Chapter 6

In Vitro Selection of RNA Aptamers to Clinically Applicable Small-Molecule Ligands

Abstract

Various platforms for constructing ligand-responsive RNA-based gene regulatory devices have been demonstrated. Although these devices utilize diverse architectures, most existing devices that can sense and respond to molecular inputs include sensor components consisting of RNA aptamers that bind to specific ligands of interest. In particular, well-characterized aptamers such as those responsive to the small molecules theophylline and tetracycline have been used in the majority of synthetic RNA control devices developed to date. Although theophylline and tetracycline have been invaluable in proof-of-concept studies, their use in practical applications such as metabolic engineering and cellular therapy is limited by their toxicity. *In vitro* selection procedures have been developed for the generation of new RNA aptamers, but few aptamers have been selected to clinically applicable small molecules that would be required for applications such as T-cell proliferation control in cellular immunotherapy. Here, we present three different *in vitro* aptamer selection procedures for the selection of aptamers to phenobarbital, vitamin B_{12}, and folinic acid. While ligand binding was observed in qualitative assays, we have been unable to quantitatively determine the binding affinities of aptamers obtained from these initial efforts. We discuss specific aspects of the selection processes—including partition efficiency evaluation for nitrocellulose membranes, format and timing of counter-selections, sequence and structural analyses of RNA pools, and techniques for characterizing putative aptamer sequences—that may contribute to the improvement of aptamer selection protocols.

Introduction

Ligand-responsive regulation over gene expression and cellular behavior has broad applicability in areas ranging from fundamental biomolecular investigation to metabolic engineering to health and medicine. However, the practicality of such regulatory schemes is dependent, in part, on the ability to tailor the input response of these control devices to each application. For example, control devices responsive to specific metabolite intermediates may be required for pathway engineering in biofuel production, whereas those responsive to pharmaceutical molecules are critical to the success of various therapeutic applications.

The RNA-based regulatory devices developed in the Smolke Laboratory and examined in the context of mammalian cell regulation in this thesis are composed of modular components, including sensor domains that can be systematically altered to program the desired input response specificity[1-3]. The aptamer sequence comprising the sensor domain specifies the input responsiveness of each regulatory device, independent of the actuator domain and the regulatory target. The work presented thus far on RNA-based regulatory systems has utilized molecular inputs such as theophylline and tetracycline, which are small molecules with well-characterized aptamer sequences. To fully realize the potential of these RNA-based regulatory devices for therapeutic applications, we investigate the generation of new RNA aptamers to clinically relevant input molecules. In particular, we focus on small-molecule ligands to avoid the use of heterologous proteins, which are prone to immunogenicity.

Although the Systematic Evolution of Ligands by EXponential enrichment (SELEX; see Chapter 1 for detailed description) has been successfully applied to the

selection of numerous ligand molecules, a number of challenges remain in generating aptamers to clinically relevant small molecules. The ability to effectively enrich ligand-binding sequences depends on efficient partition methods that can separate ligand-bound sequences from the unbound pool. In the case of protein ligands, filtration through nitrocellulose membranes, which are capable of separating proteins and protein-bound nucleic acids from free nucleic acid molecules, is the most commonly used separation method[4-8]. However, membrane filtration cannot be applied to the selection of aptamers to small-molecule ligands due to the inability of nitrocellulose membranes to retain small molecules. Instead, column chromatography, in which the small-molecule ligand is immobilized onto resin particles and ligand-bound nucleic acid sequences are specifically eluted, is the conventional separation method employed for the selection of aptamers to small-molecule ligands[9-11]. However, ligand conjugation to resin particles requires that the small molecules of interest possess suitable functional groups for the necessary chemistry. This is a requirement unmet by many clinically relevant small molecules, including phenobarbital and tamoxifen—two pharmaceutical molecules of particular interest to the development of T-cell proliferation control systems due to their known tolerance by human patients and potential for creating combinatorial regulatory systems utilizing both RNA-based switch devices and engineered cytokine receptor chains (M.C. Jensen, personal communication) that are responsive to these molecules.

To address this challenge, we examined the use of protein-conjugated small molecules for aptamer selection and developed alternative methods combining both membrane filtration and affinity elution to isolate aptamers specific for phenobarbital. Furthermore, we performed column-based SELEX to select aptamers specific for vitamin

B_{12} (cyanocobalamin) and folinic acid, two well-tolerated small molecules that are amenable to resin conjugation. While ligand binding was observed in qualitative assays, we have been unable to quantitatively determine the binding affinities of aptamers isolated from our efforts to date. Nevertheless, these early attempts led to a greater understanding of the selection procedure, which informs ongoing aptamer selection efforts in the Smolke Laboratory. Here, we present a summary of the procedures performed and discuss specific aspects of these selection processes—including evaluation of partition efficiency by nitrocellulose membranes, format and timing of negative selections, sequence and structural analyses of enriched RNA pools, and techniques for characterizing putative aptamer sequences—that contribute to the continuing improvement of aptamer selection protocols.

Results

Conventional Nitrocellulose Membrane-Based SELEX Has Low Partition Efficiency and Enriches for Nitrocellulose-Binding RNA Sequences. Phenobarbital is a barbiturate approved by the United States Food and Drug Administration for use as an anticonvulsant in human patients[12]. It is well tolerated by both mouse T cells (Appendix 1) and human natural killer cells (Appendix 2) in culture, and is a promising molecule for use in ligand-responsive RNA-based regulatory systems. As an aptamer to phenobarbital does not exist, we attempted to isolate a novel RNA aptamer to this molecule using SELEX. However, phenobarbital is a compact molecule with no appropriate functional group for conjugation to resin particles (Figure 6.1), thus precluding aptamer selection using column chromatography in the absence of chemical modification to the molecule.

Furthermore, nitrocellulose membranes cannot retain small molecules such as phenobarbital, thus eliminating the second conventional method for separating free and ligand-bound nucleic acids during SELEX. To address this challenge, we performed membrane-based selection of RNA aptamers toward phenobarbital conjugated to bovine serum albumin (phenobarbital-BSA). The BSA enables separation by nitrocellulose membranes, and rigorous negative selection against unconjugated BSA is expected to reduce enrichment of BSA-binding RNA sequences.

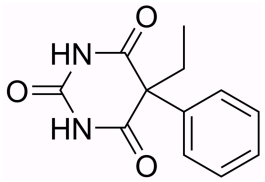

Figure 6.1. Chemical structure of phenobarbital. In the absence of chemical modification, phenobarbital contains no functional group suitable for resin conjugation chemistry.

An initial RNA library of approximately 1.2 x 10^{13} unique sequences, each containing a 30-nt randomized (N30) region, was subjected to 20 rounds of selection (Figure 6.2). Prior to the first selection cycle, the RNA library was filtered through a nitrocellulose membrane to remove membrane-binding sequences. In each selection cycle, RNA was incubated with phenobarbital-BSA (containing 12–15 phenobarbital molecules per BSA molecule) and filtered through a nitrocellulose membrane. The membrane was washed by filtering through selection buffer (20 mM Tris-HCl, pH7.5; 100 mM NaCl; 750 μM $MgCl_2$) and subsequently soaked in phenol-chloroform to elute the bound RNA. The eluted RNA was reverse transcribed to generate DNA templates for the subsequent selection cycle. Several selection conditions were gradually modified throughout the process to increase selection stringency. Specifically, the phenobarbital-to-RNA molar ratio and ligand-RNA incubation time were gradually reduced, whereas

the wash volume was gradually increased (see Materials and Methods for detailed protocol). In addition, negative selections against BSA were performed before positive selections for phenobarbital-BSA in cycles 11 to 13 and 15 to 20.

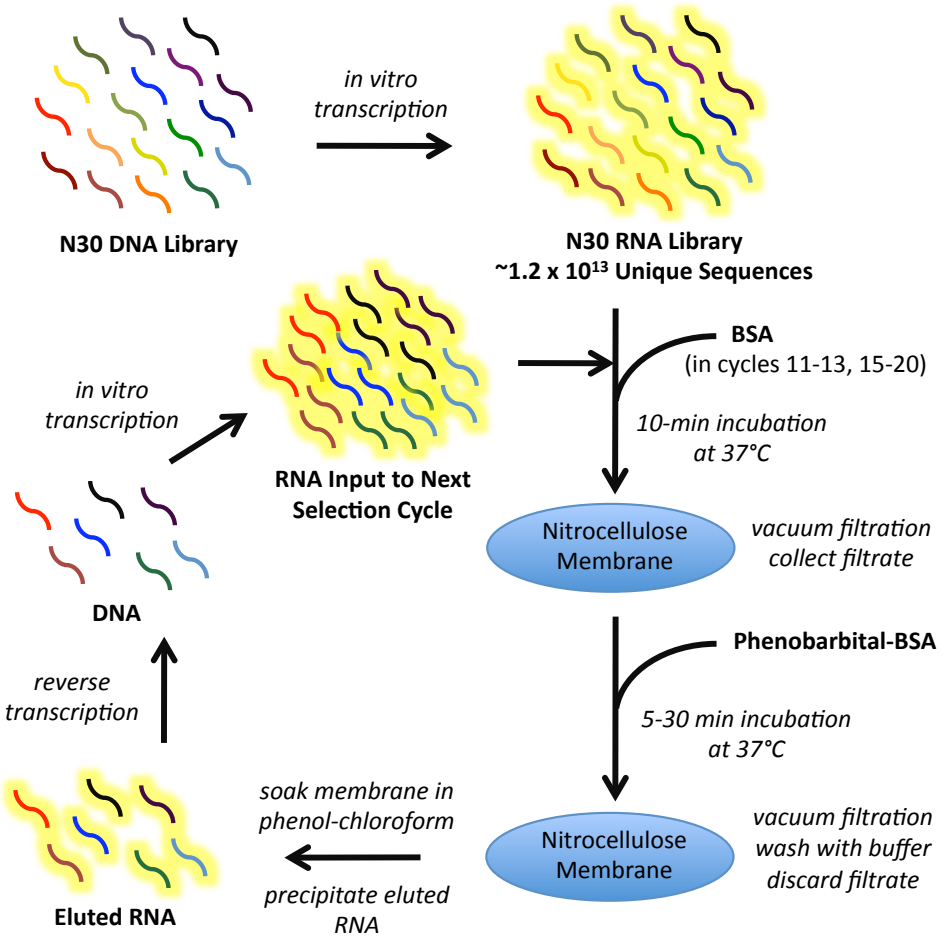

Figure 6.2. Schematic of conventional nitrocellulose membrane-based SELEX for RNA aptamer selection to BSA-conjugated phenobarbital. An RNA sequence library was generated by *in vitro* transcription and incubated with the target protein (phenobarbital-BSA). The binding mixture was filtered through a nitrocellulose membrane, which retains proteins and protein-bound RNA molecules. The membrane was washed with selection buffer to remove non-specifically or weakly binding RNA sequences. Protein-bound RNA sequences were eluted from the membrane by phenol-chloroform and reverse transcribed into cDNA, which served as the transcription template for the next selection cycle. In cycles 11-13 and 15-20, an additional negative selection step against BSA-binding sequences was inserted before the positive selection step with phenobarbital-BSA.

RNA pools obtained after cycles 15 and 20 of the selection were examined for ligand-binding specificity. ^{32}P-labeled RNA sequences were incubated with

phenobarbital-BSA, BSA (unconjugated), lysozyme, or no protein, and the binding mixture was filtered through nitrocellulose membranes as in regular selection cycles. The initial RNA flow-through was collected, and the membrane was washed with 1.8 ml of selection buffer. Radioactivity in the initial filtrates and on the washed membrane was quantified by scintillation counting to measure the extent of RNA binding to target proteins. RNA pools from both cycles show no specific binding to phenobarbital or to either of the proteins tested (Figure 6.3). Furthermore, the majority of RNA was retained on the membrane even in the absence of protein input, indicating strong affinity of the RNA library for the nitrocellulose membrane.

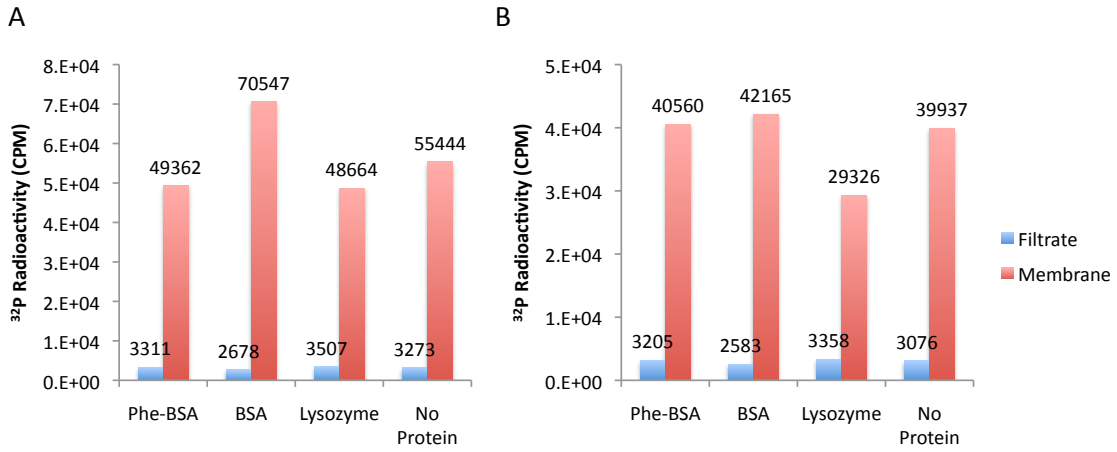

Figure 6.3. RNA sequence pools obtained after multiple selection cycles show strong affinity for nitrocellulose membranes and no specific binding for phenobarbital-BSA. Radiolabeled RNA was transcribed from cDNA obtained after (A) 12 and (B) 15 selection cycles and incubated with phenobarbital-BSA, BSA alone, lysozyme, or no protein. The binding solutions were filtered through nitrocellulose membranes, followed by washes with selection buffer. Radioactivity in the initial filtrates and on the washed membranes was quantified by scintillation counting. Regardless of the type of protein input, the majority of radioactivity is retained on the membrane, with a small fraction found in the initial filtrate, indicating strong binding to the nitrocellulose membrane.

To verify that RNA in general does not have non-specific affinity for nitrocellulose, we performed the radioactive binding assay on the initial N30 RNA library. The N30 RNA pool shows minimal retention by the membrane (Figure 6.4),

suggesting the membrane-binding activities observed in post–cycle 15 and post–cycle 20 RNA pools are specific to RNA sequences that have been enriched through multiple rounds of membrane-based separation.

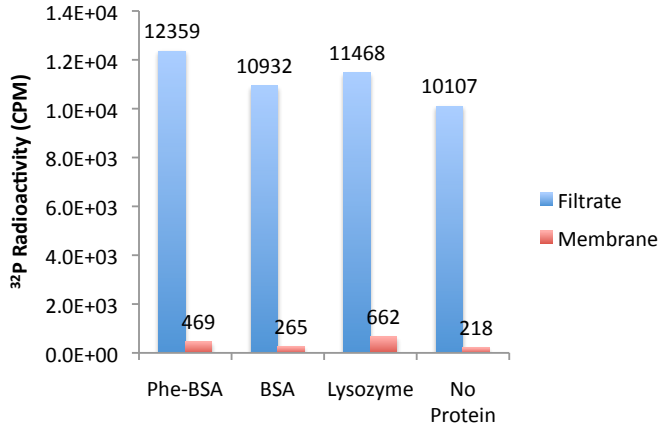

Figure 6.4. Randomized RNA sequence pool has minimal affinity for nitrocellulose membranes. Radiolabeled RNA was transcribed from randomized N30 DNA library and incubated with phenobarbital-BSA, BSA alone, lysozyme, or no protein. Filtration was performed as described in Figure 6.3. The majority of radioactivity is found in the initial filtrate, indicating low binding affinity for nitrocellulose membranes.

RNA pools obtained after cycles 1, 4, 8, 12, and 16 were analyzed using the radioactive binding assay without protein incubation to better understand the process by which nitrocellulose-binding sequences were enriched. Results indicate a noticeable increase in nitrocellulose binding starting in cycle 8, and affinity for nitrocellulose is prominent by cycle 12 (Figure 6.5). Although we did perform negative selection against the nitrocellulose membrane in cycle 1, these observations suggest that the negative selections should be continued in subsequent cycles to prevent the enrichment of nitrocellulose-binding sequences.

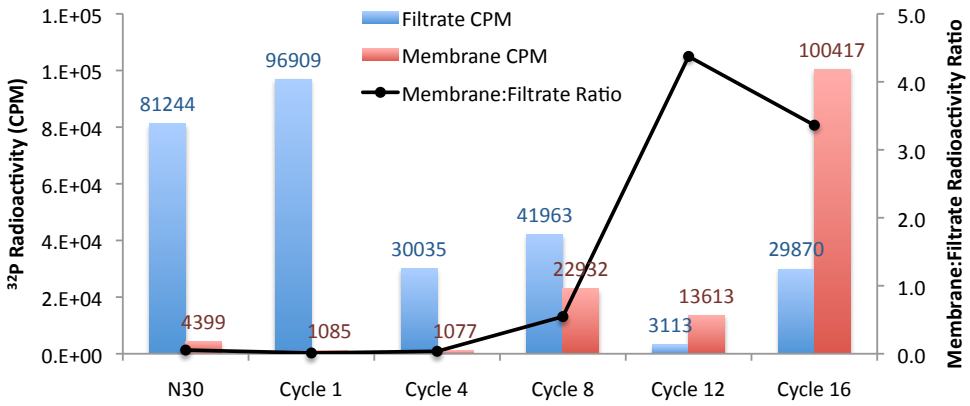

Figure 6.5. Nitrocellulose membrane-binding RNA sequences are enriched through selection cycles. Radiolabeled RNA was transcribed from either the randomized N30 DNA library or cDNA obtained after various selection cycles. The RNA was filtered through nitrocellulose membranes without prior incubation with protein targets, and radioactivity was measured as described in Figure 6.3. The black curve indicates the ratio of radioactivity on the membrane to radioactivity in the filtrate. Results reveal a significant increase in membrane-binding affinity in later selection cycles.

The enrichment of membrane-binding RNA sequences suggests a more detailed investigation on the performance characteristics of nitrocellulose membranes may be beneficial to subsequent endeavors in membrane-based SELEX. We first examined non-specific binding interactions between RNA and nitrocellulose membranes to determine the wash volume needed for complete removal of RNA from the membranes in the absence of protein components. A radioactive preparation of the N30 RNA library was filtered through a nitrocellulose membrane without prior protein binding, and the membrane was washed by filtering through five 1-ml aliquots of selection buffer. Radioactivity in the initial filtrate, each wash fraction, and the washed membrane was measured by scintillation counting. Results indicate that approximately 85% of all RNA passed through the membrane upon initial filtration, and the first 1-ml wash was sufficient to remove almost all residual RNA (Figure 6.6). Filtrates from subsequent wash steps and the washed membrane yielded minimal or undetectable levels of radioactivity, suggesting that RNA has low non-specific binding affinity for nitrocellulose membranes.

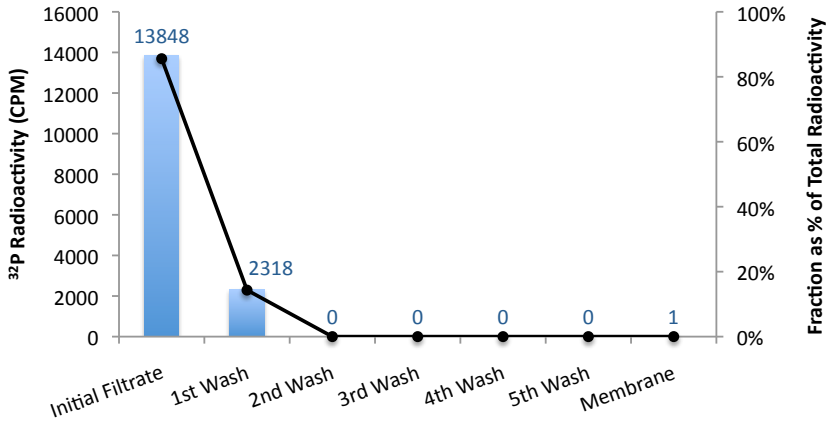

Figure 6.6. Removal of non-specifically bound RNA molecules from nitrocellulose membranes can be achieved with small wash volumes. Radiolabeled RNA was transcribed from randomized N30 DNA library and filtered through nitrocellulose membranes without prior binding incubation with protein targets. The membrane was washed with five 1-ml aliquots of selection buffer, and radioactivity in the initial filtrate, each 1-ml wash fraction, and the washed membrane was quantified by scintillation counting. The black curve indicates the percent of total measured radioactivity in each fraction.

We next examined the efficiency of nitrocellulose membranes in separating free and protein-bound RNA sequences by performing radioactive binding assays on known RNA aptamers incubated with either their cognate protein targets or with a mismatched protein (BSA). The lysozyme RNA aptamer, which has a K_D of 31 nM[13], was incubated with 300 nM of either lysozyme or BSA. The NF-κB RNA aptamer, which has a K_D of 5.4 nM[14], was incubated with 24 nM of either NF-κB or BSA. The binding solution was filtered through a nitrocellulose membrane, follow by washing with three 1-ml aliquots of selection buffer. Radioactivity in the initial filtrate, each wash fraction, and the washed membrane was measured by scintillation counting. The majority of RNA was found in the initial filtrate and never retained by the membrane, possibly due to a molar excess of RNA relative to protein targets (Figure 6.7). Incubating RNA aptamers with their cognate proteins did result in greater initial RNA retention on the nitrocellulose membrane compared to incubation with a mismatched protein, indicating protein-specific binding activity by the aptamers. However, the retained RNA was rapidly lost during the

subsequent wash steps, suggesting that the ligand-specific binding interaction is easily disrupted by buffer filtration.

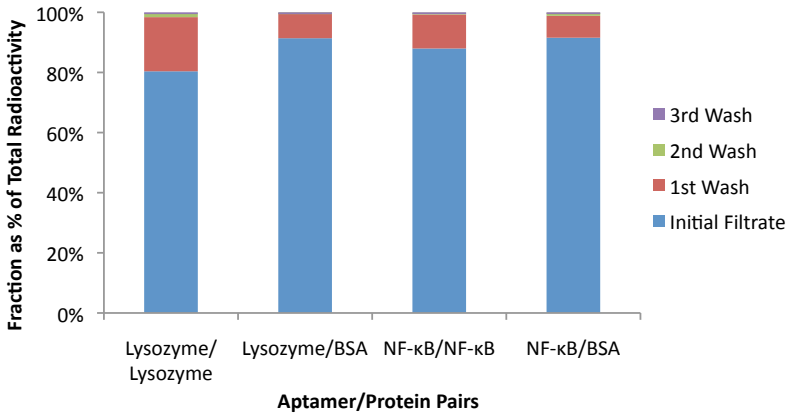

Figure 6.7. Nitrocellulose membranes show weak retention of RNA aptamers bound to their specific protein ligands. RNA aptamer sequences were incubated with either their cognate protein or a mismatched target (BSA) and filtered through nitrocellulose membranes. Incubation with cognate proteins results in greater RNA retention on the membrane in the initial filtration, but the retained RNA is easily removed by subsequent wash steps.

The observation that even pure RNA aptamer sequences with high affinities for their target proteins cannot withstand relatively gentle wash conditions suggests that the conventional membrane-based SELEX protocol—in which membranes are loaded with RNA, washed extensively with buffer, and eluted by soaking in solutions such as phenol-chloroform or urea—may not be the most efficient method for recovering and enriching protein-bound RNA sequences. Specifically, protein-bound RNA sequences initially retained on the membrane are likely to be lost during washing, while only strongly nitrocellulose-binding sequences are able to withstand the wash steps and be recovered in the elution step. In light of these observations, we modified the membrane-based selection procedure to incorporate an alternative elution strategy that is similar in principal to affinity elution in column chromatography.

RNA Recovery by Affinity Elution Prevents Enrichment of Nitrocellulose-Binding Sequences. In a second attempt to select for phenobarbital-binding RNA aptamers, we modified the nitrocellulose membrane-based SELEX procedure to perform ligand-specific elution by filtering concentrated phenobarbital solutions through the RNA-loaded membrane (Figure 6.8). Unlike the previous RNA recovery method, in which washed nitrocellulose membranes were soaked in phenol-chloroform to remove membrane-bound RNA molecules, the modified protocol is expected to release phenobarbital-specific RNA sequences from the membrane through competitive binding between free phenobarbital and phenobarbital-BSA molecules. RNA sequences released from the protein can flow through the membrane and be collected in the eluate fraction.

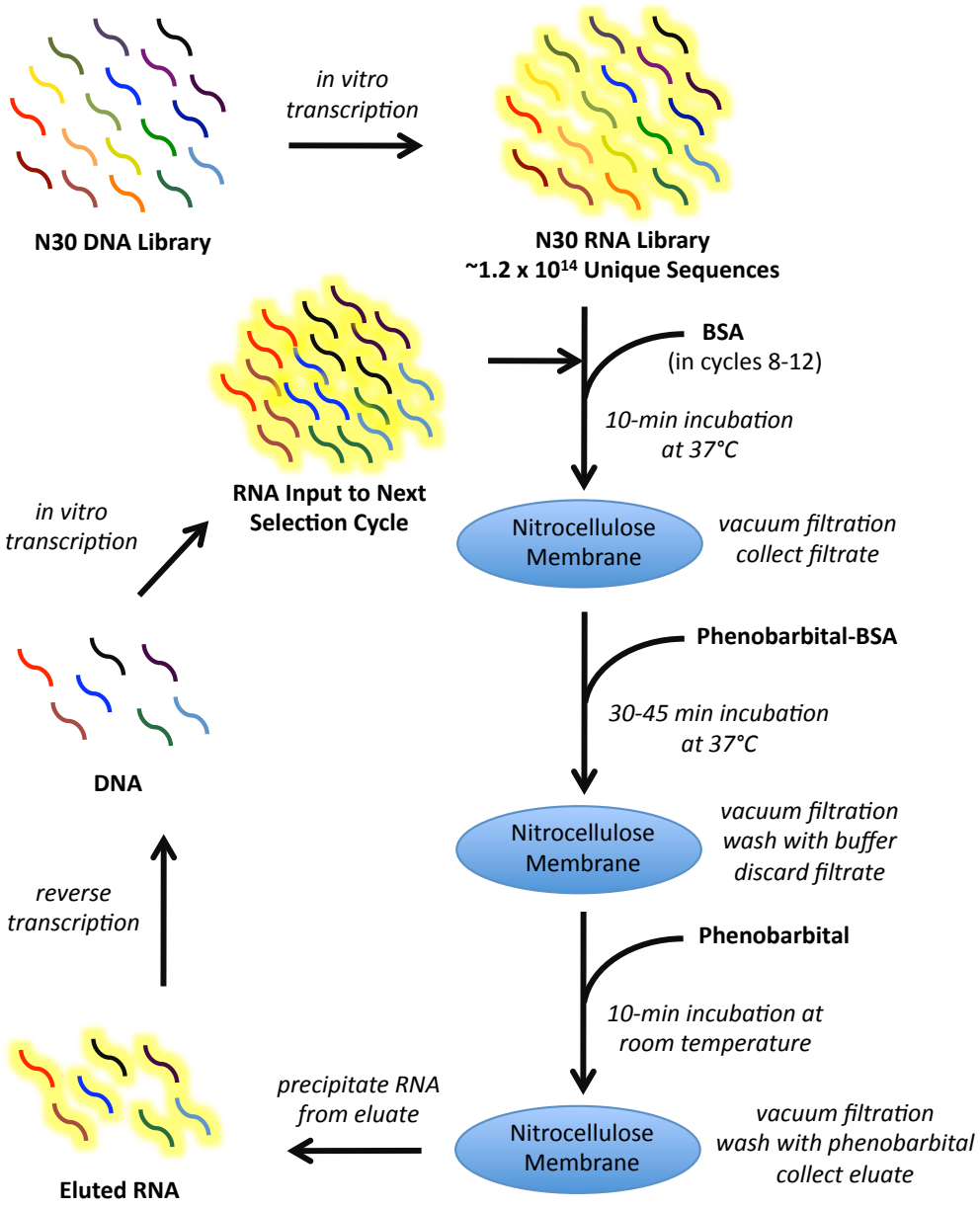

Figure 6.8. Schematic of nitrocellulose membrane-based SELEX with ligand-specific elution for the selection of RNA aptamers to BSA-conjugated phenobarbital. The selection procedure depicted in Figure 6.2 was modified such that ligand-bound RNA was specifically eluted by filtration with concentrated phenobarbital solutions rather than non-specific elution by soaking the membrane in phenol-chloroform.

An initial N30 RNA library containing approximately 1.2 x 10^{14} unique sequences (ten times more than the previous library) was employed to broaden the selection pool. Twelve selection cycles were performed, with the phenobarbital-to-RNA ratio fixed at 14:1 throughout the selection. The decision not to reduce the ligand-to-RNA ratio was

informed by the observation that even high-affinity RNA aptamer sequences incubated with their cognate proteins are easily lost during the filtration process (Figure 6.7), suggesting that a low ligand-to-RNA ratio may impose an unproductively stringent selection condition. The wash volume was maintained at 1 ml for cycles 1 through 9 and increased to 2 ml for cycles 10 through 12. Negative selection against BSA was performed before positive selection with phenobarbital-BSA in cycles 8 through 12.

RNA pools obtained after cycles 6 and 12 of the selection were examined for ligand-binding specificity, and the N30 RNA library was included as a control. ^{32}P-labeled RNA sequences were incubated with phenobarbital-BSA, BSA, or no protein, and the binding mixture was filtered through nitrocellulose membranes as in regular selection cycles. The initial RNA flow-through was collected, and the membrane was washed with 1.2 ml of selection buffer followed by elution with 200 μl of 1 mM phenobarbital and 800 μl of 5 mM phenobarbital. Radioactivity in the initial filtrate, wash fraction, combined eluate fraction, and the washed membrane was quantified by scintillation counting. In all three RNA pools tested, the presence and identity of protein have minimal impact on the binding and elution behavior of the RNA (Figure 6.9). Compared to the N30 library, RNA pools from cycles 6 and 12 show significantly more RNA retention by the membrane upon initial filtration, but the great majority of retained RNA is removed by the wash step. The small percentage of RNA found on the final washed membrane indicates that the modified protocol succeeded in preventing the enrichment of nitrocellulose-binding sequences. However, the minimal amount of RNA found in the eluate fraction also indicates that the RNA pool has not been sufficiently enriched for phenobarbital-binding species.

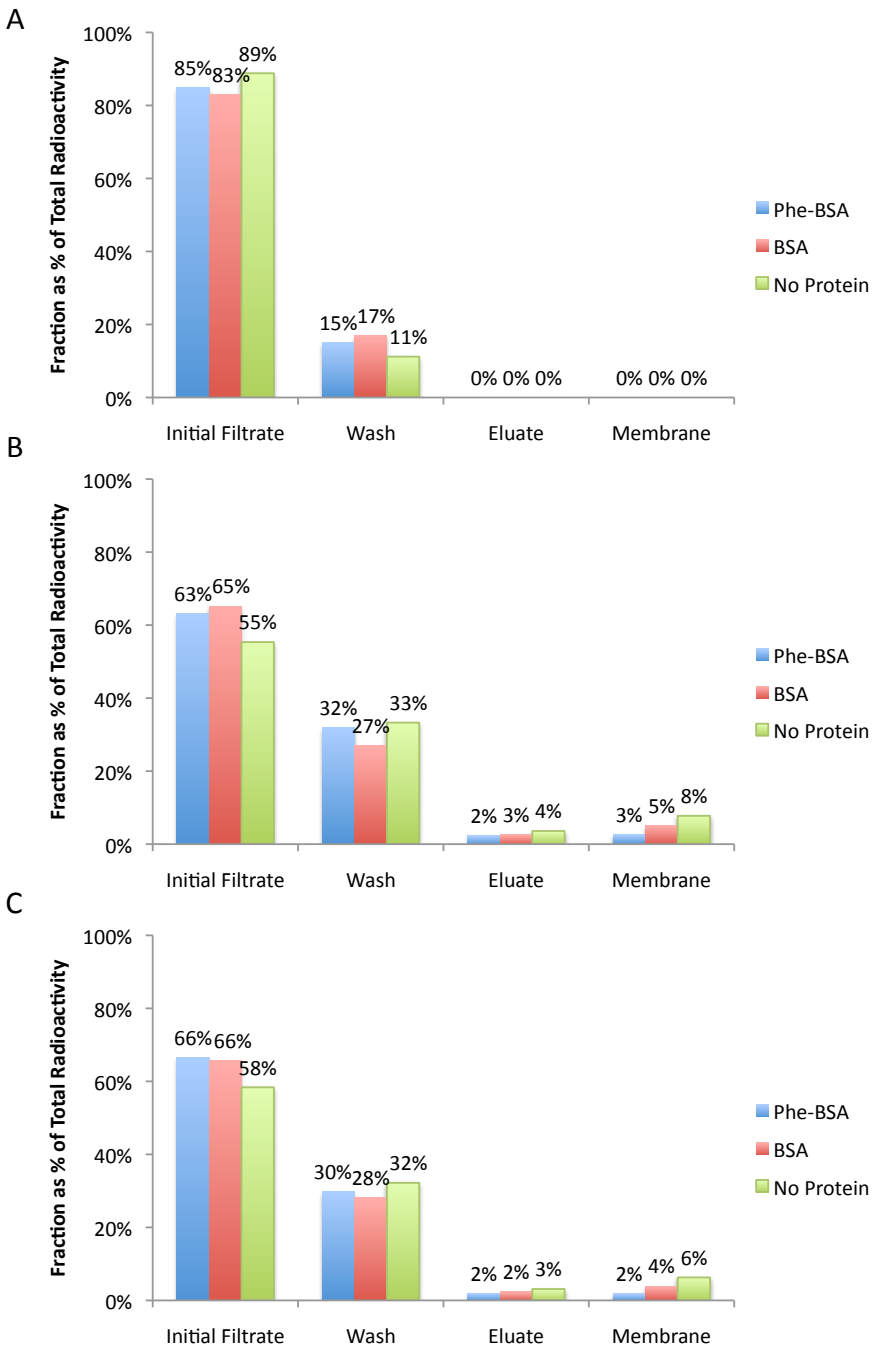

Figure 6.9. RNA sequence pools obtained after multiple selection cycles show no specific binding for phenobarbital-BSA (phe-BSA) or BSA alone. (A) The N30 DNA library and cDNA obtained after (B) six and (C) twelve selection cycles served as templates for the transcription of radiolabeled RNAs, which were incubated with phenobarbital-BSA, BSA alone, or no protein. Filtration was performed as described in Figure 6.3, with an added step of elution with phenobarbital after the wash step with selection buffer. Radioactivity in each fraction was analyzed by scintillation counting. Radioactivity found in each fraction is reported as the percent of total measured radioactivity. Sequence pools obtained after cycles 6 and 12 show increased retention by the membrane upon initial filtration, but the RNA-nitrocellulose binding interaction is sufficiently weak for the majority of retained RNA to be removed by the wash step. No increase in RNA retention is seen with either phenobarbital-BSA or BSA alone.

We sequenced the cycle 12 pool to better understand the extent of sequence enrichment at the end of the selection process. Surprisingly, only six unique and highly related sequences were found, with two-thirds (20 of 29) of the sequences being identical (Cycle 12 Sequence, Figure 6.10) We then sequenced pools from the initial N30 library and cycles 1, 4, 7, and 8 (the first cycle after negative selection began). Results from the N30 library confirm that the initial pool is diverse, with no repeats or detectable motifs found among 14 sequenced samples. However, the dominant sequence found in cycle 12 (SVC12.6) is already present in the cycle 1 pool. The cycle 4 pool is dominated by two sequences, including SVC12.6, and this sequence accounts for the great majority of sequences found in cycles 7 and 8.

ID	N30 Library Sequence	Frequency
N30.1	*AGCTAGCCAGG*AGCGCAGCGCGCAACACGGAGAGGTTGATC*GAGGCAAAGCTTCCG*	1 of 14
N30.2	*AGCTAGCCAGG*CAGTGTGATACGCACTGAGTTCCGATGGGGG*AGGCAAAGCTTCCG*	1 of 14
N30.6	*AGCTAGCCAGG*ATCTTAGGTTTACCCCTGGAGGTAGTATGGG*AGGCAAAGCTTCCG*	1 of 14
N30.10	*AGCTAGCCAGG*TGGGATACGGCCCATGAAGGCGGGCGGGAT*GAGGCAAAGCTTCCG*	1 of 14
N30.11	*AGCTAGCCAGG*ATCCTAGATTAGCGCAGGTAGCGTTAGTGGG*AGGCAAAGCTTCCG*	1 of 14
N30.13	*AGCTAGCCAGGGG*AGCAAAAGAGTCCCGGACTAATACAGGGG*AGGCAAAGCTTCCG*	1 of 14
N30.14	*AGCTAGCCAGG*TGTGCCGTCAGGAAAAGTGAATCACCTGTGG*AGGCAAAGCTTCCG*	1 of 14
N30.20	*AGCTAGCCAGGGG*TGGATCGTGGAATGTATCCGCACGACGG*GAGGCAAAGCTTCCG*	1 of 14
N30.31	*AGCTAGCCAGG*AAAACACCAAAAGGTGGCCAGCATCTACAG*GAGGCAAAGCTTCCG*	1 of 14
N30.32	*AGCTAGCCAGG*AGGCCGAAAGCGGAAGAAAACAAGTAGGAT*GAGGCAAAGCTTCCG*	1 of 14
N30.33	*AGCTAGCCAGG*CAAACCGGTTACGTATCGTCTACGTACGGG*GAGGCAAAGCTTCCG*	1 of 14
N30.191	*AGCTAGCCAGG*AACAGGCTGCGAAGGGGTATATGATTTAGA*GAGGCAAAGCTTCCG*	1 of 14
N30.192	*AGCTAGCCAGG*ATAGTTCGGGTTGTATGGTTAAGTGCTTGG*GAGGCAAAGCTTCCG*	1 of 14
N30.193	*AGCTAGCCAGG*AACAGAAGATCGAGGATCGATGAATCATG-*GAGGCAAAGCTTCCG*	1 of 14

ID	Cycle 1 Sequence	Frequency
SVC1.28	*AGCTAGCCAGG*CTGGGCGTCATCAGGAGTGTCGGGTCGGTG*GAGGCAAAGCTTCCG*	1 of 13
SVC1.30	*AGCTAGCCAGG*AATGGGGGACCCCAGATTAGTACCTGTGGGG*AGGCAAAGCTTCCG*	1 of 13
SVC1.34	*AGCTAGCCAGG*CATATCGGGGATCAAAGAATCGGTGGAGGG*GAGGCAAAGCTTCCG*	1 of 13
SVC1.36	*AGCTAGCCAGG*GCATTACCGAGCTACACTCAACGCCTCTGGG*AGGCAAAGCTTCCG*	1 of 13
SVC1.38	*AGCTAGCCAGG*AGTAAGTGGGAGGAGAACGTAGGGGTCTGGG*AGGCAAAGCTTCCG*	1 of 13
SVC1.40	***AGCTAGCCAGG*AAAGTGAGGAGGTGGTGAAGTACTGGTGTA*GAGGCAAAGCTTCCG***	1 of 13
SVC1.42	*AGCTAGCCAGG*CGCAAGCAGGAGGGATAACAGTACGGCGGG*GAGGCAAAGCTTCCG*	1 of 13
SVC1.44	*AGCTAGCCAGG*GCTTCGGATCGAATCTAATGCTTGATTGAT*GAGGCAAAGCTTCCG*	1 of 13
SVC1.46	***AGCTAGCCAGG*AAAGTGAGGAGGTGGTGAGGTACTGGTGTA*GAGGCAAAGCTTCCG***	1 of 13
SVC1.50	*AGCTAGCCAGG*AGGAATCATACGATGTATTCGTAAGGCATG*GAGGCAAAGCTTCCG*	1 of 13
SVC1.321	*AGCTAGCCAGG*TGGCGAACAGGAGAACTGAGCGGTGAGGGT*GAGGCAAAGCTTCCG*	1 of 13
SVC1.322	*AGCTAGCCAGG*CTGAAGATGCAAGTAGGTAGGCTATGTGTT*GAGGCAAAGCTTCCG*	1 of 13
SVC1.323	*AGCTAGCCAGG*GAAGCCGGGGTGGGCAAACCAGGTGCGTG*G-AGGCAAAGCTTCCG*	1 OF 13

ID	Cycle 4 Sequence	Frequency
SVC4.8	***AGCTAGCCAGG*AAAGTGAGGAGGTGGTGAAGTACTGGTGTA*GAGGCAAAGCTTCCG***	8 of 19
SVC4.11	*AGCTAGCCAGG*GAACGTGACAGGAAAACTGCGTCTCGGCTG*GAGGCAAAGCTTCCG*	1 of 19
SVC4.16	***AGCTAGCCAGG*TTGAAGGTGGAGGAGGAGTAATCTTGGTA*GAGGCAAAGCTTCCG***	8 of 19
SVC4.28	*AGCTAGCCAGG*CAATGTATTGACCTGGTATACGGGGTGGG-*GAGGCAAAGCTTCCG*	1 of 19
SVC4.242	*AGCTAGCCAGG*GTTGAAAGTGGACGAGGATTGTTGCGGGTA*GAGGCAAAGCTTCCG*	1 of 19

ID	Cycle 7 Sequence	Frequency
SVC7.27	***AGCTAGCCAGG*AAAGTGAGGAGGTGGTGAAGTACTGGTGTA*GAGGCAAAGCTTCCG***	10 of 13
SVC7.40	*AGCTAGCCAGG*AGAATCGTGGACTTGAGATCGTGCGCGTGG*GAGGCAAAGCTTCCG*	1 of 13
SVC7.45	*AGCTAGCCAGG*GTTGAAGGTGGAGGAGGAGTAATCTTGGTA*GAGGCAAAGCTTCCG*	1 of 13
SVC7.46	*AGCTAGCCAGG*GGCAAAAGCAATTCTTGTGTTGTTGCTGTT*GAGGCAAAGCTTCCG*	1 of 13

ID	Cycle 8 Sequence	Frequency
SVC8.2	***AGCTAGCCAGG*AAAGTGAGGAGGTGGTGAAGTACTGGTGTA*GAGGCAAAGCTTCCG***	13 of 14
SVC8.16	***AGCTAGCCAGG*AAAGTGAGGAGGAGGTGAAGTACTGGTGTA*GAGGCAAAGCTTCCG***	1 of 14

ID	Cycle 12 Sequence	Frequency
SVC12.6	***AGCTAGCCAGG*AAGTGAGGAGGTGGTGAAGTACTGGTGTA*GAGGCAAAGCTTCCG***	20 of 29
SVC12.53	*AGCTAGCCAGG*AAGTGAGGAGGAGGTGAAGTACTGGTGTA*GAGGCAAAGCTTCCG*	2 of 29
SVC12.60	*AGCTAGCCAGGG*CTTCGAGGGAGGAGGAGGAGTGAAGTGTT*GAGGCAAAGCTTCCG*	4 of 29
SVC12.70	***AGCTAGCCAGGG*TTGAAGGTGGAGGAGGAGTAATCTTGGTA*GAGGCAAAGCTTCCG***	1 of 29
SVC12.402	*AGCTAGCCAGG*AAGTGAGCAGGCGCTGAAGTACTCGTGTA*GAGGCAAAGCTTCCG*	1 of 29
SVC12.482	*AGCTAGCCAGG*ATTGAAGGTGGAGGAGGAGTAATCTTGGTA*GAGGCAAAGCTTCCG*	1 of 29

Figure 6.10. Sequences obtained after various selection cycles show a rapid decrease in sequence diversity and the early appearance of a dominant sequence. Italicized nucleotides constitute the constant regions. Bolded sequences of the same color are identical, except where blue highlights mark point mutations that distinguish the two red-colored sequences in cycles 1 and 8. Green and yellow highlights mark sequence motifs among sequences found in the cycle 12 pool.

These results indicate that the selection conditions rapidly eliminated sequence diversity from the RNA library. The sequence pool was nearly homogenous by the time negative selection against BSA commenced in cycle 8, likely rendering subsequent cycles ineffectual. However, it is curious that the SVC12.6 sequence accounts for 77% (10 of 13) of the sequenced cycle 7 pool and 93% (13 of 14) of the cycle 8 pool but only 69% (20 of 29) of the cycle 12 pool, suggesting a resurgence of less prevalent sequences during the later cycles. The six unique sequences found in the cycle 12 pool are all highly enriched in G and show conserved motifs. The AGG motif (alone or in repeats) was found in all sequences at similar positions, and the AAGT sequence was found in all but two (Figure 6.10). The 5' and 3' ends of all six sequences also show convergence upon similar bases. A purine occupies the first nucleotide after the 5' constant region in all six sequences, and KKGTA (K = G or T) is found immediately before the 3' constant region in all but one sequence (which has KKGTT). Such sequence similarities are typical of enriched aptamer pools, yet no ligand-binding activity was detected in radioactive binding assays performed on the final sequence pool (Figure 6.9C), suggesting that the binding characterization method may not be ideal. As an alternative method, capillary electrophoresis (CE) was employed for binding affinity evaluation.

Capillary Electrophoresis (CE) Assay Shows No Ligand-Binding Activity by Putative Phenobarbital Aptamers. CE is an automated process that separates mixture components based on differences in electrophoretic mobility, which is dependent on the size-to-charge (approximated by the mass-to-charge, or m/z) ratios of the various species within a mixture[15]. A capillary, which is typically made of fused silica and carries

negative charges on its surface, connects an inlet anode and an outlet cathode. As a run buffer containing background electrolytes passes through the capillary, an electroendoosmotic flow (EOF) toward the cathode is transmitted throughout the diameter of the capillary, creating a nearly perfect plug flow down the length of the tube. When a sample mixture is injected into the capillary, components are simultaneously separated by charge due to the EOF and by size due to frictional drag in accordance with Stoke's Law.

Since RNA molecules are highly negatively charged, a mixture of free protein, free RNA, and protein-RNA complexes can be separated by CE based on differences in their m/z ratios. Free protein molecules are eluted first, followed by protein-RNA complexes and finally the free RNA molecules. For the purpose of characterizing ligand-binding behavior of putative phenobarbital aptamers, RNA molecules were transcribed from single-sequence DNA templates and incubated with an equimolar amount of phenobarbital-BSA (5.24 μM each). The three most prevalent sequences from cycle 12 (SVC12.6, SVC12.60, and SVC12.70) were examined. The resulting elution profiles show clear peaks corresponding to free phenobarbital-BSA and free RNA but no detectable protein-RNA complex peak (Figure 6.11), indicating a lack of phenobarbital- and BSA-binding activities by the RNA sequences. Together with the radioactive binding assay results, these observations suggest that the selection process failed to enrich for phenobarbital-binding sequences, and alternative aptamer selection protocols or molecular targets need to be identified.

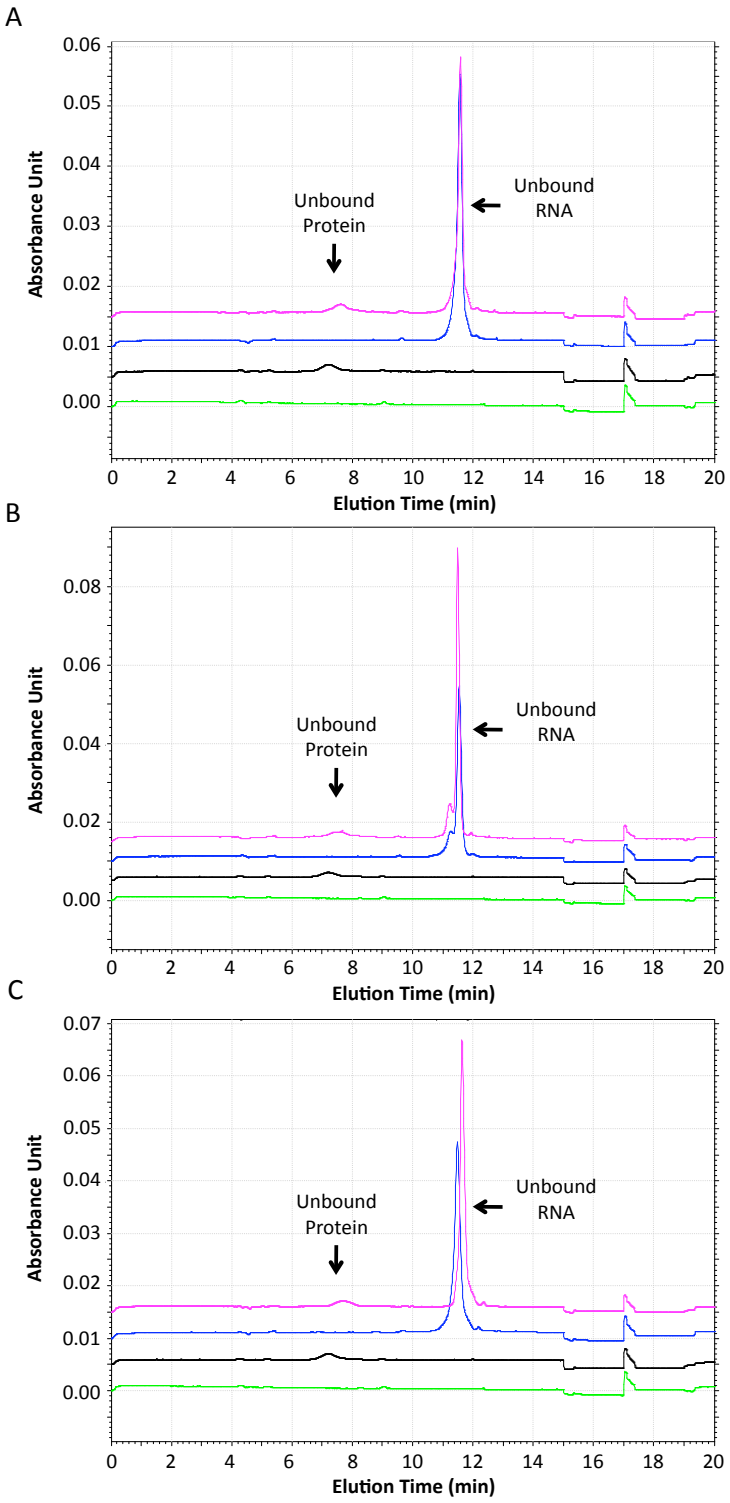

Figure 6.11. Capillary electrophoresis shows no evidence of ligand binding by putative phenobarbital aptamer sequences. Selection buffer alone (green), phenobarbital-BSA alone (black), RNA alone (blue), and a binding solution containing both RNA and phenobarbital-BSA (pink) were analyzed by CE. The RNA sequences (A) SVC12.6, (B) SVC12.60, and (C) SVC12.70 were characterized, with none showing specific binding to the phenobarbital-BSA molecule.

Column-Based SELEX for Vitamin B_{12} Fails to Enrich for Ligand-Binding RNA Sequences. Unlike nitrocellulose membrane–based separation, column chromatography allows for the selection of aptamers to small molecules without protein conjugation. However, phenobarbital does not possess appropriate functional groups for resin-conjugation chemistry and thus cannot be immobilized onto columns for nucleic acid separation. As a result, alternative small-molecule ligands were evaluated as SELEX candidates. Vitamin B_{12} (cyanocobalamin) and folinic acid are two small molecules commonly used as a nutritional supplement and an adjuvant in cancer chemotherapy[16], respectively (Figure 6.12). We have also demonstrated that these molecules have low or no toxicity toward mouse T cells (Appendix 1) and human natural killer cells (Appendix 2), and would be suitable input molecules for ligand-responsive RNA-based regulatory systems. Importantly, both vitamin B_{12} and folinic acid can be conjugated to agarose resin molecules through standard chemistry, thus enabling the use of column-based SELEX.

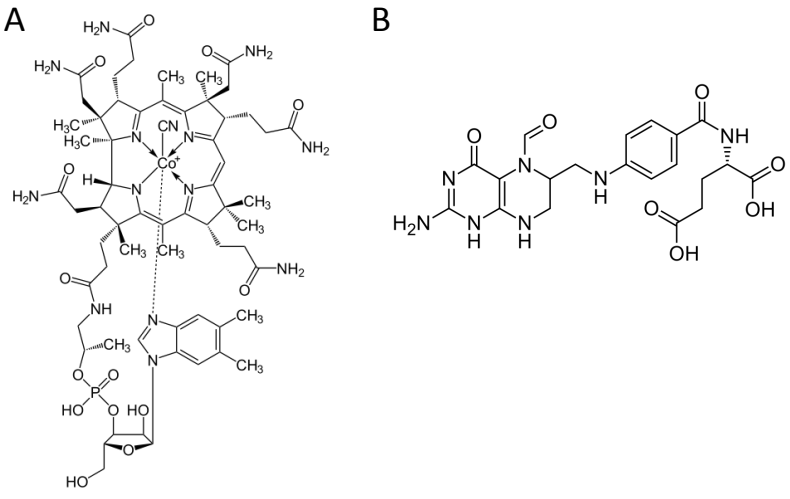

Figure 6.12. Chemical structures of (A) vitamin B_{12} (cyanocobalamin) and (B) folinic acid. Vitamin B_{12} can be conjugated to Sepharose 6B resin through its hydroxyl group. Folinic acid can be conjugated to EAH Sepharose 4B resin through its carboxyl groups.

An RNA aptamer for vitamin B_{12} had previously been isolated by column-based SELEX[17]. However, the binding activity of this aptamer is dependent on the presence of high concentrations of Li^+, making it unsuitable for *in vivo* applications. Furthermore, this aptamer has a complex tertiary structure, including pseudoknots required for ligand binding[18]. Such structural complexities render the incorporation of this aptamer into RNA-based regulatory devices difficult, if not impossible. Nevertheless, the previous example of vitamin B_{12} aptamer selection indicates that it is possible to isolate vitamin B_{12}–binding RNA sequences by column-based SELEX, and that the ion dependence of the isolated sequences is partially dictated by the composition of the selection buffer[17]. To generate a vitamin B_{12}–specific RNA aptamer capable of *in vivo* functionality, we performed a modified SELEX protocol in which the selection buffer contained a more physiological ion concentration (250 mM NaCl and 2.5 mM $MgCl_2$ in contrast to 1 M LiCl and 5 mM $MgCl_2$ in the published selection process). Furthermore, the randomized RNA library was designed to contain 5' and 3' constant regions that formed a stem at the base of all sequences to facilitate future integration into RNA-based regulatory devices (Figure 6.13).

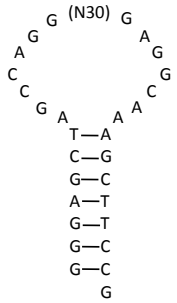

Figure 6.13. The randomized RNA library contains constant regions that form a stable basal stem. The 5' and 3' constant regions flanking the N30 sequence form a stable hairpin through Watson-Crick base pairing to ensure that aptamer sequences obtained from the selection process have structures appropriate for integration into RNA-based regulatory devices.

An initial RNA library containing approximately 2.4 x 10^{14} unique sequences was subjected to 15 cycles of selection (Figure 6.14). In each cycle, a column packed with commercially available vitamin B_{12}–conjugated agarose (same as used in Ref. 17) was equilibrated with selection buffer, loaded with RNA, washed with selection buffer, and eluted with 5 mM vitamin B_{12}. Eluted RNA was precipitated and reverse transcribed to produce DNA templates for the next selection cycle. Negative selection against an adipic acid dihydrazide agarose column was performed immediately before positive selection for vitamin B_{12} in cycles 1 through 3 to eliminate agarose-binding sequences. The wash volume was increased from 5 column volumes (CV) in cycle 1 to 10 CV in cycle 4. The volume was subsequently increased in 10-CV intervals from cycle 5 to cycle 10, then in 20-CV intervals from cycle 10 to cycle 15. Mutagenic PCR was performed after cycle 10 to diversify the selection pool.

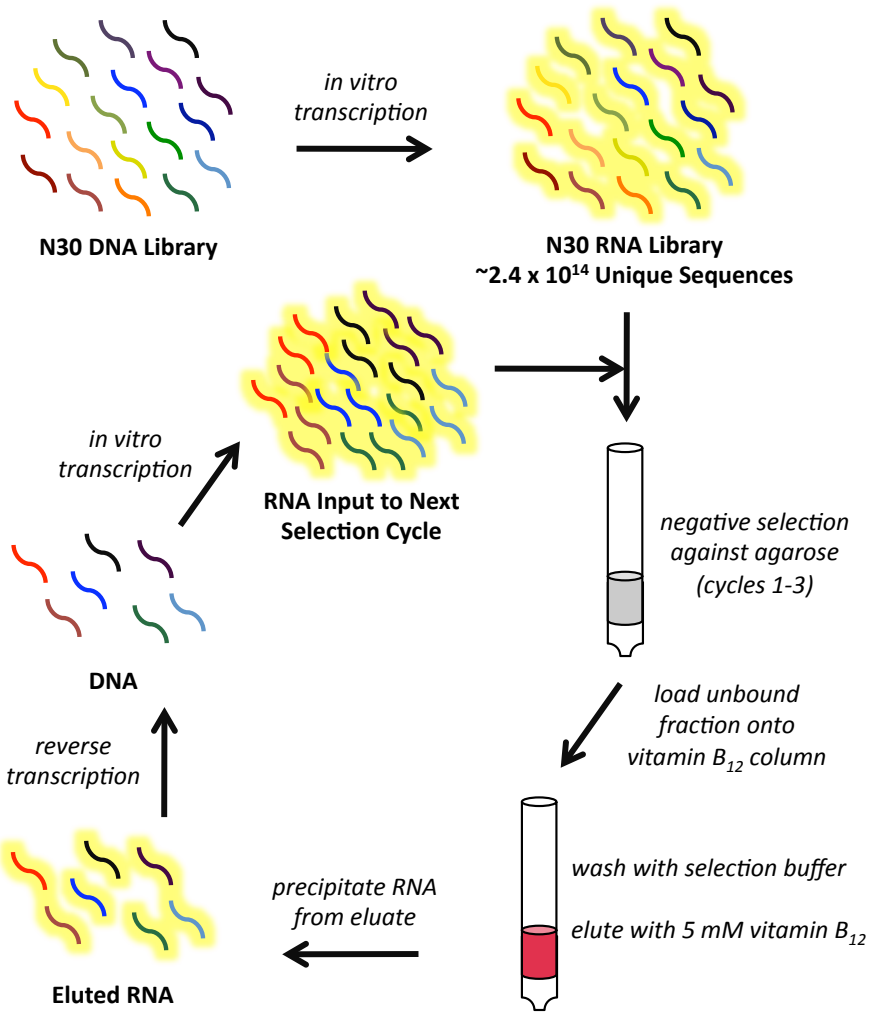

Figure 6.14. Schematic of column-based SELEX used to select for RNA aptamers to vitamin B_{12}. An RNA sequence library was generated by *in vitro* transcription and filtered through a vitamin B_{12}–conjugated agarose column. The column was washed with selection buffer, and specifically bound RNA was eluted with concentrated vitamin B_{12} solution. Eluted RNA was reverse transcribed into cDNA, which served as the transcription template for the next cycle's RNA pool. In cycles 1-3, an additional negative selection step against agarose resin was inserted before the positive selection step with vitamin B_{12}–conjugated agarose.

The RNA pool obtained after 15 selection cycles was sequenced, and no repeat was observed among 43 sequenced samples. Analyses were performed to investigate potential sequence and structural relationships among the sequences, and a number of conserved motifs were manually identified (Figure 6.15). Several sequences form stem-

loop structures, likely due to structural constraints imposed by the constant regions in the sequence library, but no single structure dominates the sequence pool.

A

B12-4
>AAGGATTCAATGCTAGTTTCCCGCCACCAT<
B12-44
>AGCTTAGGTGCTAGTTCTCCCGCTTCTGTAAAGGCAAAGCTTCCG

B12-2
>CAAATAGTCTGTGTAACCCGTTCCCGTGTT<
B12-23
>AGAGACTTGTCTGGCAATGTTCGTTCCCAT<

B12-6
>TGTTATTAGGCGAGTACTCCCGTCTCTGTA<
B12-10
>TTTATTAGGCGGTAGTTCCCCTCCGTAGAT<

B12-7
>ATCCTACCTCTGGCGCTTCCACGTCCTGTA<
B12-66
>CGAGCAGTATCCCACCTAGGCACTCCTGTA<

B12-15
>GTTCTTCCGGTTGGCGTACCCATGCACTAT<
B12-45
>TATTCTTGTGGTTAGCGCTTTCCATACTAT<
B12-11
>TATCATCTCTGGTTAGCGGTACCACTGTAT<

B12-19
>TATGTTAGCTCGCCATTCGTCTTCATGCAT<
B12-48
>TGCTAGTTCGATCCAGATCGCTCATGTGTT<
B12-71
>TTCTCGAGAATCTTAGTTTCGTCATGTGTA<

B12-1
>TGTCCGCGTTGGCTGGTTCCGTACCTTGTA<
B12-12
>TCCGACATCACTGGTGTGGTCCCTGCTGTA<
B12-49
>CCGATTGGGGTCGGGTAGTCCCATGCTGTA<

B

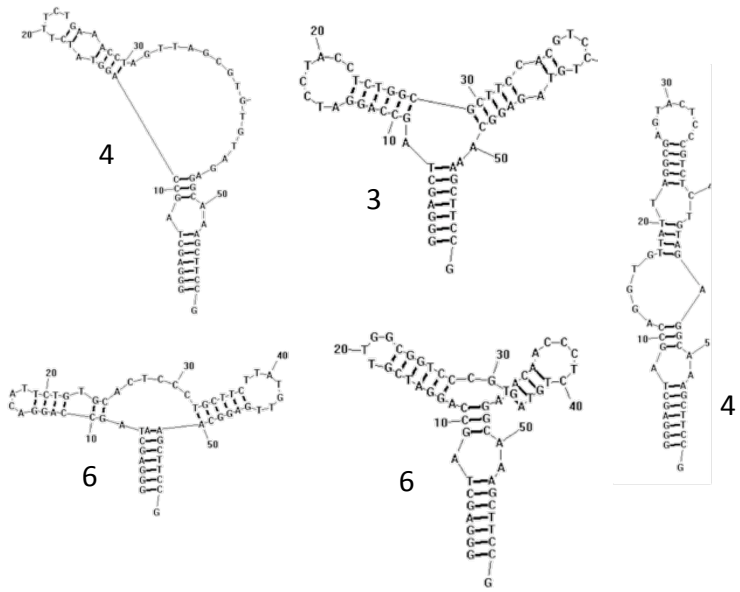

Figure 6.15. Vitamin B_{12} SELEX resulted in a diverse sequence pool with several sequence and structural motifs after 15 selection cycles. (A) No sequence appears more than once in the pool, but similar motifs can be found among the diverse sequences. Colored highlights mark motifs found within each group of sequences. (B) The secondary structures of sequenced samples were determined using the RNAstructure software[19], and structural motifs were identified. Structural elements including loop size, loop number, stem shape, and stem number were analyzed manually, and five structure groups were found to include multiple sequences. The number next to each structure denotes the number of sequences for which the shown structure is representative.

Several groups of similar sequences were identified (Figure 6.15A), and one sequence from each of three such groups was chosen for evaluation by radioactive binding assays. Radiolabeled RNA was loaded onto a vitamin B_{12}–conjugated agarose column, washed with three 500-μl aliquots of selection buffer, and eluted with six 500-μl aliquots of 5 mM vitamin B_{12}. Radioactive quantification of wash and eluate fractions shows weak specific elution by vitamin B_{12} (Figure 6.16A). More importantly, the overwhelming majority (>97%) of RNA remains bound to the column, indicating strong binding affinity toward resin particles. However, the same sequences tested on adipic acid dihydrazide agarose columns show minimal RNA retention on the column (Figure 6.16B), suggesting that the adipic acid dihydrazide agarose may not have been sufficiently similar to the vitamin B_{12}–conjugated agarose to serve as an effective negative selection target, even though dihydrazide agarose was used for this purpose in the previously published selection for vitamin B_{12} aptamers[17].

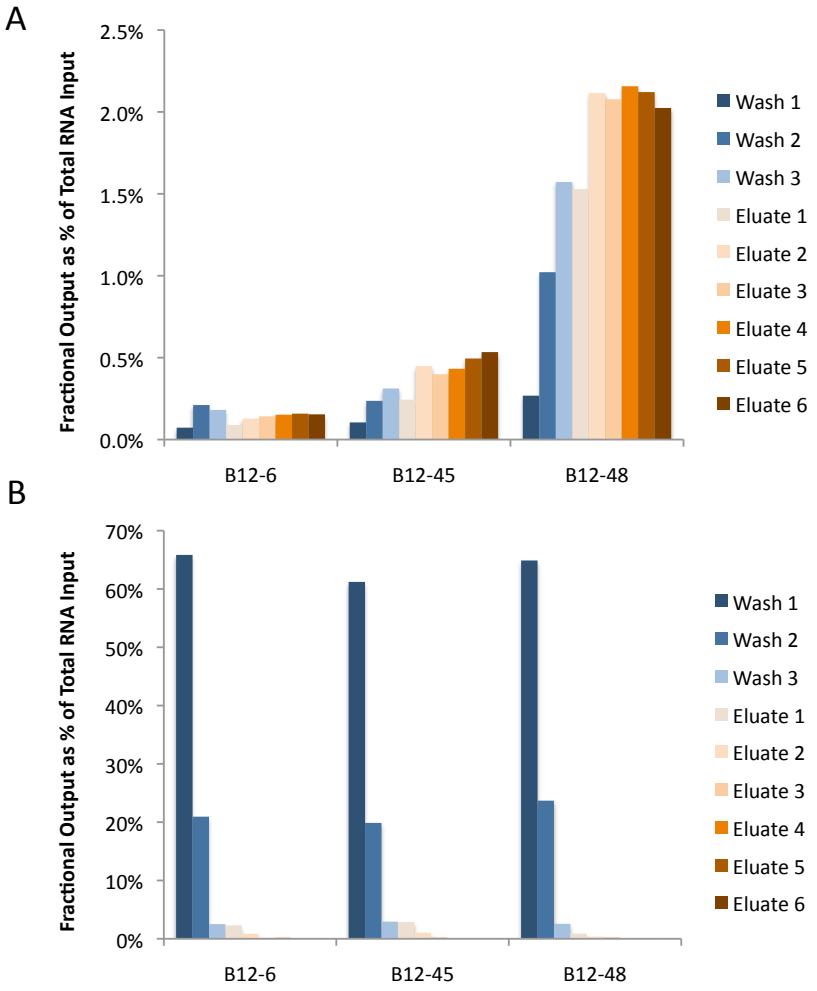

Figure 6.16. Putative vitamin B_{12} aptamer sequences show weak binding affinity for vitamin B_{12}, strong affinity for vitamin B_{12}–conjugated agarose, and no affinity for dihydrazide agarose. Radiolabeled, single-sequence RNAs were filtered through (A) vitamin B_{12}–conjugated or (B) adipic acid dihydrazide agarose columns. The columns were washed with selection buffer and eluted with 5 mM vitamin B_{12}. Each 500-µl aliquot of wash and eluate was collected and analyzed for radioactivity by scintillation counting. Values are reported as a percent of total radioactivity loaded onto each column. The B12-45 and B12-48 sequences show slight preferences for specific elution by vitamin B_{12} compared to selection buffer. All three sequences show strong binding affinity toward vitamin B_{12}–conjugated agarose as indicated by the small percentage of radioactivity removed from the B_{12}–conjugated column through wash and elution. The sequences show no affinity for dihydrazide agarose and are efficiently removed from those columns.

Eight additional sequences were evaluated by a modified radioactivity binding assay, in which alternate cycles of wash and elution steps were applied to vitamin B_{12}–conjugated agarose columns loaded with RNA. This alternating sequence was designed to rule out the possibility that any increase in RNA recovery during the elution steps was

simply due to non-specific removal by increased liquid volumes flowing through the column. None of the sequences evaluated by this method show specific elution by vitamin B_{12}, and all of them show strong binding affinity toward the column matrix (Table 6.1).

Table 6.1. Radioactive binding assay for putative vitamin B_{12} aptamer sequences with vitamin B_{12}–conjugated agarose columns

	% of Total RNA Input[a]							
Seq #	**2**	**4**	**7**	**12**	**15**	**19**	**23**	**44**
Wash 1[b]	0.06	0.08	0.24	0.05	0.09	0.91	0.18	0.12
Wash 2	0.20	0.22	0.67	0.22	0.22	N/A	0.39	0.34
Wash 3	0.17	0.20	1.20	0.22	0.23	1.30	0.72	0.40
Eluate 1	0.15	0.16	1.64	0.17	0.24	1.06	0.76	0.33
Eluate 2	0.14	0.14	2.07	0.15	0.26	0.80	0.87	0.32
Eluate 3	0.14	0.13	2.40	0.15	0.29	0.66	0.91	0.31
Wash 4	0.13	0.13	2.35	0.13	0.29	0.59	0.98	0.31
Wash 5	0.12	0.12	2.30	0.13	0.31	0.55	1.03	0.32
Eluate 4	0.13	0.13	2.40	0.13	0.36	0.51	1.12	0.34
Eluate 5	0.15	0.15	2.32	0.13	0.41	0.45	1.21	0.34
Total	**1.38**	**1.47**	**17.58**	**1.48**	**2.70**	**6.83**	**8.16**	**3.13**

[a]Value indicates the radioactivity of each wash or eluate fraction as a percentage of the total radioactivity loaded onto each column.
[b]Each wash and eluate fraction is 500 µl in volume.

Affinity toward agarose does not negate the potential usefulness of these putative aptamer sequences, provided they exhibit sufficient binding affinity for vitamin B_{12}. However, the minute amount of RNA recoverable from the vitamin B_{12}–conjugated agarose used in the selection process compromises the accuracy of these binding assays. To better characterize the isolated RNA sequences, we performed radioactive binding assays using an alternative separation medium—columns with Sepharose 6B resin particles that were either unmodified or conjugated to vitamin B_{12} through its hydroxyl group. The five unique sequences tested show varying degrees of non-specific binding to the Sepharose 6B resin, but all to a considerably lesser extent compared to the previous agarose resin (Table 6.2). However, the results also provide no evidence of specific

elution by vitamin B_{12}. These findings indicate that the SELEX procedure performed has failed to enrich for vitamin B_{12}–binding sequences.

Table 6.2. Radioactive binding assay for putative vitamin B_{12} aptamer sequences with Sepharose 6B columns

		% of Total RNA Input[a]	
RNA Sequence #	**Sample**	**Unmodified Sepharose 6B**	**Vitamin B_{12}–Conjugated Sepharose 6B**
5	**Wash**	77.92	66.79
	Eluate	0.35	0.28
6	**Wash**	67.74	54.96
	Eluate	0.20	0.20
9	**Wash**	78.04	67.09
	Eluate	0.17	0.27
45	**Wash**	50.92	45.85
	Eluate	0.13	0.16
48	**Wash**	69.57	66.81
	Eluate	0.13	0.13

[a]Value indicates the radioactivity of each wash or eluate fraction as a percentage of the total radioactivity loaded onto each column.

Column-Based SELEX Isolates Aptamer Sequences with Binding Affinity for Both Folinic Acid and Sepharose Matrix. We also performed column-based SELEX for RNA aptamers to folinic acid. Folinic acid is a 5-formyl derivative of folic acid and comprises 10%–20% of total cellular folates[20]. The 6S stereoisomer is biologically active and found in natural systems. Although not present in natural physiological environments, 6R–folinic acid is equally well tolerated *in vivo*, and racemic 6(R,S)–folinic acid is commonly used in cancer treatments either as an adjuvant in methotraxate therapy or in combination with 5-fluorouracil in the treatment of colorectal cancer and other neoplasms[20-22]. Pharmacokinetics studies have shown that 6R–folinic acid does not undergo metabolism and has a significantly longer serum half-life than 6S–folinic acid[23-25]. This combination of low toxicity, biological stability, and absence in natural systems makes 6R–folinic acid an ideal input signal for ligand-responsive regulatory systems.

Since racemic folinic acid is the most commonly used and thus widely available therapeutic agent, we began our aptamer selection process using the racemic mixture as the molecular target. Subsequent efforts to isolate aptamers specific to the 6R stereoisomer are described in a later section in this chapter.

The same selection procedure as described for vitamin B_{12} (Figure 6.14) was performed using racemic folinic acid as the target molecule. Folinic acid was conjugated through its carboxyl groups to EAH Sepharose 4B resin, and unmodified EAH Sepharose 4B resin was used for negative selection against the column matrix. The RNA pool obtained after 15 selection cycles was sequenced. In marked contrast to vitamin B_{12}, the folinic acid pool yielded only three unique sequences among 30 sequenced samples (Cycle 15 Sequence, Figure 6.17). One sequence (FA15.1) accounts for 93% (28 of 30) of the pool, and all three sequences share similar motifs. To better understand the sequence enrichment process, we sequenced RNA pools obtained after cycles 5, 10, and 11. Sequencing results indicate a diverse sequence pool after ten selection cycles, with no repeat observed among ten sequenced samples (Figure 6.17). Surprisingly, the dominant sequence makes its first appearance in cycle 11, after the mutagenic PCR step, accounting for two of ten sequenced samples. These results suggest that mutagenic PCR may have biased the sequence library, even though a similar effect was not observed in the vitamin B_{12} sequence pool.

ID	Cycle 5 Sequence	Frequency
FA5.30	*GGGAGCTAGCCAGG*CCTTCGGGTCTCGCCGGTCCCCTTCATCGT*GAGGCAAAGCTTCCG*	1 of 8
FA5.37	*GGGAGCTAGCCAGG*ATAATGGGATGGGGGCTCCGGTCGCTTGTT*GAGGCAAAGCTTCCG*	1 of 8
FA5.39	*GGGAGCTAGCCAGG*TCCACAAGGCTGTTTCCACTCTCGCGTGAT*GAGGCAAAGCTTCCG*	1 of 8
FA5.40	*GGGAGCTAGCCAGG*TGTACTGGCGTGGCTCGGCGGTTCTCGTGAT*GAGGCAAAGCTTCC G*	1 of 8
FA5.41	*GGGAGCTAGCCAGG*CGCCATAACCCAGCACCTCGGCTTACCCCT*GAGGCAAAGCTTCCG*	1 of 8
FA5.42	*GGGAGCTAGCCAGG*GTCTTGTTTAAGGACTTTCGGCTAGTTGTT*GAGGCAAAGCTTCCG*	1 of 8
FA5.43	*GGGAGCTAGCCAGG*CGAGATGCTTGCGCAGGCAACCACTTTGAT*GAGGCAAAGCTTCCG*	1 of 8
FA5.44	*GGGAGCTAGCCAGG*TTGAGCTGGCTAAACGATTGTGTAGGTGGT*GAGGCAAAGCTTCCG*	1 of 8

ID	Cycle 10 Sequence	Frequency
FA10.3	*GGGAGCTAGCCAGG*TTCCCTGACAGTTGCTTATAGTTCATTCTA*GAGGCAAAGCTTCCG*	1 of 10
FA10.5	*GGGAGCTAGCCAGG*CTCTCTGGTGCTTCCTTACTTGATTGTGAT*GAGGCAAAGCTTCCG*	1 of 10
FA10.6	*GGGAGCTAGC*----------TTGCATTAGAACTCGGTA*GAGGCAAAGCTTCCG*	1 of 10
FA10.7	*GGGAGCTAGCCAGG*ATGATGCTTGGGGAGCTCTGTCTCTGCTGT*GAGGCAAAGCTTCCG*	1 of 10
FA10.9	*GGGAGCTAGCCAGG*CTTCCTTAGTGCTTCGTGGATTTATGTGAT*GAGGCAAAGCTTCCG*	1 of 10
FA10.10	*GGGAGCTAGCCAGG*GTCTCTAGATTCTGTTACAGCGTTTATGTA*GAGGCAAAGCTTCCG*	1 of 10
FA10.11	*GGGAGCTAGCCAGG*TTGATACTCTAGCTCTTTAGTTGTCTTGAT*GAGGCAAAGCTTCCG*	1 of 10
FA10.12	*GGGAGCTAGCCAGG*TTACTAGTTAGCTCTGCGCTGAGATGTTAT*GAGGCAAAGCTTCCG*	1 of 10
FA10.13	*GGGAGCTAGCCAGG*TATTTAAGGGCTCCTTGTGTTCGTACATAT*GAGGCAAAGCTTCCG*	1 of 10
FA10.14	*GGGAGCTAGCCAGG*ATCTGGTCTTGGTGTTGCTCCACCTCGTGT*GAGGCAAAGCTTCCG*	1 of 10

ID	Cycle 11 Sequence	Frequency
FA11.3	***GGGAGCTAGCCAGG*AATGGTAGCTCGACTTCATGCTTCAGTGAT*GAGGCAAAGCTTCCG***	2 of 10
FA11.4	*GGGAGCTAGCCAGG*TGTTTCAGGCTAGATTCTTAACTCGTGCAT*GAGGCAAAGCTTCCG*	1 of 10
FA11.6	*GGGAGCTAGCCAGG*GCTCCGTCCTTTCCATTCTATTTTCGTGTA*GAGGCAAAGCTTCCG*	1 of 10
FA11.7	*GGGAGCTAGCCAGG*TTCCCGCTTCCACTCAGTCGATGTTGTGAT*GAGGCAAAGCTTCCG*	1 of 10
FA11.8	*GGGAGCTAGCCAGG*TTGGTGCAGGCTCCGTCCTTTGTCGGCTA*GAGGCAAAGCTTCCG*	1 of 10
FA11.16	*GGGAGCTAGCCAGG*AGACTGGTTAGACTCGTCCAACCCGTTGTA*GAGGCAAAGCTTCCG*	1 of 10
FA11.17	*GGGAGCTAGCCAGG*TATGGTTCTAGCTTCGGCTTCTGGATTGTA*GAGGCAAAGCTTCCG*	1 of 10
FA11.18	*GGGAGCTAGCCAGG*ACTTGGTTAGCGTCATAGTTTGCTGTAGTA*GAGGCAAAGCTTCCG*	1 of 10
FA11.21	*GGGAGCTAGCCAGG*TGTCGCACCTGCTAATCCCACTTGCTGCAT*GAGGCAAAGCTTCCG*	1 of 10

ID	Cycle 15 Sequence	Frequency
FA15.1	***GGGAGCTAGCCAGG*AATGGTAGCTCGACTTCATGCTTCAGTGAT*GAGGCAAAGCTTCCG***	28 of 30
FA15.5	*GGGAGCTAGCCAGG*AATTTGATAGCTCCGTTGGTGTTCTCATAT*GAGGCAAAGCTTCCG*	1 of 30
FA15.35	*GGGAGCTAGCCAGG*TTTCTGGTACTCCGTGTTCATGTCTCGTGT*GAGGCAAAGCTTCCG*	1 of 30

Figure 6.17. Sequence diversity in folinic acid SELEX is dramatically reduced after mutagenic PCR. RNA pools obtained after various selection cycles were cloned and sequenced. The sequence pools remained diverse through cycle 10, but one sequence, FA15.1 (red and bolded), began to emerge in cycle 11 and eventually dominated the pool after 15 cycles. Italicized nucleotides constitute the constant regions. The yellow and green highlights mark conserved motifs among the three sequences found in the cycle 15 pool.

A radioactive binding assay was performed to evaluate the binding affinity of the dominant FA15.1 sequence for both racemic and stereospecific isomers of folinic acid. Columns packed with EAH Sepharose 4B resin conjugated to racemic folinic acid were loaded with radiolabeled FA15.1 RNA and treated with alternate cycles of wash with selection buffer and elution with 5 mM racemic folinic acid, 6R–folinic acid, or 6S–folinic acid. Radioactivity measurement of each wash and eluate fraction indicates specific elution by folinic acid, with no preference for either stereoisomer (Figure 6.18).

Importantly, the sequence has a strong affinity for the Sepharose 4B resin, as more than 90% of the RNA input remain bound to the column after extensive wash and elution steps.

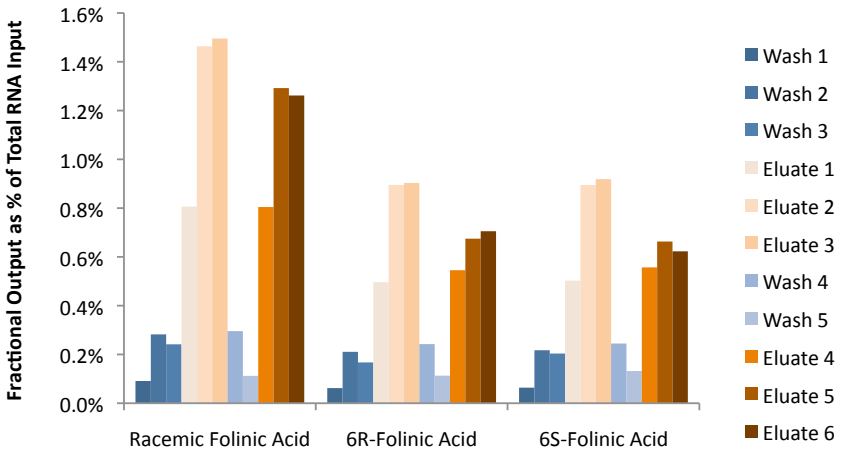

Figure 6.18. The putative folinic acid aptamer sequence shows folinic acid–specific binding, no stereospecific binding preference, and strong affinity for the column resin. The FA15.1 RNA sequence was loaded onto columns packed with resin conjugated to racemic folinic acid and washed with selection buffer. RNA elution was performed with 5 mM solutions of racemic folinic acid, 6R–folinic acid, or 6S–folinic acid. Radioactivity in each 500-µl aliquot of wash or eluate fraction was quantified by scintillation counting. Values are reported as a percent of total radioactivity loaded onto each column. The RNA sequence shows specific elution with all three ligands, with no preference for either stereoisomer. The great majority of RNAs remain bound to the column despite the large wash and elution volumes applied.

To circumvent challenges posed by the RNA sequence's affinity for Sepharose resin, we repeated the radioactivity binding assay using a vitamin B_{12}–conjugated agarose column. Over 98% of the RNA loaded onto the column remains bound after washing with three 500-µl aliquots of selection buffer, suggesting that the sequence also has non-specific affinity for the agarose resin (Figure 6.19). However, over 60% of the RNA is eluted from the column with three 500-µl aliquots of racemic folinic acid, indicating a significantly stronger affinity for folinic acid than for the column matrix. This difference in RNA elution is observed again in the second round of wash and elution steps,

confirming that elution with folinic acid is ligand-specific and not simply due to RNA being removed from the column by additional flow-through volumes.

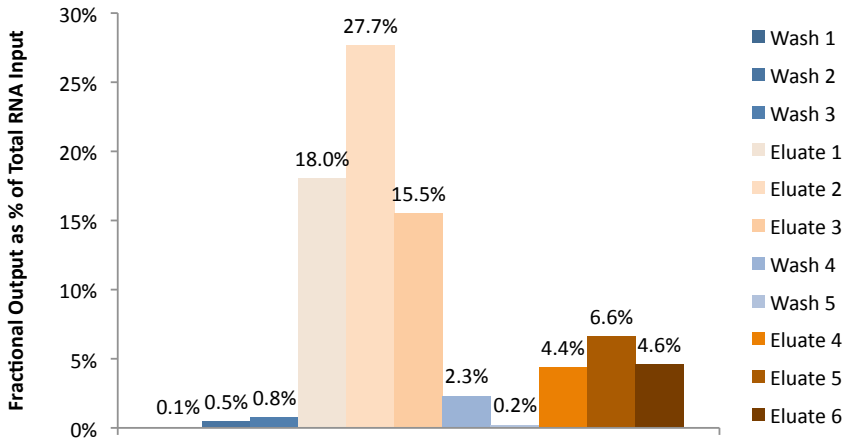

Figure 6.19. The putative folinic acid aptamer shows ligand-specific elution. The FA15.1 RNA sequence was loaded onto a vitamin B_{12}–conjugated agarose column and washed with selection buffer. RNA elution was performed with 5 mM racemic folinic acid, and radioactivity in each 500-µl aliquot of wash or eluate fraction was quantified by scintillation counting. Values are reported as a percent of total radioactivity loaded onto each column. The RNA shows specific elution with folinic acid and only weak affinity for the vitamin B_{12}–conjugated agarose resin.

The two remaining sequences found in the cycle 12 sequence pool were also characterized by the radioactive binding assay using Sepharose resin conjugated to racemic folinic acid. Results indicate that both sequences have strong affinity for the resin, and only one of the sequences (FA15.5) shows specific elution by folinic acid (Table 6.3). Since the dominant FA15.1 sequence shows greater elution by folinic acid and a slightly weaker affinity for the Sepharose resin compared to FA15.5, subsequent characterization efforts were focused on FA15.1.

Table 6.3. Radioactive binding assay for putative folinic acid aptamer sequences with folinic acid–conjugated and unmodified Sepharose 6B columns

	% of Total RNA Input[a]			
Sequence #	**FA15.5**		**FA15.35**	
Column	**Folinic Acid**	**Unmodified**	**Folinic Acid**	**Unmodified**
Wash 1[b]	0.03	0.00	0.00	0.03
Wash 2	0.09	0.00	0.01	0.09
Wash 3	0.11	0.01	0.03	0.10
Eluate 1	0.56	0.03	0.05	0.13
Wash 4	0.28	0.03	0.04	0.08
Wash 5	0.18	0.02	0.02	0.06
Wash 6	0.18	0.01	0.02	0.06
Wash 7	0.18	0.01	0.02	0.06
Eluate 2	1.06	0.03	0.05	0.12
Eluate 3	1.44	0.05	0.06	0.16
Eluate 4	1.40	0.04	0.05	0.15
Total	**5.51**	**0.24**	**0.35**	**1.04**

[a]Value indicates the radioactivity of each wash or eluate fraction as a percentage of the total radioactivity loaded onto each column.
[b]Each wash and eluate fraction is 500 µl in volume.

LC-MS Provides Qualitative Evaluation of Ligand-Binding Activity by Putative Aptamer Sequences. In light of the substantial affinity toward column resins exhibited by the putative aptamer sequences described above, binding assays utilizing alternative separation methods are desired. Various affinity assays have been devised for the evaluation of aptamers to small-molecule ligands[9, 11, 17, 26], but quantitative measurements of dissociation constants have generally been obtained through either surface plasmon resonance (SPR) analysis or methods employing radiolabeled ligand molecules. We have examined the use of SPR analysis in our efforts to characterize RNA aptamers. However, the protocol is still in the optimization stage as of this writing, and the results will be presented in later work. An alternative "equilibrium filtration" method that is simple and potentially effective in aptamer evaluation was presented by Jenison and colleagues in the characterization of the theophylline aptamer[9]. In this assay, known concentrations of aptamer RNA and radiolabeled theophylline are co-incubated and filtered through a

membrane-based size-exclusion column. Free theophylline is sufficiently small to pass through the membrane while RNA-bound theophylline is retained. Theophylline concentrations in the filtrate and the retentate are measured by scintillation counting, and their difference represents the concentration of RNA-bound theophylline. The K_D value is obtained by finding the RNA concentration at which 50% of the theophylline is bound.

Radiolabeled folinic acid and vitamin B_{12} are not commercially available, thus preventing the evaluation of putative aptamers to these ligands by the method described above. However, a slightly altered protocol may be performed using liquid chromatography (LC) combined with mass spectrometry (MS). LC-MS is a powerful tool for the quantitative analysis of chemical mixtures, and we applied this analytical tool to the evaluation of aptamer sequences. In this protocol, aptamer RNA is incubated with unlabeled ligand molecules at a known concentration and filtered through a size-exclusion column. Ligand concentration in the filtrate, which is the final free-ligand concentration, is quantified by LC-MS. The percentage of ligand bound is calculated from the known initial and measured final free-ligand concentrations. A range of RNA input concentrations is tested with a fixed folinic acid input concentration to generate a binding curve, from which the K_D value can be obtained by finding the RNA concentration at which 50% of the ligand molecules are bound.

This characterization method was first demonstrated using theophylline and its RNA aptamer, which has a reported K_D value of 0.1 μM[9]. RNA at concentrations ranging from 1.25 μM to 10 μM was incubated with 5 μM theophylline and processed as described above. Ligand concentration in the filtrate was calculated based on the intensity of the theophylline peak in the extracted ion chromatogram, which was calibrated to a

standard curve generated with pure theophylline solutions at known concentrations. The resulting binding curve is incomplete in that ligand binding does not reach saturation even at the highest RNA input concentration tested (Figure 6.20). However, the results show a clear ligand-RNA binding response, with approximately 50% of theophylline bound at RNA input concentrations between 1.5 μM and 2.5 μM. These observations suggest that the LC-MS method is able to reveal aptamer-binding activity, even though accurate quantification of the K_D value remains challenging.

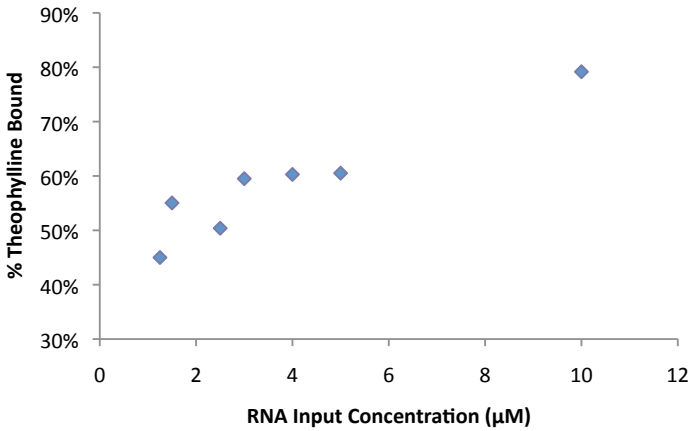

Figure 6.20. LC-MS and size-exclusion columns can be used to verify binding interactions between RNA aptamers and their cognate small-molecule ligands. The theophylline RNA aptamer was incubated with 5 μM theophylline and filtered through a size-exclusion column. Theophylline concentration in the filtrate was measured by LC-MS, and the percent of theophylline bound to RNA (and thus retained by the membrane) was calculated based on a one-to-one theophylline-to-RNA binding assumption.

We next examined the FA15.1 RNA sequence for binding affinity toward racemic folinic acid. RNA at concentrations ranging from 0 μM to 90 μM was incubated with 5 μM folinic acid and processed as described above. The resulting binding curve shows clear concentration-dependent binding between folinic acid and the RNA sequence, suggesting the FA15.1 sequence may have specific binding toward folinic acid (Figure 6.21). However, only 33% of the folinic acid was bound at the maximum RNA input concentration tested. Higher RNA input concentrations could not be achieved due to

limitations in *in vitro* transcription efficiency and RNA solubility, thus preventing accurate quantification of the K_D value by this method.

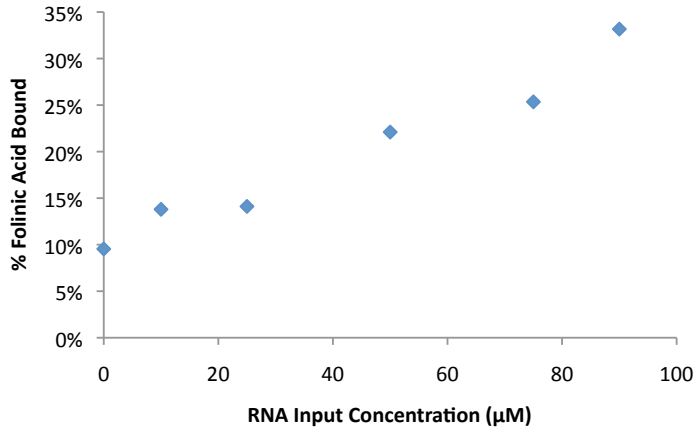

Figure 6.21. The putative folinic acid aptamer shows ligand-specific binding by LC-MS analysis. The FA15.1 RNA sequence was incubated with 5 μM racemic folinic acid, and the sample was processed and analyzed as described in Figure 6.20. Increasing RNA input concentration corresponds to increasing percent of folinic acid bound, indicating specific ligand-RNA binding interaction.

The observation that almost 10% of folinic acid was missing from the filtrate even in the absence of RNA input (Figure 6.21) suggests that the size-exclusion column retains some free folinic acid despite the molecule's being well below the molecular weight cutoff of the column membrane. One possible explanation is that the ligand concentration is above the carrying capacity of the membrane and causes membrane fouling. This hypothesis is supported at high folinic acid concentrations by the observation that yellow residues are retained on the column membrane for solutions at 500 mM and above. In addition, standard curves generated with folinic acid that has been filtered through the column in the absence of RNA deviate from linearity at concentrations beyond 100 μM (Figure 6.22A). In comparison, standard curves generated with unfiltered folinic acid remain linear up to 400 μM (Figure 6.22B). Even unfiltered folinic acid deviates from the linear trend line beyond 400 μM, indicating a limited concentration range that can be

accurately quantified by the LC-MS system. This constraint in the LC-MS detection range can be circumvented by careful sample dilution. However, limits in the folinic acid concentration that can be properly filtered by column membrane undermines the feasibility of an alternative binding assay in which a fixed RNA input concentration is paired with varying folinic acid input concentrations.

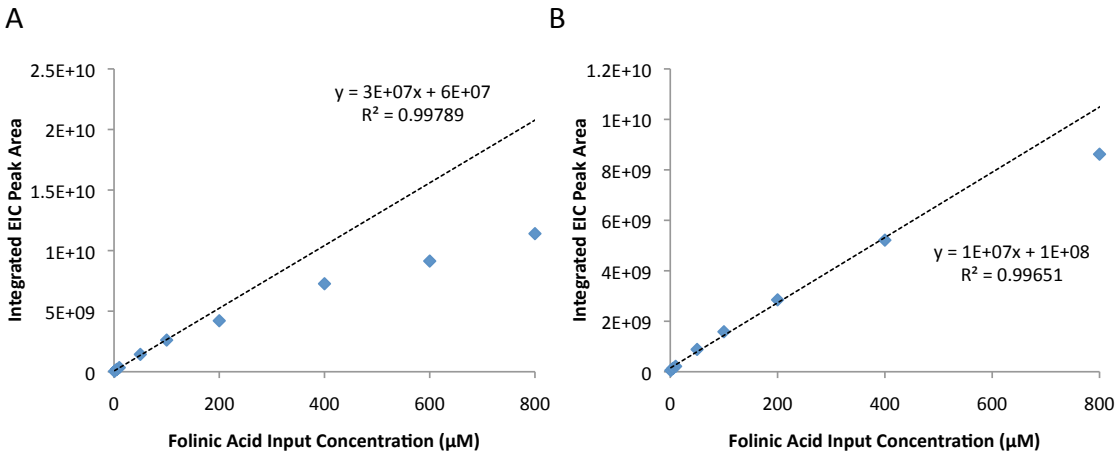

Figure 6.22. LC-MS binding assays cannot be performed at high folinic acid concentrations due to errors associated with inefficient membrane filtration. LC-MS analysis was performed on racemic folinic acid solutions that are either (A) filtered through size-exclusion columns or (B) unfiltered. The integrated peak areas of extracted ion chromatograms at a molecular weight of 474 are shown. Filtered samples with concentrations above 100 μM deviate from a linear relationship between detected signal strength and folinic acid input concentration, whereas unfiltered samples remain linear through 400 μM. The difference between filtered and unfiltered samples indicates errors introduced by the membrane filtration step.

Although the binding curve shown in Figure 6.21 suggests ligand-specific binding by the putative folinic acid aptamer, it does not rule out non-specific binding between folinic acid and RNA in general. To evaluate this possibility, the binding assay was repeated using the theophylline RNA aptamer instead of the FA15.1 RNA. Results indicate that there is nonspecific loss of folinic acid from the filtrate with increasing RNA input concentration (Theo Aptamer, Figure 6.23), either due to non-specific RNA-folinic acid interaction or due to concentrated RNA contributing to membrane fouling. Nevertheless, the extent of folinic acid "binding" observed with the theophylline aptamer

is significantly less than that with the FA15.1 RNA, suggesting that the FA15.1 sequence has specific affinity for folinic acid.

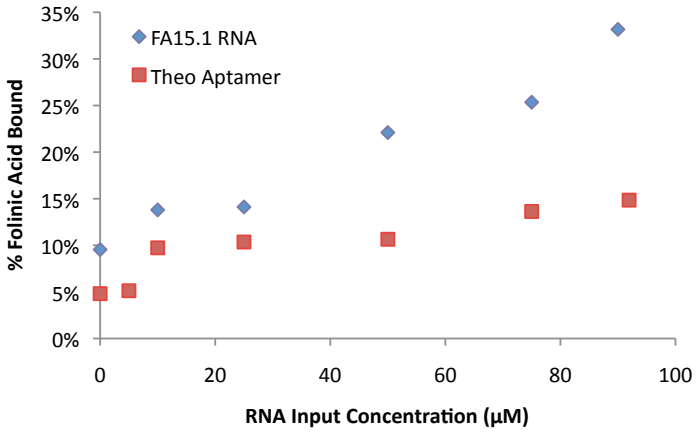

Figure 6.23. Folinic acid binding to the putative folinic acid aptamer is specific to the RNA sequence. The FA15.1 RNA sequence and the theophylline RNA aptamer were each incubated with 5 μM racemic folinic acid, and the samples were processed and analyzed as described in Figure 6.20. Folinic acid shows weak non-specific binding to the theophylline aptamer, but at noticeably lower levels than to the FA15.1 RNA, suggesting that the latter binding interaction is sequence specific.

Selection for Stereoisomer-Specific Folinic Acid Aptamer Results in Diverse Sequence Pool. As described previously, 6R–folinic acid is an unnatural stereoisomer of folinic acid whose low toxicity, relatively long serum half-life, and absence in natural systems make it an ideal molecular input for ligand-responsive gene regulatory systems. To isolate RNA aptamers with binding specificity for 6R–folinic acid, we used the RNA pool obtained after cycle 10 of the racemic folinic acid aptamer selection as the starting material. This choice was made for two reasons. First, negative selection against chemically similar ligands are typically performed after several cycles of positive selection in order to prevent premature elimination of sequence diversity prior to enrichment of ligand-binding sequences. Therefore, cycle 10 is a reasonable point to begin stereospecific selection for the 6R isoform of folinic acid. Second, sequencing results from the racemic folinic acid aptamer selection suggest that the mutagenic PCR

step performed after cycle 10 in that selection process may have biased the sequence pool. Therefore, it is of interest to repeat the selection without the mutagenic PCR step to determine whether sequence diversity would follow the same pattern as observed in the previous selection. In light of the observation that all isolated sequences from the previous selection show significant binding affinity toward the Sepharose 4B matrix, we performed negative selection against unmodified Sepharose 4B resins in every cycle prior to positive selection using a 6R–folinic acid–conjugated Sepharose 4B column.

In this selection process, RNA was filtered through an unmodified Sepharose column and the unbound fraction was collected and loaded onto a 6R–folinic acid–conjugated column. The column was washed with 4 CV of selection buffer followed by 5 CV of 5 mM 6S–folinic acid and another 10 CV of buffer to select against the natural stereoisomer. The column was eluted with 7 CV of 5 mM 6R–folinic acid, and the eluted RNA was prepared as previously described for the next selection cycle. We performed five rounds of selection and sequenced the RNA pool obtained after the last cycle. Results indicate a diverse sequence pool with no repeats found among 21 sequenced samples (Figure 6.24A). FA15.1, the dominant sequence isolated from the racemic folinic acid selection, is present in the 6R–folinic acid pool (as 6RFA15.55) but shows no obvious relation with the remaining sequences in the pool. Short sequence motifs were identified among the sequenced samples, but most contain slight variations among sequences and none are truly conserved in more than two sequences (Figure 6.24B). As of this writing, characterization of these isolated sequences awaits results from ongoing efforts in the development of more accurate, high-throughput assays for ligand-RNA binding affinity.

A

ID	Cycle 15 Sequence	Frequency
6RFA15.1	*GGGAGCTAGCCAGG*CTCCGCACAAATGTATCCTTACATCGTGTA*GAGGCAAAGCTTCCG*	1 of 21
6RFA15.45	*GGGAGCTAGCCAGGG*TTAGCTTCAGTGAGTTTCTTATTGTTGTC*GAGGCAAAGCTTCCG*	1 of 21
6RFA15.48	*GGGAGCTAGCCAGG*CTTCCCTGCTAACTGTATCTCTCCGGTATA*GAGGCAAAGCTTCCG*	1 of 21
6RFA15.49	*GGGAGCTAGCCAGG*CTCCTCCTGTTGACTGTTCATTCAGTTGGC*GAGGCAAAGCTTCCG*	1 of 21
6RFA15.50	*GGGAGCTAGCCAGG*CTCTTGCTAGTTCCGCGCTATCTTGTGGTT*GAGGCAAAGCTTCCG*	1 of 21
6RFA15.52	*GGGAGCTAGCCAGG*ATTATCCCGGTGTCTCCTATCTTGCGTTAT*GAGGCAAAGCTTCCG*	1 of 21
6RFA15.53	*GGGAGCTAGCCAGGG*CGCATAAAATTTTAATCGCAAACTTATAT*GAGGCAAAGCTTCCG*	1 of 21
6RFA15.55	***GGGAGCTAGCCAGGAATGGTAGCTCGACTTCATGCTTCAGTGAT*GAGGCAAAGCTTCCG**	1 of 21
6RFA15.57	*GGGAGCTAGCCAGG*ATTTGCTTGGCGAACCCCGATACCTTTGTA*GAGGCAAAGCTTCCG*	1 of 21
6RFA15.66	*GGGAGCTAGCCAGG*TCCGGCAACAGGCCCTGGCCTAAATTCGAT*GAGGCAAAGCTTCCG*	1 of 21
6RFA15.67	*GGGAGCTAGCCAGG*TATTCCTCTTGGCTAACCCGTTGTCTCTAT*GAGGCAAAGCTTCCG*	1 of 21
6RFA15.68	*GGGAGCTAGCCAGG*TTGGACAAGCCCCAACGATTAATCTAGGAA*GAGGCAAAGCTTCCG*	1 of 21
6RFA15.69	*GGGAGCTAGCCAGG*TTGTGCTGTAGCTCACCACTCAACATGTAT*GAGGCAAAGCTTCCG*	1 of 21
6RFA15.70	*GGGAGCTAGCCAGG*TGTTGTTGCTGCTCCGATGTCGTACTTCAT*GAGGCAAAGCTTCCG*	1 of 21
6RFA15.72	*GGGAGCTAGCCAGG*CTCCGTGAGTGCTTTTACAAGTGGTTGTGT*GAGGCAAAGCTTCCG*	1 of 21
6RFA15.74	*GGGAGCTAGCCAGG*TAAAAGGTTACGGACACGCCGAGGGTCAGG*GAGGCAAAGCTTCCG*	1 of 21
6RFA15.77	*GGGAGCTAGCCAGG*TCTTTGGCGGCTCGCGAGTGTCTTCTAGGT*GAGGCAAAGCTTCCG*	1 of 21
6RFA15.80	*GGGAGCTAGCCAGG*TATGGCTAGGGGCTCTCGGTCTCTGAGTAT*GAGGCAAAGCTTCCG*	1 of 21
6RFA15.84	*GGGAGCTAGCCAGG*CTACTCACGATAACTGGTTCGCAACCTTGT*GAGGCAAAGCTTCCG*	1 of 21
6RFA15.85	*GGGAGCTAGCCAGG*CTCCTCATATTTGGCTTTCAGATGTGTA*GAGGCAAAGCTTCCG*	1 of 21
6RFA15.88	GGGAGCTAGCGAACGCCAGTTTCAGTTGTGTT*GAGGCAAAGCTTCCG*	1 of 21

B

6RFA15.84
>CTACTCACGATAACTGGTTCGCAACCTTGT<
6RFA15.53
>GCGCATAAAATTTTAATCGCAAACTTATAT<
6RFA15.1
>CTCCGCACAAATGTATCCTTACATCGTGTA<

6RFA15.48
>CTTCCCTGCTAACTGTATCTCTCCGGTATA<
6RFA15.67
>TATTCCTCTTGGCTAACCCGTTGTCTCTAT<

6RFA15.1
>CTCCGCACAAATGTATCCTTACATCGTGTA<
6RFA15.72
>CTCCGTGAGTGCTTTTACAAGTGGTTGTGT<
6RFA15.49
>CTCCTCCTGTTGACTGTTCATTCAGTTGGC<
6RFA15.45
>GTTAGCTTCAGTGAGTTTCTTATTGTTGTC<

Figure 6.24. 6R–folinic acid SELEX yields a diverse sequence pool with conserved motifs. (A) The sequence pool remains diverse after 10 selection cycles toward racemic folinic acid and 5 subsequent cycles toward 6R–folinic acid. The RNA pool was cloned and sequenced. Results indicate a diverse sequence pool, even though the dominant sequence found in the racemic folinic acid selection (FA15.1) also appears in the 6R–folinic acid pool (6RFA15.55, red and bolded). (B) No sequence appears more than once in the pool, but similar motifs can be found among the diverse sequences. Italicized nucleotides constitute the constant regions. Colored highlights mark motifs found within each group of sequences.

Discussion

RNA aptamers constitute the sensor domain of most existing ligand-responsive RNA-based regulatory devices, including the switch systems presented in this thesis. Our efforts in selecting new RNA aptamers toward therapeutically applicable small molecules have met with several challenges, leading to a close examination of different selection protocols and the potential pitfalls associated with each separation method. Studies

detailed in this chapter show that nitrocellulose membranes, though widely used for aptamer selection to protein targets, have surprisingly low partition efficiencies. Specifically, nitrocellulose membranes cannot effectively retain even high-affinity RNA aptamers in the presence of their cognate protein targets, thus undermining the usefulness of nitrocellulose membranes as a separation method in aptamer selection.

An alternative selection method employs column chromatography, which requires target immobilization on resin particles. However, many small molecules suitable for clinical applications lack appropriate functional groups for resin conjugation. Although synthetic chemistry has been used to generate modified small molecules for resin conjugation in some instances[9, 27], such modifications are not always possible and may affect the binding specificity of the aptamer sequences isolated from such selection processes. In collaboration with the Chemistry Core Facility at City of Hope, we have examined the possibility of chemically modifying phenobarbital for resin conjugation, but our efforts were unsuccessful due to phenobarbital's compact structure. Furthermore, any modification to phenobarbital would have represented a significant change to its chemical makeup, and aptamer sequences isolated for such modified molecules may not have the same binding affinity for the intended target.

Another challenge associated with both membrane- and column-based selections is the enrichment of sequences with high affinity for the solid support itself. We repeatedly isolated sequences that bind strongly to either nitrocellulose or the column resin used for separation. These findings indicate that stringent negative selections against the solid support must be applied throughout the selection process. Combining affinity elution with membrane filtration was effective in preventing the enrichment of

membrane-binding sequences, but it is unclear whether the hybrid method is effective for aptamer selection. The ability to use more than one separation method in a selection process (e.g., alternating between membrane- and column-based selection for each cycle) would shift the selection bias away from sequences with strong binding affinity for a specific solid support. Alternative selection methods that do not require solid supports, including the use of capillary electrophoresis[28-30] and direct ribozyme switch isolation through *in vitro* and *in vivo* screening, are also being explored in the Smolke Laboratory.

Our aptamer selection efforts yielded diverse pools of potential aptamers for vitamin B_{12} and 6R–folinic acid. The binding characterization assays developed thus far—including radioactive binding assays and LC-MS analyses—have been qualitative and low throughput, making them unsuitable for screening through large sequence pools. Development of more quantitative and higher throughput methods, including the use of SPR-based technology such as Biacore (GE Healthcare), is ongoing and will be applied to the sequence pools obtained from these selection processes.

The racemic folinic acid selection yielded a dominant sequence that shows specific binding to its intended target by both radioactive binding assays and LC-MS analyses. However, initial characterizations by Biacore have shown no quantitative evidence for binding between the putative aptamer and racemic folinic acid (J. C. Liang, personal communication). The putative aptamer sequence has been integrated into the ribozyme switch platform and tested both by *in vitro* cleavage assays and in yeast cultures, but no functional switch construct has been developed thus far (J. C. Liang and M. N. Win, personal communication). The ribozyme switches may be improved by randomizing the transmitter domain and performing a library screen for effective switch

activity. Furthermore, the putative aptamer sequence will have to be characterized in greater detail as more quantitative evaluation methods become available.

SELEX technology was first developed in 1990 and has been used widely for the *de novo* generation of diverse DNA and RNA aptamer sequences. However, no aptamer with *in vivo* activity and specificity for clinically suitable ligand molecules has been isolated thus far. Our attempts at selecting novel RNA aptamers toward small-molecule targets have generated mixed results but also provided insights into the optimization of various selection parameters. Informed by past challenges, ongoing efforts in the Smolke Laboratory continue to explore the intricacies of aptamer selection, with the aim of developing more efficient and reliable methods for the isolation of new aptamers.

Materials and Methods

Nitrocellulose membrane-based SELEX for phenobarbital aptamer. A N30 DNA library was generated by PCR using forward primer N30 Fwd (5'TTC*TAATACGACTCA CTATA*GGGAGCTAGCCAGG), reverse primer N30 Rev (5'CGGAAGCTTTGCCTC), and the N30 Template (5' TTCTAATACGACTCACTATAGGGAGCTAGCCAGG (N30) GAGGCAAAGCTTCCG), where the italicized sequence denotes the T7 promoter and underlined sequences denote the restriction sites NheI and HindIII used for cloning. All oligonucleotides were synthesized by Integrated DNA Technologies. The initial PCR amplification was performed with 20 pmol of the N30 Template, corresponding to approximately 1.2×10^{13} unique sequences, assuming complete randomization in the oligonucleotide synthesis process. A N30 RNA library was generated by *in vitro* transcription using the N30 DNA library as template. The reaction was performed with

40 mM Tris-HCl, pH 7.9, 16 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 2 mM spermidine, 3 mM of ATP, CTP, and UTP (Epicentre), 300 μM of GTP (Invitrogen), 10 μCi of [α-^{32}P]-GTP (GE Healthcare), 50 U of T7 RNA polymerase (New England Biolabs), and 40 U of RNaseOUT ribonuclease inhibitor (Invitrogen) and incubated at 37°C overnight. The transcription product was treated with 2 U of DNaseI (New England Biolabs) at 37°C for 15 min and purified with a NucAway column (Ambion) hydrated with selection buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 750 μM $MgCl_2$). RNA was denatured by heating to 75°C for 3 min and allowed to renature at room temperature for 30 min.

Membrane filtration was performed using a Multiscreen$_{HTS}$ Vacuum Manifold (Millipore) with a 96-well Multiscreen$_{HTS}$-HA filter plate (Millipore). In all filtration steps, the membrane wells were pre-wetted by incubation with 100 μl of selection buffer for 1 min before buffer removal by vacuum filtration at a pressure of 5 in. Hg. In cycle 1, the N30 RNA library was filtered through a membrane well and the filtrates were collected for incubation with 75 pmol of phenobarbital-BSA (Meridian Life Science) at 37°C for 30 min. The binding reaction was filtered through a new membrane well, and the membrane was washed with 800 μl of selection buffer. The membrane was removed from the plate and soaked in 600 μl of Tris-buffered phenol-chloroform, pH 7.9, at room temperature for 30 min. RNA was extracted by adding 200 μl of RNase-free water and collecting the aqueous layer. A second extraction step was performed with phenol-chloroform before RNA precipitation with 2 volumes of 95% ethanol, 0.1 volumes of 3 M sodium acetate, and 0.1 mg/ml glycogen (Ambion) followed by a wash step with 500 μl of 70% ethanol. Reverse transcription and cDNA amplification was performed in a single step with a 50-μl RT-PCR reaction containing 20 mM Tris-HCl, pH8.4, 50 mM

KCl, 5 mM DTT, 200 μM dNTP, 0.2 μM each of the N30 Fwd and N30 Rev primers, 40 U of RNaseOUT, 200 U of SuperScriptIII reverse transcriptase (Invitrogen), and 5 U of *Taq* DNA polymerase. The reaction was incubated at 55°C for 35 min (for reverse transcription) and immediately subjected to 15 cycles of PCR amplification. The cDNA product was used for transcription in the next selection cycle.

The process was repeated as described for 20 selection cycles with the following changes. First, negative selection against nitrocellulose membrane was not performed beyond cycle 1. Second, starting in cycle 3, transcriptions were performed with 3 mM of each rNTP with no radioactivity, and the reaction was allowed to proceed at 37°C for 2 hours. RNA concentration was quantified using a Nanodrop spectrophotometer, and the amount of phenobarbital-BSA added to the binding reaction was adjusted accordingly to achieve the desired ligand-to-RNA ratio. The phenobarbital-BSA stock used contains 12–15 phenobarbital molecules per BSA molecule, and the BSA:RNA ratio used in each cycle is summarized in the table shown on the following page. Third, the ligand-RNA incubation time was gradually decreased and the volume of selection buffer filtered through the membrane during the wash step gradually increased through the selection cycles to increase selection stringency (see table on next page). Fourth, negative selection against unconjugated BSA was performed prior to positive selection with phenobarbital-BSA in cycles 11-13 and 15-20. In these negative selections, renatured RNA was first incubated with unconjugated BSA (New England Biolabs) at a BSA:RNA ratio of 1:10 at 37°C for 10 min. The binding mixture was filtered through a membrane well, and the filtrate was collected for incubation with phenobarbital-BSA as in a typical selection cycle.

Cycle #	Negative Selection	BSA:RNA Ratio (for positive selection)[a]	Binding Incubation Time (min)	Wash Volume (ml)
1	Nitrocellulose	1:1	30	0.8
2	None	1:1	30	0.8
3	None	1:1	30	0.8
4	None	1:10	30	0.8
5	None	1:1	30	0.8
6	None	1:10	15	1.2
7	None	1:10	15	1.6
8	None	1:20	15	2.0
9	None	1:50	15	2.0
10	None	1:100	15	2.0
11	BSA	1:10	15	2.0
12	BSA	1:10	15	2.0
13	BSA	1:10	15	2.0
14	None	1:100	10	2.0
15	BSA	1:100	10	2.0
16	BSA	1:133	5	4.0
17	BSA	1:100	5	4.0
18	BSA	1:100	5	4.0
19	BSA	1:100	5	4.0
20	BSA	1:100	5	4.0

Nitrocellulose membrane-based SELEX for phenobarbital aptamer with ligand-specific elution. A N30 DNA library was generated by PCR as described above using 200 pmol of template input for a total of approximately 1.2 x 10^{14} unique sequences. Overnight radioactive RNA transcription was performed for cycle 1 as described previously, and all subsequent transcriptions were performed at 37°C for 2 hours without radiolabeling. The selection buffer composition was changed to 20 mM Tris-HCl, Ph7.5, 100 mM NaCl, and 1 mM $MgCl_2$ for this selection process. Twelve selection cycles were performed as described using phenobarbital-BSA, with the BSA:RNA ratio fixed at 11:1 throughout the selection. The binding reaction was performed at 37°C for 45 min in cycles 1–5 and for 30 min in cycles 6–12. Wash volume was maintained at 1 ml for cycles 1–9 and increased to 2 ml for cycles 10–12. Negative selection against BSA was

performed with a BSA:RNA ratio of 1:1 before positive selection with phenobarbital-BSA in cycles 8–12. No explicit negative selection against nitrocellulose was performed.

Nitrocellulose membrane-based radioactive binding assay. Radioactive RNA was transcribed, purified, denatured, and renatured as described above. Radiolabeled RNA was incubated with either no protein or the specified protein target at 37°C for 10 min. The binding mixture was filtered through a membrane well as described above, and the membrane was washed with selection buffer or eluted with concentrated ligand solutions as specified by the experiment. Collected samples (either liquid filtrate or washed nitrocellulose membrane) were added to 5 ml of Safety-Solve scintillation liquid (Research Products International) and analyzed using a liquid scintillation counter (Beckman Coulter).

Column-based SELEX for vitamin B_{12}. Chromatography matrices were prepared by packing 850 μl of vitamin B_{12}–agarose (Sigma) or adipic acid dihydrazide-agarose (Sigma) into columns with a column volume (CV) of approximate 500 μl following manufacturer's protocol (Pierce). The packed columns were washed with 25 ml of RNase-free water and 10 ml of selection buffer and wrapped in foil (to prevent photo-degradation of vitamin B_{12}) prior to use. A N30 DNA library was generated by PCR as described above using 400 pmol of template input for a total of approximately 2.4×10^{14} unique sequences. The initial N30 RNA library was generated by a ten 100-μl transcription reactions each containing 40 mM Tris-HCl, pH 7.9, 16 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine, 25 mM of each rNTP, 200 U of T7 RNA polymerase, and 120 U

of RNaseOUT ribonuclease inhibitor and incubated at 37°C for 2 hours. Each 100 μl of transcription product was treated with 4 U of DNaseI at 37°C for 1 hour and purified with a NucAway column hydrated with selection buffer (20 mM Tris-HCl, pH7.5, 250 mM NaCl, 2.5 mM $MgCl_2$).

The resulting RNA was split into two equal aliquots: one for vitamin B_{12} selection and the other for folinic acid selection. The RNA was loaded onto the adipic acid dihydrazide-agarose column, incubated for 20 min, and eluted with selection buffer. This negative selection against unmodified column matrix was performed in cycles 1 through 3. The unbound fraction was subsequently loaded onto the vitamin B_{12}–agarose column, incubated for 20 min, and washed with 5 CV of selection buffer. The column was incubated with 5 mM vitamin B_{12} (Sigma) dissolved in selection buffer for 30 min and the RNA was eluted in a total of 7 CV of 5 mM vitamin B_{12}. The entire column chromatography procedure was performed at room temperature. Eluted RNA was precipitated with 2 volumes of 95% ethanol, 0.1 volumes of 3 M sodium acetate, and 10 μg/ml of glycogen followed by a wash step with 500 μl of 70% ethanol. Reverse transcription and cDNA amplification was performed in a single step with a 100-μl RT-PCR reaction containing 20 mM Tris-HCl, pH8.4, 50 mM KCl, 0.1 M DTT, 200 μM dNTP, 0.2 μM each of the N30 Fwd and N30 Rev primers, 80 U of RNaseOUT, 400 U of SuperScriptIII reverse transcriptase, and 10 U of *Taq* DNA polymerase. The reaction was incubated at 55°C for 35 min (for reverse transcription) and immediately subjected to 10 cycles of PCR amplification. Transcription for subsequent cycles was performed as described but in one 50-μl reaction for each cycle using cDNA from the previous cycle as template.

Fifteen selection cycles were performed, with the wash volume increasing from 5 CV to 10 CV in cycle 4. The wash volume was subsequently increased in 10-CV intervals from cycle 5 to cycle 10, then in 20-CV intervals from cycle 10 to cycle 15. The cDNA obtained after cycle 10 served as template for a 15-cycle, 100-μl mutagenic PCR reaction containing 10 mM Tris-HCl, pH8.3, 50 mM KCl, 7 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.4 μM each of the N30 Fwd and N30 Rev primers, 0.2 mM of dATP and dGTP, 1 mM of dTTP and dCTP, and 10 U of *Taq* DNA polymerase. This PCR product was used as the template for RNA transcription in cycle 11 of the selection process.

Column-based SELEX for racemic folinic acid. EAH Sepharose 4B resin (GE Healthcare) washed with water at pH 4.5 and 0.5 M NaCl before overnight incubation with 0.1 M EDAC (Invitrogen) and 5 mM racemic folinic acid (Sigma) at room temperature in the dark on a rotating rack. Unreacted functional groups on the resin were blocked by incubation with 1 M glacial acetic acid at room temperature for 3 hours. The ligand-conjugated resin was packed into columns with a column volume of approximate 500 μl following manufacturer's instructions. Three wash cycles each consisting of 10 CV of low pH salt solution (0.1 M sodium acetate, pH 4.0, 0.5 M NaCl) followed by 10 CV of high pH salt solution (0.1 M Tris-HCl, pH 8.3, 0.5 M NaCl) were performed on the packed columns to remove unconjugated folinic acid that may have remained in the column through weak ionic interactions. Columns were then washed with 20 CV of selection buffer and wrapped in foil (to prevent photo-degradation of folinic acid) prior to use. Unmodified EAH Sepharose 4B resin was washed with water and NaCl as described above and used to pack unmodified columns used for negative selections. Packed

columns were washed only with selection buffer prior to use. The selection process was performed exactly as described for vitamin B_{12}, except racemic folinic acid was used instead of vitamin B_{12}.

Column-based SELEX for 6R–folinic acid. EAH Sepharose 4B resin was conjugated to 5 mM 6R–folinic acid (Schircks Laboratories) and used to pack columns as described for racemic folinic acid. cDNA obtained after cycle 10 of the racemic folinic acid aptamer selection was used as template for *in vitro* transcription to generate the starting RNA pool for this selection. In each of 5 selection cycles, RNA was first loaded unto an unmodified Sepharose 4B column, incubated for 20 min, and eluted with selection buffer. The unbound fraction was subsequently loaded onto the 6R–folinic acid column, incubated for 20 min, and washed with 5 CV of selection buffer followed by 5 CV of 5 mM 6S–folinic acid (Schircks Laboratories) and another 10 CV of selection buffer. The column was incubated with 5 mM 6R–folinic acid dissolved in selection buffer for 30 min and the RNA was eluted in a total of 7 CV of 5 mM 6R–folinic acid. The entire column chromatography procedure was performed at room temperature, and the eluted RNA was precipitated and reverse transcribed as described for the vitamin B_{12} selection process.

Column-based radioactive binding assay. RNA was transcribed in a 20-μl reaction using the AmpliScribe T7 High Yield Transcription Kit (Epicentre) following manufacturer's protocol, except only 750 μM of GTP was used and 10 μCi of [α-^{32}P]-GTP was added to generate radiolabeled RNA. The transcription product was treated with 2 U of DNaseI at 37°C for 1 hour and purified with a NucAway column hydrated

with selection buffer. RNA was loaded onto the appropriate column as specified by the experiment, incubated at room temperature for 20 min, and the unbound fraction was collected. The column was subsequently washed with selection buffer or eluted with dissolved ligand as specified by the experiment. Collected samples were added to 5 ml of Safety-Solve scintillation liquid and analyzed using a liquid scintillation counter.

Sequence and structural analyses. RNA pools from the specified selection cycles were reverse transcribed into cDNA and amplified by PCR as described. The PCR products were digested with the NheI and HindIII restriction enzymes and cloned into pcDNA3.1(+) (Invitrogen) for sequencing. Sequence results were aligned with the Clustal X software (University College Dublin) to assist with identifying sequence motifs. RNA folding was determined using the RNAstructure software (University of Rochester Medical Center), and structural motifs were identified by manual sorting.

Capillary electrophoresis binding assay. DNA templates encoding the SV12.6, SVC12.60, and SVC12.70 sequences were synthesized by Integrated DNA Technologies and PCR amplified using the N30 Fwd and N30 Rev primers. RNA transcription was performed in 20-μl reactions using the AmpliScribe T7 High Yield Transcription Kit following manufacturer's protocol. The transcription product was treated with 2 U of DNaseI at 37°C for 1 hour and purified with a NucAway column hydrated with RNase-free water. For RNA-only samples, RNA was diluted to 100 ng/μl (5.24 μM) with selection buffer (20 mM Tris-HCl, Ph7.5, 100 mM NaCl, 1 mM $MgCl_2$). For ligand-binding reactions, 2 μg of RNA was diluted to a total volume of 17.72 μl with selection

buffer, denatured in a PCR block at 75°C for 3 min, renatured by cooling to 25°C at 0.3°C/sec, and added to 2.28 μl of 45.81 μM phenobarbital-BSA to produce a 20-μl mixture with a final concentration of 5.24 μM RNA and 5.24 μM phenobarbital-BSA (molar concentration for BSA).

Capillary electrophoresis was performed with a P/ACE MDQ Capillary Electrophoresis System (Beckman Coulter) using a photodiode array (PDA) detector. For each run, 110 nl of sample was injected at 2 psi over 8 sec, and separation was performed at 22.5 kV over 14 min. The capillary was filled with run buffer (25 mM sodium tetraborate, pH 9.1) prior to each sample injection. Capillary regeneration was performed by rinsing with 0.1 M HCl, followed by 0.1 M NaOH, water, and finally the run buffer. Each rinse step was performed for 2 min at 20 psi. Elution profiles were analyzed using the 32Karat software (Beckman Coulter).

LC-MS binding assay. RNA was transcribed in five 100-μl reactions using the AmpliScribe T7 High Yield Transcription Kit following manufacturer's protocol. Each 100-μl transcription product was treated with 20 U of DNaseI at 37°C for 1 hour and purified with a NucAway column hydrated with RNase-Free water. The eluted RNA was extracted with phenol-chloroform and precipitated with 2 volumes of 95% ethanol and 0.1 volume of 3 M sodium acetate followed by a wash step with 500 μl of 70% ethanol. The precipitated RNA was resuspended in 100 μl of selection buffer (20 mM Tris-HCl, pH7.5, 250 mM NaCl, 2.5 mM $MgCl_2$), denatured at 75°C for 3 min, and renatured at room temperature for 30 min. Binding reactions were set up with the RNA and ligand (folinic acid or theophylline) concentration specified by the experiment. The binding

mixture was incubated at 37°C for 30 min and loaded onto a Microcon YM-10 column (Millipore). The column was centrifuged at maximum speed for 30 min, and the filtrate was analyzed by LC-MS. Standard curves for concentration calculation were generated with pure ligand solutions prepared at known concentrations (16 μM to 1 μM in two-fold dilutions for typical binding assays) and filtered in the same way as the RNA-ligand binding mixtures.

LC-MS analysis was performed with an Agilent 6320 Ion Trap LC/MS system using either an Agilent ZORBAX SB-Aq, 3.0 x 250 mm, 5 μm column (for folinic acid) or an Agilent ZORBAX Eclipse XDB-C18, 2.1 x 50 mm, 3.5 μm column (for theophylline). The method for analyzing folinic acid is shown below, where solvent A is 0.1% acetic acid and 0.1% methanol in water, solvent B is 0.1% acetic acid in methanol, and solvents A and B always sum to 100% of the liquid flux.

Time (min)	% Solvent B	Flow Rate (ml/min)
0.00	45	0.500
6.00	45	0.500
6.50	60	0.500
7.00	75	0.500
7.50	100	0.500
13.00	100	0.500
13.50	45	0.500
38.00	Method Ends	Method Ends

The method for analyzing theophylline is shown on the following page, where the solvent setups are as described for the folinic acid protocol.

Time (min)	% Solvent B	Flow Rate (ml/min)
0.00	15	0.350
2.50	15	0.350
3.00	100	1.000
4.50	100	1.000
5.00	15	1.000
6.50	15	1.000
6.60	15	0.350
7.00	Method Ends	Method Ends

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