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J. Am. Chem. Soc., **2005**, 127 (38), 13220-13226• DOI: 10.1021/ja052143x • Publication Date (Web): 01 September 2005

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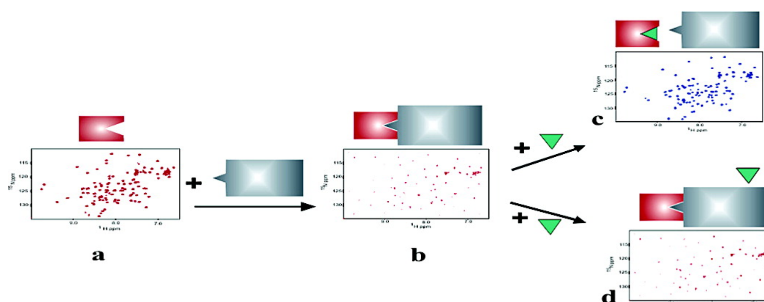

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Monitoring the Effects of Antagonists on Protein−Protein Interactions with NMR Spectroscopy

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Abstract: We describe an NMR method that directly monitors the influence of ligands on protein−protein interactions. For a two-protein interaction complex, the size of one component should be small enough (less than ca. 15 kDa) to provide a good quality ^{15}N (^{13}C) HSQC spectrum after ^{15}N(^{13}C) labeling. The size of the second unlabeled component should be large enough so that the molecular weight of the preformed complex is larger than ca. 40 kDa. When the smaller protein binds to a larger one, broadening of NMR resonances results in the disappearance of most of its cross-peaks in the HSQC spectrum. Addition of an antagonist that can dissociate the complex would restore the HSQC spectrum of the smaller component. The method directly shows whether an antagonist releases proteins in their wild-type folded states or whether it induces their denaturation, partial unfolding, or precipitation. We illustrate the method by studying lead compounds that have recently been reported to block the MDM2−p53 interaction. Activation of p53 in tumor cells by inhibiting its interaction with MDM2 offers new strategy for cancer therapy.

Introduction

The NMR chemical shift perturbation methods have been successfully used for mapping binding interfaces in proteins and for screening small molecule lead compounds in ligand−protein interactions.[1−7] The most popular protocol has been based on the use of chemical shift perturbations in 2D ^{1}H−^{15}N HSQC spectra of ^{15}N-labeled proteins upon addition of ligands or peptides/proteins.[1,2,4,8−11] In general, a prerequisite for mapping these interactions is that the assignment of the NMR spectrum of the protein is known, at least for the nuclei that exhibit chemical shift changes, although a method has recently been described that allows mapping interfaces of protein complexes without the knowledge of chemical shift assignments provided that the 3D structures are known.[12] The assignment is not needed if the only purpose of the NMR experiment is detecting the binding of ligands to target proteins.

The NMR screening studies for lead compounds concentrated so far on binary interactions of lead compounds with small to middle size domains of target proteins,[1,2,9−11] a small size of the protein being crucial for obtaining a good quality HSQC spectrum. The principal purpose of an antagonist compound discovery, however, is determining whether a lead compound inhibits or dissociates protein−protein interactions. Here, we describe an NMR method that allows for direct monitoring of the influence of a ligand on protein−protein binding. Importantly, our method shows whether a small molecule compound is capable of releasing proteins in their wild-type folded states or whether it induces their denaturation, partial unfolding, or precipitation.

We illustrate our method with the p53−MDM2 interaction.[13−23] The human p53 protein is a tumor suppressor transcription factor, and loss of p53 function through mutation is involved in 50% of human cancer. The remainder retains wild-type p53, but the p53 pathway is inactivated through, for example, interaction with the MDM2 protein. MDM2 is a principal

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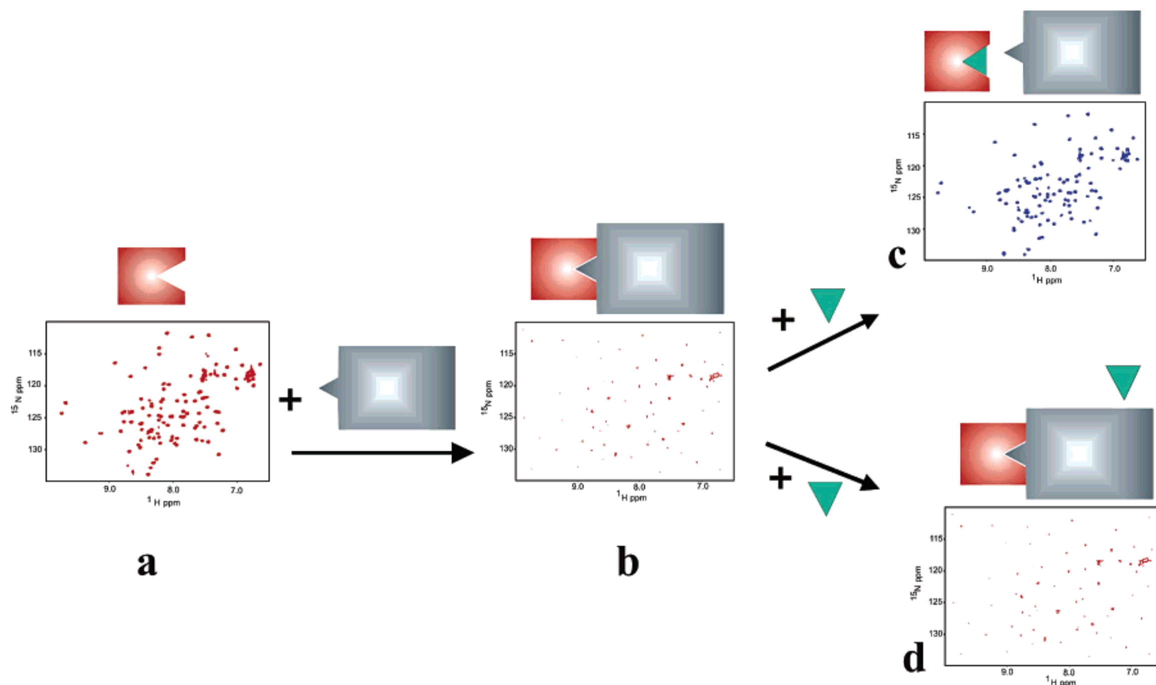

Figure 1. Schematic representation of our method for studying the effect of an antagonist on the interface between two proteins. (a) An ^{15}N HSQC spectrum of a ca. 10 kDa uniformly ^{15}N labeled protein (each amino acid gives a cross-peak for the N−H pair. The side-chain N−H resonances are observed at around 7 ppm ^{1}H and at 120 ppm ^{15}N chemical shifts). (b) The cross-peaks disappear on addition of a large protein (ca. 35 kDa) that forms a complex with the smaller one. (c) The cross-peaks reappear on addition of a strong inhibitor ligand that dissociates the complex. (d) A weak inhibitor does not dissociate the complex.

cellular antagonist of p53 that interacts through its 100 residue amino terminal domain with the N-terminal transactivation domain of p53. The rescue of the impaired p53 function by disrupting the MDM2−p53 interaction offers new avenues for anticancer therapeutics,[13−15] and several lead compounds have recently been reported to inhibit the p53−MDM2 interaction in assays based on tumor cell cultures or immunoprecipitation techniques. All of these compounds were claimed to bind to the p53-binding site on MDM2.

Results

A schematic representation of our method for a two-protein complex is shown in Figure 1. At least two protein components that make up a multiprotein complex are monitored. The size of one component should be small enough (less than ca. 15 kDa) to provide a good quality HSQC spectrum after ^{15}N or ^{13}C labeling of the protein (Figure 1a). The size of the second component should be large enough so that the molecular weight of the preformed complex is larger than ca. 40 kDa. When the smaller protein binds to a larger one, the observed $1/T_2$ transverse relaxation rates of the bound protein in the complex increase significantly and broadening of NMR resonances results in the disappearance of most of the cross-peaks in the HSQC spectrum[9,24−26] (Figure 1b). Addition of an antagonist that dissociates the complex would restore the HSQC spectrum of the smaller component (Figure 1c), whereas a nonbinder would not affect the HSQC spectrum of the complex (Figure 1d). A weak inhibitor could partially release the labeled protein when added in large excess (for example, an inhibitor of K_D 5 μM would release about 40% of the protein at 250 μM concentration, assuming 100 μM each of proteins and a K_D of 0.7 μM for the complex).

We tested our method on the p53−MDM2 interaction. Structure-based screens for this interaction utilized an N-terminal domain of MDM2 of ca. 100 amino acids and short peptides of p53 (refs 22, 27−30). The primary binding site of MDM2 on the p53 protein has been mapped to residues 18−26 (refs 16−21). In our study, we use a 118 amino acid N-terminal domain of MDM2 and the N-terminal 312-residue fragment of p53, which encompasses the transactivation and DNA binding domains (a schematic of the full-length proteins is shown in Supporting Information Figure 1). The binary complex has a total molecular weight of 45 kDa. Isothermal titration calorimetry (ITC) measurements indicated a K_D of 0.77 μM in the buffer solution used in our NMR experiments (see Supporting Information for ITC), which agrees well with that of ca. 0.7 μM reported in the literature.[21,27,31]

In the first experiment, the isotopically enriched ^{15}N-MDM2 (Figure 2a) was titrated against unlabeled p53, and the complex formation was observed by the disappearance of most of the MDM2 peaks, as seen in Figure 2b. The majority of the backbone ^{15}N−^{1}H resonances of structured regions of the molecule broadened and/or disappeared. The leftover peaks originate from flexible residues of the complex and/or free MDM2 (for the complex MDM2−p53 with a K_D of 0.77 μM

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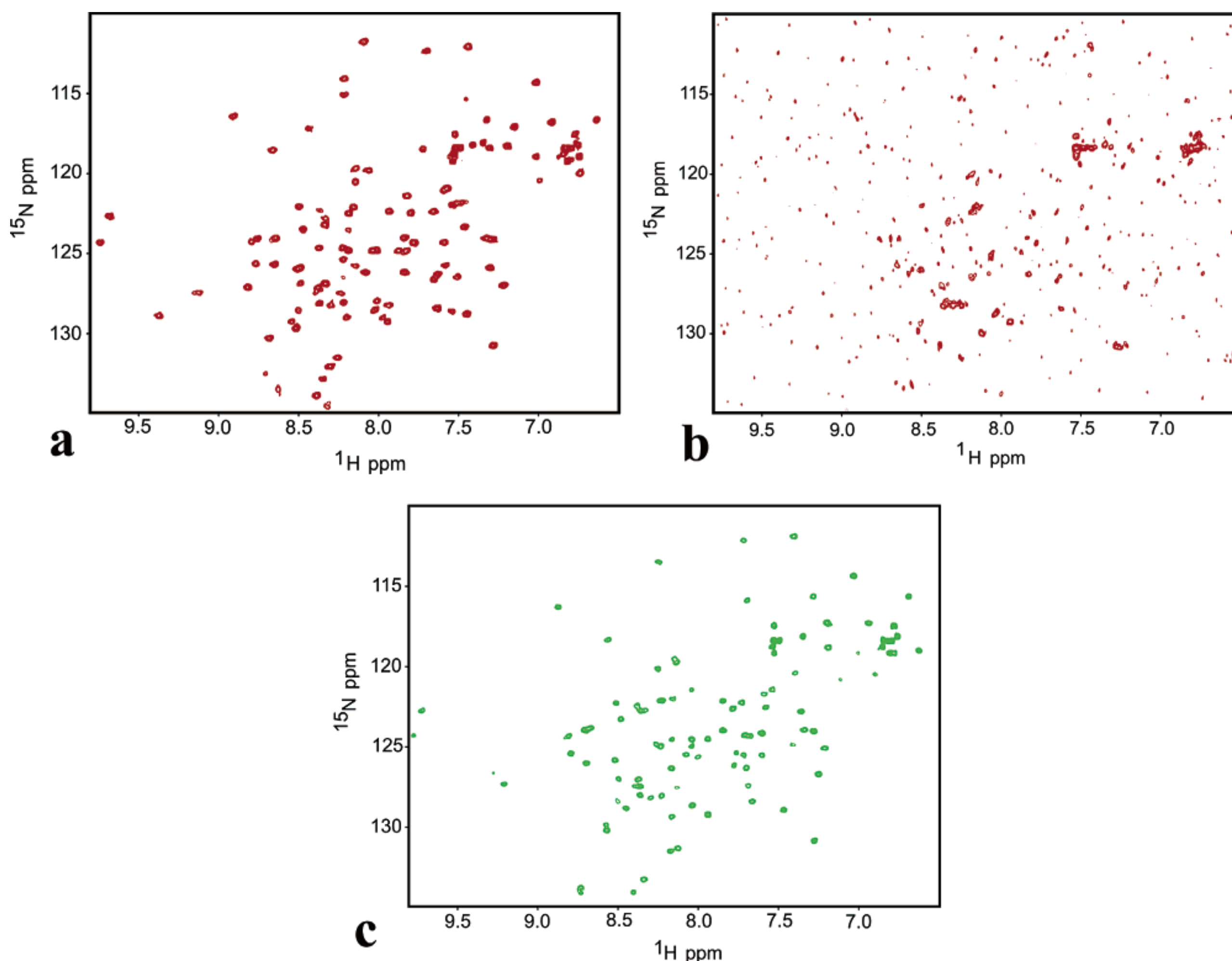

Figure 2. Spectra of the ^{15}N uniformly labeled MDM2. (a) ^{1}H$-^{15}$N HSQC spectrum of ^{15}N-MDM2. (b) ^{1}H$-^{15}$N HSQC spectrum of ^{15}N MDM2 complexed with p53. Most of the cross-peaks disappear from the reference MDM2 spectrum, indicating complex formation. (c) ^{1}H$-^{15}$N HSQC spectrum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

and a protein concentration of 0.1 mM, there is still 8.4% free MDM2 present, and the residual observed sharp signals could arise from this free protein). All of these signals are located in the spectrum at the "central 8.3 ppm NH amide" region, diagnostic for unstructured residues, plus flexible side chains at 7 and 7.5 ppm (for details on how to use NMR to assess the extent of folding in proteins, see ref 25 and the Supporting Information).

Nutlins are a class of *cis*-imidazoline compounds that were recently reported to inhibit the p53$-$MDM2 interaction.[22] The most potent among them, nutlin-3, was reported to displace recombinant p53 protein from its complex with MDM2 with an inhibitory concentration (IC_{50}) value of 0.09 μM (ref 22). A stepwise 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 being, however, shifted. The freed p53 is folded (the core domain) as judged by NMR (Supporting Information Figure 2). The experiment also shows that the MDM2/nutlin complex is soluble and that nutlin did not induce precipitation of MDM2. Supporting Information Figure 3 shows the cross section from the ^{1}H$-^{15}$N HSQC spectra of one of the peaks of ^{15}N-MDM2/p53 complex titrated at increasing concentrations of nutlin-3.

We also performed the experiment with the ^{15}N-labeled p53 and unlabeled MDM2. Figure 3a shows the ^{1}H$-^{15}$N HSQC spectrum of the uniformly labeled p53. The spectrum indicates that the first 93 residues are flexible and mostly unstructured. The residues of the p53 DNA core domain are mostly not seen because of their broad line widths as compared to those of the flexible part. Complex formation can be monitored from the NMR spectrum, as the three primary binding sites of p53 to MDM2 are known (Phe19, Trp23, and Leu26 (ref 27)). The cross-peaks of these residues begin to disappear on stepwise addition of MDM2, and they completely disappeared on the complex formation as seen in Figure 3b. The experiment unequivocally shows that the N-terminal residues are still not structured when bound to the MDM2 domain, with the exception of a 10 residue-binding site (residues 17$-$26). Nutlin-3 was then added to the MDM2/p53 complex in a stepwise manner, and the reappearance of the three peaks (Phe19, Trp23, and Leu26) was monitored. Figure 3c shows the spectra of ^{15}N-p53 and the reappearance of the three binding sites, indicating that nutlin-3 dissociates the p53$-$MDM2 complex by binding to MDM2.

We checked our methodology on another small molecule compound, a sulfonamide compound NSC 279287, recently reported to inhibit the MDM2/p53 interaction with IC_{50} of 32

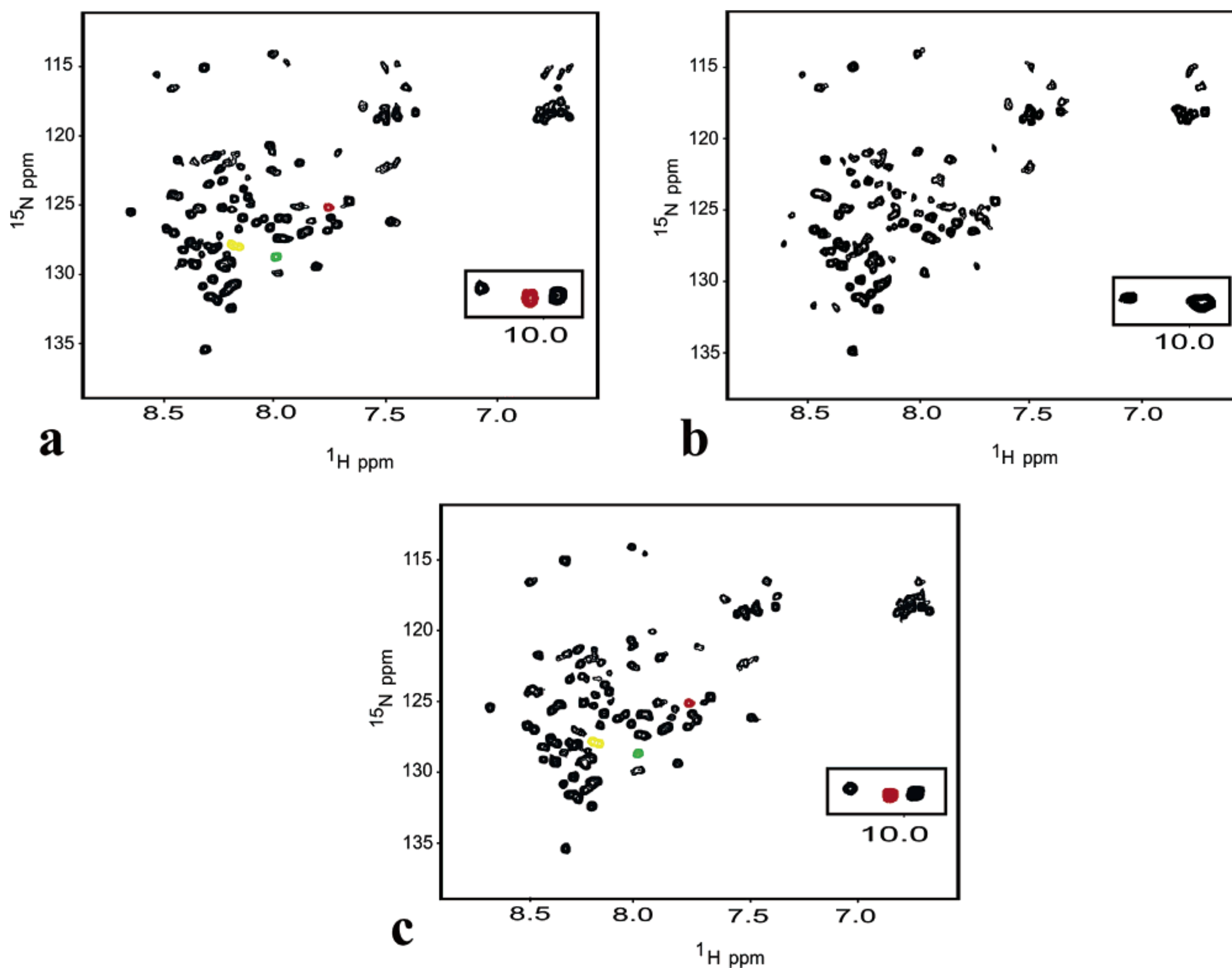

Figure 3. Spectra of the ^{15}N uniformly labeled p53. (a) ^{1}H−^{15}N HSQC spectrum of p53 (for assignments, see refs 36,37). The cross-peak of Phe19 is shown in yellow, and Leu26 is in green. The two cross-peaks in red are from Trp23, with the side-chain cross-peak at the ^{1}H chemical shift of 10.10 ppm (inset). (b) ^{1}H−^{15}N HSQC spectrum of ^{15}N-p53 complexed with MDM2. The complex formation is monitored by the disappearance of peaks corresponding to Phe19, Trp23, and Leu26. (c) ^{1}H−^{15}N HSQC spectrum of free p53 after addition of nutlin-3. The spectrum corresponds to that of Figure 3a, with all of the "bound" peaks being restored, indicating the dissociation of the complex.

μM (ref 32). A stepwise addition of the sulfonamide to p53/^{15}N-MDM2 resulted in the release of the folded p53. The release of p53 could also be monitored from the 1D proton NMR spectra. Supporting Information Figure 4 shows the 1D spectra of the region where the side chain of Trp23 resonates at 10.10 ppm. We also added sulfonamide to the free ^{15}N-MDM2 with the intention of adding p53 after the preincubation of MDM2 with the compound; however, we observed only very small, insignificant induced chemical shift changes in the ^{15}N HSQC spectrum of MDM2 on titration with sulfonomide (see Supporting Information Figure 5). We thus conclude that the compound sulfonamide works by precipitating MDM2, as no signals from MDM2 were present in the final NMR 1D proton spectrum. MDM2 begins to precipitate at about 0.3 mM sulfonamide concentration, which is about 3 times the protein concentration, and the precipitation was complete at about 1 mM, which is about 10 times the protein concentration.

Recently, novel boronic-chalcone derivatives have been described as MDM2 antagonists with antitumor effect against cultured tumor cells.[33] Employing our methodology by using ^{15}N-labeled MDM2, the derivative **3b** of the boronic chalcone (Table 1, ref 33) was titrated to study its effect on the MDM2/p53 complex. This boronic chalcone did not dissociate the MDM2−p53 complex even at concentrations of 2 mM, that is, 20 times that of the protein complex (data not shown; the HSQCs were all equivalent to those of Figure 2b). At this concentration of the ligand, the complex completely precipitated in the NMR tube. However, direct titration of ^{15}N-MDM2 with chalcone showed that they bind to the tryptophan-binding subsite of the p53-binding cleft of human MDM2 (refs 27−29) with very low, that is, high micromolar, affinity, indicating that these compounds are extremely weak inhibitors for the MDM2/p53 interaction.

Discussion

We have tested three lead compounds that have recently been reported to block the p53−MDM2 interaction: nutlin-3 (ref 22), a sulfonamide compound NSC 279287 (ref 32), and a boronic chalcone.[33] Among these three candidates, only nutlin-3 was shown to indeed be an inhibitor of the p53−MDM2 complex and therefore is a potential candidate for drug development in cancer treatment. Our assay showed that nutlin-3 releases p53 from the complex by competing with p53 for binding to MDM2.

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The important outcome of our experiment is that it shows that the freed p53 is folded. It also shows that the MDM2/nutlin complex is soluble and that nutlin did not induce precipitation of MDM2. The dissociation constant (K_D) of nutlin-3 for MDM2/p53 complexes estimated from NMR is in the low micro-molar range (<1 μM), indicating tight binding.

The sulfonamide compound precipitates MDM2 and releases folded p53. The boronic chalcone precipitated p53 and MDM2 at high ligand concentrations. This boronic chalcone is an extremely weak binder for MDM2, inconsequential for the MDM2/p53 interaction, and probably expresses its antitumor effect in tumor cell lines via inhibition of interactions other than the primary p53−MDM2 interaction. In case of sulfonamide, the precipitation was selective for MDM2 but not for p53. Direct treatment of MDM2 with sulfonamide gave the same results as that of the complex.

The two last examples indicate that our assay provides for a more rigorous detection of inhibition of protein−protein interactions than the approaches based on affinity chromatography pull down assays, and immunoprecipitation, and similar to these methods they are known to give false positive results. In addition, these methods provide very limited information about the structural status of proteins. The correct structure of a protein is a universal requirement for its function.

An interesting side-result of our experiments is the determination of the folding status of our p53 free and bound to MDM2. Characterization of this status has been a subject of several recent studies.[34−37] Our NMR spectra indicate that the first 93 residues are flexible and unstructured, in agreement with the findings that showed that the full-length p53 contains large unstructured N- and C-terminal regions in its native state.[35−37] Because the HSQC spectrum of these 93 residues is almost identical to that of the isolated N-terminal domain,[37] the conformations of these residues have to be the same as those found in ref 37; that is, although the p53 transactivation domain does not have tertiary structure, it is nevertheless populated by a nascent helix and turns.[36−38] A new finding is that our NMR spectra unequivocally show that, with the exception of a 10 residue-binding site (residues 17−26), the bulk of the N-terminal residues are still not structured when bound to the MDM2 domain.

Our method should provide an important extension to the traditional "SAR by NMR".[1,9−11] One weakness of the SAR approach is that small structured fragments of large proteins have to be found although only larger fragments are usually available at initial studies of protein−protein bindings. 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. A remedy would be to prepare a ^{15}N selectively labeled protein with one amino acid type and obtain a less crowded NMR "subspectrum".[12]

An example of spectral overlap is provided by spectra of the retinoblastoma protein (pRb) shown in Figure 4. The smallest functional fragment of pRb, which is also structured, is the so-called small pocket domain of 361 residues (pRb-AB)(ref 39). The retinoblastoma tumor suppressor protein is a fundamental negative regulator of cell proliferation that is frequently targeted in human cancer. Many viral oncoproteins (for example, HPV E7, E1A) are known to bind to the pRb pocket domain via a LXCXE binding motif.[39−42] The HSQC spectrum of the ^{15}N/^{2}H uniformly labeled pRb-AB contains NH resonances from 361 residues (except prolines) and is too crowded for detecting interactions with the pRb binding peptides. Figure 4a shows the spectra of ^{15}N-labeled pRb small pocket domain titrated with the HPV E7 peptide of 20 amino acids. Figure 4b shows that the complex formation E7−pRb can easily be detected with NMR if a larger fragment of the E7 protein is used.

Another weakness of the traditional "SAR by NMR" approach arises when NMR cross-peaks of several resonances disappear upon ligand binding. This happens for intermediate exchanges when the lifetime of the free and bound states is approximately equal to the differences in chemical shift and/or transverse relaxation rates between the free and ligand-bound forms. These peaks are usually those residues located at the binding interface; however, it is difficult in practice to interpret these data, unless additional information from the structure of the protein is available.

Several variants of our approach are possible. In few favorable cases (flexible residues), 1D proton NMR spectra may suffice for monitoring the states of proteins in complexes upon treatment with ligands. The regions of the proton NMR spectra at ppm 8.7 to 12 and 0.0 to −0.5 could be used for these purposes.[24,25] We have used 1D spectra of the NH side chains of Trp residues of p53. In general, however, the highly flexible feature of the N-terminal domain of p53 is an exception rather than a rule.[34]

A protocol that would start first with the titration of the small ^{15}N-labeled component of the complex would correspond to the traditional "SAR by NMR" approach. Adding the second larger protein would then follow. Finally, a part of the large protein fragment could be replaced by GST, which has 226 amino acids. For example, a GST tagged N-terminal 93-residue p53 domain could replace the 1−312 residue p53 fragment.

Methods

Protein Expression and Purification. The recombinant human MDM2 (residues 1−118) was overexpressed at 30 °C in *E. coli* BL21 (DE3) using the pQE-40 vector (Qiagen). The protein was renatured from *E. coli* inclusion bodies as previously published.[29] Refolded MDM2 was first applied to the butyl Sepharose 4 Fast Flow (Amersham) and second to HiLoad 16/60 Superdex75 gel filtration (Amersham) columns. The recombinant human p53 protein (residues 1−312) was overexpressed at 37 °C overnight in *E. coli* BL21 (DE3) using a modified pQE-40 with N-terminal His-tag and T5 promotor as described in ref 34. The protein was purified under denaturing conditions using a NiNTA (Qiagen) column, refolded, and further purified using a Heparin Sepharose 6 Fast Flow (Amersham) column. Final purification was done via a HiLoad 16/60 Superdex75 gel filtration column. The uniformly ^{15}N enriched protein samples were prepared by growing the bacteria in minimal media containing ^{15}N-NH_4Cl (ref 29).

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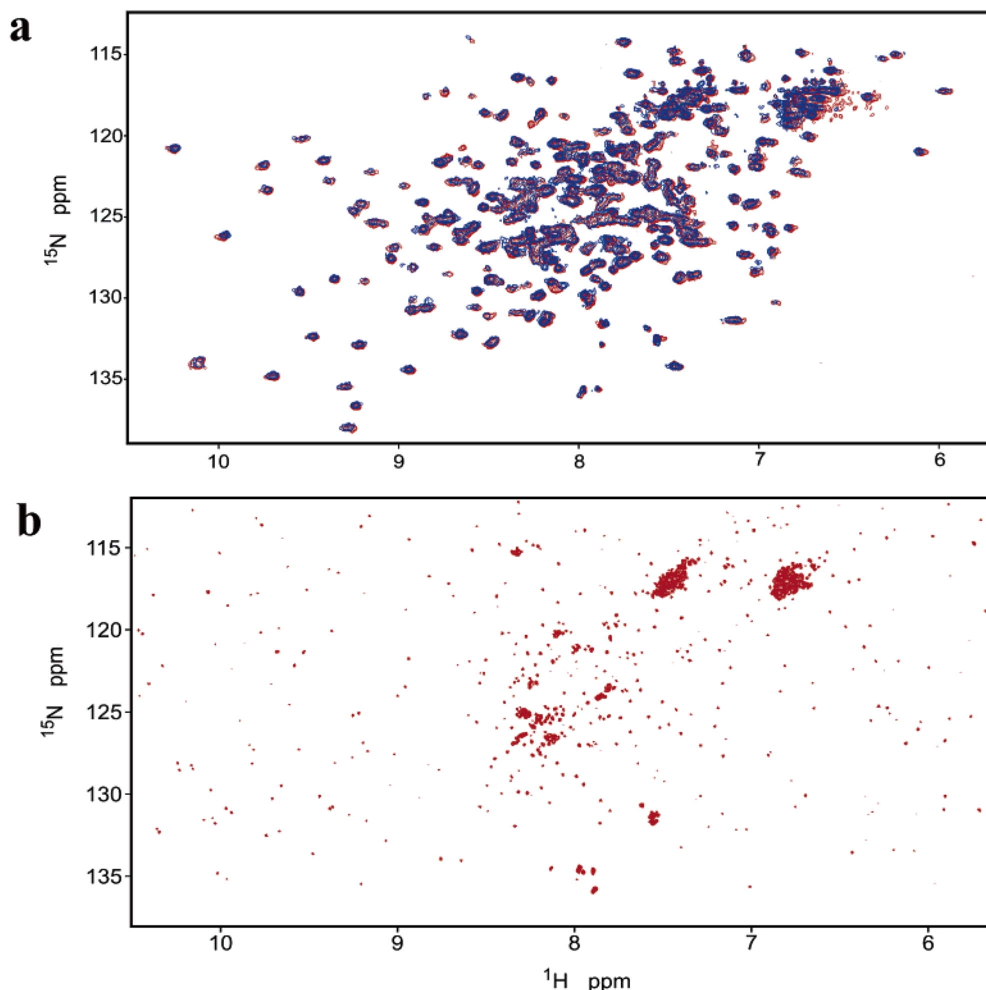

Figure 4. (a) ^{15}N-HSQC spectra of the small pocket domain of [^{15}N, ^{2}H]-pRb. The reference spectrum is shown in red, and the one in blue is after titration with the E7 peptide. (b) On addition of the dimeric full-length E7 protein (104 residues for a monomer), the pRb spectrum disappears due to the formation of the complex.

The small pocket domain of pRb (pRb-AB) was cloned into the pET30 LIC/Xa vector and consisted of an N-terminal 11 amino acid long 6-His-tag followed by the pocket domain without spacer region (from amino acid 379−578 and 642−791). For expression of the protein, the *E. coli* strain BL21 STARTM (DE3) (Invitrogen) was used. Uniformly ^{15}N and specifically ^{15}N lysine labeled pRb-AB were prepared following standard procedures,[43] except that protein induction was carried out at 18 °C overnight. For the perdeuterated sample preparation, cells were first adapted by growing them on small culture with 30%, 60%, 75%, and 90% before growing them in 99% $^{2}H_2O$ containing media. In this case, induction was carried out for 18 h at 18 °C.

In the first step of purification, the crude cell lysate, after the sonication, was passed through the Ni-NTA column. The eluent from the Ni column was subjected to the MonoQ (Amersham-Pharmacia) anionic exchange chromatography column. The final buffer of the protein solution was 50 mM $NaH_2PO_4 \cdot H_2O$, 10 mM βME, pH 7.8. The identity of protein was confirmed with the SDS-PAGE, Western blot, N-terminal sequencing, and mass spectrometry. Final protein samples used for all of the studies were more then 95% pure as judged by SDS-PAGE analysis. In case of the perdeuterated sample, the protein was >95% perdeuterated as judged by mass spectrometry.

NMR Spectroscopy. All NMR spectra were acquired at 300 K on a Bruker DRX 600 MHz spectrometer equipped with a cryoprobe. Typically, NMR samples contained up to 0.1 mM of protein in 50 mM KH_2PO_4, 50 mM Na_2HPO_4, 150 mM NaCl, pH 7.4, 5 mM DTT, 0.02% NaN_3, and protease inhibitors. For the ^{1}H−^{15}N HSQC spectrum,[44] a total of 1024 complex points in t_2 and 128 t_1 increments were acquired. Water suppression was carried out using the WATERGATE sequence. NMR data were processed using the Bruker program Xwin-NMR version 3.5.

Ligand Binding. Ligand binding experiments were carried out in an analogous way to that described in Stoll et al.[29] 500 μL of the protein sample containing 10% D_2O, at a concentration of about 0.1 mM, and a 20 mM stock solution of each compound in DMSO-d_6 were used in all of the experiments. Titrations were carried out with three inhibitors, nutlin-3 (nutlin-3 was purchased from Cayman Chemical, MI (Catalog No. 10004372) and comprised an enantiomer with the most potent binding activity toward MDM2 (ref 22)), sulfonamide (obtained from the National Cancer Institute, NSC 279287), and a boronic chalcone (derivative **3b**, Table 1, ref 33). In case of the experiments with nutlin-3, the titration was carried out until no further change in the 2D spectrum was observed, thus indicating saturation. The maximum concentration of DMSO at the end of titration experiments was about 2−3%. The pH was maintained constant during the entire titration.

As controls, to check the effect of DMSO on the proteins, we titrated the protein complex and proteins with DMSO. We found no significant changes in terms of chemical shifts, precipitation, or denaturation of

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the proteins for DMSO concentrations used in the compound titrations (up to 3%). Supporting Information Figure 6 shows the $^{1}H-^{15}N$ HSQC spectra of MDM2 titrated with DMSO. The p53/MDM2 complex titrated up to 20% with DMSO partially precipitated (40% after 10 h). Preincubation of the p53/MDM2 complex with 15% DMSO at 310 K for 1.5 h, 10 deg higher than the temperature at which NMR experiments were carried out (300 K), resulted in increased precipitation (50%), but no other changes in the spectrum of the soluble fraction were observed. The soluble complex was reacting with nutlin-3. The p53 peaks of the ^{15}N-labeled p53 shift little with higher concentrations of DMSO (ca. 0.1 ppm in proton dimension for the Trp 23 side-chain signal at 20% DMSO). We thus conclude that the DMSO effect on the proteins while titrating with different compounds can be neglected.

Acknowledgment. We thank the National Cancer Institute (Chemistry and Synthesis Branch) for providing us with the sulfonamide NSC 279287 compound. We thank Dr. Heiko Hermeking for the p53 constructs.

Supporting Information Available: A discussion on understanding the extent of protein folding, a schematic representation of the full length proteins used in our experiments, ITC binding result for p53 and MDM2, a ^{15}N spectrum of p53 showing the structured parts of the protein, cross sections of the ^{15}N HSQC spectrum of the complex of p53/MDM2 titrated with nutlin-3, 1D proton spectra of the side chains of tryptophans of p53, ^{15}N spectra of MDM2 titrated with the sulfonamide compound, ^{15}N spectra of MDM2 titrated with DMSO, and complete refs 23 and 29. This material is available free of charge via the Internet at http://pubs.acs.org.

JA052143X