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Diamidinium and iminium aromatics as new aggregators of the bacterial signaling molecule, c-di-GMP

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ABSTRACT

C-di-GMP has emerged as an important bacterial signaling molecule that is involved in biofilm formation. Small molecules that can form biologically inactive complexes with c-di-GMP have the potential to be used as anti-biofilm agents. Herein, we report that water-soluble diamidinium/iminium aromatics (such as berenil), which are traditionally considered as minor groove binders of nucleic acids, are capable of aggregating c-di-GMP into G-quadruplexes via π-stacking interactions.

Bacterial resistance to antibiotics is increasingly becoming problematic as more bacteria develop ways to counter most drugs yet only a handful of new antibiotics are introduced in clinical use.[1] Very few drugs exist for the treatment of patients who have been infected with highly virulent strains of most human pathogens and the recent example of *Escherichia coli* outbreak in Europe that claimed a few lives is a reminder to us that despite years of active research into antimicrobials, we still do not have a good handle on how to treat some bacterial infections.[2] Recent advances in our understanding of bacterial signaling networks have revealed that virulence factors in bacteria are under the control of both extracellular and intracellular small molecules.[3] Recently, it has been shown that dinucleotides, such as 3′,5′-cyclic diguanylic acid (c-di-GMP) and 3′,5′-cyclic diadenylic acid (c-di-AMP) play important roles in the pathogenesis of most bacteria.[4] Simple nucleotides have, in the past, been assumed to mainly play the role of being precursors to polynucleotides but it is now well appreciated that they also have important signaling roles in both prokaryotes and eukaryotes.[4,5] C-di-GMP, which was first shown to be a an allosteric regulator molecule in *Gluconacetobacter xylinus* in 1987, has since been established as an important universal second messenger, uniquely found in bacteria.[6]

While the importance of c-di-GMP in bacterial 'lifestyle' is now generally acknowledged, a complete classification of the effector proteins and nucleic acids that bind to it is lacking. C-di-GMP is synthesized by diguanylate cyclases (DGCs) containing a GGDEF domain, and is broken down by phosphodiesterases (PDEs), which typically contain an EAL domain, although some contain an HD-GYP domain.[7,8] In several bacteria, there exist multiple DGCs and PDEs, which are dedicated to the metabolism of c-di-GMP. For example, *Salmonella typhimurium* harbors twelve DGC proteins and fourteen PDE proteins; *Pseudomonas aeruginosa* harbors 33 DGC proteins and 21 PDE proteins; *E. coli* has 19 DGC proteins and 17 PDE proteins; and *Vibrio vulnificus* contains 66 DGC proteins and 33 PDE proteins.[9] The redundancy of the c-di-GMP signaling pathway in several bacteria has made it extremely difficult to intercept this signaling pathway, as it is challenging to design a small molecule that can simultaneously inhibit several different proteins, which have the same function but might be structurally different.

We recently proposed a new paradigm for interrupting the c-di-GMP signaling pathway whereby the signaling molecule itself and not the receptor proteins or nucleic acids is targeted with small molecules.[10] In this approach, c-di-GMP is made to form G-quadruplex aggregates, in the presence of small molecules (see Fig. 1) and because most receptors bind to either monomeric or dimeric c-di-GMP (at least those that have been characterized so far), the formation of higher order aggregates of c-di-GMP by these small molecules would effectively sequester the 'active' signaling molecule into 'inactive' forms. In our previous communication, we disclosed that aromatic intercalators (such as acridines) facilitated the formation of stable c-di-GMP G-quadruplexes at physiological conditions.[10] The majority of the intercalators that promoted higher-order aggregate formation in c-di-GMP, however had poor water solubility and a few even had to be added to c-di-GMP as

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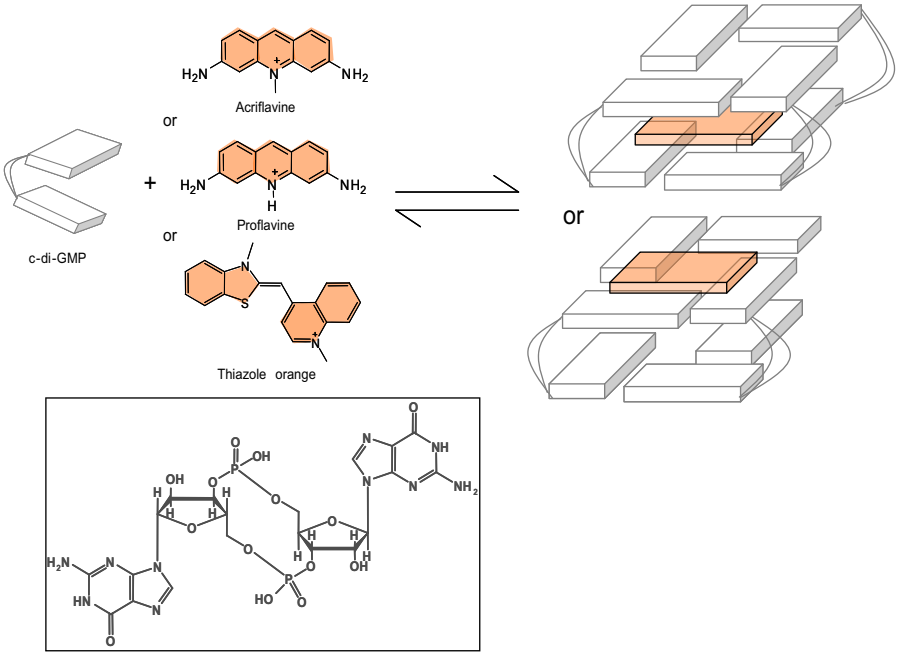

Figure 1. At physiological conditions and low micromolar concentrations of c-di-GMP, acridine derivatives (acriflavine, proflavine) and thiazole orange aggregate with c-di-GMP to form stable G-quadruplexes.[10]

solutions in DMSO. As DMSO is a chaotropic solvent, these compounds that have poor water solubility would not be appropriate for further biological investigations. We therefore embarked on a search for new water-soluble aggregators of c-di-GMP. Amidine or amino-containing compounds are usually protonated at physiological pH and are typically water-soluble. Herein, we disclose the identification of a new class of c-di-GMP aggregators that are water-soluble.

We examined five compounds: pentamidine isethionate (PTM, **1**), diminazene actuate (DMZ, **2**), 2-hydroxystilbamidine bis(methanesulfonate) (HSB, **3**), auramine O (ARO, **4**), 4,4′-diaminodiphenylamine (DDA, **5**), see Figure 2. These molecules contain aromatic moieties and so could potentially intercalate into c-di-GMP. Furthermore, the positively charged diamidinium or iminium moieties could neutralize the phosphate charges on the c-di-GMP upon intercalation into c-di-GMP, in addition to their water-solubilizing role. Previous work had established that circular dichroism (CD) could be used to determine if c-di-GMP formed G-quadruplex via the appearance of a signature CD peak at around 300 nm.[11] In Jones' important work, which corroborated CD data with NMR studies, it was established that monomeric c-di-GMP had a positive CD peak around 260 nm and a negative CD peak around 280 nm whereas G-quadruplex formation in c-di-GMP resulted in the appearance of positive CD peaks centered around 300 nm.[11] Therefore, one can deduce if c-di-GMP is forming G-quadruplexes by monitoring the signature CD peak around 300 nm.

In the presence of 250 mM KCl, but without any diamidinium/iminium compound, c-di-GMP (100 μM) has no CD peak at around 300 nm, indicating that at this concentration of c-di-GMP, G-quadruplex formation does not occur. However, upon the addition of diminazene aceturate (DMZ) and auramine O (ARO), positive CD peaks formed at around 295 and 310 nm, respectively (see Fig. 3a). Some complex formation could also be seen to form between pentamidine (PTM) or 4,4′-diaminodiphenylamine (DDA) and c-di-GMP when these compounds were added to a solution of this dinucleotide (see Fig. 3a). The intensities of the new positive

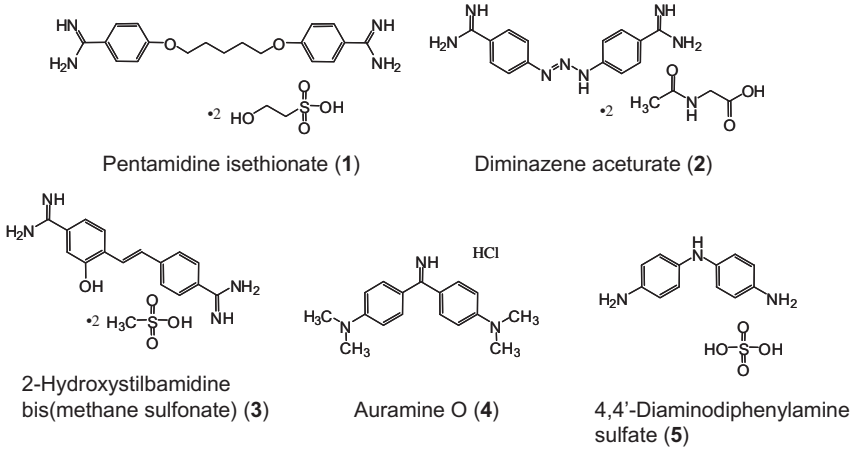

Figure 2. Structures of aromatics used in this study. Compounds **1**–**4** are water-soluble.