Binding Affinity

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Ranking of High-Affinity Ligands by NMR Spectroscopy**

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NMR spectroscopy is a powerful biophysical technique to detect and characterize molecular interactions. Its high sensitivity and robustness to detect weakly binding ligands makes it an attractive tool, particularly for the early phases of drug-discovery research that comprise hit finding and hit validation.[1–3] Several methods are available to determine dissociation constants (K_D) by NMR spectroscopy,[4–7] including the direct titration of protein target with increasing amounts of ligand and competition-based experiments, such as NMR reporter screening.[8–11] However, these methods generally work only for weakly or moderately binding ligands, but not for tightly binding ligands. This situation precludes application of these methods to the later lead-optimization stage of drug-discovery projects. Potent compounds are generally evaluated in a functional assay, or by using different biophysical techniques, such as isothermal titration calorimetry (ITC) or surface plasmon resonance (SPR). In practice, however, data are sometimes conflicting, and ITC and SPR may not always be applicable. In these cases, application of an independent biophysical technique, such as NMR spectroscopy, would be desirable. Herein we describe a new NMR spectroscopic method that allows the precise determination of relative binding affinities of two tightly binding ligands. This approach is a valuable tool for the lead optimization process.

Direct titration in NMR spectroscopy is not applicable for high-affinity ligands for two reasons: First, high-affinity ligands generally have slow dissociation kinetics (slow k_{off}), so that upon titration of a ligand, a second signal set corresponding to the complexed state gradually appears while the signal set for the unbound state gradually disappears. In contrast, in the low-affinity case, fast k_{off} rates generally lead to the shifting of signals as the fraction of complexed state increases, and the extent of chemical shift change can be conveniently plotted as a function of ligand concentration in order to determine the dissociation constant, K_D. Second, it is a fundamental principle in biophysics that dissociation constants can be precisely measured only for protein concentrations in the range of K_D. The high protein concentrations (typically double-digit micromolar) required for NMR spectroscopic measurements therefore allow the precise measurement of K_D values in the micromolar or millimolar range, but not much lower. This is because the K_D is encoded in the curvature of the titration curve, and the curvature is not precisely measurable for high affinities (Figure 1). Reporter screening extends this limit to the high-nanomolar range, but not further since the dynamic range of quantification is about an order of magnitude around the reporter ligand, and the reporter ligand must show weak or intermediate binding.

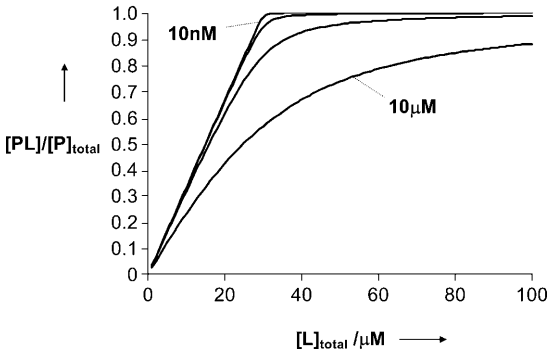

Figure 1. Calculated binding curves for an assumed one-to-one protein–ligand complex, for $K_D = 10$ nM, 100 nM, 1 μM, and 10 μM. The K_D is encoded in the curvature of the binding curve, and cannot be determined precisely for high affinities. The fraction of bound protein, $[PL]/[P]_{total}$, is plotted versus total ligand concentration, $[L]_{total}$. The calculation assumes a protein concentration of 30 μM.

The methods we present herein extend the limit of K_D determination to the high-affinity (nanomolar and sub-nanomolar) range, although no absolute affinities but only relative affinities of two competitive ligands can be determined. The methods are competition-based and can be carried out by using protein observation or ligand observation (Figure 2). Both methods take advantage of the high spectral resolution that NMR spectroscopy offers, which enables the detection of individual components and their binding state directly within the mixture. For both detection methods, the underlying principle is to offer two ligands to the protein target, and let the protein select the one with higher binding affinity. Similar competition formats have been proposed for ITC[12] and HPLC-MS,[13] although the latter detection method requires separation of the protein–ligand complexes and unbound ligands.

For protein observation, reference ^{15}N- or ^{13}C- HSQC spectra are recorded for both ligands. This step is followed by

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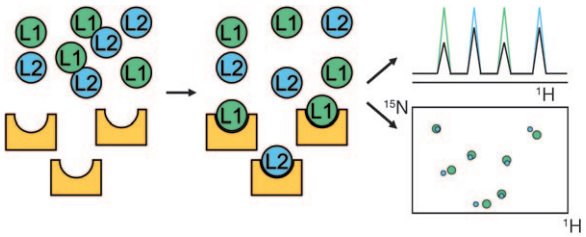

Figure 2. Principle of the experiment. An equimolar mixture of both ligands (L1, L2) under investigation is incubated and equilibrated with a sub-stoichiometric amount of protein receptor (yellow). The percentage of complexation for each ligand is then measured either by ligand observation, where the decrease of ligand signal intensity reflects complexation with protein, or by protein observation where both complexes can be distinguished by their different chemical shifts.

an HSQC spectrum with a one-to-one mixture of both ligands, wherein the concentration of each tightly binding ligand exceeds the protein concentration, so that each ligand would independently saturate the protein binding site. The protein can therefore choose the ligand for complexation, and after appropriate equilibration, it will preferentially complex with the higher-affinity ligand according to their relative K_D values. The fraction of protein bound to ligand 1 or to ligand 2 can be readily extracted from the HSQC spectrum of the mixture since both HSQC spectra show ligand-dependent chemical shift differences. All signal intensities should be normalized with respect to the intensities of the reference spectra. The fraction of protein bound to ligand 1 or to ligand 2 can then be translated into relative K_D values by the graph presented in Figure 3.[13]

For ligand observation, a one-to-one mixture of both ligands is prepared and a 1D proton spectrum is recorded of the mixture and of both individual compounds. Subsequently, a sub-stoichiometric amount of protein, corresponding to roughly half the concentration of each individual ligand, is added to the mixture and another 1D proton spectrum is recorded. Again, the protein chooses the ligand for complexation, and after appropriate equilibration, the relative complexation of both ligands will reflect the relative K_D values of both ligands. Quantification of the fraction of bound ligand can be carried out simply by measuring the fraction of unbound ligand as the percentage of remaining ligand signal. This approach takes advantage of the fact that ligand signals broaden and often change their chemical shifts upon complexation. For large proteins, the broadening effect is so strong that the signal from bound ligand completely disappears after the signal of the protein envelope (from a 1D protein spectrum of uncomplexed protein) is subtracted. For small proteins, application of a T1ρ filter removes all signals from bound ligand. Note that this ligand observation method works only for tightly binding ligands that have no contributions to the line width from chemical exchange.

The method is illustrated with the N-terminal p53-interaction domain of hdm2, a small protein domain that is considered an attractive drug target.[14] In the course of early lead optimization, discrepancies were observed between a Förster resonance energy transfer (FRET) binding assay and ITC or SPR measurements, and NMR spectroscopic measurements were needed to clarify the situation. Figure 4 (left) shows protein-observation spectra and ligand-observation spectra of a pair of hdm2 ligands (**1** and **2**). Figure 4 (left top) shows parts of HSQC spectra of ^{15}N-hdm2 in the presence of **1** in blue, in the presence of **2** in green, and in the presence of both **1** and **2** in red. Clearly, the red HSQC spectrum coincides with the green HSQC spectrum, and no red peak occurs where a blue peak occurs. This result indicates that in the presence of both **1** and **2**, hdm2 picks only ligand **2** but not ligand **1** for complexation, indicating a higher binding affinity for **2**. Estimating that 10% hdm2 complexed to **1** could be detected, the ratio of binding affinities must be at least 20-fold, as seen from Figure 3B. This result is in line with ITC measurements which indicated a 20-fold higher affinity (K_D = 5 nM versus 90 nM) for **2**.

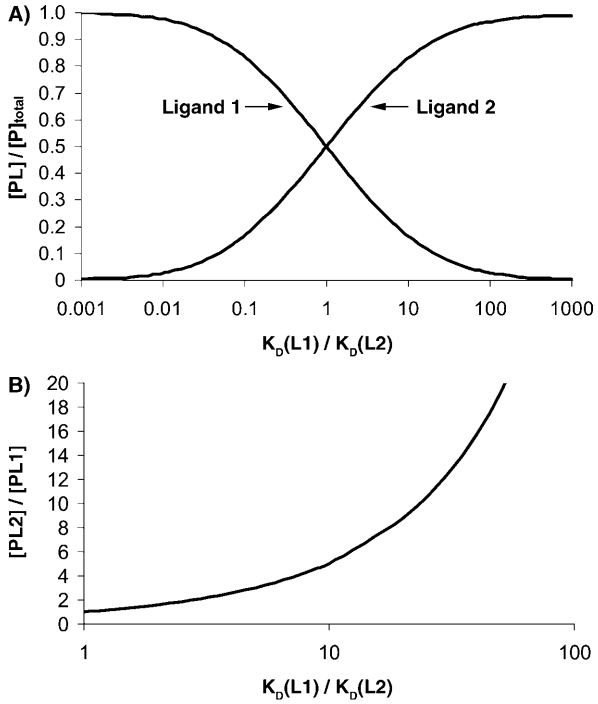

Figure 3. A) Fractions of protein complexed to ligand 1 and protein complexed to ligand 2, as a function of relative ligand affinities, calculated for equimolar ligand concentrations.[13] These curves are independent of the absolute K_D values, as long as [ligand 1] = [ligand 2] > [protein] $\gg K_D$(ligand 1), K_D(ligand 2). B) Ratio between protein complexed to ligand 2 and protein complexed to ligand 1, as a function of relative K_D value.

The same conclusions are drawn from the ligand-observed spectra shown in Figure 4 (left bottom). The upper and middle spectra show the resonance positions of **1** and **2**, respectively. The lower panel shows a mixture of **1** and **2** in the absence (black) and presence (red) of sub-stoichiometric amounts of hdm2. Only the resonances arising from **2** decrease as a result of complexation with hdm2. These spectra show that from the mixture of **1** and **2**, hdm2 picks only ligand **2** for complexation, indicating the higher binding affinity of **2**, as concluded from the protein-observation spectra.

A pair of ligands with similar binding affinities (**3** and **4**) is shown in the right panels of Figure 4. The protein-observation

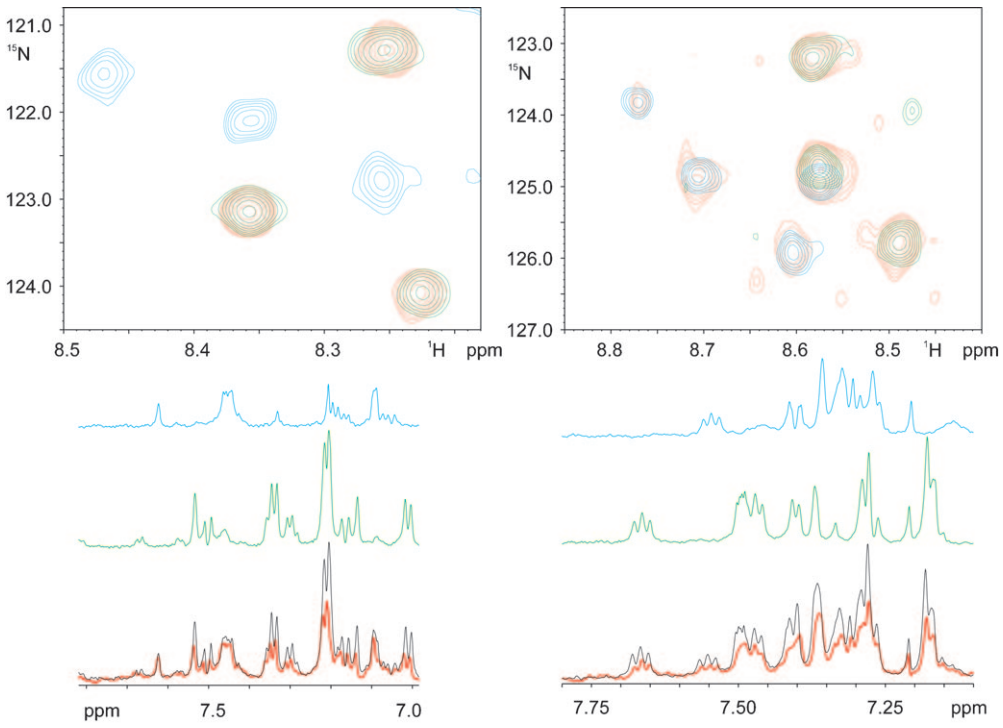

Figure 4. Left: Affinity ranking of two compounds with a large difference in K_D. Top: HSQC spectra of hdm2 in the presence of ligand **1** (blue), ligand **2** (green), and a one-to-one mixture of ligands **1** and **2** (red). Note that the red spectrum is essentially identical to the green spectrum, showing that when both ligands are offered, hdm2 complexes only ligand **2**, because of its higher binding affinity. Bottom: 1D ^{1}H spectra of ligands **1** and **2** (top and middle spectra), and a mixture of **1** and **2** (bottom spectra) in the absence (black) and presence (red) of hdm2. Note that only ligand **2** is complexed in the presence of both ligands and hdm2, indicating its higher binding affinity. A 200 ms spinlock filter was applied to attenuate protein signals, and the remaining protein envelope has been subtracted in the red spectrum of the lower panel. Right panels: Affinity ranking of two compounds with similar K_D values, see text for details.

spectra in Figure 4 (right top) show that in the mixture of **3** and **4**, 70% of hdm2 is complexed to **4** (green), and 30% is complexed to **3** (blue). This spectrum implies that the binding affinity of **4** is three-times higher than that of **3** (Figure 3B). Similarly, in the ligand-observation spectra (Figure 4; right, bottom), signals of **4** disappear slightly more as a result of complexation than the signals of **3**, indicating a similar but slightly higher binding affinity for **4**. This result is in line with the K_D values determined by ITC (460 nM and 250 nM for **3** and **4**, respectively), but is not compatible with the IC50 values determined by FRET (20 nM and 2 nM respectively).

The precision of the relative K_D values depends mainly on the precision of the relative peak intensities. Although other factors contribute,[15] this primarily depends on the signal-to-noise ratio in the NMR spectra, which in turn depends mainly on protein concentration and measuring time. If both ligands vary in their K_D by a factor of 10, then a spectrum with 15% of the maximum signal intensity must be quantified (Figure 3A). This can generally be accomplished with sufficient precision, so that the usable range of this experiment is for 0.1 to 10-fold relative binding affinity. If a higher range is required, spectra with a higher signal-to-noise ratio have to be recorded, although the associated error can become prohibitively large (Figure 3B).

We consider protein observation the more robust method, but it is also more time-consuming, requires isotope-labeled protein, and works only for those proteins that give good HSQC or TROSY spectra, typically in the molecular-weight range below 30 kDa, or 50 kDa for deuterated proteins. Protein observation reveals unexpected ligand behavior, for example, non-competitive, simultaneous binding of both ligands. It is also applicable for ranking weakly binding ligands, as long as the solubility of both ligands is much higher than their binding affinity, so that both ligands can independently saturate the protein. Ligand observation is much faster, does not require labeled protein, and works also (and actually better) for large proteins. If one of the ligands, or ideally both, contain fluorine atoms, ^{19}F spectroscopy can be advantageously used to minimize signal overlap.[8] However, it can be applied only to tightly binding and slowly exchanging ligands with dissociation rates much slower than chemical shift differences between free and bound states, so that no exchange phenomena distort the disappearance of signal, which must only be due to complexation. Contributions from fast or intermediate exchange would lead to misinterpretations, as would non-specific, simultaneous (non-competitive), or non-stoichiometric binding. If the binding kinetics are unknown, T1ρ relaxation experiments can reveal fast or intermediate exchange. Alternatively, titration of protein to single ligand shows a linear decrease of ligand signal if, and only if, slow dissociation kinetics occur. We generally prefer the protein observation method for small- to mid-size targets for which ^{15}N-labeled protein can be supplied in sufficient quantities, and perform ligand observation for larger targets, targets that cannot be prepared with isotope labels, or targets for which a large number of ligands should be ranked. Furthermore, we apply the ligand-observation method also for the mere detection of tightly binding ligands, by titrating protein to a single ligand or mixtures of ligands and observing its signal decrease.

The proposed method is significant for drug discovery since it adds NMR spectroscopy as an independent biophysical technique to the lead-optimization stage of drug discovery. In this stage, accurately ranking ligand binding potencies

is often more critical than the determination of absolute potencies, but other biochemical or biophysical methods often have an experimental error of a factor of 2–5, or even higher. As with most NMR spectroscopic applications, protein demands are higher compared to other biophysical techniques. However, in comparison with ITC, the new NMR spectroscopic method is faster and works also in cases of small binding enthalpies. In comparison with SPR, the NMR spectroscopic method does not require immobilization and is a label-free, solution-state method. While other biochemical or biophysical methods have higher throughput, the NMR method has the highest accuracy and highest precision in ranking ligand binding affinity, and thus further expands the applicability and versatility of NMR spectroscopy.[16]

Experimental Section

^{15}N-labeled hdm2(17–111) was prepared as described in the literature[17] and in the Supporting Information. The final buffer contained 25 mM d-Tris (deuterated 2-amino-2(hydroxymethyl)-1,3-propanediol), pH 8.0, 100 mM NaCl, 0.25 mM TCEP (tris(2-carboxyethyl)-phosphine). All NMR spectroscopic experiments were carried out at 298 K on a Bruker AV600 spectrometer with a TCI cryoprobe. Protein and ligand concentrations were 12 μM and 20 μM, respectively. HSQC spectra were recorded with 180 *t*1 increments with 128 transients each (7 h), and 1D ^{1}H spectra were recorded with 1024 transients (50 min). The same samples were used to obtain the protein- and ligand-observed spectra in Figure 4, although the latter would not need ^{15}N-labeling of hdm2.

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