LETTER

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A Fragment-Based Approach to Identifying Ligands for Riboswitches

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ABSTRACT Riboswitches are regions of mRNA that directly bind metabolites, leading to alteration of gene expression. We have developed fragment-based methods to screen for compounds that bind the *Escherichia coli thiM* riboswitch. Using complementary biophysical techniques we have identified several ligands with K_D <100 μM. From these there is the potential to develop potent and selective modulators of riboswitch function.

RNA is involved in biological processes as diverse as protein synthesis, gene regulation, information storage, and catalysis, so consequently it is an attractive target for chemical intervention (*1*). Notable small molecules that bind to RNA include the erythromycin, aminoglycoside, and (synthetic) linezolid antibiotics (*2*). However general methods for the development of new specific ligands for RNA are not well established. In this paper we describe the application of fragment-based approaches to the identification of small molecules that bind to riboswitches, structured regions of mRNA that regulate the expression of genes by selectively binding cellular metabolites with high affinity (*3*).

Riboswitches are found in many prokaryotic and some eukaryotic organisms (*4*–*6*). They are usually composed of two functionally distinct domains: the aptamer, where the riboswitch binds its ligand, and the expression platform, which transduces the binding event into an effect on gene expression. As riboswitches control essential metabolic pathways, they are potential candidates for modulation by small molecules. The *Escherichia coli thiM* riboswitch resides within the 5′ untranslated region of the *thiMD* operon involved in thiamine pyrophosphate (TPP, coenzyme B1) biosynthesis (*7*). TPP binds to the riboswitch (K_D = 200 nM (*8*)), triggering a structural rearrangement that masks the ribosome binding site and so inhibits translation of the ThiM and ThiD proteins (*9*). In turn, this modulates the intracellular level of TPP. Herein we describe the discovery of novel fragment ligands for the aptamer of the *E. coli thiM* riboswitch.

Fragment-based methods have proven to be effective for the development of ligands against protein targets (*10*). The screening of low molecular weight compounds (MW <250 Da) allows an efficient exploration of chemical space, increasing the likelihood of finding a hit while using relatively small compound libraries (of the order of 10^2-10^3) (*11*). Fragment hits typically achieve only low millimolar to high micromolar affinities, as a result of unfavorable entropic contributions that mask their intrinsic binding energies. Subsequent elaboration allows the generation of more potent ligands by enhancing the binding energy while incurring only a small additional entropic penalty.

The fragment approach applied to the discovery of enzyme inhibitors uses a battery of biophysical techniques, including Thermofluor, NMR spectroscopy, isothermal titration calorimetry (ITC), and X-ray crystallography, to identify fragments that bind to the protein (*12*). Each method examines a different aspect of the binding, *e.g.* Thermofluor detects changes in protein stability, ITC gives the thermodynamic parameters, and X-ray crystallography shows the binding mode. By using them in combination, a more complete picture of the ligand−macromolecule interaction can be obtained. The potential of using this

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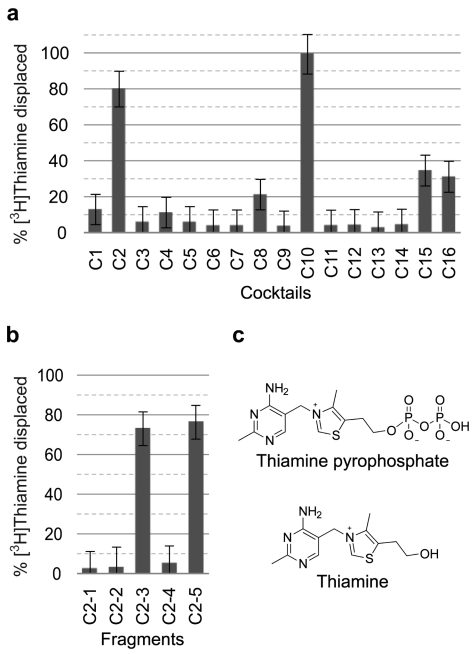

Figure 1. Representative results obtained by equilibrium dialysis screening showing normalized percentage displacement of radioligand. a) Fragments assayed in cocktails. b) Deconvoluting a cocktail hit, C2; in this case the cocktail contained two distinct hits. c) Natural ligands of the *E. coli thiM* riboswitch.

approach to develop ligands against RNA is in its infancy. While NMR spectroscopy (*13–15*) and mass spectrometry (*16*) have been used individually to screen fragments against RNA, the process of using multiple complementary techniques to validate and quantify binding has not been reported. There is also a lack of a general screening method for RNA that is simple to setup and can be used as an initial screen equivalent to the way Thermofluor screening is often used for proteins.

We found that equilibrium dialysis can fulfill the role of an initial screen with sufficient throughput and used it to screen a fragment library against the *thiM* riboswitch (*7*). Equilibrium dialysis relies on the differential distribution of radiolabeled ligand molecules between two chambers separated by a dialysis membrane, where only one chamber contains the riboswitch. In a competition format, [^{3}H]thiamine (K_D = 1.5 μM, Supplementary Figure 1) was employed as a reporter ligand, permitting hits to be observed by their displacement of thiamine from the chamber containing *thiM* RNA. The displacement can be measured quantitatively as a percentage relative to a negative control with no fragments and a positive control with saturating amounts of TPP. This has the advantage of allowing ligands to be ranked according to their potency.

A library of ~1300 commercially available fragments was screened in cocktails of 5 compounds at 1 mM each (Figure 1, panel a). Those that gave ≥30% displacement of the radioligand were deemed hits. Cocktail hits were then deconvoluted by testing the constituent fragments individually (Figure 1, panel b). Of the 252 cocktails tested, 32 scored as hits. After deconvolution and retesting of the individual fragments, 20 hits from 16 cocktails were identified.

To confirm binding of each fragment, a ligand-based NMR spectroscopic technique, waterLOGSY (ligand observed via gradient spectroscopy) was used (*17*). In these experiments, a proton spectrum of the test compound was recorded with and without *thiM* RNA. In the absence of RNA, the fragment peaks were negative, whereas in the presence of RNA, they became less negative or even positive, which is characteristic of binding to a macromolecule (Supplementary Figure 2). All 20 fragments found to bind the *thiM* aptamer by equilibrium dialysis were also seen to bind by NMR spectroscopy.

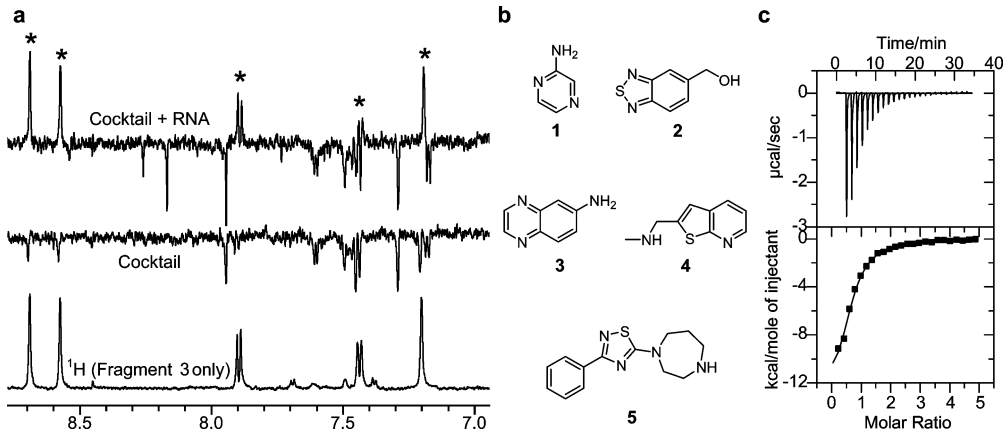

Figure 2. Validation of a fragment hit. a) NMR spectroscopy data for the cocktail (C10, see Figure 1, panel a) containing fragment hit 3. The waterLOGSY spectra of the cocktail ± RNA are shown above the 1D spectrum of 3 by itself. Asterisks denote peaks belonging to the binding fragment. b) Fragment ligands of the *E. coli thiM* riboswitch. c) Isothermal titration calorimetry data obtained for 3.

WaterLOGSY can also be used to screen cocktails of compounds (Figure 2, panel a.) Moreover, it is possible to identify the active component(s) directly in the mixture by comparison with the 1D proton spectra of the individual fragments. However NMR spectroscopy was used primarily as a tool for validating hits from equilibrium dialysis rather than for initial screening, since it uses 10 times more RNA per experiment than equilibrium dialysis. Also, although NMR spectroscopy is a highly sen-

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sitive and robust way of detecting binding, waterLOGSY has the drawback of not being quantitative, so strong binders cannot be differentiated from weak ones.

Our screening of the fragment library resulted in 20 hits from ~1300 compounds, giving a 2% hit rate. In published studies using solely NMR spectroscopy for screening, hit rates of up to 21% have been reported (*14*). However these screens were carried out on small (<120) focused libraries of fragments and identified all RNA binders. The use of equilibrium dialysis selects only for fragments capable of displacing thiamine from the riboswitch, and the quantitative data acquired allows flexibility in setting an appropriate threshold so that only the most potent compounds are identified. Therefore, although the hit rate is lower, the hits are likely to be of better quality.

The binding affinities of all 20 hits were then examined by ITC; 17 of these showed significantly negative enthalpies of binding (ΔH). Five representative examples of moderate to strong displacers of [^{3}H]thiamine, fragments **1**−**5** (Figure 2, panel b) were found to have dissociation constants (K_D) ranging from 280 to 22 μM (Figure 2, panel c, Supplementary Figure 3). These values correspond to high ligand efficiencies (*18*) (defined as ΔG/no. of heavy atoms) of up to 0.69 (Supplementary Table 1).

To eliminate generic RNA binders, all *thiM* hits were counter-screened against the aptamer of a second riboswitch (the lysine-responsive *lysC* (*19*) from *Bacillus subtilis*) by equilibrium dialysis. For these experiments, [^{3}H]lysine was used as the reporter molecule. Seven fragments tested gave >10% displacement (the approximate experimental error) and were considered binders of the *lysC* riboswitch, including **3** (90% [^{3}H]lysine displacement). Since their natural ligands are so dissimilar, hits common to both riboswitches are most probably binding by nonspecific interactions such as intercalation or to common RNA features such as loops and bulges. It is perhaps significant that **3** has some structural similarity to known RNA intercalators such as safranin and acridine orange (*20*). Fragments **1**, **2**, **4**, and **5**, however, are among the 10 selective hits. Fragment **1** might reasonably be expected to mimic the H-bonding and π-stacking interactions to the *thiM* riboswitch that the aminopyrimidine moiety of TPP forms (*21*) and that may be the source of its selectivity.

In summary, the fragment-based approach has great potential for discovering new small molecule (ant)agonists of riboswitch function. A key advantage is its ability to find structurally and chemically diverse ligands. By combining well-established biophysical techniques, we have found an efficient method for rapidly identifying new motifs that bind to riboswitches and quantifying their interactions. Riboswitches are model candidates for equilibrium dialysis experiments, as many of their ligands are commercially available in radiolabeled form; however, any RNA with known ligands that can be labeled in some way can likewise be scrutinized. In the event that the target RNA has no known small molecule binders or the labeled ligand is not available, NMR spectroscopy can be employed as the primary screen to observe binding.

From our collection of hits, we have several promising candidates to progress to structural studies by X-ray crystallography, including the aforementioned fragments **1**−**5**. The ease with which we have found fragment hits against riboswitches has positive implications on how tractable they are likely to be as biological targets.

METHODS

RNA Preparation. RNA obtained by *in vitro* transcription was purified by phenol/chloroform extraction followed by urea-PAGE. All RNA-ligand binding experiments were carried out in Binding Buffer (*thiM*: 50 mM Tris-HCl pH 7.6, 50 mM NaCl, 5 mM $MgCl_2$; *lysC*: 50 mM Tris-HCl pH 7.6, 100 mM KCl, 5 mM $MgCl_2$).

Equilibrium Dialysis. Equilibrium dialysis was carried out in DispoEquilibrium Dialyzers (*7*), (Harvard Apparatus) in Binding Buffer with a maximum of 5% (v/v) DMSO. Starting concentrations: Chamber A, 200 nM radioligand and 2 mM fragment (× 5 if cocktail) *or* 1 mM TPP *or* 2 mM L-lysine for positive controls *or* no added ligand for negative controls; Chamber B, 10 μM RNA. Dialyzers were allowed to equilibrate at 4 °C (pH of Binding Buffer at 4 °C is 8.2) overnight before scintillation counting. To calculate the percentage radioligand displaced, first the *cpmB*/(*cpmA* + *cpmB*) ratio was calculated for each sample. This number was then normalized against the positive control (100% displacement, TPP or L-lysine added) and the negative control (0% displacement, no ligand added).

NMR. Samples consisted of Binding Buffer in H_2O, 10% (v/v) D_2O, DMSO-d_6 (3% (v/v) max.), 20 μM 3-(trimethylsilyl)propionic acid-d_4 (internal standard), 5 × 500 μM fragments (for cocktails) ± 15 μM RNA. For individual 1D ^{1}H spectra, 750 μM fragment was used, with no RNA present. NMR data processed using Topspin 1.3 (Bruker).

ITC. Typical experiments consisted of 25 injections of 2.5−10 mM fragment (5 mM in the case of **3**) into 100 μM RNA (both solutions contained 1% (v/v) DMSO in Binding Buffer) at 25 °C. All ITC data were processed using MicroCal Origin 7 software (MicroCal).

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Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

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