

## An NMR-Based Antagonist Induced Dissociation Assay for Targeting the Ligand–Protein and Protein–Protein Interactions in Competition Binding Experiments

Marcin Krajewski,<sup>†</sup> Ulli Rothweiler,<sup>†</sup> Loyola D'Silva,<sup>†</sup> Sudipta Majumdar,<sup>†</sup> Christian Klein,<sup>‡</sup> and Tad A. Holak<sup>\*,†</sup>

Max Planck Institute for Biochemistry, D-82152 Martinsried, Germany, and Roche Diagnostics GmbH, Pharmaceutical Research, D-82377 Penzberg, Germany

Received March 28, 2007

We present an NMR-based antagonist induced dissociation assay (AIDA) for validation of inhibitor action on protein–protein interactions. As opposed to many standard NMR methods, AIDA directly validates the inhibitor potency in an in vitro NMR competition binding experiment. AIDA requires a large protein fragment (larger than 30 kDa) to bind to a small reporter protein (less than 20 kDa). We show here that a small fragment of a protein fused to glutathione *S*-transferase (GST) can effectively substitute the large protein component. We successfully used a GST-tagged N-terminal 73-residue p53 domain for binding studies with the human MDM2 protein. Other interactions we studied involved complexes of CDK2, cyclin A, p27, and the retinoblastoma protein. All these proteins play a key role in the cell division cycle, are associated with tumorigenesis, and are thus the subject of anticancer therapy strategies.

### Introduction

NMR-based methods for screening for drug candidate compounds that bind protein targets can be divided into two main classes. In the first class, NMR signals of low molecular weight compounds are monitored by utilizing NOE<sup>a</sup> effects or differential relaxation rates of their free and bound states.<sup>1–6</sup> The second approach focuses on the changes in the ligand-induced NMR chemical shifts of the protein.<sup>7–9</sup> The latter class of the methods requires larger amounts of isotopically labeled proteins but can provide more detailed information on the ligand–protein interaction by showing where compounds bind on their target proteins. For proteins of small size (i.e., less than 20 kDa) the most popular protocol has been based on the use of chemical shift perturbations in 2D <sup>1</sup>H–<sup>15</sup>N HSQC spectra of uniformly <sup>15</sup>N-labeled proteins.<sup>7,10–12</sup> For larger proteins, when <sup>15</sup>N labeling is not sufficient to resolve spectral overlap in 2D <sup>1</sup>H–<sup>15</sup>N spectra, selective amino acid labeling<sup>13,14</sup> (<sup>15</sup>N or <sup>13</sup>C) or protein perdeuteration may be necessary and TROSY<sup>10,15</sup> type of experiments are advantageous over “traditional” HSQCs.

In principle, the assignment of NMR resonances is not required if the only purpose of the NMR experiment is to detect the binding of ligands to target proteins. To monitor a specific binding site on a protein, active site residues must be identified, for example, by experiments with known ligands,<sup>13</sup> or for a more detailed structural interpretation, the interface can be determined on the basis of a known 3D structure<sup>14</sup> and NMR assignment procedures.<sup>16</sup>

The NMR screening techniques concentrated so far on monitoring binary ligand–protein interactions. For protein–protein interactions, the ultimate goal of an antagonist compound discovery is to find a lead compound that inhibits or dissociates

these interactions. Binding to a target protein does not directly imply this desired result. On the other hand, a compound that does not bind to one component of the complex can still suppress complex formation by interacting with the second protein; thus, screening for both targets would be necessary. We have recently developed an in vitro NMR method for studying the effect of antagonists on protein–protein interactions.<sup>17,18</sup> The method, named AIDA NMR (for antagonist induced dissociation assay by NMR), requires the protein complex of interest to be formed between proteins with considerably varying molecular weights, one of which is labeled and should give a good quality S/N NMR spectrum and would serve as an NMR reporter protein. Formation of the complex should result in higher transverse relaxation rates R<sub>2</sub> of the reporter protein, which causes a decrease in its NMR intensities to a point that they may completely disappear. This clear indication of the interaction can be easily detected and this part of the experiment must be performed only once, at the time of the system and conditions selection.

The spectrum of the protein–protein complex changes dramatically if an inhibitor that breaks the complex is added. Two outcomes are possible in the case of the complex dissociation: either the reporter spectrum is recovered unchanged, when the inhibitor binds to the larger protein, or the recovered spectrum displays modifications caused by the inhibitor binding. It is noteworthy to point out that the dissociation is detected irrespective of the protein the compound acts on; thus, there are two targets checked simultaneously and the selection of the right binding site and the inhibition strength is obtained in principle without any prior knowledge of these parameters. Our approach therefore targets protein–protein interactions and not a single protein. A schematic representation of our method for a two-protein complex is shown in Figure 1.

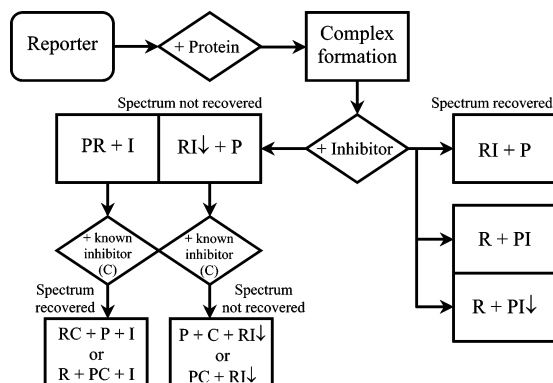
AIDA also provides information whether its action is through denaturation, precipitation, or release of the protein in its functional folded state. We have checked this method on three lead compounds that have recently been reported to inhibit the p53–MDM2 interaction;<sup>19–24</sup> only one of them turned out to be a potent inhibitor of this interaction.<sup>25</sup> Disrupting the MDM2–p53 interaction (and more general also MDMX–p53)<sup>26–28</sup> would rescue the impaired p53 function, and thus

\* To whom correspondence should be addressed. Phone: ++49 89 8578 2673. Fax: ++49 89 8578 3777. E-mail: holak@biochem.mpg.de.

<sup>†</sup> Max Planck Institute for Biochemistry.

<sup>‡</sup> Roche Diagnostics GmbH.

<sup>a</sup> Abbreviations: AIDA, antagonist induced dissociation assay; GST, glutathione *S*-transferase; MDM2, murine double minute 2; CDK2, cyclin dependent kinase 2; p53(1–312), a domain of human p53 between residues 1–312; NOE, nuclear Overhauser effect; HSQC, heteronuclear single quantum coherence; TROSY, transverse relaxation-optimized spectroscopy; pRb-C, the C-terminus of the retinoblastoma protein; ITC, isothermal titration calorimetry.



**Figure 1.** Schematic representation of the AIDA method for studying the effect of an antagonist on the interaction between two proteins.

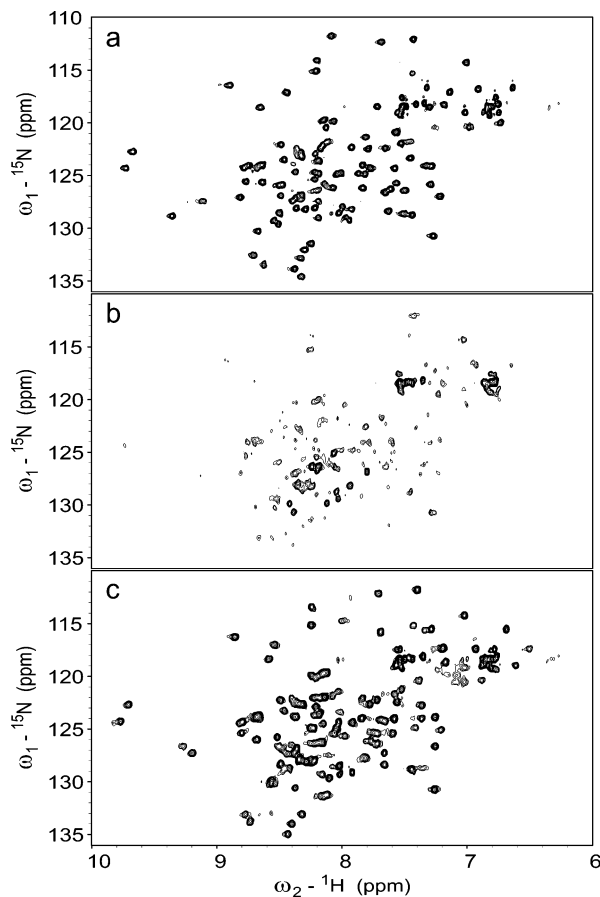
inhibitors of the p53–MDM2 interaction offer a new avenue for cancer therapy.<sup>20,25,29</sup> To monitor the influence of potential antagonists on the p53–MDM2 interaction,<sup>19–22,25,30,31</sup> we used a <sup>15</sup>N-labeled 118 amino acid N-terminal domain of MDM2 and a 312-residue N-terminal fragment of p53. In the present report, we describe our NMR experiments for the GST-tagged N-terminal 73-residue p53 domain, which replaces the 1–312 residue p53 fragment. The possibility to work with the GST-tagged domains would make our NMR protocol universal, as these are protein constructs that are routinely obtained in molecular biology preparations. We further tested our protocol on pure multiprotein interactions, which involved competition of two small protein fragments, the C-terminal domain of the retinoblastoma protein, and the N-terminal domain of p27, for binding to a large complex of CDK2/cyclinA.<sup>32–35</sup> All these proteins play a fundamental role in the regulation of the human cell division cycle.<sup>36</sup>

## Results

In the first experiment the isotopically <sup>15</sup>N enriched fragment of MDM2 (residues 1–118) (Figure 2a) was titrated against the unlabeled GST-p53 (residues 1–73). Complex formation was observed by the disappearance of most of the MDM2 peaks, as seen in Figure 2b. The leftover peaks originate from flexible residues of MDM2 in the complex and are located in the spectrum at the “central 8.3 ppm NH amide” region, diagnostic for unstructured residues, plus side chains at 7 and 7.5 ppm.<sup>37</sup>

We have used nutlin-3 as a positive control for our NMR assay. Nutlin-3 is the most potent inhibitor out of the three nutlins, a class of *cis*-imidazoline compounds recently reported as inhibitors of the p53–MDM2 interaction.<sup>38</sup> Addition of nutlin-3 to the MDM2/p53 complex restores the MDM2 spectrum, as seen in Figure 2c, with the sites involved in binding to nutlin-3 being shifted. The freed GST-p53 is not precipitated; this could be also seen in the 1D NMR spectrum (data not shown). The experiment also shows that the MDM2/nutlin complex is soluble, and that nutlin-3 did not induce precipitation of MDM2.

The second system studied comprises the interaction of p27/pRb with cyclinA/CDK2.<sup>36</sup> The C-terminal domain of pRb (pRb-C) does not adopt a structured conformation. Upon binding of the <sup>15</sup>N-labeled pRb-C to the cyclinA/CDK2 complex, the NMR signals of several residues are shifted (Figure 3a), indicating the binding to the complex. However, most of the cross-peaks of <sup>15</sup>N pRb-C did not disappear, despite the large molecular weight of the pRb-C/cyclinA/CDK2. Thus, the binding did not significantly change the dynamics of the unstructured pRb-C. We have used the N-terminal domain of



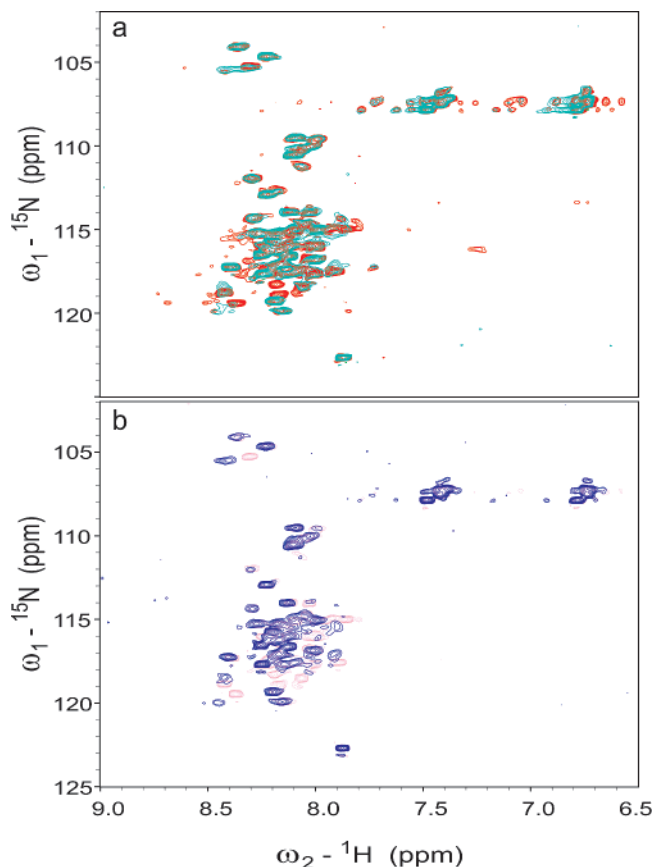
**Figure 2.** Spectra of the <sup>15</sup>N uniformly labeled MDM2. (a) <sup>1</sup>H–<sup>15</sup>N HSQC spectrum of <sup>15</sup>N-MDM2. (b) <sup>1</sup>H–<sup>15</sup>N HSQC spectrum of <sup>15</sup>N-MDM2 complexed with p53. Most of the cross-peaks disappear from the reference MDM2 spectrum, indicating complex formation. (c) <sup>1</sup>H–<sup>15</sup>N HSQC spectrum of MDM2 in complex with nutlin-3. Some cross-peaks are shifted (compared to Figure 2a) due to binding of nutlin to MDM2.

p27 to compete for the pRb-C binding to cyclinA/CDK2. Addition of p27 to the fully complexed pRb-C causes the dissociation of pRb-C from the complex. The initial spectrum of pRb-C is restored (Figure 3b). In a separate experiment, we also tested the interaction of the <sup>15</sup>N-labeled N-terminal domain p27 with CDK2/cyclinA (Figure 4). The N-terminal domain (residues 1–96) of p27 by itself is unstructured. Most of the NMR peaks from <sup>15</sup>N-p27 disappeared on addition of the CDK2/cyclinA complex. The final spectrum consists of peaks arising from the backbone and side chain NHs of residues that are the most flexible. p27 binds tightly to CDK2/cyclinA ( $K_D = 3.5$  nM)<sup>39</sup> and the addition of an unlabeled p27 could not replace the bound <sup>15</sup>N-labeled p27, even after incubating the sample for several hours at 37 °C.

Analogous experiments were performed on the <sup>15</sup>N-labeled p27 and CDK2 as the only binding partner ( $K_D = 70$  nM).<sup>39</sup> The formation of the complex was clearly seen; addition of an unlabeled p27 did not release the <sup>15</sup>N-labeled p27. The complex did not exchange bound p27 even after the 80 min incubation at 40 °C followed by a 1-day measurement. This data indicates that p27 in both p27–CDK2 and p27/cyclinA/CDK2 interactions forms high-affinity complexes.

## Discussion

The benefit of our method is the direct observation of the action of an inhibitor or a protein without prior knowledge of structure/assignment information. The method covers also large

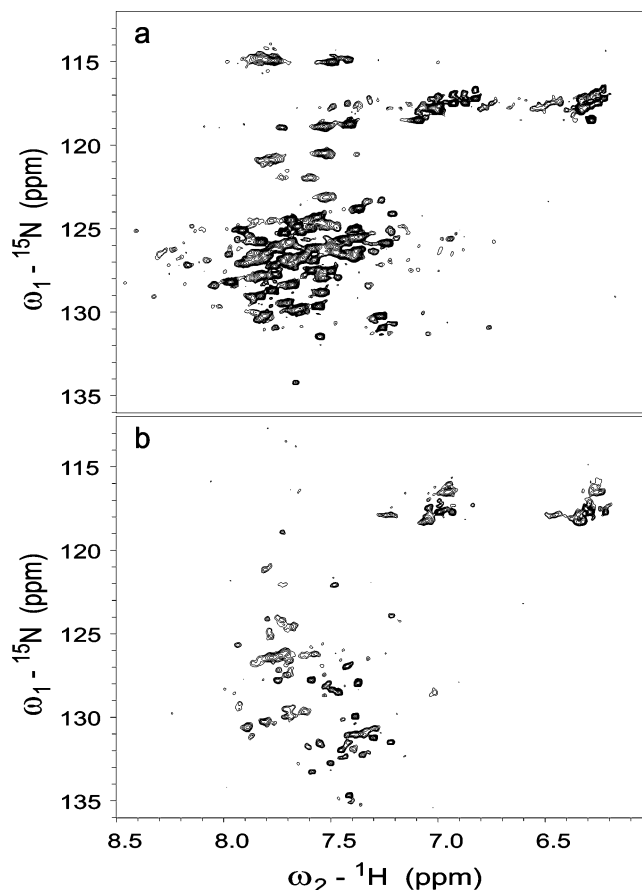


**Figure 3.** Spectra of the  $^{15}\text{N}$  uniformly labeled pRb-C. (a) effects of cyclinA/CDK2 addition: red, initial spectrum; green, ca. 50% complexed. Double peaks indicate slow exchange. (b) Fully complexed pRb-C (blue) and after addition of p27 initial spectrum is restored (pink).

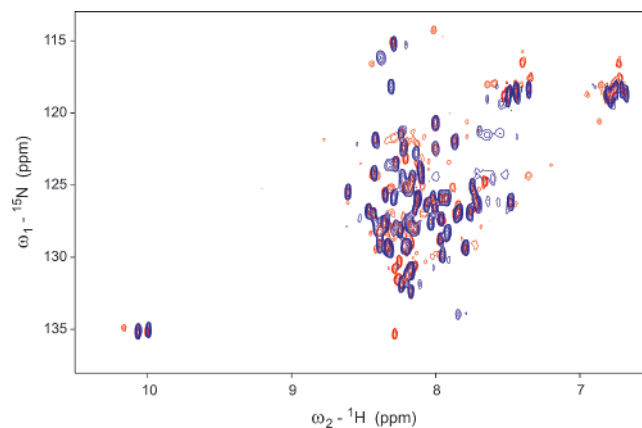
protein complexes and protein/polypeptide inhibitors, encountering the limit rather in sample concentration (or the spectrometer sensitivity) than in the multiprotein complex size, provided that one component of the complex alone gives a good spectrum. A dramatic change in the spectrum in the case of ligand binding offers comfortable and reliable automation opportunities.

In the current study, we have used a 118 amino acid N-terminal domain of MDM2 and the GST fusion N-terminal 73-residue fragment containing the transactivation domain of p53 (a schematic of the full-length proteins is shown in Figure 5). The binary complex has a total molecular weight of 50 kDa and a  $K_D$  of ca.  $0.7 \mu\text{M}$ .<sup>40</sup> We show experimentally in this report that GST-p53(1–73) can substitute for p53(1–312) in our assay. Although GST is known to form dimers, GST-p53 eluted as a monomer in gel filtration experiments.

Since our method is a competition assay, it is essential to understand how strong a binder the ligand must be in order to dissociate the protein–protein interaction. In principle, the effect of a ligand will depend on the strength of the protein–protein interaction, concentrations of the ligand and protein, and the inhibitor–protein affinity. The sensitivity of the NMR spectrum is also important, as we have to know how much of the protein–protein complex must be dissociated in order to detect partial recovery of the reporter protein in the NMR spectra. We have used ca. 0.1 mM concentrations for protein complexes, and in our practice we found that 30% of the dissociated MDM2 is required for a clear-cut detection by  $^{15}\text{N}$  HSQCs; thus, we will use this value in further discussion. An exact analytical solution to the competition problem was published by Wang<sup>41</sup> and was used for applications in the isothermal titration calorimetry (ITC)



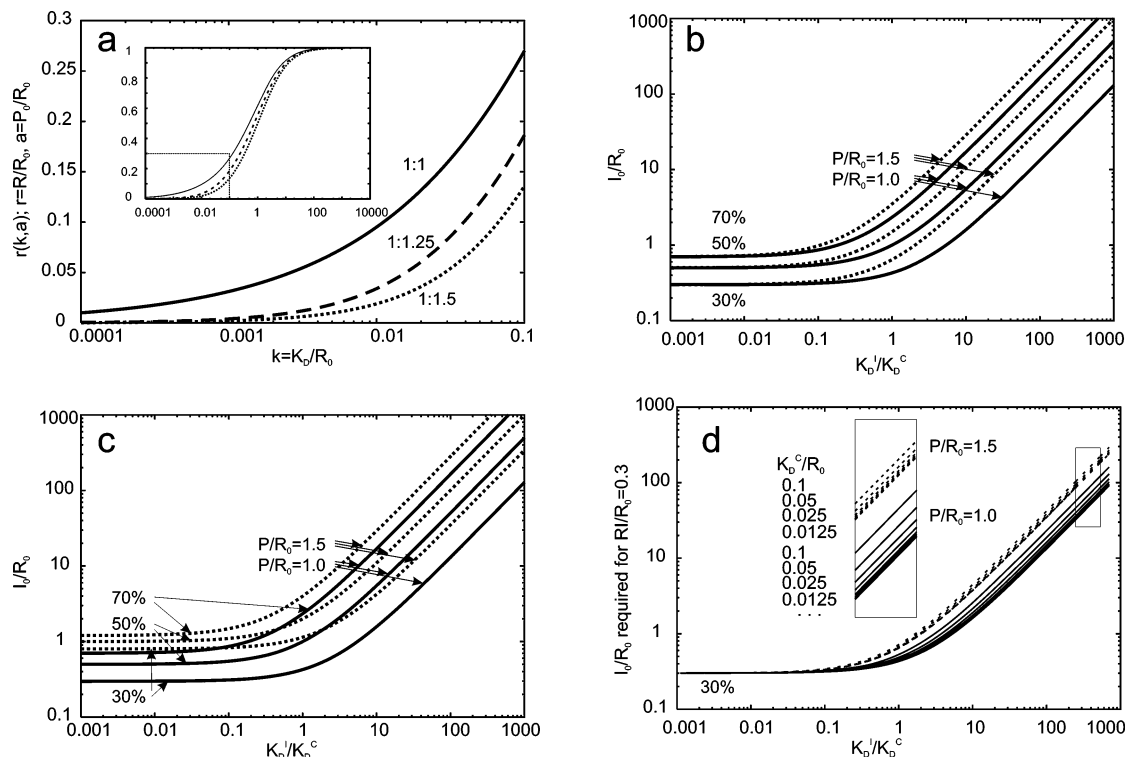
**Figure 4.** (a) A  $^{15}\text{N}$ -HSQC spectrum of  $^{15}\text{N}$ -labeled p27 (a) and after addition of cyclinA/CDK2 complex (b). Most of the peaks disappeared. Addition of the substance that dissociates the complex should restore the spectrum. The system seems to be well-suited for the inhibitor research.



**Figure 5.** Superposition of HSQC spectra of  $^{15}\text{N}$ -labeled p53 (residues 1–312, red) and GST-p53 (residues 1–73, blue).

by Sigurskjold.<sup>42</sup> Below we present considerations for the conditions specific to our NMR experiments.

First, we have to know how much of a free reporter protein is present in the sample prior to the ligand addition. The equilibrium concentrations of the reporter, the binding protein, and the protein–reporter complex are denoted  $[R]$ ,  $[P]$ , and  $[PR]$ , respectively.  $K_D^C$  is the dissociation constant of the complex, and  $[R]_0$  and  $[P]_0$  are the total concentrations of the reporter and protein ( $[R]_0 = [R] + [PR]$ ,  $[P]_0 = [P] + [PR]$ ). The ratio of the free reporter concentration to the total reporter concentration  $r = [R]/[R]_0$  is  $r = \frac{1}{2}(\sqrt{(k+\alpha-1)^2+4k} - (k + \alpha -$



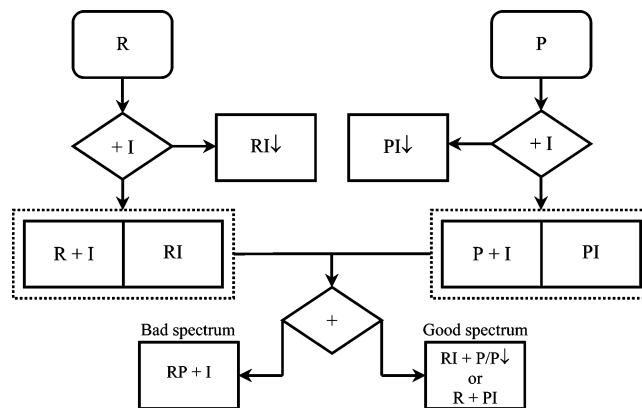
**Figure 6.** Equilibrium concentrations of selected compounds. (a) Binary mixture, free reporter concentration versus different  $K_D$  to reporter concentration ratio, for 1.0, 1.25, and 1.5 molar ratio of the complex substrates. (b) Ternary mixture. Amount of inhibitor (in  $[R]_0$  units) necessary to bind 30%, 50%, or 70% of reporter molecules for different binding strength ratio, and 1.0 or 1.5 molar ratio of complex substrates, found numerically at  $K_D/[R]_0 = 0.001$ . (c) Amount of inhibitor (in  $[R]_0$  units) necessary to reach 30%, 50%, or 70% of free reporter molecules for different binding strength ratio, found numerically at 1.0 and 1.5 molar ratio of complex substrates and  $K_D/[R]_0 = 0.001$ . (d) Illustration on how the curve from part b depends on  $K_D/[R]_0$ .

1)), where  $k = K_D^C/[R]_0$  and  $\alpha = [P]_0/[R]_0$ . It can be found by solving a quadratic equation. It is illustrated in Figure 6a.

Typically, the NMR sample concentration is higher than or close to 0.1 mM, while the  $K_D$  is below 10  $\mu\text{M}$ ; thus,  $k < 0.1$ . For  $\alpha = 1.0$ , one obtains  $r = 0.27$ , which is near to the 30% detection threshold but decreases with higher binding strengths and sample concentrations. Figure 6a shows how the free reporter concentration drops for smaller  $k$ . One can also consider using an excess of the binding protein, thus increasing parameter  $\alpha$  in the above equation.

The second question is how much of an inhibitor should be added to cause a detectable effect. Two cases can be considered. In the first case, when the inhibitor binds to the reporter there are two types of molecules that potentially can be detected (they have molecular weight smaller than 30 kDa): an inhibitor reporter complex and the free reporter protein. For simplicity, we assume that free reporter is undetectable in this case. In the second case, when inhibitor binds to the protein interacting with the reporter, the only detectable molecule is free reporter. For further discussion we found it convenient to express the total ligand concentration  $[I]_0 = [I] + [RI]$ , as a parameter,  $\beta = [I]_0/[R]_0$ . One can then plot a curve of  $\beta$  necessary to release a given percentage (30%) of the reporter (or inhibitor reporter complex) for different  $K_D^C/K_D^C$  ratio (Figure 6b,c). The shape of this curve depends on  $K_D^C/[R]_0$ ; however, for NMR conditions, where this value is usually below 0.1, it is almost universal (Figure 6d).

The first conclusion is that the concentration of an uncomplexed reporter protein often can be neglected. The second is that if the large binding component protein or the reporter protein have considerably higher affinity to the ligand than to the other binding partner, it will bind mostly to the added inhibitor and dissociation of the reporter protein complex will



**Figure 7.** An AIDA protocol for an irreversible reaction.

be detected at total concentration of an inhibitor similar to the total concentration of the protein–reporter complex component which is replaced by the inhibitor. If, on the other hand, the affinity to the ligand is 10 times, or more, weaker than the binding between the two proteins, then the complex can be dissociated at detectable levels only when a huge excess of the ligand is used. Note that a ligand that binds to the target protein “tightly”, e.g. 0.5  $\mu\text{M}$ , might require an excess of a ligand exceeding its solubility limit, if the  $K_D$  of the complex is, for example, below 10 nM, and thus would be deemed to be ineffective. Our experimental protocol can be still adapted for the case of an extremely tight binding or an irreversible chemical reaction. In such cases, effective inhibition requires the presence of the tested inhibitor compound prior to the complex formation (Figure 7). In this variant, the chemical shift changes caused by the compound in the reporter spectrum can be measured on the same sample. Regardless of which protocol was used for

the complex formation, a known strong inhibitor that dissociates the complex can be used for a positive control if the tested compound did not cause detectable effects (see Krajewski et al.<sup>18</sup>). Our method tests this property directly, while the “SAR by NMR” approach requires comparison of the dissociation constants of the protein complex and ligand–target interaction. Such information can be considerably difficult to obtain at required precision in the case of tight binding, whereas AIDA-NMR can provide hints about how much the affinity must be improved.

We would like to note that our method is neither in competition with the traditional SAR by NMR<sup>7,10–12</sup> nor can it be considered as a variant of methods described in the Introduction. Although the common final goal of all these methods is to find a potential inhibitor, they use different paradigms to achieve it. The AIDA-NMR and SAR by NMR methods provide different types of information, and being suitable for different situations, they well supplement each other. The SAR by NMR approach cannot be used when only large fragments of proteins are available, which may be the case at the beginning of the characterization of protein–protein binding. Also, many important minimal domains of proteins are about 300 amino acids in length. For large proteins of ca. 30 kDa, the HSQC spectra are normally too crowded to be of practical use in these types of experiments. Such systems are often perfect for the method we present. AIDA-NMR, on the other hand, does not provide directly structural information on how to design or improve the inhibitor.

Increased  $T_2$  relaxation rates in our NMR assays were achieved through the formation of large molecular weight complexes. Several other methods may be used to achieve a similar effect, like, for example, complexing with paramagnetic probes. The method can possibly be used for monitoring factors necessary for a polymerization or depolymerization reaction. The requirement is that one of the complex members gives a good NMR spectrum when free in solution and that its transverse relaxation rates would be distinctly higher after the reaction.

## Methods

**Protein Expression and Purification.** The recombinant human MDM2 protein was obtained from the *Escherichia coli* BL21(DE3) RIL expression system and contains the first 118 N-terminal residues of human MDM2 cloned in a pET46 Ek/LIC vector (Novagen). The inclusion bodies were washed with the PBS buffer containing 0.05% Triton X-100 with subsequent centrifugation at 12 000g and solubilized with 6 M guanidine hydrochloride in 100 mM Tris-HCl, pH 8.0, including 1 mM EDTA and 10 mM DTT. After lowering the pH to 3–4, the protein was dialyzed overnight at 4 °C, against 4 M guanidine hydrochloride, pH 3.5, including 10 mM DTT. For renaturation, the protein was diluted (1:50–1:100) into 10 mM Tris-HCl, pH 7.0, including 1 mM EDTA and 10 mM DTT by adding the protein in several pulses to the refolding buffer. Refolding was performed overnight at room temperature. Ammonium sulfate was added to a final concentration of 1.5 M and after ca. 1 h butyl sepharose 4 Fast Flow (Pharmacia, FRG) was added to the refolded human MDM2. We used a glass-frit funnel as a filter to collect MDM2–butylsepharose. The protein was eluted with 0.1 M Tris-HCl, pH 7.2, supplied with 5 mM DTT. Finally, all fractions containing MDM2 were pooled, concentrated, and applied to a HiLoad Superdex 75 pg gel filtration column (Pharmacia, FRG). The running buffer contained 50 mM  $\text{KH}_2\text{PO}_4$ , 50 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.4, 150 mM NaCl, 5 mM DTT. All fractions with the monomeric human MDM2 were pooled and concentrated for NMR spectroscopy.

The recombinant human GST-p53 protein (residues 1–73) was overexpressed at 37 °C in *E. coli* BL21 (DE3) using a pGEX vector. The protein was purified using a GST Sepharose Fast Flow column

(Amersham). The recombinant p27 (residues 1–96) was overexpressed at 37 °C in *E. coli* BL21 (DE3) using a pET28 vector. The protein was purified under native condition using Ni-NTA Agarose (Quiagen). The C-terminal construct of pRb residues (residues 801–890) was overexpressed in *E. coli* BL21 (DE3) overnight at 20 °C using a pET 46 vector and purified under native condition using Ni-NTA Agarose (Quiagen). The final purification of all proteins was done with a HiLoad 16/60 Superdex75 gel filtration column (Amersham). CDK2 and cyclinA were expressed separately in *E. coli* BL21 (DE3) at 20 °C using a pET 46 vector. They were first purified under native condition using Ni-NTA Agarose (Quiagen) and second a HiLoad 16/60 Superdex75 gel filtration column (Amersham). The two purified proteins were mixed in equimolar concentrations, and the complex was separated from the monomers by a second gel filtration. The uniformly  $^{15}\text{N}$ -enriched protein samples were prepared by growing the bacteria in minimal medium containing  $^{15}\text{N}$ -labeled  $\text{NH}_4\text{Cl}$ .

**NMR Spectroscopy.** All NMR spectra were acquired on a Bruker DRX 600 MHz spectrometer equipped with a cryoprobe except the pRb-C titration experiment, which was done on the DRX 500 MHz with a standard triple resonance probe. Typically, NMR samples contained up to 0.2 mM (0.5 mM at the 500 MHz spectrometer) of protein in 50 mM  $\text{KH}_2\text{PO}_4$ , 50 mM  $\text{Na}_2\text{HPO}_4$ , 150 mM NaCl, pH 7.4, 5 mM DTT, 0.02%  $\text{NaN}_3$ , and protease inhibitors or 40 mM HEPES, 200 mM NaCl, 5 mM DTT for the experiments with CDK2/cyclinA. The MDM2/p53 system was measured at 300 K, while titration of pRb-C with CDK2/cyclin was carried out at 283 K. For the  $^1\text{H}$ – $^{15}\text{N}$  HSQC spectrum,<sup>43</sup> a total of 2048 complex points in  $t_2$  and 128  $t_1$  increments were acquired. Water suppression was carried out using the WATERGATE 5 sequence. NMR data were processed using the Bruker program Xwin-NMR version 3.5. Titration experiments were performed using a series of  $^1\text{H}$ – $^{15}\text{N}$  HSQC of labeled p53 or MDM2 along with the unlabeled partner. By monitoring the 1D proton spectra, care was taken to prevent overtitration of the unlabeled sample.

**Supporting Information Available:** An analytical solution for a chemical equilibrium in the case of competitive binding. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## References

- (1) Jahnke, W.; Widmer, H. Protein NMR in biomedical research. *Cell. Mol. Life Sci.* **2004**, *61*, 580–599.
- (2) Lepre, C. A.; Moore, J. M.; Peng, J. W. Theory and applications of NMR-based screening in pharmaceutical research. *Chem. Rev.* **2004**, *104*, 3641–3675.
- (3) Stockman, B. J.; Dalvit, C. NMR screening techniques in drug discovery and drug design. *Prog. NMR Spectrosc.* **2002**, *41*, 187–231.
- (4) Klages, J.; Coles, M.; Kessler, H. NMR-based screening: A powerful tool in fragment-based drug discovery. *Mol. Biosys.* **2006**, *2*, 319–331.
- (5) Peng, J. W.; Moore, J.; Abdul-Manan, N. NMR experiments for lead generation in drug discovery. *Prog. NMR Spectrosc.* **2004**, *44*, 225–256.
- (6) Dalvit, C.; Flocco, M.; Knapp, S.; Mostardini, M.; Perego, R.; Stockman, B. J.; Veronessi, M.; Varasi, M. High-throughput NMR-based screening with competition binding experiments. *J. Am. Chem. Soc.* **2002**, *124*, 7702–7709.
- (7) Shuker, S. B.; Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. Discovering high-affinity ligands for proteins: SAR by NMR. *Science* **1996**, *274*, 1531–1534.
- (8) Dehner, A.; Furrer, J.; Richter, K.; Schuster, I.; Buchner, J.; Kessler, H. NMR chemical shift perturbation study of the N-terminal domain of Hsp90 upon binding of ADR AMP-PNP, geldanamycin, and radicicol. *Chem. Biol. Chem.* **2003**, *4*, 870–877.
- (9) Prestegard, J. H.; Valafar, H.; Glushka, J.; Tian, F. Nuclear Magnetic Resonance in the era of structural genomics. *Biochemistry* **2001**, *40*, 8677–8685.
- (10) Pellecchia, M.; Sem, D. S.; Wüthrich, K. NMR in drug discovery. *Nat. Rev. Drug Discovery* **2002**, *1*, 211–219.

- (11) Kriwacki, R. W.; Hengst, L.; Tennant, L.; Reed, S. I.; Wright, P. E. Structural studies of p21(Waf1/CipSdi1) in the free and Cdk2-bound state: Conformational disorder mediates binding diversity. *Proc. Nat. Acad. Sci. U.S.A.* **1996**, *93*, 11504–11509.
- (12) Stoll, R.; Renner, C.; Hansen, S.; Palme, S.; Klein, C.; Belling, A.; Zeslawski, W.; Kamionka, M.; Rehm, T.; Muhlhahn, P.; Schumacher, R.; Hesse, F.; Kaluza, B.; Voelter, W.; Engh, R. A.; Holak, T. A. Chalcone derivatives antagonize interactions between the human oncoprotein MDM2 and p53. *Biochemistry* **2001**, *40*, 336–344.
- (13) Singh, M.; Krajewski, M.; Mikolajka, A.; Holak, T. A. Molecular determinants for the complex formation between the retinoblastoma protein and LXCXE sequences. *J. Biol. Chem.* **2005**, *280*, 37868–37876.
- (14) Reese, M. L.; Doetsch, V. Fast mapping of protein-protein interfaces by NMR spectroscopy. *J. Am. Chem. Soc.* **2003**, *125*, 14250–14251.
- (15) Pervushin, K. V.; Wider, G.; Wüthrich, K. Single transition-to-single transition polarization transfer (ST2-PT) in [<sup>15</sup>N,<sup>1</sup>H]-TROSY. *J. Biomol. NMR* **1998**, *12*, 345–348.
- (16) Wüthrich, K. *NMR of Proteins and Nucleic Acids*. Wiley: New York, 1986; pp 130–161.
- (17) D’Silva, L.; Ozdowy, P.; Krajewski, M.; Rothweiler, U.; Singh, M.; Holak, T. A. Monitoring the effects of antagonists on protein–protein interactions with NMR spectroscopy. *J. Am. Chem. Soc.* **2005**, *127*, 13220–13226.
- (18) Krajewski, M.; Ozdowy, P.; D’Silva, L.; Rothweiler, U.; Holak, T. A. NMR indicates that the small molecule RITA does not block p53-MDM2 binding in vitro. *Nat. Med.* **2005**, *11*, 1135–1136.
- (19) Chen, J.; Marechal, V.; Levine, A. J. Mapping of the p53 and mdm-2 interaction domains. *Mol. Cell Biol.* **1993**, *13*, 4107–4114.
- (20) Chene, P. Inhibiting the p53–MDM2 interaction: An important target for cancer therapy. *Nat. Rev. Cancer* **2003**, *3*, 102–109.
- (21) Kussie, P. H.; Gorina, S.; Marechal, V.; Elenbaas, B.; Moreau, J.; Levine, A. J.; Pavletich, N. P. Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science* **1996**, *274*, 948–953.
- (22) Ayed, A.; Mulder, F. A. A.; Yi, G. S.; Lu, Y.; Kay, L. E.; Arrowsmith, C. H. Latent and active p53 are identical in conformation. *Nat. Struct. Biol.* **2001**, *8*, 756–760.
- (23) Blommers, M. J. J.; Fendrich, G.; Garcia-Echeverria, C.; Chene, P. On the interaction between p53 and MDM2—Transfer NOE study of a p53-derived peptide ligated to MDM2. *J. Am. Chem. Soc.* **1997**, *119*, 3425–3426.
- (24) Dawson, R.; Mueller, L.; Dehner, A.; Klein, C.; Kessler, H.; Buchner, J. The N-terminal domain of p53 is natively unfolded. *J. Mol. Biol.* **2003**, *332*, 1131–1141.
- (25) Vassilev, L. T.; Vu, B. T.; Graves, B.; Carvajal, D.; Podlaski, F.; Filipovic, Z.; Kong, N.; Kammlott, U.; Lukacs, C.; Klein, C.; Fotouhi, N.; Liu, E. A. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* **2004**, *303*, 844–848.
- (26) Toledo, F.; Wahl, M. Regulating the p53 pathway: In vitro hypotheses, in vivo veritas. *Nat. Rev. Cancer* **2006**, *6*, 909–923.
- (27) Toledo, F.; Krummel, K. A.; Lee, C. J.; Liu, C. W.; Rodewald, L. W.; Tang, M.; Wahl, G. M. A mouse p53 mutant lacking the proline-rich domain rescues Mdm4 deficiency and provides insight into the Mdm2-Mdm4-p53 regulatory network. *Cancer Cell* **2006**, *9*, 273–285.
- (28) Böttger, V.; Böttger, A.; Garcia-Echeverria, C.; Ramos, Y. F.; van der Eb, A. J.; Jochemsen, A. G.; Lane, D. P. Comparative study of the p53-mdm2 and p53-MDMX interfaces. *Oncogene* **1999**, *18*, 189–199.
- (29) Klein, C.; Vassilev, L. T. Targeting the p53-MDM2 interaction to treat cancer. *Br. J. Cancer* **2004**, *91*, 1415–1419.
- (30) Picksley, S. M.; Vojtesek, B.; Sparks, A.; Lane, D. P. Immunochemical analysis of the interaction of p53 with MDM2—Fine mapping of the MDM2 binding site on p53 using synthetic peptides. *Oncogene* **1994**, *9*, 2523–2529.
- (31) Fischer, P. M.; Lane, D. P. Small-molecule inhibitors of the p53 suppressor HDM2: Have protein–protein interactions come of age as drug targets? *Trends Pharm. Sci.* **2004**, *25*, 343–346.
- (32) Toyoshima, H.; Hunter, T. P27, a novel inhibitor of G1 cyclin-cdk protein kinase activity, is related to p21. *Cell* **1994**, *78*, 67–74.
- (33) Polyak, K.; Kato, J. Y.; Solomon, M. J.; Sherr, C. J.; Massague, J.; Roberts, J. M.; Koff, A. P27(KIP1), a cyclin-cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev.* **1994**, *8*, 9–22.
- (34) Polyak, K.; Lee, M. H.; Erdjument-Bromage H.; Koff, A.; Roberts, J. M.; Tempst, P.; Massague, J. Cloning of p27(KIP1), a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* **1994**, *78*, 59–66.
- (35) Russo, A. A.; Jeffrey, P. D.; Pavletich, N. P. Structural basis of cyclin-dependent kinase activation by phosphorylation. *Nat. Struct. Biol.* **1996**, *3*, 696–700.
- (36) Ruas, M.; Peters, G. The p16(INK4A)/CDKN2A tumor suppressor and its relatives. *Biochem. Biophys. Acta* **1998**, *1378*, F115–F177.
- (37) Rehm, T.; Huber, R.; Holak, T. A. Application of NMR in structural proteomics: Screening for proteins amenable to structural analysis. *Structure* **2002**, *10*, 1613–1618.
- (38) Fry, D. C.; Emerson, S. D.; Palme, S.; Vu, B. T.; Liu, C. M.; Podlaski, F. NMR structure of a complex between MDM2 and a small molecule inhibitor. *J. Biomol. NMR* **2004**, *30*, 163–173.
- (39) Lacy, E. R.; Filippov, I.; Lewis, W. S.; Otieno, S.; Xiao, L. M.; Weiss, S.; Hengst, L.; Kriwacki, R. W. p27 binds cyclin-CDK complexes through a sequential mechanism involving binding-induced protein folding. *Nat. Struct. Mol. Biol.* **2004**, *11*, 358–364.
- (40) Böttger, V.; Böttger, A.; Howard, S. F.; Picksley, S. M.; Chene, P.; Garcia Echeverria, C.; Hochkeppel, H. K.; Lane, D. P. Identification of novel Mdm2 binding peptides by phage display. *Oncogene* **1996**, *13*, 2141–2147.
- (41) Wang, Z. An exact mathematical expression for describing competitive binding of two different ligands to a protein molecule. *FEBS Lett.* **1995**, *360*, 111–114.
- (42) Sigurskjold, B. W. Exact analysis of competition binding by displacement isothermal titration calorimetry. *Anal. Biochem.* **2000**, *277*, 260–266.
- (43) Mori, S.; Abeygunawardana, C.; Johnson, M. O.; van Zijl, P. C. Improved sensitivity of HSQC spectra of exchanging protons at short interscan delays using a new fast HSQC (FHSQC) detection scheme that avoids water saturation. *J. Magn. Reson. B* **1995**, *108*, 94–98.

JM070365V