Supplemental Information for article entitled

Discovery of Potent, Non-Carbonyl Inhibitors of Fatty Acid Amide Hydrolase (FAHH)

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Contents:

FAAH assay protocol:

HEK293-TRex cells (Invitrogen) stably transfected with hFAAH or rFAAH in the pCDNA5-Tet-off vector were used for microsomal enzyme preparation. For homogenization, the cell pellets are thawed on ice at room temperature and resuspended in homogenization buffer (50 mM HEPES (pH 7.4), 1 mM EDTA, 1 μM Pepstatin A, 100 μM Leupeptin, 0.1 mg/mL aprotinin). Cell suspensions are then homogenized on ice using the Polytron 1200C at setting 6 for three 30-second intervals with 30-second rests. The suspension is centrifuged at 1000g for 10 minutes at 4°C and the supernatant is collected and further centrifuged at 24,000 rpm for 30 minutes at 4°C using an ultracentrifuge. Pellets are resuspended by adding in cold microsomal buffer (50 mM HEPES (pH 7.4) and 1 mM EDTA) and sheared through a 23-gauge needle five times, keeping the suspension on ice. Protein concentrations are determined using the BCA assay and aliquoted preparations are stored at -80°C until needed. Compound potency against hFAAH or rFAAH is determined using an enzymatic assay with a fluorescence readout. Briefly, experiments were carried out in a 96-well plate format (Corning Costar, # 3370) with a total well volume of 160 μL with components added in the following order: assay buffer (50 mM HEPES (pH 7.4), 1 mM EDTA, 1.4 mg/mL BSA), compound solutions (7 different concentrations per compound in duplicate), microsomal enzyme preparation (10 μg per well) and substrate [AA-AMC (arachadonyl 7-amino 4-methyl coumarin amide), 2 μM]. After a brief shaking, a kinetic read of the plate is obtained using a Tecan Safire II in kinetic mode for 275 cycles with excitation and emission wavelengths of 355 and 460 nm, respectively. Raw data obtained between 2,000 and 5,000 seconds is then processed and analyzed using Assay Explorer and GraphPad Prism. URB-597 (**3**) was used as a control and displayed an average IC_{50} of 3 nM under these conditions.

Notes on biochemical experiments in Figure 2:

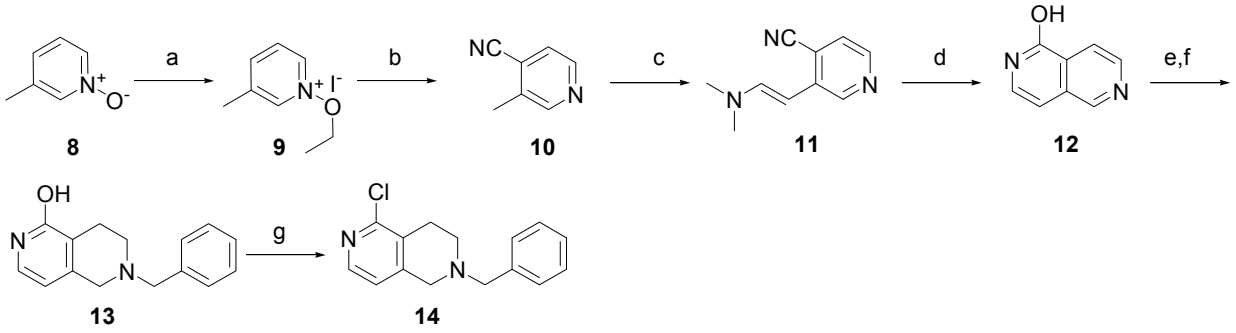
Biochemical experiments shown in Figure 2 were conducted using a microsomal preparation of full length hFAAH expressed in mammalian cells coupled with a radioactive enzymatic assay measuring the hydrolysis of ^{3}H-AEA.

Notes on mass spectrometry experiments in Figure 3:

Based on protocols from reference 5 (K. Ahn, D. S. Johnson, L. R. Fitzgerald, M. Liimatta, A. Arendse, T. Stevenson, E. T. Lund, R. A. Nugent, T. K. Nomanbhoy, J. P. Alexander, B. F. Cravatt *Biochemistry* **2007**, *46*, 13019), rat FAAH (Δ1-31) protein expressed in *E. coli* was incubated with an excess of either RN-450 **29** (500 μM), PF-750 **4** (100 μM) or with an equal volume of DMSO for 1 hour at room temperature. Modifications to rFAAH protein were determined using tryptic digestion followed by mass spectrometry at the Protein Core Facility of Columbia University.

Synthesis of final compound 29:

All final compounds detailed in this paper were purified to >95% as determined by high performance liquid chromatography.



***N*-Ethoxy-3-methylpyridinium iodide 9**:

A round bottom flask was charged with 3-picoline-*N*-oxide **8** (50.00 g, 458 mmol) and anhydrous methylene chloride (90 mL). To this was added ethyl iodide (715.57 g, 4587 mmol) and stirred overnight at room temperature. After completion of the reaction (TLC), the white solid formed was filtered and washed with ethyl acetate(2 x 50 mL) and dried to afford the desired product (102 g, 86 %). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 1.53 (t, J = 6.9 Hz, 3H), 2.76 (s, 3H), 5.00 (q, J = 6.9 Hz, 2H), 8.22-8.27 (m, 1H), 8.44 (d, J = 7.8 Hz, 1H), 9.37 (d, J = 6.3 Hz, 1H), 9.57 (s, 1H).

3-Methylpyridine-4-carbonitrile 10:

A round bottom flask was charged with *N*-ethoxy-3-methylpyridinium iodide **9** (74.00 g, 536 mmol), potassium carbonate (148.00 g, 1072 mmol) and water (350 mL). To this was slowly added a solution of sodium cyanide (49.9 g, 1018 mmol) in water (200 mL) over a period of 30 min. The resultant reaction mixture was heated to 50 ^{o}C for 2 hours. After completion of the reaction (TLC), the reaction mixture was extracted with ethyl acetate (3 x 500 mL). The combined organic layers were dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure to obtain 28.5 g of the product (mixture of 2- and 4- isomers) which was used in the next step without further purification. *m/z* = 119 $(M+H)^+$.

3-(2-(dimethylamino)vinyl)pyridine-4-carbonitrile 11:

A round bottom flask was charged with 3-methylpyridine-4-carbonitrile **10** (52 g, 440 mmol), anhydrous *N,N*-dimethylformamide (100 mL), *N,N*-dimethylformamide dimethylacetal (314 g, 2.64 mol) and pyrrolidine (31 g, 440 mmol) under an inert atmosphere and heated at 130°C for 16 hours. After completion of the reaction (TLC), the reaction was treated with ice-cold water (300 mL) and then extracted with ethyl acetete (3 x 500 mL). The combined organic layers were dried over anhydrous sodium sulfate and the solvent was removed to get 72 g of the desired product (mixture of 2- and 4-isomers) which was used as such in the next step without further purification. *m/z* = 174 $(M+H)^+$.

2,6-Naphthyridin-1-ol 12:

A round bottom flask was charged with 3-(2-(dimethylamino)vinyl)pyridine-4-carbonitrile **11** (40.0 g, 231 mmol) and ethanol (100 mL). 48% aq. HBr (277 g) was added and the resultant reaction mixture was refluxed for 16 hours. After completion of the reaction (TLC), the solvents were removed under vacuum and the residue was filtered and washed with cold ethanol to afford the desired product (40%). ^{1}H-NMR (CD_3OD, 300 MHz) δ 6.94 (d, *J* = 7.2 Hz, 1H), 7.58 (d, *J* = 7.2 Hz, 1H), 8.67 (d, *J* = 6.0 Hz, 1H), 8.74 (d, *J* = 6.0 Hz, 1H), 9.45 (s, 1H); *m/z* = 147 $(M+H)^+$.

6-benzyl-5,6,7,8-tetrahydro-2,6-naphthyridin-1-ol 13:

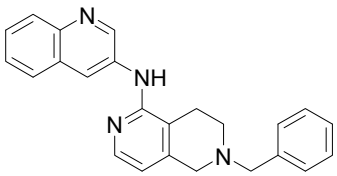
A round bottom flask was charged with 2,6-naphthyridin-1-ol **12** (17 g, 116 mmol), ethanol-water (1:2, 200 mL) and benzyl bromide (99.52 g, 582 mmol) and potassium carbonate (8.5 g, 61 mmol) were added . The resultant reaction mixture was then refluxed for 3 hours. After completion of the reaction (TLC), the reaction mixture was cooled to 0°C, sodium borohydride (17.6 g, 465 mmol) was added portion-wise and allowed to stir at room temperature for 16 hours. After completion, the reaction was quenched with 6N HCl. The solid obtained was filtered and the filtrate was basified with 10% NaOH solution to obtain a solid. The solid thus obtained was triturated with ethyl acetate and petroleum ether

to afford 9 g of the desired product. (32% yield). ^{1}H-NMR (DMSO-d_6, 300 MHz) δ 2.39 (t, J = 5.1 Hz, 2H), 2.62 (t, J = 5.4 Hz, 2H), 3.62 (s, 2H), 5.87 (d, J = 6.6 Hz, 1H), 7.11 (d, J = 6.6 Hz, 1H), 7.24-7.35 (m, 1H); m/z = 241(M+H)$^+$.

2-Benzyl-5-chloro-1,2,3,4-tetrahydro-2,6-naphthyridine 14:

A round bottom flask was charged with 6-benzyl-5,6,7,8-tetrahydro-2,6-naphthyridin-1-ol **13** (7 g, 29 mmol) and phosphorous oxychloride (80 mL) and heated to reflux for 16 hours. The progress of the reaction was monitored by TLC. After completion of the reaction, $POCl_3$ was distilled and the residue was quenched with ice-cold water and then extracted with dichloromethane (3 x 100 mL). The combined organic layers were dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure. The residue was purified by column chromatography (methanol/dichloromethane, 5:95) to afford the desired product (80 % yield). ^{1}H-NMR (DMSO-d_6, 300 MHz) δ 3.00-3.15 (m, 2H), 3.47-3.49 (m, 2H), 4.30 (s, 2H), 4.41 (s, 2H), 7.31 (d, J = 4.8 Hz, 1H), 7.46-7.50 (m, 3H), 7.67-7.68 (m, 2H), 8.25 (d, J = 4.8 Hz, 1H); m/z = 259 (M+H)$^+$.

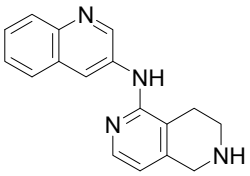
Synthesis of 6-Benzyl-*N*-(quinolin-3-yl)-5,6,7,8-tetrahydro-2,6-naphthyridin-1-amine 15:



A microwave vial was charged with 2-benzyl-5-chloro-1,2,3,4-tetrahydro-2,6-naphthyridine **14** (300 mg, 1.2 mmol), 3-quinolinamine (201 mg, 1.4 mmol), sodium *tert*-butoxide (167 mg, 1.7 mmol), Xantphos (30 mg, 0.06 mmol), tris(dibenzylideneacetone)dipalladium(0) (110 mg, 0.12 mmol), toluene (1 mL) and *tert*-butyl alcohol (0.2 mL). The mixture was heated at 120 °C for 2 hours, whereupon the reaction went to completion. After allowing to cool, the mixture was filtered, washed with methanol and dichloromethane and the solvent removed under reduced pressure and the residue purified by flash

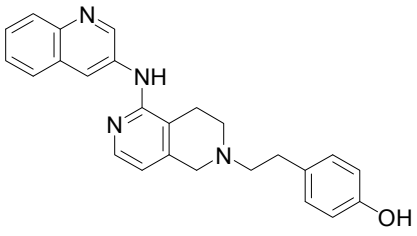
chromatography to obtain the product as a orange solid. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 9.09 (s, 1H), 8.70 (s, 1H), 8.28 (s, 1H), 7.96 (d, J = 5.5 Hz, 1H), 7.90 (d, J = 8.2 Hz, 1H), 7.83 (d, J = 7.6 Hz, 1H), 7.56-7.48 (m, 2H), 7.40-7.26 (m, 5H), 6.61 (d, J = 5.5 Hz, 1H), 3.69 (s, 2H), 3.52 (s, 2H), 2.79 (bs, 4H); m/z = 367.4 $(M+H)^+$.

Synthesis of *N*-(quinolin-3-yl)-5,6,7,8-tetrahydro-2,6-naphthyridin-1-amine 21:



A round bottom flask was charged with 6-benzyl-*N*-(quinolin-3-yl)-5,6,7,8-tetrahydro-2,6-naphthyridin-1-amine **15** (100 mg, 0.27 mmol), ethanol (5 mL) and palladium, 10% weight on charcoal (6.7 mg). The reaction flask was evacuated and flushed with hydrogen three times and the reaction stirred at room temperature overnight under an atmosphere of hydrogen. The reaction mixture was filtered through celite, washed with methanol and solvent removed under reduced pressure and the residue purified by prep HPLC to obtain the product as a white solid. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 9.10 (d, J = 2.5 Hz, 1H), 8.71 (d, J = 2.5 Hz, 1H), 8.24 (s, 1H), 7.96 (d, J = 5.2 Hz, 1H), 7.99 (d, J = 8.8 Hz, 1H), 7.82 (d, J = 7.5 Hz, 1H), 7.55-7.44 (m, 2H), 6.61 (d, J = 5.2 Hz, 1H), 3.79 (s, 2H), 3.04 (t, J = 5.5 Hz, 2H), 2.62 (t, J = 5.5 Hz, 4H); m/z = 277.4 $(M+H)^+$.

Synthesis of 4-{2-[5-(quinolin-3-ylamino)-3,4-dihydro-1*H*-[2,6]naphthyridin-2-yl]-ethyl}-phenol 29.



A reaction vial was charged with *N*-(quinolin-3-yl)-5,6,7,8-tetrahydro-2,6-naphthyridin-1-amine **21** (75 mg, 0.27 mmol), 4-(2-bromo-ethyl)-phenol (54.6 mg, 0.27 mmol), *N,N*-diisopropylethylamine (95 µL, 0.54 mmol) and acetonitrile (5 mL) and the reaction heated in a microwave at 120 °C for 1h. The reaction was then quenched with water and extracted with dichloromethane. The solvent was removed and the residue purified by prep HPLC to get the product as very light brown solid. ^{1}H-NMR (400 MHz, d_6-DMSO) δ 9.09 (d, J = 2.1 Hz, 1H), 8.70 (d, J = 1.9 Hz, 1H), 8.26 (s, 1H), 7.98 (d, J = 5.6 Hz, 1H), 7.90 (d, J = 8.1 Hz, 1H), 7.83 (d, J = 7.6 Hz, 1H), 7.66-7.48 (m, 2H), 7.05 (d, J = 8.4 Hz, 2H), 6.68-6.65 (m, 3H), 3.59 (s, 2H), 2.81 (m, 2H), 2.77-2.72 (m, 4H), 2.67-2.63 (m, 2H); *m/z* = 397.3 $(M + H)^+$; elemental CHN analysis ($C_{25}H_{24}N_4O$): 75.73% C, 6.10% H, 14.13% N (calculated: 75.73% C, 6.10% H, 14.13% N, 4.04% O).