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Structural computational modeling of RNA aptamers

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Abstract

RNA aptamers represent an emerging class of biologics that can be easily adapted for personalized and precision medicine. Several therapeutic aptamers with desirable binding and functional properties have been developed and evaluated in preclinical studies over the past 25 years. However, for the majority of these aptamers, their clinical potential has yet to be realized. A significant hurdle to the clinical adoption of this novel class of biologicals is the limited information on their secondary and tertiary structure. Knowledge of the RNA's structure would greatly facilitate and expedite the post-selection optimization steps required for translation, including truncation (to reduce costs of manufacturing), chemical modification (to enhance stability and improve safety) and chemical conjugation (to improve drug properties for combinatorial therapy). Here we describe a structural computational modeling methodology that when coupled to a standard functional assay, can be used to determine key sequence and structural motifs of an RNA aptamer. We applied this methodology to enable the truncation of an aptamer to prostate specific membrane antigen (PSMA) with great potential for targeted therapy that had failed previous truncation attempts. This methodology can be easily applied to optimize other aptamers with therapeutic potential.

Keywords

RNA aptamers; RNA secondary and tertiary structure; Structural algorithms; Vfold2D model; Vfold3D model; Prostate specific membrane antigen (PSMA); A9 aptamer; A9g aptamer; NAALADase Assay

1. Introduction

RNA aptamers are short single-stranded oligonucleotide ligands that can bind with high affinity and specificity to target molecules. These RNA ligands are derived through a process of directed chemical evolution known as SELEX (systematic evolution of ligands by exponential enrichment), which was first described 25 years ago by Tuerk and Gold [1] and Ellington and Szostak [2]. The SELEX approach has since yielded a vast number of

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aptamers for a wide range of applications, including *in vitro* diagnostics, biomarker discovery, biosensor technology and therapeutics [3–11]. The first aptamer therapy, Pegaptanib (Macugen), was approved in 2004 by the US Food and Drug Administration for the treatment of wet (neovascular) age-related macular degeneration [5]. Today the pipeline of therapeutic aptamers extends to at least 10 candidates, which are undergoing clinical trials for ocular diseases, cancer, cardiovascular disease, and type II diabetes [7–11]. Despite these successes, the path to the clinic for aptamer biologics is slow given the costs of manufacturing of long RNA sequences and the slow post-selection optimization process, which is primarily based on trial and error.

In a recent study we used computational RNA structural and RNA/protein docking modeling to guide the truncation of the A9 prostate-specific membrane antigen (PSMA) RNA aptamer (70 nucleotides long) [12–13]. This analysis resulted in shorter versions of the PSMA A9 aptamer that retain the ability to bind to and inhibit PSMA, as well as internalize into PSMA-expressing cells, but are now amenable to large-scale chemical synthesis for therapeutic applications. One of these second-generation truncated aptamers, termed A9g, has recently been evaluated in preclinical models of prostate cancer metastasis and shown to reduce metastatic spread in mice [14].

In conclusion, the methodologies and assays described herein, with the exception of the NALAADase assay, can be broadly applied to determining the structure of other aptamers and are poised to expedite the optimization of aptamers for clinical applications. Aptamer-specific functional assays (e.g. binding assays, inhibition assays) can be developed and optimized for any given aptamer and used in place of the NAALADase assay. Below is a detailed description of the methods for guiding RNA aptamer truncations. We also included methods for the NAALADase assay that was used for the PSMA aptamer. This will be of interest to those who wish to truncate other aptamers to PSMA or aptamers to other protein targets with carboxypeptidase activity. A detailed description of the NAALADase assay can be also found in Dickey et al. 2016 [15].

2. Reagents

2.1. Reagents for NAALADase assay

1. RNA aptamers can be *in vitro* transcribed as previously described [13–16]. Alternatively, chemically synthesized RNA aptamers can be purchased from TriLink Biotechnologies (<http://www.trilinkbiotech.com>).
2. 10× binding buffer: 200 mM HEPES pH 7.4, 1.5 M NaCl and 20 mM CaCl₂. 10× binding buffer can be stored at room temperature. Make 10× binding buffer by combining 10 mL of 1 M HEPES pH 7.4 (product # 15620-080), 15 mL of 5 M NaCl (product # 59222C-500ML, Sigma-Aldrich). Bring to a final volume of 50 mL with UltraPure DNase/RNase-Free Distilled Water (product # 10977-023, Invitrogen).
3. 1× binding buffer: The working solution of 1× binding buffer can be made by performing a 1:10 dilution of the 10× binding buffer with UltraPure water. To stabilize the PSMA protein, add Bovine Serum Albumin (product #

A9418-10G, Sigma-Aldrich) to a final concentration of 0.01% BSA (for example: to 50 mL of 1× binding buffer add 0.05 g BSA).

4. 1× RNA folding buffer: To fold the RNA use 1× binding buffer without BSA.
5. 200 mM Tris-HCl, pH 7.5: 200 mL of UltraPure 1 M Tris-HCl Buffer, pH 7.5 (product # 15567-027, Invitrogen) plus 800 mL of UltraPure H₂O.
6. 10 mM CoCl₂: 1189.6 mg of Cobalt (II) chloride hexahydrate (Product # 255599-100G, Sigma-Aldrich) and 500 mL Ultra-Pure H₂O.
7. Recombinant human PSMA/FOLH1/NAALADase I (rhPSMA; product # 4234-ZN-010, R&D Systems).
8. 100 μM NAAG (product # A5930, Sigma-Aldrich).
9. [Glutamate-3,4-H³]-NAAG (product # 1082050UC, Perkin Elmer).
10. AG 1-X8 Resin (product #140-1454, Bio-Rad).
11. Flint glass Pasteur pipettes, 9 in. (product # 22-230490, Fisher Scientific).
12. Custom made rack to hold 9 in. Pasteur pipettes in place and in alignment with racks scintillation vials [15].
13. Borosilicate glass beads, 3 mm diameter (product # Z143928-1EA, Sigma-Aldrich).
14. 0.1 M Na₃HPO₄: Mix 5.7 g of Na₃HPO₄ Anhydrous (product # 0404-500G, ISC BioExpress) in UltraPure H₂O. Bring to a final volume of 400 mL, and store at 4 °C until ready to use.
15. 1 M formic acid: 383 mL of UltraPure H₂O and 17 mL of 88% formic acid (product # 399388-500ML, Sigma-Aldrich).
16. Bio-Safe II scintillation fluid (product # 111195, Research Products International Corp.).
17. Polyethylene scintillation vials (product # M2026-1000EA, Sigma-Aldrich).
18. Beckman LS6500 scintillation counter (Beckman Coulter, Inc.).

3. Methods

The Vfold2D RNA folding computational model was used to predict the two-dimensional (2D) structure of a 70 nucleotide (nt) long RNA aptamer (A9) to prostate specific membrane antigen (PSMA) and identify key sequence/structure motifs (Fig. 1). Based on the predicted secondary structure of A9, we systematically modified the original aptamer sequence to include base deletions/insertions/mutations that would alter the aptamer's overall predicted secondary structure. For each modified sequence, we predicted the 2D and three-dimensional (3D) RNA structures using Vfold2D and Vfold3D modeling algorithms (*see* Note 1). The altered RNA sequences were then *in vitro* transcribed and validated experimentally to assess activity in an established functional assay (NAALADase assay).

From the structure–activity relationship for the different sequences and structures, we identify the sequence and structural motifs that are essential for aptamer activity.

3.1. Search for the minimal functional structure

1. Use the Vfold2D model [17–22] (see Note 2) to predict the 2D structure for the full-length A9 aptamer sequence (Fig. 1, see Note 3 and 4).
2. Based on the predicted 2D structure of the A9 aptamer, reduce the original structure. Specifically, for A9 (Fig. 1), we gradually shortened the stem (strands G1-U29 and A43-C68) from the stem terminal (see Note 5).
3. For each shortened sequence, run Vfold2D to predict the 2D structure to ensure that the shortened sequence retains the basic folding structure of the original full-length sequence.
4. From the functional inhibition data, identify the minimal aptamer sequence that retains the same activity as the original full-length sequence. Using this approach, the full-length A9 was truncated down to 43nt (Fig. 1). The truncated aptamer is referred to as A9g. Importantly, we confirmed that A9g has comparable inhibitory function as the full-length A9 aptamer (see Note 6).

3.2. Probing the critical nucleotides and structure features

The overall stem-loop motif (four helices, a hairpin loop, a bulge loop and two internal loops, shown in Fig. 1) of A9g may be important to the function. To further identify the critical nucleotides and essential structural features, we performed systematic base deletion/insertion/mutation of A9g and computed the structure for each altered sequence. The altered RNAs were *in vitro* transcribed as previously described by us [13,16]. The transcribed RNAs were then evaluated in the NAALADase functional assay to identify critical sequential/structural elements necessary that contribute to the inhibitory activity of A9g.

¹A two-dimensional (2D) structure is defined by the base pairs in the structure and a three-dimensional (3D) structure is defined by the 3D coordinates of all the atoms in the RNA.

²The Vfold RNA folding model relies on a coarse-grained (virtual bond) representation of RNA structures. In the model, each nucleotide has two backbone virtual bonds P-C4-P and a base/sugar virtual bond C4-N1 for pyrimidine or C4-N9 for purine. The model gives RNA loop entropy parameters from explicit conformational sampling in the 3D space. The entropy parameters for the different motifs are precomputed and tabulated [18–22]. Vfold2D is a 2D structure-based folding model. By employing the Vfold-based loop free energy parameters and the experimental thermodynamic data for the different base stacks, Vfold2D predicts RNA 2D structures from the partition function calculations from the RNA sequence.

³Other RNA secondary structure prediction models, such as Mfold [27–28], RNAstructure [29–30], RNAfold [31–32], and MC-Fold [33] can also be used.

⁴RNAs often display multiple, heterogeneous conformational distributions with the formation of multiple stable and metastable structures. Vfold2D model predicts two slightly different 2D structures. The two structures differ by a single nucleotide shift, resulting in the presence/absence of the single-nucleotide B1 bulge in A9g and the resultant rearrangement of the S4 helix (See Fig. 1). Protein binding may stabilize one structure over the other. The experimental data can provide information to help us determine the relevant 2D structure. For A9g, the functional study results for the different mutants, such as the non-functionality of the B1 bulge-free mutant A9g.5 in Fig. 1, support the presence of the B1 bulge and the S4 helix in the functional A9g. Therefore, we use the A9g 2D structure shown in Fig. 1 for the 3D structure prediction. The predicted 3D structure is then used for A9g aptamer-PSMA docking.

⁵To maintain a T7 transcription start-site (5'GGG) and base-pairing complementarity at the 3' end, selective base changes at the 5' and 3' ends may be needed.

⁶The experimental conditions as well as the details of other activity assays (filter binding assay, and cell binding/internalization assays) can be found in Rockey et al. 2011 [13].

1. To test the effects of helix lengths, delete and/or insert base pairs in helices (*see* Note 7). A9h shown in Fig. 1 has different helix lengths compared with A9g.
2. To test the effects of helix rigidity, replace the non-canonical base pairs in the 1 $\times$ 1 internal loops and/or insert a complementary nucleotide to the one-bulge loops. With U39 replaced with G39, the non-canonical base pair 5C-U39 of L1 internal loop in A9g is transformed to a canonical base pair 5C-G39 in A9g.3 in Fig. 1.
3. To identify the key local elements that are directly involved in the target-aptamer interaction, we separate the overall motif into several individual local motifs (*see* Note 8). For example, A9i shown in Fig. 1 has only the hairpin loop and the bulge loop of A9g.
4. To identify the specific sequence/structural elements necessary for inhibition of NAALADase activity, we introduce a series of mutations (*see* Note 9). As shown in Fig. 1, A9g.5 has a different arrangement of the intra-loop structures due to the mutation of A31 to G31.

The above analyses suggest that for the A9 PSMA aptamer, the sequence identity of helix S2 is not important but the formation of the loops is required for its inhibitory function.

3.3. Predicting the three-dimensional structure

To gain additional insights into the interaction of the RNA aptamer with its target, we use the Vfold3D [17·23·24] (*see* Note 10 and 11) model to predict the aptamer 3D all-atom structures. Here we used A9g shown in Fig. 1 to illustrate how to use Vfold3D to predict the 3D structures from the given 2D structure.

1. Identify the structure motifs (such as hairpin loop, internal loop, pseudoknot loop, and three-way junctions) from the given 2D structure. A9g aptamer has a hairpin loop (H1), a bulge-C loop (B1), two internal loops (L1 and L2) and four helices.
2. Build the virtual bond-based coarse-grained 3D structure for helices according to the A-form helix template (*see* Note 2).
3. Based on the length (primary) and the sequence (secondary) matches, Vfold3D searches for the best templates from the library of known structures for each non-helix motif. The optimal template for the hairpin loop H1 was found in the

⁷Nucleotide changes in a helix are made by changing A-U pairs to G-C and changing the order of the base pairs.

⁸For a multi-branched junction, by replacing the helix stems with the closing base pairs of the helices would convert a multi-branched junction into a hairpin loop.

⁹The nucleotide changes in a helix may not change the global folding, but the change of the nucleotides in loops, especially in junction regions, might lead to a significant change in the global structure.

¹⁰To predict the 3D structures for a given 2D structure, the Vfold3D model assembles motif-specific structural templates into a 3D scaffold structure, followed by the refinement of AMBER energy minimization. Currently, due to the limited structural template database, Vfold3D can only predict the 3D structures with hairpin loops, internal/bulge loops, multi-branched junctions and pseudoknots.

¹¹Instead of generating an ensemble of 3D structures, Vfold3D determines the 3D templates according to the best match for the loop size and loop sequence similarity. Therefore, the output from the Vfold3D is deterministic.

PDB structure 2gya. B1 was found in 2gdi. The internal loops L1 and L2 were found in 1kp7 and 1j5a, respectively.

4. Build the virtual bond 3D structures of each motifs from the (all-atom) templates found in the previous step.
5. Assemble the virtual bond-based motif structures to construct the 3D scaffold of the whole RNA.
6. Add bases to the virtual bond scaffold structure according to the templates for base configurations.
7. Refine the 3D structure using AMBER energy minimization (*see* Note 12). See Fig. 2 for the predicted 3D structures for the designed sequence variants.

The 3D structures are provided to protein–RNA docking program, MdockPP [25·26] (*see* Note 13 and 14), to further show/verify the key structural features discovered in the above structure–activity analysis. For example, for A9g, the sequence conservation (Uridine) at position 39 may be more important than the overall structure of the L2 loop for conferring the RNA aptamer's inhibitory function.

3.4. NAALADase assay

A functional assay should be performed in order to confirm that the aptamer retains its activity following truncation. Here we describe a functional assay which measures PSMA enzymatic activity (NAALADase).

1. Fold each aptamer in 1× RNA folding buffer without BSA (obtained by doing a 1:10 dilution of the 10× binding buffer) at a concentration of up to 5 μM. The folding protocol is as follows: incubate up to 500 μL of the 5 μM aptamer solution at 65 °C for 10 min, followed by incubating at 37 °C for 10 min. The A9g aptamer can be freeze–thawed several times after the folding protocol and still retain its activity.
2. The NAALADase assay is performed as follows: each folded full length and truncated aptamer is diluted to 0.333 μM in 1× binding buffer (with BSA). 120 μL of each sample is used. 1× binding buffer (with BSA) alone is also included as a negative control.
3. A Tris/CoCl₂ master mix is made by adding 500 μL of 200 mM Tris–HCl, pH 7.5 to 250 μL of 10 mM CoCl₂. 60 μL of the Tris–HCl/CoCl₂ master mix is added to each sample.
4. Aliquot the rhPSMA into 2 μL aliquots under sterile conditions and store the aliquots at –80 °C upon arrival. The rhPSMA aliquots should be diluted by adding 375 μL of UltraPure H₂O and 125 μL of 200 mM Tris–HCl, pH 7.5 to

¹²Currently, the energy minimization has not been automated in the Vfold3D server. We manually perform AMBER energy minimization for the Vfold3D predicted structures.

¹³The crystal structure of PSMA was downloaded from the Protein Data Bank (PDB id: 1z8l).

¹⁴If the crystallographic or NMR structure of the protein is not available, one may use protein structure modeling software to build a model for the protein structure.

the 2 μL of the PSMA solution before using in the NAALADase assay. Add 10 μL of the diluted rhPSMA mixture to the RNA mixtures, and gently mix by pipetting. Incubate at 37 °C for 5 min, and start the timer after adding PSMA to the first sample.

5. Make a NAAG solution consisting of 992 μL of UltraPure H_2O , 5.5 μL of 100 μM NAAG, and 2.5 μL of ^3H -NAAG. Add 10 μL of the NAAG solution to each sample and incubate at 37 °C for 15 min. Mix the reaction by gently pipetting up and down after 7.5 min has past. Start the timer after the NAAG solution is added to the first sample.
6. Add 200 μL of ice cold 0.1 M Na_3HPO_4 to each sample to stop the reaction.
7. Prepare columns by placing as many Pasteur pipettes as needed into the plastic rack, with one 3 mm borosilicate glass ball in each pipette [15].
8. Make a 1:1 mixture of UltraPure H_2O and AG 1-X8 resin to create a slurry of resin. Add 2 mL of the resin slurry to each Pasteur pipette. After all of the liquid has drained from the columns, add 2 mL of UltraPure H_2O to the columns. Allow the liquid to flow through the columns and discard.
9. After the liquid has drained from the columns, place the scintillation vials under the columns. Add 190 μL of each reaction mixture to separate columns, and allow it to completely soak into the resin.
10. Add 2 mL of 1 M formic acid to each column. Allow the liquid to completely flow through the columns and collect 2 mL of eluent into each scintillation vial.
11. Add 10 mL of Bio-Safe II scintillation fluid to each scintillation vial and count in a liquid scintillation counter.

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NAALADase inhibition %	A9	A9g	A9h	A9g.3	A9i	A9g.5
	80 to 90%	80 to 90%	< 20%	0%	< 10%	0%

Fig 1.
The Vfold2D-predicted secondary structures of A9 (70nt), A9g (43nt), A9h (37nt), A9g.3 (43nt), A9i (24nt), and A9g.5 (43nt). The overall stem-loop motif (four helices, a hairpin loop H1, a bulge loop B1 and two internal loops L1 and L2) of A9g may be important to the function of the full-length of A9. Nucleotides different from A9g for A9h, A9g.3, A9i, and A9g.5 are shown in red. The table inset below the structures indicates the amount of inhibition of PSMA NAALADase activity by each aptamer [13].

Fig 2.

The Vfold3D-predicted 3D structures of A9g, A9h, A9g.3, A9i, and A9g.5 for the given 2D structures shown in Fig. 1. Nucleotides in cyan in the predicted structure of A9g are functionally important.