### Title
Fluorine NMR-Based Screening on Cell Membrane Extracts




### Journal
ChemMedChem, 9(2)

### ISSN
1860-7179



### Authors
Veronesi, Marina
Romeo, Elisa
Lambruschini, Chiara
et al.



### Publication Date
2014-02-01

### DOI
10.1002/cmdc.201300438

ChemPubSoc Europe

DOI: 10.1002/cmdc.201300438

# Fluorine NMR-Based Screening on Cell Membrane Extracts

Marina Veronesi,[a] Elisa Romeo,[a] Chiara Lambruschini,[a] Daniele Piomelli,[a, b] Tiziano Bandiera,[a] Rita Scarpelli,[a] Gianpiero Garau,*[a] and Claudio Dalvit*[c]

The possibility of measuring the action of inhibitors of specific enzymatic reactions in intact cells, cell lysates or membrane preparations represents a major advance in the lead discovery process. Despite the relevance of assaying in physiological conditions, only a small number of biophysical techniques, often requiring complex set-up, are applicable to these sample types. Here, we demonstrate the first application of *n*-fluorine atoms for biochemical screening (*n*-FABS), a homogeneous and versatile assay based on $^{19}F$ NMR spectroscopy, to the detection of high- and low-affinity inhibitors of a membrane enzyme in cell extracts and determination of their $IC_{50}$ values. Our approach can allow the discovery of novel binding fragments against targets known to be difficult to purify or where membrane-association is required for activity. These results pave the way for future applications of the methodology to these relevant and complex biological systems.

Monitoring enzyme-mediated substrate metabolism in intact cells, cell lysates, or membrane preparations provides a more physiological environment for analyses and can lead to relevant advances in lead discovery. However, despite several examples of enzyme assays reported in literature, only a small number of methodologies are compatible with such biological samples.[1] These are generally restricted to fluorescence-based detection methods, and the technologies upon which these methods are based include the need for reporter assays. Importantly, the use of cell lysates or membrane preparations to monitor the enzyme activities is poorly applicable to highly sensitive label-free spectroscopic methods or biosensor-based technologies, which mainly rely on isolated biochemical systems containing purified enzyme targets.

[a] *M. Veronesi,[+] Dr. E. Romeo,[+] C. Lambruschini, Prof. Dr. D. Piomelli, Dr. T. Bandiera, Dr. R. Scarpelli, Dr. G. Garau*
*Department of Drug Discovery & Development*
*Fondazione Istituto Italiano di Tecnologia*
*Via Morego 30, 16163 Genoa (Italy)*
*E-mail: gianpiero.garau@iit.it*

[b] *Prof. Dr. D. Piomelli*
*Department of Anatomy & Neurobiology*
*School of Medicine, University of California, Irvine*
*Irvine, CA 92697 (USA)*

[c] *Dr. C. Dalvit*
*Institute of Chemistry, Faculty of Science*
*University of Neuchâtel*
*Avenue de Bellevaux 51, 2000 Neuchâtel (Switzerland)*
*E-mail: claudio.dalvit@unine.ch*

[+] *These authors contributed equally to this work.*

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201300438.

*n*-Fluorine atoms for biochemical screening (*n*-FABS)[2–4] is an NMR functional assay based on fluorine spectroscopy that requires the labeling of the substrate (or cofactor) of the enzyme with a fluorine-containing group and allows the direct measurement of the conversion of the substrate (S) into the product (P). *n*-FABS is a homogeneous assay and does not need radioactive labeling, secondary coupled chemical or enzymatic reactions, or labeled antibodies. *n*-FABS has been successfully applied to several pharmaceutically relevant targets resulting in the identification of novel inhibitors.[5–11] In a recent work, we reported its application to the screening of chemical fragments against a purified recombinant membrane enzyme.[12] Indeed, membrane proteins are challenging targets for biochemical assays. Often, modification of membrane enzymes with fusion partners, such as maltose binding protein (MBP), or point mutations are required to enhance the level of expression of these proteins, their solubility and stability in solution.[13–18] Here, we show that *n*-FABS method can be extended to cell extracts, therefore using the enzyme of interest in more physiological conditions. As a model protein, we used fatty acid amide hydrolase (FAAH), a membrane-bound serine hydrolase responsible for the catabolism of a class of endogenous bioactive lipids called fatty acid ethanolamides (FAEs). FAEs, including *N*-arachidonylethanolamine (AEA, anandamide) and *N*-palmitoylethanolamine (PEA),[19] are involved in the regulation of a wide range of physiopathological conditions, such as pain, inflammation, and cognitive/emotional states. AEA is an endogenous agonist of the cannabinoid receptors, $CB_1$ and $CB_2$, and FAAH inhibition in vivo leads to the increase of AEA levels in neural synaptic space, inducing analgesic effects comparable to those of marijuana.[20] Therefore, FAAH has been proposed as a relevant therapeutic target for the treatment of pain and central nervous system (CNS) disorders.[21–25]

Recently, we identified and synthetized a soluble fluorinated substrate analogue (ARN1203)[12] of AEA. Using ARN1203, we successfully performed *n*-FABS against FAAH resulting in the identification of inhibitors with novel chemical scaffolds.[12] This study was carried out using a purified recombinant truncated form of rat FAAH (lacking the first 32 residues of the N-terminal transmembrane $\alpha$-helix) fused to MBP as proposed by Labar et al.[26] Herein, we demonstrate the feasibility of performing an efficient functional screening by *n*-FABS method using cell membrane preparations of human FAAH (hFAAH)-overexpressing HEK293 cells, and the subsequent $IC_{50}$ determination of the identified hits.

First, we checked whether the hFAAH-enriched membrane preparation (for details, see the Materials and Methods Section in the Supporting Information) was able to cleave the fluorinated substrate ARN1203 (Figure 1a) and whether the known

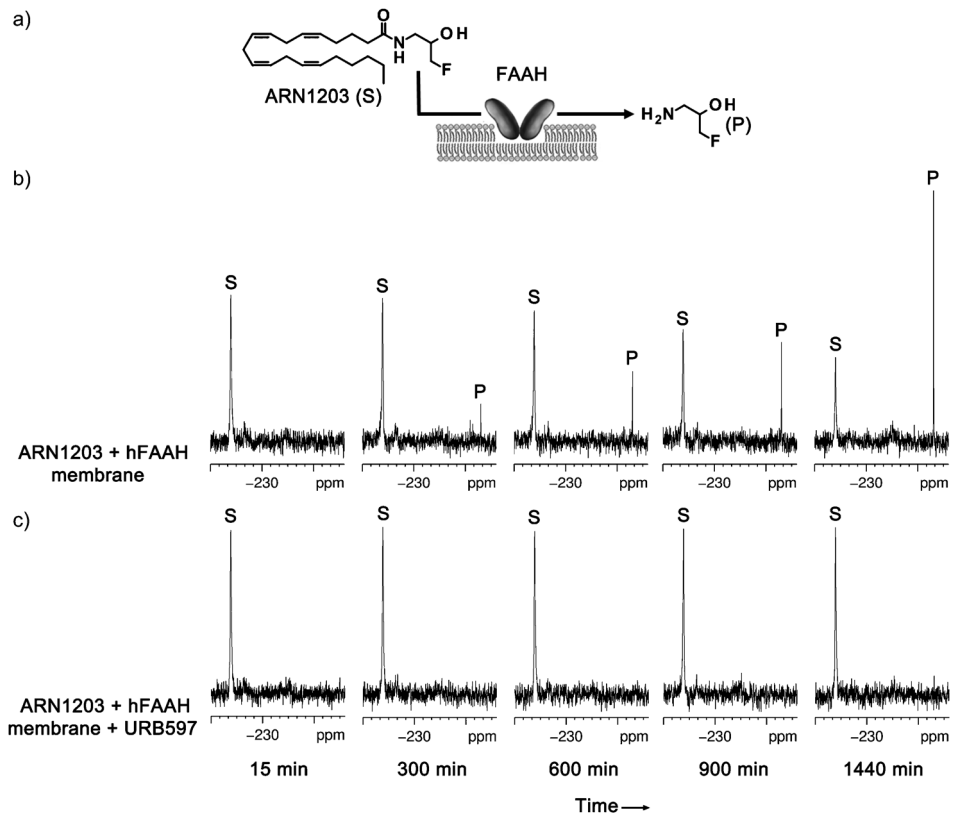



**Figure 1.** a) Enzymatic degradation of the substrate (ARN1203) by hFAAH membrane preparation (of the two products of the enzymatic reaction, only the fluorinated product is shown). b) $^{19}$F NMR spectra of ARN1203 (60 μM) in the presence of total protein extract (0.008 mg mL$^{-1}$) from FAAH-enriched HEK293 membrane preparation. c) The same sample as shown in panel b) but in the presence of URB597 (40 μM). S and P indicate the $^{19}$F NMR signals of substrate and product, respectively. The $^{19}$F NMR spectra were recorded at different time intervals over 24 h, as indicated below the spectra.

FAAH inhibitor URB597[23] completely quenched the reaction. The enzymatic activity of the hFAAH preparation on ARN1203 (60 μM) was monitored over 24 h by recording the $^{19}$F NMR spectra with $^{1}$H decoupling. The same sample was also analyzed in the presence of URB597 (40 μM). Changes in the $^{19}$F NMR signals of the substrate (S) and product (P) over time are shown in Figure 1b. The $^{19}$F NMR signal of the substrate (S) decreases in intensity, whereas the $^{19}$F NMR signal of the fluorinated product (P) increases in intensity with time. In the presence of URB597, no product signal is observable even after 24 hours, indicating that URB597 completely inhibits the enzyme (Figure 1c).

In order to rule out the cleavage of the substrate by other endogenous enzymes, we compared the activity of the preparation using cell extracts from untransfected (control) and transfected HEK293 cells. The enzymatic reactions were quenched with URB597 (40 μM) after 2, 4, 6 and 24 hours, and $^{19}$F NMR spectra were recorded for the eight samples (see Figure S2 in the Supporting Information). No modification of the substrate occurred in the presence of the control (Figure 2a), while the reaction in the presence of hFAAH membrane preparations went almost to completion after 24 h (Figure 2b). The 1D $^{1}$H NMR spectra of this last preparation after 2 and 24 hours of incubation were also recorded (Figure 2c). Although the $^{19}$F NMR spectra were different for the two samples at different time points (Figure 2b), the corresponding $^{1}$H NMR spectra were indistinguishable. The presence of large signals from detergent and many signals from endogenous components present in the membrane preparations hinders

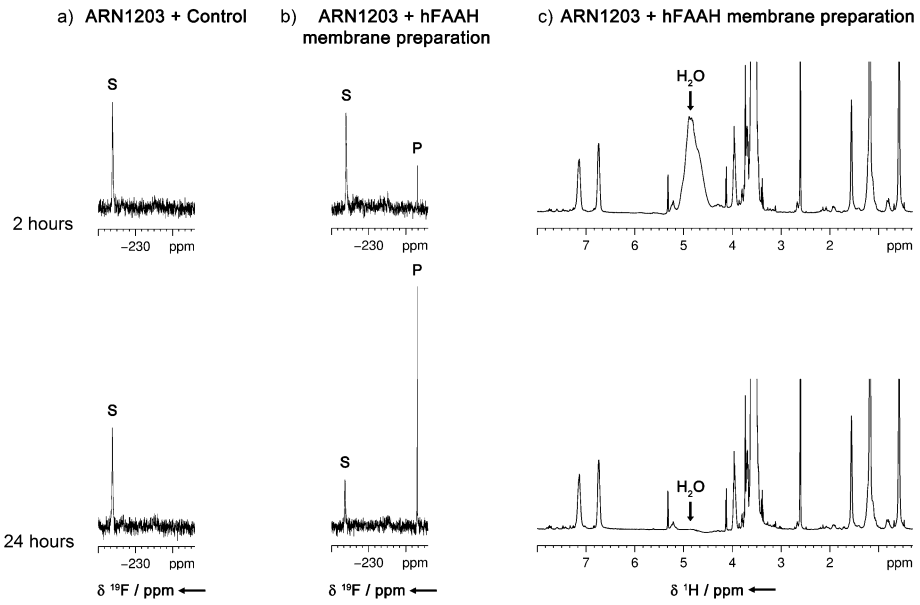
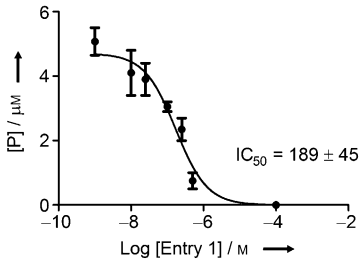



**Figure 2.** $^{19}$F NMR spectra of ARN1203 (60 μM) in the presence of total protein extracts (0.016 mg mL$^{-1}$) from a) wild-type HEK293 membrane preparation and b) FAAH-enriched HEK293 membrane preparation after 2 h (top) and 24 h (bottom) of reaction. The respective $^{1}$H NMR spectra recorded for the sample in panel b) after 2 h (top) and 24 h (bottom) of reaction are shown in panel c). S and P indicate the $^{19}$F NMR signals of substrate and product, respectively.

the observation of the substrate and product signals thus preventing their quantification. This evidence underlines the enormous advantage of $^{19}F$ NMR spectroscopy compared with $^{1}H$ NMR spectroscopy when working with cellular extracts.

*n*-FABS was then applied to hFAAH membranes preparation for inhibitor screening and $IC_{50}$ measurements. Two known FAAH inhibitors (**1** and **2**, Figure 3b)[27] and two fragments previously identified (**3** and **4**, Figure 3b)[12] were used for validation. The experiments were performed in an end-point format, and the integrals of the product signal in the presence and absence of the test molecules were measured and compared. This format of the assay, using the fluorinated substrate of Figure 1a, allows for the screening of approximately 150–200 samples per day. The compounds can be tested as single compounds or in mixtures for increased throughput. As expected, all four compounds were found to be active in our assay. Determination of the $IC_{50}$ values was then performed at increasing inhibitor concentrations. The plot of the integrals of the $^{19}F$ NMR product signal as a function of the concentration of the test molecules, as shown for entry **1** in Figure 3a, allowed the calculation of the $IC_{50}$ values for the four molecules (Figure 3b).

a)




b)

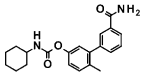
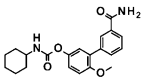
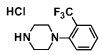
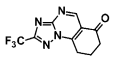
| Entry | Structure | *n*-FABS $IC_{50}$ [μM] | |
|---|---|---|---|
| | | MBP-rFAAH[a] | hFAAH membrane preparation[b] |
| **1** |  | 0.060 ± 0.010 | 0.189 ± 0.045 |
| **2** |  | 0.137 ± 0.031 | 0.292 ± 0.060 |
| **3** |  | 4.2 ± 1 | 29 ± 6 |
| **4** |  | 3 ± 1 | 31 ± 7 |

**Figure 3.** a) $IC_{50}$ determination using *n*-FABS for entry **1** in panel b) (below). The integral of the product $^{19}F$ signal (*y* axis) is plotted as a function of the compound concentration (*x* axis). b) Chemical structures and $IC_{50}$ values for four molecules determined using two different protein preparations. Footnotes: [a] values taken from Ref. [12]; [b] this work.

Remarkably, the $IC_{50}$ values obtained for entries **1** and **2** in Figure 3b (0.189 μM and 0.292 μM, respectively) showed a rank of potency similar to that obtained previously using samples of recombinant purified MBP-FAAH,[12] and were in line with those obtained by radiometric assays.[27] Moreover, our approach allowed us to measure accurately the $IC_{50}$ values of the two fragments **3** and **4**, despite their relatively weak potencies (29 μM and 31 μM, respectively). Figure 3b shows that the $IC_{50}$ values obtained with the membrane preparations were generally higher than those measured with the recombinant enzyme. The differences between the two protein samples with respect to the source (human versus rat), length (full versus truncated), and construct type (native versus fused with MBP), overall might explain the differences in terms of $IC_{50}$ values. In addition, it should be pointed out that in the FAAH-enriched membrane preparations, the presence of other proteins and metabolites, and thus possible off-target interactions, can result in a weaker affinity of the compound for the desired target. This effect is expected to be more pronounced for fragments that, due to their small size, can promiscuously bind to several proteins. Therefore, some of the very-low-affinity inhibitors could escape detection resulting in "false negatives".

In conclusion, our findings demonstrate that *n*-FABS using cell extracts is feasible. This approach is particularly useful for the discovery of inhibitors against enzymes that cannot be easily overexpressed or purified as recombinant proteins in a functional and pure form, or that require membrane components for their activity. Both potent inhibitors and weakly binding fragments could be identified, and their $IC_{50}$ values reliably measured. Of note, the use of membrane preparations allowed us to run the screening against hFAAH, which is known to have unfavorable biochemical properties and very low-expression yields in recombinant systems.[28] The successful implementation of the methodology in cell extracts opens novel perspectives for its application in the drug discovery process for human diseases. Finally, this work encourages us to explore the possibility to develop *n*-FABS directly on living cells.

## Experimental Section

Detailed descriptions of the h-FAAH-enriched HEK293 membrane preparation and of the NMR experiments are provided in the Supporting Information.

## *Acknowledgements*

*The authors thank Dr. Benedetto Grimaldi Italian Institute of Technology (IIT) for helpful scientific discussions.*

**Keywords:** drug discovery · fragments · functional screening · inhibitors · membrane proteins · 19F NMR spectroscopy

[1] J. Inglese, R. L. Johnson, A. Simeonov, M. H. Xia, W. Zheng, C. P. Austin, D. S. Auld, *Nat. Chem. Biol.* **2007**, *3*, 466–479.
[2] C. Dalvit, E. Ardini, M. Flocco, G. P. Fogliatto, N. Mongelli, M. Veronesi, *J. Am. Chem. Soc.* **2003**, *125*, 14620–14625.
[3] C. Dalvit, E. Ardini, G. P. Fogliatto, N. Mongelli, M. Veronesi, *Drug Discovery Today* **2004**, *9*, 595–602.
[4] C. Dalvit, *Prog. Nucl. Magn. Reson. Spectrosc.* **2007**, *51*, 243–271.
[5] R. Fattorusso, D. W. Jung, K. J. Crowell, M. Forino, M. Pellecchia, *J. Med. Chem.* **2005**, *48*, 1649–1656.

[6] M. Forino, S. Johnson, T. Y. Wong, D. V. Rozanov, A. Y. Savinov, W. Li, R. Fattorusso, B. Becattini, A. J. Orry, D. W. Jung, R. A. Abagyan, J. W. Smith, K. Alibek, R. C. Liddington, A. Y. Strongin, M. Pellecchia, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 9499 – 9504.
[7] C. Dalvit, G. Papeo, N. Mongelli, P. Giordano, B. Saccardo, A. Costa, M. Veronesi, S. Y. Ko, *Drug Dev. Res.* **2005**, *64*, 105 – 113.
[8] S. Frutos, T. Tarrago, E. Giralt, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2677 – 2681.
[9] N. Kichik, T. Tarrago, E. Giralt, *ChemBioChem* **2010**, *11*, 1115 – 1119.
[10] T. Tarragó, S. Frutos, R. A. Rodriguez-Mias, E. Giralt, *ChemBioChem* **2006**, *7*, 827 – 833.
[11] B. J. Stockman, *J. Am. Chem. Soc.* **2008**, *130*, 5870 – 5871.
[12] C. Lambruschini, M. Veronesi, E. Romeo, G. Garau, T. Bandiera, D. Piomelli, R. Scarpelli, C. Dalvit, *ChemBioChem* **2013**, *14*, 1611 – 1619.
[13] J. A. Christopher, J. Brown, A. S. Dore, J. C. Errey, M. Koglin, F. H. Marshall, D. G. Myszka, R. L. Rich, C. G. Tate, B. Tehan, T. Warne, M. Congreve, *J. Med. Chem.* **2013**, *56*, 3446 – 3455.
[14] F. Magnani, Y. Shibata, M. J. Serrano-Vega, C. G. Tate, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 10744 – 10749.
[15] M. J. Serrano-Vega, F. Magnani, Y. Shibata, C. G. Tate, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 877 – 882.
[16] Y. Shibata, J. F. White, M. J. Serrano-Vega, F. Magnani, A. L. Aloia, R. Grisshammer, C. G. Tate, *J. Mol. Biol.* **2009**, *390*, 262 – 277.
[17] G. Lebon, K. Bennett, A. Jazayeri, C. G. Tate, *J. Mol. Biol.* **2011**, *409*, 298 – 310.
[18] N. Robertson, A. Jazayeri, J. Errey, A. Baig, E. Hurrell, A. Zhu-kov, C. J. Langmead, M. Weir, F. H. Marshall, *Neuropharmacology* **2011**, *60*, 36 – 44.
[19] B. F. Cravatt, D. K. Giang, S. P. Mayfield, D. L. Boger, R. A. Lerner, N. B. Gilula, *Nature* **1996**, *384*, 83 – 87.
[20] A. Calignano, G. La Rana, A. Giuffrida, D. Piomelli, *Nature* **1998**, *394*, 277 – 281.
[21] B. F. Cravatt, K. Demarest, M. P. Patricelli, M. H. Bracey, D. K. Giang, B. R. Martin, A. H. Lichtman, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 9371 – 9376.
[22] C. J. Fowler, M. Borjesson, G. Tiger, *Br. J. Pharmacol.* **2000**, *131*, 498 – 504.
[23] S. Kathuria, S. Gaetani, D. Fegley, F. Valino, A. Duranti, A. Tontini, M. Mor, G. Tarzia, G. La Rana, A. Calignano, A. Giustino, M. Tattoli, M. Palmery, V. Cuomo, D. Piomelli, *Nat. Med.* **2003**, *9*, 76 – 81.
[24] K. Otrubova, C. Ezzili, D. L. Boger, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 4674 – 4685.
[25] L. Bertolacci, E. Romeo, M. Veronesi, P. Magotti, C. Albani, M. Dionisi, C. Lambruschini, R. Scarpelli, A. Cavalli, M. De Vivo, D. Piomelli, G. Garau, *J. Am. Chem. Soc.* **2013**, *135*, 22 – 25.
[26] G. Labar, F. V. Vliet, J. Wouters, D. M. Lambert, *Amino Acids* **2008**, *34*, 127 – 133.
[27] J. R. Clapper, G. Moreno-Sanz, R. Russo, A. Guijarro, F. Vacondio, A. Duranti, A. Tontini, S. Sanchini, N. R. Sciolino, J. M. Spradley, A. G. Hohmann, A. Calignano, M. Mor, G. Tarzia, D. Piomelli, *Nat. Neurosci.* **2010**, *13*, 1265 – 1270.
[28] M. Mileni, D. S. Johnson, Z. G. Wang, D. S. Everdeen, M. Liimatta, B. Pabst, K. Bhattacharya, R. A. Nugent, S. Kamtekar, B. F. Cravatt, K. Ahn, R. C. Stevens, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 12820 – 12824.

Received: November 1, 2013
Published online on December 11, 2013