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Robust NMR Screening for Lead Compounds Using Tryptophan-Containing Proteins

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We have recently described an NMR-based assay for studying the effect of antagonists on protein−protein interactions.[1,2] The method, named AIDA-NMR (for the Antagonist Induced Dissociation Assay-NMR), belongs to the target protein-detected NMR screening methods[3] and provides unambiguous information on whether an antagonist of a protein−protein interaction is strong enough to dissociate the complex and whether its action is through denaturation, precipitation, or release of a protein in its functional folded state. For effective antagonists, AIDA can also quantitatively characterize antagonist-protein and antagonist-protein−protein interactions in the form of K_D's and fractions of the released proteins from their mutual binding. AIDA requires a large protein fragment (larger than 30 kDa) to bind to a small protein (less than 20 kDa). The 2D NMR version relies on monitoring HSQC spectra of the ^{15}N and/or ^{13}C labeled reporter protein (Figure 1S in the Supporting Information).[1] For its 1D proton NMR variant (on unlabeled proteins, i.e., no ^{15}N or ^{13}C labeling required), one must have or introduce an amino acid "reporter" that has at least one nonoverlapped NMR signal which is sensitive to the binding of a ligand to the investigated protein (Figure S2). Since signal overlap in proton 1D spectra of proteins may present a problem, we have chosen to use tryptophan; this is because it is the only amino acid whose $^{N}H^{\epsilon}$ indole side chain gives an NMR signal at ∼10 ppm at physiological pH; the signal is hence well separated from the bulk of amide protons and can be easily monitored. To be used in our NMR assay, the Trp residue must be positioned near a potential antagonist binding site and, importantly, its indole side-chain must be flexible so that its high motion gets restricted upon binding of the Trp-reporter protein to its proteinous target (Figure S2). By using tryptophan-bearing proteins, we showed that a 1D proton NMR version of AIDA-NMR is faster than the 2D version and can be used universally in competition experiments for monitoring ligand/protein−protein complexes.[1,2] In addition, such tryptophan-containing proteins can be used for studying binary interactions between ligands and target proteins with 1D NMR.[2] For a 1D version of AIDA, we found that a reliable K_D can be achieved typically for protein concentrations as low as 40 μM for a ca. 0.5 h experiment in a 5 mm NMR tube using a cryogenically cooled probehead on the 600 MHz spectrometer (Figure S3).[2] Here, we present a method that combines the 1D Trp screening technique with a selective NMR pulse sequence (abbreviated further as the SEI, for Selective Excitation-Inversion), thus increasing the sensitivity of a 1D ^{1}H NMR experiment ca. 3 times and reducing the experimental time by an order of magnitude compared to the AIDA parameters given above (Figure 1).

Spin relaxation is the main factor limiting the sensitivity of NMR experiments.[4] Fast transverse relaxation rates cause spectral line broadening, whereas slow longitudinal relaxation requires introduction of long magnetization recovery delays in NMR experimental schemes. Since biomolecular NMR experiments are performed in aqueous solutions, the repetition rate in a 1D ^{1}H NMR experiment is determined by longitudinal relaxation of water, and thus the highest sensitivity per time unit is achieved when the sum of acquisition and relaxation periods included in the experiment is ca. 3 s.[4]

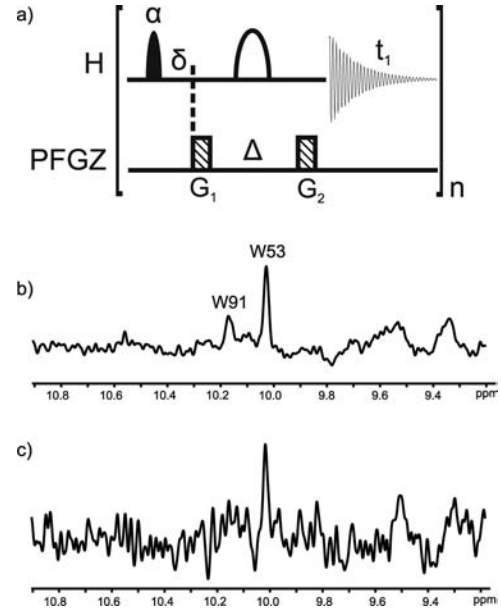

Figure 1. (a) Experimental scheme of the SEI experiment. The pulse sequence incorporates a Gaussian-shaped pulse of angle $\alpha \leq 90°$, followed by a modified WATERGATE sequence for residual water signal suppression. The rSnob profile[9] is used for selective refocusing of downfield shifted protein resonances in the spin−echo part of the experiment (the 2 ms long rSnob is placed in the middle of the $\Delta = 4$ ms delay). The Gaussian pulse has the duration of 1 ms and α of 77°. Selective pulses are applied at 10 ppm. Gradient strengths are 22% and 20% for G1 and G2, respectively; δ was set to 100 μs, the gradient recovery delay to 100 μs, and the acquisition time t_1 to 300 ms, giving the total scan time $T_{scan} \sim 300$ ms. (b) (c) 1D ^{1}H NMR spectra of the 35 μM Mdm2−p53 complex; two $^{N}H^{\epsilon}$ signals of Trp of p53 are visible (for the NMR properties of the complex see Figure S2). Both spectra were acquired in 2.5 min using (b) the SEI pulse sequence and (c) a 90° hard pulse followed by the WATERGATE-W3 sequence.

The SEI pulse sequence, shown in Figure 1a, employs selective pulses only on well-separated NMR signals (e.g., Trp $^{H}N^{\epsilon}$ signals). Application of this pulse sequence has several advantages over a corresponding 1D ^{1}H sequence with a 90° hard pulse. First, it is possible to adjust selective pulses' lengths and offsets so that the water magnetization is not moved from the $+Z$ axis and excellent water suppression is achieved in a single scan. Moreover, since the slowly relaxing water magnetization is not saturated, no long

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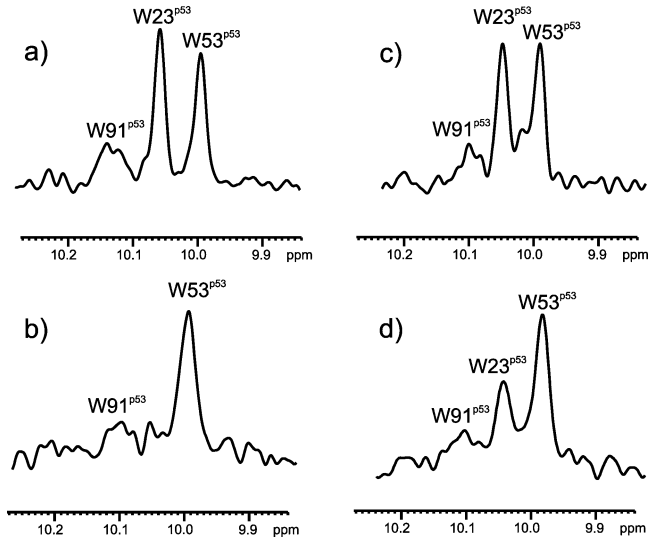

Figure 2. $^{N}H^{\varepsilon}$ indole Trp region of 1D ^{1}H NMR spectra of the Mdm2−p53 complex. The signal of $W23^{p53}$ is used for monitoring the ligand/Mdm2−p53 interactions (details of the diss/reappearance of $^{N}H^{\varepsilon}$ signals are explained in Figure S2). (a) Free p53. (b) The Mdm2−p53 complex. (c) The Mmd2-p53 complex titrated with nutlin-3; the protein: inhibitor molar ratio 1:2. (d) The Mmd2-p53 complex titrated with compound PB10; the protein: inhibitor molar ratio 1:2. All the spectra were acquired using the SEI pulse sequence, complex concentration was 36 μM, acquisition time was 2 min. Corresponding 1D ^{1}H spectra recorded using hard pulses are shown in Figure S5.

recycle delays are necessary and the Ernst angle excitation[4a] is possible. Considering that only a small fraction of spins of a macromolecule is in a nonequilibrium state, the dipole interactions between the excited spins and other spins in the protein and in bulk water significantly speed up the longitudinal relaxation.[5] Thus for the SEI sequence, the duration of a single scan can be decreased down to 300 ms without significant reduction in the signal-to-noise ratio per scan. With faster acquisition, the experimental time can be reduced by an order of magnitude or protein concentration may be decreased by ca. 70% compared our 1D proton "Trp" version of AIDA described previously.[1c,2]

We illustrate this method with lead compounds that block the Mdm2−p53 interaction in humans and with inhibitors of human cyclin-dependent kinase 2 (CDK2).[6,7] In tumors that retain the wildtype p53, the p53 pathway is mostly inactivated by its negative regulator, the human Mdm2 protein, a principal cellular antagonist of p53. Mdm2 interacts through its ca. 118-residue amino terminal domain with the N-terminal transactivation domain of p53 (Figure S4).[8] The restoration of the impaired function of a single gene, p53, by disrupting the p53−Mdm2 interaction, offers a profound new avenue for anticancer therapy across a broad spectrum of cancers.[6] Figure 2 shows examples of our assay for two antagonists of the Mdm2−p53 interaction: nutlin-3[6a] and compound PB10 (for formulas see Figures S6). Since AIDA is a competition experiment, the K_D of protein−antagonist interaction can be determined in a single measurement as described in ref 1c. For nutlin-3, our assay produces the K_D of binding to Mdm2 of 90 nM, in agreement with the literature data.[6] The SEI AIDA of Figure 2d indicates that compound PB10 binds weakly to Mdm2, with an estimated K_D of 3 μM in agreement with the binary titration on the Mdm2 T101W mutant using also the SEI pulse sequence (Figures S7a-d).

CDK2, together with its associated cyclin, controls the passage of the cell through different phases in cell division. Inhibiting CDKs in tumor cells should arrest or stop the progression of the uncontrolled tumor cell division.[2,7] Figure S7e−g show the application of the SEI to roscovitine (Figure S6), an extensively characterized small molecule inhibitor of CDK2 with nanomolar affinity.[2,7]

The SEI experiment gives spectra with a reduced bandwidth and significantly increased signal-to-noise ratio. Since only well separated downfield or upfield shifted signals are recorded, the experiment is insensitive for common additives (buffering substances, detergents, etc.) and thus their perdeuteration is not required. The water suppression scheme employed in the SEI sequence is insensitive for pulse miscalibration. The method may be therefore routinely applied without the need for precise pulse length calibration; i.e., it is possible to measure NMR samples with different solvent compositions without changing the experimental setup. Since only relatively short and low power pulses are employed, the method is safe for superconducting low-temperature NMR probes. The SEI pulse sequence can be in principle also used to improve the sensitivity of other separated proton signals (e.g., the aliphatic signals of proteins, if resolved).

In summary, we show that the combination of a simple SEI pulse sequence with 1D AIDA NMR screening is a straightforward, robust alternative to traditional NMR screening methods. Due to increased sensitivity, the SEI experiment is beneficial for the NMR of proteins difficult to obtain, whereas the reduction of experimental time can significantly increase the throughput of NMR screening.[3] SEI AIDA-NMR can be used universally for monitoring ligand/protein−protein complexes because by introducing tryptophan residues through site-directed mutagenesis the method can also be applied to proteins that do not contain tryptophan in their natural amino acid sequence. Our method is suitable also for chromophoric and fluorescent aromatic small molecule compounds and, thus, may complement assays based on the intrinsic fluorescence of tryptophan, which usually fail in these cases.

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Supporting Information Available: Experimental details, formulas, and further examples of ligand screening using well separated proton signals. This material is available free of charge via the Internet at http://pubs.acs.org.

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