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Selective binding of 2′-F-c-di-GMP to Ct-E88 and Cb-E43, new class I riboswitches from *Clostridium tetani* and *Clostridium botulinum* respectively†

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C-di-GMP is a second messenger in bacteria and partly regulates bacterial physiology by binding to class I and II riboswitches. Four class I c-di-GMP riboswitch aptamer candidates, Ct-E88, Cb-17B, Cb-E43 and Cd-630 RNAs, selected from a GEMM RNA sequence motif in the Rfam database, were expressed and experimentally verified to bind to c-di-GMP. The two newly characterized c-di-GMP riboswitches, Ct-E88 and Cb-E43, bound c-di-GMP with nanomolar K_d whereas the affinities of Cb-17B and Cd-630 for c-di-GMP were at least a 100-fold weaker. Interestingly, whereas the three riboswitches (Vc2, Et-E88 and Cb-E43) bound c-di-GMP with similar K_d values, 2′-modified analogs of c-di-GMP differentially bound to these three class I aptamers. For example, 2′-F-c-di-GMP bound Vc2 with a K_d value of 102 nM whereas the K_d value of 2′-F-c-di-GMP–Ct-E88 is 43 μM (422× higher than that for Vc2 RNA), revealing that there are differences in the binding sites of functional class I c-di-GMP riboswitches.

Introduction

C-di-GMP functions as an important second messenger in bacteria. Recent studies have shown that many critical biological functions are controlled *via* c-di-GMP signaling, including exopolysaccharide synthesis, cellular motility, virulence and biofilm formation.[1] Although c-di-GMP has been studied for more than two decades,[2] it is only recently that some of the molecular details of the interactions of c-di-GMP with macromolecular targets have been uncovered. Until the recent discovery by Breaker and co-workers that c-di-GMP binds to RNA riboswitches to regulate gene expression and translation,[3j] it had largely been assumed that c-di-GMP only binds to effector proteins, such as transcription factors, to regulate bacterial physiology.[3] The discovery of these c-di-GMP responsive riboswitches expands the role of RNA in the c-di-GMP signaling pathway. Thus, inhibiting these c-di-GMP riboswitches with small molecules could potentially lead to the inhibition of biofilm formation or virulence factors production in bacteria. Motivated by potential promise of c-di-GMP riboswitch inhibitors, we and others have embarked on structure–activity-relationship (SAR) studies of c-di-GMP binding to riboswitches.[4-6]

To this end, we demonstrated that 2′-biotinylated analogs of c-di-GMP could bind to class II c-di-GMP riboswitches, CdA RNA from *Clostridium acetobutylicum*, but not class I riboswitches, such as Vc2 RNA from *Vibrio cholerae*.[4] Others have also shown that other c-di-GMP analogs could exhibit differential binding to class I riboswitches (Vc2) and class II CdA RNAs.[5,6]

Given these previous promising results and the preponderance of c-di-GMP class I over class II riboswitches, we are interested in the identification of small molecules that could discriminate between different c-di-GMP class I riboswitches. C-di-GMP class I riboswitches can be found in both symbiotic and pathogenic bacteria[7] and therefore being able to discriminate between class I riboswitches, found in symbiotic and pathogenic bacteria, will be critical for the maintenance of natural microbiota during antibacterial therapy. However, the paucity of structural data for different c-di-GMP class I riboswitches makes it difficult to design selective binders of c-di-GMP class I riboswitches *a priori*. We therefore investigated the SAR of c-di-GMP binding to different class I riboswitches (Fig. 1), using c-di-GMP analogs (see Fig. 2 for structures of analogs) to determine if different c-di-GMP class I riboswitches used different recognition elements for binding to c-di-GMP.

Results and discussion

Although over 500 examples of c-di-GMP class I riboswitch RNAs have been predicted computationally,[7] only a few have

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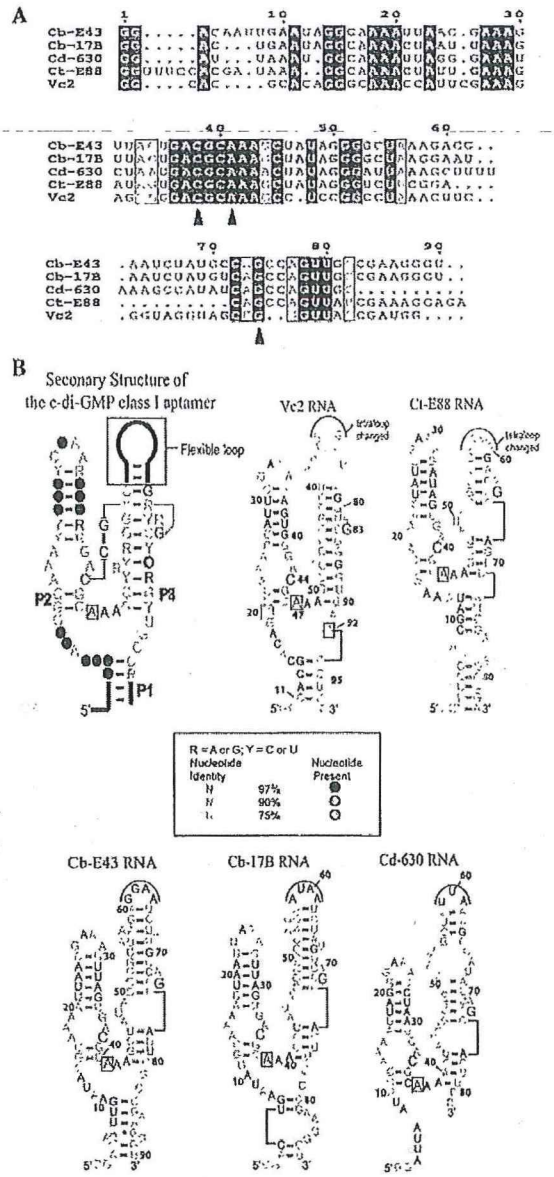

Fig. 1 (A) Alignment of predicted c-di-GMP riboswitch sequences. Alignment was done using Clustal W2[15] and ESPript.[16] (B) Predicted structures of the selected c-di-GMP-I riboswitch aptamers are based on the structural model of the c-di-GMP class I aptamer.[7] In Vc2 RNA structure, C44 on P2 stem (colored blue) and G83 on P3 stem (colored blue) are expected to form an interhelical Watson-Crick base pairing in the ligand bound structure and A47 (colored blue and boxed) is expected to intercalate into the two bases of c-di-GMP in the bound form.[7,13] C92 (colored red and boxed) and G20 (colored green and boxed) are two important residues that base pair with the two bases of c-di-GMP.[7,13]

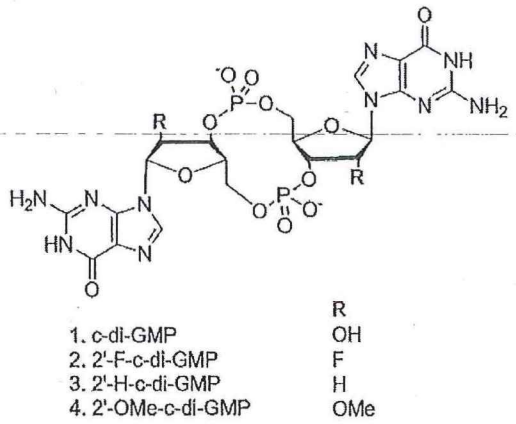

Fig. 2 Family of c-di-GMP analogs, which were designed and synthesized.

been experimentally verified to bind to c-di-GMP.[3j,5,7,8] We selected four of the computationally predicted c-di-GMP class I riboswitch aptamer candidates (Ct-E88, Cb-17B, Cb-E43 and Cd-630 RNAs) from the GEMM RNA sequence motif in the Rfam database (Fig. 1).[9] These putative c-di-GMP binding riboswitches are found in different pathogenic bacteria that cause human diseases, including *Clostridium tetani*[10] (Ct-E88), *Clostridium botulinum*[11] (Cb-17B and Cb-E43) and *Clostridium difficile*[12] (Cd-630). Vc2 RNA, from *Vibrio cholerae*, which has been biochemically and structurally characterized as a c-di-GMP binding riboswitch was used as a positive control.[3j,7,13]

C-di-GMP class I riboswitch aptamers are predicted to form a three-helix Y-shaped structure, with the ligand-binding site at the junction of helices P1, P2 and P3.[7] The crystal structure of the Vc2–c-di-GMP complex[5,7] reveals that 5′- and 3′-flanking nucleotides extended the putative P1 helix, to include a Watson–Crick G:C base pair between the C92 residue of the RNA (boxed red in the Vc2 model, Fig. 1B) and one of two guanosines of c-di-GMP (Gβ).[7,13] Helices P2 and P3 are parallel to each other in the presence of Mg^{2+}, while helix P1 forms only when c-di-GMP is present.[14] Residue G20 (boxed green in the Vc2 model, Fig. 1B) in helix P2 of Vc2 contacts the top nucleobase of c-di-GMP (Gα) along its Hoogsteen face in the bound form.[7,13] The highly conserved adenosine A47 (colored blue and boxed in the Vc2 model, Fig. 1B) intercalates between the two nucleobases of c-di-GMP, resulting in extensive stacking interactions that bridge the P1 and P2 helices.[7,13] Several nucleotides are conserved in the selected class I aptamers (Fig. 1A) and the predicted structures of the selected riboswitches are similar, so we would expect that they all bind to c-di-GMP.

Experimental validation of selected computationally predicted class-I RNA aptamers binding to c-di-GMP

To verify if the predicted class I riboswitches could bind to c-di-GMP, an equilibrium microdialysis experiment was performed for each aptamer RNA (Vc2, Cd-630, Cb-17B, Ct-E88 and Cb-E43) with different ligands. Details of the preparation of the various RNAs can be found in the ESI.† Equilibrium dialysis is a simple but effective assay to quickly determine if a ligand binds to a macromolecular target.[4,6b] One of the advantages of equilibrium dialysis assay is that sampling is done at equilibrium so it is possible to detect even the low affinity

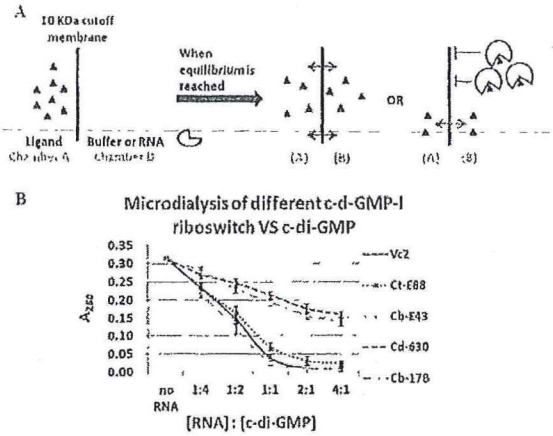

Fig. 3 Equilibrium microdialysis of selected c-di-GMP class I riboswitch aptamers with a ligand. (A) Schematic diagram for equilibrium microdialysis. (B) A plot of the concentration of c-di-GMP in chamber A *versus* the amount of different c-di-GMP class I riboswitch aptamers added in chamber B. Error bars are generated based on standard deviation between three experiment repeats.

interactions that are difficult to detect using non-equilibrium methods, such as gel shift assays. Herein, the binding of each class I aptamer to c-di-GMP was initially directly measured qualitatively by this method as described previously.[4] A plot of the UV absorbance at 260 nm (A_{260}) of the ligand in chamber A *versus* the concentration of RNA in chamber B measures the relative binding affinities of selected c-di-GMP class I RNAs with c-di-GMP (Fig. 3B). The equilibrium dialysis results showed that c-di-GMP could bind to Cb-E43, Ct-E88 and Vc2 RNAs with similar affinities, whereas Cb-17B and Cd-630 RNAs have relatively low binding affinities to c-di-GMP (Fig. 3 and Table 1). We postulate that the low binding affinity of Cd-630 or Cb-17B RNA with c-di-GMP is likely due to the lack of a P1 helix in these two RNAs (Fig. 1). Our microdialysis data suggest that if Cb-E43 and Ct-E88 are expressed in bacteria, then they would likely be functional c-di-GMP riboswitches in *Clostridium tetani* (Ct-E88) and *Clostridium botulinum* (Cb-E43).

Recognition of 2′-position modified c-di-GMP analogs

Having identified Ct-E88 and Cb-E43 as potential functional c-di-GMP riboswitches, we next sought to investigate further their binding modes. Previous reports have demonstrated that modification of the 2′-position of c-di-GMP differentially affected binding to different classes of c-di-GMP riboswitches.[5] The X-ray crystal structure of the c-di-GMP–Vc2 complex[5,7] indicated that one of the 2′-OH of c-di-GMP interacts with a nonbridging phosphate oxygen of A47 in Vc2 (Fig. 1B)[7,13] and this might partly explain why c-di-GMP class I riboswitches are sensitive to 2′-modification of c-di-GMP.[4,5,6b]

However, different c-di-GMP class I riboswitches have different residues in the predicted ligand binding pocket. Thus we hypothesized that discrimination between these riboswitches, which belong to the same class, could be achieved using c-di-GMP analogs that provide different puckering modes, or hydrogen bonding capabilities, or both, at the 2′-position. To test this hypothesis, we synthesized three different c-di-GMP analogs with the 2′-position modified, including 2′-F-c-di-GMP (2),[6b] 2′-H-c-di-GMP (3)[17] and 2′-OMe-c-di-GMP[5,6b] (4) (Fig. 2) and investigated the binding affinities of these analogs for different c-di-GMP class I aptamers (Vc2, Cd-630, Cb-17B, Ct-E88 and Cb-E43) using equilibrium dialysis and electrophoretic mobility shift assay (EMSA).

Affinity measurements of c-di-GMP and analogs for different class I aptamers

We used gel-shift and quantitative equilibrium dialysis assays to determine the K_d value for c-di-GMP/2′-analogs and the various class I riboswitches. The binding affinity of various class I riboswitch aptamers to c-di-GMP was measured by titrating a radiolabeled c-di-GMP probe with increasing concentration of RNA. The fraction of radiolabeled c-di-GMP that was bound was

Table 1 Binding affinities of c-di-GMP and analogs measured for various class I riboswitches (Vc2, Ct-E88, Cb-E43, Cb-17B and Cd-630 RNAs)

		Gel competition			Microdialysis		
RNA	Ligand	K_d (nM)	$\Delta\Delta G_{bind}$ (kcal mol^{-1})	Fold loss	K_d (nM)	ΔG_{bind} (kcal mol^{-1})	Fold loss
Vc2	c-di-GMP	47 ± 4			43 ± 6		
	2′-F-c-di-GMP	176 ± 7	0.8	3.7	102 ± 10	0.5	2.4
	2′-H-c-di-GMP	8560 ± 980	3.0	182	12 600 ± 1160	3.3	293
	2′-OMe-c-di-GMP	>1 mM	>5.8	>21 000	>1 mM	>5.9	>23 000
Ct-E88	c-di-GMP	60 ± 5			90 ± 7		
	2′-F-c-di-GMP	45 000 ± 9400	3.9	750	43 400 ± 9100	3.6	482
	2′-H-c-di-GMP	52 000 ± 5700	4.0	867	67 000 ± 9800	3.9	744
	2′-OMe-c-di-GMP	n.b.[a]			>1 mM		
Cb-E43	c-di-GMP	128 ± 18			58 ± 7		
	2′-F-c-di-GMP	996 ± 10	1.2	7.8	1260 ± 180	1.8	21.7
	2′-H-c-di-GMP	12 200 ± 6200	2.7	95.3	17 100 ± 3500	3.3	295
	2′-OMe-c-di-GMP	n.b.[a]			>1 mM		
Cb-17B	c-di-GMP	>1 μM			>1 μM		
Cd-630	c-di-GMP	>1 μM			>1 μM		

[a] No detectable binding at highest ligand concentration (600 μM) tested.

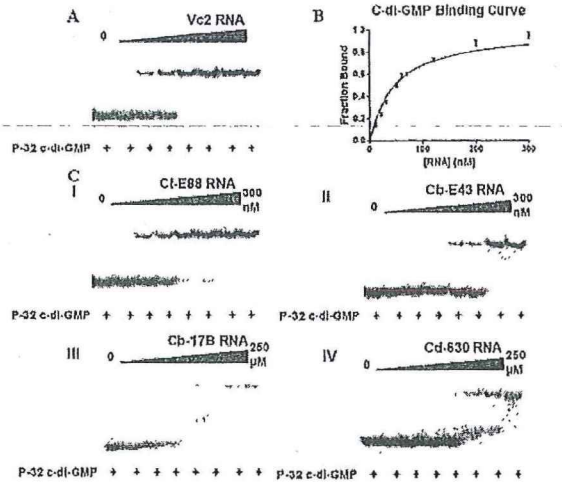

Fig. 4 K_d measurement of c-di-GMP by gel-shift assay, using radiolabeled c-di-GMP. (A) Representative gel-shift experiment for measuring the K_d of c-di-GMP with Vc2 RNA by direct binding. (B) Representative c-di-GMP binding curve for class I aptamer RNAs. (C) Gel-shift experiments for measuring the dissociation constants (K_d) of c-di-GMP with putative class I aptamers (Ct-E88, Cb-E43, Cb-17B and Cd-630 RNA).

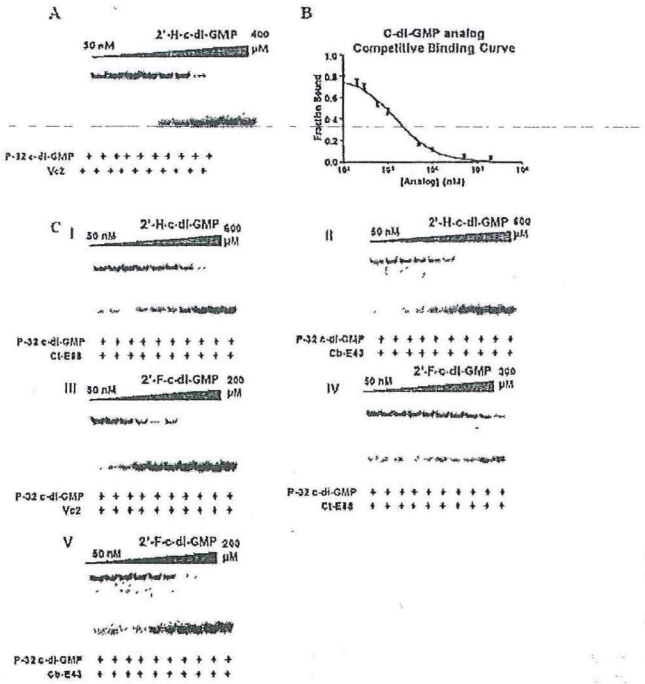

Fig. 5 K_d measurement of c-di-GMP analogs using competition gel-shift assay with radiolabeled c-di-GMP. (A) Representative competition gel-shift experiment of Vc2 RNA with 2′-H-c-di-GMP. (B) Representative binding curve from the competition gel-shift assay. (C) Gel-shift experiments for measuring the K_d of 2′-H-c-di-GMP and 2′-F-c-di-GMP for each class I aptamer (Vc2, Ct-E88, Cb-E43 RNA) by competition assay. RNA, radiolabeled c-di-GMP, and increasing concentrations of competitor analogs are incubated at room temperature until equilibrium. Free P-32 c-di-GMP is separated from RNA-bound c-di-GMP by 10% native PAGE.

determined from the gel shift (Fig. 4) or from the dialysis experiment and the data were fitted to a quadratic equation (3) (see ESI†) to obtain the K_d value (Fig. 4 and Table 1). The K_d values obtained from equilibrium microdialysis were similar to those obtained from the gel shift assay (Table 1). C-di-GMP bound to Vc2, Cb-E43 and Ct-E88 RNAs tightly with nanomolar K_d values (Table 1), whereas no binding was observed for Cb-17B and Cd-630 RNAs, even at ligand concentrations of 1 μM or more. To measure the affinities of the unlabeled c-di-GMP analogs, a competition gel-shift assay or equilibrium dialysis with radiolabeled c-di-GMP was used since the preparation of radiolabeled c-di-GMP analogs is non-trivial. Class I aptamers and labeled c-di-GMP were incubated in the presence of increasing concentrations of the unlabeled competitor analogs until equilibrium was achieved. The fraction of free radiolabeled c-di-GMP, which was displaced from the bound riboswitch was monitored to determine the affinity of the competitor (Fig. 5 and Table 1).[5,6b]

Our data suggest that deletion of the 2′-OH group in c-di-GMP to give the 2′-H-c-di-GMP was detrimental for class I riboswitch binding. The loss in binding energy was highest for Ct-E88 RNA (3.9–4.0 kcal mol^{-1}), compared to Vc2 and Cb-E43 RNAs (loss of 3.0–3.3 kcal mol^{-1} and 2.7–3.3 kcal mol^{-1}, respectively). The loss in binding energy seen with 2′-H-c-di-GMP could be due to an abrogation of hydrogen bonding interactions between the RNA and c-di-GMP. Alternatively, it is also possible that replacing the 2′-OH group with hydrogen affects the ribose puckering, which then affects the relative orientation of the different moieties, including the nucleobases of c-di-GMP, to offset important interactions between c-di-GMP and RNA. To differentiate the sugar pucker effect from the hydrogen bonding effect in c-di-GMP binding to these RNAs, the binding affinities of 2′-F-c-di-GMP to Vc2, Ct-E88 and Cb-E43 RNAs were measured. The fluoro group is a good isostere for the OH group and it has been demonstrated that 2′-fluoro nucleotides likely exist in the 3′-endo sugar puckering mode.[18] Although fluorine is more electronegative than oxygen, when bonded to carbon, fluorine is not as good a hydrogen bond acceptor as oxygen is.[19] Secondly, the OH group can act as a donor or acceptor of a hydrogen bond whereas the fluoro group can only accept a hydrogen bond. The loss in binding energy when the 2′-OH in c-di-GMP was replaced with a fluoro group was 3.6–3.9 kcal mol^{-1} for Ct-E88 RNA, resulting in 482–750-fold loss in binding affinity compared to c-di-GMP. Interestingly, the loss in binding affinity was only 2.4–3.7-fold and 7.8–21.7-fold for Vc2 and Cb-E43 RNA respectively.

Conclusions

This work has identified two new c-di-GMP riboswitches, Ct-E88 and Cb-E43. C-di-GMP binds to Vc2, Ct-E88 and Cb-E43 RNAs with similar affinities but these RNAs bind to c-di-GMP analogs with different affinities. For example, Vc2 and Cb-E43 bind to 2′-F-c-di-GMP better than Ct-E88. Cb-E43 also binds to 2′-H-c-di-GMP slightly better than the other RNAs (Vc2, Ct-E88, Cd-630 and Cb-17b).

All of the tested c-di-GMP class I riboswitches are sensitive to steric encumbrance at the 2′position because none of the RNAs bind to the 2′-OMe-c-di-GMP. Strobel *et al.* have suggested that the deleterious effects of 2′-deoxy or 2′-methoxy modifications are probably due to their inability to maintain a favored 3′-*endo* conformation.[6b] The 2′-fluoro modification however maintains this favoured conformation, and yet this particular analog binds strongly to Vc2 and Cb-E43 RNAs but not Ct-E88. The differential binding of 2′-F-c-di-GMP to c-di-GMP class I riboswitches Vc2, Cb-E43 and Ct-E88 suggests that the hydrogen bonding interactions in the binding pocket of some class I riboswitches, such as Ct-E88 RNA, are very different to that of others, such as Vc2 and Cb-E43 RNAs. Future structural studies should help delineate the factors that contribute to the differential binding of c-di-GMP and analogs to these tested RNAs. Our biochemical data suggest that although the c-di-GMP class I riboswitches use some common binding determinants (such as sensitivity to steric bulk at the 2′-position), there are unexpected differences in the binding modes with c-di-GMP and these differences could be utilized for the development of selective small molecules that disrupt c-di-GMP signaling in some bacteria and not others. The ability to selectively target one particular class I riboswitch, while not affecting others, could be used to affect c-di-GMP signalling in some pathogenic but not symbiotic bacteria. Although this dream remains to be realized, our results provide some optimism for the discovery of such selective inhibitors.

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