Reliable high-throughput functional screening with 3-FABS

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An NMR method called 3-FABS has extended the capabilities of NMR, enabling rapid, efficient and reliable high-throughput functional screening for the identification of inhibitors and for measuring their 50% mean inhibition concentration (IC_{50}) with accuracy. The substrate is tagged with a CF_3 moiety and ^{19}F NMR spectroscopy is used for the detection of the substrate and product components. We provide comprehensive insight into 3-FABS, a discussion of its strength and weakness when compared with other HTS techniques and a presentation of some of its applications to the screening of different enzymes and to multiple screening.

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▼ High-throughput screening (HTS) is nowadays the most widely used approach in the pharmaceutical industry for identifying potential lead molecules [1–4]. Advances in HTS using 96-, 384- or even 1536-well plates enable the screening of many tens of thousands of samples in a short period of time [5–11]. The need for increasingly higher-throughput screening is dictated both by the increasing number of drug targets made available through genomics and the increasing number of chemical molecules generated through combinatorial chemistry [3]. Unfortunately, the huge amount of data generated with HTS since its first applications in the early 1990s has not translated into a significant increase of late-phase drug candidates [12]. Several important issues concerning the quality of the assays and the quality of the compound collections have emerged. Many functional assays used in HTS are rather complex and performed with multi-component systems. Some of the identified hits inhibit or interact with an assay component other than the target of interest. In addition, interference with the colorimetric or fluorimetric detection methods or compound aggregation can result in the generation of 'false positives'. Target-specific secondary assays are then used to eliminate some of the useless false positives that often plague an HTS effort. The issue of false positives has been discussed extensively in recent publications [13–17]. In some cases, false-positive rates as high as 95–99% were found for a variety of coupled enzymatic, cellular and fluorescence-based assays [14]. However, the issue concerning 'false negatives', that is, inhibitors that escape detection, has not received much attention. Sometimes HTS performed on a target results in the identification of a limited number of hits. Plausible explanations for the poor outcome are the intrinsic difficulties of the systems investigated (e.g. protein targets with large, solvent-exposed flat-binding surfaces). However, one likely source for the unsuccessful HTS results can be ascribed to the failure of the assay to detect potential inhibitors. Primary HTS can fail to identify inhibitors because of the complexity of the assay, the large experimental errors in the measurements, the presence of bovine serum albumin (BSA) and the near-impossibility of directly characterizing the concentration, purity, stability and aggregation state of the screened compounds. Therefore, the emphasis in HTS today has shifted toward the design of biochemical assays that are more robust and reliable, that is, a quality imperative has arisen. The value of focusing purely on the volume of data produced drops when the amount of knowledge gained from each data point increases [12]. As a result, the collection of high-quality data has become more important.

Over the past few years, NMR screening has emerged as a powerful and reliable

1359-6446/04/$ – see front matter PII: S1359-6446(04)03161-7

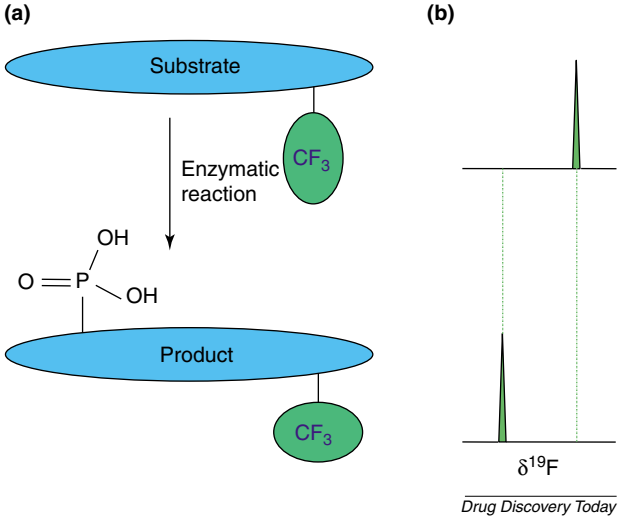

Figure 1. Schematic diagram of the three fluorine atoms for biochemical screening (3-FABS) method. The enzyme in the example is a kinase that phosphorylates the substrate. **(a)** The chemical modification of the substrate by the enzyme changes the electronic cloud of the CF_3 moiety, **(b)** resulting in distinct chemical shifts for the product and substrate ^{19}F signals.

methodology for the identification of lead molecules. The technique is now recognized for its impact on the drug discovery process and has become an established tool for hit identification and validation in many pharmaceutical companies. NMR-based screening methodologies identify molecules that bind to the target of interest by detecting changes in an NMR parameter either of the test molecules, reference molecule or protein resonances. As this methodology is becoming increasingly important, several review articles on its applications have appeared in the literature [17–33].

More recently, NMR has been extended to functional screening. The proposed technique identifies, in an efficient and reliable way, inhibitors of an enzymatic reaction and measures their inhibitory activity. The focus of this comprehensive article is to provide insight into this approach, discuss its strengths and weaknesses compared with other techniques used in HTS and show some of its applications to the screening of different enzymes.

NMR-based functional assay

NMR has been extensively used for characterizing the product or products of an enzymatic reaction and for gaining insight into the kinetics of the reaction (e.g. see references [34–39]). High substrate concentrations were necessary for these studies owing to the low sensitivity of the NMR technique. NMR-based biochemical screening that measures the inhibition or activation of an enzyme instead has found limited applications [40]. One of the main explanations for the lack of its use is evident from Equation 1.

$$K_I = \frac{IC_{50}}{1 + \frac{[S]}{K_M}} \qquad \text{[Eqn 1]}$$

The equation describes the dissociation binding constant K_I of the identified inhibitor as a function of the concentration [S] of the substrate, the Michaelis constant K_M and the concentration of the inhibitor at which a 50% inhibition of the enzymatic reaction is achieved, (IC_{50}) [41]. Equation 1 is valid only for a substrate-competitive inhibitor. The Michaelis constant is given by Equation 2.

$$K_M = \frac{K_{off} + K_{cat}}{K_{on}} \qquad \text{[Eqn 2]}$$

where K_{cat} is the catalytic rate constant and K_{off} and K_{on} are the off-rate and on-rate constants of the substrate binding to the enzyme, respectively. For rapid equilibrium conditions K_M corresponds to the dissociation binding constant K_D of the substrate.

Substrates typically have K_M (or K_D) in the low micromolar range. Consider an hypothetical screening situation where we assume a K_M of 2 μM and a concentration for the screened molecules of 10 μM. In addition, a substrate concentration of 400 μM is used to detect the NMR signals of the substrate and product (or products) in a reasonable acquisition time. We will consider as hits molecules with an $IC_{50} \leq 10$ μM. According to Equation 1, only strong inhibitors (with a $K_I \leq 50$ nM) will be detected. This is clearly not acceptable for primary screening, where the goal is to identify weak- and medium-affinity inhibitors derived from a diversity of chemical classes in addition to any high-affinity hits.

3-FABS

A way to overcome these limitations is to tag the substrate with CF_3 moieties and use ^{19}F NMR (with proton decoupling) as the method of detection. The principle of this approach, named three fluorine atoms for biochemical screening (3-FABS) [42], is described in Figure 1. The high receptivity of fluorine NMR spectroscopy, the 100% natural abundance of the isotope ^{19}F and the presence of three fluorine atoms, result in ^{19}F NMR signals of high intensity. In addition, the isotropic chemical shift of the fluorine resonances is highly sensitive to the chemical environment [43–45]. Modification of the substrate through the enzymatic reaction results in perturbations of the fluorine electronic cloud, even when the CF_3 moiety is distant from the reaction site. Therefore, distinct ^{19}F signals for the substrate and product(s) are observed.

CF_3- and CF-labelled substrates

Fluoro-containing moieties (CF or CF_3) can easily be inserted at different positions of a molecule by chemical synthesis, because of the low reactive nature of the fluorocarbon bond.

When the substrate is a peptide or peptidomimetic, several different strategies can be followed and many established synthetic reactions are available for the preparation of CF_3-labelled peptides [46–48]. The N-terminal can be trifluoroacetylated by reacting the free N-terminal with trifluoroacetic anhydride during the solid-phase synthesis and then detaching the peptide from the resin with trifluoroacetic acid. The CF_3 group can be inserted at the C-terminus via amidation of the carboxy group with a trifluoromethyl-containing amine [49,50]. S-trifluoromethyl-cysteine and homocysteine [51], trifluoronorvaline, trifluoronorleucine [52] and the α-trifluoromethyl-substituted α-amino acids [47] are just a few examples of available CF_3 modified amino acids that can be incorporated into peptides. Magnetically equivalent multiple CF_3 moieties {e.g. 3,5 bis (trifluoromethyl) phenylalanyl (unpublished data) or 4-perfluoro-tert-butyl-phenyliodoacetamide used originally to label cysteins in proteins [53]} can also be used for improving the sensitivity of the experiment. We will refer to these variants of the experiments as the 3(x2) and 3(x3) FABS.

K_M measurement

The measurement of K_M of the substrate and cosubstrate is important for two reasons: it enables the determination of the substrate and cosubstrate concentrations to be used in 3-FABS; and the binding constant of the identified inhibitor can be calculated according to Equation 1 in the presence of a competitive mechanism. The K_M of the substrate (or cosubstrate) is measured using Equation 3.

$$V = \frac{[S]}{K_M + [S]} V_{\max} \qquad \text{[Eqn 3]}$$

where V is the initial speed of the reaction and V_{max} is the maximum speed that would be observed if all the enzyme [E_{tot}] is complexed with the substrate ($V_{max} = K_{cat}$ x [E_{tot}]). V is obtained experimentally by measuring the integral of the ^{19}F signal of the product divided by the incubation time of the reaction. A plot of these values as a function of [S] enables the determination of K_M and V_{max}. The incubation time can be the same for all the different values of [S], but it often differs. In fact, for high [S] it is better to reduce the incubation time to keep the reaction in the linear region of the kinetics curve [42]. The concentration of the substrate used then in 3-FABS is typically 2–5 times higher than the K_M, which allows the possibility of also detecting weak- and medium-affinity inhibitors. For ranking the very strong inhibitors among themselves (IC_{50} <10 nM) it is sometimes necessary to use a higher substrate concentration, owing to the limit in IC_{50} values determined by the enzyme concentration (e.g. low nM).

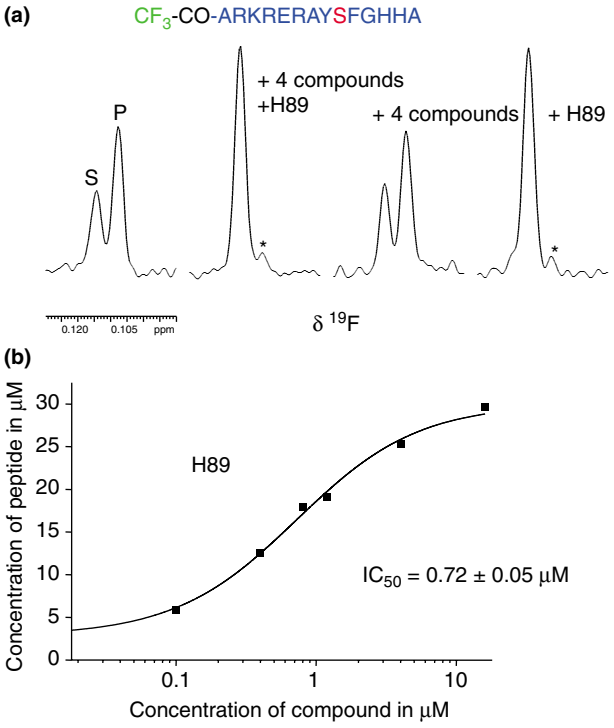

Figure 2. Screening and deconvolution **(a)** and IC_{50} measurement **(b)** for compound H89 [N-(2-[[(2E)-3-(4-bromophenyl)prop-2-enyl]amino]ethyl)] [59] performed with the three fluorine atoms for biochemical screening (3-FABS) method. The substrate is the N-terminal trifluoracetylated AKTide [60], the enzyme is the Ser/Thr kinase AKT1 [61]. The four compounds are: 2-amino-6 methylquinazolin-4 ol, ethyl 2-quinoxalinecarboxylate, 5-methylbenzimidazole and methyl isoquinoline-3-carboxylate. The activated enzyme AKT1, the peptide, ATP and compound concentrations were 25 nM, 30 µM, 131 µM (~2K_M) and 10 µM, respectively. S and P represent the CF_3 signal of the peptide and the phosphorylated peptide, respectively. The IC_{50} value was measured with Equation 5, but the same value was obtained with Equation 6.The asterisks indicate the tiny amount of phosphorylated peptide in the presence of H89. Reprinted with permission from Reference [42].

Screening

The screening of a large, proprietary compound collection with 3-FABS can be performed using either single molecules or compound mixtures. An end point format is used where the enzymatic reaction is quenched after a defined delay. This delay is established from the enzyme and substrate concentration and the speed of the enzymatic reaction. The quenching is achieved by adding either a denaturant, a chelating agent or a strong inhibitor. In every screening run,

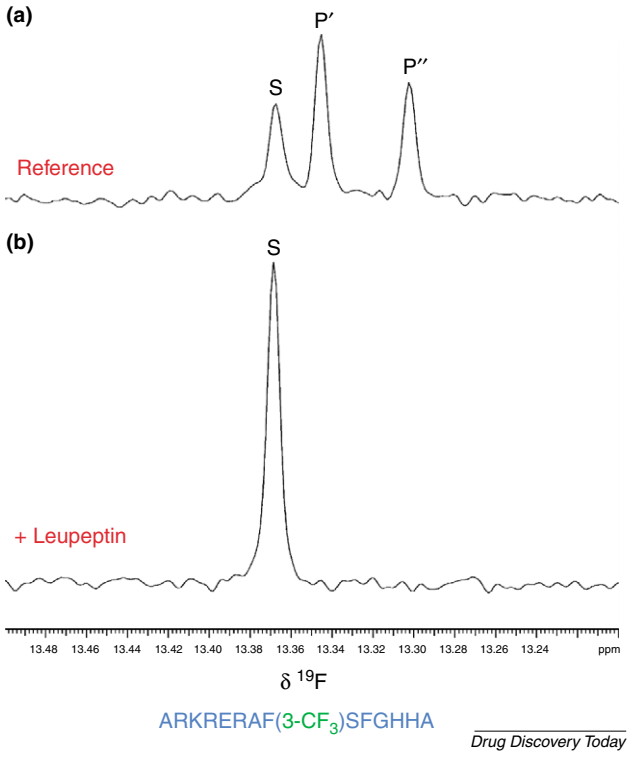

Figure 3. Three fluorine atoms for biochemical screening (3-FABS) performed with the protease trypsin (Roche Molecular Biochemical Cat. No. 1418475) at an enzyme concentration of only 50 pM. **(a)** The substrate concentration was 30 μM. The reaction was performed in 50 mM Tris pH 7.5 and 0.03% Triton X-100 (Sigma X-100) at 20°C and quenched after 6 hours and 40 minutes with 0.5mM phenylmethylsulfonyl fluoride. S is the original peptide and P′, P″ are two different products of the enzymic hydrolysis. In the presence of 20 μM leupeptin **(b)**, no product formation is observed.

one of the samples is the reference sample – that is, a sample without test molecules. This represents 0% inhibition.

The analysis of the data is straightforward, as shown in Figure 2. When the same substrate concentration is used in all the samples, it is sufficient to take the intensity ratio (or better the integral ratio) of the product and substrate signals. This process is automated and therefore analysis of even a large screening run is fast. It is then sufficient to concentrate the deconvolution efforts on those mixtures in which the intensity ratio value differs from that of the reference. The percentage of inhibition is calculated according to Equation 4:

$$\%\,Inhibition = 100 * \left(1 - \frac{[P_w]}{[P_{w/o}]}\right) \qquad \text{[Eqn 4]}$$

where $[P_w]$ and $[P_{w/o}]$ are given by the integrals of the product (or products) signal in the presence and absence of the inhibitor, respectively.

The IC_{50} value of the identified inhibitor is obtained by simply recording experiments at different inhibitor concentration and by monitoring the integral of the product or substrate ^{19}F signal, as shown in Figure 2. A plot of these values as a function of the inhibitor concentration enables the IC_{50} value to be determined according to Equations 5 and 6 below.

a) monitoring the substrate signal

$$[S_w] = \frac{[S_{w/o}] - [S_{TOT}]}{1 + \left(\frac{[I]}{IC_{50}}\right)^n} + [S_{TOT}] \qquad \text{[Eqn 5]}$$

b) monitoring the product signal

$$[P_w] = \frac{[P_{w/o}]}{1 + \left(\frac{[I]}{IC_{50}}\right)^n} \qquad \text{[Eqn 6]}$$

where $[S_w]$ and $[S_{w/o}]$ are given by the the integrals of the substrate signal in the presence and absence of the inhibitor, respectively and $[S_{TOT}] = [P_{w/o}] + [S_{w/o}] = [P_w] + [S_w]$. [I] is the concentration of the inhibitor, IC_{50} is the concentration of the inhibitor at which 50% inhibition is observed and n is the cooperativity factor. In the absence of allosteric effects (i.e. $n = 1$), a meaningful value for IC_{50} can be derived with a single experimental point. This is possible because the values for both plateaus are known. These are $[S_{w/o}]$ and $[S_{TOT}]$ if Equation 5 is used and $[P_{w/o}]$ and the 0 value if Equation 6 is used.

Typically, concentration in the low nM is used, but if the reaction is fast and the enzyme is stable for a few hours the concentration can be further reduced. This can be appreciated in the example of Figure 3, where screening against the protease trypsin is performed at an enzyme concentration of only 50 pM. In this particular case, a mere 0.6 mg enzyme is required, using a 5mm probe, to screen one million samples. The amount of enzyme can be further reduced to 0.2 mg with the use of 3 mm or flow-injection probes.

At the low enzyme concentration used with 3-FABS and other techniques, problems are encountered with enzyme adsorption to the solid surface. Often, BSA or detergents (e.g. Triton X-100) are added to the solutions to prevent reagent coating. The influence of BSA on the IC_{50} measurements is discussed in detail in reference [42].

Multiple screening

With 3-FABS it is possible to screen multiple enzymes at the same time using different CF_3-containing substrates. This might be relevant in measuring the selectivity of an inhibitor for one target enzyme in the presence of other enzymes of the same family (e.g. kinases). For this purpose,

the selected CF_3 substrates should be specific for the different enzymes. A simple analysis of the products and substrates ^{19}F signals enables a direct measurement of the selectivity of the inhibitor.

In addition, enzymes that are involved in a common enzymatic pathway can also be screened simultaneously with this method. For this purpose it is necessary to insert the CF_3 moiety on the part of the substrate molecule that is recognized by the different enzymes involved in the cascade.

An example of multiple screening is shown in Figure 4, where the screening is performed in the presence of two enzymes, the Ser/Thr kinase, AKT1 and the protease, trypsin. The substrate is first phosphorylated by the kinase and subsequently, upon addition of trypsin, cleaved by the protease. This is a challenging test case owing to the multiple cleavages performed by trypsin on both the substrate and product. Therefore, the resulting spectra are complicated by the presence of several resonances. Despite this difficulty, 3-FABS works efficiently, demonstrating the robustness of the method. Simple analysis of the ^{19}F chemical shift and relative intensities of the substrate and the different product resonances enables detection of the inhibitors, measurement of their inhibitory activity and direct identification of the specific enzyme of the cascade that is inhibited. Inhibition of a pathway can also be studied using only a CF_3-labelled substrate of the last enzyme in the cascade. In this case it is then necessary to perform the experiments with the single enzymes to identify the inhibited enzyme.

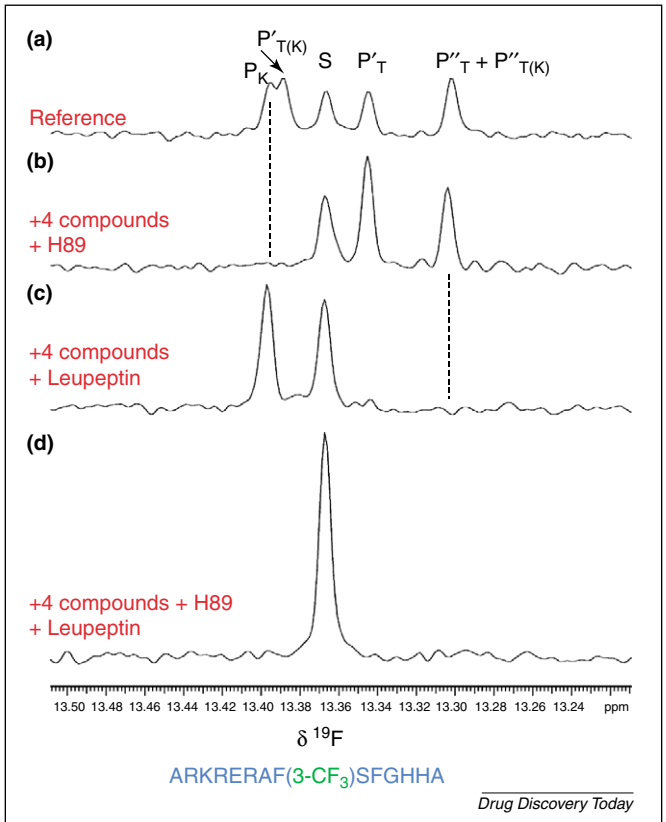

Figure 4. Multiple screening performed with the three fluorine atoms for biochemical screening (3-FABS) method. The first enzymatic reaction was performed with AKT1 and quenched after 3h 40m with the potent staurosporin inhibitor. Subsequently trypsin was added to the solution and the enzymatic reaction was quenched after 15 minutes with 0.5 mM PMSF. The enzymes AKT1, trypsin, the peptide, ATP and compounds concentrations were 25 nM, 1nM, 30 μM, 250 μM and 20 μM, respectively. S, P_K, P'_T and P''_T, $P'_{T(K)}$ and $P''_{T(K)}$ are the substrate, product of the kinase, products of the protease and products of both enzymatic reactions, respectively **(a)**. The 4 compounds are represented in Figure 2. In the presence of the kinase inhibitor H89 **(b)**, only the products of the protease are observed; in the presence of the protease inhibitor leupeptin **(c)**, only the product of the kinase is observed; and in the presence of both H89 and leupeptin **(d)**, no products are observed.

Other NMR techniques for functional screening

(1) ^{13}C NMR spectroscopy has also been proposed as a method for performing a functional assay [40]. The substrate is ^{13}C labelled at one carbon position and ^{13}C spectra are recorded for monitoring the signals of the substrate and product. The sensitivity of the proposed method [40] is only 1/156-fold when compared with the 3-FABS and can be improved to 1/39-fold when an 'insensitive nucleus enhancement by polarization transfer' (INEPT) [54] can be applied (^{13}C is not a quaternary carbon). Therefore, the method requires, even in the best experimental conditions, a 39-times higher substrate concentration to achieve, in the same measuring time, the same signal to noise ratio of the 3-FABS method. Although the method is very elegant for studying kinetic processes, it is clearly not suitable for screening purposes in the context of Equation 1.

(2) ^{31}P NMR spectroscopy has been used extensively for studying the turnover of ATP and other phosphorus-containing metabolites *in vitro* and *in vivo* [36,39]. Although ^{31}P isotope is present at 100% natural abundance and the isotropic chemical shift is sensitive to the chemical environment, ^{31}P NMR spectroscopy suffers, like ^{13}C spectroscopy, from the intrinsic low sensitivity (i.e. 1/37-fold compared with 3-FABS) and therefore is not appropriate as an assay for detecting weak- and medium-affinity inhibitors.

(3) ^{1}H NMR spectroscopy would be the most attractive technique owing to its high sensitivity. Monitoring the proton resonance of a CH_3 moiety results in a sensitivity that is 1.2-fold when compared with the 3-FABS method if the CH_3 is a singlet resonance, but which drops to 0.6-fold when the CH_3 is a doublet resonance.

Noise introduced by the solvent signals and complex pulse sequences used to suppress the large solvent signals reduce the theoretical ^{1}H sensitivity. In addition, there are some major problems associated with ^{1}H detection: (1) the strong solvent, buffer and detergent signals present at molar and mM concentration can hamper the detection of the weak signals of the substrate present at only few micromolar concentration; (2) the signals of the molecules to be screened, the cofactors, cosubstrates and detergents can easily overlap with the signals of the substrate and product, thus making the quantitation of the product formation a difficult task; (3) the proton chemical shift dispersion is very small and therefore the isotropic chemical shift of the monitored CH_3 resonance can often be identical for the substrate and product. To partly overcome problems (1) and (2), a ^{13}C isotopically labelled substrate can be used at one of its non-quaternary carbons. ^{13}C-edited 1D proton experiments are then used for the selection of only the protons attached at the ^{13}C nucleus.

Comparison of 3-FABS with other techniques used in HTS

The strengths and weaknesses of 3-FABS when compared with the other well-established methods are discussed in detail in the following section.

(1) The sensitivity of the fluorescence techniques enables ultra-HTS and miniaturized format using operating working volumes of only 2 µl for each sample [8,10]. The 3-FABS method is clearly less sensitive compared with fluorescence readouts and consequently it requires larger volumes. This results in a larger amount of enzyme, test molecules and substrate necessary for the screening. With the use of a ^{19}F flow-injection probe or a 3 mm probe the volume required for each sample is in the range of 100–200 µl. The recording time for each sample with the current set-up is a few minutes [42]. Developments are currently underway to build a probe with cryogenic technology optimized for ^{19}F detection. Analogous to cryogenic probes developed for ^{1}H detection, this new technology will enable a 10–16-fold reduction in the measurement time. In addition, it will be possible in some fortunate cases to use substrate molecules with a CF moiety (1-FABS).

To achieve the high throughput required to screen a large proprietary compound collection (>100,000 compounds) in a few days it is necessary to perform the 3-FABS using mixtures of test compounds. Chemical mixtures comprised of many compounds can also be used with 3-FABS because of the lack of spectral interferences. Overlap with the CF_3 signals of the substrate and product even in the presence of CF_3-containing test molecules is rare because of the large dispersion of the ^{19}F chemical shift. Throughput can also be increased by simultaneous data collection on multiple samples using capillary lines formed into a bundle that is 5 mm in diameter [21], or using a recently introduced four-coil, flow-through multiplex sample probe [55].

(2) When compared with all other techniques used in HTS, the main advantage of 3-FABS is the direct characterization of the screened compound. After 3-FABS and deconvolution of the mixtures, 1D ^{1}H spectra are recorded for every identified NMR hit. The spectra are recorded in the same buffer solution used for the screening, but without substrate, cosubstrate, cofactors and enzyme and in the presence of a reference molecule of known concentration. This allows a measurement of the real sample concentration, sample purity, compound identity, solubility in aqueous solution, aggregation state and chemical stability. The characterization of the chemical properties is of fundamental value for deriving accurate values of IC_{50} (necessary for building a meaningful SAR table) and for identifying potential inhibitors that would otherwise be discarded (false negatives) with the other techniques. In our experience using 3-FABS, we identified several inhibitors with IC_{50} values in the nM range that appeared inactive (at 10 µM concentration) in a scintillation proximity assay (SPA). Analysis of the ^{1}H spectra provided explanations for the failure of SPA in detecting these inhibitors.

(3) Some fluorescence techniques require major structural modifications of the substrate to allow the insertion of one or two (e.g. donor and acceptor in the FRET experiments) large and bulky fluorophores. These modifications of the original substrate can significantly alter the binding properties compared with 3-FABS, where it is sufficient merely to insert a CF_3 moiety. For peptide or peptido-like substrates, combinatorial chemistry can introduce different types of CF_3 residues efficiently in one or more positions of the sequence. These peptide mixtures can then be tested with 3-FABS. The analysis of ^{19}F chemical shifts, changes in signal intensities and appearance of new signals enable the direct identification of the most efficient substrate. The selected peptide will then be synthesized in large amounts for the 3-FABS experiments. In this respect, the development of a functional assay with 3-FABS is simple, straightforward and fast.

(4) Some of the assays used in HTS can be rather complex, requiring secondary reactions, labelled specific antibodies, washing steps to remove the substrate or cosubstrate. The complexity of an assay results in measurements

subject to larger experimental errors and to an increasing number of sources of potential interferences. Other assays use radioactive isotopes (e.g. SPA) and, consequently, introduce all of the health and safety issues related to the handling and disposal of radionuclides. 3-FABS is probably the simplest conceivable functional assay because it is homogeneous and detects directly both substrate and product. Its simplicity results in the reliable detection of lead molecules and precise quantification of their inhibitory strength. Even weak inhibitors can be identified unambiguously. This is important when only a limited chemical library of compounds is available for screening. Minor chemical modifications of the weak inhibitor [56] or the selection of similar molecules bearing the same scaffold might result in the identification of potent inhibitors.

Although 3-FABS is not subject to interferences originating from the method of detection, artefacts deriving from compound aggregation and protein denaturation will plague 3-FABS just as effectively as they plague any other enzymatic assay. However, the possibility of directly characterizing the compound chemical properties with ^{1}H NMR spectroscopy enables the identification of many of the false positives.

Conclusion and outlook

The initial results and the high-quality data generated with 3-FABS and its variants are encouraging. Although the method cannot compete with the ultra-high-throughput achieved with fluorescence spectroscopy, it scores highly on quality. The approach is particularly suited for a primary assay of focused targeted-directed libraries and for a reliable secondary assay.

The development of the ^{19}F probes with cryogenic technology will further improve the sensitivity of the 3-FABS and will enable, in some cases, the use of the 1-FABS.

The method can find many useful applications in different drug discovery projects.

(1) HTS of natural product extracts is easily achieved with 3-FABS. The substances of the active extracts are then separated via HPLC and tested with 3-FABS as single compounds for the identification of the active ingredient.

(2) Determination of the function of a newly sequenced protein for addressing its potential as a drug target. A library of CF_3- and CF-labelled substrates of known enzymes is created and the ^{19}F chemical shift of the substrates and their products are measured. These substrates are then used to screen novel proteins for determining their function [42]. For these experiments it is also possible to use CF-labelled substrates because there are no limitations on the protein and substrate concentrations – the goal is simply the detection of product formation. The method is clearly limited to activated enzymes. For a more general application a library of CF- and CF_3-labelled known ligands is also created. FAXS [57,58] and FABS are then both used for the screening.

(3) HTS within living cells can also be performed with 3-FABS. Cells do not contain fluorinated compounds and therefore the only observable signals are those of the product and substrate. For this purpose, the CF_3-labelled substrate is inserted into the cells. Rapid degradation of the CF_3-labelled substrate could represent a problem. Proteases present in the cell can rapidly cleave the substrate if it is a peptide, thereby not allowing its use for screening purposes. An approach to enhance the resistance to enzymatic hydrolysis is the use of CF_3-labelled β-peptides, cyclic peptides or peptides composed of *D* amino acids.

It is envisioned that the speed and easy set-up of 3-FABS, together with its broad range of applications and its reliable and rich information-content, will have a major impact in the drug discovery process for discovering potent, bioavailable and safe clinical candidates.

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