Synthetic ligands for PreQ₁ riboswitches provide structural and mechanistic insights into targeting RNA tertiary structure

Colleen M. Connelly¹, Tomoyuki Numata²,³, Robert E. Boer¹, Michelle H. Moon¹, Ranu S. Sinniah¹, Joseph J. Barchi¹, Adrian R. Ferré-D’Amaré² & John S. Schneekloth Jr.¹

Riboswitches are naturally occurring RNA aptamers that regulate gene expression by binding to specific small molecules. Riboswitches control the expression of essential bacterial genes and are important models for RNA-small molecule recognition. Here, we report the discovery of a class of synthetic small molecules that bind to PreQ₁ riboswitch aptamers. These molecules bind specifically and reversibly to the aptamers with high affinity and induce a conformational change. Furthermore, the ligands modulate riboswitch activity through transcriptional termination despite no obvious chemical similarity to the cognate ligand. X-ray crystallographic studies reveal that the ligands share a binding site with the cognate ligand but make different contacts. Finally, alteration of the chemical structure of the ligand causes changes in the mode of RNA binding and affects regulatory function. Thus, target- and structure-based approaches can be used to identify and understand the mechanism of synthetic ligands that bind to and regulate complex, folded RNAs.
A n explosion of interest in RNA biology in recent years has revealed a multitude of new regulatory functions for noncoding RNA. Many noncoding RNAs regulate gene expression or protein function and are often dysregulated in cases of infectious disease or cancer. The observation that regulatory, noncoding RNAs can directly influence disease states has led to the suggestion that such RNAs could be suitable as targets for small molecules. Thus, the goal of developing RNA-binding small molecules as both therapeutics and chemical probes is an area of increasing interest. However, the discovery of molecules that bind to RNA with good affinity and specificity is a major challenge owing to RNA’s highly flexible, dynamic structure and largely solvent-exposed binding pockets, in addition to the fact that many RNAs are actively unfolded in the cell. Further, understanding the function of noncoding RNA has proven highly challenging in many cases. Although it is now routine to utilize structure to rationalize the function of protein-binding small molecules, high-resolution structures of RNA are often difficult to determine. The challenge in characterizing the specific contacts that small molecules make with RNAs is often a substantial impediment to understanding and developing small molecule probes of RNA.

One class of regulatory, noncoding RNAs where major advances in structure determination have been seen is riboswitches. Riboswitches are structured RNA elements that occur in the untranslated regions of mRNA, most often in bacteria. Riboswitches act as regulators of gene expression through recognition of a small-molecule ligand that induces a conformational change in the RNA, thereby regulating downstream gene expression. Riboswitches often recognize ligands related to the function of the associated gene product and, in some examples, the ligand can be a product of the biosynthetic pathway regulated by the riboswitch. Molecules recognized can range from nucleobases, cofactors, and amino acids to metal ions. These structured RNA elements have two domains, an aptamer domain that recognizes the cognate ligand through specific interactions, and an expression platform that changes conformation upon binding to modulate gene expression. Riboswitch-mediated gene regulation can occur at either the transcriptional or translational level. For transcriptional riboswitches, cognate ligand binding usually promotes an RNA conformation that contains a terminator helix that triggers dissociation of RNA polymerase and leads to transcription termination upstream of the coding sequence. In the absence of the ligand, the RNA folds into an alternate antiterminator, therefore, allowing transcription of the downstream gene. In translational riboswitches, ligand binding causes a conformational change that produces a helix that occludes the Shine-Dalgarno sequence and prevents ribosomal binding. In the absence of the ligand, an alternate structure forms, increasing the availability of the ribosomal binding region and facilitating translation initiation. The ability of riboswitches to control essential bacterial genes has stimulated their evaluation as facilitating translation initiation. The ability of riboswitches to increasing the availability of the ribosomal binding region and binding. In the absence of the ligand, an alternate structure forms, causing a conformational change that produces a terminator hairpin and downregulates transcription. Similarly, the PreQ1 riboswitch from *Thermoanaerobacter tengcongensis* (Tt) also adopts an H-type pseudoknot, causing a conformational change that induces a terminator hairpin and downregulates transcription.

In this study, we report the discovery of a new class of synthetic small molecules that bind directly to PreQ1 riboswitches. We performed a small molecule microarray (SMM) screen on the aptamer domain of the PreQ1 riboswitch found in *B. subtilis*. Hit compounds that showed selective binding to the riboswitch over other RNAs were validated through a series of biophysical experiments. One hit compound from this screen exhibits a dissociation constant of ~500 nM to the PreQ1 riboswitch aptamer by multiple orthogonal fluorescence titrations. Further, in-line probing suggests that the synthetic ligand induces a riboswitch conformation that is different from that of the cognate ligand-bound form. The compound is also shown to bind the structurally similar, but functionally distinct TtPreQ1 riboswitch. Co-crystal structures with this aptamer show that the compound binds in the PreQ1-binding site but makes key interactions with conserved nucleotides that are different from those of the cognate ligand. Importantly, in vitro transcription termination assays demonstrate that the small molecule is capable of regulating riboswitch function upon binding. Structure-guided alteration of the chemical structure of the ligand impacts both the mode of binding and activity of the ligands. The co-crystal structures will further be used for developing additional compounds that target the PreQ1 riboswitch and the Q biosynthetic pathway.

## Results

### SMM screening of a PreQ1 riboswitch aptamer.

To identify drug-like small molecules that bind to the PreQ1 riboswitch, we used a SMM-screening strategy. In this approach, small molecules are spatially arrayed and covalently linked to a glass surface. Next, a fluorescently tagged RNA of interest is incubated with the arrays. Slides are washed, imaged, and the fluorescence intensity is measured for each location on the array. For each compound (printed in duplicate), a composite Z-score is generated, reflecting the increase in fluorescence upon addition of labeled RNA. In parallel, other RNAs may be counter screened to evaluate selectivity and prioritize hit compounds that arise from the screen.

Toward this end, we designed a 5′-Cy5-labeled RNA consisting of the 34-nt aptamer domain of the PreQ1 riboswitch from *B. subtilis* (5′-Cy5-BSPreQ1-RS, Fig. 1A)30. Once purity was confirmed by gel electrophoresis, the RNA was annealed and incubated on SMM slides to perform the screen. In parallel, we also screened analogous 5′-Cy5-labeled constructs consisting of aptamer domains from S-adenosylmethionine (SAM-II) and thiamine pyrophosphate (TPP) riboswitches (Supplementary Table 1).
Each of these three aptamer domains are known to recognize different biologically active ligands and have well-defined, complex three-dimensional structures, making them suitable controls for selectivity. To further measure selectivity, the Z-score for each compound is compared across many different SMM screens (in this case 29 different oligonucleotides, Supplementary Table 2). We identified 243 hits from a collection of 26,227 compounds screened in the SMM, for an initial hit rate of 0.93%. After ruling out compounds that bound promiscuously to other riboswitches or other RNAs and DNAs previously screened against the library, we generated a list of 86 candidate compounds for further study (Supplementary Table 3). The TPP and SAM-II riboswitches had slightly fewer numbers of “selective” hits, with rates of 51 hits (0.19%) and 61 hits (0.23%), respectively. Twenty hits identified as selective for the PreQ1 aptamer were purchased for further analysis (Supplementary Table 4 and Supplementary Fig. 1). As a representative example, direct binding on SMM slides (Fig. 1B) and selectivity data (Fig. 1C) are shown for compound 1.

**Ligand observed NMR validates binding.** Each of the purchased compounds was evaluated using Water-Ligand Observed via Gradient Spectroscopy (WaterLOGSY) NMR43. In this experiment, each compound was subjected to a standard 1H NMR pulse sequence (to assess solubility in aqueous buffer), WaterLOGSY without RNA (to assess aggregation), and WaterLOGSY in the presence of unlabeled BsPreQ1-RS RNA (to assess binding to the riboswitch aptamer) (Fig. 2A). For compounds that bind directly to the RNA, peaks phase positively only in the presence of RNA. In contrast, aggregating compounds phase positively even in the absence of RNA. Peaks for compounds that are soluble but do not bind to the RNA are phased negatively in both cases (as can be seen with N-methyl-L-valine, included here as a convenient internal, non-binding control). From these experiments, we identified five compounds with suitable solubility and RNA binding in the solution phase. We also evaluated binding of each compound against 31 different structured RNAs and DNAs from previous SMM screens, where compound 1 was found to have the best profile of selective binding (Fig. 1C).
ligand and has a mostly conserved binding site. Gratifyingly, I also bound directly to the TtPreQ₁ aptamer domain by WaterLOGSY (Fig. 2A). In addition to WaterLOGSY experiments, we evaluated the behavior of I in Carr-Purcell-Meiboom-Gill (CPMG) ¹H NMR experiments, which can be used to observe alterations in T₂ relaxation times of small molecules that bind large molecules in solution (with binding shortening relaxation time)⁴⁵,⁴⁶. Here, I was subjected to a CPMG pulse sequence in the presence and absence of both the Bs and Tt aptamers used in the WaterLOGSY experiments. In both cases, substantial peak attenuation of I with no attenuation of the control was observed in the presence of RNA, indicating shortening of the T₂ relaxation time of protons in I (Fig. 2B). Thus, CPMG experiments further validate the binding of I to both aptamers. Because the two aptamers have conserved binding sites but diverge moderately in structure, this suggested that I could potentially share a binding site with the cognate ligand.

**Fluorescence titrations demonstrate submicromolar binding.** To estimate the affinity of I to the PreQ₁ riboswitch aptamer domain, we used orthogonal fluorescence titrations. First, we measured changes in fluorescence of the Cy5-labeled Bs aptamer domain construct used for SMM screening (5'-Cy5-BsPreQ₁-RS) as a function of compound concentration. In parallel, we evaluated effects on an AlexaFluor 647-labeled Tt aptamer domain construct (5'-AF647-TtPreQ₁-RS, Supplementary Table 1) to both measure affinity and further rule out effects associated with the fluorophore (Fig. 3A). In the case of the Bs aptamer, we measured an apparent dissociation constant (Kᵦ) of 534 ± 123 nm for I. Similarly, I had a Kᵦ of 457 ± 202 nm for the Tt aptamer domain. For comparison, we also measured the affinity of PreQ₁ itself in this assay. The affinity of PreQ₁ for the Bs aptamer was 4.1 ± 0.6 nM, and for the Tt aptamer it was 2.8 ± 0.4 nM (Supplementary Fig. 2). This is in good agreement with literature values measured by other methods²⁹,³⁰,⁴⁴. Owing to the presence of a conjugated π-electron system, we evaluated I for inherent fluorescence and found that I was fluorescent, with λₑₓ = 300 nm and λₑₘ = 340 nm. Thus, by holding the concentration of I constant and titrating in increasing quantities of unlabeled riboswitch aptamers, we could measure Kᵦ values by observing changes in fluorescence as a function of RNA concentration (Fig. 3B). Here, we measured a Kᵦ of 490 ± 368 nM for the Bs aptamer domain and 99 ± 38 nM for the Tt aptamer domain, respectively. Thus, there is good agreement for Kᵦ measurements between multiple different techniques. In addition to the selectivity measured by comparing different SMM screens (Fig. 1C), we also used fluorescence titration to evaluate the binding of I to tRNA. Upon titrating tRNA into a solution of I, only non-specific binding was observed, and fluorescence quenching did not fit to a 1:1 binding model, indicating only weak binding to tRNA at high micromolar levels (Supplementary Fig. 3).

**Ligand induces conformational change to riboswitch aptamers.** A key aspect of riboswitch biochemistry is that biological effects are driven by ligand-induced conformational changes in the RNA upon binding. Thus, we aimed to evaluate the effects of I on the conformation of the PreQ₁ aptamers by in-line probing experiments³⁰. In-line probing experiments are routinely used to monitor alterations of riboswitch conformation and can report on ligand-induced effects on RNA structure. First, we performed in-line probing using a fluorescently tagged 36-mer Bs aptamer domain (5'-AF647-BsPreQ₁-RS, Supplementary Table 1) with PreQ₁. Consistent with previous reports using a radiolabeled 36-mer construct³⁰, we observed an increase in cleavage at C12 and a corresponding decrease in cleavage at U32 (based on 34-
mer numbering, Fig. 1D). Thus, in-line probing using a fluoro-
cently tagged target accurately matches literature reports on
ligand-induced conformational changes. Next, we performed an
analogous experiment in the presence of synthetic ligand 1
(Fig. 4A). In contrast to the cognate ligand, 1 caused an increase
in cleavage at C8, U9, U13, C15, U22, and U24, whereas it did not
induce prominent increase in cleavage at C12. Although these
changes were consistent from experiment to experiment, smaller
changes in cleavage at other residues (for example nucleotides
17–19) were less consistent from experiment to experiment and
could not be attributed to a specific binding interaction. Thus,
although the compound binds directly to the Bs aptamer domain
and alters the conformation of the RNA, the effects of 1 in this
assay are distinct from those of PreQ1. We also performed in-line
probing with the Tt riboswitch (5’-AF647-TtPreQ1-RS, Supple-
mentary Table 1). In this case, with PreQ1 the most notable
increase in cleavage occurred at U12 (Fig. 4B). Upon addition of
compound 1, a similarly small increase in cleavage occurred at
U12 and A13. Thus, 1 may induce a conformational change in the
Tt aptamer similar to PreQ1. The Bs and Tt riboswitches have
been previously described to have similar conformational
ensembles but differ in their ability to recognize ligands by
conformational selection and induced fit mechanisms, respec-
tively44. This distinction may be reflected in the effects observed
in in-line probing experiments.

Ligand modulates riboswitch transcriptional termination.
Having demonstrated that 1 binds and causes conformational
changes in both the Bs and Tt aptamers, we next evaluated 1 in
a functional assay. As described above, the Bs riboswitch regulates
the transcription of downstream genes upon ligand binding.
Thus, we examined the capacity of 1 to modulate transcriptional
termination. DNA templates were designed containing the apta-
meter domain and terminator hairpin of the BsPreQ1 riboswitch,
followed by an elongation sequence (BsPreQ1-TTA, Supple-
mentary Table 5). In vitro transcription of these templates produces
an RNA sequence containing the riboswitch. This transcription
can either be halted at the terminator hairpin in the presence of
the cognate ligand or produce a read-through product that is
transcribed to the end of the template. Despite repeated attempts,
efforts to observe transcriptional termination of the Bs riboswitch
by PreQ1 were unsuccessful in this assay. *Staphylococcus sapro-
phyticus* (Ss) also contains a related PreQ1 riboswitch sequence
with a conserved binding site that functions by transcriptional
termination (Fig. 1D). Dose-dependent transcriptional termina-
tion was observed with a template containing the Ss riboswitch
(Ss PreQ1-TTA, Supplementary Table 5). Near-complete termi-
nation was observed at the highest PreQ1 concentrations tested
with an EC50 for PreQ1 of 36 ± 5 nM (Fig. 5). Similarly, addition of
increasing concentrations of 1 also resulted in near-complete
termination of transcription with an EC50 of 359 ± 23 μM. Thus, 1
functions by a mechanism similar to PreQ1 itself. We also con-
firmed that 1 binds to the Ss aptamer by WaterLOGSY experi-
ments (Supplementary Fig. 4). In addition, the KD of 1 for the Ss
aptamer was determined to be by 42 ± 6 nM by fluorescence
titration (Supplementary Fig. 5). As a further control, the che-
ically unrelated ligand 5-aminoimidazole-4-carboxamide ribo-
nucleotide, which binds to other riboswitches45, was tested in
transcriptional assays and had no effect on the Ss reporter con-
struct (Supplementary Fig. 7).

X-Ray crystal structure of the ligand/aptamer complex. To
establish a molecular basis for its selective binding, we solved
crystal structures of 1 bound to the TtPreQ1 riboswitch aptamer
domain. Initial efforts using the wild-type TtPreQ1 riboswitch
aptamer domain failed, yielding structures indistinguishable from
the previously reported ligand-free aptamer domain structure of
the RNA, in which the nucleobase of A14 occupies the PreQ1-
binding site29. To alleviate competition by the intramolecular
interaction with binding of the exogenous ligand, we designed
aptamer domains in which the nucleobase at position 14, as well
as one or two adjacent disordered loop nucleobases were

![Fig. 3 Affinity of 1 for PreQ1-RS aptamers. a Fluorescence intensity assay of 5’-Cy5-labeled BsPreQ1-RS or 5’-AlexaFluor 647-labeled TtPreQ1-RS RNA in the presence of increasing concentration of 1. b Inherent fluorescence titration of 1 with increasing concentration of unlabeled BsPreQ1-RS or TtPreQ1-RS RNA. Error bars indicate the standard deviation determined from three independent measurements. Source data are provided as a Source Data file.](https://doi.org/10.1038/s41467-019-09493-3)
Treatment with PreQ1 at a concentration of 10 μM is used as a positive control. OH and Tt are a partial alkaline hydrolysis ladder and ribonuclease A DMSO control in the absence (−) or presence (+) of 1 mM MgCl₂. Treatment with PreQ₁ at a concentration of 10 μM is used as a positive control. OH and Tt are a partial alkaline hydrolysis ladder and ribonuclease T₁ digestion, respectively. Arrows designate nucleotide positions where the cleavage efficiency was significantly altered by compound treatment (blue) or preQ₁ treatment (red).

**Fig. 4** Ligand-induced conformational changes. In-line probing of a 5′-AlexaFluor 647-labeled BsPreQ₁-RS RNA and b 5′-AlexaFluor 647-labeled TtPreQ₁-RS RNA after treatment with 1 at increasing concentrations or a DMSO control in the absence (−) or presence (+) of 1 mM MgCl₂. Treatment with PreQ₁ at a concentration of 10 μM is used as a positive control. OH and T₁ are a partial alkaline hydrolysis ladder and ribonuclease T₁ digestion, respectively. Arrows designate nucleotide positions where the cleavage efficiency was significantly altered by compound treatment (blue) or preQ₁ treatment (red).

removed. (ab13_14 and ab13_14_15, Supplementary Table 1). Co-crystal structures of these abasic TtPreQ₁ riboswitch aptamer domains complexed with 1 (ab13_14-1, and ab13_14_15-1) were determined by the molecular replacement (MR) method and both refined at 1.8 Å resolution. These two structures are near-identical (r.m.s.d. is 0.14 Å for 638 non-hydrogen atom pairs). The following discussion focuses on the ab13_14-1 complex. We also determined the crystal structure of ab13_14 in complex with PreQ₁ (ab13_14-PreQ₁) at 1.69 Å resolution, for comparison (Methods and Supplementary Table 7).

Unbiased ΔF₀|ΔF₁ residual electron-density maps unambiguously located 1 at the interhelical interface of stems S₁ and S₂, surrounded by the L₂ and L₃ loops, in both co-crystal structures (Fig. 6A, ab13_14-1; Supplementary Fig. 8, ab13_14_15-1). Thus, the synthetic ligand occupies a position similar to that of the cognate ligand PreQ₁. The dibenzofuran of 1 is sandwiched between G11 and the G5+C6 pair, with the furan oxygen engaging in a hydrogen bond (3.2 Å) with the N₆ atom of A29 (Fig. 6B and Supplementary Fig. 5). The same oxygen is in van der Waals contact with the N₁ atom of A29 (3.7 Å). The nucleotides that interact with 1 are phylogenetically conserved among PreQ₁ riboswitches (Fig. 1D), being responsible for PreQ₁ recognition (Supplementary Fig. 9). In contrast to the solvent-inaccessible heterocycle of 1, its amine-bearing sidechain (largely coplanar with the dibenzofuran heterocycle) is solvent exposed, with the amine hydrogen bonding to water, and therefore not directly recognized by the RNA (Fig. 6B). However, to avoid steric clash with the L₂ loop, 1 must bind with its sidechain emerging from the PreQ₁-binding pocket away from L₂ loop of the riboswitch. Of note, lack of direct recognition of the amine of 1 provides a plausible explanation for how the binding event occurred when 1 was chemically conjugated to the SMM slide. Although the sugar of residue 13 of the ab13_14-1 structure lacked electron density and is presumed disordered, in the ab13_14_15-1 structure, the ribose ring of residue 13 is ordered and in van der Waals contact with ring C of the dibenzofuran of 1 (Supplementary Figure 8).

Structure-guided changes alter mode of binding and activity. Next, we asked whether alteration of the chemical structure of 1 would have effects on riboswitch recognition or activity. We hypothesized that alteration of the basicity of the pendant amine of 1 might enable it to engage in hydrogen-bonding contacts with the RNA. Thus, we prepared the dimethylamino derivative 2, containing a more basic tertiary amine (Fig. 7A). We evaluated the affinity of 2 for both the Bs and Tt aptamers by monitoring changes in fluorescence of the 5′-Cy5-BsPreQ₁-RS and 5′-AF647-TtPreQ₁-RS RNAs (Supplementary Table 1) upon titration with 2 and determined affinities of 0.4 ± 0.1 μM and 0.6 ± 0.2 μM, respectively (Supplementary Fig. 10). In addition to the pendant amine, we asked whether alteration of the core heterocyclic scaffold would have effects on RNA binding. We noted that in the structure of 1 bound to the riboswitch, the exocyclic amine of A29 makes a hydrogen bond with the oxygen atom of the dibenzofuran. In this structure, A29 is not coplanar with 1 and sits at an angle of 40°, where N1 and N6 bisect the plane of the dibenzofuran core. Nucleotides are amphiphilic and are capable of serving as both hydrogen bond donors and acceptors. We therefore synthesized a carbazole derivative of 2, compound 3 (Fig. 7A), replacing the dibenzofuran oxygen with a nitrogen atom (that may potentially donate a hydrogen bond). Affinities of 3 for the Bs and Tt riboswitch aptamer domains were measured to be 0.1 ± 0.08 μM and 0.1 ± 0.04 μM, respectively, using fluorescence titrations (Supplementary Fig. 10). Thus, all three compounds have similar affinities for the riboswitch aptamers. Finally, 2 and 3 were evaluated in transcriptional termination assays (Fig. 7B, Supplementary Fig. 11). Here, 2 behaved similarly to 1 in terms of maximal effect. However, 3 was markedly inferior in the functional assay.

To further understand the interactions of 2 and 3 with the riboswitches, we also solved crystal structures of each ligand in complex with the Tt riboswitch. The co-crystal structures of abasic mutant riboswitch aptamer domains bound to 2 and 3
(ab13_14-2 and ab13_14_15-3, respectively) were solved by the MR method and refined at 1.94 Å and 2.56 Å resolution, respectively (Methods and Supplementary Table 7). The overall structure of ab13_14-2 is similar to that of the riboswitch bound to 1, with 2 in a similar binding pose, directly in the PreQ1 binding site (Fig. 8A). In this structure, the sidechain of 2 bends upwards at the methylene preceding the amine, bringing the pyramidal tertiary amine of 2 within hydrogen-bonding distance (2.9 Å) of the N7 atom of the phylogenetically conserved G5 of the riboswitch (Fig. 1D). In contrast, the primary amine of 1 lies 3.6 Å from the same RNA next. We solved the crystal structure of 3 in complex with the Tt riboswitch aptamer domain. Similar to 1 and 2, compound 3 occupies the PreQ1-binding site (Fig. 8B). However, in this structure, 3 has shifted by ~1 Å in the direction of RNA loop L2 (Fig. 8C) to support a hydrogen bond between the new donor atom in the heterocycle and the RNA (the distances between the carbazole nitrogen atom of 3 and the N1 and N6 atoms of A29 are 3.2 Å and 3.5 Å, respectively). This confirms that alteration of the chemical structure of the initial hit can lead to changes in binding modes and somewhat altered affinity. Possibly owing to the ~1 Å shift in its binding pose (Fig. 8C), the conformation of the sidechain of the bound 3 resembles that of the bound 1 rather than that of 2. The sidechain of the bound 3 is largely coplanar with the carbazole, exposed to solvent, and does not lie within hydrogen-bonding distance of G5 of the RNA.
The striking contrast between the ab13_14-I and WT-PreQ1 structures is predominantly attributable to the binding of the bulkier compound to the riboswitch. Consistent with this, the crystal structure of ab13_14-PreQ1 showed a similar conformation to WT-PreQ1, rather than ab13_14-I, where C15 in ab13_14-PreQ1 is well superimposed to that in WT-PreQ1 and A32 and G33 in ab13_14-PreQ1 shift toward the ligand-binding site compared with the ab13_14-I structure (Fig. 5D-G and Supplementary Fig. 5). In addition, U12 of ab13_14-PreQ1 is placed in a similar location to A14 of WT-PreQ1 and hydrogen bonds with G11 in order to compensate for the absence of the base at position 14. Therefore, PreQ1 binding to the ligand binding site results in a conformation similar to WT-PreQ1 despite the absence of the bases at positions 13 and 14. From these findings, I binding to the wild-type TtPreQ1 riboswitch may induce a suboptimal formation of the continuous base stack that transmits the ligand binding information to the expression platform of the riboswitch. This suboptimal conformation may lead to the decreased maximum termination efficiency in the transcription termination assay for I in comparison with the cognate ligand.

Like the translational class I PreQ1 riboswitch, transcriptional class I PreQ1 riboswitches also have a continuous base stack from the ligand-binding site to the expression platform. Given that the transcriptional and translational PreQ1 riboswitches control ON/OFF switching using similar structural motifs, the I-bound TtPreQ1 riboswitch structure can explain why this compound has an EC₅₀ of 359 ± 23 μM in transcription termination assays (Ss aptamer). This value is considerably higher than that for PreQ1 (36 ± 5 nM) despite affinities of 0.5 ± 0.1 μM and 4 ± 0.6 nM, respectively (Bs aptamer). These findings suggest that the region of I situated near C15 would be a good candidate for synthetic efforts to improve the potency for downregulating gene expression. Our co-crystal structures are the starting point for developing additional compounds that target the bacteria-specific Q biosynthetic pathway.

Although 1, 2, and 3 have similar binding affinities for the Tt and Bs aptamers, they do not have identical activity in...
transcription termination with the 5S riboswitch. This difference in activity may be due to subtle differences in binding modes to the RNA, reflected in the lack of a hydrogen bond to G5 and the altered pose of ligand 3. Importantly, the synthetic ligand has considerably decreased solubility at concentrations needed for transcription termination, which may also play a role in decreased function. Although it has been previously demonstrated that binding affinity is not the sole parameter that governs the activity of RNA-binding compounds, these observations are often not accompanied by mechanistic or structural rationalization. Our work demonstrates that subtle alterations in the mode of recognition of ligands may also have a role in the ability of a compound to induce conformational or functional effects. This work highlights a challenge in developing ligands for functional RNAs, namely that affinity is not the only property that governs the activity of a compound. Finally, the work described herein illustrates the role structure has in understanding the behavior of small synthetic compounds that bind to and modulate the function of complex RNAs.

**Methods**

**General RNA methods.** To avoid RNase contamination, all buffers were prepared with diethyl pyrocarbonate-treated water and all surfaces and equipment were decontaminated with RNaseZap (Ambion) prior to RNA handling. Deprotected and high-performance liquid chromatography purified oligonucleotides were purchased from Dharmacon (ThermoFisher) or IDT DNA. Sequences of the RNA and high-performance liquid chromatography purified oligonucleotides used for biochemical and biophysical experiments can be found in Supplementary Table 1.

**SMM screening.** SMM slides were prepared according to established procedures. In brief, y-aminopropyl silane microscope slides (Corning) were functionalized with an Fmoc-protected amino polyethylene glycol spacer (Fmoc-8-amino-3,6-dioxoanonic acid) in N,N-dimethylformamide (DMF). Following piperidine deprotection, 1,6-diisocyanatohexane was coupled to the surface to provide isocyanate-functionalized microarray slides for immobilization of small molecule library members. A total of 26,227 small molecules (10 mM in DMSO) containing at least one primary or secondary alcohol or amine were purchased from commercial vendors including ChemBridge and ChemDiv. The libraries were printed on seven array slides containing ~ 3940 distinct molecules printed in duplicate, in addition to dyes and controls used for quality-control validation. The arrays were exposed to pyridine vapor to facilitate covalent attachment to the isocyanate-functionalized slides. Slides were then incubated with a 1:20 polyethylene glycol/DMF (v/v) solution to quench unreacted isocyanates. The 5′-Cy5-3′PreQ1-RS RNA was dissolved in 50 mM Tris, 100 mM KCl, 1 mM MgCl2, pH 7.5, diluted to 5 μM, and was annealed by heating to 75 °C for 5 min, followed by slowly cooling to room temperature for 30 min. The annealed RNA was then further diluted to 1 μM in 50 mM Tris, 100 mM KCl, 1 mM MgCl2, pH 7.5 for screening. Printed microarray slides were incubated with the RNA at a concentration of 1 μM for 2 h using a LifterSlip (Electron Microscopy Sciences). Following incubation, slides were washed three times with 4 mL of 50 mM Tris, 100 mM KCl, 1 mM MgCl2, pH 7.5 buffer for 2 min in a four-well Nunc plate, and the slides were dried by centrifugation for 2 min at 4000 × g. Slides were imaged for fluorescence (650 nm

![Fig. 8](https://i.imgur.com/4yQ5.jpg)

**Fig. 8** Structural analysis of the bound TPreQ1 aptamers. a Ligand-binding site of the ab13_14-2 co-crystal structure, superimposed on the [Fo]−|Fc| electron-density map calculated before addition of the ligand to the crystallographic model (blue mesh, 3.0 σ contour). Hydrogen bonds are indicated as dotted lines. b Ligand-binding site of the ab13_14-15-3 co-crystal structure. The [Fo]−|Fc| electron-density map for the compound is colored blue and contoured at 3.0 σ. c Comparison of binding modes of 1 (magenta), 2 (cyan), and 3 (green). Ligand-binding site and continuous base stack of the aptamer domains of the d WT-PreQ1, e WT free, f ab13_14-PreQ1, and g ab13_14-1 forms. (Upper) Cartoon representations with ligands and key nucleotides labeled and colored cyan, magenta, yellow, and green in the WT-PreQ1, WT free, ab13_14-PreQ1, and ab13_14-1 forms, respectively. To compare the locations of A32 and G33 among these structures, dashed lines are indicated. (Lower) Detail of the ligand-binding site. PreQ1 and 1 are in yellow. Key nucleotides responsible for forming the base stack are colored cyan. U12 in the ab13_14-PreQ1 structure, which occupies a similar location to A14 in the WT-PreQ1 structure, is in green. Hydrogen bonds between PreQ1 and C15 are indicated as dotted lines.
excitation, 670 nm emission) on an Innopysc Immnoscan 1100 AL Microarray Scanner with a resolution of 5 μm. The scanned image was aligned with the corresponding GenoGlycoList (GAL) file to identify individual features. Hits were determined based on signal-to-noise ratio (SNR), defined as (mean foreground−mean background)/standard deviation of background, and Z-score, defined as Z = (Mean SNR33compound−Mean SNR33library)/SD SNR33library with the following criteria: (a) coefficient of variance of duplicate spots <10%, (b) Average Z-score for a compound > 3, (c) [Z-score<0.5−ZscoreControl Array]/ZscoreControl Array > 3, (d) no activity with any other nucleic acid structures screened in parallel. Other Cy5-labeled riboswitches screened in parallel included TPP and SAM-II, which were screened using the same method described above for the PreQ1 riboswitch. Hits were further validated by visual inspection of array images and compounds for further study were purchased from original suppliers.

**General ligand observed NMR methods.** All NMR spectra were recorded at 293 K on a Bruker AVANCE III 500 MHz spectrometer equipped with a TCI cryoprobe. NMR buffer was composed of 50 mM Tris-d_1, pH 7.5, 100 mM KCl, 1 mM MgCl_2 in 100% UltraPure distilled H_2O (Invitrogen). Subsequently prepared NMR samples contained 10% D_2O and 5% DMSO-d_6 to improve compound solubility. All compounds were first dissolved to a 10 mM stock concentration in 100% DMSO-d_6 prior to NMR sample preparation. Purchased RNA was buffer exchanged (3 kDa MWCO spin column, EMD Millipore) into the NMR buffer prior to use. For WaterLOGSY and CPMG experiments, a reference 1D-1H spectrum was acquired to confirm the sample’s integrity. The scanned image was aligned with the sample’s 1D-1H spectrum. The reference 1D-1H spectrum was then measured on a Synergy Mx microplate reader (BioTek) at an excitation wavelength of 300 nm and an emission wavelength of 400 nm (gain = 120). The fluorescence intensity was then normalized to the values obtained for I in the absence of RNA and was plotted against RNA concentration. The dissociation constants were determined using single site binding models.

**Analysis of Mg^{2+}-induced in-line cleavage.** This procedure was adapted from a related procedure that used radiolabeled oligonucleotides. 5′-AF647-BePreQ1-RS or 5′-AF647-TPreQ1-RS RNA was diluted into 50 mM Tris, 100 mM KCl, pH 7.5 to a concentration of 10 μM and were annealed by heating to 75 °C for 5 min, followed by cooling on ice for 10 min. The annealed RNA was incubated at a final concentration of 1 μM in 50 mM Tris, pH 8.3, 10 mM KCl buffer with either a DMSO control (5% final concentration) or compound 1 at a concentration of 0.1, 1, 3, 10, 30, 100, or 300 μM. For 5′-AF647-BePreQ1-RS, MgCl_2 was added at a final concentration of 2 mM, and the reactions were incubated at room temperature in darkness for 40 hrs. For 5′-AF647-TPreQ1-RS-RS, MgCl_2 was added at a final concentration of 2 μM, and the reactions were incubated at room temperature in darkness for 72 h to reach the desired level of cleavage. Alkaline hydrolysis was performed in 50 mM NaOH, pH 9.0 at 95 °C for 5 min. Ribonuclease T1 digestion was carried out with 0.11 U of ribonuclease T1 (Ambion) in 20 mM Tris, pH 7.5, 50 mM NaCl, 0.1 mM MgCl_2 at room temperature for 20 min. The RNase T1 reaction was stopped by adding 0.2 volumes of 50 mM EDTA. Equal volumes of loading buffer containing 7 μl urea, 1 × Tris-borate-EDTA (TBE), and 0.01% direct red dye was added to each reaction, and the samples were heated to 95 °C for 5 min. Each sample was analyzed by electrophoresis on a 3% polyacrylamide sequencing gel (20% polyacrylamide, 19:1 crosslinking, 7 μM urea) at 60 W, 45 °C for 3.5 hrs. The gel was visualized by fluorescence of the 5′-Cy5 label (630 nm excitation, 670 emission) with Typhoon FLA 9500 Phosphorimager (GE Healthcare Life Sciences) and was analyzed with ImageQuant software.

**X-ray crystallography RNA preparation.** Sequences of the RNAs employed in this study are based on BePreQ1 riboswitch aptamer domain and listed in Supplementary Table 1. RNAs were purchased from Dharmaco and deprotected according to the manufacturer’s instructions. After lyophilization, RNAs were dissolved in water and stored at −25 °C.

**Cryoelectron microscopy data collection.** Data from the cryo-electron microscopy investigations were collected at 11.8 m sodium cacodylate (pH 7.0) were heated at 65 °C for 2 min. MgCl_2 and synthetic compound were then added to the final concentrations of 10 mM and 0.5 mM, respectively. The reactions were incubated at 65 °C for 3 min, and then cooled down gradually to room temperature. TPreQ1 riboswitch was crystallized by the hanging-drop vapor diffusion method at 21 °C, under conditions containing 5–15 mM Mg acetate, 50 mM MES (pH 5.6), and 2.3–2.7 mM ammonium sulfate (Condition I) and containing 0.1–0.3 mM potassium sodium tartrate, 100 mM sodium cacodylate (pH 5.6), and 0.01% direct red dye (Condition II). Hanging drops were prepared by mixing 1 μl of the RNA solution (0.38 mM RNA in 10 mM sodium cacodylate, pH 7.0, 10 mM MgCl_2, and 0.5 mM synthetic compound) with 1 μl of the reservoir solution and were equilibrated against 400 μl of reservoir solution. The RNA crystals grew within 2 weeks to maximum dimensions of 300 × 100 × 100 μm. For a data collection, the RNA crystals were transferred to the cryoprotectant solutions containing 10 mM Mg acetate, 27.5 mM MES (pH 5.6), and 2.15 mM lithium sulfate for the crystals obtained with the Condition I, and 60 μM

**Inherent ligand fluorescence titration.** Fluorescence titrations were performed using either unlabeled BePreQ1-RS or TPreQ1-RS. Each RNA was resuspended to a concentration of ~ 500 μM in 50 mM Tris, pH 7.5, 100 mM KCl, 1 mM MgCl_2, and annealed by heating to 75 °C for 5 min, followed by slowly cooling to room temperature for 30 min. Serial dilutions of each RNA were prepared in 50 mM Tris, pH 7.5, 100 mM KCl, 1 mM MgCl_2. Inherent fluorescence titrations were performed with compound I at a final concentration of 500 μM in 50 mM Tris, pH 7.5, 100 mM KCl, 1 mM MgCl_2 with 5% DMSO. For BePreQ1-RS, in a black 384-well plate, I was diluted to a final concentration of 500 nM in buffer with a 5% final DMSO concentration. RNA was added to final concentrations ranging from 0–50 μM in triplicate, the plate was centrifuged (1000 rpm, 2 min), and samples were allowed to incubate at room temperature under shaking. Fluorescence intensity was then measured on a Synergy Mx microplate reader (BioTek) at an excitation wavelength of 300 nm and an emission wavelength of 340 nm (gain = 120). The fluorescence intensity was then normalized to the values obtained for I in the absence of RNA and was plotted against RNA concentration. The dissociation constants were determined using single site binding models.

**Fluorescence intensity assay.** Fluorescence titrations were performed using either a 5′-Cy5-BePreQ1-RS or AF647-TPreQ1-RS. Each RNA was diluted into 50 mM Tris, 100 mM KCl, 1 mM MgCl_2 in Ultrapure distilled H_2O using centrifugal filtration (3 kDa MWCO, EMD Millipore) and were annealed by heating to 75 °C for 5 min, followed by slowly cooling to room temperature for 30 min. NMR samples containing each SM compound at a concentration of 375 μM and a negative control N-methyl-L-valine (Chem-Impex International) at 375 μM were prepared in 50 mM Tris-d_1, pH 7.5, 100 mM KCl, 1 mM MgCl_2 in Ultrapure distilled H_2O containing 10% D_2O and a final concentration of 5% DMSO-d_6. For each compound, samples were prepared with and without each RNA at a concentration of 15 μM. Samples were incubated on ice for 10 min, degassed at 20 °C for 15 min, and transferred to a Shigeniu NMR tube. A reference 1D-1H and 1D WaterLOGSY spectra with and without RNA were recorded. NMR spectra were recorded at 293 K on a Bruker AVANCE III 500 MHz spectrometer equipped with a TCI cryoprobe.
potassium sodium tartrate, 25 mM sodium citrate (pH 5.6), and 2.15 M lithium sulfate for the crystals under the Condition II. The crystals were mounted in a nylon loop and flash frozen by plunging into liquid nitrogen. X-ray diffraction data were collected at the beamlines 5.0.1 and 5.0.2 of the Advanced Light Source (ALS), Lawrence Berkeley National Laboratory. Diffraction data were integrated and scaled with the program DIALS\(^49\). Data processing statistics are summarized in Supplementary Table 7.

**Structure determination and refinement.** The structures were solved by the MR method using the previously determined \( T. congolens\)s PreQ\(_1\) aptamer structure (PDB ID: 3Q51 [https://doi.org/10.2210/pdb3Q51/pdb]) as a search model with the program PHASER\(^50\). The solutions were subjected to simulated annealing, energy minimization, restrained isotropic B-factor, and TLS refinement with PHENIX\(^51\), and the resulting electron-density maps revealed the locations of the small molecules. Iterative cycles of refinement and manual rebuilding\(^52\) produced the current co-crystal structures of ab13\(_{14-1}\), ab13\(_{14-15-1}\), ab13\(_{14-2}\), ab13\(_{14-15-3}\), and ab13\(_{14-14}\)-PreQ\(_1\) with R\(_\text{free}\) of 20.5, 20.3, 24.8, and 21.0\% at 1.80, 1.80, 1.94, 2.56, and 1.69 Å resolution, respectively. Reflection statistics are summarized in Supplementary Table 7. Molecular graphics were produced with PyMol (http://www.pymol.org/). Stereo images are provided in Supplementary Figure 12 and 13.

**Single-round transcription termination assay.** The transcription termination assays were carried out according to established protocols\(^53,54\) with several modifications. The DNA plasmid containing \( h_{bg}\) promoter and 26-nt C-less sequence followed by the \( Staphylococcus\) \( suprophylactic\) PreQ\(_1\) riboswitch and its downstream sequence cloned into pDTSMART-AMP was purchased from Integrated DNA Technologies. The DNA template was amplified by PCR from the plasmid using forward and reverse primers (Supplementary Table 6), and then was gel-extracted in order to prevent undesired non-specific interactions between the 26-nt C-less sequence and riboswitch. Elongation was restarted by combining 9 µL of halted transcription complexes in 1 × transcription buffer and incubated at room temperature for 5 min, in order to prevent undesired non-specific interactions between the 26-nt C-less sequence and riboswitch.

**Synthetic procedures and characterization.** Chemical synthesis and compound characterization are provided in the Supplementary Methods.

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Author contributions
C.C. designed experiments, performed SMM screens, florescence titrations, in-line probing, NMR experiments, and transcription termination assays. T.N. designed and performed transcription termination assays and crystallography. R.B. performed synthetic chemistry and characterization. M.M. performed SMM screens and affinity titrations. R.S. and J.B. performed NMR experiments. A.F. designed experiments and helped write the manuscript. J.S. conceived the project, designed experiments, and helped write the manuscript. All authors contributed in editing the manuscript.

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