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In vivo cleavage of a target RNA by copper kanamycin A. Direct observation by a fluorescence assay†

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A novel fluorescence assay to monitor *in vivo* cleavage chemistry of RNA target sequences has been established and used to demonstrate the activity of copper aminoglycoside mediated degradation of RNA in bacterial cells.

Pathogenic bacteria give rise to infections such as pneumonia, meningitis, and septicemia through the agency of protein toxins.[1] The emergence of resistance to antimicrobial agents has led to a need for new families of antibiotics and/or targets. In this regard, the production of virulence factors is often controlled by regulatory RNA sequences,[1,2] and so such structured RNA motifs can be viewed as potential drug targets. The diversity and complexity of RNA tertiary structure provides for high fidelity recognition by small molecule agents such as aminoglycosides, which have a well recognized propensity to bind to cognate RNAs.[3–9] Aminoglycosides are permeable to both Gram-positive and Gram-negative bacteria,[10,11] while the lack of a cellular repair system for RNA provides further impetus for drug targeting to cognate RNA motifs. Herein we report the first demonstration of *in vivo* recognition and cleavage of an RNA target sequence. These results suggest copper aminoglycosides to be a potential source of new metal-based drugs that both recognize and destroy RNA targets *in vivo*. A novel fluorescence assay for monitoring intracellular cleavage chemistry is also described.

Previously we have demonstrated copper derivatives of aminoglycosides (Fig. 1)[12] to mediate highly specific cleavage of cognate RNA targets at concentrations as low as picomolar levels at physiological pH and temperature.[3] Structural and thermodynamic analyses of the aminoglycoside–RNA complex,[4] and the cleavage chemistry of the complex under hydrolytic and oxidative conditions[3] have been assessed. However, demonstration of the efficacy of such reagents *in vitro* is only a first step. To demonstrate *in vivo* cleavage chemistry we have designed a fluorescence assay based on use of the green fluorescent protein (GFP),[12–14] a molecular probe for analysis of cellular gene expression. The active chromophore of GFP from the jellyfish *Aequorea victoria* is generated *via* the

† Electronic supplementary material (ESI) available: experimental details for plasmid construction, and the fluorescence and assay measurements. See http://www.rsc.org/suppdata/cc/b1/b108439a/

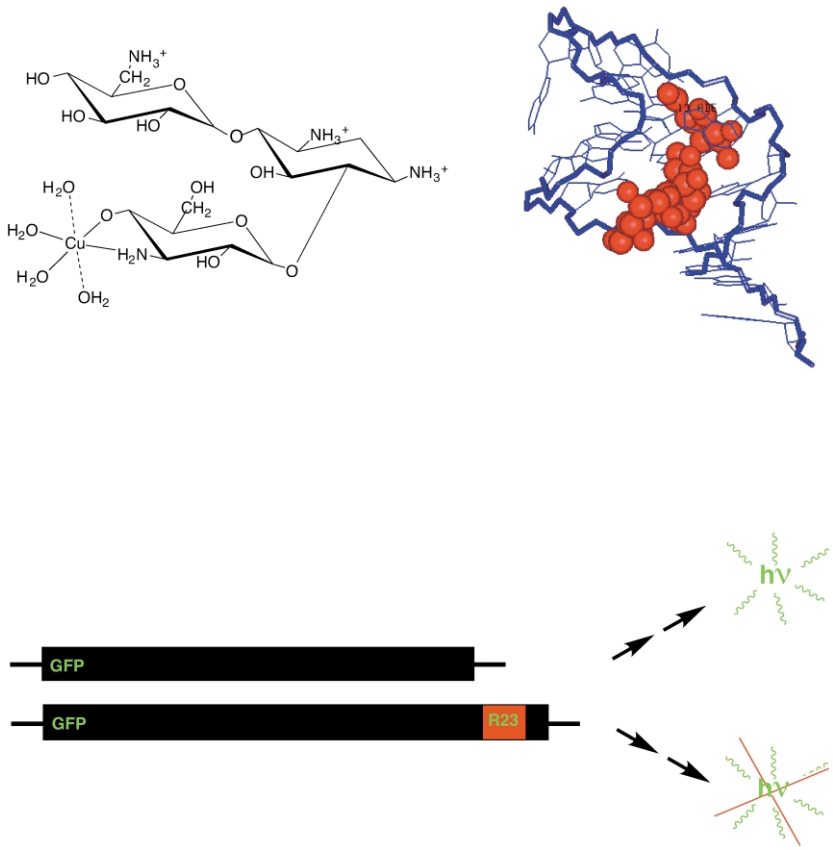

Fig. 1 (Top) Copper kanamycin A (left) and an experimentally determined structure[4] (right) of aminoglycoside bound to target R23 RNA. (Bottom) Schematic illustration of GFP mRNA transcripts. The upper sequence is translated to GFP with retention of fluorescence. The lower sequence is cleaved before the stop signal and translation of the polypeptide chain is aborted.

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spontaneous cyclization and oxidation of a serine–dehydrotyrosine–glycine trimer within a defined hexapeptide sequence of the protein and shows a green fluorescence with $\lambda^{em}_{max} \sim 509$ nm.[13] We have utilized the advantages of GFP as a reporter gene following introduction of a deoxyribonucleotide sequence encoding the 23-mer target RNA aptamer (R23)[3] at the 3′-end of the GFP gene prior to the stop codon (Fig. 1). Recognition of this R23 sequence in the transcribed mRNA results in cleavage *in vivo*, and the fluorescence intensity of GFP (Figs. 1 and 2) decreases as a consequence of the destruction of translatable mRNA for GFP.[15,16] Such a strategy provides a framework for the screening of novel drugs in the cleavage of pathogenic mRNA sequences. Plasmid constructs containing the control and modified GFP genes were cloned by standard methods.[14] These two resulting plasmids were transformed into a bacterial expression host and expression efficiency was readily quantitated by following the emission maximum at 509 nm. The optimal conditions for GFP overexpression were found to be 4 h after IPTG induction, which showed the highest emission intensity at 509 nm. The fluorescence spectra (Fig. 2) of GFP that were measured either under whole cell conditions, or from cell extracts (with $\lambda_{ex} = 395$ nm and $\lambda_{em} = 509$ nm) are similar to previous reports.[10] Further fluorescence characterization of the GFP-R23 fusion protein (Fig. 2) from cell extracts shows similar spectral properties to that of the control GFP.

To test whether the R23 sequence had been cleaved by copper kanamycin A *in vivo*, we utilized a 96-well reader format that would provide high throughout screening. The cells were grown to log phase and induced with IPTG to provide mRNA transcripts. In contrast to eukaryotic organisms, bacterial cells are permeable to low molecular weight reagents carrying high charge densities, and so addition of such to a growth medium is followed by uptake into the cell. In our case the growth medium was supplemented with copper kanamycin A, varying from 1 to 80 μM, and following incubation at 37 °C for 4 h the cell extracts were collected and the fluorescence at 509 nm was monitored for each reaction (with λ_{ex} of 395 nm). The results of the presumed copper kanamycin A cleavage of GFP-R23 mRNA transcripts have been plotted as fluorescence intensity *vs.* the concentration of copper kanamycin A in the growth medium (Fig. 3). The results show that translation of GFP mRNA carrying the R23 insert is significantly reduced with increasing concentration of copper kanamycin A, reflected by the decrease in fluorescence intensity. These results are consistent with *in vivo* cleavage of the R23 RNA target sequence by copper kanamycin A resulting in a decrease in translatable mRNA transcript. Moreover, the significant reduction in fluorescence indicates that almost all of the mRNA transcript is inactivated. Control experiments following addition of either the metal-free ligand kanamycin A (Fig. 3), or with

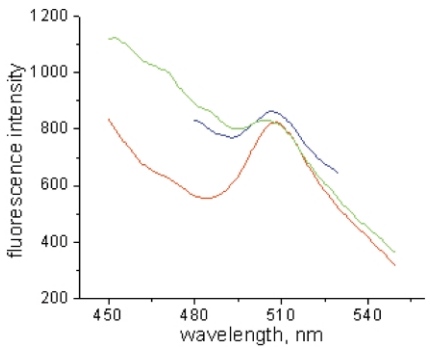

Fig. 2 Fluorescence spectra of cells harboring plasmid with normal GFP (blue), with R23-tagged GFP (red), and from cell extracts containing normal GFP (green). Data was obtained from similar numbers of cells grown under similar conditions. Fluorescence spectra were obtained with $\lambda_{ex} = 395$ nm. The characteristic GFP emission with $\lambda_{em} \sim 509$ nm was observed.

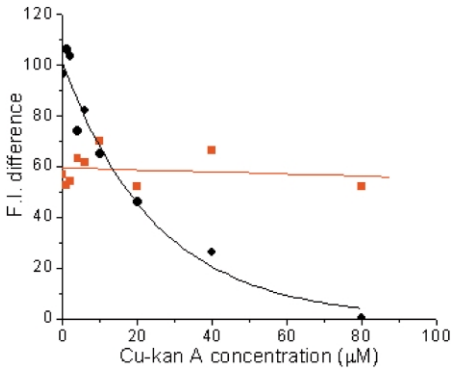

Fig. 3 Intracellular cleavage of GFP-R23 mRNA transcripts by the copper complex of kanamycin A (black) resulting in a decrease in GFP production and fluorescence intensity. The difference in fluorescence intensity (F.I. difference), relative to background, is plotted against the concentration of copper kanamycin A added to the culture medium. Control assay with kanamycin A (red).

either the ligand or copper derivative against GFP lacking the R23 insert, show no change in emission intensity with increasing concentration of ligand or complex. These data eliminate the possible inhibition of translation by kanamycin A itself, or of any other influence on cellular chemistry by the ligand or complex that might prevent formation of GFP. The absence of significant inhibition by the control kanamycin A ligand over the concentration range employed is significant insofar as it demonstrates that mere inhibition of translation through binding of small molecule inhibitors to mRNA target sequences is relatively ineffective at the low concentration regimes required of a drug molecule. A strategy combining recognition, binding, and degradation of a target sequence would appear more effective.

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