**Conformational readout of RNA by small ligands**

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RNA molecules have highly versatile structures that can fold into myriad conformations, providing many potential pockets for binding small molecules. The increasing number of available RNA structures, in complex with proteins, small ligands and in free form, enables the design of new therapeutically useful RNA-binding ligands. Here we studied RNA ligand complexes from 10 RNA groups extracted from the protein data bank (PDB), including adaptive and non-adaptive complexes. We analyzed the chemical, physical, structural and conformational properties of binding pockets around the ligand. Comparing the properties of ligand-binding pockets to the properties of computed pockets extracted from all available RNA structures and RNA-protein interfaces, revealed that ligand-binding pockets, mainly the adaptive pockets, are characterized by unique properties, specifically enriched in rare conformations of the nucleobase and the sugar pucker. Further, we demonstrate that nucleotides possessing the rare conformations are preferentially involved in direct interactions with the ligand. Overall, based on our comprehensive analysis of RNA-ligand complexes, we suggest that the unique conformations adopted by RNA nucleotides play an important role in RNA recognition by small ligands. We term the recognition of a binding site by a ligand via the unique RNA conformations “RNA conformational readout.” We propose that “conformational readout” is a general way by which RNA binding pockets are recognized and selected from an ensemble of different RNA states.

**Introduction**

Recent discoveries in RNA biology underscores the importance of RNA in normal and aberrant cellular functions and highlights the potential of RNA as a drug target for many diseases. There are several advantages in targeting RNA over traditional protein targets. For example, RNA targets have the potential for slower development of drug resistance against small molecules. More sites are accessible at the RNA level, whereas the “active site” is often the only target on the protein. Moreover, unlike DNA, RNA produces unique three-dimensional (3D) pockets suitable for specific binding of small ligands. Overall, RNA molecules have highly versatile structures that can fold into different conformations, providing specific recognition sites for diverse drugs.

With the increasing number of available RNA structures in the protein structure data base (PDB), the design of new therapeutically useful RNA-binding ligands is now technically feasible. Since the majority of drugs are ligands for proteins, RNA provides a unique niche for pharmacological development. Thus far, small molecule ligands for RNA have been developed toward three major classes of targets: antibacterial targets (such as bacterial ribosome); antiviral targets [such as trans-activating response RNA (TAR) in HIV]; and human mRNA targets (such as protein-mediated translation control).1,3,6

RNA ligand interactions are generally divided into three types. The first type includes nonspecific electrostatic interactions between the positively charged ligand and the negatively charged RNA phosphate backbone that account for at least one-half of the total binding energy in certain aminoglycoside-RNA interactions. The second type includes specific interactions generally involving direct hydrogen bonding or van der Waals (VDW) interactions with nucleic acid bases in the deep major groove or the wide shallow minor groove of the RNA helix. In a recent study of RNA-ligand complexes, interactions between the ligands and the Watson-Crick edge of the RNA were frequently observed. These interactions were proposed to play a key role in ligand selectivity. In addition, RNA-ligand interactions via the Hoogsteen and/or sugar edge were also observed, though the latter were found to be much less frequent. A third type of interaction includes stacking interactions between RNA bases and aromatic ligands. In a study by Kondo et al., it was observed that pseudo base pairs using the Watson-Crick edge in combination with stacking interactions were frequently involved in specific ligand recognitions. They showed that binding in the deep major groove was the most preferred recognition mode for bulky sugar ligands such as aminoglycosides.

In a previous study conducted on the ribosome, we demonstrated that known antibiotic-binding sites, mainly in the large ribosomal subunit, share several unique properties that we defined as the “RNA signature.” The most noticeable features found in the majority of known antibiotic-binding sites on the ribosome were the prevalence of non-paired bases, a high frequency of unusual syn conformation and an unusual ribose sugar pucker. The prevalence of syn conformation was also shown in...
active sites of functional RNAs. We previously proposed that the unique nucleotide conformation and the sugar pucker may reflect higher flexibility of rRNA bases in antibiotic-binding sites, which may contribute to antibiotic selectivity and action. In an attempt to ask whether unique RNA conformations are a general phenomenon, here we studied ligand-binding sites from diverse (non-ribosomal) RNA targets. Consistent with previous results, we found that in general ligand-binding pockets in RNA, mainly the adaptive pockets, are characterized by the abundance of unique properties, specifically rare sugar pucker and nucleobase conformations. Interestingly, these features were also enriched in the majority of ligand-free (apo) structures, available in the database. Furthermore, we show that nucleotides which adopt rare conformations are indeed involved in direct interactions with the ligand. Our results reinforce the hypothesis that the unique conformations adopted by nucleotides in ligand-binding pockets in RNA contribute to the specific recognition of the binding pockets via an “RNA conformational readout” mode.

Results and Discussion

The unique properties of the ligand binding pockets on RNA. To study the unique properties of ligand binding pocket on RNA we extracted a set of RNA-ligand complexes from the PDB solved by X-ray crystallography or NMR, as described in detail in the Materials and Methods section. Among the complexes, we included “rigid” targets and “adaptive” binding targets (aptamers, riboswitches) (Table S1). From the extended list of complexes we selected a set of 33 representative pockets from 10 RNA target groups (highlighted in gray in Table S1), on which the statistical analyses were conducted (see Materials and Methods). Subsequent to the data extraction, we computed the physical, chemical and structural features of the ligand-binding pockets in RNA compared with the same features in a large set of predicted pockets (extracted from all RNA structures in PDB). The predicted pockets were calculated using two different pocket extraction methods: Solvent and Fpocket (for details see Materials and Methods). Based on the higher overlap between the pockets predicted by the Solvent program and the known binding pockets in comparison to the overlap obtained between the pockets predicted by the Fpocket program and the real binding pockets (Tables S2 and S3), we chose the pockets predicted by the Solvent program as the preferred background set. Overall, the two different approaches for predicting pockets produced very similar results.

Physicochemical properties. When analyzing the physicochemical properties of the ligand-binding pockets on RNA in comparison to the background, we noticed an overall increased representation of atoms belonging to the nitrogen bases rather than atoms of the sugar-phosphate backbone in the known binding pockets (Tables S2 and S3). The significant enrichment of nucleobases in the known binding pockets relative to the computed pockets was a global phenomenon and was not unique to a specific target group. This phenomenon was also observed for the antibiotic binding sites on the ribosome.

Structural properties. To characterize the structural properties of RNA within the binding pockets, we used the MC-annotate program. We calculated the structural properties of the nucleotides in the known ligand-binding pockets on RNA compared with properties in the predicted pockets. Notably, since the complexes in our data set were solved by different techniques (X-ray crystallography and NMR) to ensure that the properties of the pockets are not biased by the technique used to solve the structure, we analyzed the structural properties of the pockets independently, i.e., pockets solved by X-ray crystallography and pockets solved by NMR were analyzed separately. As demonstrated in Figure 2, we noticed an overall increased representation of unique properties such as non-paired nucleotides, and a decreased representation of other properties such as standard Watson-Crick pairing (WWcis). Interestingly, the overrepresentation of unique...
properties in the pockets was more pronounced in the subset of pockets belonging to aptamers and riboswitches (Fig. 2B).\textsuperscript{12-14} Importantly, while conformation differences in ligand binding pockets that were solved by different techniques have been previously observed (as for example, in the PreQ1 riboswitch\textsuperscript{16}), our comprehensive analysis shows that, in general, the abundance of unique RNA conformations is common to both X-ray and NMR structures of RNA and does not seem to be influenced by the technique used to solve the structure.

Furthermore, we observed a preference for \textit{syn} over \textit{anti} conformation of RNA bases in the majority of the known ligand-binding pockets in RNA (Fig. 2A). As depicted in Figure 2 and Figure S2 while the preference for the \textit{syn} over the \textit{anti} conformation was not detected in all complexes the majority of pockets showed a clear preference for the \textit{syn} conformation. Similar results were previously observed in antibiotic binding sites on the ribosome\textsuperscript{20} and in the active sites of functional RNAs.\textsuperscript{11} \textit{Syn} and \textit{anti} conformations are defined by glycosidic torsion angle $\chi$. The \textit{anti} conformation is usually more stable, while the \textit{syn} conformation requires an external stabilizing force. Rotation about the glycosidic bond is hindered, with purines being usually less hindered than pyrimidines.\textsuperscript{17} As shown in Figure 2A, we also observed an overabundance of the unusual sugar pucker conformation (both C2 endo and C4 exo) in the ligand-binding pockets on RNA over the more common C3 endo form found typically in RNA structures. Notably, while as can be expected from the weak correlation between the torsion angels ($\chi, \delta$),\textsuperscript{14} several of the nucleotide in the \textit{syn} conformation adopted a C2 endo sugar pucker, in the majority of pockets we did not observe a strong dependency between the two properties (Table S4). Overall, the C2 endo conformation provides less steric hindrance compared with the C3 endo, meaning that the C2 endo conformer is inherently more flexible, accommodating a wider range of allowed $\chi$ values and involving a lower energy cost for the aforementioned \textit{syn} conformation.\textsuperscript{17-19} While the C2 endo conformation is relatively rare, it has been shown to play functionally important roles in RNA. For example, C2 endo nucleotides have been previously suggested to function as molecular timers in RNA folding and ligand recognition reactions.\textsuperscript{20} Moreover, deletion of one C2 endo nucleotide at RNase P was shown to accelerate RNA folding by an order of magnitude.\textsuperscript{21} C2 endo nucleotides were also shown to play a functional role in the spliceosome and in the ribosome.\textsuperscript{22,23} In the latter, the flexibility of glycosidic bonds of bases in the antibiotic-binding sites of the large ribosomal subunit (specifically at the PTC and the tunnel region) was suggested to explain antibiotic selectivity and action.\textsuperscript{24,25}

As demonstrated in Figure 2A, in addition to the high frequency of rare RNA conformations in the ligand binding pockets, we also noticed that non-paired bases were overrepresented in most of the ligand-binding pockets on RNA. This could likely be due to the greater variation in chemical groups available for structure-specific interactions compared with the nitrogen bases, which are engaged in inter-nucleotide interactions. In addition, we observed a bias for non-canonical WH pairing in the known ligand-binding pockets on RNA. Formation of non-canonical base pairs in RNA is essential because, in contrast to

**Figure 2.** (A) Enrichment of structural properties in 33 representative structures of ligand binding pockets on RNA. Heatmap demonstrates over- and under-representations of the different structural properties in the binding pockets relative to background. The color scheme refers to the standardized score calculated against a background of computed pockets (calculated by the Solvent program). Scores were scaled to range from $-1$ to $1$. Significant preferences of properties relative to the background of all RNA pockets are colored red ($1$) while blue denotes under-representation ($-1$). Numbers represent the index of the complex listed in Table S1. (B) Hierarchical clustering of the 33 representative structures of RNA ligand complexes based on their structural properties. The colors of the dots represent different RNA groups (7SK SNRNA – bright green, Aptamer – red, DIS-HIV1 – pink, Duplex – brown, HIV Helix – azure, HCV IRES Domain IIa – gray, Riboswitch – yellow, Ribozyme – blue, TAR-purple, Splicing Regulatory – dark green).
Watson-Crick base pairs in RNA, non-canonical base pairs often play an important functional role, for example, in catalytic RNA. Structural roles for non-canonical pairing was exemplified in the stabilization and formation of the RNA tertiary structure.26 Overall, our results show that the ligand-binding pockets in RNA are characterized by rare structural properties of the RNA. Further, when clustering the ligand-binding pockets on RNA according to their structural properties, we noticed that, in general, the artificial RNA aptamers and the different subgroups of the natural RNA riboswitches were clustered together (Fig. 2B), showing a similar pattern of enrichment of the rare properties, specifically the nucleotides in syn conformation, the C2 endo/C4 exo sugar pucker and the non-paired and non-canonical nucleotides.

Unique RNA properties are also found in apo structures. To examine whether the rare RNA conformations are found in the bound (holo) state only we extracted a set of 10 representative RNAs, which their structures were available both in the apo and holo states (see “Material and Methods” section for details). The distributions of the structural properties were calculated independently for the pockets of the bound states and their corresponding nucleotides in the unbound states. As can be noticed from Table 1, overall, the properties which were significantly high in the bound pockets (highlighted in bold in Table 1) were usually enriched in the unbound state. For example, as demonstrated in Figure 3, the three nucleotides in the structures of the lysine riboswitch (3DOU/3DOX, for the bound and unbound, respectively) were found to adopt the syn conformations in both the free and bound form (Fig. 3, Table 1). These results are consistent with Garst et al., observations that the lysine confers only limited local changes upon binding to the RNA pocket.27 Nevertheless, in the case of preQ1 riboswitch, which was shown to be unstructured in its free form28 we indeed noticed a significant difference in the frequencies of the unique RNA properties (Table 1). Interestingly, the relative frequencies of the unique RNA conformations were usually higher in the unbound state.

Overall, in the majority of the representative pockets non-paired nucleotides were frequent in both the bound and unbound state. One exception was the SAMI riboswitch in which the frequency of non-paired nucleotides was only significant in the bound state. Interestingly, in the latter example the free-state structure revealed that the nucleotide A46 occupies the binding site in place of the SAM ligand.29 Recent dynamic studies of riboswitches suggest that in the absence of ligands, the riboswitch may adopt an ensemble of different states from which the ligand selects a ligand-binding competent conformation, namely a “conformational selection.”29,30 Our results which show that the unique and rare RNA conformations tend to be found in unbound states of different binding pockets, support the “conformational selection” hypothesis, further suggesting that the unique RNA conformations are recognized and selected by the ligand.

### Table 1: Unique RNA conformations in the bound and unbound structures

| Name             | PDB   | C2endo/C4exo | Syn  | Non-paired | Non-canonical |
|------------------|-------|--------------|------|------------|---------------|
| Lysine riboswitch| 3DOX  | 0.27         | 0.27 | 0.27       | 0.18          |
| Riboswitch       | 3DOU  | 0.27         | 0.27 | 0.27       | 0.18          |
| FMN              | 2YIF  | 0.14         | 0.00 | 0.38       | 0.00          |
| Riboswitch       | 2YE   | 0.14         | 0.05 | 0.38       | 0.10          |
| SAMI             | 3IQP  | 0.06         | 0.00 | 0.18       | 0.00          |
| Riboswitch       | 3IQN  | 0.00         | 0.00 | 0.29       | 0.00          |
| Glycine riboswitch| 3OX0  | 0.25         | 0.08 | 0.33       | 0.17          |
| PreQ1            | 3QS1  | 0.25         | 0.08 | 0.25       | 0.50          |
| Riboswitch       | 3GCA  | 0.00         | 0.00 | 0.42       | 0.33          |
| Ribozyme         | 2GCS  | 0.06         | 0.11 | 0.44       | 0.06          |
| DIS              | 1XP7  | 0.13         | 0.00 | 0.31       | 0.00          |
| Duplex           | 1KP5  | 0.00         | 0.00 | 0.10       | 0.20          |
| Riboswitch       | 1FU1  | 0.10         | 0.00 | 0.10       | 0.20          |
| Splicing regulatory| 1OC8  | 0.17         | 0.00 | 0.33       | 0.00          |
| TAR              | 1ANR  | 0.07         | 0.00 | 0.36       | 0.00          |
|                  | 1UUI  | 0.14         | 0.00 | 0.14       | 0.14          |

Each row represents an RNA structure, unbound on the top (light) rows and bound on the bottom (dark) rows. Values which are greater than the average value of the property in the background pockets are highlighted in bold.
and protein-binding interfaces, we found over-representation of the non-paired conformation over the canonical WWW cis base-pairings compared with all RNA (Fig. 4H). This phenomenon of favoring non-paired conformation over canonical Watson-Crick base-pairing was also observed by Gupta and Gribskov in a recent comprehensive study comparing RNA-protein interfaces to all RNA.39 Taken together, we found that ligand-binding sites in RNA have unique properties that usually differ from RNA-protein interfaces.

**Interactions between nucleotides with unique RNA conformations and ligand atoms.** Many structural studies of RNA ligand complexes have pointed out that RNA ligands tend to bind non-paired nucleotides or nucleotides in non-canonical pairing conformations (as, for example, in aptamer ligand structures38). In order to examine whether in general nucleotides with unique RNA structural properties (i.e., C2 endo/C4 exo, syn, non-paired, non-canonical base pairing) are involved preferentially in direct interactions with the ligand, we concentrated on four types of contacts: hydrogen bond, VDW, hydrophobic and aromatic. As shown in Figure 5, our analysis reinforces that non-paired nucleotides are involved preferentially (statistically significant) in hydrogen bonds with the ligands. Interestingly, among the other contacts (VDW, hydrophobic and aromatic), we did not notice a significant enrichment of contacts toward non-paired nucleotides (Fig. 5A; Table S5). This seems reasonable considering that non-paired nucleotides are not engaged in inter-nucleotide hydrogen bond interactions and, thus, offer more conformational flexibility compared with paired nucleotides. On the contrary, the ability of nucleotides to interact with the ligand via hydrophobic contacts is not expected to be affected by its pairing status.

Furthermore, we observed that nucleotides with the unique property syn were preferentially (statistically significant) involved in interactions with the ligand via hydrogen bonds, VDW, and hydrophobic interactions while nucleotides in either C2 endo or C4 exo conformations were significantly more involved in VDW contact in general and in hydrophobic interactions specifically (Fig. 5A; Table S5). Notably, while nucleotides possessing both syn and C2 endo or C4 exo conformations were preferentially (statistically significant) involved in interactions with the ligand, in many cases the nucleotides involved in binding had only one of the unique conformations (Table S5). Moreover, nucleotides involved in non-canonical interactions within the RNA were found to interact preferentially (statistically significant) with ligands via VDW contacts (Fig. 5A; Table S5). An example of a typical RNA-ligand complex exhibiting preferred interactions between nucleotides having unique RNA features and the ligand is shown in Figure 5B and C. As shown, nucleotides possessing rare structural properties are involved in direct interactions with the ligand.

**Materials and Methods**

**Data extraction.** RNA-ligand complexes. RNA-ligand structures in the holo state were obtained from the PDB (January 2011 Release). Selected structures were solved either by X-ray crystallography (< 3 Å) or NMR. Only structures with > 12 nucleotides were included, removing structures with a high structural similarity (RMSD ≤ 1 Å) that bind the same ligand. In addition RNA-ligand complexes including proteins were removed from the data set. The final set, included 77 RNA-ligand structures. Among them we selected 33 representative (non-redundant) pockets from 10 different RNA groups (highlighted in gray in Table S1) for further analyses.

**Apo and holo structures of binding pockets on RNA.** Ten pairs of apo and holo structures of RNA (one representative from each RNA group) were obtained from the PDB. The structures were solved either by X-ray crystallography (< 3.3 Å) or NMR.

**All RNA.** All RNA structures (excluding ribosomal structures) were obtained from the PDB (January 2011 Release). Data included RNA structures that were solved either by X-ray crystallography (< 3 Å) or NMR. A total set of 776 structures was obtained, including 225 protein-RNA complexes. Consistent with the RNA-ligand data set, structures with < 12 nucleotides were removed from the “all RNA” control set. In addition, water molecules and edge nucleotides (the last nucleotide in each RNA chain) were not included in the structural analysis. Interface residues were calculated using the Intervor web server56 excluding water molecules.

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**Figure 3.** Demonstration of the unique conformations found in the holo and apo states of the lysine riboswitch. (A) Lysine riboswitch in the unbound state (apo), (PDB ID 3D0X). Nucleotides which adopt the syn conformation are highlighted in color: Nucleotide A8 (orange), Nucleotide A9 (green), Nucleotide A77 (purple). (B) Lysine riboswitch in the bound state (holo), (# 52 PDB ID 3D0U). Nucleotides in syn conformation are highlighted, coloring scheme is as in A. The ligand is shown as red spheres and the hydrogen bonds are represented as blue dashed lines.
Binding site extraction. A binding site was defined to include all atoms within a radius of 6 Å from any atom of the ligand. The water molecules were excluded, as well as all edge nucleotides.

Putative pockets extraction for the background model. Putative pockets were extracted from all RNA structures using two different methods:

1. Using the Solvent program: The Solvent program from the 3V package gets as an input a PDB file and the radius of a small and a large probe sphere, and outputs the void in the structures that can accommodate a small probe size (1.5 Å) but not a large probe size (5.5 Å). The void itself, which corresponds to a potential binding pocket, is represented by the oxygen atoms of water molecules that define the borders of the void. The procedure for extracting putative pockets was performed as in David-Eden et al. The pocket was defined to include all atoms within a radius of 9 Å from the computed oxygen atom. Only pockets that comprise 12 nucleotides and differ in at least one nucleotide were retained, resulting in 70,912 pockets.

2. Using the Fpocket program: The Fpocket program relies on the concept of α spheres. The Fpocket core can be resumed in three major steps. During the first step, the whole ensemble of α spheres is determined from the structure, resulting in a pre-filtered collection of spheres. The second step involves identifying clusters of spheres close together to identify pockets and remove clusters of poor interest. The final scoring step in Fpocket calculates the atomic properties of the pocket and is not applied to RNA structures. The program takes a PDB file as input and outputs a PDB file containing only the atoms defining the pocket. Running Fpocket on all RNA structures resulted in 2,938 computed pockets.

Sensitivity and positive predictive value (PPV) of binding site identification. Sensitivity and PPV were calculated based on the degree of overlap between computed pockets and known binding sites (Tables S2 and S3). Sensitivity equals the fraction of common nucleotides divided by the number of nucleotides in the known binding site. PPV equals the fraction of common nucleotides divided by the number of nucleotides included in the putative (computed) pocket.

Sensitivity = number of true positive/number of true positives+number of false negatives
PPV = number of true positives/number of true positives+number of false positives

Physicochemical and structural properties calculations. The nitrogen and oxygen atoms in the base were classified as donors or acceptors. Backbone oxygen atoms were labeled as sugar or

Figure 4. Comparison of the different physicochemical and structural property frequencies between known ligand-binding pockets on RNA to RNA-protein interfaces and to overall RNA. The known ligand-binding pockets in RNA are presented as black bars. RNA-protein interfaces are presented as gray bars. Overall RNA is presented as white bars. Bar height represents the normalized frequency of the different properties in each group (known ligand binding pockets on RNA, RNA-protein interfaces, overall RNA). Groups were compared using Fisher exact tests. The stars denote statistical significance (p value < 0.001), NS denotes insignificance. Each panel represents different properties: (A) phosphate, (B) sugar, (C) base donors, (D) base acceptors, (E) C2 endo + C4 exo, (F) syn, (G) non-canonical, (H) non-paired.
interactions were categorized as stacking interactions between adjacent and non-adjacent nucleotides. The features were extracted as described in reference 42 using an in-house Perl script converting the MC-Annotate output files into binary format, i.e., each nucleotide was given a score of “1” when a specific property was present and a score of “0” when

phosphate. The MC-Annotate program was employed for classifying the RNA structural properties, including base-pairing and base-base stacking interactions. Base-pairing was defined according to three edges of the corresponding RNA bases available for H-bonding interactions: the Watson-Crick (W) edge, the Hoogsteen edge (H) and the sugar edge (S). Base-base stacking interactions were categorized as stacking interactions between adjacent and non-adjacent nucleotides.

The features were extracted as described in reference 42 using an in-house Perl script converting the MC-Annotate output files into binary format, i.e., each nucleotide was given a score of “1” when a specific property was present and a score of “0” when
it was absent. To calculate the relative abundance of a specific property, the fraction of nucleotides in the pocket possessing that property was calculated.

**Analysis of RNA-ligand interactions.** Intermolecular hydrogen bonds and VDW, hydrophobic and aromatic contacts were calculated for each RNA-ligand complex using the LIGPLOT program.43 LIGPLOT uses the HBPLUS44 program for calculating the hydrogen bonds. The program computes all possible positions of hydrogen atoms (H) attached to donor atoms (D) that satisfy specified geometrical criteria with acceptor atoms (A) in the vicinity. The criteria used to define a hydrogen bond were H-A distance < 2.7 Å, D-A distance < 3.35 Å and D-H-A angle > 90°. VDW contacts were defined as all contacts between carbon atoms of the RNA and atoms of the ligand not involved in hydrogen bonds that were < 3.9 Å apart. Hydrophobic contacts were defined as all contacts between carbon atoms of the RNA and carbon atoms of the ligand not involved in hydrogen bonds that were < 3.9 Å apart. Aromatic contacts were defined as all contacts between aromatic carbon atoms of the RNA and aromatic carbon atoms of the ligand not involved in hydrogen bonds that were < 3.9 Å apart. Aromatic carbon atoms of the ligand were determined according to the PDB chemical component dictionary.

**Enrichment analysis.** The relative abundance of each property in a known site was evaluated relative to the background of putative pockets (from the same technique X-ray or NMR) calculated using the PDB chemical component dictionary.

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**Supplemental Material** Supplemental material may be found here: http://www.landesbioscience.com/journals/rnabiology/article/24682/
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