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Targeting zinc finger domains with small molecules: solution structure and binding studies of the RanBP2-type zinc finger of RBM5

Dr. Biancamaria Farina^{[a],[b]}, Prof. Roberto Fattorusso^[c], and Prof. Maurizio Pellecchia^[a]

Maurizio Pellecchia: mpellecchia@sanfordburnham.org

^[a]Dr. B. Farina, Prof. M. Pellecchia Infectious Diseases and Cancer Center Sanford-Burnham Medical Research Institute 10901 North Torrey Pines Rd., La Jolla, CA Fax: (858-795-5225)

^[c]Prof. R. Fattorusso Dipartimento di Scienze Ambientali Seconda Università di Napoli Via Vivaldi 43, 81100, Caserta, Italy

Abstract

The RNA Binding Motif protein 5 (RBM5), also known as Luca15 or H37, is a component of prespliceosomal complexes, that regulates the alternative splicing of several mRNAs, such as Fas and caspase-2.

The *rbm5* gene is located at the 2p21.3 chromosomal region, which is strongly associated with lung cancer and many other cancers. Both increased and decreased levels of RBM5 can play a role in tumor progression. In particular, down-regulation of *rbm5* is involved in lung cancer and other cancers upon Ras activation, and, also, represents a molecular signature associated with metastasis in various solid tumors. On the other hand, up-regulation of *rbm5* occurs in breast and ovarian cancer. Moreover, RBM5 was also found to be involved in the early stage of the HIV-1 viral cycle, representing a potential target for the treatment of the HIV-1 infection.

While the molecular basis for RNA recognition and ubiquitin interaction have been structurally characterized, small molecules binding this ZF domain that may contribute to characterize their activity and to develop potential therapeutic agents have not been yet reported. Via an NMR screening of a fragment library we identified several binders and the complex of the most promising one, named compound **1**, with the RBM5 ZF1 was structurally characterized in solution. Interestingly, the binding mechanism reveals that compound **1** occupies the RNA binding pocket and is therefore able to compete with the RNA to bind RBM5 RanBP2-type ZF domain, as indicated by NMR studies.

Keywords

NMR; Fragment-based drug discovery; Zn-finger; RanBP2; RBM5; NMR spectroscopy; protein-ligand interactions

Introduction

The RNA Binding Motif protein 5 (RBM5), also known as Luca15 or H37, is a component of prespliceosomal complexes^[1] that regulates the alternative splicing of several mRNAs, such as Fas^[2] and caspase-2.^[3] RBM5 full length is a 815 residue protein that contains two

Correspondence to: Maurizio Pellecchia, mpellecchia@sanfordburnham.org.

^[b]Present address Dr. B. Farina, Istituto di Biostrutture e Bioimmagini C.N.R. Via Mezzocannone 16, 80134, Naples, Italy

RNA binding domains, also recognized as RRM (RNA Recognition Motif), two zinc fingers, one arginine-serine (RS) domain, and one glycine (G) patch (Figure S1). The first zinc finger (ZF) corresponding to the 181–210 region is located between the two RRMs and belongs to the RanBP2-type zinc finger sub-family. This motif occurs in at least 21 human proteins,^[4] playing different biological roles. Despite the diversity of the proteins containing this motif, two distinct functions are observed: ubiquitination/SUMOylation and RNA metabolism.^[5] The RanBP2-type ZF motif is defined by the consensus sequence Trp-X-Cys-X₂₋₄-Cys-X₃-Asn-X₆-Cys-X₂-Cys and contains a single zinc ion coordinated by the four cysteine sulphurs. The fold has been structurally characterized in the free and/or the bound form with ubiquitin or RNA and is composed by two distorted β -hairpin sandwiching the conserved tryptophan and the zinc ion.^[6]

The *rbm5* gene is located at the 2p21.3 chromosomal region^{[7] [8]}, which is strongly associated with lung cancer and many other cancers. Both increased and decreased levels of RBM5 can play a role in tumor progression.^[2] In particular, down-regulation of RBM5 is involved in lung cancer^{[7] [9]} and other cancers upon Ras activation, and, also, represents a molecular signature associated with metastasis in various solid tumors,^{[10] [11]}. On the other hand, up-regulation of RBM5 occurs in breast and ovarian cancer.^[12]

More recently, RBM5 was also found to be involved in the early stage of the HIV-1 viral cycle^[13], suggesting that the modulation of the activity of the RBM5 protein may provide novel strategies for the treatment of the HIV.

Finally and very recently, the RBM5 RanBP2-type ZF has been demonstrated to bind in a stoichiometric and sequence-specific manner single stranded RNA, suggesting an important role for this domain in the protein function.^[4] Interestingly, very few molecules able targeting ZFs have been discovered and characterized^[14] and particularly binders able to modulate the activity of this zinc finger subfamily have not yet been reported. Therefore, the identification of small molecules able to bind the RBM5 RanBP2-type ZF would greatly contribute to characterize the activity of the RBM5 protein and to develop potential therapeutic agents.

In the present study we first determined the high resolution NMR structure of the RBM5 RanBP2-type ZF domain. Subsequently, via an NMR screening of a fragment library several binders were identified and the complex of the most promising one, compound **1**, with the RBM5 ZF was structurally characterized in solution. Interestingly, the binding mechanism reveals that compound **1** occupies the RNA binding pocket and is therefore able to compete with the RNA to bind RBM5 RanBP2-type ZF domain, as indicated by NMR studies.

Results and discussion

NMR structure of the RBM5 RanBP2-type ZF

The one-dimensional ¹H NMR spectra of the RBM5 RanBP2-type zinc finger domain (residues 181–210) upon titration with ZnCl₂ showed an increased spread of the amide and aliphatic resonances and confirmed the formation of a 1:1 complex (RBM5-ZF1) (Figure S2). Proton spin system identification and assignment of individual resonances of RBM5-ZF1 were carried out by using a combination of TOCSY^{[15] [16]} and DQF-COSY^[17] spectra. Sequence-specific assignment was obtained by NOESY experiments,^[18] according to the standard procedures.^[19] Using the CYANA software,^[20] the solution structure of RBM5-ZF1 was preliminary calculated by means of 309 NOE restraints and 12 distance restraints for the tetrahedral zinc coordination. No zinc ion was included during these calculations. To further refine the NMR structure, amide temperature coefficients were measured to include additional hydrogen bond restraints in the calculation. The final

ensemble of the best 20 CYANA RBM5-ZF1 conformers was energy minimized, including Zn(II) and no NOE and hydrogen bond restraints. The NMR structure of RBM5-ZF1, reported in Figure 1, is well defined with the average rms deviations to mean of the backbone and of the heavyatoms (residues 4-29) of 0.33 Å and 0.98 Å, respectively (Table S3).

Residues 4-29 of RBM5-ZF1 adopt a compact fold that incorporates a zinc ion coordinated by thiolate groups of the cysteine residues Cys⁷, Cys¹⁰, Cys²¹ and Cys²⁴ (Figure 1). Cys¹¹ is not implicated in the coordination of the zinc and is solvent exposed. The zinc finger fold is also stabilized by a small hydrophobic core, constituted by the indole ring of the conserved Trp⁵, which makes hydrophobic contacts with the side chains of Asn¹⁴ from strands 2 and of Arg²⁸, as confirmed by the upfielded chemical shift of one Asn¹⁴ H β and the side chains protons of Arg²⁸.

The fold is consistent with that of a RanBP2-type ZF, consisting of two distorted hairpins, each providing two of the zinc coordinating cysteine residues (Figure 1b). The first (residues Asp⁴-Phe¹⁵) is a canonical β -hairpin formed by two anti-parallel β -strands; strand 1 comprises residues Trp⁵-Leu⁶ and strand 2 residues Asn¹³-Asn¹⁴. Three hydrogen bonds are observed, Trp⁵ H^N $\rightarrow$ Asn¹⁴ CO, Asn¹⁴ H^N $\rightarrow$ Trp⁵ CO and Cys⁷ H^N $\rightarrow$ Leu¹² CO. The second hairpin consists of residues Leu¹⁹-Lys²⁸ but in none of the final conformers β -strands are properly formed. Indeed, no hydrogen bonds are observed between Leu¹⁹ H^N and Lys²⁸ CO, as confirmed by the amide temperature coefficient of the Leu¹⁹ (Table S1), whereas hydrogen bonds are established between the H^N of Cys²¹ and the CO of Ala²⁶, and the H^N of Gly²⁵ and the CO of Cys²¹. Nevertheless, this difference in the hydrogen bond pattern between the two hairpin is also observed in other RanBP2 like ZFs. [21] [22] [23]

The two turns of the hairpins represent the typical “knuckles” as observed in the rubredoxins and in the other ZFs (ref). Indeed, in the first “knuckle” (Cys⁷-Leu¹²) the H^Ns of the Lys⁹ and Cys¹⁰ form hydrogen bonds with the S γ of the Cys⁷, as confirmed by amide temperature coefficients (Table S1), whereas the H^N of the Leu¹² establishes an hydrogen bond with the S γ of the Cys¹⁰. Similarly, in the second “knuckle” (Cys²¹-Ala²⁶) the H^Ns of the Arg²³ and Cys²⁴ form hydrogen bonds with the S γ of the Cys²¹, whereas the H^N of the Ala²⁶ establishes an hydrogen bond with the S γ of the Cys²⁴. Also in this case, the involvement in hydrogen bonds is confirmed by the amide temperature coefficients of those residues (table S1). The hairpin are connected by a type III β -turn (residues Phe¹⁵-Arg¹⁸), as confirmed by the backbone dihedral angles of Arg¹⁶ and Lys¹⁷ residues (table S2) and by the intramolecular 4₁ hydrogen bond, involving the H^N of Arg¹⁸ and the CO of Phe¹⁵.

Finally, residues in the Asp²⁷-Asp³⁰ segment, judging from the ϕ and ψ dihedral angles of Lys²⁸ and Phe²⁹ (table S2), form a type I β -turn characterized by a hydrogen bond i,i+3 between the CO of Asp²⁷ and the H^N of Asp³⁰.

Screening of a fragment library and binding studies

To identify small molecules that could be used as chemical probes for target validation and functional studies of the RBM5-ZF1, we performed a NMR-based screening of a 500 compounds fragment-based library by using 1D ¹H NMR experiments. The screen was conducted using protein samples at 10 μ M concentration and mixture of 6-7 compounds per sample (100 μ M each). Compound mixtures producing chemical shift perturbation in the 1D ¹H NMR spectrum of RBM5-ZF1 were subsequently deconvoluted.. Totally, nine compounds (1-9, Figure 2a) emerged as able to produce significant chemical shift variations in the 1D spectrum of the polypeptide. Comparison of 1D ¹H NMR spectrum of RBM5-ZF1 in presence of the ligands and that of RBM5 without Zn(II) suggests that compounds 8 and 9 induced Zn ejection presumably by interacting directly with the coordinating Cys residues.

Hence, we focused on compounds that interact with the protein and preserve its integrity (compounds **1-7**). In particular, compounds **1** (Figure 2b) and **2** produced the largest chemical shift variations upon titration. Both compounds bind the RBM5-ZF1 in the micromolar range, compound **1** exhibiting the highest affinity for the zinc finger (K_D 1/RBM5-ZF1 = 82 μ M and K_D 2/RBM5-ZF1 = 214 μ M, see Figure 2c and Figure S3). Moreover, to further validate the binding to RBM5-ZF1 of compound **1**, we carried out a 2D [1 H, 1 H] NOESY experiment, measured in presence and absence of the polypeptide. As a result, intense negative NOEs were observed (Figure 2d) in presence of the RBM5-ZF1, which is a typical behavior for small molecule binding to a molecule with a slow tumbling such as the target polypeptide and larger proteins.

To define the binding site of the compound **1** on RBM5-ZF1, we performed chemical shift perturbation studies, monitoring the chemical shift variations in 2D [1 H, 1 H] NOESY spectrum. Interestingly, the NOESY spectrum, reported in Figure 3a, showed large variations in chemical shifts after the addition of the compound **1**. Virtually complete assignment of the proton resonances of **1**-RBM5-ZF1 complex were obtained by analysis of NOESY and TOCSY spectra based on the previous obtained assignments of the free polypeptide. The residues most affected upon addition of **1** were found to be Leu¹², Phe¹⁵, Lys¹⁷, Arg¹⁸ and Phe²². The cross-peaks for the aromatic protons of Phe¹⁵ and Phe²² and the methyl protons of Leu¹² were shifted upfield upon complex formation, whereas the aliphatic side chains protons of Arg¹⁸ and the H^N protons of Lys¹⁷ and Phe²² were downfield shifted (Figure 3b). Mapping the chemical shift changes onto the NMR structure and the surface of RBM5-ZF1 (Figure 3c and 3d) revealed that the residues most affected upon complexation were clustered in a contiguous region. A closer inspection of the RBM5-ZF1 NMR structure (Figure 3c) shows that these residues constitute a cleft in which the side chain of the Arg¹⁸ were the most disordered. In particular, Arg¹⁸ side chain can be clustered in two different conformations (Figure S4), an open one (Figure S4a and c) in which the Arg¹⁸ side chain is more distant to the two Phe rings and a close one (Figure S4b and d) in which the Arg is positioned between the two Phe rings. Interestingly, the most representative structure of Arg¹⁸ side chain (Figure 3c) is the open one which gives rise to a larger cleft. Interestingly, this cleft represent, as very recently demonstrated, a central region of the RNA binding site for RBM5-ZF1 and other RanBP2-type ZF [4].

Structure of the complex between RBM5-ZF1 and compound **1**

The 2D [1 H, 1 H] NOESY spectral quality of RBM5-ZF1 remained high upon complexation with the compound **1** (Figure 4a). Moreover, 9 intermolecular NOEs were found between RBM5-ZF1 and **1**, allowing to better define the intermolecular interface. Indeed, as shown in figure 4b, interactions were observed between the protons of the compound **1** and the Leu¹² methyl group, the Asp¹⁴ H α , the Phe¹⁵ H δ and H ϵ aromatics, the Arg¹⁸ H δ proton and the Phe²² H ϵ aromatic. Interestingly, all the observed intermolecular NOEs involved the most affected residues of the peptide upon the addition of the compound **1**, except the Asp¹⁴, indicating that the perturbations observed in the NOESY spectrum were due to direct interaction with the fragment and no to long range effects.

The three-dimensional structure of the complex between RBM5-ZF1 and compound **1** was calculated using 292 NOEs, 12 distance restraints for the tetrahedral zinc coordination and 9 distance restraints for intermolecular interactions. The ensemble of the 20 best energy minimized CYANA structures, depicted in Figure S5, is of good quality with the rms deviation values over RBM5-ZF1 residues 4-29 of the backbone and of the heavy atoms equals to 0.28 Å and 0.89 Å, respectively. Moreover, the rms deviation of the compound **1** atoms is 0.31 Å. Most of backbone ϕ/ψ pairs fall well within the most favoured and additional allowed regions of the Ramachandran plot (Table S4).

In the **1**-RBM5 ZF1 complex, the small molecule fills, as expected, the hydrophobic cleft of the open conformation constituted by residues Leu¹², Phe¹⁵, Arg¹⁸, Phe²², with the Arg¹⁸ side chain pointing away from the aromatic rings of the two Phe (Figure 4c and d). Overall, a relative small change of RBM5-ZF1 surface to accommodate compound **1** is observed as indicated by superposition of RBM5-ZF1 structure in the free and bound form (Figure 4c). Specifically, the most significant changes involve the backbone conformation of Arg¹⁶ and Lys¹⁷, causing a small distortion of the type III β -turn, and of Phe²² and Arg²³ in the second “knuckle”, that change the position of the Phe²² aromatic ring. In the binding site, the anthraquinone ring of **1** makes hydrophobic interaction with the Leu¹² side chain and π - π stacking interactions with the Phe¹⁵ aromatic ring, whereas the anionic sulphonate group exhibits packing interactions with the Phe²² aromatic ring and electrostatic interactions with Arg¹⁸ guanidine group.

Competition studies of ssRNA and compound **1** binding to RBM5-ZF1

Since the binding site of the compound **1** corresponds to the central region of the RNA binding surface of the RBM5-ZF1, we performed competition binding studies of ssRNA and compound **1** to RBM5 ZF1. In particular, increasing amounts of the ssRNA AGGUAA^[4] were added to RBM5-ZF1. The complex formation was monitored via 1D ¹H NMR spectra, observing during the titration a number of changes as expected for the interaction with a medium-size molecule like a RNA (Figure 5a). The binding affinity of the RNA was also measured and confirmed to be in the low micromolar range for RBM5 ZF1 (Figure 5b). Successively, increasing quantities of compound **1** were added to ssRNA-RBM5-ZF1. The ¹H NMR spectra showed that at higher concentration we observed the formation of the **1**-RBM5-ZF1 complex (Figure 6). On the other hand, when the ssRNA was added to the **1**-RBM5-ZF1 complex, it is able to provoke the **1**-RBM5-ZF1 complex dissociation and the formation of ssRNA-RBM5-ZF1 complex (Figure 7). These results clearly indicate that compound **1** competes with the ssRNA AGGUAA for the same RBM5-ZF1 binding site and represents a good starting molecule to design new binders able to finely modulate the RNA-RBM5-ZF1 interactions.

Discussion

In the realm of drug discovery, the identification of initial validated hits is the most essential step for the subsequent, albeit lengthily and intensive, optimizations steps. Often times, drug discovery campaigns fail either for lack of such viable hits or because the selection of not suitable or artefacts hits from HTS campaigns. Fragment-based screening is a relative young and promising alternative to these problems, combining the power of biophysical approaches to ligand screening with structure based strategies for optimizations.^{[24] [25]} NMR has been often shown to be a critical method for both fragment screenings and to guide the optimizations campaigns.^[25d] Another aspect of fragment based screening is that it is rapid and provides critical information on the “druggability” of a given target.^[26] If a given protein target is not very receptive to bind small molecule ligands, such observation will rapidly emerge in a fragment based screen. For such targets than it is not advisable to embark in lengthy and costly HTS campaigns. Based on these principles, we sought here to assess the druggability of Zn-fingers by using a fragment-based screen. Zn-finger proteins are involved in various cellular processes, such as replication, transcription, metabolism, signalling, cell proliferation and apoptosis, via a broad range of molecular interactions, including protein-protein interactions, DNA and RNA recognition, and lipid binding.^[27] Thus, in principle they represent a large class of targets for a variety of diseases from cancer to neurodegeneration, to anti-viral and anti-bacterial agents. An example of the latter statement is the nucleocapsid protein 7 (NCp7) CCHC zinc finger that represent a potential target for the development of anti-HIV drugs. Several compounds were designed to inhibit NCp7 and most of them act by ejecting the metal ion from the ZF (zinc ejectors), destroying

the three-dimensional structure of the protein and leading to a loss of function. Among these inhibitors, the zinc ejector compound azodicarbonamide (ADA) [28] [29] was tested in phase I/II clinical trial.[30] However, obtaining a specific Zn-ejector is not a trivial task and the inherently reactive nature of such ligands preclude their use as chemical tools or drug leads. In fact when we tested the ADA compound against the RBM5 ZF1, we observed zinc ejection (Figure S6). Unfortunately, apart from these reactive molecules, to date no reports have been published assessing the druggability of such potentially rich class of targets by drug-like molecules that don't work by ejecting the metal ion.

We focused our attention on the first ZF of RBM5 that represents a potential therapeutic target for HIV1 and cancer. We determined the high resolution structure of NMR structure of RBM5-ZF1 which provided an important support for the fragment-based screening. The FBDD screen performed revealed several novel and interesting elements. First, unlike catalytic metal ions, the coordination of the metal ion by the four Cys residues precludes the selection of metal chelating compounds to anchor on the metal ion. Second, negatively charged molecules, however, can anchor via electrostatic interactions with the positively charged side chains, typical with RNA and DNA binding proteins. Third, the binding of compounds is complemented by interactions within a hydrophobic pocket that likely is important for recognition of the RNA molecule. In fact, displacement studies with an RNA fragment confirms that the compounds identified compete for binding to the same site of binding for the nucleic acid. Fourth, and maybe most important observation, is that the ligands that are found are specific, in that do not interact significantly (or interact with a significantly reduced affinity) to other ZF, as we demonstrate with NCp7 (Figure S7).

Hence, we believe that our studies should provide the impetus and confidence to perform further drug discovery efforts aimed at obtaining lead like molecules against ZF. These compounds would constitute unprecedented molecular probes to interrogate a large class of drug targets and potentially may result in novel classes of drugs against a variety of human disease.

Experimental Section

NMR samples

The RanBP2-type zinc finger of the human RBM5 is located at the residues 181-210 (UniProtKB/Swiss-Prot accession code: P52756). The 30-residue peptide was synthesized and purchased from Abgent (San Diego, CA). The RBM5-ZF1 was dissolved in 20 mM d_{13} -MES at pH 5.7, 1 mM d_{10} -DTT, in 90% H_2O /10% D_2O (NMR buffer), in absence and in presence of increased amount of $ZnCl_2$. For structure determinations, the peptide concentration was 100 μM in the NMR buffer in presence of 150 μM of $ZnCl_2$.

Data acquisition and resonance assignments

All the NMR experiments were carried out on a Bruker Avance 700 spectrometer equipped with a TCI cryoprobe at $T=293$ K. One-dimensional (1D) 1H spectra were acquired with a spectral width of 8417.51 Hz, relaxation delay 1.0 s, 8k data points for acquisition and 16k for transformation. The two-dimensional (2D) $[^1H, ^1H]$ spectra: double quantum filtered correlated spectroscopy (DQF-COSY),^[17] total correlation spectroscopy (TOCSY),^[15] nuclear Overhauser effect spectroscopy (NOESY)^[18] were acquired using the time-proportional phase-incrementation (TPPI) method to obtain complex data points in the t1 dimension. Typically, 32 or 64 scans per t1 increment were collected with a spectral width of 8417.51 Hz along both f1 and f2, 2048x256 data points in t2 and t1 respectively and recycle delay 1.0 s.

Water suppression was achieved using 3-9-19 pulse sequence with gradients^[31]. TOCSY experiments were recorded using a DIPSI-2 mixing scheme of 70 ms with 8.5 KHz spin-lock field strength. The NOESY spectra were carried out with a mixing time of 150 ms. The data were typically apodized with a square cosine window function and zero filled to a matrix of size 8192x2048 prior to Fourier transformation and baseline correction. Chemical shifts were referenced to the water residual peak, 4.831 ppm at 293 K. All NMR data were processed with Topspin 2.1 software and analyzed using Homoscope and NEASY,^[32] tools of CARRA (<http://www.nmr.ch>).

Structure determination

Experimental distance restraints for structure calculation were derived from the manual integration of the NOESY cross-peaks using the NEASY software and their conversion to upper distance limits by using the automatic calibration module of the CYANA v.2.1 program^[39]. Distance constraints were then used by the gridsearch module, implemented in CYANA, to generate a set of allowable dihedral angles; the structure calculations, using the torsion angle dynamics protocol of CYANA, were then started from 100 randomized conformers. Zinc ions were not included in CYANA structure calculations, but distance restraints for the tetrahedral zinc coordination, derived from the x-ray three-dimensional structure of ZNF265/ZRANB2 ZF2 RanBP2-type ZF domain (PDB code: 3G9Y) were introduced in the calculations.

Temperature dependence of NH chemical shift was determined in order to probe the possible existence in hydrogen bonds from TOCSY spectra recorded in the temperature range of 293 – 303 K. Additional H-bond constraints were introduced if the amide proton showed a $-\Delta\delta/\Delta T$ value lower than 6.0 ppb/K and if it were involved in H-bond in more than 6 structures calculated without these constraints.

The molecular graphics program MOLMOL^[33] was used to analyze and represent the 20 lowest CYANA target function structures of RBM5 RanBP2-type ZF. The coordinates for free and bound RanBP2-Zn-finger have been deposited in the PDB (PDB codes 2lk0 and 2lk1, respectively).

NMR Structure refinement

The ensemble of the best 20 CYANA structures were minimized with the software package Sybyl (Tripos, St Louis, MO, USA). The calculations were performed by using an AMBER 7 FF02 force field parameters for all the peptide atoms and distance-dependent dielectric constant. The non-bonded interaction cutoff was set to 8 Å. Zinc ions were included in the structures by the superimposition with the x-ray tridimensional structure of ZNF265/ZRANB2 ZF2 RanBP2-type ZF domain (PDB code 3G9Y) and distance constraints between zinc ion and sulphur cysteines were included in the calculations. For energy minimizations 200 steps of steepest descent method with a maximum step size of 0.05 Å were performed.

NMR small molecules screenings and binding studies

RBM5-ZF1 peptide was screened against 500 compounds from an in-house assembled fragment library. 10 µM zinc-peptide was analyzed in presence of mixtures of 6-7 compounds (100 µM each). Ligand binding was detected by comparing the aliphatic region of 1D ¹H NMR spectra of RBM5-ZF1 in presence and absence of small molecules mixtures. Hits were identified by deconvolution of the mixtures that caused significant perturbation in the 1D ¹H NMR spectra. Such hits were further characterized by 2D-NOESY experiments acquired with mixing times of 150–500 ms.

Determination and refinement of the structure of the complex RBM5-ZF1- compound 1

The structure of the intermolecular complex between RBM5-ZF1-compound **1** was determined using the upper distance restraints derived from the intra- and inter-molecular cross-peaks of the NOESY spectrum (mixing time 150 ms) of the zinc-peptide 100 μ M in presence of 5 equivalents of the compound **1**. All intermolecular upper distance constraints were set to 4.5–5.5 Å. Structure calculations were performed using CYANA software like for the RBM5-ZF1 in the free form, except that the compound **1** was inserted in the cyana library and included in the calculations as a residue bound to the C-terminus of the peptide by 26 linker dummy residues.

The 20 lowest cyana target function structures of the complex were subject to energy minimization by means of Sybyl software including zinc ions like for the free peptide. In this case, the energy minimization of the complex was performed first with 50 steps of Powell algorithm fixing the peptide and 200 steps of steepest descent fixing the ligand. After that the whole system was subject to 100 steps of conjugated gradient minimizations. For all the calculations Tripos force field as implemented in Sybyl was used. Gasteiger-Huckel partial charges were assigned to the ligand atoms, whereas Amber partial charges were assigned to the peptide atoms. The formal charge of the zinc ion was manually set to +2.

ssRNA binding studies to RBM5 ZF1 and competition studies with the compound 1

The single stranded RNA (ssRNA) AGGUAA was purchased by Integrated DNA technologies. A stock solution was prepared by dissolving 310 nmol of the ssRNA in 500 μ L of 20 mM d₁₃-MES at pH 5.7 with water RNase-free. Increasing amounts of AGGUAA, dissolved in 20 mM d₁₃-MES at pH 5.7 with water RNase-free, were added to RBM5-ZF1 10 μ M until an excess of 5 times. The chemical shift perturbations were followed by 1D ¹H NMR spectrum and quantified by calculating the chemical shift difference with respect to the free polypeptide ($\Delta\delta$ H). To obtain the dissociation constant (K_D) of ssRNA-RBM5-ZF1 $\Delta\delta$ H values of RBM5-ZF1 fast-exchanging protons were plotted versus ssRNA concentrations and fitted to a 1:1 binding model by means of GraphPad Prism program. The final estimation of the K_D was an average value over the K_D obtained from each fitting and the error is given by the standard deviation.

Displacement studies of the ssRNA AGGUAA from RBM5-ZF1 with compound **1** were performed by adding 100 and 300 μ M of compound **1** to RBM5-ZF1 (10 μ M) in presence of ssRNA AGGUAA (5 μ M). On the other hand, displacement studies of the compound **1** from RBM5-ZF1 with ssRNA AGGUAA were carried out by adding ssRNA AGGUAA (5 μ M) to RBM5-ZF1 (10 μ M) and of **1** (500 μ M).

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Figure 1.

The solution structure of RBM5-ZF1. (a) Superimposition over the backbone atoms of residues 4-29 of the best 20 energy minimized CYANA structures of RBM5-ZF1. The disordered residues 1-3 are omitted for clarity (b) Ribbon diagram of the most representative structure of RBM5-ZF1. The side chains of the cysteines involved in the zinc(II) coordination are shown in gold, whereas the zinc ion is displayed as sphere in magenta.

Figure 2.

NMR screening of a fragment-based library against RBM5-ZF1. (a) Chemical structures of the compounds positive to the screening. (b) 1D ^1H NMR spectrum of RBM5-ZF1 in presence (top) and in absence (bottom) of the compound **1**. Significant variations in the ^1H NMR spectrum of the polypeptide are indicative of the binding of the compound **1**. (c) NMR titration of RBM5-ZF1 with **1**. Chemical shift perturbations of RBM5-ZF1 fast exchanging protons are shown. The curves represent the best fit to a 1:1 binding model. The dissociation constant (K_D) of **1**-RBM5-ZF1 is an average value and the error is given by the standard deviation. (d) 2D NOESY spectrum sections at 500 ms of the compound **1** (500 μM) in presence (bottom) and in absence (top) of RBM5-ZF1 (100 μM). trNOEs of the compound **1** observed in the presence of the polypeptide are indicated with the arrows.

Figure 3.

Structural binding studies of the compound **1** with RBM5-ZF1. (a) 2D $[^1\text{H}, ^1\text{H}]$ NOESY (150 ms) sections of the RBM5-ZF1 free (black) and bound to the compound **1** (grey). The most significant shifts observed in the **1**-RBM5-ZF1 complex are indicated with the arrows. (b) Chemical shift differences for RBM5-ZF1 in presence of 5 equivalents of compound **1** against residue number. (c) Chemical shift mapping on the ensemble of the NMR RBM5-ZF1 structure represented as neon. (d) Chemical shift mapping on the surface of the most representative structure of the free RBM5-ZF1. Regions most affected by the binding of compound **1** are highlighted in red. In (c) the side chains of the cysteine residues involved in the zinc(II) coordination are shown in gold, whereas the zinc ion is displayed as sphere in magenta.

Figure 4. Structural studies of the complex between RBM5-ZF1 and compound **1**. a) 2D $[^1\text{H}, ^1\text{H}]$ NOESY (150 ms) sections of the amide-aliphatic (left) and the amide-aromatic (right) regions of the RBM5 RanBp2 Zinc finger bound to the compound **1**. Intermolecular NOEs between the polypeptide and the compound **1** are circled. (b) Schematic representation of the intermolecular NOEs observed between the protons of the two molecules. (c) e (d) Binding site of the RanBp2 ZF for the compound **1** indicated as capped stick and surface, respectively. These figures were generated by Molmol^[33] (c) and SYBYL (d, Tripos. St. Louis, MO, USA). The color code of the surface is according to cavity depth: blue, shallow; yellow, deep.

Figure 5.

a) NMR titration of RBM5-ZF1 with the ssRNA AGGUAA. Chemical shift perturbations of RBM5-ZF1 fast-exchanging protons are plotted versus ssRNA concentrations and fitted to a 1:1 binding model. The reported dissociation constant (K_D) of ssRNA-RBM5-ZF1 is an average value and the error is given by the standard deviation.

Figure 6.

Displacement studies of the ssRNA AGGUAA from RBM5-ZF1 with compound **1**. ^1H NMR spectra of RBM5-ZF1 (10 μM) in presence of ssRNA AGGUAA (5 μM) (a–c). 100 and 300 μM of compound **1** are present in (b) and in (a) respectively. In (a) symptomatic signals of the formation of the **1**-RBM5 ZF1 complex are indicated with the asterisk. (d) ^1H NMR spectra of the free RBM5-ZF1 (10 μM).

Figure 7.

Displacement studies of the compound **1** from RBM5-ZF1 with ssRNA AGGUAA. ^1H NMR spectra of RBM5-ZF1 (10 μM) and **1** (500 μM) in absence (bottom) and in presence (top) of ssRNA AGGUAA (5 μM). Signals characteristic of the **1**-RBM5 ZF1 complex are indicated with the asterisk.