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Development of Fragment-Based *n*-FABS NMR Screening Applied to the Membrane Enzyme FAAH

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Despite the recognized importance of membrane proteins as pharmaceutical targets, the reliable identification of fragment hits that are able to bind these proteins is still a major challenge. Among different ^{19}F NMR spectroscopic methods, *n*-fluorine atoms for biochemical screening (*n*-FABS) is a highly sensitive technique that has been used efficiently for fragment screening, but its application for membrane enzymes has not been reported yet. Herein, we present the first successful application of *n*-FABS to the discovery of novel fragment hits, targeting the membrane-bound enzyme fatty acid amide hydrolase (FAAH), using a library of fluorinated fragments generated based on the different local environment of fluorine concept. The use of the recombinant fusion protein MBP-FAAH and the design of compound **11** as a suitable novel fluorinated substrate analogue allowed *n*-FABS screening to be efficiently performed using a very small amount of enzyme. Notably, we have identified 19 novel fragment hits that inhibit FAAH with a median effective concentration (IC_{50}) in the low mM–μM range. To the best of our knowledge, these results represent the first application of a ^{19}F NMR fragment-based functional assay to a membrane protein.

Introduction

Over the last decade, the fragment-based approach (FBA) has become an established and efficient method for hit identification and optimization as an appealing alternative to high-throughput screening (HTS).[1] The central idea in FBA is to identify fragments that bind to target proteins from a relatively small library of compounds (100–1000) complying with the rule of three.[2] Such libraries are able to cover the chemical space more thoroughly than libraries of larger molecules and are more likely to contain cores that fit into the target binding site.[3] Due to their low molecular weight, fragments usually bind weakly to the target macromolecules (mM–μM range). In order to detect such weak binding, sensitive biophysical techniques such as NMR, fluorescence spectroscopy, surface plasmon resonance, and X-ray crystallography[4] have been applied.

Although FBAs have been successfully applied against soluble protein targets, and several compounds have advanced to clinical trials,[5] the use of this approach on membrane-bound targets is still in its infancy. These proteins constitute more than 40% of drug targets, demonstrating their notable relevance for the treatment of different diseases. The development of drugs targeting membrane proteins has been based predominantly on the screening of large compound libraries.[6]

In general, major challenges in targeting membrane proteins include: 1) production and purification of sufficient quantity of functional target; 2) stabilization of the protein in a homogeneous system suitable for the assay; and 3) high levels of false positives due to nonspecific partitioning of fragments into detergent systems. Although different new reagents and techniques have enabled the production and purification of a variety of membrane proteins,[7] continuous efforts are dedicated to the development of efficient and reliable assays for the investigation of this class of challenging targets.

n-Fluorine atoms for biochemical screening (*n*-FABS)[8] has been demonstrated to be a powerful and sensitive method for performing biochemical assays in an FBA.[9] *n*-FABS is a biochemical methodology which requires the labeling of the enzyme substrate (or cofactor) with a fluorine-containing group. It is based on the extreme sensitivity of ^{19}F isotropic chemical shift to small perturbations, resulting in different ^{19}F NMR signals for the starting substrate and the enzymatically modified substrate (or cofactor), even when the modification occurs far from the fluorine moiety. Its robustness results in the detection of even weak inhibitors and in reliable IC_{50} determinations of the hits.

Recently, attention has been also dedicated to the development of more sophisticated approaches to build efficient fragment libraries. In this context, a strategy based on the local en-

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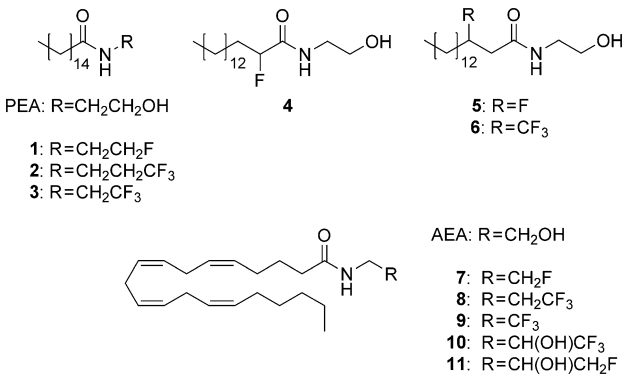
vironment of fluorine concept has been recently proposed by Vulpetti et al.[10] The component fragments of such libraries are selected based on their different fluorine local environmental fingerprints, which allows for better detection of putative fluorophilic hot spots on the desired biological target.[11]

Our main interest was focused on the development of an *n*-FABS assay for fragment screening against membrane proteins. For this purpose, we selected the fatty acid amide hydrolase (FAAH) enzyme[12] and screened a small fluorinated library built in-house. FAAH is a membrane-bound serine hydrolase, mainly expressed in brain, testis, and liver, responsible for the catabolism of a class of endogenous bioactive lipids called fatty acid ethanolamides (FAEs), such as *N*-arachidonylethanolamine (AEA, anandamide) and *N*-palmitoylethanolamine (PEA; Scheme 1). FAEs are involved in several physiological functions



Scheme 1. Selected natural substrates (PEA and AEA) and fluorinated substrate analogues (compounds **1**–**11**).

and in the regulation of a wide range of mammalian behaviors, including pain, inflammation, and cognitive/emotional states. Inhibition of FAAH raises the extracellular levels of AEA and leads to indirect activation of cannabinoid receptors.[13] Over the last decade, a significant number of reversible and irreversible FAAH inhibitors have been reported.[14] These molecules might be useful in the treatment of pain, inflammation, drug addiction, appetite regulation, sleep disorders, anxiety, and depression.[15]

The requirement of a purified stable functional enzyme and the identification of a suitable fluorinated enzyme substrate has rendered *n*-FABS assay development particularly challenging when applied to FAAH, for two main reasons: 1) FAAH is a membrane-bound enzyme; and 2) the natural substrates of FAAH are characterized by high lipophilicity and low solubility in aqueous solutions.

In the present work, we report on the development of an NMR-based fragment screening assay that can overcome the challenges posed by targeting FAAH. We demonstrate the performance of our methodology through the screening of our in-house fluorinated fragment library and identification of novel fragment hits, with a good hit rate (16.5%). To the best of our knowledge, the present study represents the first application of ^{19}F NMR fragment-based biochemical screening for a membrane protein.

Results and Discussion

The aim of this work was the development of a fast, efficient, and reliable biochemical fragment screening method applicable to FAAH, combining *n*-FABS methodology with the use of a fragment library. In order to apply this approach, three main requirements had to be properly fulfilled: 1) availability of a stable and active form of FAAH in aqueous/detergent solution; 2) identification of a suitable fluorinated substrate of FAAH; and 3) design and construction of a library of fragments.

Expression of the recombinant membrane enzyme FAAH

FAAH is a 63 kDa protein whose structure was first solved by Bracey et al. in 2002,[16] expressed in *E. coli* as a truncated functional form of the enzyme (ΔTM-FAAH) which lacks 29 residues at the N terminus. The crystal structure showed that this enzyme anchors cell membranes through the elongated transmembrane α-helix of the N terminus and two highly hydrophobic α-helices (α18 and α19). The enzyme presents two internal channels: the membrane access channel, which allows the substrate to access the active site catalytic triad (Lys 142, Ser 217, and Ser 241), and the acyl chain binding pocket, which accommodates the fatty acid chain of the substrate during hydrolysis.

The insertion of an N-terminal fusion protein increases the amount of membrane protein that is heterologously expressed, and maltose-binding protein (MBP) is particularly effective at promoting overexpression of soluble well-folded proteins.[17] Hence, we generated a construct of ΔTM-*r*FAAH (rat FAAH residues 33–574) bearing an MBP at the protein N terminus as a fusion protein, and a hexahistidine (His_6) tag at the C terminus to optimize the performance of the recombinant protein (MBP-*r*FAAH) purification. This system allowed us to produce this membrane protein with a yield of ~5 mg L^{-1} of culture medium and purity exceeding 80% after a single affinity purification step using Ni–NTA agarose. Importantly, activity of recombinant MBP-*r*FAAH was stable over time (see the Supporting Information). Moreover, WaterLOGSY[18] experiments on MBP-*r*FAAH in the presence of a five-fragment mixture from our fluorinated library demonstrated that the enzyme did not aggregate (data not shown).

Identification of fluorinated FAAH substrates

One of the challenges we encountered in the setup of the *n*-FABS assay was the identification of an aqueous soluble fluorinated FAAH substrate. It has been reported that FAAH catalyzes the hydrolysis of several FAEs.[19] We selected AEA and PEA as our starting point for the design of new fluorinated analogues, which are shown in Scheme 1.

Vandevoorde et al.[20] reported that several PEA analogues bearing different substituents in the amine moiety (head chain), including the fluoroethyl group of compound **1**, are recognized by FAAH, demonstrating that the OH group of the head chain in the natural substrate is not fundamental for FAAH recognition. On the other hand, the authors also ob-

served a low solubility of those compounds in aqueous buffers. For this reason, we designed analogues bearing the fluorinated moiety not only in the head chain, such as in compounds **2** and **3**, but also in the acyl portion (tail chain), such as in compounds **4**–**6**, aiming to enhance the solubility of the fluorinated PEA analogues by maintaining the polar hydroxy group of the head chain.

With regard to the fluorinated AEA analogues, we decided to introduce the fluorinated moiety only in the head chain (compounds **7**–**11**) for easier synthetic accessibility. Among these, compounds **7** and **9** are known FAAH substrates[20–21] in which the CH_2OH group is replaced by a CH_2F and a CF_3 moiety, respectively. Compound **8** is a close analogue of compounds **7** and **9**, while compounds **10** and **11**, bearing a polar OH group in the head chain, were designed with the aim of enhancing the solubility of known FAAH substrates **7** and **9**.

To select a suitable fluorinated substrate, we applied three criteria: 1) solubility and stability; 2) lack of aggregation in assay buffer; and 3) affinity profile (K_m and enzyme efficiency). Solubility, stability, and aggregation state were determined in phosphate-buffered saline (PBS, pH 7.4) with 8% D_2O and 0.05% Triton X-100 using ^{19}F NMR experiments (see the Supporting Information for experimental details). Under these conditions, compounds **1**–**6** demonstrated low solubility (< 5 μM) and a strong tendency to aggregate. On the other hand, compounds **7**–**11** showed improved solubility in PBS. Unfortunately, compounds **8** and **9** aggregate at concentrations higher than 10 μM (Figure S1 in the Supporting Information).

Therefore, compounds **7**, **10**, and **11** were selected as possible substrates of FAAH in the setup of the *n*-FABS assay. The stability and K_m of the three candidates were then evaluated. To determine the stability of **7**, **10**, and **11**, we recorded their ^{19}F NMR spectra in PBS at room temperature every 60 min over 14 h. No differences in ^{19}F NMR signals were observed, indicating that the three compounds are sufficiently stable at room temperature (Figure S2).

To calculate K_m values and enzyme efficiencies of **7**, **10**, and **11**, we determined the concentrations of the enzymatic products by analyzing the integral values of ^{19}F NMR signals of the products at increasing concentrations of the substrates (in Figure 1 A), data at 40 μM are shown as an example). The reactions were quenched after 50 min for **7** and 70 min for **10** and **11** by adding the known potent FAAH inhibitor URB597.[13] The rate of the reaction, obtained by the measurement of the inte-

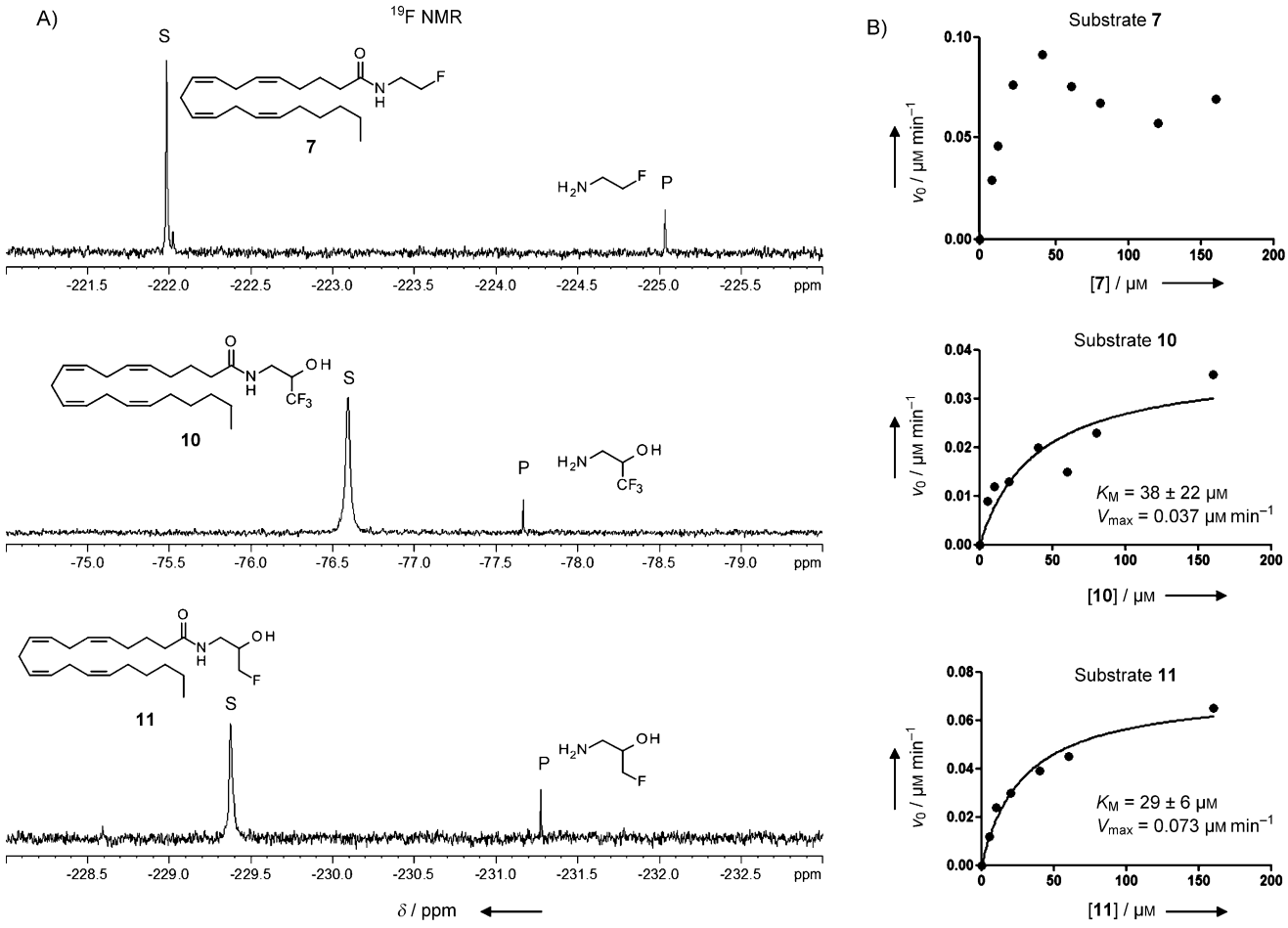
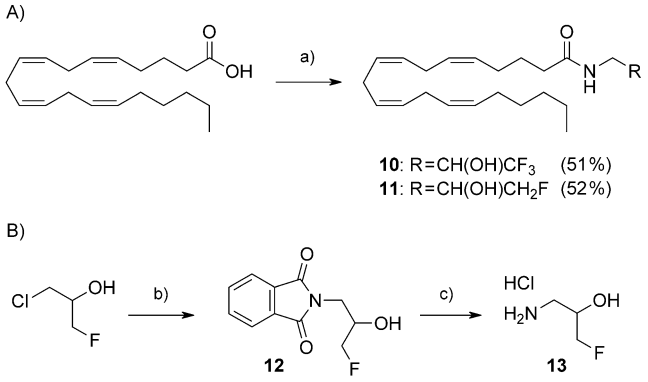


Figure 1. A) ^{19}F NMR spectra of substrate **7** (40 μM) in the presence of 10 nM MBP-*r*FAAH, and substrates **10** and **11** (40 μM) in the presence of 30 nM MBP-*r*FAAH. S and P indicate the ^{19}F NMR signals of substrate and product, respectively. B) Michaelis–Menten plots. The best data fit afforded the K_m and V_{max} values.

gral of the ^{19}F NMR signal of the product divided by the incubation time, as a function of the substrate concentration, was then plotted. The best fit of these data afforded K_m and V_{max} values and, consequently, also the enzyme efficiency (k_{cat}/K_m) values. (Figure 1 B). Unfortunately, it was not possible to accurately calculate the K_m for compound **7** due to aggregation at concentrations higher than 40 μM. As can be observed in Figure 1, there is a large difference in the ^{19}F chemical shift between the substrate and product signals for both CF_3- and CH_2F-containing compounds.

Finally, compound **11** was selected as suitable fluorinated substrate for the *n*-FABS assay based on its superior enzyme efficiency ($k_{cat}/K_m = 1407\ M^{-1}s^{-1}$) compared to compound **10** ($k_{cat}/K_m = 542\ M^{-1}s^{-1}$). To the best of our knowledge, the ability of compounds **10** and **11** to behave as FAAH substrates is an unprecedented result. It is worth noting that compound **7**, which is an efficient substrate according to Figure 1 despite the uncertainty of its K_m value, can also be used for *n*-FABS. This would require working at low-μM substrate concentrations, in order to avoid aggregation, which is currently possible with an NMR spectrometer equipped with ^{19}F cryogenic probe technology.

Compounds **10** and **11** were synthesized using standard reaction conditions (Scheme 2 A) by treatment of arachidonoyl



Scheme 2. Synthesis of fluorinated AEA analogues **10** and **11**. Reagents and conditions: a) i: DMF (cat.), $(COCl)_2$, CH_2Cl_2, 0 °C to RT, 3 h; ii: Et_3N, RCH_2NH_2, CH_2Cl_2, 0 °C to RT, 12 h; b) potassium phthalimide, DMF, 80 °C, 4 h; 61%; c) 13% HCl, 100 °C, 12 h, 92%.

chloride with the appropriate fluorinated amine, which was either commercially available or obtained from the corresponding chloride by Gabriel synthesis, as reported in Scheme 2 B). See the Supporting Information for experimental details of the synthesis of all substrates.

Construction of the fluorinated fragment library

We built our fluorinated fragment library according to the methodology developed by Vulpetti et al.[10] We analyzed about 7000 commercial fluorinated compounds containing CF or CF_3 moieties and clustered them as a function of the fluorine environments. Out of the 160 ordered compounds, 115 compounds passed the SPAM filter,[22] showing solubility ≥100 μM in PBS (pH 7.4) with 8% D_2O and 5 μM EDTA, purity ≥75% and lack of aggregation. For experimental details on compounds characterization, see the Supporting Information. The final set of 115 compounds was used to generate 23 mixtures of five compounds each to screen against FAAH.

n-FABS assay, deconvolution, and IC_{50} determination

Our *n*-FABS assay was first validated by determining IC_{50} values of known FAAH inhibitors. The derived IC_{50} values showed the same rank order of potencies reported in literature, obtained using different substrate and assay conditions (for experimental details, see the Supporting Information).

The 23 mixtures in our fluorinated library were tested at a concentration of 200 μM using enzyme and substrate concentrations of 15 nM and 30 μM, respectively, as described in the Experimental Section. This substrate concentration that corresponds to the K_m is suitable for a balanced assay, thus allowing the efficient detection of competitive, uncompetitive, and noncompetitive inhibitors. In this particular case, the inhibition binding constant, K_i, corresponds to the $IC_{50}/2$ value for both competitive and uncompetitive inhibitors and to the IC_{50} value for noncompetitive inhibitors. Nine mixtures showed inhibition >15% and were deconvoluted for hit identification. An example of screening and the following deconvolution of an active mixture are shown in Figure 2. The disappearance of the ^{19}F signal of the product (P) with tested mixture 17 indicates the presence of inhibitor(s); on the other hand, no changes in intensities were observed in the presence of mixture 9, which is therefore an inactive mixture (Figure 2 A). The deconvolution consists in a stepwise process that allows for the identification of an active fragment(s). Mixture 17 was first split into two sets of three and two compounds, respectively, and assays were carried out on these new sets. Only the mixture containing fragments 4 and 5 was active (Figure 2 B). Fragments 4 and 5 were then tested as single compounds to identify the active one: fragment 5 (Figure 2 C). Through this process, of the nine mixtures showing inhibition >15%, we identified 19 active fluorinated fragments. In some mixtures, more than one hit could be detected.

Determination of the IC_{50} values of our fragment hits was performed with *n*-FABS experiments at different concentrations of the inhibitors in triplicate samples by measuring the integral values of the ^{19}F NMR signal of enzymatic product (P). IC_{50} values were then calculated by plotting these values as a function of the inhibitor concentration, as shown for hit 1 and hit 6 in Figure 3. It is worth noting that the IC_{50} value can also be determined by the ^{19}F NMR signal of the substrate. This is possible because the *n*-FABS assay allows for simultaneous detection of the substrate and product, which is not feasible with many biophysical methods applied to biochemical assays. Based on the conditions of the assay, our methodology allowed for the measurement of IC_{50} values ranging from low nM (limit determined by protein concentration) to mM values (limit determined by compound solubility). The structures of the fragment hits, the corresponding *r*FAAH IC_{50} values, and the binding efficiency index (BEI)[23] are reported in Table 1.

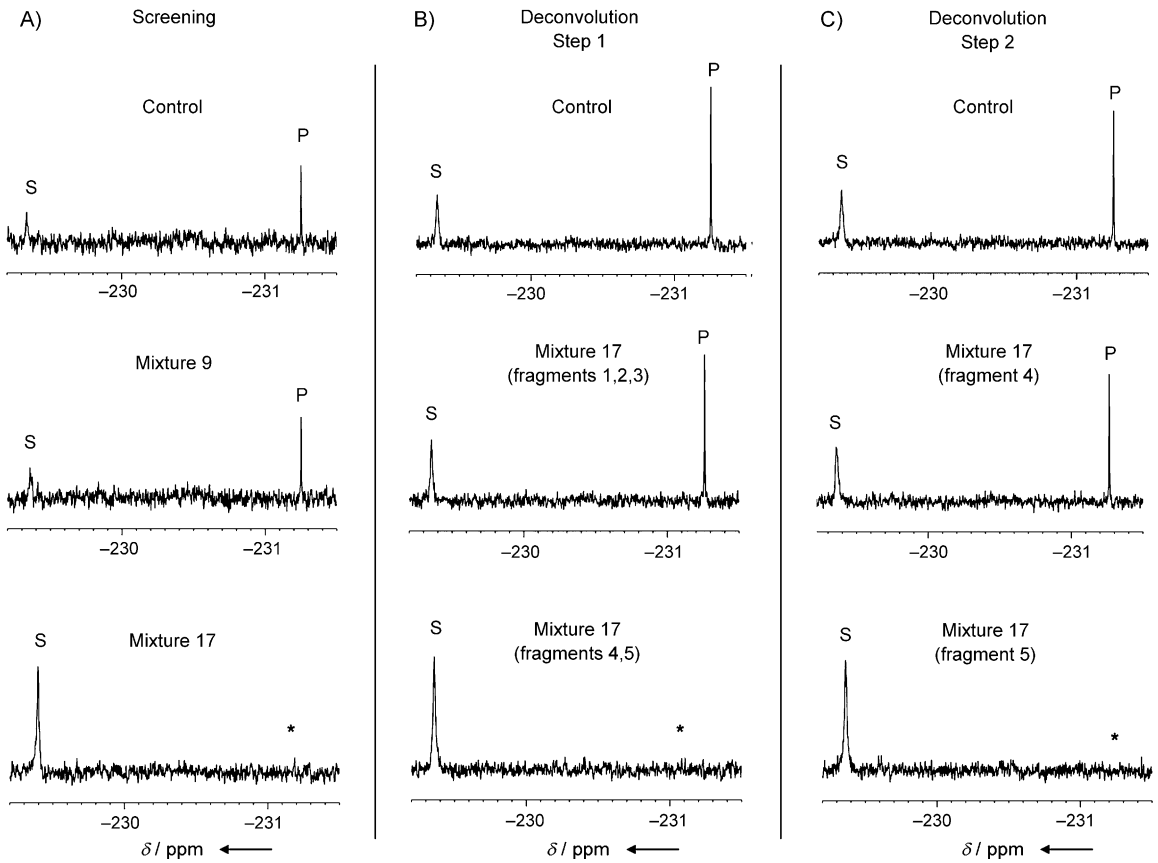


Figure 2. *n*-FABS screening and deconvolution. S and P indicate the ^{19}F NMR signal of substrate **11** and product, respectively. The asterisk indicates the absence of product and, consequently, the presence of an active compound. A) ^{19}F NMR spectra of an inactive (mixture 9) and active (mixture 17). B) Only the set of mixture 17 containing fragments 4 and 5 was active. C) Fragment 5 was identified as active compound.

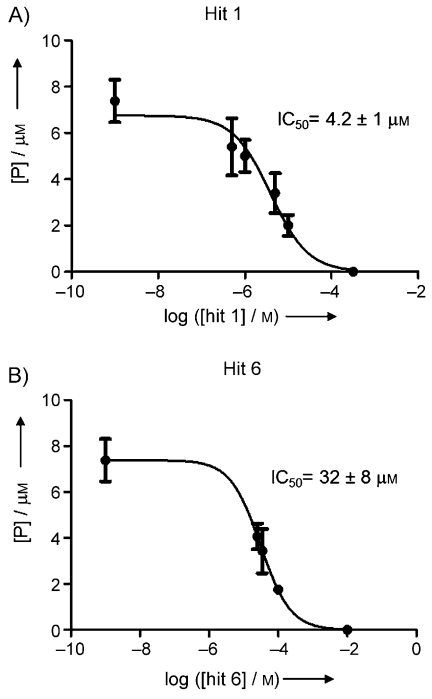


Figure 3. IC_{50} determination for two fragment hits: A) hit 1 and B) hit 6. Their chemical structures are reported in Table 1.

Notably, structural analysis of the identified fragment hits revealed that our scaffolds, except for the trifluoromethyl ketone (hit 3) and trifluoroacetyl benzotriazole (hit 14), do not contain evident chemical overlapping substructures with any of the published FAAH inhibitors.[14a] A common structural leitmotif may be highlighted between hits 2, 15, and 17, bearing a fused bicyclic 6,5-ring system, and hits 18 and 19 as anilide derivatives of five-membered heteroaromatic rings. Interestingly, hit 16, a known fluorinated selective COX-2 inhibitor (niflumic acid), was previously found to inhibit FAAH activity.[24] It is also worth noting the presence of several CF_3 aromatic-containing molecules. It has been shown that these moieties are often found in proximity of α-helices (a secondary structure element abundant in FAAH), with the CF_3 group making close interactions with the side chain of hydrophobic residues in positions *i* and $i+3$ or $i+4$ of the helix.[11b,25] It is also worth noting the identification of very weak inhibitors with IC_{50} in the mM range, as it is for hit 17 and hit 18, despite the low concentration at which the fragments were tested. At the 200 μM concentration used for screening, the two hits showed only a ~15% inhibition. Often, these hits would be discarded in HTS assays because these small values of inhibition are contained in the error bars of the measurements. However, the robustness and reliability of the *n*-FABS allowed their selection for IC_{50} determination. In general, most of the identified hits are

Table 1. Structures and IC_{50} and BEI values of the identified hits.

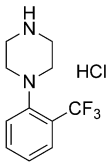
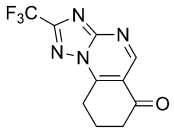
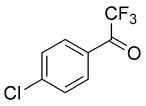
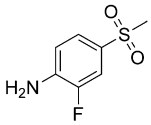
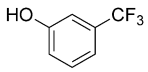
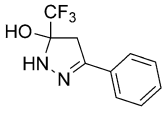
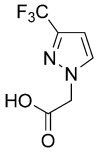
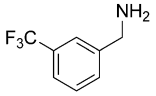
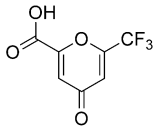
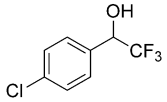
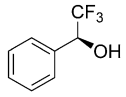
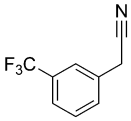
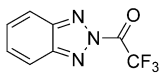
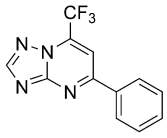
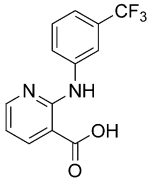
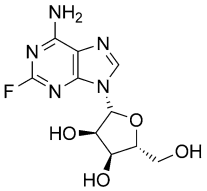
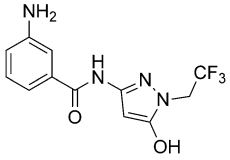
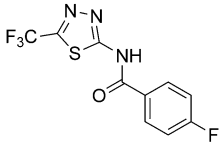
Hit	Structures	FAAH IC_{50} [μM]	BEI[a]
hit 1		4.2 ± 1	23
hit 2		3 ± 1	22
hit 3		26 ± 6	22
hit 4		58 ± 21	22
hit 5		450 ± 45	21
hit 6		32 ± 8	20
hit 7		147 ± 24	20
hit 8		270 ± 60	20
hit 9		465 ± 60	20
hit 10		137 ± 31	19
hit 11		143 ± 47	18
hit 12		563 ± 36	18
hit 13		720 ± 90	17
hit 14		400 ± 65	16

Table 1. (Continued)

Hit	Structures	FAAH IC_{50} [μM]	BEI[a]
hit 15		110 ± 27	15
hit 16		162 ± 44	13
hit 17		1125 ± 230	10
hit 18		1315 ± 260	10
hit 19		53% at 100 μM	

[a] BEI (binding efficiency index) = pIC_{50} [M]/MW [kDa].

amenable to chemical structural evolution which, in combination with the novelty of the structural frameworks, may allow for the identification of new FAAH inhibitors.

Conclusions

Membrane proteins are challenging targets in drug discovery and, therefore, the application of an FBA is often not straightforward. Herein, we report the use of the *n*-FABS method for screening a library of 115 fluorinated fragments, targeting the membrane-bound enzyme FAAH. The screening was performed by using recombinant MBP-*r*FAAH and compound **11** as a novel fluorinated and soluble substrate analogue. The small amount of enzyme required for NMR screening of the fragment library (15 nM concentration for each mixture) indicates that the method can be extended to efficient screening against those membrane enzymes that can only be produced in very small quantities. The low concentration required for *n*-FABS allows for better solubility of the membrane protein and reduces aggregation. In addition, screening with *n*-FABS applied to membrane proteins does not require the use of expensive deuterated detergents, because the ^{19}F NMR spectra are recorded. We identified 19 novel fragment hits that inhibit

the enzyme with IC_{50} values in the low mM–μM range. The fragment evolution of selected hits is currently ongoing, and the results will be described in due course. To the best of our knowledge, our results represent the first application of ^{19}F NMR fragment-based screening to a membrane protein using a functional assay. We are confident that these results will contribute to future application of the *n*-FABS methodology to other membrane proteins.

Experimental Section

FAAH construct generation: the encoding sequence of rat *r*FAAHΔTM (97–1722 bp) was amplified from cDNA clone 7370226 (Open Biosystem) using the following primer pair: forward 5′-GGGAAT TCCATA TGGGGC GCCAGA AGGCCC-3′ (NdeI site is underlined); reverse 5′-ATAGTT TAGCGG CCGCTC AATGAT GATGAT GATGAT GAGGGG TCATCA GCT-3′ (NotI site is underlined). A His_6 tag was introduced in the reverse primer sequence. To obtain the construct MBP-*r*FAAH-His_6 pMALc5x, the amplified *r*FAAHΔTM was cloned in a pMALc5x vector in frame with N-terminal MBP.

Protein expression and purification: Expression of the MBP-*r*FAAH-His_6 protein was carried out in the *E. coli* Rosettagami 2 (DE3)pLysS strain (Novagen). At an optical density (OD_{600}) of 0.6, bacteria were induced by the addition of 0.25 mM IPTG for 16 h at 25 °C. Cells were then harvested by centrifugation, resuspended in buffer (50 mM sodium phosphate, 0.3 M NaCl, 10 mM imidazole, pH 7.4), and lysed by sonication and addition of 1% Triton X-100. The lysate was incubated for 1 h with benzonase nuclease and centrifuged at 15000 *g* for 30 min. The clarified supernatant was incubated for 2 h with Ni–NTA agarose (Qiagen) and washed with buffer containing increasing concentrations of imidazole. Elution was performed with buffer containing 0.25 M imidazole. The protein was then concentrated, and the buffer was exchanged with a solution of 50 mM sodium phosphate, 0.3 M NaCl, and 0.1% CHAPS, pH 7.4. Protein aliquots were stored at −80 °C until use.

Chemistry: For experimental and analytical details, see the Supporting Information.

Synthesis of (5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(2-fluoroethyl)icosa-5,8,11,14-tetraenamide (7): DMF (1 μL, 0.01 mmol) and oxalyl chloride (43 μL, 0.45 mmol) was added to a solution of arachidonic acid (75 mg, 0.25 mmol) in dry CH_2Cl_2 (1.5 mL) at 0 °C under N_2 atmosphere. After stirring for 3 h, the solvent was removed under reduced pressure, and the crude product was washed with dry CH_2Cl_2 (3×2 mL, evaporation after each step). The residue was then dissolved in dry CH_2Cl_2 (1.5 mL) under N_2 atmosphere and added through a cannula to a solution of dry Et_3N (80 μL, 0.55 mmol) and 2-fluoroethylamine hydrochloride (30 mg, 0.31 mmol) in dry CH_2Cl_2 (2.5 mL) at 0 °C. After stirring overnight at room temperature, 2 N HCl solution (15 mL) was added. The reaction mixture was extracted with CH_2Cl_2 (3×15 mL), and the organic layer was washed with brine and dried over Na_2SO_4. After evaporation of the solvent, the crude product was purified by column chromatography using a SI (5 g) cartridge, eluting with CH_2Cl_2 (100%) to afford **7** (26 mg, 31%) as a colorless oil: $R_f = 0.28$ (CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) $\delta = 0.89$ (t, $J = 6.5$ Hz, 3H), 1.23–1.42 (m, 6H), 1.73 (p, $J = 7.4$ Hz, 2H), 2.06 (q, $J = 6.9$ Hz, 2H), 2.12 (q, $J = 6.8$ Hz, 2H), 2.21 (t, $J = 7.6$ Hz, 2H), 2.77–2.87 (m, 6H), 3.57 (dq, $J = 28.4$, 5.1 Hz, 2H), 4.49 (dt, $J = 47.4$, 4.7 Hz, 2H), 5.27–5.48 (m, 8H), 5.76 ppm (s, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) $\delta = 14.2$, 22.7, 25.6, 25.8, 26.8, 27.4, 29.5, 31.7, 36.1, 40.0 (d, $J = 19.5$ Hz), 83.0 (d, $J = 166.0$ Hz), 127.7, 128.0, 128.3, 128.4, 128.8, 129.0, 129.2, 130.7, 173.1 ppm.

Synthesis of (5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(3,3,3-trifluoro-2-hydroxy-propyl)-icosa-5,8,11,14-tetraenamide (10): DMF (1 μL, 0.01 mmol) and oxalyl chloride (55 μL, 0.66 mmol) were added to a solution of arachidonic acid (100 mg, 0.33 mmol) in dry CH_2Cl_2 (2 mL) at 0 °C under N_2 atmosphere. After stirring for 3 h, the solvent was removed under reduced pressure, and the crude product was washed with dry CH_2Cl_2 (3×2 mL, evaporation after each step). The residue was then dissolved in dry CH_2Cl_2 (2 mL) under N_2 atmosphere and added through a cannula to a solution of dry Et_3N (70 μL, 0.49 mmol) and 3-amino-1,1,1-trifluoropropan-2-ol (55 mg, 0.43 mmol) in dry CH_2Cl_2 (2.5 mL) at 0 °C. After stirring overnight at room temperature, a 2 N HCl solution (15 mL) was added. The reaction mixture was extracted with CH_2Cl_2 (3×15 mL), and the organic layer was washed with brine and dried over Na_2SO_4. After evaporation of the solvent, the crude product was purified by preparative HPLC-MS to afford **10** (70 mg, 51%) as a colorless oil: $R_f = 0.56$ (CH_2Cl_2/MeOH, 98:2); ^1H NMR (400 MHz, [D_6]DMSO) $\delta = 0.85$ (t, $J = 6.9$ Hz, 3H), 1.21–1.37 (m, 6H), 1.54 (p, $J = 7.6$ Hz, 2H), 2.02 (q, $J = 6.8$ Hz, 4H), 2.10 (t, $J = 7.5$ Hz, 2H), 2.72–2.86 (m, 6H), 3.06 (ddd, $J = 13.6$, 7.6, 5.7 Hz, 1H), 3.37–3.45 (m, 1H), 3.93–4.05 (m, 1H), 5.26–5.41 (m, 8H), 6.39 (d, $J = 6.4$ Hz, 1H), 8.03 ppm (t, $J = 5.6$ Hz, 1H); ^{13}C NMR (101 MHz, [D_6]DMSO) $\delta = 13.9$, 21.9, 25.2, 26.2, 26.6, 28.7, 30.9, 34.6, 39.1, 67.4 (q, $J = 28.6$ Hz), 125.3 (q, $J = 283.4$ Hz), 127.5, 127.6, 127.8, 128.0, 128.1, 129.4, 129.9, 172.4 ppm; MS (ESI): *m/z* calcd. for $C_{23}H_{36}F_3NO_2$: 416 $[M+H]^+$, found: 416.

Synthesis of 2-(3-fluoro-2-hydroxypropyl)isoindoline-1,3-dione (12): to a suspension of potassium phthalimide (300 mg, 1.60 mmol) in dry DMF (3.2 mL) was added 1-chloro-3-fluoropropan-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 product was purified by automated column chromatography, eluting with cyclohexane/EtOAc (from 100:0 to 75:25) to afford **12** (180 mg, 61%) as a white solid: ^1H NMR (400 MHz, $CDCl_3$) $\delta = 2.71$ (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 ppm (dd, $J = 5.5$, 3.1 Hz, 2H); MS (ESI): *m/z* calcd. for $C_{11}H_{10}FNO_3$: 224 $[M+H]^+$, found: 224.

Synthesis of 1-amino-3-fluoro-propan-2-ol hydrochloride (13): A suspension of **12** (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 (2 mL) was added. The mixture was washed with EtOAc (3×15 mL), and the aqueous phase was evaporated under reduced pressure to dryness to afford **13** (66 mg, 92%) as a white solid: ^1H NMR (400 MHz, [D_6]DMSO) $\delta = 2.68$–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 ppm (s, 3H); MS (ESI): *m/z* calcd. for C_3H_8FNO: 94, found: 94.

Synthesis of (5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(3-fluoro-2-hydroxy-propyl)icosa-5,8,11,14-tetraenamide (11): DMF (1 μL, 0.01 mmol) and oxalyl chloride (55 μL, 0.66 mmol) were added to a solution of arachidonic acid (100 mg, 0.33 mmol) in dry CH_2Cl_2 (2 mL) at 0 °C under N_2 atmosphere. After stirring for 3 h, the solvent was removed under reduced pressure, and the crude product was washed with dry CH_2Cl_2 (3×2 mL, evaporation after each step). The residue was then dissolved in dry CH_2Cl_2 (2 mL) under N_2 atmosphere and added through a cannula to a solution of dry Et_3N (120 μL, 0.82 mmol) and **13** (55 mg, 0.43 mmol) in dry CH_2Cl_2 (2.5 mL) at 0 °C. After stirring overnight at room temperature, a 2 N HCl solu-

tion (15 mL) was added. The reaction mixture was extracted with CH_2Cl_2 (3×15 mL), and the organic layer was washed with brine and dried over Na_2SO_4. After evaporation of the solvent, the crude product was purified by preparative HPLC-MS to afford **11** (64 mg, 51%) as a colorless oil: ^{1}H NMR (400 MHz, [D_6]DMSO) 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 ppm (t, J = 5.5 Hz, 1H); ^{13}C NMR (101 MHz, [D_6]DMSO) δ = 13.9, 21.9, 25.2, 25.2, 25.2, 26.3, 26.6, 28.7, 30.9, 34.7, 40.8 (d, J = 8.1 Hz), 68.2 (d, J = 18.6 Hz), 85.1 (d, J = 168.3 Hz), 127.5, 127.7, 127.8, 128.0, 128.1, 129.4, 129.9, 172.3 ppm; MS (ESI): *m/z* calcd. for $C_{23}H_{38}FNO_2$: 381 $[M+H]^+$, found: 380; 378 $[M-H]^-$, found: 378.

NMR experiments: all NMR screening experiments were recorded at 298 K with a Bruker FT NMR Avance III 600 MHz spectrometer, equipped with a 5 mm CryoProbe QCI ^{1}H/^{19}F–^{13}C/^{15}N–D quadruple resonance, a shielded z-gradient coil, and an automatic sample changer SampleJet NMR system. All *n*-FABS experiments were performed using low nM enzyme concentration in PBS (pH 7.4) with 8% D_2O in the presence of 0.05% Triton X-100, required for increasing the solubility of the substrate and enzyme.

***n*-FABS assay setup, K_m, and V_{max} determination**: MBP-*r*FAAH (10 or 30 nM) in the presence of increasing concentrations of substrate **7**, **10**, or **11** (from 5 to 180 μM) were incubated in Eppendorf vials for 50 min (substrate **7**) and 70 min (substrates **10** and **11**) at 25 °C in a Thermomixer. The reactions were then quenched by adding URB597 (40 μM), and ^{19}F NMR spectra with proton decoupling (256 scans) were recorded. The initial rate values, obtained from integral values of the product ^{19}F signal divided by the incubation time, were plotted as a function of the substrate concentration. The best data fit, using the GraphPad Prism 5 software package, gives the Michaelis–Menten parameters K_m and V_{max}.

***n*-FABS screening and deconvolution**: 115 fragments were screened in mixtures containing five compounds each at a concentration of 200 μM in an endpoint format in the presence of substrate **11** (30 μM) and MBP-*r*FAAH (15 nM). The reactions were incubated at 25 °C in a Thermomixer incubator and quenched after 3.5 h with URB597 (40 μM). In every run, two controls (samples without inhibitor but with the same amount of [D_6]DMSO), were recorded in order to have a value of 0% inhibition.

***n*-FABS IC_{50} determination**: the known inhibitors and fragment hits were tested at different concentrations under the same experimental conditions as the screening run. Three replicates of these measurements were performed on different days. The integral values of the ^{19}F product signal were measured and plotted as a function of the inhibitor concentration in order to obtain the IC_{50} value. The best data fit was performed using GraphPad Prism 5.

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Keywords: FAAH · fluorine NMR screening · fragment-based approach · membrane proteins

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