 0 °C. After stirring overnight at room temperature, a 2N HCl solution (15 mL) was added. The reaction mixture was extracted with DCM (3 × 15 mL), and the organic layer was washed with brine and dried over Na_2SO_4. After evaporation of the solvent, the crude was purified by preparative HPLC-MS to afford (5Z, 8Z, 11Z, 14Z)-N-(3-fluoro-2-hydroxy-propyl)icosa-5,8,11,14-tetraenamide (ARN1203) (64 mg, 51%) as colorless oil. ^1H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, *J* = 6.8 Hz, 3H), 1.20–1.37 (m, 6H), 1.54 (p, *J* = 7.5 Hz, 2H), 1.97–2.06 (m, 4H), 2.09 (t, *J* = 7.5 Hz, 2H), 2.71–2.88 (m, 6H), 3.00–3.17 (m, 2H),

3.62–3.78 (m, 1H), 4.24 (ddd, J = 47.9, 9.6, 5.7 Hz, 1H), 4.31 (ddd, *J* = 47.6, 9.6, 3.6 Hz, 1H), 5.18 (d, *J* = 5.2 Hz, 1H), 5.27–5.43 (m, 8H), 7.83 (t, *J* = 5.5 Hz, 1H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 13.89, 21.94, 25.18, 25.20, 25.23, 26.27, 26.59, 28.69, 30.86, 34.73, 40.75 (d, *J* = 8.1 Hz), 68.23 (d, *J* = 18.6 Hz), 85.10 (d, *J* = 168.3 Hz), 127.49, 127.65, 127.76, 127.98, 128.10, 129.39, 129.92, 172.25; MS (ESI) *m/z*: $[M+H]^+$ calcd for $C_{23}H_{39}FNO_2$ 380.30; found 380.4; $[M–H]^-$ calcd for $C_{23}H_{37}FNO_2$ 378.28; found 378.2.

NMR. All spectra were recorded at 298 K using a Bruker FT-NMR 600 MHz UltrashieldTM Plus ADVANCE III equipped with a Cryoptobe QCI ^{1}H/^{19}F – ^{13}C/^{15}N – D and a Samplejet autosampler with temperature control. Water suppression in all proton experiments was achieved with the excitation sculpting sequence. WaterLOGSY experiments were carried out using a 25 ms 1800 Gaussian pulse. SPAM approach [Dalvit C et al. *Curr Drug Disc. Technol.* 2006, 3, 115-124] was applied to test solubility, purity and aggregation of the molecules. For analysis, protein samples of MBP-fusion protein in [PBS pH 7.4, 0.05% Triton-X100] buffer was added with D_2O (8% final concentration) for the lock signal. Before use, inhibitors were dissolved in DMSO at 100 mM, and the substrate-analogue ARN1203 in DMSO-d6 at 5 mM. The n-FABS screening was performed in [PBS pH 7.4, 0.05% Triton, 8% D2O] buffer in an end-point format using 15nM protein and 30 μM ARN1203. The reactions were performed (at 25°C and 350 rpm) in vial tubes in the absence or presence of 200 μM of each inhibitor. After 310 minutes, reactions were quenched adding 40 μM of URB597. Samples were then transferred to 5 mm NMR tubes for analysis. ^{19}F spectra were recorded with proton decoupling [spectral width 45,556 Hz, acquisition time 0.4 s, relaxation delay 2.5 s] in an automated way. Actual concentration of the screened compounds in the sample was monitored recording a ^{1}H ^{1}D NMR spectrum after each ^{19}F-based analysis. Concentration of the reaction product in each sample was calculated from the integrals of the ^{19}F NMR signals of substrate and product. Data were analyzed with GraphPad Prism 5 software package.

In vitro assay. FAAH activity was measured by incubating for 30 min at 37°C the substrate (1μM cold anandamide and 0.6 nM [^{3}H]-anandamide in the presence of either 50 μg of total rat brain homogenate or 100ng of recombinant 6xHis-rFAAH-6xHis (wild type or mutated) protein, in assay buffer containing 50mM TRIS pH 6.0, 0.05% fatty acid free BSA (Item No. A-8806, Sigma-Aldrich). Rat brain homogenates were obtained from male Sprague Dawley rats (100-125g). Samples were homogenized in Tris HCl 20mM, sucrose 0.32 M, pH 7.4 using glass potter. The homogenate was centrifuged at 1000xg for 15 minutes and the supernatant was recovered for the assay.
FAAH activity reactions were stopped with cold 1:1 CHCl3/methanol. The aqueous phase was counted by liquid scintillation (Microbeta2 Lumijet, Perkin Elmer Inc., MA-USA: adapted from Kathuria et al, 2003). Inhibitors were pre-incubated in assay at appropriate concentration for 10 minutes prior to substrate addition.

Structure determination. Protein was dialyzed to remove the excess of imidazole, concentrated to 20 mg/ml, and supplemented to give final concentration of 1 mM of carprofen, 3% xylitol, and 1.6 % benzyl-dimethyl-dodecyl-ammonium bromide (Sigma). Crystals were grown at 16°C by sitting drop vapor diffusion in 96-well plates, using a reservoir containing 30% polyethylene glycol-400, 100 mM TRIS-HCl pH 7.5, and 100 mM magnesium chloride. Crystals were flash frozen, and data were collected at the BM14 beamline of the European Synchrotron Radiation Facility (ESRF) in Grenoble (France). The structure of the complex was determined at 2.25 Å resolution by molecular replacement, using the (monomer A) protein coordinates from the isomorphous humanized rFAAH-URB597 complex (3LJ7) as a search model, and the program MOLREP [Vagin A & Teplyakov A. *J. Appl. Cryst.* 1997, 30, 1022-1025]. The structure was refined using WinCOOT [Emsley P, Lohkamp B, Scott WG & Cowtan K. *Acta Cryst. D*66, 2010, 486-501], Refmac5 [Vagin A, Stiner RS, Lebedev AA, Potterton L, McNicholas S, Long F & Murshudov GN. *Acta Cryst D*60, 2004, 2284-2295], and other programs included in the CCP4 package suite [Winn MD, Ballard CC, Cowtan KD, Dodson EJ, Emsley P, Evans PR, Keegan RM, Krissinel EB, Leslie AGW, McCoy A, McNicholas SJ, Murshudov GN, Pannu NS, Potterton EA, Powell HR, Read RJ, Vagin A & Wilson KS. *Acta Cryst D*67, 2011]. The final refinement statistics of the complex is shown in Supplementary Results, Table 1. The atomic coordinates and structure factors have been deposited in the Protein Data Bank (http://www.rcsb.org) with accession code 4DO3. Figures were prepared using PyMOL (http://pymol.sourceforge.net).

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