Exploring Weak Ligand–Protein Interactions by Long-Lived NMR States: Improved Contrast in Fragment-Based Drug Screening**

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Abstract: Ligands that have an affinity for protein targets can be screened very effectively by exploiting favorable properties of long-lived states (LLS) in NMR spectroscopy. In this work, we describe the use of LLS for competitive binding experiments to measure accurate dissociation constants of fragments that bind weakly to the ATP binding site of the N-terminal ATPase domain of heat shock protein 90 (Hsp90), a therapeutic target for cancer treatment. The LLS approach allows one to characterize ligands with an exceptionally wide range of affinities, since it can be used for ligand concentrations [L] that are several orders of magnitude smaller than the dissociation constants $K_D$. This property makes the LLS method particularly attractive for the initial steps of fragment-based drug screening, where small molecular fragments that bind weakly to a target protein must be identified, which is a difficult task for many other biophysical methods.

Over the last decade, fragment screening has emerged as a powerful way to identify new lead compounds[1] and fragment-based drug discovery (FBDD) has gained wide acceptance in pharmaceutical industry, as evidenced by the significant number of fragments that have been developed into lead series and clinical candidates.[1,2] For this purpose, relatively small libraries of carefully chosen compounds with low molecular weights (120–250 Da) are screened to identify fragments that can weakly bind to a protein target. Useful fragments typically have dissociation constants $K_D$ ranging from 0.1 to 10 mM or greater. Structural biology is usually employed to establish their binding mode and guide their optimization. Techniques that can detect ligand–protein complexes, such as X-ray crystallography, surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), and high-concentration assays can be used for fragment screening. The output of these target-based methods depends on the fraction of bound protein with respect to the total protein concentration. If the binding affinities are weak, the equilibrium can only be shifted by increasing the concentration of the fragments, which must therefore be highly soluble, a requirement that is difficult to meet.

In ligand-based methods the output is given by the fraction of bound ligands with respect to total ligand concentration. So despite its low intrinsic sensitivity, the detection of ligands by NMR spectroscopy can be used over an extremely wide dynamic range of dissociation constants $K_D$ while requiring only relatively low protein and ligand concentrations. In contrast to the above-mentioned biophysical techniques, one can perform screening with ligand concentrations [L] that are orders of magnitude lower than the corresponding dissociation constants $K_D$. Among the best-known NMR methods, one should mention the transfer of magnetization from the solvent to protein-bound ligands (“Water-LOGSY”),[4] magnetization transfer from the protein to the ligand by saturation transfer difference (STD),[5] the accelerated transverse relaxation of $^1$H or $^{19}$F nuclei attached to bound ligands measured by Carr–Purcell–Meiboom–Gill (CPMG) spin echo sequences, and selective measurements of relaxation rates of $^1$H nuclei of bound ligands.[6] These methods exploit a difference in relaxation rates between bound and free ligands [Eq. (1)].

$$\Delta R_i = R_i^{\text{bound}} - R_i^{\text{free}}$$ (1)

Here $i = 1$ stands for longitudinal relaxation, $i = 2$ for transverse relaxation, $i = 1\rho$ for relaxation in the rotating frame, $i = \text{LLS}$ for long-lived states, $i = \text{LLC}$ for long-lived coherences, etc. The difference $\Delta R_i$ determines the observed relaxation rate $\langle R_i^{\text{obs}} \rangle$, which in the fast-exchange regime (provided the exchange rates are faster that the difference in chemical shifts) is a weighted average of $R_i^{\text{bound}}$ and $R_i^{\text{free}}$ determined by the molar fractions [Eq. (2)].

$$\langle R_i^{\text{obs}} \rangle = R_i^{\text{bound}} f_{\text{bound}} + R_i^{\text{free}} f_{\text{free}}$$ (2)
Long-lived states (LLS) can be best excited within dipole–dipole interactions between the two spins involved.\cite{9} As their name implies, LLS have the property that their magnetization decays with a low rate constant $R_{LLS}$ that is often much smaller than the longitudinal relaxation rate $R_I$. Ratios $R_I/R_{LLS}$ up to 37 have been measured for pairs of protons\cite{10} making LLS valuable probes to study slow diffusion\cite{11} and slow exchange phenomena\cite{12} and to preserve hyperpolarization induced by DNP.\cite{13} It has been shown that even a small change of the chemical shifts of the nuclei that carry the LLS, $\Delta \gamma$, can be detected. The experimental conditions must be chosen to yield a sufficiently large contrast $C_{LLS}$, and hence increase the contrast $C_{LLS}$ due to differences in the two chemical shifts $\Delta \gamma$ to render the two spins magnetically equivalent. When this rf field is effective, $R_{LLS}$ is substantially larger than the longitudinal relaxation rate $R_I$.\cite{14}

\begin{equation}
C_{LLS} = \frac{R_{obs}^{LLS} - R_{free}^{LLS}}{R_{obs}^{LLS}} \times 100\%
\end{equation}

It has been proposed recently that LLS can be used for ligand–protein screening.\cite{15} The difference $\Delta R_{LLS}$ can be much larger than $\Delta R_I$, $\Delta R_S$, etc., so that it is possible to achieve a high contrast $C_{LLS}$ even for high ligand/protein ratios, making LLS-based screening particularly attractive for a fragment-based approach that seeks to identify weakly binding ligands.

LLS are nuclear spin states that are immune to dipole–dipole interactions between the two spins involved.\cite{9} As their name implies, LLS have the property that their magnetization decays with a low rate constant $R_{LLS}$ that is often much smaller than the longitudinal relaxation rate $R_I$. Ratios $R_I/R_{LLS}$ up to 37 have been measured for pairs of protons\cite{10} making LLS valuable probes to study slow diffusion\cite{11} and slow exchange phenomena\cite{12} and to preserve hyperpolarization induced by DNP.\cite{13} It has been shown that even a small change of the chemical shifts of the nuclei that carry the LLS upon binding can boost $R_{LLS}^{bound}$, and hence increase the contrast $C_{LLS}$ since the radio-frequency (rf) field that must be applied to sustain the LLS becomes inefficient if it is not applied exactly halfway between the two chemical shifts. The combination of a large contrast $C_{LLS}$ and a slow relaxation rate $R_{LLS}^{free} < R_I^{free}$ makes the dynamic range of LLS screening particularly attractive, since one can detect the binding of fragments with dissociation constants $K_D$ that cover a wide range. In this work we demonstrate that it is possible to measure dissociation constants up to 12 mm, where all other known biophysical techniques fail, including conventional NMR methods based on the observation of ligands.

To illustrate the advantages of LLS screening, we determined the contrast $C_{LLS}$ for the LLS signals of the aromatic protons $I$ and $S$ of vanillic acid diethylamide (ligand II), represented by bold red letters $H$ in Figure 1, during a titration against the N-terminal ATPase domain of heat shock protein 90 (Hsp90). Excitation of long-lived states (LLS) was achieved as described by Sarkar et al.\cite{10} Three nonselective “hard” pulses $90^\circ$, $-180^\circ$, $-45^\circ$ were used to generate zero-quantum coherences and $S_I S_S$ terms. A monochromatic continuous-wave (CW) radio-frequency field (rf) was applied during a delay $\Delta$ exactly halfway between the two chemical shifts\cite{14} to render the two spins $I$ and $S$ magnetically equivalent. When this rf field is effective, the dipolar interaction between spins $I$ and $S$ does not contribute to the LLS relaxation, hence $R_{LLS} < R_I$. Finally, two more pulses $45^\circ$, $-180^\circ$, $-45^\circ$ convert the LLS into observable $I$ and $S$ terms.

Table 1 shows mole fractions $X_{bound}$ of bound ligands for different ligand/protein ratios and the corresponding contrast $C_{LLS}$. Even for a large ligand-to-protein ratio $[L]/[P] = 272$, one observes a dramatic 45% contrast.

A contrast $C_{LLS} = 23\%$, corresponding to a ratio $R_{LLS}^{free}/R_{LLS}^{free} = 1.3$ could be achieved with a ratio $[L]/[P]_{free} = 707$. In other words, under conditions where less than 0.2% of the ligand was bound to the protein. Compared to other $^1H$-detected NMR methods, which suffer from lower contrast, this method allows ligand binding to be detected for low protein concentrations and/or low binding affinities. One can thus more easily adjust the concentrations of proteins and ligands to study very weak affinities in screening assays. For example, to detect ligands with $K_D < 1$ mM and $[L] = 500$ mM, one would require a protein concentration $[P] = 3$ $\mu$m; alternatively, if $[P] = 20$ $\mu$m one can detect binding if $K_D > 10$ mM. Such weak affinities are typically encountered for fragments that bind protein–protein interfaces. This offers considerable advantages over fragment screening by traditional ligand-based NMR methods.

Long-lived states (LLS) can be best excited within isolated two-spin systems, although larger spin systems can also support LLS.\cite{15} Many small fragments contain suitable pairs of $^1H$ or $^13C$ nuclei. LLS screening is most effectively run in competition mode, as proposed by Dalvit et al.\cite{16} for traditional $R_I$ and $R_S$ experiments: a strongly binding ligand
partly displaces a weakly binding “spy” ligand from the binding site, so that one observes a decrease of the relaxation rate \( R_{\text{LLS}} \) of the displaced spy ligand. Thus, it is possible to determine the affinity of strongly binding ligands by monitoring the rate \( R_{\text{LLS}} \) of a spy ligand. Furthermore, by keeping the concentration of the spy ligand low, one can study competing ligands with limited solubility. This is a major advantage not only for screening mixtures, but also for determining the affinities of weakly binding fragments.

Prior to starting a fragment screening campaign, a small number of fragments are typically screened using different NMR methods. The NMR assay can then be customized to a specific protein target. During this phase a number of hits may be identified so that one can select a spy ligand that is suitable for competitive screening. To show the applicability of the method, we measured relaxation rates \( R_{\text{LLS}} \) in a group of three ligands that were known to bind Hsp90 in its ATP binding site, located in the N-terminal ATPase domain of the protein.[16] Adenosine diphosphate (ADP, ligand I) is the product of the ATPase reaction. Vanillic acid diethylamide (ligand II) and 2-aminopyrimidine (ligand III) have been identified as weakly binding ligands by fragment screening.[16]

LLS experiments were performed by focusing attention on pairs of scalar-coupled protons in these three compounds (in bold red letters in Figure 1). The two diastereotopic protons H5 and H5' of the ribose group were selected to lose LLS in ADP, while pairs of aromatic protons were selected for compound II (for ligand III, we could not excite any LLS with a useful ratio \( R_{\text{LLS}}/R_{\text{LLS}} > 1 \)). To measure the rates \( R_{\text{LLS}} \), direct titration curves were measured for 0.3 mM < \([L]_{\text{tot}} < 6 \) mM in the presence of \([P]_{\text{tot}} = 10 \) mM Hsp90. The curves were fitted to the following function[17] given in Equation (4) to extract \( K_D \) and \( R_{\text{LLS}} \).

\[
\langle R_{\text{LLS}} \rangle = \frac{[P]}{[L]_{\text{tot}}} \left( R_{\text{LLS}}^{\text{bound}} - R_{\text{LLS}}^{\text{free}} \right) + R_{\text{LLS}}^{\text{free}} \tag{4}
\]

Here the ratio \([P]/[L]_{\text{tot}}\) is a function of the dissociation constant \( K_D \,[17] \). Table 2 also gives dissociation constants \( K_D \) determined by isothermal titration calorimetry (ITC)[16] for comparison.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>( K_D ) ([\mu M] )</th>
<th>( K_D ) ([\mu M] )</th>
<th>ITC ( R_{\text{LLS}}^{\text{obs}} ) ([\text{s}^{-1}] )</th>
<th>ITC ( R_{\text{LLS}}^{\text{obs}} ) ([\text{m} \text{s}^{-1}] )</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>15 ± 10</td>
<td>10</td>
<td>77 ± 6</td>
<td>731 ± 7</td>
</tr>
<tr>
<td>II</td>
<td>708 ± 97</td>
<td>790</td>
<td>94 ± 3</td>
<td>228 ± 11</td>
</tr>
<tr>
<td>III[4]</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>1000</td>
<td>1000</td>
</tr>
</tbody>
</table>

[a] For ligand III no useful LLS signal could be observed.

The equilibrium constants determined by LLS and ITC are in fair agreement. In order to detect fragments with 100 \( \mu M < K_D < 10 \) mM in competitive binding experiments, the weakest ligand (ligand II) that could be identified by ITC was chosen as a spy molecule. Figure 2 shows the LLS signals of ligand II in the absence (top) and in the presence (middle) of Hsp90. When Astex’s clinical Hsp90 inhibitor AT13387 is added (bottom), the signal is almost completely restored, demonstrating that both ligand II and the high-affinity inhibitor bind Hsp90 to the same ATP binding site.

If a library of, say, 1000 compounds is to be screened against a protein target, it is most efficient to screen “cocktails” containing typically three to ten ligands, to reduce experimental time and protein consumption. We tested the performance of LLS screening in competition mode with a mixture containing known binders and known nonbinders. Ligands V, VI, and VII (see Figure 3) had previously been identified as weak ADP-competitive binders during a screening campaign at Astex.[16]

In the absence of competing binders, the interaction between the spy ligand and the protein leads to rapid LLS relaxation and hence to the attenuation of the LLS signal (spectrum 1 in Figure 4); conversely, the presence of a competitor leads to a partial displacement of the spy ligand, hence to slower LLS relaxation and a partial restoration of the LLS signal of the spy (spectrum 2 in Figure 4). This change in LLS

![Figure 2](image-url)
Once weak binders have been identified, their dissociation constants \( K_d \) can be determined from \( K_{D_{\text{LLS}}}^{\text{avg}} \) of the weakly binding ligand \( \text{[IV]} \). \( K_{D_{\text{LLS}}}^{\text{avg}} \) was used to measure the dissociation constant of the four fragments shown in Figure 2. The problem could be further reduced by selecting a weak ligand with a larger difference in chemical shifts, in order to decrease the likelihood that the two doublets stemming from the LLS overlap with other signals.

Once weak binders have been identified, their dissociation constants \( K_d \) can be determined from \( K_{D_{\text{LLS}}}^{\text{avg}} \) of the weakly binding ligand in the presence of a constant amount of a weak binder or vice versa.\(^{[11]}\) Titration of a spy ligand makes it possible to use the same experimental setup for different fragments. The highest concentrations of the competing ligands are limited only by their aqueous solubility. At each concentration, the rates \( R_{\text{LLS}} \) can be obtained from the ratio of LLS signal intensities observed with two different sustaining delays \( \tau_a \) and \( \tau_b \). In order to verify that our procedures are self-consistent, the dissociation constant \( K_d \) of ADP (ligand I) was determined by monitoring \( R_{\text{LLS}} \) in vanillic acid diethylamide (ligand II) while titrating 500 \( \mu \)M [I] with 10 \( \mu \)M Hsp90 and a fixed ADP concentration \( [L_1] = 15 \mu \)M. The resulting \( K_d(L_1) = 8 \pm 3 \mu \)M is in reasonable agreement with \( K_d(L_1) = 15 \pm 10 \mu \)M determined by direct titration of ADP (ligand I) against Hsp90 (Table 2). Following a similar procedure, the affinity of 2-aminopyrimidine (ligand III) (Figure 5) was determined with 10 \( \mu \)M Hsp90, using a fixed concentration [I] = 7 mM, and titrating 500 \( \mu \)M [I] with 5 mM [II]. The fitted dissociation constant \( K_d(L_{\text{III}}) = 11 \pm 2 \) mM suggests very weak binding of this ligand to the protein, which must, however, clearly be specific to explain these observations. The fragment bound to Hsp90 was also observed by X-ray crystallography, as reported by Murray et al.\(^{[16]}\).

This approach was used to measure the dissociation constants \( K_d \) of the four fragments shown in Figure 2. The values derived from full titrations and from experiments with a single concentration are in good agreement (Table 3).

**Table 3:** Dissociation constants of fragments measured by LLS competition binding experiments.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>( K_d^{\text{comp}} ) [mM]</th>
<th>( K_d^{\text{LLS}} ) [mM] (from first point of titration)</th>
<th>Concentration [L] of competing ligands (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.008 ± 0.003</td>
<td>0.010</td>
<td>0.015</td>
</tr>
<tr>
<td>II</td>
<td>(spy)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>III</td>
<td>11 ± 2</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>IV</td>
<td>0.9 ± 0.2</td>
<td>0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>V</td>
<td>1.4 ± 0.9</td>
<td>2.2</td>
<td>1</td>
</tr>
<tr>
<td>VI</td>
<td>1.7 ± 0.9</td>
<td>2.2</td>
<td>1.6</td>
</tr>
<tr>
<td>VII</td>
<td>7 ± 1</td>
<td>5</td>
<td>2.9</td>
</tr>
</tbody>
</table>
latter procedure is to be preferred when fragment hits are ranked according to affinity after a screening campaign.

Note that the choice of the spy molecule determines the experimental conditions of the LLS assay. With our choice of a spy molecule (ligand II, $K_D = 790 \mu M$), a concentration of 7 mM of the competing ligand III ($K_D = 12 \text{ mM}$) gives rise to 19% contrast (first point of black curve in Figure 5). This can be reduced to 3.3 mM to give rise to a 10% contrast, which is sufficient to show binding in screening experiments, as shown in Figure 4 for ligand V (difference between spectra 1 and 2). If the expected affinities of fragments for a particular target are on the order of $K_D = 5 \text{ mM}$ or higher, it is most convenient to identify and use a weaker spy molecule that would ensure a 10% contrast while working at lower fragment concentrations [see Eq. (4)]. As a consequence, one can effectively screen and identify weak fragments with very low solubility.

In summary, the detection of LLS requires ligands that contain a (preferably isolated) two-spin system, a condition that cannot easily be fulfilled for all fragments in a screening “cocktail”. We have therefore shown that the LLS method can be used very effectively in a competitive approach, where the displacement of a suitable spy molecule is detected and thus extending the sensitivity of detection compared to methods such ITC and high concentration assays may fail, in particular when the ligand solubility is limited, enables the investigation of structure–activity relationships and the guidance of initial steps of hit-optimization chemistry.

Keywords: dissociation constants · drug discovery · fragment screening · ligand binding · NMR spectroscopy


