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High-Throughput NMR-Based Screening with Competition Binding Experiments

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Abstract: The Achille's heel of ligand-based NMR screening methods is their failure to detect high-affinity ligands and molecules that bind covalently to the receptor. We have developed a novel approach for performing high-throughput screening with NMR spectroscopy that overcomes this limitation. The method also permits detection of potential high-affinity molecules that are only marginally soluble, thus significantly enlarging the diversity of compounds amenable to NMR screening. The techniques developed utilize transverse and/or selective longitudinal relaxation parameters in combination with competition binding experiments. Mathematical expressions are derived for proper setup of the NMR experiments and for extracting an approximate value of the binding constant for the identified ligand from a single-point measurement. With this approach it is possible to screen thousands of compounds in a short period of time against protein or DNA and RNA fragments. The methodology can also be applied for screening plant and fungi extracts.

Introduction

Many drugs currently on the market were developed from leads identified from high-throughput screening (HTS). Targets of therapeutic interest used in HTS are often recombinant proteins produced from cloned genes which can be expressed in different ways. A large compound collection is typically screened against these proteins for the identification of inhibitors. During the last 10 years the size of the proprietary compound collection has increased exponentially as a result of systematic application of combinatorial chemistry to different projects. Combinatorial chemistry nowadays generates large compound libraries that complement other compound libraries available from traditional medicinal chemistry and natural sources.[1] The development and application of robotics and automation have made it feasible to test large numbers of compounds in a short period of time. Several new detection systems are used for the identification of potential lead molecules. Recently, NMR has emerged as a powerful method for the detection of small molecules that interact with targets of pharmaceutical interest.[2−11] Although NMR is not a sensitive technique, it has the advantage that it is less subject to artifacts observed with other systems of detection. Recent developments in cryoprobe technology have reduced the period of time or the amount of protein necessary for the screening. Fesik and co-workers[12] have successfully implemented this technology for screening a large compound collection against isotopically labeled proteins. Chemical shift changes of cross-peaks in a $^{15}N-^{1}H$ HSQC spectrum of the target protein are monitored in the presence of a compound mixture. Deconvolution of the mixture then results in the identification of the molecule interacting with the protein (i.e. the compound responsible for the chemical shift changes). When the three-dimensional structure of the protein is known and the sequence-specific NMR assignments of the protein backbone resonances have been obtained, the method provides important structural information of the ligand binding site and ligand

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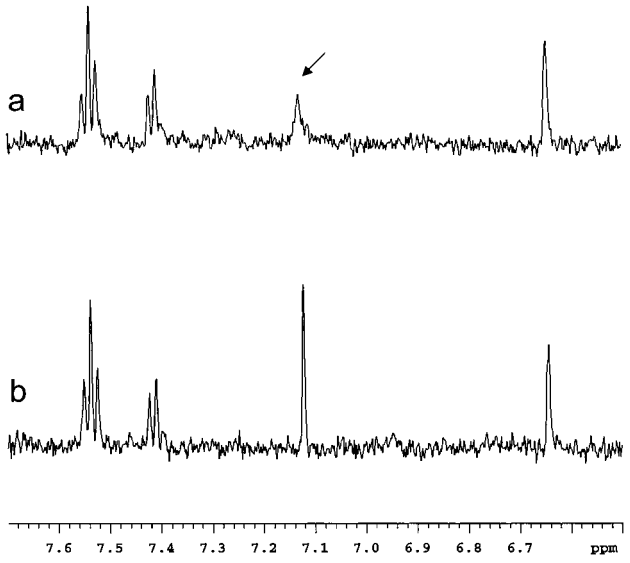

Figure 1. Expanded region of the 1D ^{1}H spectra recorded for 40 μM PHA-285283 in the presence (a) and in the absence (b) of the kinase (2 μM in PBS). The data were multiplied with a cosine window function prior to Fourier transformation. An arrow indicates the resonance undergoing significant broadening in the presence of the protein.

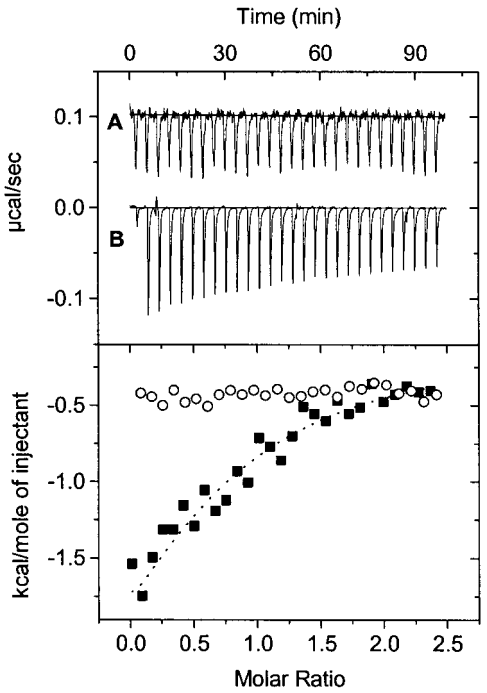

Figure 2. ITC binding experiments on the binding of PHA-285283 to the kinase. Upper panel: Injection heat effects measured during a titration of 100 μM of the protein into buffer (A) as well as into 8 μM PHA-285283 dissolved in the same buffer (B). 50 mM Tris/Cl pH 7.0, 100 mM NaCl, 1 mM DTT was used as a buffer in this experiment. Lower panel: Integrated and normalized binding heats derived from the raw data trace shown in the upper panel. Binding enthalpies measured on PHA-285283 are shown as solid squares. The corresponding fitted function is indicated as a dotted line. Dilution heat effects determined from the blank titration are shown as open circles. The binding thermodynamics are $K_B = 1.4 \pm 0.4 \times 10^5\ M^{-1}$, $\Delta H^{obs} = -3.3 \pm 0.8$ kcal/mol, $T\Delta S = 3.6$ kcal/mol, $N = 0.8 \pm 0.2$.

binding mode. Another method for performing NMR screening is based on the detection of the ligand resonances. Several NMR parameters have been proposed in the literature as a tool for ligand identification.[13−19] These methodologies permit rapid deconvolution of the screened mixtures and are particularly suited for the identification of medium- to low-affinity ligands. However, these techniques suffer from some drawbacks. First, no structural information regarding the binding site is directly available. Second, high-affinity ligands and molecules that bind covalently to the receptor escape detection because of the large excess of ligand over protein typically used in these experiments. Third, compounds with poor solubilities that are potential ligands are difficult to detect since the method requires the observation of the ligand signals.

Competition binding experiments have been used to test specificity of an identified ligand and for extracting with titration measurements the dissociation binding constant.[17c,19b] We present here a novel approach for performing efficient HTS based on properly setup competition binding experiments without the drawbacks associated with typical ligand-observed screening experiments. In addition, the methods provide an estimation of the K_D of the identified ligand with use of a single-point measurement. With this approach it is possible to screen thousands of compounds in a short period of time against protein or DNA and RNA fragments. The methodology can also be applied to the screening of plant and fungi extracts.

Results and Discussion

The first step is to identify a reference compound with medium to low affinity for the target. To accomplish this, a small library containing a few hundred very soluble and well-characterized molecules is screened by using the WaterLOGSY method.[19] The identified binders are subsequently studied with isothermal titration calorimetry (ITC) experiments to determine their binding constants. One of these compounds, based on its binding constant, is selected as the reference compound for subsequent competition binding experiments. NMR-based HTS can then be carried out by using either transverse or longitudinal relaxation experiments.

Transverse Relaxation. Figure 1 shows an expanded spectral region of a selected compound in the absence and in the presence of a Serine/Threonine p21-activated Kinase. The molecule identified with WaterLOGSY has a K_D of 7.1 μM as calculated from ITC measurements (see Figure 2). The observed transverse relaxation rate constant ($R_{2,obs} = \text{FWHH}\cdot\pi$) for a resonance of the ligand in the presence of the protein is provided by the equation[20]

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$$R_{2,obs} = \frac{[EL]}{[L_{TOT}]}R_{2,bound} + \left(1 - \frac{[EL]}{[L_{TOT}]}\right)R_{2,free} + \frac{[EL]}{[L_{TOT}]}\left(1 - \frac{[EL]}{[L_{TOT}]}\right)^2 \frac{4\pi^2(\delta_{free} - \delta_{bound})^2}{K_{-1}} \quad (1)$$

where [EL] is the concentration of bound ligand, $[L_{TOT}]$ is the total ligand concentration, $R_{2,bound}$ and $R_{2,free}$ are the transverse relaxation rate constant for the ligand in the bound and free state, respectively, δ_{bound} and δ_{free} are the chemical shifts for the resonance of the ligand in the bound and free state, respectively, and $1/K_{-1}$ is the residence time of the ligand bound to the protein. An increased broadening of the ligand resonances is observed in the spectrum of the ligand in the presence of the protein (see Figure 1a). The resonance at 7.13 ppm, representing the sharpest resonance in the spectrum of the ligand in the absence of the protein (Figure 1b), displays a significant broadening in the presence of the protein (Figure 1a). The extensive broadening results from the contribution of the third term in eq 1. The compound binds in the ATP binding pocket of the kinase. The X-ray structure of the complex reveals the close proximity of this ligand proton to a phenylalanine ring (E. Casale, personal communication). Therefore it is expected that the chemical shift difference ($\delta_{free} - \delta_{bound}$) is large for this proton resonance. The exchange term does not contribute significantly to the $R_{2,obs}$ of the other resonances.

The next step is the acquisition of NMR spectra at different ligand concentrations and fixed protein concentration or different protein concentrations and fixed ligand concentration. The latter is preferred when the reference compound is not soluble at high concentrations and when the molecule has a second low-affinity binding site that will start to be partially populated at high concentrations. Figure 3 shows the spectra of the ligand as a function of the total ligand concentration and ratio $[EL]/[L_{TOT}]$. This ratio can be calculated from the knowledge of the ligand K_D derived from ITC and the total protein $[E_{TOT}]$ and total ligand $[L_{TOT}]$ concentration used in the experiments according to the equation[20,21]

$$\frac{[EL]}{[L_{TOT}]} = \{[E_{TOT}] + [L_{TOT}] + K_D - \sqrt{([E_{TOT}] + [L_{TOT}] + K_D)^2 - 4[E_{TOT}][L_{TOT}]}\}/\{2[L_{TOT}]\} \quad (2)$$

At low $[EL]/[L_{TOT}]$ the NMR spectrum is approaching the spectrum of the ligand in the absence of the protein. The measured line width of the resonance at 7.13 ppm, or a more precise measurement, as shown in Figure 4, the signal intensity ratio of the resonance at 7.13 ppm divided by another ligand resonance, are plotted as a function of $[EL]/[L_{TOT}]$. Fitting functions are then used to extract an approximate value of the dissociation binding constant of the identified NMR hits.

The presence in a chemical mixture of a molecule competing with the reference molecule will result in a decrease in [EL]. This can be appreciated in Figure 5, which shows the NMR spectrum of the reference molecule in the presence of a seven-compound chemical mixture. The significant sharpening of the resonance at 7.13 ppm reveals the presence of a high-affinity ligand in the mixture. Deconvolution performed in the presence of the reference compound allows, as shown in Figure 5, for the identification of the high-affinity ligand. Despite the 5-fold excess of the reference molecule concentration, the NMR-identified high-affinity molecule causes almost complete displacement of the reference molecule from the receptor. Therefore, to obtain an approximate estimate of the dissociation binding constant for the high-affinity ligand and not simply a lower limit, it is necessary to record an additional experiment with an even lower concentration of the NMR hit. The signal intensity ratio of the two reference molecule resonances, or the line width of the resonance at 7.13 ppm measured from these spectra, is used as input to calculate the value $[EL]/[L_{TOT}]$ from the fitting functions of Figure 4. The knowledge of $[L_{TOT}]$ used in the NMR screening competition binding experiments permits one to calculate [EL] for the reference compound in the presence of the competing molecule. With $[L_{TOT}]$, [EL], and $[E_{TOT}]$ known it is possible to determine the apparent dissociation binding constant K_D^{app} of the reference compound according to the equation

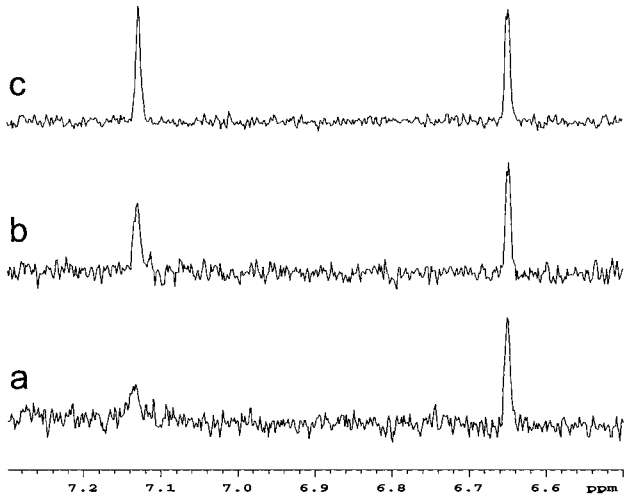

Figure 3. Expanded region of the 1D ^{1}H spectra recorded at different PHA-285283 concentrations in the presence of the kinase (1.5 μM in PBS). The spectra were acquired with 128 scans and 2.82 s repetition time. The ligand concentration was 40 (a), 80 (b) and 140 μM (c).

$$K_D^{app} = \frac{[E_{TOT}][L_{TOT}] - [E_{TOT}][EL] + [EL]^2 - [L_{TOT}][EL]}{[EL]} \quad (3)$$

In the approximation of a simple competitive mechanism the K_D^{app} is then used to extract the binding constant K_I of the NMR-identified ligand according to the equation

$$K_I = \frac{[I]K_D}{K_D^{app} - K_D} \quad (4)$$

where [I] is the concentration of the competing molecule. Table 1 shows the K_I obtained by using this approach for two different compounds with binding constants ranging from nM to μM. The good qualitative agreement between the NMR-derived and ITC-measured binding constants is very promising. It should be pointed out that the NMR-derived values were obtained from a single-point measurement. An advantage of the method proposed here is that it permits the determination of the

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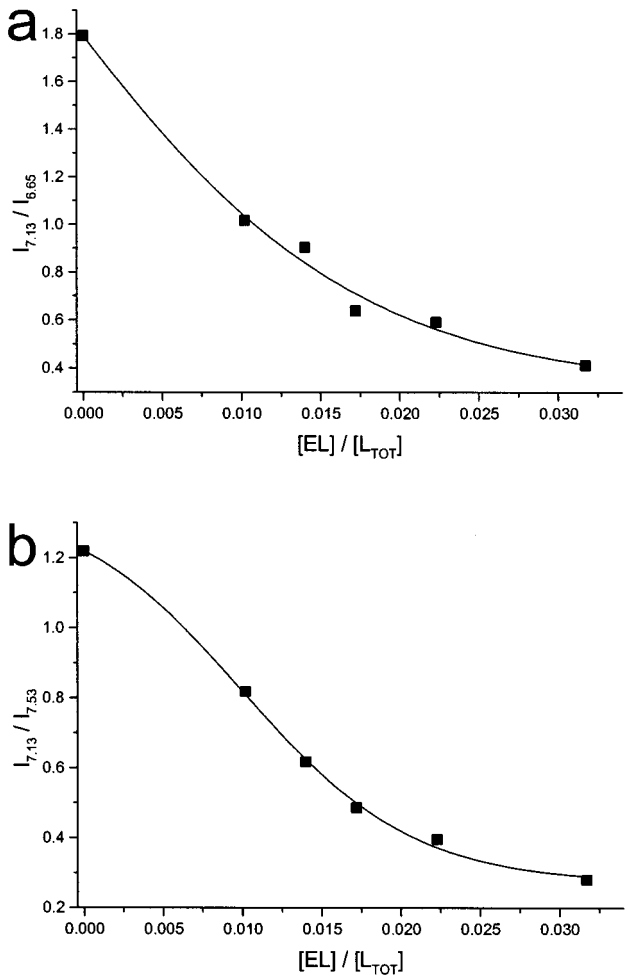

Figure 4. Plot of the signal intensity ratio of two ligand resonances $I_{7.13}/I_{6.65}$ (a) and $I_{7.13}/I_{7.53}$ (b) of PHA-285283 as a function of the ratio [EL]/[L_{TOT}]. The resonance at 7.13 ppm is the singlet (1 proton) that undergoes significant exchange broadening in the presence of the protein, the resonance at 7.53 ppm is a triplet (2 protons), and the resonance at 6.65 is a doublet (1 proton) with a small J. The ratio [EL]/[L_{TOT}] was calculated by using the ITC derived K_D of 7.1 μM for PHA-285283. The first point on the left corresponds to the value in the absence of the protein. The concentration of the protein kinase was 1.5 μM in PBS. The curves represent the best fit of the experimental points.

concentration of [I]. Such a task is achieved simply by comparing the integral of a resonance of the reference molecule for which we know the precise concentration and the integral of a resonance of the competing molecule. This is important for obtaining a meaningful value of the binding constant. An error in the effective concentration of the competing molecule due to either poor solubility of the molecule or low purity would result in a large error in the determination of the binding constant.

The example described here may represent a fortunate case since at 600 MHz one ligand resonance is in the so-called intermediate exchange regime. Working at stronger magnetic fields may be beneficial since the third term of eq 1 becomes larger and therefore many potential reference molecules may display at least one resonance in the intermediate exchange regime. However, even when the exchange term does not contribute significantly to the line width it is possible to perform similar competition binding experiments. In this case the $R_{2,obs}$ of the reference compound is measured with a Carr Purcell

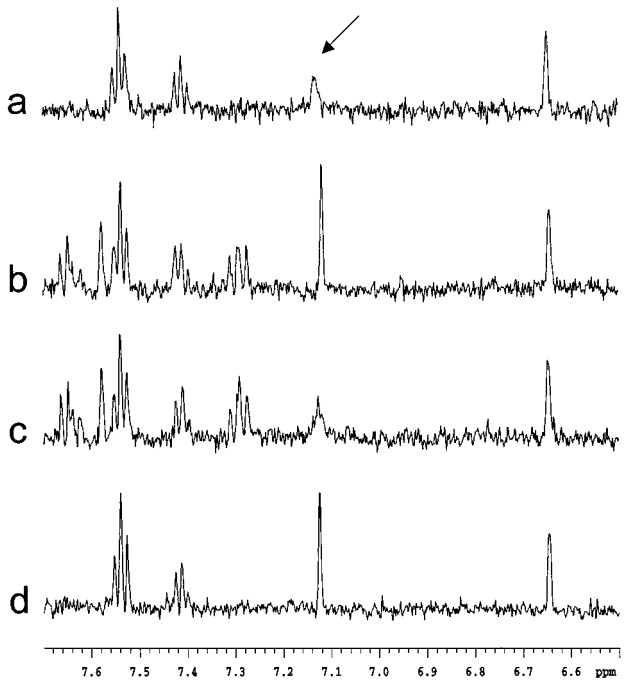

Figure 5. NMR screening and deconvolution performed with 50 μM PHA-285283 in the presence of the kinase (2 μM in PBS). Expanded region of the 1D spectra recorded in the absence (a) and in the presence (b) of a 20 μM seven-compound mixture containing the molecules SPECS AB-323/25048456, ethyl 2-quinoxalinecarboxylate, methyl isoquinoline-3-carboxylate, 7-phenyl-4-pteridinol, 2-amino-6-methylquinazolin-4-ol, 5-methylbenzimidazole, and SU-13901, (c) spectrum recorded in the presence of the chemical mixture without SU-13901, and (d) spectrum recorded in the presence of only SU-13901. The spectra were acquired with 128 scans and 2.82 s repetition time. An arrow indicates the resonance undergoing significant broadening in the presence of the protein.

Meibom Gill (CPMG) pulse sequence[22] at different [L_{TOT}] or [E_{TOT}]. The measured R_2 values or simply the signal intensity ratio of a resonance in two experiments one recorded without R_2 filter and the other recorded with a long CPMG are then plotted against [EL]/[L_{TOT}] in a similar way as the graph of Figure 4. The extent of reduction of R_2 or the change of the signal intensity ratio for the reference compound in the presence of a competing molecule permits one to extract an approximate value of the binding constant of the inhibitor. This method described here should be very sensitive in the SLAPSTIC experiment[18b] where the difference in R_2 for the bound and free state of the ligand is large. Other methods that can be used with the competition binding experiments are the single- or multiple-selective R_2 experiment (90°-τ−G-180°$_{sel}$−G-τ−water suppression) and the 1D selective TOCSY. G are gradients of the same strength, and 180°$_{sel}$ is a single- or multiple-selective inversion pulse applied at the chemical shift of the reference compound resonances. Typically, two spectra are recorded, one with $\tau = 0$ and another with a long τ period. The intensity ratios of the signals extracted from the two spectra are used for the titration measurements and for the screening process as described above. For the system used in our work, it was sufficient to record a selective R_2 experiment with a single spin−echo period and double-selective inversion of the two resonances at 7.13 and 6.65 ppm (data not shown). In cases of severe overlap, the 1D selective TOCSY can also be used. However, it is applicable only if the reference compound contains scalar-

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Table 1. Single-Point NMR-Derived Binding Constants for Two Inhibitors of the Kinase and Their Comparison with Measured ITC Values[a]

inhibitor	$I_{7.13}/I_{6.65}$	$I_{7.13}/I_{7.52}$	K_D	[I]	$[E_{TOT}]$	$[EL]/[L_{TOT}]$	[EL]	K_D^{app}	K_i^{NMR}	K_i^{ITC}
PHA-691222	1.143		5.6	40	1.5	0.0081	0.41	133.9	2.5 ± 0.7	$2.5^{(a)}$
	1.143		10.0	40	1.5	0.0079	0.39	140.0		
		0.827	5.6	40	1.5	0.0099	0.50	100.2	3.3 ± 0.9	
		0.827	10.0	40	1.5	0.0097	0.48	104.3		
PHA-691222	1.428		5.6	60	1.1	0.0041	0.21	217.4	2.2 ± 0.6	$2.5^{(a)}$
	1.428		10.0	60	1.1	0.0039	0.20	230.4		
		1.020	5.6	60	1.1	0.0059	0.30	135.6	3.7 ± 1.1	
		1.020	10.0	60	1.1	0.0058	0.29	138.8		
SU-13901	0.995		5.6	3	2.5	0.0108	0.54	180.2	0.14 ± 0.04	$0.25^{(b)}$
	0.995		10.0	3	2.5	0.0104	0.52	187.5		
		0.714	5.6	3	2.5	0.0123	0.61	152.2	0.16 ± 0.05	
		0.714	10.0	3	2.5	0.0118	0.59	159.8		

[a] $[L_{TOT}]$ is 50 μM. The K_D values of 5.6 and 10 μM of the reference compound PHA-285283 correspond to the lower and upper limits determined by the error in K_D derived from the ITC measurement. The same curves as described in Figure 4 were derived for the two K_D values. K_i^{NMR} is the binding constant in μM measured with eq 4, K_i^{ITC} is the binding constant in μM derived from ITC measurements: (a) value measured at 283 K, (b) value measured at 298 K. The binding thermodynamics parameters were $K_B = 4.0 \pm 2.0 \times 10^5$ (a), $0.4 \pm 0.02 \times 10^5$ M^{-1}(b), $\Delta H^{obs} = +1.5 \pm 0.4$ (a), -5.5 ± 0.1 kcal/mol (b), $T\Delta S = 9.1$ (a), 3.3 kcal/mol (b), $N = 1.1 \pm 0.1$ (a), 0.9 ± 0.1 (b). For PHA-691222 two experiments were recorded at different ligand and protein concentrations. The K_i^{NMR} are, from top to bottom, 2.6 ± 0.9, 3.5 ± 1.4, 2.4 ± 1.2, 4.0 ± 2.1, 0.15 ± 0.05, and 0.16 ± 0.06, with a $\pm 5\%$ error in the signal intensity ratio measurement.

coupled spin systems. The selective excitation is achieved with the same scheme used in the selective R_2 experiment. Two experiments are recorded as described above and with a fixed spin−lock period. An identical approach, but using selective R_1, is shown in the next example.

Longitutinal Relaxation. The observed longitudinal relaxation rate $R_{1,obs}$ of a molecule interacting with a macromolecule is given by the equation[23]

$$R_{1,obs} = \frac{[EL]}{[L_{TOT}]}R_{1,bound} + \left(1 - \frac{[EL]}{[L_{TOT}]}\right)R_{1,free} \qquad (5)$$

where $R_{1,bound}$ and $R_{1,free}$ are the longitudinal relaxation rate constants for the ligand in the bound and free state, respectively.

Nonselective R_1 lacks the direct dependence on τ_c (i.e. tumbling correlation time) and therefore is not suited for monitoring binding events. Selective R_1 ($R_{1,s}$) contains the direct τ_c dependence necessary for identifying small molecules interacting with macromolecules.[24−27]

$R_{1,s}$ experiments require the selective inversion of one resonance of the ligand. This technique has been used to characterize the binding of known ligands. Although these experiments have not been used in NMR screening because of the problems of achieving selectivity for a large library of chemically diverse compounds, they are particularly suited for performing HTS NMR screening with competition binding experiments. In these experiments it is sufficient to selectively invert always the same resonance of the reference molecule. Because the reference molecule is in excess compared to the protein and the molecule is a weak- to medium-affinity ligand, the observed chemical shift of the ligand resonances corresponds to the chemical shift of the free ligand. This permits the acquisition of the experiments in automation mode by using the same excitation frequency for the selective inversion. For maximum sensitivity of the experiment the resonance to be selectively inverted should be chosen from among the ligand resonances that display the largest difference of $R_{1,s}$ in the free and bound state. When possible, a singlet is preferred to obtain greater intensity and reduced problems of overlap. It should be pointed out that even in the presence of overlap it is possible to detect ligands both in the R_2 and $R_{1,s}$ experiments. In these particular cases it is necessary to record the same experiments also for the mixture in the absence of the reference compound. Subtraction of the spectra in the presence and absence of the reference compound permits one to ascertain the presence or not of a ligand. The titration experiments are performed either by keeping the protein concentration fixed and varying the reference compound concentration or by keeping the ligand concentration fixed and varying the protein concentration.

The system used in our example is human serum albumin (HSA) and the reference compound is tryptophan (Trp). The endogenous Trp and other Trp derivatives bind on Sudlow site II of HSA.[28] Therapeutic agents such as naproxen, diazepam, and ibuprofen also bind to HSA on Sudlow site II.[28,29]

An $R_{1,s}$ measurement for the C2−H resonance of Trp in the absence and presence of HSA is shown in Figure 6. In the presence of the protein the $R_{1,s}$ becomes larger. The experimental $R_{1,s}$ values of Trp are measured at different $[L_{TOT}]$ and plotted as a function of the ratio $[EL]/[L_{TOT}]$ as shown in Figure 7, where [EL] was calculated by using eq 2 with the K_D at 20 °C reported in the literature.[29] Other parameters that can be plotted as a function of $[EL]/[L_{TOT}]$ are (i) the intensity ratio of an inverted resonance with a non-inverted resonance in a $R_{1,s}$ experiment recorded with a single τ value (the time between the selective 180° pulse and the detection hard 90° pulse) as shown in Figure 7 or (ii) the intensity ratio of an inverted resonance in two $R_{1,s}$ experiments recorded with two different τ values. For better sensitivity the magnetization has to be negative with the first τ value and positive with the second τ value. In addition, these τ values should be chosen in the time region where the largest intensity difference is observed between

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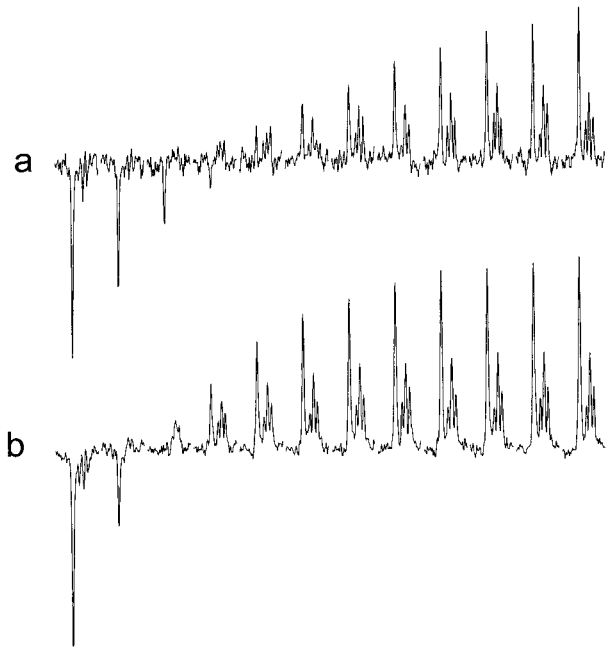

Figure 6. Selective R_1 spectra as a function of τ acquired with selective inversion of the C2−H resonance of Trp (100 μM) in the absence (a) and in the presence (b) of HSA (8 μM in PBS). Only the spectral region containing the C2−H resonance is displayed. The spectra were acquired with 8 (a) and 32 (b) scans. The repetition delay was 10.82 (a) and 8.82 s (b). An array of 16 (a) and 12 (b) τ values was used starting from 0.005 s (left) with an increment of 0.475 s. For spectrum a only the first 12 τ values are displayed for a direct comparison with spectrum b. The longer repetition time and the larger array in spectrum a was necessary because of the small $R_{1,s}$ for Trp in the absence of the protein.

spectra a and b of Figure 6. This time region can be derived from a simple simulation as shown in Figure 8. The advantage of (i) and (ii) that we define as the $R_{1,s}$ filtered experiments is the rapid data acquisition since it is not necessary to measure the $R_{1,s}$ values.

Screening is performed by using an $R_{1,s}$ experiment recorded with a single τ value or two τ values as described above. For maximum sensitivity this delay should correspond to the τ value at which the largest intensity difference is observed, as shown in Figure 8. For rapid visual inspection the τ value corresponding to the null point (i.e. $1 - 2\exp(-\tau R_{1,s}) = 0$) can also be selected as shown in Figure 9. The presence in the chemical mixture of a competing molecule will result in an $R_{1,s}$ filtered spectrum with a residual negative signal (Figure 9b) (i.e. the $R_{1,s}$ of the reference compound is smaller because of partial displacement of the reference compound from the protein). Deconvolution of the mixture carried out with the same $R_{1,s}$ experiment in the presence of the reference molecule identified diazepam as the molecule competing with Trp (Figure 9d). It is also possible to reduce the complexity of the NMR spectrum of the mixture by subtracting the $R_{1,s}$ spectrum of the mixture (Figure 10b) from the spectrum containing only the reference spectrum (Figure 10a). The resulting spectrum (Figure 10c) contains only the signals of the molecules comprising the mixture and a negative signal for the C2−H resonance of Trp. The other signals of the reference compound and the signals of the protein are absent. This strategy can be applied also in the transverse relaxation experiments described above.

Measurement of the $R_{1,s}$ or the signal intensity ratio of the inverted resonance with a non-inverted resonance permits, using

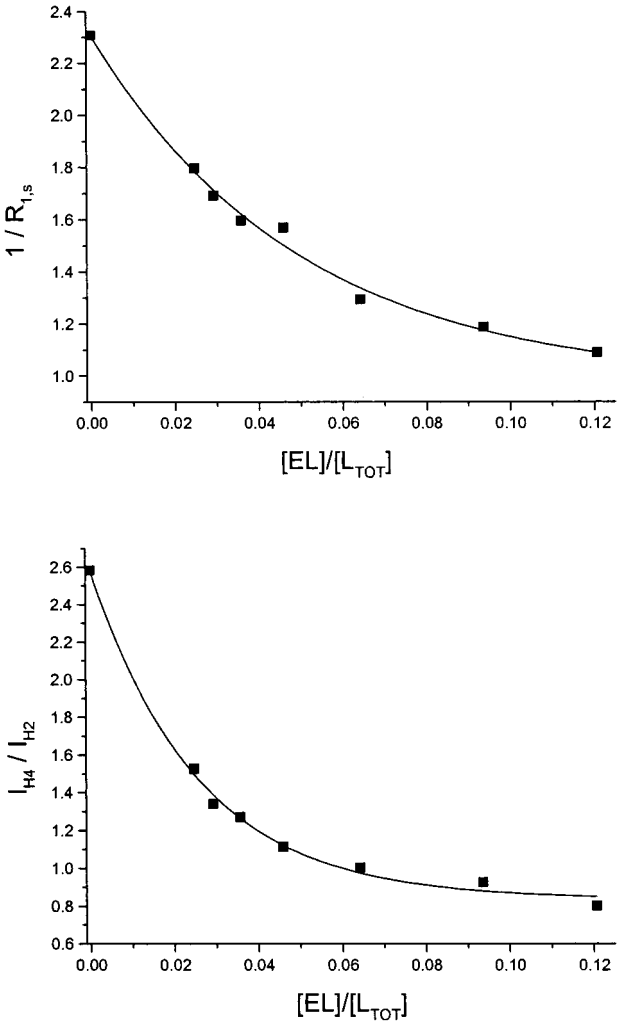

Figure 7. Top: Plot of $1/R_{1,s}$ for the C2−H resonance of Trp as a function of the ratio [EL]/[L_{TOT}]. Bottom: Plot of the signal intensity ratio for the C4−H and C2−H Trp resonances in an $R_{1,s}$ experiment recorded with selective inversion of the C2−H resonance and a single τ value ($\tau = 1.905$ s) as a function of the ratio [EL]/[L_{TOT}]. This ratio was calculated by using the equilibrium dialysis derived K_D (23 μM) for Trp measured at 20 °C. The first point on the left corresponds to the value in the absence of protein. The concentration of HSA was 8 μM in PBS. The curves represent the best fit of the experimental points. A similar diagram was obtained by plotting the signal intensity ratio for the C2−H Trp resonance in two $R_{1,s}$ experiments recorded with selective inversion of the C2−H resonance and with $\tau = 0.475$ and 1.905 s (data not shown).

the fitting functions of Figure 7 and following the same procedure described for the transverse relaxation experiments, an estimate of the binding constant of the competing molecule as reported in Table 2. The binding constant derived from NMR for diazepam although very similar in magnitude to the value measured by equilibrium dialysis reported in the literature,[29] is somewhat larger. This small difference can probably be ascribed to the presence of a low-affinity second binding site for Trp. The affinity for this site at 20 °C is of the order of several hundreds μM as measured previously by NMR. Similar findings were observed for indole-3-acetic acid (data not shown). Despite this complication, the values that are rapidly derived from the NMR screening experiments serve for most of the purposes in the early stages of drug discovery. Other techniques more suitable for precisely measuring binding constants can then be used for detailed characterization of the binding kinetics of the

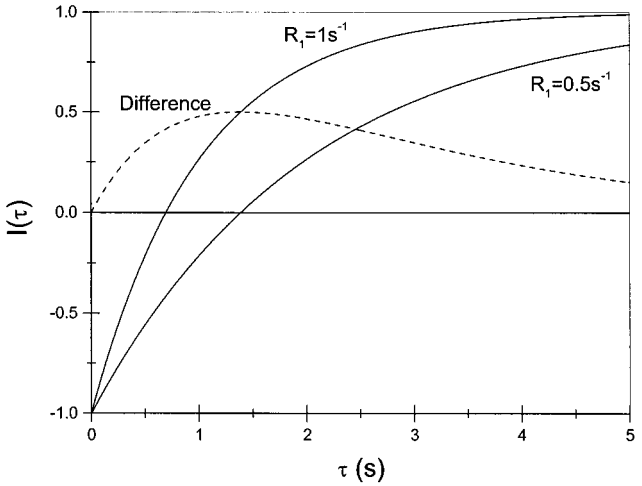

Figure 8. Signal intensity $I(\tau)$ as a function of the τ period in a $R_{1,s}$ experiment. Decays with R_1 of 0.5 and 1 s^{-1} were used in the simulation. The signal intensity difference between the two decays is shown with a dashed line.

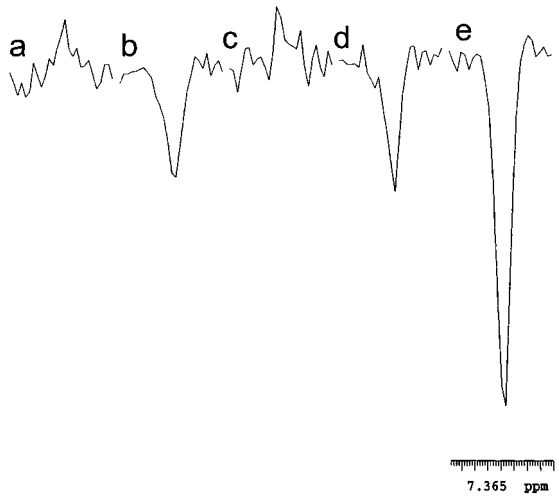

Figure 9. NMR screening and deconvolution performed with a 1D $R_{1,s}$ filtered experiment for 100 μM Trp in the presence of the protein HSA (8 μM in PBS). Expanded region containing only the selectively inverted Trp C2−H resonance recorded in the absence (a) and in the presence (b) of a 30 μM four-compound mixture containing sucrose, D-5-CH_3 Trp, L-5-CH_3 Trp, and diazepam, (c) spectrum recorded in the presence of the chemical mixture without diazepam, (d) spectrum recorded in the presence of only diazepam, and (e) spectrum recorded in the absence of HSA and the mixture. The spectra were acquired with 128 scans and 5.82 s repetition time. The τ value was 0.955 s, corresponding closely to the null point for spectrum a. Only a very weak positive signal is observed in spectra a and c.

NMR-identified ligands. A drawback of the $R_{1,s}$ competition binding experiments is the detection of only molecules competing with the reference molecule for the same protein binding site. By contrast R_2 and competition-WaterLOGSY experiments also permit the detection of weak- to medium-affinity ligands that bind at different sites. This is clearly valid only if the concentration of the compounds comprising the mixture is comparable to the concentration of the reference molecule.

Conclusion

We have shown that with properly designed competition binding experiments it is possible to perform HTS with NMR for the detection of high- to medium-affinity ligands. A drawback of the method is its limitation to compounds that compete or exert an allosteric effect on the reference compound. The initial step of the approach requires the identification of a medium- to low-affinity ligand and the determination of its binding constant with ITC measurements. Transverse or $R_{1,s}$ experiments are recorded at different $[L_{TOT}]$ or $[E_{TOT}]$ and different experimental observables are plotted as a function of $[EL]/[L_{TOT}]$. These graphs are used for designing the proper setup of the NMR experiments and for extracting an approximate value for the binding constant of the detected ligand. With this approach it is possible to rapidly screen thousands of compounds against protein or DNA and RNA fragments. The methodology can be extended also to the screening of plant and fungi extracts. Another useful application of these experiments is in the screening of a class of compounds for HSA binding affinity. Compounds that retain good activity for the desired receptor but have reduced HSA binding can be rapidly identified with this technology. This information is highly valuable in drug development.

Cryoprobe technology could further enhance the throughput of this screening process. In this case, the limiting factor will

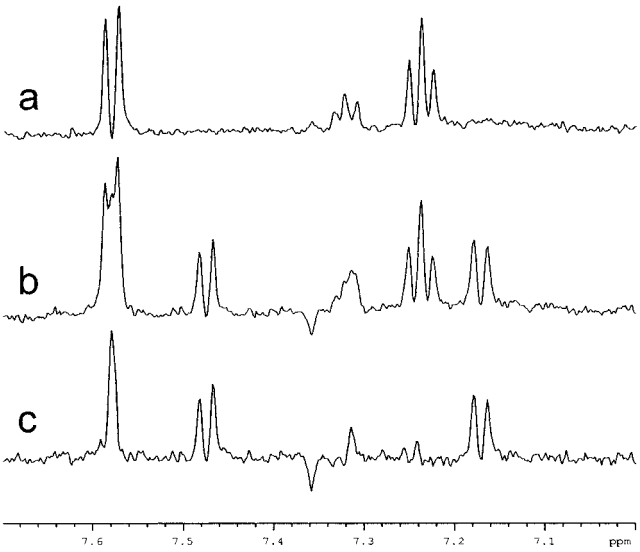

Figure 10. Expanded spectral region of the 1D $R_{1,s}$ filtered experiment for 100 μM Trp in the presence of HSA (8 μM in PBS) obtained with selective inversion of the C2−H resonance. The spectra were acquired in the absence (a) and in the presence (b) of a 30 μM four-compound mixture containing sucrose, D-5-CH_3 Trp, L-5-CH_3 Trp, and diazepam. Other experimental parameters are the same as in Figure 9. Spectrum c was obtained with the subtraction of spectrum a from spectrum b. The positive signals are the resonances of the compounds comprising the mixture; the negative signal is the C2−H resonance of Trp. The other resonances of Trp are absent in spectrum c.

Table 2. Single-Point NMR-Derived Binding Constant for Diazepam Bound to HSA and Its Comparison with the Equilibrium Dialysis Measured Value[a]

[I]	$K_D^{(a)}$	$[E_{TOT}]$	$[L_{TOT}]$	I_{4H}/I_{2H}	$1/R_{1,s}$	$[EL]/[L_{TOT}]$	[EL]	K_D^{app}	K_i^{NMR}	$K_i^{(b)}$
30	23	8	100	1.282		0.0344	3.44	127.8	6.6	2.6
30	23	8	100		1.765	0.0260	2.60	202.9	3.9	

[a] [I] is the diazepam concentration in μM, $1/R_{1,s}$ is the selective longitudinal relaxation of C2−H Trp in seconds, I_{4H}/I_{2H} is the signal intensity ratio for the C4−H and C2−H Trp resonances in an $R_{1,s}$ experiment recorded with selective inversion of the C2−H resonance and a single τ value (τ = 1.905 s). (a) Binding constant of L-Trp and (b) binding constant of diazepam in μM measured with equilibrium dialysis experiments[29] at 20 °C. Only the average value of K_D was used due to the small reported error.[29] K_i^{NMR} is the binding constant of diazepam in μM, measured with eq 4. The K_i^{NMR} are, from top to bottom, 7.0 ± 1.8 and 4.2 ± 1.6, with a ±5% error in the experimental measurement.

be the time required to change the sample, equilibrate the sample temperature, and shim the sample.

Experimental Section

Fatty acid free human serum albumin (A-3782) was purchased from Sigma and used without further purification. The kinase domain MW ~ 34000 of a Serine/Threonine p21-activated kinase was expressed as a GST fusion protein in E. Coli and purified to homogeneity after removal of GST tag.

Sucrose (S7903) and L-Trp (T0254) were purchased from Sigma, 5-CH_3-D,L-Trp (69560) was purchased from Fluka. Diazepam was purchased from Carlo Erba.

NMR samples were in phosphate-buffered saline (PBS), pH 7.4. D_2O was added to the solutions (8% final concentration) for the lock signal. The small molecules were prepared in concentrated stock solutions in deuterated DMSO and stored at 253 K.

NMR Measurements. All spectra were recorded at 293 K with a Varian Inova 600 MHz NMR spectrometer equipped with a 5 mm triple-resonance inverse probe and a Sample Management System (SMS) autosampler. Water suppression in all experiments was achieved with the excitation sculpting sequence.[30] The two water selective 180° square pulses and the four PFGs of the scheme were 2.6 and 1 ms in duration, respectively. The gradient recovery time was 0.25 ms. The data were collected with a sweep width of 7407 Hz, an acquisition time of 0.82 s, and a relaxation delay of 2.82 s. For the $R_{1,s}$ experiments, the relaxation delay ranged beween 5.82 and 10.82 s in order to achieve complete relaxation of the magnetization. Resonance selective inversion was achieved with a 24 ms duration 180° Gaussian pulse.

ITC Experiments. Calorimetric measurements were carried out with a VP-ITC titration calorimeter (MicroCal). Heats of dilution were measured in blank titrations by injecting the protein into the buffer used in the particular experiment and were subtracted from the binding heats. Thermodynamic parameters were determined by nonlinear least-squares methods, using routines included in the Origin software package (MicroCal).

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