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Fluorine nuclear magnetic resonance-based assay in living mammalian cells

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ABSTRACT

Nuclear magnetic resonance (NMR)-based screening has been recognized as a powerful approach for the identification and characterization of molecules interacting with pharmaceutical targets. Indeed, several NMR methods have been developed and successfully applied to many drug discovery projects. Whereas most of these approaches have targeted isolated biomolecular receptors, very few cases are reported with the screening performed in intact cells and cell extracts. Here we report the first successful application of the fluorine NMR-based assay n-FABS (n-fluorine atoms for biochemical screening) in living mammalian cells expressing the membrane protein fatty acid amide hydrolase (FAAH). This method allows the identification of both weak and potent inhibitors and the measurement of their potency in a physiological environment.

During recent years, there has been increasing interest in the development of methods that allow studying ligand–protein interactions directly in living cells [1–6]. Nuclear magnetic resonance (NMR)-based approaches, which are widely used for target-based screening on purified proteins [7], show promise in this regard, but their application to compound screening in intact cells is still very limited [8,9]. Recently, ^{1}H NMR spectroscopy was used to screen small molecules in intact bacterial cells [10], showing that it is possible to use this technology in living cells for the identification of inhibitors against a specific target and for the evaluation of their potency. Other NMR approaches for screening are based on the use of ^{19}F NMR spectroscopy [11,12]. One of these is the functional assay

n-FABS (n-fluorine atoms for biochemical screening) [13,14]. This methodology has important advantages such as rapid setup, versatility, and lack of interfering signals [12,15–17]. Its robustness and sensitivity have prompted its application in several drug discovery projects in both academia and industry [18]. n-FABS has been successfully used for the identification of inhibitors of different enzymes [19–25] and for the screening of natural extracts originated from traditional Chinese medicine [26]. The versatility of the methodology allows its application to complex biological systems such as one enzyme with multiple substrates, one substrate with multiple enzymes, and multiple substrates with multiple enzymes. These systems are those found in enzymatic pathways and, more generally, in system biology [14,21,27].

Recently, we successfully applied n-FABS to identify inhibitors of the membrane-bound serine amidase, fatty acid amide hydrolase (FAAH), as a purified protein [28,29] or in enriched membrane extracts [30]. FAAH is responsible for the catabolism of a class of endogenous bioactive lipids (anandamide and other fatty acid amides) [31] and represents a potentially important pharmaceutical target for the treatment of inflammation, pain, and central nervous system disorders [32–39].

In the current work, we report the first application of *n*-FABS as a valuable screening method to identify inhibitors of human FAAH (hFAAH) in intact mammalian living cells. First, we show that it is possible to determine target inhibition in intact cells using ^{19}F NMR spectroscopy. Second, we report that coupling ^{1}H NMR to ^{19}F NMR spectroscopy allows obtaining a preliminary metabolic fingerprint of the cell system, which could reveal metabolic alterations and/or toxic effects of tested compounds.

Materials and methods

hFAAH overexpressing HEK293 cells

hFAAH-overexpressing HEK293 (hFAAH–HEK293) cells were obtained as described previously [30]. The cells were cultured at 37 °C in a humidified incubator (5% CO$_2$) using Dulbecco's modified Eagle's medium (DMEM; EuroClone, code ECB7501L) containing 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin–streptomycin (complete DMEM).

n-FABS in cell assay

hFAAH–HEK293 cells (1×10^5) were plated in 1 ml of 60% complete DMEM and 40% FreeStyle 293 medium (Life Technologies, code 12338-018) with 20 U/ml heparin and 1% penicillin–streptomycin using 24-well plates. The plates were kept at 37 °C in a humidified incubator (5% CO$_2$) for approximately 48 h (grown until 80% confluence). The enzymatic reaction was started by changing the medium to FreeStyle 293 (600 μl) containing 60 μM of a fluorinated anandamide analog ARN1203 [29] and DMSO-d_6 up to the final concentration of 0.5%. Cells were returned into the incubator, and the enzymatic activities were tested in an end-point format of *n*-FABS by quenching the reactions at different times with the addition of 40 μM of the FAAH inhibitor URB597 [40]. The supernatants were transferred to vials and frozen at −80 °C. For NMR experiments, 460 μl of supernatant of each sample was transferred to NMR tubes and added with 40 μl of a 0.63% Triton X-100 solution in D$_2$O (final concentrations of D$_2$O and Triton X-100 were 8 and 0.05%, respectively) for the lock signal and for increasing substrate solubility.

Control experiments

The HEK293 cells are adherent cells when they grow in plate. All of the control experiments were performed in 24-well plates at 37 °C. Cells (8×10^4) were plated and grown as described above. Experiments in parallel were performed in the presence of HEK293 intact cells and in the presence of hFAAH–HEK293 intact cells in order to evaluate whether the substrate was metabolized only by FAAH. The reactions were quenched at different time points with URB597. Cells and supernatant together were frozen for one night at −80 °C. For the NMR experiments, 460 μl of each sample was transferred to the NMR tubes and 40 μl of a 1.275% Triton X-100 solution in D$_2$O (final concentrations of D$_2$O and Triton X-100 were 8 and 0.102%, respectively) was added for the lock signal and for increasing substrate solubility. For evaluating whether the substrate and fluorinated product could freely move across the membranes, the following control experiments were performed. In the first plate, we quenched the reaction and incubated the samples for 15 min. Next, pipetting of both cells and supernatant was carried out several times in order to carefully remove all of the cells from the wall. The samples were then frozen at −80 °C for one night. In the second plate, the enzymatic reactions were quenched at the same time as for the first plate. However, in this case we took only the supernatant, paying attention not to touch and aspirate the

cells. The samples were then frozen at −80 °C for one night. To the remaining cells, we added 200 μl of lysis buffer containing 0.5% Triton X-100. These samples were incubated for 15 min at 4 °C and then frozen at −80 °C for one night. For the first two sets of NMR samples, 460 μl of the cells plus supernatant solutions and of the supernatant solutions were transferred into the NMR tubes, and 40 μl of a 1.275% Triton X-100 solution in D$_2$O (final concentrations of D$_2$O and Triton X-100 were 8 and 0.102%, respectively) was added for the lock signal and for increasing substrate solubility. For the third set of NMR samples, 160 μl of the lysed cell solutions was transferred into the NMR tubes and 40 μl of D$_2$O was added for the lock signal. A total of 300 μl of phosphate-buffered saline (PBS, pH 7.4) was also added to reach the volume of 500 μl. The Triton X-100 concentration in the final volume was 0.102%.

n-FABS assay validation and IC$_{50}$ calculation

Cells were plated and grown as described above. Reactions were started by removing the medium and adding 600 μl of FreeStyle 293 medium containing 60 μM ARN1203 and different concentrations of the test compounds with a final DMSO-d_6 concentration of 0.5%. In each run, there were two samples without inhibitors (controls); these samples correspond to 0% of inhibition. The reactions were quenched after 2.5 h by adding 40 μM URB597. Supernatants were transferred to vials and frozen at −80 °C. The experiments were run in triplicate on different days. The samples were analyzed, as described before, by recording ^{19}F and ^{1}H NMR spectra. The integrals of the product ^{19}F signal were measured and plotted as a function of the inhibitor concentration in order to obtain the half-maximal inhibitory concentration (IC$_{50}$) value. The best data fit was performed using GraphPad Prism 5.

NMR experiments

All NMR experiments were recorded at 25 °C 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 the automatic sample changer SampleJet NMR system. The ^{19}F NMR spectra were recorded with 512 scans, a repetition time of 3 s, and proton decoupling during the acquisition period. The data were multiplied with an exponential window function with 5 Hz line broadening prior to Fourier transformation. The ^{19}F chemical shifts are referenced to CFCl$_3$. The ^{1}H NMR experiments were recorded with the one-dimensional (1D) version of the NOESY (nuclear Overhauser effect spectroscopy) pulse sequence and with H$_2$O signal presaturation. A total of 64 scans were recorded for each spectrum. The data were multiplied with an exponential window function with 0.3 or 0.1 Hz line broadening prior to Fourier transformation. The ^{1}H NMR chemical shifts are referenced to the internal signal of 3-(trimethylsilyl) propanoic acid (TSP).

Results and discussion

n-FABS in cell setup

To set up the *n*-FABS method in living cells, we generated a human FAAH-overexpressing HEK293 cell line (hFAAH–HEK293). HEK293 cells are widely used in cell biology and biotechnology for protein expression and gene therapy [41,42] because they grow easily in both plates and flasks and are simple to transfect. Therefore, they represent an ideal model for performing functional screening in living cells. To monitor the enzymatic activity of FAAH in this cell system, we used the fluorinated anandamide analog ARN1203 (S) [29], which we have reported to act as FAAH substrate,

being hydrolyzed to arachidonic acid and 1-amino-3-fluoro-propan-2-ol (P) (Scheme 1).

This is a challenging system for the application of *n*-FABS because the target protein is a membrane-bound enzyme and the substrate is sparsely soluble in water.

Initially, we tested whether ARN1203 could be cleaved by hFAAH–HEK293 cells. This is illustrated in Fig. 1, which shows the ^{19}F NMR spectra recorded as a function of incubation time.

^{19}F NMR signals labeled S and P correspond to the signal of the substrate, ARN1203, and the fluorinated product of the enzymatic reaction, 1-amino-3-fluoropropan-2-ol, respectively. As the incubation time increases, the area of the substrate signal decreases and the area of the product signal increases. After 24 h, the substrate was completely transformed into the product. The signal of the substrate is broader compared with the signal of the product due to its larger size and likely self-aggregation effects.

In addition to the ^{19}F spectrum, for each sample we also recorded the ^{1}H NMR spectrum. Fig. 2 shows the ^{19}F and ^{1}H NMR spectra along with the images of hFAAH–HEK293 intact cells recorded at two different incubation times.

It is worth noting that it was not possible to follow the enzymatic reaction by ^{1}H NMR experiments due to the extensive signal overlap and the low concentration of both the substrate and products compared with those of the components present in the medium. However, some differences in the metabolic fingerprint signals were visible at different incubation times, as indicated by the arrows in Fig. 2. For instance, after 24 h of incubation, the ^{1}H NMR signals of glucose decreased in intensity, whereas the ^{1}H NMR signals of lactate increased. This observation is particularly useful both for monitoring the viability of the cells during the experiments and for identifying possible cytotoxic effects of tested compounds.

Control experiments

To demonstrate that our substrate was metabolized only by FAAH, we ran in parallel the same experiments using the HEK293 overexpressing hFAAH and the HEK293 cells. The reactions were quenched at different time points. The ^{19}F NMR spectra after 1 and 2 h of incubation for the four samples containing the cells and the supernatant are reported in Fig. 3. The ^{19}F product signal is visible for the samples with the HEK293 cells overexpressing hFAAH, whereas no ^{19}F product signal is visible in the sample with the HEK293 cells even after 2 h of incubation. These experiments demonstrate that hFAAH is the only enzyme responsible, in these conditions, for the ARN1023's metabolism. Fig. 3 also shows the
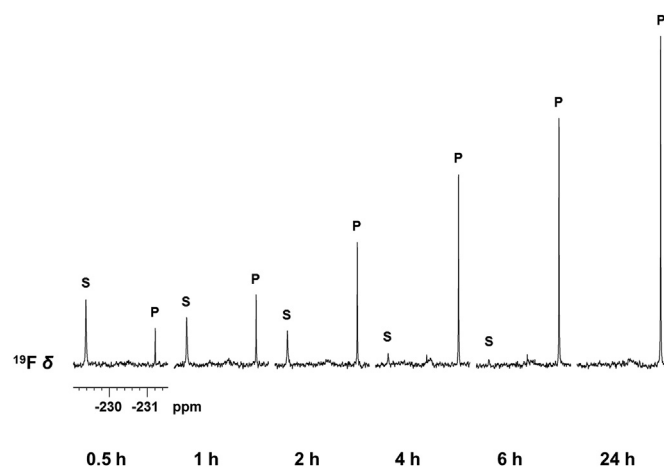

Fig.1. ^{19}F NMR spectra at different incubation times of 60 μM ARN1203 at 37 °C in the presence of hFAAH–HEK293 intact cells. ^{19}F NMR signals of both substrate (S) and product (P) of the enzymatic reaction are shown. The substrate is completely cleaved after 24 h.

images of the HEK293 and hFAAH–HEK293 intact cells recorded at the two different incubation times. No significant differences in the two cell types are visible up to 2 h of incubation.

An important issue to be considered for the assay setup was whether the product would be released into the supernatant or would remain inside the cells.

To provide the answer to this relevant question, we performed the control experiments as reported above in Materials and Methods ("Control experiments" section). The ^{19}F NMR spectra of the three different samples (i.e., cells + supernatant, lysed cells, and supernatant after 4 h of incubation) are reported in Fig. 4.

It is worth noting that the substrate (S) and fluorinated product (P) are visible in both samples containing the cells plus supernatant and only the supernatant, whereas there is no fluorinated product visible in the sample containing only the lysed cells. Our results indicate, first, that the ARN1203 substrate is able to cross the membrane and act as FAAH substrate and, second, that the substrate and fluorinated product can freely cross the membrane into and back out of the cell. It is also worth noting that some substrate is present in the cell lysed sample, indicating that probably it is partially contained in the cells and/or attached to the membranes, whereas the fluorinated product is completely released by the cells. In our screening effort, we use the ^{19}F NMR signal of the fluorinated product to obtain the percentage of inhibition and then calculate
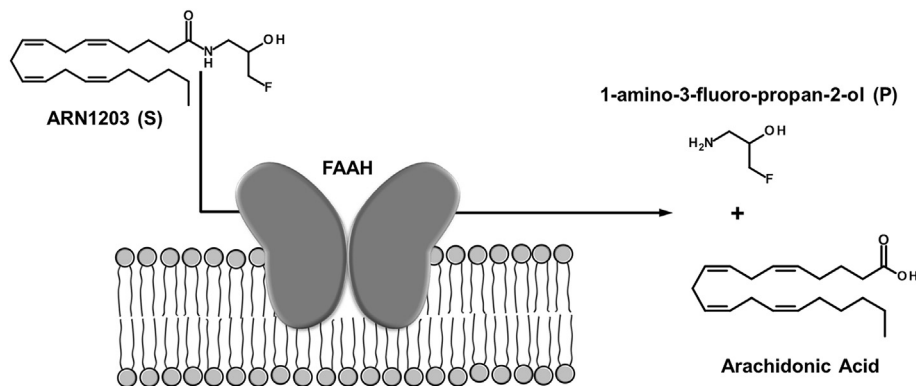
Scheme 1. In-cell enzymatic hydrolysis of the fluorinated anandamide analog ARN1203 catalyzed by hFAAH. The enzyme is bound to the internal membrane of the cell. Both products of the reaction are shown, but only the fluorinated substrate (S) and the 1-amino-3-fluoropropan-2-ol (product, P) are detected in the ^{19}F NMR spectra.
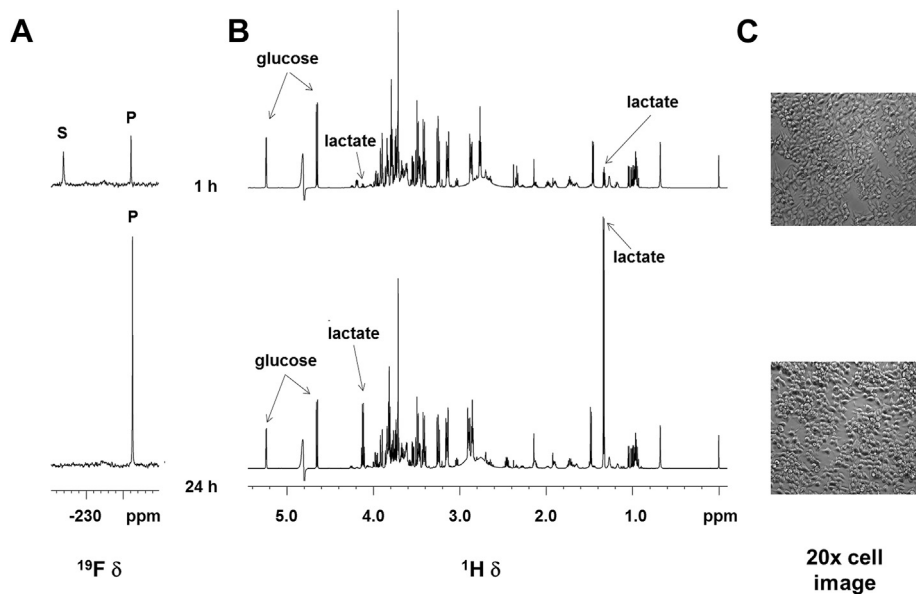
Fig.2. ^{19}F NMR (A) and ^{1}H NMR (B) spectra of 60 μM ARN1203 after 1 h (top) and 24 h (bottom) of incubation with hFAAH–HEK293 intact cells at 37 °C. S and P indicate the ^{19}F NMR signals of substrate and product, respectively. (C) Representative light microscopy images of the hFAAH–HEK293 cells in the presence of 60 μM ARN1203 taken immediately before quenching the reaction with 40 μM URB597 at 1 and 24 h. Pictures were obtained at 20× magnification using a Leica 6000 microscope with phase contrast. No significant differences were observed in cells after 24 h.

the IC$_{50}$ values. Therefore, for our screening purpose, it was sufficient to use the supernatant. If some of the product would have remained in the cells, it would have been necessary to simply perform the screening by using the cells plus supernatant, as shown in Fig. 4A.

n-FABS assay validation and IC$_{50}$ calculation

To validate the performance of *n*-FABS, for screening in living mammalian cells we tested previously identified molecules and fragment inhibitors of FAAH of widely different potencies [29].
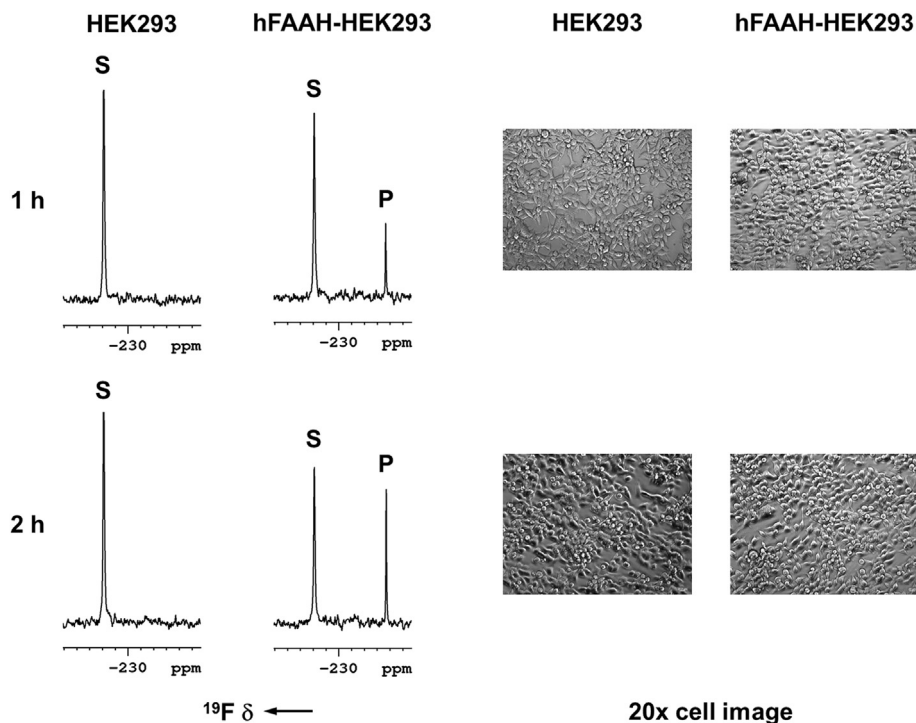

Fig.3. ^{19}F NMR spectra of 60 μM ARN1203 after 1 h (top) and 2 h (bottom) of incubation time with HEK293 intact cells and hFAAH–HEK293 intact cells at 37 °C. S and P indicate the ^{19}F NMR signals of substrate and fluorinated product, respectively. The NMR samples contain the cells and the supernatant. No product signal is visible in the presence of HEK293 cells. The corresponding light microscopy images of the intact cells in the presence of 60 μM ARN1203, taken immediately before quenching the reaction with 40 μM URB597 at 1 and 2 h, are displayed. Images were obtained at 20× magnification using a Leica 6000 microscope with phase contrast. No significant differences were observed between the different cell types at different incubation times.
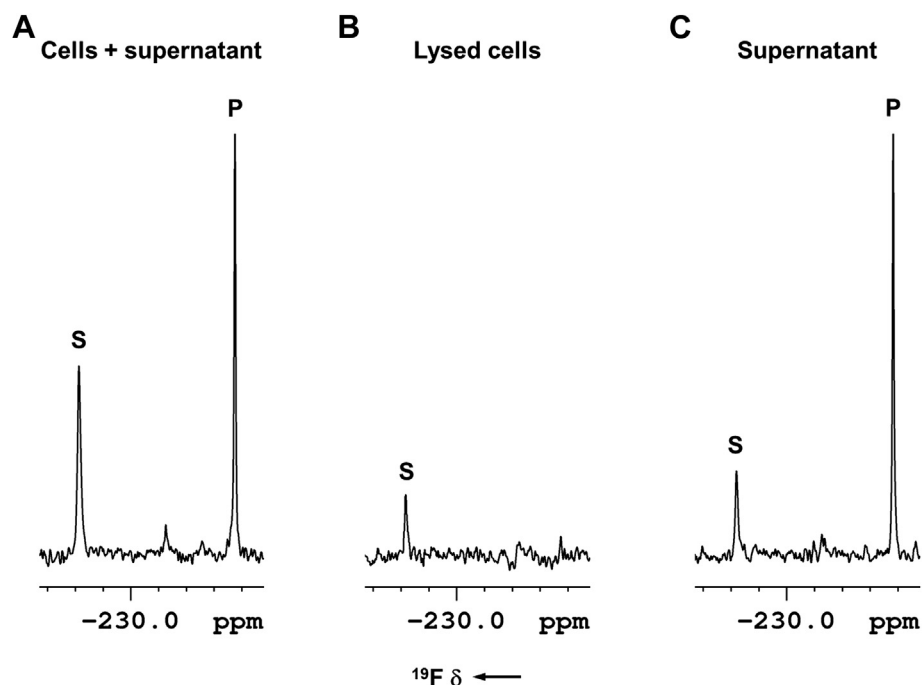
Fig.4. ^{19}F NMR spectra after 4 h of incubation of 60 μM ARN1203 at 37 °C in the presence of 8 × 10^4 hFAAH−HEK293 intact cells. The three panels indicate the samples containing both cells and supernatant (A), the lysed cells (B), and only the supernatant (C). ^{19}F NMR signals of both substrate (S) and product (P) of the enzymatic reaction are shown. No product ^{19}F NMR signal is detectable in the lysed cells (see Materials and methods for further details).
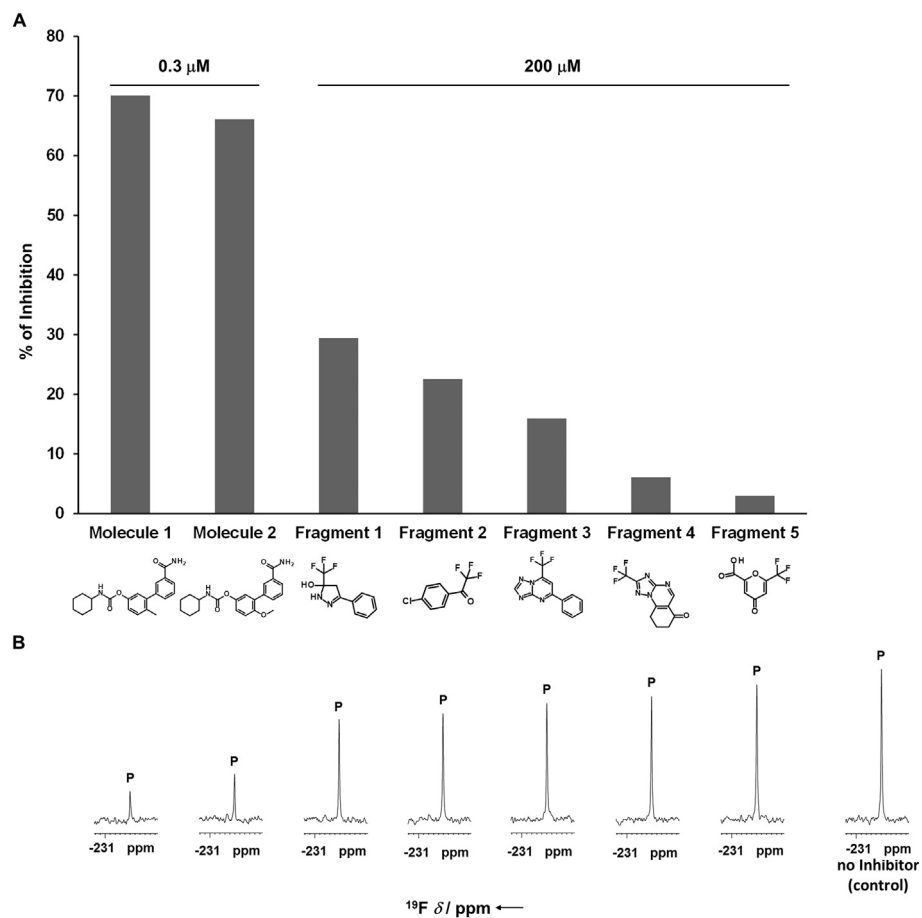

Fig.5. (A) Percentage of hFAAH inhibition in hFAAH−HEK293 intact cells in the presence of different compounds as measured by ^{19}F NMR (spectra shown below). The tested concentrations of the inhibitors are shown above the graph, and the corresponding names and molecule chemical structures are shown below it. (B) ^{19}F NMR spectra of ARN1203 incubated for 2.5 h in hFAAH−HEK293 intact cells in the absence (control) and presence of the corresponding inhibitors. Only the ^{19}F NMR signal of the product (P) is shown.

The hFAAH–HEK293 cellular screening was conducted in an end-point format in 24-well plates. We used 60 μM substrate ARN1203, whereas the potent and weak inhibitors were tested at 0.3 and 200 μM, respectively. The reactions were quenched after 2.5 h by adding 40 μM of the potent FAAH inhibitor URB597 [40] (see Materials and Methods). Two additional samples of hFAAH–HEK293 intact cells were incubated with the same amount of substrate and DMSO-d_6, but no test compounds, and were used as controls corresponding to 0% inhibition of the hFAAH activity in cells.

Fig. 5 clearly shows that the *n*-FABS method was able to detect the strong inhibition of FAAH activity in cells by molecule 1 and molecule 2, two known potent FAAH inhibitors [43]. The percentage of inhibition *F* is calculated using the equation

$$F = 100*\left(1 - \frac{[P_+]}{[P_-]}\right),$$

where $[P_+]$ and $[P_-]$ are the integrals of the ^{19}F signal of the fluorinated product in the presence and absence of the inhibitor, respectively. Experiments performed at different inhibitor concentrations, using the same experimental conditions applied for the screening run, allowed the measurement of their IC$_{50}$ values in intact living cells, as shown in Fig. 6A. The compounds were tested in independent triplicates on different days, and the reactions were quenched by adding 40 μM URB597 after 2.5 h of incubation (see Materials and Methods).

The IC$_{50}$ values of molecule 1 and molecule 2 measured in cells are consistent with those obtained with *n*-FABS in cell membrane extracts [30]. The selected fragments were tested at 200 μM and displayed different levels of inhibition (see Fig. 5A). The inhibition was small but meaningful. This can be appreciated in Fig. 6B for fragment 1 and fragment 2, where a concentration-dependent response was observed at three fragment concentrations. Although the inhibitory effects were small, it was encouraging to observe the sensitivity of our NMR approach for detecting the effect elicited by fragments in intact living cells.

n-FABS and metabolic fingerprint

Exogenous chemical compounds can alter the metabolism of cells or have cytotoxic effects, especially when they are tested at high concentrations. This is expected to result in differences in the
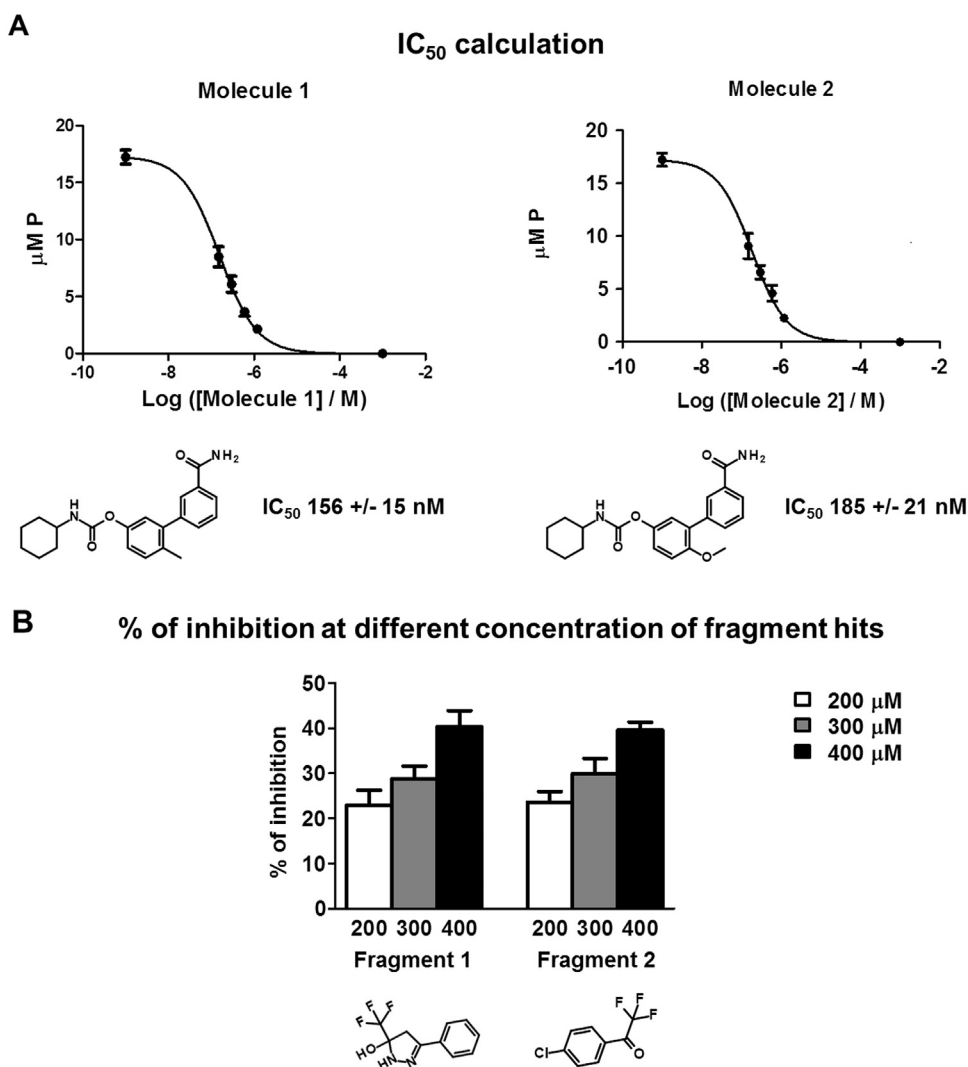

Fig.6. (A) IC$_{50}$ determination of molecule 1 and molecule 2 using *n*-FABS. The integral of the product ^{19}F signal (*y* axis) is plotted as a function of the compound concentration (*x* axis). The IC$_{50}$ values obtained from the best fit of the data are reported close to the chemical structure. (B) Percentage of inhibition at three different concentrations of fragment 1 and fragment 2. The concentrations of the hits are shown under the graphics.
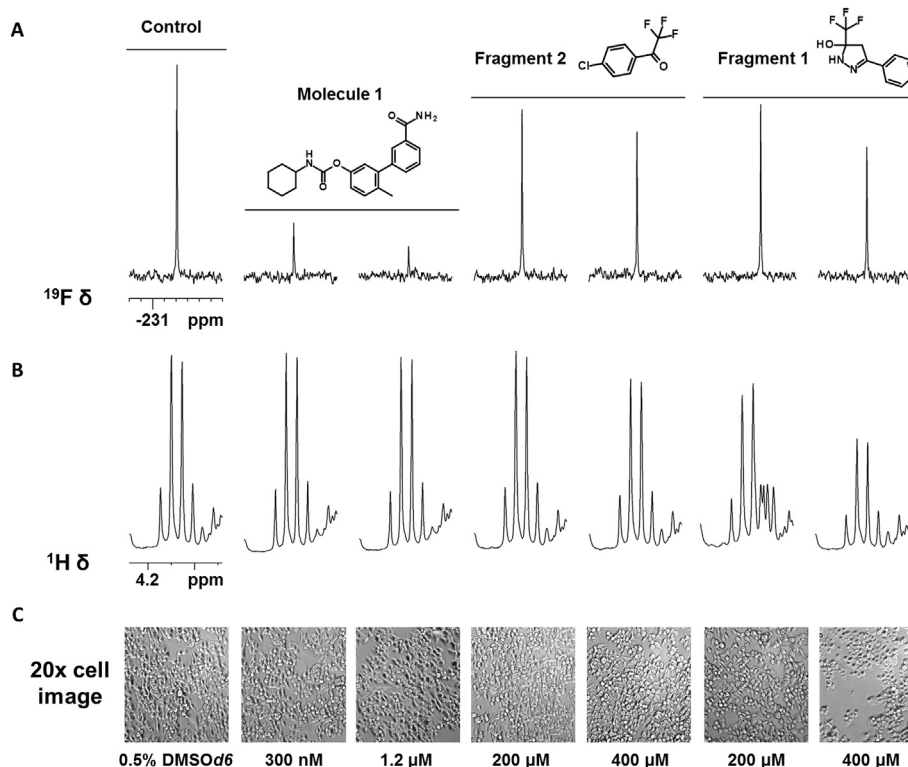
Fig.7. (A) ^{19}F NMR spectra of 60 μM ARN1203 after 2.5 h of incubation in hFAAH–HEK293 cells in the absence (control) and presence of inhibitors at the indicated concentrations. Only the product ^{19}F NMR signal is shown. (B) ^{1}H NMR signals of the $C_\alpha H$ resonance of lactate after treatment of hFAAH–HEK293 cells without and with inhibitors. (C) Light microscopy images of hFAAH–HEK293 cells taken just before quenching the reaction with 40 μM URB597. Images were obtained at 20× magnification using a Leica 6000 microscope with phase contrast. The name and structure of each compound are reported at the top of the figure, whereas the tested concentrations are reported at the bottom.

^{1}H NMR metabolic signals (for reviews, see Refs. [44–47]). Thus, by recording the ^{1}H spectra on the same samples used for *n*-FABS, it is possible to obtain a metabolic fingerprint of the system that reveals metabolic alterations and/or possible cytotoxic effects of the tested compounds. In our specific case, we have focused the attention only on the glucose uptake and lactate production because their measurement in the culture medium can be used as an indicator of the cell growth and/or of an altered cell metabolism. This is illustrated in Fig. 7. It should be pointed out that a more thorough analysis of the metabolism would require inspection and quantification of many metabolites, but this is outside the scope of this work.

The lactate signal intensity for molecule 1 and fragment 2 at different concentrations appeared almost unchanged, within the experimental error, in comparison with that of the control sample. The pictures of the cells for these samples indicated no changes in cell number or shape. On the contrary, fragment 1 led to a substantial reduction of the lactate signal at 400 μM, indicating that this compound, at high concentration, affects cell metabolism, resulting in reduced lactate production. The picture of the cells, taken immediately before the quenching of the enzymatic reaction, showed a reduced cell number, indicating a cytotoxic effect for fragment 1. These findings suggest that the method that combines the analysis of the ^{19}F and ^{1}H NMR spectra of cell systems in the presence of inhibitors for a specific protein target might be generally applied to measure potency while identifying potentially undesired cellular effects.

Conclusions

We have demonstrated that the ^{19}F NMR-based functional assay *n*-FABS can be successfully applied in drug discovery for the screening and identification of inhibitors for a specific enzymatic target and for the determination of their IC_{50} values in mammalian living cells. This method is widely applicable and should facilitate the identification and characterization of small-molecule inhibitors in a more physiological environment. With its easy setup, versatility, and lack of interfering signals, the method could play an important role in drug discovery.

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