NMR Spectroscopy

International Edition: DOI: 10.1002/anie.201411424
German Edition: DOI: 10.1002/ange.201411424

Affinity Screening Using Competitive Binding with Fluorine-19 Hyperpolarized Ligands**

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Abstract: *Fluorine-19 NMR and hyperpolarization form a powerful combination for drug screening. Under a competitive equilibrium with a selected fluorinated reporter ligand, the dissociation constant (K_D) of other ligands of interest is measurable using a single-scan Carr–Purcell–Meiboom–Gill (CPMG) experiment, without the need for a titration. This method is demonstrated by characterizing the binding of three ligands with different affinities for the serine protease trypsin. Monte Carlo simulations show that the highest accuracy is obtained when about one-half of the bound reporter ligand is displaced in the binding competition. Such conditions can be achieved over a wide range of affinities, allowing for rapid screening of non-fluorinated compounds when a single fluorinated ligand for the binding pocket of interest is known.*

Fluorine atoms rarely occur in biology, but are used in pharmaceuticals. Consequently, NMR spectroscopy of ^{19}F is often part of the screening method for protein–ligand interactions in drug discovery.[1–4] Detection of ^{19}F spectra is convenient because they are free of background signals from the protein or even the solvent. Owing to the high gyromagnetic ratio and 100% abundance of the ^{19}F isotope, the NMR detection sensitivity is nearly as high as for protons. Nevertheless, a relatively high ligand concentration is required to obtain a sufficient signal-to-noise (S/N) ratio for identification of the protein–ligand interaction. Recently, we demonstrated that dissolution dynamic nuclear polarization (D-DNP), a hyperpolarization technique to increase the NMR sensitivity by several orders of magnitude,[5] is applicable to ^{19}F NMR in screening experiments.[6] A limitation of ^{19}F NMR detection lies in the omission of non-fluorinated drug candidates. However, if a suitable fluorinated ligand is known for a protein of interest, this ligand can be used in competitive binding experiments to report on other, non-fluorinated ligands. Competitive binding experiments have been demonstrated using proton- or fluorine-based NMR spectroscopy,[7–10] as well as with hyperpolarized long-lived spin states.[11] If the reporter ligand is in fast exchange with the protein, ligands with a range of binding affinities can thus be identified, such as using the "fluorine chemical shift anisotropy and exchange for screening" method.[10] Herein, we demonstrate the measurement of dissociation constants of a non-fluorinated ligand from a rapid, single-scan measurement of the ^{19}F spin–spin relaxation rate (R_2) of a DNP hyperpolarized reporter ligand.

A CF_3-functionalized ligand, TFBC (Figure 1a), acts as a reporter ligand for binding to the serine protease trypsin from *B. taurus*. Binding of a second, non-fluorinated ligand causes changes in the fraction of bound reporter ligand, which are manifested as changes in R_2, observed in single-scan Carr–Purcell–Meiboom–Gill (CPMG) experiments.[12] For demonstration, Figure 1b shows spin-echo intensities of TFBC recorded without hyperpolarization. A measurement under competition with the trypsin inhibitor benzamidine is complemented by two reference experiments.

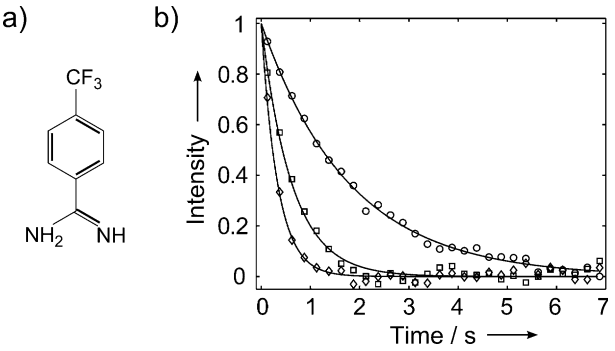

Figure 1. a) Structure of reporter ligand 4-(trifluoromethyl)benzene-1-carboximidamide (TFBC). b) Single-scan CPMG spin-echo intensities, averaged 128 times, of 100 μM TFBC without hyperpolarization, obtained in absence (○) and presence (◇) of 1.8 μM trypsin, and in the presence of both 1.8 μM trypsin and 40 μM benzamidine (□). Each point shown is the average of 581 successive data points, which were measured at time intervals of 420 μs. R_2 relaxation rates were obtained from the fit to a single exponential; $R_{2,r}^{(f)} = 0.60\ s^{-1}$ (○), $R_{2,r}^{(nc)} = 3.00\ s^{-1}$ (◇), $R_{2,r}^{(c)} = 1.61\ s^{-1}$ (□).

The relaxation rate at the competitive equilibrium ($R_{2,r}^{(c)}$) is in-between those of free reporter ligand ($R_{2,r}^{(f)}$) and of the reporter in the presence of protein without competition ($R_{2,r}^{(nc)}$). The dissociation constant of the competing ligand of interest ($K_{D,c}$) can be determined from the measured R_2 values, which depend on the fraction of bound reporter ligand $p_{b,r} = [RP]/[R]_{tot}$ using a model for competitive binding to a single site (concentrations $[RP]$ = reporter-ligand–protein complex and $[R]_{tot}$ = total reporter ligand).[13–15] For small $p_{b,r}$, $R_{2,r}$ is a $p_{b,r}$-weighted average of relaxation rates of free and bound ligand (see Supporting Information).[1] Practically,

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[**] Financial support from the National Institutes of Health (Grant 5R21GM107927) is gratefully acknowledged.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201411424.

it is convenient to introduce a parameter depending on measured relaxation rate differences [Eq. (1)]

$$\alpha = \frac{R_{2,r}^{(c)} - R_{2,r}^{(f)}}{R_{2,r}^{(nc)} - R_{2,r}^{(f)}} \quad (1)$$

which also cancels paramagnetic contributions from free radicals used in DNP. Under the above assumption, α relates the fractions of bound reporter ligand with and without competition ($p_{b,r}^{(c)}$ and $p_{b,r}^{(nc)}$, respectively) [Eq. (2)]

$$p_{b,r}^{(c)} = \alpha \cdot p_{b,r}^{(nc)} \quad (2)$$

Consequently, $p_{b,r}^{(c)}$ can be found using the result from the R_2 measurements in combination with Equation (3)

$$p_{b,r}^{(nc)} = \frac{[R]_{tot}+[P]_{tot}+K_{D,r} - \sqrt{\left([R]_{tot}+[P]_{tot}+K_{D,r}\right)^2 - 4[R]_{tot}[P]_{tot}}}{2[R]_{tot}} \quad (3)$$

which depends only on known parameters (dissociation constant of reporter $K_{D,r}$, and total concentrations of protein, $[P]_{tot}$, and reporter $[R]_{tot}$).[16] By solving the equations for the binding equilibrium, an apparent dissociation constant for the reporter ligand ($K_{D,app}$) can first be calculated [Eq. (4)].[7]

$$K_{D,app} = [P]_{tot}\left(\frac{1}{p_{b,r}^{(c)}} - 1\right)[R]_{tot}\left(1 - p_{b,r}^{(c)}\right) \quad (4)$$

This expression can be written in terms of the free competing ligand $[C]$ [see Eq. (S17) in the Supporting Information], and solved for the dissociation constant of the competing ligand, resulting in Equation (5).

$$K_{D,c} = \frac{[C] \cdot K_{D,r}}{K_{D,app} - K_{D,r}} \quad (5)$$

The data from the non-hyperpolarized measurement in Figure 1b, and knowledge of $K_{D,r} = 142\ \mu M$,[6] yields $K_{D,c} = (15.7 \pm 1.9)\ \mu M$ for benzamidine.

By utilizing D-DNP, ^{19}F NMR signal enhancements of over 1000-fold were achieved. Hyperpolarized ligands were injected into mixtures of competing ligand and protein, monitored by acquisition of single-scan CPMG data. It was possible to reduce the final reporter ligand and protein concentrations considerably to 1 μM. Data for three ligands at three different concentrations is shown in Figure 2.

Information on binding is obtained in two different regimes. A simple confirmation of binding can be made if the reporter ligand is almost completely displaced, by observing $R_{2,r}^{(c)} \approx R_{2,r}^{(f)}$ (panels in upper right corner in Figure 2). A numerical value for $K_{D,c}$ can be calculated in the case of partial displacement, when $R_{2,r}^{(nc)} > R_{2,r}^{(c)} > R_{2,r}^{(f)}$ (panels on the upper left to lower right diagonal in Figure 2). The R_2 and $K_{D,c}$ values obtained from these experiments are summarized in Table 1. For validation, non-hyperpolarized single-scan (Tables 1 and Table S1) and multi-scan (Figure S1 and Table S2) CPMG experiments were conducted using higher sample concentrations. The DNP NMR measurements are in good agreement with those, as well as with literature values under similar, but not identical conditions (leupeptin, 0.031–0.4 μM; benzamidine and benzylamine, 18 μM and 300 μM at 100 mM Tris, pH 8.0, 298 K).[17–19] Lowering the final concentrations of protein and competing ligand to 0.5 μM, still resulted in a $K_{D,c}$ value that is within error limits of the value from Table 1 (Figure S6).

Whereas in non-hyperpolarized NMR spectroscopy, calibrated peak intensities in single NMR spectra can be used to determine K_D,[10] the single-scan CPMG experiment employed herein appears ideal for D-DNP. Data acquisition is completed rapidly, and the measurement of echo intensities does not require narrow spectral lines, simplifying shimming procedures that need to be done prior to sample injection in D-DNP.

Unlike many other methods for K_D determination, this experiment also does not require a titration in the case of competitive binding to a single site. For this binding mode, the known $K_{D,r}$ and $p_{b,r}^{(c)}$ provide sufficient information for determination of $K_{D,c}$. More complicated models, such as those involving cooperativity or allosteric interactions, may also be evaluated. The fraction of bound ligand in this case would need to be determined at multiple concentrations.

Since the reporter ligand is in fast exchange between free and bound states, even strongly binding ligands of interest, which can present a challenge in ligand detected screening at

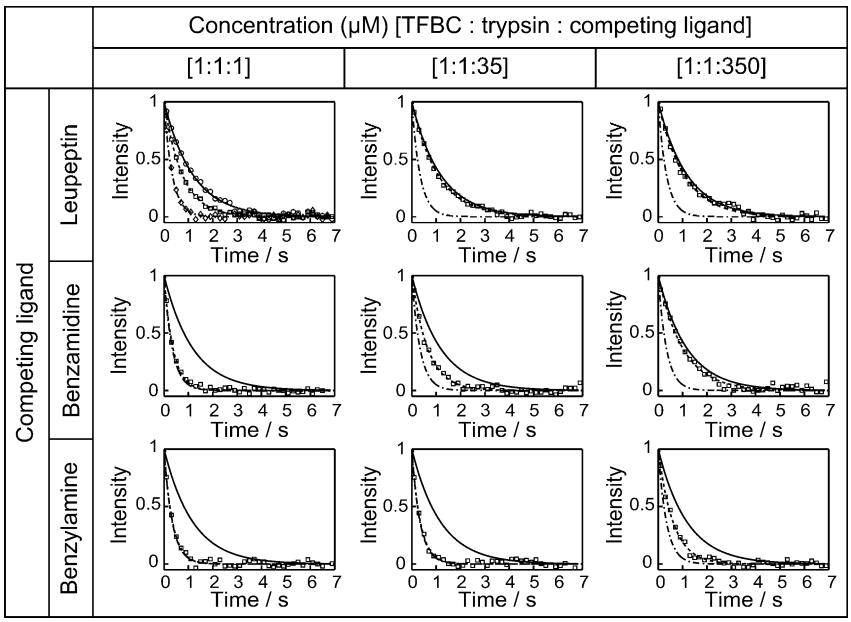

Figure 2. CPMG spin-echo intensities of 1 μM hyperpolarized TFBC ($p_{b,r}^{(nc)} = 0.7\%$). The data points for reference experiments with TFBC in the absence (○) and presence (◇) of 1 μM trypsin are only included in the first graph, but the fit curves are shown in all panels. The data points of hyperpolarized TFBC in the competition experiments are shown in all graphs (□). Each point shown is the average of 465 successive data points measured at time intervals of 420 μs. NMR probe-head background has been subtracted and data before 42.4 ms discarded.

Table 1: Summary of $R_{2,r}$ and $K_{D,c}$ determination.

Competing ligand	$R_{2,r}^{(c)}/s^{-1}$[a] ($[R]_{tot}=[P]_{tot}=1$ μM)			$K_{D,c}$/μM	
	$[C]_{tot}=$ 1 μM	$[C]_{tot}=$ 35 μM	$[C]_{tot}=$ 350 μM	DNP NMR	NMR[c]
Leupeptin	1.39 ± 0.08 (3)[b]	0.94, 0.93 (2)	0.93, 0.94 (2)	0.09 ± 0.03	0.08 ± 0.02 (3)
Benzamidine	2.75, 2.53 (2)	1.52 ± 0.03 (3)	0.99, 0.94 (2)	16.3 ± 1.6	15.7 ± 1.9 (3)
Benzylamine	2.54, 2.90 (2)	2.86, 2.53 (2)	1.73 ± 0.11 (3)	258.1 ± 56.6	217.9 ± 43.1 (3)

[a] $R_{2,r}^{(c)}$ are results from fits of DNP NMR echo intensities to a single exponential, processed as in Figure 2. Mean and standard deviations of $K_{D,c}$ are from calculations with all possible combinations of measured R_2 values. Reference values, $R_{2,r}^{(f)}=0.85\pm0.03\ s^{-1}$ and $R_{2,r}^{(nc)}=2.92\pm0.12\ s^{-1}$, were determined each from three DNP NMR measurements. [b] In parentheses is the number of repetitions performed. [c] Single-scan CPMG experiments without hyperpolarization are from samples of $[[R]_{tot}, [P]_{tot}, [C]_{tot}]=[100, 1, 1]$ μM for leupeptin, [100, 1.8, 40] μM for benzamidine and [100, 1.8, 400] μM for benzylamine.

excess concentration, are readily identified. For this screening application, throughput could further be increased by using mixtures of putative ligands. For determination of $K_{D,c}$, a single competing ligand should be used together with the reporter, and a kinetic equilibrium is required prior to data acquisition. Numerical solutions of the rate equations indicate that assuming $k_{on}=10^8\,M^{-1}s^{-1}$,[20] a typical sample settling time for D-DNP of 400 ms is sufficient for equilibration of the ligands used herein. Under the same assumption for k_{on}, $K_{D,c}$ values as low as 10 nM can be determined with an equilibration time of 1 s, still without prohibitive relaxation losses in the DNP experiments (Figure S2–S5).

Sensitivity and throughput could be further optimized through improvements in solid-state polarization and sample injection. Current developments of D-DNP may be applicable, such as using a polarizer with dual center magnet,[21] increasing the magnetic field for polarization[22] or sample transfer,[11] or a multiplexed polarization process.[23] ^{19}F NMR detection could be improved for increased sensitivity and reduced probe background. A dedicated cryogenically cooled ^{19}F NMR probe at 14.1 T[24] instead of the broadband probe used in this case at 9.4 T in combination with DNP would itself reduce the limit of detection by at least 7-fold through improvements in noise, NMR coil filling factor, and detection field.

The effect of ligand concentration on the parameters observed is illustrated in Figure 3a. The relationship between $K_{D,c}$ and α is plotted for the three concentrations used, along with the experimental data points. The highest accuracy is expected where the slopes of curves are smallest, which occurs when the reporter ligand is partially displaced. The accuracy of the $K_{D,c}$ determination was further assessed in terms of errors in α, by performing Monte Carlo simulations. Using 10^6 random samples, normal distributions of an α value were created with a standard deviation of 0.04, from which unphysical values outside of $0<\alpha<1$ were removed. An exact normal distribution of α would occur for normally distributed R_2 values in its numerator and an exactly known denominator. Assuming a higher accuracy in the denominator appears reasonable; it is composed of reference values valid for an entire set of screening experiments. The interval containing 80% of the resulting $K_{D,c}$ is shaded in Figure 3a, indicating that an error in α is least

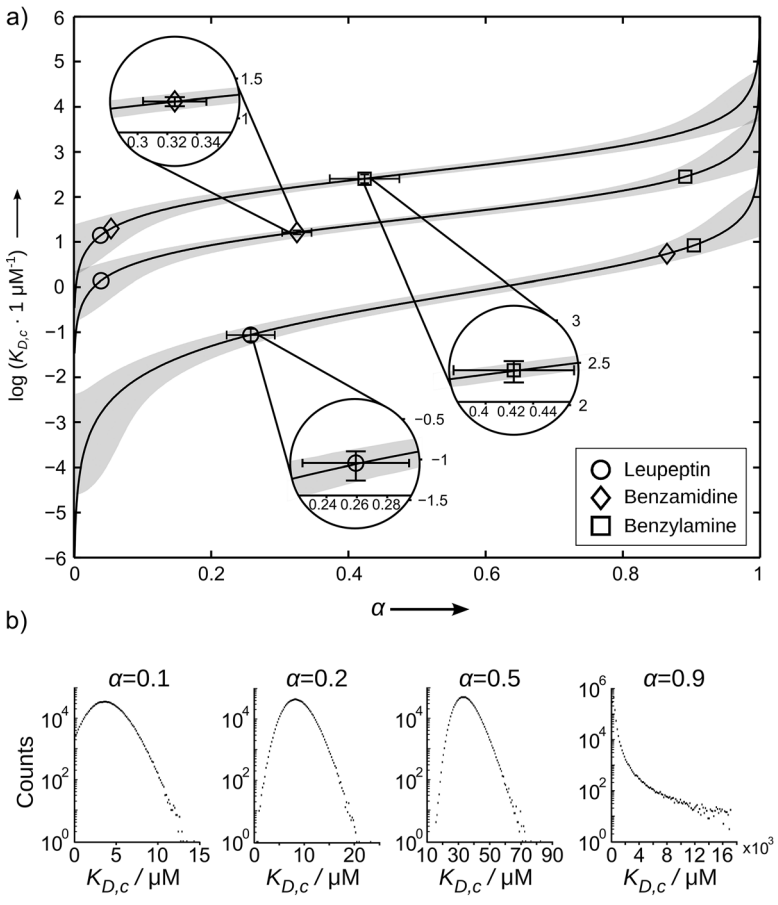

Figure 3. a) Dependence of $\log(K_{D,c})$ on α, for $[[R]_{tot}, [P]_{tot}, [C]_{tot}]=[1, 1, 1]$ μM (bottom curve), [1, 1, 35] μM (middle curve) and [1, 1, 350] μM (top curve) and $K_{D,r}=142$ μM. Points indicate the DNP NMR measurements from Table 1. Shaded areas contain 80% of $K_{D,c}$ values obtained from the Monte Carlo simulations. b) Logarithmic histograms of $K_{D,c}$ from the Monte Carlo simulations, for selected values of α on the middle curve in (a).

affecting $K_{D,c}$ when α is close to 0.5. Simulation results for four values of α are plotted as histograms in Figure 3b. The long tail of the distributions present when the mean of α is close to 1, and the non-vanishing probability for $K_{D,c} = 0$ as the mean of α approaches 0 are the cause for the increasing error under these conditions.

In summary, making use of the significant signal gain of ^{19}F DNP NMR spectroscopy, R_2 relaxation rates of a fluorinated reporter ligand obtained from single-scan CPMG experiments were used to determine the dissociation constants of competitive ligands. Monte Carlo simulations can assist in the choice of experimental conditions. The DNP-based method may be of interest for screening experiments in drug discovery, because ligand binding can be identified and K_D determined, from a single, rapid experiment.

Experimental Section

Experiments without hyperpolarization, used TFBC (100 μM; TFBC·HCl·H_2O, Maybridge, UK), trypsin (1.8 μM; AMRESCO, Solon) and benzamidine (40 μM; Sigma Aldrich, St. Louis) in buffer (50 mM Tris, 100 mM NaCl and 5 mM $CaCl_2$, pH 8.0). NMR data was acquired using a broadband observe (BBO) probe with ^{19}F tuning capability on the ^{1}H channel, on a 400 MHz spectrometer (Bruker Biospin, Billerica). For D-DNP experiments, TFBC (8 μL, 0.134 mM) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (2 μL, 150 mM; Sigma Aldrich, St. Louis) in 80% [D_6]dimethyl sulfoxide and 20% D_2O *v/v* were irradiated in a HyperSense DNP polarizer (Oxford Instruments, Abingdon, UK), with 100 mW power of 94.005 GHz microwaves, for 30 min at $T = 1.4$ K. The hyperpolarized sample was dissolved in pre-heated buffer (4 mL) and injected into a 5 mm NMR tube,[25,26] where 25 μL buffer, trypsin solution, or mixture of trypsin and non-fluorinated ligand was pre-loaded. Final NMR sample volume was 500 μL. NMR spectra were acquired 400 ms after injection at $T = 304$ K. The concentration of trypsin was determined by UV spectrophotometry, and of TFBC by ^{19}F NMR spectroscopy. Sample dilution during dissolution (average 1:100) was determined by NMR spectroscopy using a known standard. Single-scan CPMG experiments collected 16384 data points at intervals of 420 μs. Background signal from the NMR probe was obtained in a separate measurement, and subtracted. Influence from background signal was further reduced by processing data only after an initial delay of 42.4 ms. Monte Carlo simulation was performed using Matlab (MathWorks, Natick, MA).

Keywords: drug discovery · hyperpolarization · NMR spectroscopy · protein–ligand interaction

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 4941–4944
Angew. Chem. **2015**, *127*, 5023–5027

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Received: November 25, 2014
Published online: February 20, 2015

