Molecular BioSystems

This article was published as part of the

2009 Emerging Investigators Issue

Highlighting the work of outstanding young scientists at the chemical- and systems-biology interfaces

Please take a look at the full table of contents to access the other papers in this issue.
Screening of a branched peptide library with HIV-1 TAR RNA†‡

David I. Bryson,a Wenyu Zhang,a W. Keith Rayb and Webster L. Santos*a

Received 2nd March 2009, Accepted 11th June 2009
First published as an Advance Article on the web 7th July 2009
DOI: 10.1039/b904304g

The recognition that RNA is more than just an intermediate in the information transfer from genetic code to fully functional protein has placed it at the forefront of chemical research. RNA is important because of its vital role in regulating transcription, translation, splicing, replication and catalysis. Consequently, molecules that can bind to RNA and control its function have potential as powerful tools in biology and medicine. Herein, we report the discovery of HIV-1 TAR RNA-selective ligands using an on-bead screening of a library of 4096 branched peptides.

Introduction

The recognition that RNA is more than just an intermediate in the information transfer from genetic code to fully functional protein has placed it at the forefront of chemical research. RNA is important because of its vital role in regulating transcription, translation, splicing, replication and catalysis. Consequently, molecules that can bind to RNA and control its function have potential as powerful tools in biology and medicine.

In principle, the tertiary structure of RNA can provide a scaffold in which molecules can dock and form the necessary intermolecular interactions to become highly selective. The formation of unique three dimensional architecture, often generated through formation of local structures such as bulges, stem-loops, pseudoknots and turns, differentiates RNA from DNA and allows it to become a therapeutic target. In fact, antibacterial drugs (aminoglycoside, macrolide, oxazolidinone and tetracycline) have cemented the drug-feasibility of RNA. These molecules exert their antibacterial activity by binding to specific regions of rRNA. However, it is clear that targeting generic RNA with small molecules is difficult and requires further studies. Still, the difficult task is designing ligands independent of the canonical Watson–Crick base-pairing on RNA primary structure. Thus, we became interested in the widely recognized problem of developing RNA-selective molecules.

Many important functions of RNA result from specific proteins binding to complex tertiary structures of RNA. One such example is evident in the HIV-1 transactivation response element region (TAR) RNA, a conserved 59-nucleotide stem loop located at the 5′ end of all nascent transcribed HIV-1 mRNA.5 The TAR structure is comprised of a highly conserved hexanucleotide loop and a three-nucleotide bulge flanked by two double stranded stems (Fig. 1). Binding of the transcriptional activator protein Tat and the cyclin T1–cdk1 kinase complex promotes efficient transcriptional elongation. Tat is an 86 amino acid protein that contains an arginine rich motif (ARM), which specifically interacts with the TAR tri-nucleotide bulge (U23, C24, and U25). Disruption of Tat–TAR interaction results in the blockade of viral replication and thus represents a viable strategy in developing new anti-HIV therapeutics. Several ligands (aminoglycosides, argininamide, peptides, peptidomimetics, small molecules, and others) are continuously being developed to inhibit Tat–TAR interaction (Fig. 1).

Owing to the complexity, the lack of designable ligands and a growing interest in developing new molecules that can selectively interact with RNA, we aimed to develop a strategy that allows for the rapid screening of a short branched peptide library and determine their capacity as possible ligands for HIV-1 TAR RNA (Fig. 2). Branched peptides have found extensive use in biological systems including synthetic peptide vaccines (multiple antigen peptides), drug delivery vehicles and therapeutics for a variety of disease states. To date, the potential of using branched peptides for the selective targeting of structured RNA targets has not been realized. Because multivalency is often described to increase the affinity of a ligand to a particular receptor, we wanted to determine whether branched molecules are capable of forming multivalent interactions with RNA and whether presentation...
peptides were attached to TentaGel NH2 beads on-bead screening required resin-tethered probes. Thus, after deprotection of the side chain residues because the important that the peptides were not released from the beads technique in a few peptide coupling steps (ESI groups, 4096 compounds were generated by the split and pool spectrometry (MS) and were branched through the in order to simplify the architectures, and their synthetic accessibility is straightforward. An attractive feature of this strategy is the synthesis of the library on solid support, allowing for rapid generation of a large number of peptides using the split and pool technique, and subsequent on-bead screening against TAR. We designed our initial branched peptide library with biased parameters that enhance binding and selectivity to RNA (Fig. 2). These interactions include electrostatics (positively charged residues interacting with the negatively charged phosphate), π–π interactions and hydrogen bonding. In principle, the diversity of possible peptide structures can be immense because of the commercial availability of unnatural amino acid monomers.

Results and discussion

The branched peptide library was prepared such that each variable position contained one of four possible amino acid monomers (Fig. 2). Branches A1–A3 had an identical sequence in order to simplify the de novo sequencing by MALDI/mass spectrometry (MS) and were branched through the ε-amino groups on lysine. Using orthogonal protecting groups, 4096 compounds were generated by the split and pool technique in a few peptide coupling steps (ESI†). It was important that the peptides were not released from the beads after deprotection of the side chain residues because the on-bead screening required resin-tethered probes. Thus, peptides were attached to TentaGel NH2 beads via a photo-cleavable linker (3-amino-3-(2-nitrophenyl)propionic acid, ANP), which allowed the efficient release of hit molecules by UV-irradiation, post screening (Scheme 1).

Previous high-throughput screening methods suggested that the on-bead screening method can be adapted with our branched peptide library.4,14 Following this protocol, we transferred three copies of the library in an Eppendorff tube and washed them with water (5×) and buffer (3×) containing 50 mM Tris HCl, 20 mM KCl, and 0.1% Triton X-100 at pH 7.4.15 In order to address the issue of selectivity at the outset, we decided to preclude the beads containing promiscuous RNA binders. A simple process that can increase assay selectivity involves the addition of competitor RNA. In this case, unlabeled α-synuclein mRNA, a 340-nucleotide long RNA, was added in the incubation mixture. We hypothesized that longer RNA sequences are likely to bind nonspecifically to our library, and abolishing promiscuous binders can decrease the potential for off-target binding. Thus, we performed a preliminary incubation of a small subset of beads (∼2000) in BSA (1 mg ml−1) and 500 nM unlabeled α-synuclein mRNA at 4 °C for 3 hours and washed (3×) the beads to remove unbound BSA and RNA in solution. Finally, TAR RNA screening was effected by incubating the 29-nucleotide TAR RNA-labeled with DY547 fluorescent tag on the 5'-end at 3.4 nM concentration for 3 hours at 4 °C. The solution was then filtered, washed successively with buffer, placed in a 96-well plate and analyzed using confocal microscopy. It was evident from the initial experiment that the stringency of the assay was inadequate because out of ~2000 beads, six beads were sufficiently fluorescent to isolate them.

The results of the preliminary incubation studies suggested that a more stringent protocol is necessary to decrease the number of hit compounds that will have to be analyzed. To achieve this goal, the second incubation time was reduced to 1 hour. Following this protocol for the remaining library members, 10 additional beads were selected. Fig. 3 shows three representative fluorescent beads visualized under laser irradiation (Fig. 3A) and transmitted light with laser irradiation (Fig. 3B). These beads were washed with buffer and organic solvents to remove the bound RNA until fluorescence was no longer visible under the confocal microscope. As expected, rescreening of the same beads with DY547-TAR RNA under a more stringent condition confirmed RNA binding (ESI†). Finally, labeled RNA was removed with copious buffer and organic solvent washes, which was followed by photolytic release of branched peptides from the resin by exposure to UV irradiation (365 nm, handheld UV lamp, 4 W) for 1 hour.

The identities of the peptide hits were determined by de novo sequencing of the resulting solution by MALDI/MS (Table 1). Sequence homology analysis of the hit peptides revealed a

![Scheme 1](image1.png)

**Scheme 1** On-bead screening of branched peptide library against 5'-DY547-labeled TAR RNA and hit identification using MALDI/MS.

![Fig. 3](image2.png)

**Fig. 3** Visualization of hit beads under confocal microscope (A) and transmitted light with laser irradiation (B); arrows point to additional beads. Sequence alignment of 16 hits compounds generated a LOGO shown in (C).
LOGO shown in Fig. 3C.18 Of the 16 branched peptides, twelve contained Arg–Arg on the N-terminus—an unsurprising outcome given that RNA binding can be mediated by electrostatic interactions. This result suggests that a positive charge is favored both in the A1 and A2 positions. While no overwhelming preference for a specific amino acid was observed at any other positions, interestingly, arginine was not the preferred amino acid in position A5. Because the propensity for RNA binding is expected to increase in the presence of an electrostatic interaction, albeit nonselectively, the preference for tyrosine in position A5 is noteworthy. It appears that a favorable electrostatic force can be adequately replaced with potential hydrogen bonding and hydrophobic interactions. Consistent with this observation, hydrophobic residues, Phe and Leu, dominate position A6 when possible amino acids such as Ser and Asp that can hydrogen bond are present. It was also gratifying to find that one of the beads collected from the library screening, BP16, contained the sequence with the highest amino acid frequency as indicated in the LOGO illustration in Fig. 3C.

To evaluate whether the branched peptide hits bound TAR RNA, biophysical characterization was undertaken. We selected BP15, BP16 and BP17 ([RRL]2*HRF) for analysis. Validation of the activities of these compounds was paramount because false positives were possible, as in any high-throughput screening assay. BP15 was randomly selected from the hit peptides, and although BP17 was not a sequence derived from the library screening, we hypothesized that this sequence should have good binding affinity for HIV-1 TAR based on the consensus sequence analysis. For this purpose, the dissociation constants for these compounds were determined using fluorescence polarization (FP). This technique required the resynthesis of select peptides with FITC on one of the N-terminal branches, which was accomplished using orthogonally protected lysine (Fmoc-Lys(ivDde)-OH) as the branching unit (Fig. 4). The resulting branched peptides contained a N-terminal acetyl group on one branch. After HPLC purification and characterization by MALDI/MS, FP experiments were performed in triplicate for each peptide (Fig. 5). In each case, 0.5 nM FITC-peptide was incubated with increasing concentration of TAR RNA (up to 100 μM). The results reveal that BP15 has a Kd of 27 μM while BP16–17 have affinity for TAR RNA in the μM range (Fig. 5). Because the FP technique required the use of high concentration of RNA to reach full saturation, in this case > 100 μM, we were not able to determine the accurate dissociation constants for BP16–17. Gratifyingly, when a mutant version of TAR (U24 → C24, ESIz) was titrated against BP15–17, it is clear that their binding affinity is severely decreased, suggesting that these peptides are selective towards the TAR RNA used in the screening. As a control, we also synthesized a linear peptide version of BP15, T15, to investigate whether branching has an effect on the binding affinity towards TAR RNA. T15 has a sequence of RRAGVRD, where the branching unit Lys was replaced with Gly. The data indicate a marked loss of binding affinity (>10 fold) suggesting that the additional functional groups present on the branch aid in anchoring to TAR RNA.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP1</td>
<td>(RRL)2*WYL</td>
</tr>
<tr>
<td>BP2</td>
<td>(YRA)2*HRF</td>
</tr>
<tr>
<td>BP3</td>
<td>(HRW)2*HRF</td>
</tr>
<tr>
<td>BP4</td>
<td>(RRW)2*HAL</td>
</tr>
<tr>
<td>BP5</td>
<td>(RRL)2*NRF</td>
</tr>
<tr>
<td>BP6</td>
<td>(RRY)2*VRL</td>
</tr>
<tr>
<td>BP7</td>
<td>(RRW)2*HYD</td>
</tr>
<tr>
<td>BP8</td>
<td>(YRL)2*WRL</td>
</tr>
<tr>
<td>BP9</td>
<td>(RRA)2*NYF</td>
</tr>
<tr>
<td>BP10</td>
<td>(DNL)2*HYF</td>
</tr>
<tr>
<td>BP11</td>
<td>(RRA)2*VYF</td>
</tr>
<tr>
<td>BP12</td>
<td>(RRW)2*HAS</td>
</tr>
<tr>
<td>BP13</td>
<td>(RRY)2*NQD</td>
</tr>
<tr>
<td>BP14</td>
<td>(RRY)2*VQL</td>
</tr>
<tr>
<td>BP15</td>
<td>(RRA)2*VRD</td>
</tr>
<tr>
<td>BP16</td>
<td>(RRL)2*HYL</td>
</tr>
<tr>
<td>BP17</td>
<td>(RRY)2*VQL</td>
</tr>
<tr>
<td>BP18</td>
<td>(RRA)2*VRD</td>
</tr>
<tr>
<td>BP19</td>
<td>(RRL)2*HYL</td>
</tr>
</tbody>
</table>

Fig. 4 Synthesis of FITC-labeled peptides. SPPS, solid-phase peptide synthesis; Ac, acetyl; FITC, fluorescein isothiocyanate.

Fig. 5 Fluorescence polarization binding curves of branched peptides with HIV-1 TAR RNA and mutant (U24 → C24) TAR RNA. T15 is a linear peptide (RRAGVRD) version of BP15. Each experiment was done in triplicate.
Conclusion

In conclusion, we discovered selective binders for HIV-1 TAR RNA using an on-bead screening of a focused library of branched peptides. The use of branched structures with diverse functional groups and capacity to form multivalent interactions is important in defining new molecular entities that can be selective for the tertiary structure of target RNA. In addition to synthesizing a more complex branched peptide library, future work in our laboratory will focus on extensive characterization including mapping of TAR–branched peptide interactions, cell permeability and in vitro inhibition of Tat–TAR RNA interactions.

Acknowledgements

We thank Prof. Richard Helm at VA Tech mass spectrometry incubator and Jason Crumpton for help with sequencing branched peptides. We also thank Dr Philippe Bissel for synthesizing Fmoc-ANP and Dr Kristi DeCourcy at the Fralin Life Science Institute for assistance with confocal microscopy. Support for this project was provided by the Department of Chemistry at Virginia Tech. This material is based upon work supported in part by the Macromolecular Interfaces with Life Sciences (MILES) Integrative Graduate Education and Research Traineeship (IGERT) of the National Science Foundation under Agreement No. DGE-0333378.

References