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TINS, Target Immobilized NMR Screening: An Efficient and Sensitive Method for Ligand Discovery

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Summary

We propose a ligand screening method, called TINS (*t*arget *i*mmobilized *N*MR *s*creening), which reduces the amount of target required for the fragment-based approach to drug discovery. Binding is detected by comparing 1D NMR spectra of compound mixtures in the presence of a target immobilized on a solid support to a control sample. The method has been validated by the detection of a variety of ligands for protein and nucleic acid targets (K_D from 60 to 5000 μM). The ligand binding capacity of a protein was undiminished after 2000 different compounds had been applied, indicating the potential to apply the assay for screening typical fragment libraries. TINS can be used in competition mode, allowing rapid characterization of the ligand binding site. TINS may allow screening of targets that are difficult to produce or that are insoluble, such as membrane proteins.

Introduction

Ligand screening assays play a significant role in the early stages of drug discovery. High-throughput screening (HTS) methods have been developed that allow very large libraries of compounds to be screened for specific and tight binding to a target molecule. To maximize the chance of finding such a compound in a random search as well as to increase the diversity of the collection, HTS libraries have become more "druglike" [1, 2], that is, the complexity and lipophilicity have increased. Optimization of lead compounds for affinity and specificity typically involves further increases in molecular weight, complexity, and lipophilicity [1, 2], with the resulting compounds often violating Lipinski's "rule of five" [3]. An alternate approach has been proposed by the group of Fesik in which multiple, independent, weak, but specifically binding compounds are linked to ultimately provide a high-affinity lead compound [4]. The so-called fragment-based approach has several advantages over HTS-based methods. The compounds in the library can be more "leadlike," that is, more hydrophilic and simpler [1, 2], allowing greater flexibility during the optimization process. Subsequent analysis of the structure activity relationships (SAR) of "hits" is greatly simplified in comparison to the analysis of HTS hits. The number of compounds in the fragment library can be small compared to typical HTS libraries (10^4 fragments versus 10^6 compounds) while greatly exceeding the diversity of the HTS collection due to the combinatorial nature of the process [5]. However, the typical binding affinity of components of these simpler libraries to the target is significantly weaker (K_D values in the μM to mM range) than HTS libraries. The types of assays normally used in HTS are not well suited for detecting such weak interactions.

Nuclear magnetic resonance (NMR) is an ideal tool for detecting weak binding of low-molecular-weight compounds to biomacromolecules [6]. Recent years have witnessed increased interest in NMR as a ligand screening tool along with the development of a wide array of new methods and enabling hardware. NMR may be used to observe changes in the spectrum of either the target or the ligand that occur upon binding. In the first case, known as SAR by NMR, the target is labeled with ^{15}N or ^{13}C in order to exclusively observe the spectrum of the target in the presence of multiple compounds being assayed for binding [5, 7]. Using mixtures of compounds, libraries of up to 10,000 components can be efficiently screened. SAR by NMR has the advantage that it also provides information as to where a compound binds if the sequential assignment and 3D structure of the target are known or if a reference compound is available. In this incarnation, SAR by NMR has been used successfully to develop small molecule inhibitors to a wide variety of protein targets [8]. However, SAR by NMR is generally applicable to targets for which the sequential assignment is available and therefore are less than roughly 40 kDa. Recent developments have, however, enabled experimentally guided docking of ligands into the 3D structure of even very large protein targets [9, 10]. More stringent is the requirement for isotopic labeling of the target, which preferably needs to be available in large (typically >200 mg) quantities.

An alternative approach to SAR by NMR is to observe changes in the NMR spectrum of the ligand itself. These changes may be manifested directly as a change in the diffusion constant [11–13] or indirectly via an effect on the correlation time τ_c. Numerous elegant techniques have been elaborated that detect changes in τ_c, including line broadening [11, 14], the presence of intramolecular transfer NOEs (trNOE) [15], or intermolecular trNOEs [16–19], which we collectively refer to as small molecule NMR methods. These techniques alleviate the requirement for isotopically labeled protein while, at the same time, reducing the amount of target needed to screen a compound library. An additional benefit is that these techniques have the potential to directly identify the binder even in complex mixtures and can therefore

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alleviate subsequent deconvolution steps. Small molecule NMR methods do not provide the structural information that the SAR by NMR method does. However, they do provide at least a limited characterization of the ligand binding site with modifications that allow the assay to be performed in a competition mode [20], or when combined with molecular modeling [21]. One limit of small molecule NMR methods is that they are generally insensitive to tight binding ligands. This insensitivity is due to the reliance on rapid exchange on and off the receptor to detect spectral characteristics of the bound state that are transferred to the free state. Tight binding ligands, which are in slow exchange by definition, do not provide the requisite averaging throughout the ligand population (competition mode experiments [22, 23] are an exception). Spectroscopic analysis of ligands alleviates restraints on the size and chemical nature of the target, however, so that binding to very large proteins or nucleic acids [24] can be detected. In an impressive first, the group of Meyer has even detected binding to a membrane protein [25] using saturation transfer difference spectroscopy. However, to screen a 10,000 compound library 10 compounds at a time with a 50 kDa membrane protein using the conditions described would require 50 mg of protein and nearly 3 months. Many small molecule NMR methods have been put to good use in actual drug discovery programs, yet they too have limitations with respect to the range of targets to which they can be applied. In particular, screening complete compound libraries using targets that are only available in low mg quantities (as is typically the case with integral membrane proteins) is not possible using presently available methods.

One way to further reduce the amount of target required for small molecule NMR methods would be to use a single sample of the target to screen an entire compound library. This could be accomplished through immobilizing the target and utilizing a flow-injection NMR instrument. The group of Meyer was indeed able to detect binding of one oligosaccharide from a mixture of seven in the presence of a lectin bound to controlled pore glass (CPG) beads [19]. However, in this study, it was necessary to use magic angle spinning (MAS) NMR in order to overcome the line broadening induced by the magnetic field gradients resulting from the difference in magnetic susceptibility of the glass beads and the surrounding aqueous solution. The requirement for MAS necessitates a batch mode that is not well suited to screening even moderately large compound libraries.

Here, we demonstrate a technique called TINS, for *t*arget *i*mmobilized *N*MR *s*creening, in which the target is immobilized on a support that allows one to record high-resolution spectra in the static mode. This arrangement should allow the assay to be performed in a flowthrough manner using only one sample of target. Target-ligand interactions with a wide range of affinities can be detected using TINS, which, like other small molecule NMR methods, can provide limited structural information when used in a competition assay with a known ligand. However, unlike other NMR screening techniques, we show that TINS can in principle be used to screen an entire library with a single sample of 3–5 mg of the target.

Results and Discussion

Effect of Solid Support on NMR Linewidth

In order to enable a flow-injection, ligand binding assay, it was first necessary to find a solid support that was compatible with static NMR methods. We chose agarose- and Sepharose-based media because of their high solvent content and ready availability in formats appropriate for immobilization of proteins and nucleic acids. Comparison of spectra recorded on homogenous and heterogenous systems consisting of small molecules in solution in the presence of a Sepharose resin indicated that the linewidth increased from about 1 Hz to about 25 Hz at 14.1 T (400 MHz ^{1}H frequency, Figure 1). In comparison, CPG beads result in susceptibility mismatch-induced linewidths that are on the order of 100s of Hz. As seen in Figure 1C, these linewidths severely limit the usefulness of the experiment. Additionally, we find only a marginal dependence of the linewidth on field strength (7 Hz difference between 9.4 and 14.1 T). Based on these results, we were encouraged to see if we could detect binding to a target immobilized on a Sepharose support.

Detection of Binding

For a simple test system, we chose the well-characterized binding of spermidine to the phosphate backbone of DNA [26]. We synthesized a 5′ biotinylated oligonucleotide and immobilized it on streptavidin Sepharose. The spectrum of imidazole (Im), phosphotyrosine (pY), arginine, and spermidine at 1 mM each in solution (Figure 2A) is shown for reference. We prepared two samples of streptavidin Sepharose in standard NMR tubes, one of which contained the 5′ biotinylated oligonucleotide. Control and experimental resins were equilibrated with the mix of compounds, and 1D ^{1}H spectra of each were recorded (Figures 2B and 2C, respectively). Subtraction of the spectrum recorded in the presence of the oligonucleotide from the control spectrum yields what we refer to as the TINS spectrum (Figure 2D), which contains only resonances of spermidine, known to bind the phosphate backbone of DNA. The affinity of the purely electrostatic interaction is highly dependent on salt concentration and pH [26]. Under the conditions we have used, the spermidine binding should be weak (K_D approximately 5 mM). However, the presence of multiple binding sites (about nine per DNA molecule) ensures that the binding equilibrium is shifted toward the complex. The spermidine-DNA interaction demonstrates that weak binding of one compound in a mixture to an immobilized target can readily be detected using simple, static NMR methods.

We next turned to a protein target for which drugs have been successfully developed. The immunophilin FKBP12, originally used to develop the SAR by NMR method [4], is a well-characterized target for which a wide variety of ligands are available covering a range of binding constants. We immobilized FKBP12 to a commercially available Sepharose resin via Schiff-base

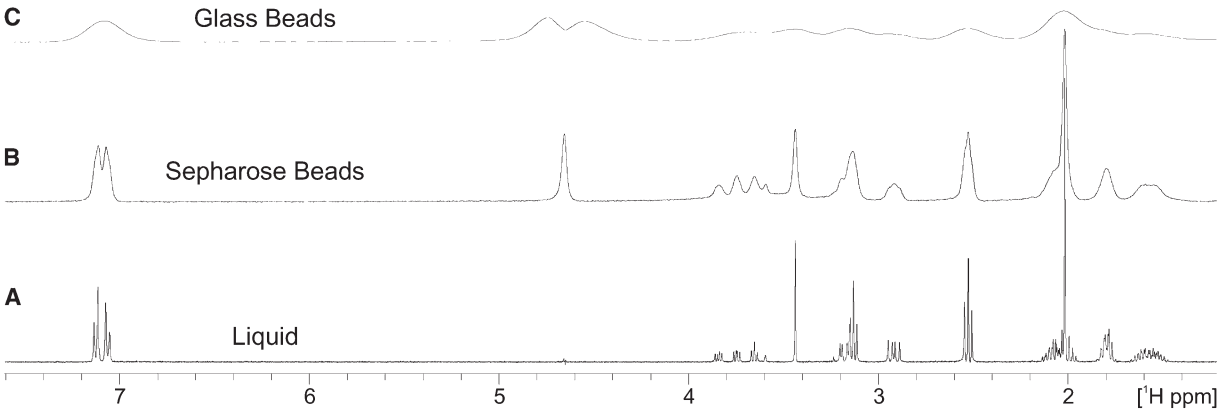

Figure 1. Effect of the Susceptibility Mismatch on the NMR Linewidth in Different Solid Supports

A 1D ^{1}H spectrum of a mixture of low molecular weight compounds (<200 Da) was recorded in solution (A) in the presence of a Sepharose support (B) or in the presence of controlled pore glass (CPG) beads (C) at a ^{1}H frequency of 400 MHz (9.1 T). The fitted linewidths are 1 Hz, 28 Hz, and ~120 Hz in the three spectra, respectively. Note that the linewidth in the CPG beads is approximate due to severe signal overlap.

formation to primary amines. This results in efficient immobilization of protein randomly oriented with respect to the bead. We chose readily available, well-characterized [27] ligands with known dissociation constants (K_D) ranging from 60 μM–500 μM. Figure 3A shows the overlaid 1D ^{1}H spectra of a mix of **2**-(2-Pyridyl)benzimidazole (**1**), Im, pY, and methionine (Met) in the presence of a control resin and a resin to which FKBP12 was immobilized. Differences in peak intensity for aromatic resonances at 7.3, 7.45, 7.75, and 8.6 ppm, which all belong to **1** (K_D 60 μM), are immediately obvious even upon casual inspection. Further, resonances from nonbinding compounds are essentially identical in the two spectra, allowing one to readily identify the known binder. In an assay being developed for medium-throughput use, a method of reliable automated data analysis would be important. We have used the simple but robust binning method [28] to analyze the TINS spectrum (upper trace of Figure 3A). Binning is less sensitive to minor artifacts due to small changes in the resonance position such as that seen for imidazole (8–8.1 ppm). Indeed, significant positive intensity is only detected in the aromatic region corresponding primarily to genuine differences observed in the overlaid spectra. While some contribution to this intensity clearly derives from the change in the imidazole resonance, this is small compared to that from the difference in the intensity of resonances from **1**. In Figure 3B, the same experiment was repeated with N-cyclohexyl-p-toluenesulphonamide(**2**), a ligand that binds to FKBP12 approximately 8 times weaker than **1** (K_D 500 μM). Nonetheless, clear differences in intensity of peaks in both aliphatic (1.1, 1.5, and 2.3 ppm) and aromatic regions (7.35 and 7.7 ppm) can be seen. Again, binning of the TINS spectrum reduces small artifactual differences and emphasizes real differences in peak intensity between the two spectra in both the aromatic and aliphatic regions. Thus, it is likely that automated integration of binned difference spectra could be used to reliably detect binding in a mixture of compounds, while the actual identity of the ligand can be obtained by careful inspection of the NMR spectra.

Physical Basis for TINS

The common method for detecting intermolecular interactions via NMR is to observe changes in the resonance position and linewidth of nuclei at the intermolecular interface. The manner in which these chemical-shift perturbations are manifested in the NMR spectrum is directly related to the affinity of the interaction, with weak interactions being characterized by gradual shifts and tight interactions giving rise to separate peaks from the free and bound forms. However, in Figures 2 and 3 there is an apparent absence of chemical-shift perturbations due to interaction of the ligands with the target (the small variations in the imidazole resonance in Figure 3 are likely due to experimental variations and not interaction with the target). These observations cannot be explained by the conventional NMR theory that describes the effect of exchange between a homogeneously dissolved free and bound form of a ligand.

The linewidth of a (nonexchanging) NMR resonance is a direct result of the rate of transverse relaxation (R_2) of the excited nuclear spins. R_2 is generally much smaller (inefficient relaxation) for the nuclei of a molecule in solution, giving rise to sharper peaks than when the same molecule is bound to a solid support. In a homogenous, rapidly exchanging system, the broad resonances of the bound state (assuming the target is large and slowly tumbling) exchange throughout the entire population of ligand such that the NMR spectrum reflects both the bound and free states (cf. Fejzo et al., 1999 [12]). In a situation such as occurs with TINS, the ligand is exchanging between a dissolved free form and a bound state on a solid matrix. The NMR parameters (linewidth and chemical shift) of such a heterogeneous system can only be described properly when self-diffusion between spatially remote free and bound states is explicitly taken into account. We have incorporated the effect of a ligand binding to an immobilized receptor on

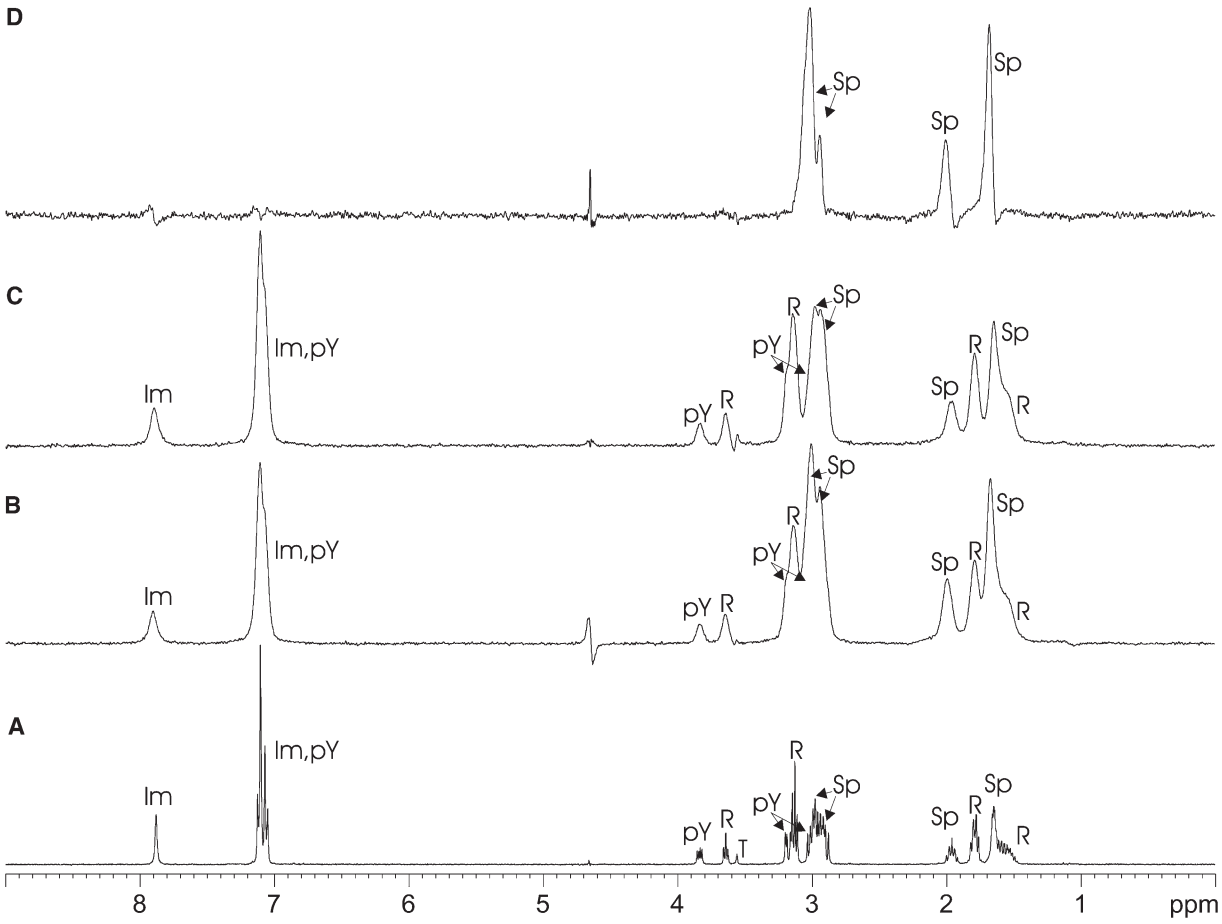

Figure 2. 1D ^{1}H Spectra of a Mixture of Small Compounds in the Presence of an Immobilized Oligonucleotide

^{1}H spectrum of a mixture of 1 mM each of imidazole (Im), phosphotyrosine (pY), arginine (R), and spermidine (Sp) in 25 mM D_{11}-Tris (pH 7.5) and 140 mM NaCl dissolved in D_2O (A). The same mixture was equilibrated with streptavidin Sepharose (B) and streptavidin Sepharose to which a 5′ biotinylated, double-stranded oligonucleotide was bound (C). ^{1}H NMR spectra were recorded of each with identical parameters (9.4 T, 256 transients, total recording time approximately 4.5 min). The difference spectrum ([B] and [C]) showing only peaks from spermidine, a known binder of the DNA backbone, is shown in (D).

the evolution of magnetization in an analytical model (unpublished data). Simulation results for this model are shown in Figure 4, which depicts the effect that varying the R_2 and interbead spacing has on the appearance of the NMR spectrum.

In the left panel of Figure 4A, R_2 of the bound ligand (R_{2b}) is set to an artificially low value (2 s^{-1}). In this case, peaks are observed in the NMR spectrum deriving from both the bound and free states as well as exchange between them. This does not correspond to the situation encountered in real TINS experiments (Figures 2 and 3). When R_{2b} is set to a realistic value (150 s^{-1} in Figure 4A, middle panel), the peak corresponding to the bound state is strongly broadened, and the spectrum closely resembles those observed experimentally in Figures 2 and 3. Why is there apparently no exchange broadening of the peak for the free ligand even though it is in rapid exchange with the bound state? The answer lies in the fact that diffusion is a stochastic process, and therefore the chance that a ligand in solution encounters an immobilized target decreases with the square of the initial distance to the target. So ligands that are initially sufficiently far away will not interact with that target during the course of an NMR experiment (approximately 80 ms). The NMR signal from ligands that do interact with the immobilized target will be broadened beyond detection. This conclusion is supported by the right panel of Figure 4A, where the size of the interbead space is reduced to 20 μm so that diffusion ensures that nearly all of the ligand encounters the immobilized target. In this case, which resembles a homogenous solution, the expected line broadening and chemical-shift perturbation are observed.

The proportion of the total amount of ligand that interacts with the immobilized target is a function of the ligand concentration, K_D, the diffusion rate, and the pore size. In Figure 4B, we have calculated the expected size of the TINS (difference) signal for ligand binding over a wide range of binding affinities. As ex-

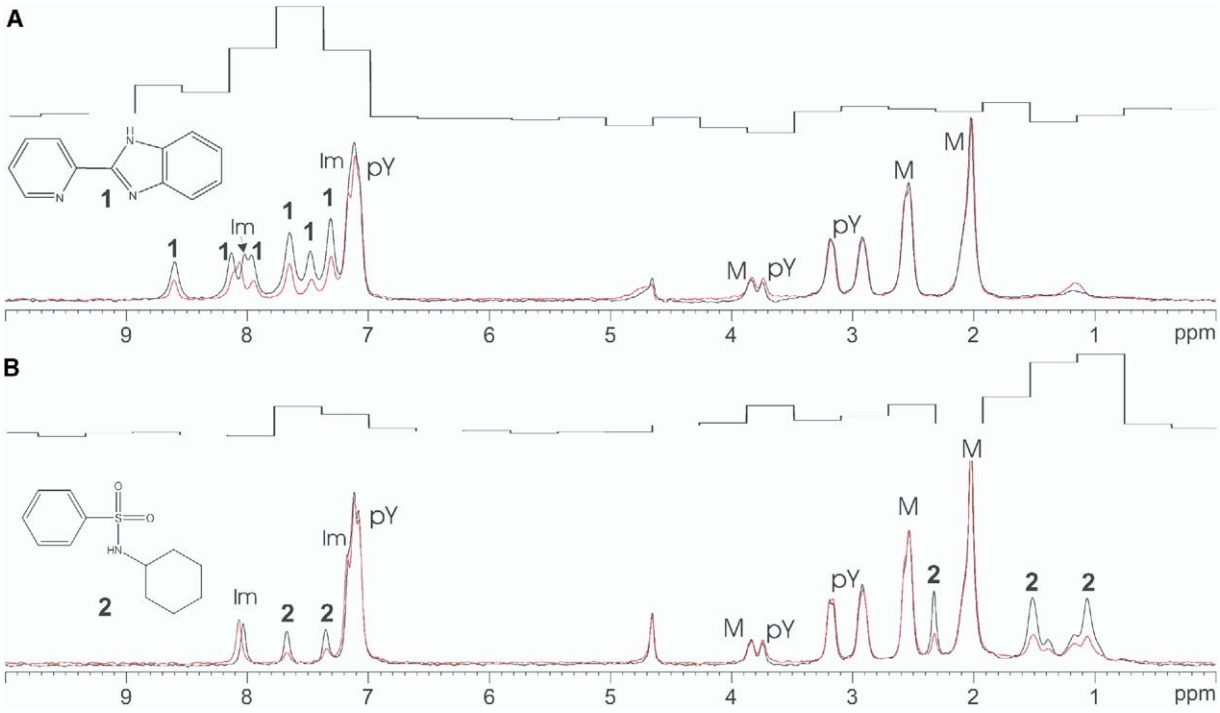

Figure 3. 1D ^{1}H Spectra of a Mixture of Small Compounds in the Presence of Immobilized FKBP

A mixture of 1 mM each of **1** (A) or **2** (B) and imidazole (Im), phosphotyrosine (pY), and methionine (M) dissolved in deuterated PBS was equilibrated with a control Sepharose resin (black trace) or a resin to which FKBP was immobilized at a solution equivalent of 0.89 mM (red trace). Spectra were recorded at 9.4 T with 64 transients each, for a total experiment time of 1.5 min. The difference spectrum was divided into 64 bins and is presented above the overlaid spectra. The vertical scale of the spectra in (A) and (B) is identical, as is the scale of the two binned difference spectra.

pected, tight binders will result in almost complete disappearance of the signal in the presence of an immobilized receptor, resulting in a large difference signal. When other factors are held constant, weaker binding results in less loss of signal due to broadening, and therefore signals in the difference spectrum are smaller. However, even for binding as weak as 1 mM, the expected difference signal is about 35% of the control signal. Such a large difference is easily detectable. Integration of peaks in Figure 3 leads to experimentally determined values of about 54% and 62% for the difference spectra of **2** and **1**, respectively, in comparison to calculated values of 55% and 75%. Considering the simplifications (to be published elsewhere) in our current model, these numbers are reasonably accurate. The surprising sensitivity of TINS to weak binding is due to the fact that a substantial portion of the total ligand population does interact with the immobilized receptor via diffusion and exchange. Thus, a balance is struck whereby a large portion, but not all, of the ligand in solution interacts with the immobilized target and undergoes severe line broadening. By detecting only that portion of the population that does not interact with the bound receptor, the TINS experiment is sensitive, easy to interpret, and amenable to automated data analysis. Further, this analysis implies that the sensitivity of the TINS experiment could, in principle, be modulated by changing the size of the beads, which would change the size of the interbead spacing.

Further Characterization of TINS

For in-depth characterization of TINS, we chose a target that had proven difficult to attack using standard HTS methods. We used the C-terminal SH2 domain (CSH2) of the p85α subunit of bovine phosphoinositide 3′ kinase, for which the phosphotyrosine (pY) ligand specificity and the 3D structure are well characterized [29, 30]. We used the SAR by NMR experiment to determine solution binding constants for the amino acid constituents of the high-affinity phosphotyrosine peptide ligand (pY and Met). pY and Met induced concentration-dependent chemical-shift changes in the spectra (see Supplemental Figure S1) indicative of rapidly exchanging, weak binding ligands. Analysis of the chemical-shift perturbations yielded dissociation constants of 0.6 and 10 mM for pY and Met, respectively (data not shown). The Met dissociation constant is approximate, since limited solubility of methionine resulted in significant dilution of the protein.

Next, we tested whether we could detect ligand binding to CSH2 when immobilized on a solid support identically to FKBP12. As with FKBP12, the binding of the known ligand, pY, could be readily detected from a mixture of nonbinding compounds in the difference spectrum

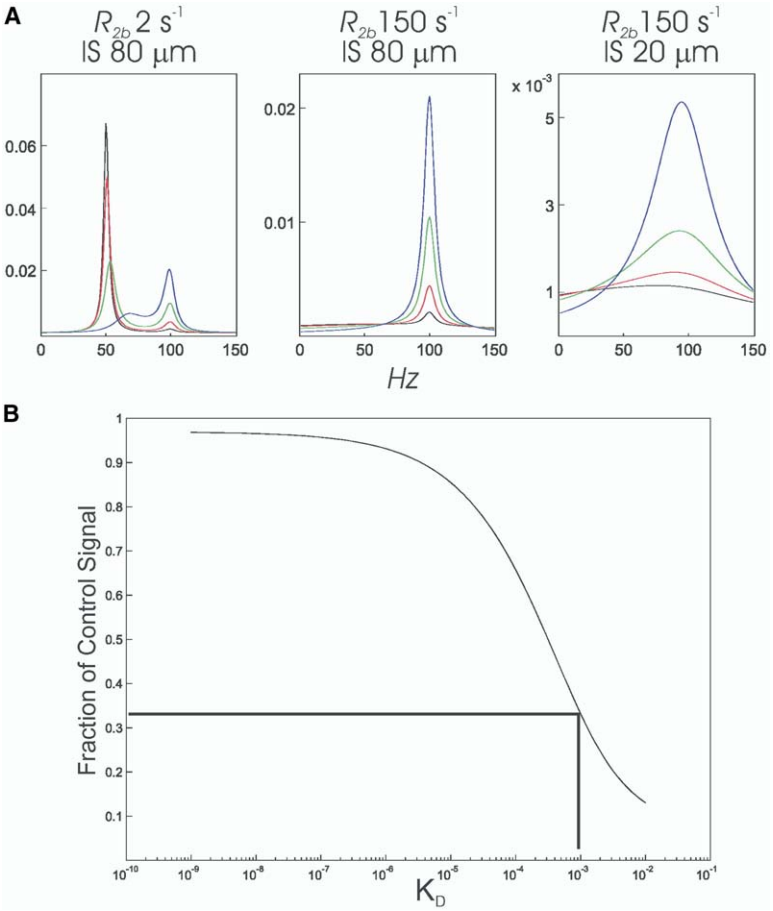

Figure 4. Simulation of the Effect on the NMR Spectrum of Ligand Binding to an Immobilized Receptor

(A) A typical peak from the ^{1}H spectrum of a low-molecular-weight compound in solution (resonance frequency 100 Hz) is depicted. The resonance frequency in the bound state is 50 Hz. In the panel on the left, the transverse relaxation rate of the bound ligand, R_{2b}, is set to an artificially slow rate of 2 s^{-1} (approximately what it would be in a pure solution state), while in the other two panels, R_{2b} is set to 150 s^{-1}, approximately what it is when bound to an immobilized receptor. The space between the beads (IS, defined as the one-dimensional distance from the wall of one bead to the next bead) is 80 μm in the left and middle panels and 20 μm in the right panel. In each panel, the spectrum is simulated for a ligand and the bound receptor at 0.5 mM with a K_D = 10^{-6} M (black), 10^{-5} M (red), 10^{-4} M (green), and 10^{-3} M (blue). The units of intensity values are the same for all three panels, but note the difference in scaling for presentation.

(B) The difference signal in the simulated spectra is presented as a function of the dissociation constant. The difference signal, the amplitude of a signal in the control spectrum minus that in the experimental spectrum normalized by the amplitude of the control spectrum (as in Figure 7), and the concentration of both ligand and target are held constant at 0.5 mM.

(Supplemental Figure S2). Further, the TINS signal is 43% (Supplemental Figure S2) of the control signal, which is in relatively good agreement with the calculated value of 50% for a ligand with K_D 0.6 mM. pY is a poor basis for a lead compound for a number of well-known reasons, such as susceptibility to phosphatases [31]. We therefore synthesized L-O-(2-malonyl) tyrosine (**3**), which is known to bind SH2 domains [31]. Chemical-shift perturbation analysis of [^{15}N,^{1}H] HSQC spectra of CSH2 indicated that **3** was in rapid exchange with the protein and bound the pY pocket with a K_D of 2.4 mM (data not shown). Repetition of the TINS experiment at 9.4 T (400 MHz) with **3** yielded only the expected peaks in the difference spectrum (Supplemental Figure S2).

Sensitivity

The data presented above utilize a solution of 1 mM of each component in the ligand mixture and a solution equivalent of 1 mM target. These concentrations are relatively high with respect to the conditions more typically used for ligand screening. We have titrated both the concentration of immobilized target and the mixture of ligands to determine the limits of sensitivity (Figure 5). These data indicate that a solution equivalent of target at 250 μM with ligands at 250–500 μM should be sufficient to rapidly collect TINS spectra (measurement times of ~10 min) and identify ligands with dissociation constants less than 1 mM.

Binding Site Identification

In order to rapidly generate high-affinity leads from the low-affinity hits, the fragments are typically linked in a manner compatible with simultaneous target binding. In most cases, this process can be efficiently achieved using 3D structural analysis of the ternary complex. However, this information would likely not be available for targets for which TINS might be applied. Therefore, it is important to be able to perform a competition binding assay in order to group the hits from a screen according to where on the target they bind. The solution titration data indicate that pY and **3** bind to the same site on CSH2. Using a sample of immobilized CSH2 (0.5 mM solution equivalent), we observed a decrease in the intensity of peaks from pY in the TINS spectrum when the concentration of **3** reached its K_D (Figure 6). The simplest explanation is that, as a substantial portion of the target is bound by **3**, pY is displaced. These data indicate that site-specific binding can be detected using TINS in competition mode with a reference ligand.

Potential for Medium-Throughput Implementation as a Ligand Screening Tool

Clearly, in order for TINS to be a useful ligand screening tool, it must be possible to screen a reasonably sized

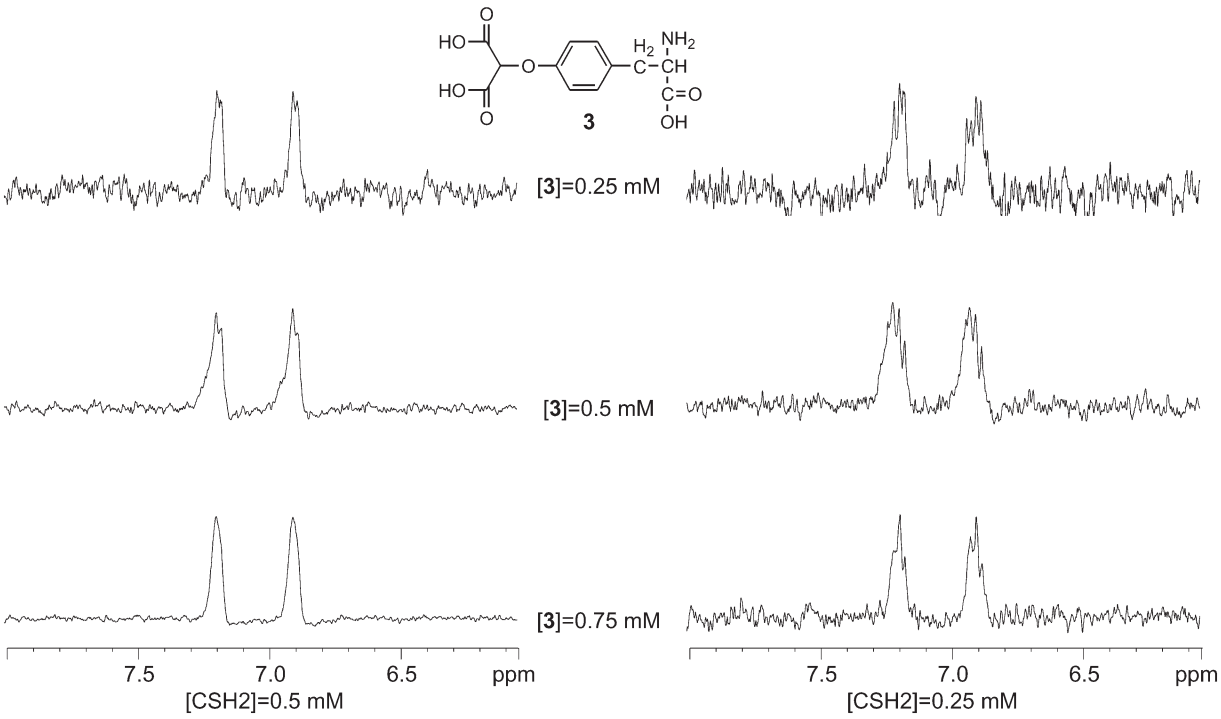

Figure 5. Sensitivity of the TINS Experiment

The CSH2 domain was immobilized on a Sepharose resin at a solution equivalent of 0.5 mM (left column) or 0.25 mM (right column). The portion of the TINS (difference) spectrum containing the aromatic resonances of 3 at different concentrations is shown. All spectra were recorded using 512 transients at 9.4 T (400 MHz), for a total recording time of about 9 min each.

compound library with a single sample of the target. We tested this possibility by applying mixtures of ten compounds at a time from the SAR by NMR screening library [8] to control and CSH2 immobilized resin samples in a column. The columns were washed and a set of ten new compounds was applied. Samples were removed at various points and tested for pY binding using TINS. Figure 7 demonstrates that the amount of pY that can bind to the immobilized CSH2 does not change even after application of 2000 different compounds. Based on these results, we feel that it is reasonable to conclude that, at least for soluble targets such as those used here, TINS could be used to screen an entire fragment library (typically containing 10,000 compounds) using only a single sample of the target.

The TINS technology has the potential to be combined with flow-injection NMR spectroscopy. A flow-injection system based on small molecule NMR has been described, but it requires substantially more target [28]. To use TINS in a flow-injection mode, both the control and target immobilized resins need to be simultaneously held inside an appropriately modified probe. A standard liquid-handling station could then be used to alternately apply compound mixes and wash them off automatically, resulting in a medium-throughput assay. Arranged in this manner, screening an entire compound library would require approximately 400 μl of a 250 μM solution equivalent of the target, which is one tenth that required using a typical STD approach [25]. The screening process using TINS would could be accomplished in 1 week, where it would require more than 3 months using the STD approach [25]. We are currently constructing such a flow-injection probe, and our preliminary data (to be reported elsewhere) indicate that the concept is indeed feasible.

Membrane proteins are a particularly attractive target for the flow-injection TINS methodology, as most are notoriously difficult to produce in large quantities. In addition, if the control resin is derivatized with a similar protein in a similar lipid/detergent environment, then false positives resulting from nonspecific interaction of the compounds with the resin or components of the immobilization system can be eliminated. While we have yet to demonstrate TINS on a membrane protein, the system appears to be quite reliable and general. Thus far, four different resins have been used, all of which have shown similar results and, importantly, none of which has demonstrated any nonspecific binding.

Significance

Fragment-based drug discovery is becoming an important additional tool in the search for fundamentally new lead compounds. This approach generally produces lead compounds that are chemically very different than those derived from high-throughput screening, which should result in higher success rates for finding new drugs. However, fragment-based methods have been limited to pharmaceutical targets that are readily available in large quantities. We describe

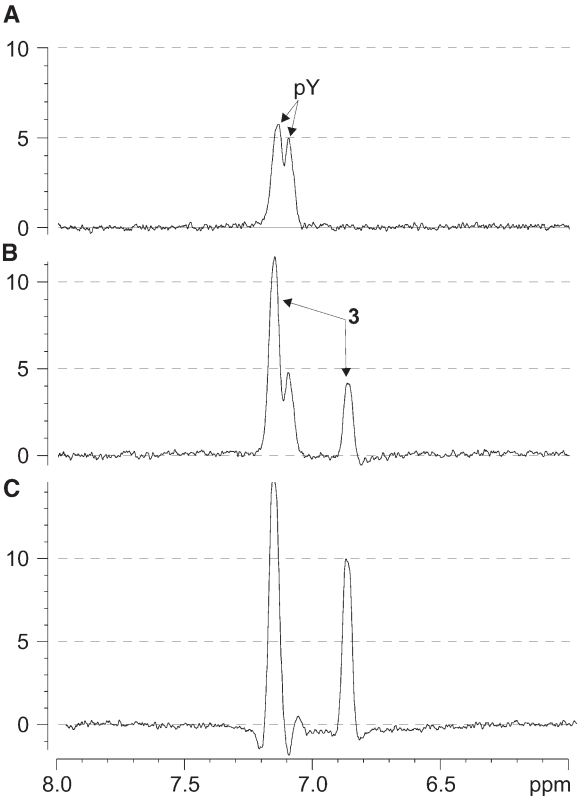

Figure 6. Competition Mode TINS Experiment

The portion of the TINS (difference spectra) containing the aromatic resonances of pY and **3** is shown. A diminution in the height of a peak reflects reduced binding of that compound to the immobilized CSH2 domain. The CSH2 domain was immobilized at a solution equivalent of 0.25 mM. pY (0.5 mM) binding was competed by an increasing amount of **3**, present at 0 mM (A), 1 mM (B), and 2.5 mM (C). The amplitude is given in arbitrary units that are constant between each spectrum. Competitive binding by **3** is most clearly seen in the reduced intensity of the pY peak at 7.1 ppm.

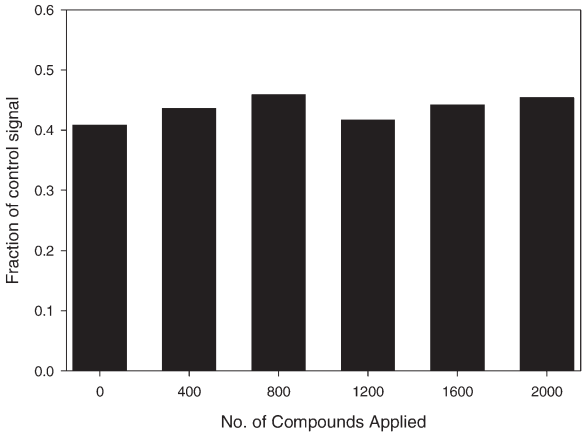

Figure 7. Reusability of the Immobilized Target

The CSH2 domain was immobilized on a Sepharose solid support at a solution equivalent of 0.5 mM. Compounds from the SAR by NMR library were applied, in mixtures of ten at a time, to either a column of the solid support to which the CSH2 domain was immobilized or a control resin. Samples were taken after every multiple of 400 compounds had been applied and tested for pY using the TINS experiment. The aromatic peaks from phosphotyrosine were integrated in spectra from control and CSH2 immobilized resins. The fraction of control signal represents the ratio $(\int_c - \int_i)/\int_c$, where $\int_c$ refers to the integral of the aromatic peaks of phosphotyrosine in the control spectrum, and $\int_i$ in the spectrum of the CSH2-bound resin.

here a variation on the fragment-based approach which will allow the screening of fragment libraries typically containing up to 10,000 members using only a single sample of the target. The method, called TINS, works by immobilizing the sample on a solid support that is compatible with high-resolution, static NMR methods. Mixes of compounds from the library can then be applied and binding detected by comparison of a simple, 1D NMR spectrum with that of an appropriately prepared control sample.

TINS holds significant advantages over other NMR-based ligand screening methods that focus on the compounds. Since the target is reused to screen the compound library, significantly less is required than for any other fragment-based approach. By careful selection of the control sample, a very high level of specificity is built into the assay. Further, since the compounds and the target are in roughly stoichiometrically equal amounts, binding to low-affinity second sites and other types of extremely weak binding interactions are eliminated. TINS is also sensitive to binding across a much greater range of affinities and therefore less likely to miss interesting "hits." Finally, the screening procedure is fast, sensitive, and identical for every target regardless of the size or even chemical composition.

Membrane proteins are one class of pharmaceutical targets that hold great promise but have generally remained beyond the capabilities of fragment-based methods. By greatly reducing the quantity of the target and by eliminating the need for solubilization, it may prove possible to use TINS for compound screening of some membrane proteins.

Experimental Procedures

Target Immobilization

FKBP12 was expressed and purified essentially as described [32]. The protein (12 mg) dissolved in phosphate buffered saline (PBS) was immobilized on 1 ml of Actigel ALD as recommended by the manufacturer (Sterogene, CA). The yield was approximately 90%, giving a solution equivalent concentration of 0.89 mM. The recombinant expression and purification of CSH2 was performed as described [33]. The Tris buffer was removed by gel filtration in a buffer consisting of 25 mM HEPES (pH 7.5), 100 mM NaCl, and 1 mM DTT. Column fractions were used directly for coupling according to the manufacturer's protocol. The CSH2 domain was immobilized at a ratio of 12 mg protein/ml resin, equivalent to a solution concentration of 1 mM. Blocking of unbound sites of experimental and control resins was accomplished using D_{11}-Tris, Cambridge Isotope Laboratories (Andover, MA).

A 5′ biotinylated oligonucleotide (sequence ATGGCGAATCC GTAATCGGATTCGCC) was synthesized by standard solid-state methods. Four milligrams of the unpurified oligonucleotide was incubated with 1 ml of streptavidin Sepharose (Amersham Biosciences, Freiburg, Germany) and extensively washed. Unmodified streptavidin Sepharose was used as the control resin.

All compounds used for screening were commercially available

and used without further purification, except for L-O-(2-malonyl) tyrosine (**3**), which was synthesized as described [31].

Sample Preparation

Due to the linewidth of the water signal in the presence of the Sepharose resin, standard solvent suppression schemes such as WATERGATE [34] do not achieve sufficient results. Therefore, all experiments are performed in deuterated solvents. All resins were washed extensively in D_2O until no further changes were observed in 1D ^1H NMR spectra. Experiments using immobilized FKBP12 were performed in PBS dissolved in D_2O. Experiments on the CSH2 derivatized resin were performed in 25 mM D_{11}-Tris (pH 7.5) and 100 mM NaCl dissolved in D_2O. Experiments using the biotinylated oligonucleotide bound to streptavidin Sepharose were performed in 25 mM D_{11}-Tris (pH 7.5), 140 mM NaCl dissolved in D_2O. Since a flow-injection probe capable of holding the resin is not currently available, all experiments were performed in batch mode using standard 5 mm NMR tubes. In all cases, the resins were equilibrated in the compound mixes three times by resuspending the resin in the NMR tube. NMR spectra were then directly recorded on the solution resin mixture.

NMR Spectroscopy

NMR experiments were performed on Bruker AV 400 and DMX 600 MHz spectrometers at 298 K. A simple 1D ^1H experiment was used in most cases, where residual water suppression was achieved via the WATERGATE method [34]. Typical acquisition times were about 60–70 ms. The number of transients used for each experiment is indicated in the figures. The FIDs were apodized with an exponential function with a decay constant of 5–8 Hz. All data manipulation and analysis, including transformation, baseline correction, spectral overlay, subtraction, and integration, was performed using either Bruker TOPSPIN software or self-written routines in MATLAB.

Supplemental Data

Two supplemental figures are available online at http://www.chembiol.com/cgi/content/full/12/2/207/DC1/.

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