ChemComm

Novel reagents for targeted cleavage of RNA sequences: towards a new family of inorganic pharmaceuticals

Alavattam Sreedhara, Anjali Patwardhan and J. A. Cowan*

Evans Laboratory of Chemistry, The Ohio State University, 100 West 18th Avenue, Columbus, Ohio 43210 USA

Received (in Bloomington, IN, USA) 10th February 1999, Accepted 27th April 1999

Copper kanamycin degrades cognate RNA targets at concentrations as low as picomolar levels at physiological pH and temperature, but shows no chemistry with random RNA or DNA molecules, thereby demonstrating potential for development as a novel antiviral agent.

RNA-binding ligands that selectively disrupt RNA–protein interactions are of potential therapeutic value.[1] Both the absence of a cellular repair mechanism for RNA and the structural diversity of RNA motifs make these attractive drug targets.[1,2] Aminoglycoside antibiotics have been demonstrated to inhibit protein translation by recognition of 16S rRNA,[3,4] to inhibit the binding of the HIV Rev protein to its viral RNA recognition element, the Rev response element (RRE),[5] and also to inhibit the activity of hammerhead ribozymes,[6] group I intron ribozymes,[7] and the binding of HIV Tat peptide to TAR-RNA.[8] Solution structures have been determined for RNA complexed with a variety of aminoglycosides (paromomycin, ribostomycin, neamine, gentamicin).[9] Given the cognitive qualities of these molecules, we sought to develop metal derivatives that would not only inhibit protein binding to target RNA molecules, but better, would mediate destruction of the target. Hydrolysis of RNA by metal ion complexes has attracted considerable attention, since sequence-specific agents that catalyze the destruction of mRNA represent a new therapeutic approach.[10] Typically, high concentrations of complex, reaction times of several hours to days, and extremes of temperature and pH are used.[10b,11] Recently, RNA cleavage under milder conditions has been described.[12]

Herein, we report our finding that copper complexes of the aminoglycosides neomycin B and kanamycin A (Fig. 1) exhibit efficient RNA cleavage activity at physiological pH and temperature. Acetylation protection and ^{13}C NMR studies have been reported for the reaction of Cu^{2+} and kanamycin A that support the structure shown.[13] We have isolated the blue Cu^{2+}(kanamycin) complex **1** from the reaction of 1 : 1 kanamycin A and $CuSO_4$, and also Cu^{2+}(kanamycin)$_2$ **2** and Cu^{2+}-neomycin **3** complexes from reaction mixtures of 2 : 1 kanamycin A and $CuSO_4$, and 1 : 1 neomycin B and $CuSO_4$, respectively, as previously described.[14] Complex **1** was found to be stable in aqueous solution for more than one day, however, complexes **2** and **3** were less stable and formed precipitates within 4 h. For this reason, our attention focused on **1**.

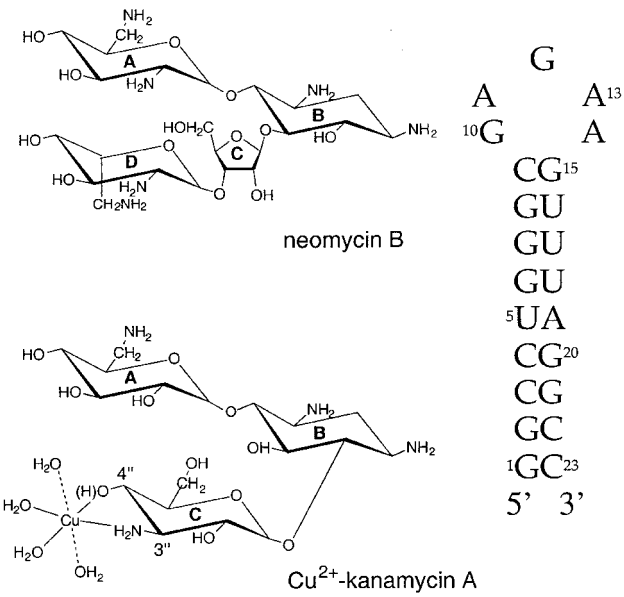

Fig. 1 Structures of the aminoglycosides used in this study (the copper complex of kanamycin A is shown), and a schematic illustration of the stem loop structure adopted by R23 (ref. 16).

A modified 23-mer RNA aptamer (hereafter termed R23) with a high binding affinity for neomycin B[15] (Fig. 1) was selected for a preliminary investigation of cleavage chemistry. The NMR solution structure of the neomycin B complex of R23 shows the aminoglycoside bound to the major groove through electrostatic and hydrogen bonding interactions with the amine and hydroxy groups of rings A and B, while the C and D sugar rings are pendant and can potentially chelate metal ions (Fig. 1).[16] Kanamycin A lacks the ribose ring C and two amine groups in ring D of neomycin B; however, neither ring contributes significantly to binding,[16] and so it is presumed that kanamycin A and neomycin B bind in a similar manner. In fact a comparison with published data indicates that rings A and B appear to form a conserved structural motif for recognition and binding to ribosomal RNA.[9]

Cleavage chemistry of **1** with either 5′-^{32}P end- or body-labeled R23 was examined in the absence and presence of the coreactants, H_2O_2 and ascorbate. R23 was incubated at 37 °C for 30 min to 1 h with various concentrations of **1**, and products were separated on a 19% denaturing polyacrylamide sequencing gel (Fig. 2). The cleavage sites were assigned by comparison with products generated by both alkaline hydrolysis and G-specific RNase T1 digestion. Data in Fig. 2(*a*) shows that cleavage of 5′-end-labeled R23 by **1** alone is not random, but rather specific cleavage sites were observed (lanes 2–4). Two sites of cleavage could be identified: one in the loop region at A^{14}/G^{15}, and the other in the stem region at C^4/U^5. This cleavage data is consistent with the NMR solution structure of R23 RNA bound to neomycin B, which shows binding of the aminoglycoside antibiotic in the loop region $A^{13}A^{14}G^{15}$, and in the stem region $U^5G^6G^7G^8$. The cleaved products co-migrate with products generated by partial alkaline hydrolysis [Fig. 2(*a*), lane 8]. Under these conditions, almost 70% cleavage of R23 is observed under hydrolytic conditions within 1 h.†

Although the hydrolysis of RNA by **1** is appreciable, addition of H_2O_2 or ascorbic acid increase the cleavage efficiency dramatically. Addition of 10 or 100 μM H_2O_2 to 30 μM R23 in the presence of as little as 50 pM **1** led to extensive cleavage—a truly catalytic multiturnover reaction with a ratio of catalyst to substrate of almost 1 : 10^6. Control experiments carried out in the absence of **1** and the presence of up to 100 μM H_2O_2 showed no background cleavage. Moreover, use of aqueous Cu^{2+} and H_2O_2 at concentrations where **1** demonstrated cleavage of R23 were ineffective for the former. Addition of 200 μM ascorbic acid to R23 treated with 20 nM **1** also showed extensive cleavage with almost 100% digestion of the parent RNA [Fig. 2(*b*], however, the relative concentrations of **1** required to effect cleavage of R23 in the presence of H_2O_2 and ascorbate indicates

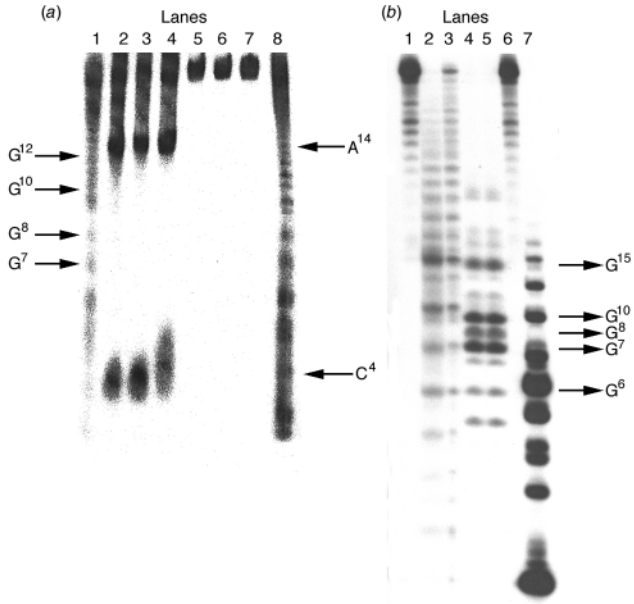

Fig. 2 (*a*) Autoradiograph of a 19% denaturing polyacrylamide electrophoretic gel for the cleavage of 5′-end-labeled R23 RNA by **1** at 37 °C for 2 h (ref. 18). Lane 1, R23 + 0.5 units RNase T 1; lane 2, R23 + 10 μM **1**; lane 3, R23 + 50 μM **1**; lane 4, R23 + 100 μM **1**; lane 5, R23; lane 6, R23 + 100 μM $CuSO_4$; lane 7, R23 + 100 μM kanamycin. Cleavage reactions were carried out in Tris-HCl (20 mM; pH 7.5) and contain *ca.* 1 μM RNA. Captions on the left indicate G-specific cleavage by RNase T1, while those on the right indicate sites giving rise to cleavage products of R23 following reaction with **1**. (*b*) Autoradiograph of a 19% denaturing polyacrylamide electrophoretic gel for the cleavage of uniformly-labeled R23 RNA by **1** at 37 °C for 1 h. Lane 1, R23; lane 2, R23 + 0.1 M NaOH; lane 3, R23 + Na_2CO_3/$NaHCO_3$ buffer; lane 4, R23 + 0.25 units RNase T 1; lane 5, R23 + 0.5 units RNase T1; lane 6, R23 + 1 μM $CuSO_4$/100 μM ascorbate; lane 7, R23 + 20 nM **1**/100 μM ascorbate. Cleavage reactions were carried out in Tris-HCl (20 mM; pH 7.5) and contain *ca.* 10 nM RNA. Captions on the right indicate G-specific cleavage by RNase T1 in lanes 4 and 5. Standard procedures for ^{32}P-labeling of R23 RNA, alkaline and RNase T1 digestion, and SDS-PAGE experiments were used (ref. 18).

that the former is more efficient in promoting oxidative damage. The products generated from ascorbate induced cleavage with **1** [Fig. 2(*b*), lane 7] were more numerous (most likely indicative of limited hydroxyl radical migration away from the copper ion), and offset relative to bands generated by backbone hydrolysis, consistent with a distinct oxidative cleavage pathway.‡ Again, control experiments show no background cleavage in the absence of **1** but in the presence of aqueous Cu^{2+} and ascorbate [Fig. 2(*b*), lane 6]. Recently we have demonstrated that **1** mediates oxidative cleavage of DNA through reactive copper-oxo or copper-hydroxo species.[14] Similarly, here we have used a spectrophotometric assay to identify formation of hydroxyl radical by use of rhodamine B.[14] The change in the λ_{552} absorbance from rhodamine was monitored in the presence of **1** (or reducing agents) alone, and with added ascorbate or H_2O_2. While **1** (or reducing agents) alone did not result in degradation of the dye, a combination of **1** with ascorbate or H_2O_2 led to a rapid decrease in λ_{552}, demonstrating that **1** is capable of producing reactive oxygen species only in the presence of reducing agents. These reactive oxygen species are capable of mediating oxidative damage to nucleic acids,[14] however, such hydroxyl radicals could not be trapped by rhodamine in the presence of a nucleic acid substrate. This indicates that the radicals formed are localized on or close to the copper ion, and are non-diffusible. Thus, the products from reaction of **1** with R23 RNA in the presence of H_2O_2 or ascorbic acid are indicative of an oxidative mechanism, mediated by a copper redox couple, with the concomitant release of hydroxyl radicals.

A primary reason for cleavage efficiency at low complex concentration arises from the high binding affinity for an R23 target site.[3,15,16] No cleavage products were observed after treating R23 with comparable concentrations of either aqueous Cu^{2+} or metal-free aminoglycoside alone (Fig. 2). To determine if cleavage by **1** was selective toward this particular structured RNA (R23), we examined the cleavage efficiency of linear oligo($A_{12\text{-}18}$) and poly(C), and double-strand poly(A).poly(U), each of which binds aminoglycosides weakly. No cleavage was detected by an FPLC assay, supporting the notion that tight binding of **1** to a target RNA is required for cleavage. Similar cleavage efficiencies for plasmid DNA were observed only at much higher complex concentrations (0.1 to 0.5 μM) as a result of the significantly lower binding affinity relative to a cognate structured RNA motif.[14] Artificial ribonucleases based on oligonucleotide-appended metal-based Lewis acidic functional groups represent an antisense approach for effective RNA cleavage.[12] Recognition of structured RNA targets[17] over linear RNA and DNA sequences by aminoglycoside antibiotics, coupled with the site-directed degradative capacity of their copper complexes, may render metalloaminoglycosides as novel and attractive therapeutic agents for the treatment of viral disease, and provide an alternative to the antisense approach. Experiments to test the antiviral activity of such complexes are in progress.

Supported in part by the National Science Foundation (CHE-9706904). J. A. C. is a Camille Dreyfus Teacher-Scholar (1994–1999). A. S. thanks OSU for the award of a University Postdoctoral Fellowship (1997–1998). We thank D. R. Schoenberg and K. Cunningham (Department of Pharmacology, OSU), and M. Foster (Department of Chemistry, OSU) for experimental advice and assistance with gel electrophoresis.

Notes and references

† Also consistent with efficient cleavage, under both oxidative and hydrolytic conditions, is the rapid increase in absorbance in UV–VIS spectra resulting from loss of hypochromism from interacting base pairs.
‡ Strand cleavage by a hydrolytic mechanism that is mediated through a 2′-OH leads to formation of 5′-OH and a terminal phosphate at the cleavage site. Cleavage by an oxidative path can lead to destruction of the ribose ring and base release, resulting in band positions that are offset from hydrolytic controls, as observed in Fig. 2(*b*) (lane 7).

1 T. Hermann, and E. Westhof, *Curr. Opin. Biotechnol.*, 1998, **9**, 66; K. Michael and Y. Tor, *Chem. Eur. J.*, 1998, **4**, 2091.
2 N. D. Pearson and C. D. Prescott, *Chem. Biol.*, 1997, **4**, 409.
3 P. Purohit and S. Stern, *Nature*, 1994, **370**, 659.
4 D. Moazed and H. F. Noller, *Nature*, 1987, **327**, 389.
5 M. L. Zapp, S. Stern and M. R. Green, *Cell*, 1993, **74**, 969.
6 T. K. Stage, K. J. Hertel and O. C. Uhlenbeck, *RNA*, 1995, **1**, 95.
7 U. Von Ahsen, J. Davies and R. Schroeder, *J. Mol. Biol.*, 1992, **226**, 935.
8 H.-Y Mei, A. A. Galan, N. S. Halim, D. P. Mack, D. W. Moreland, K. B. Sanders, H. N. Truong and A. W. Czarnik, *Bioorg. Med. Chem. Lett.*, 1995, **5**, 2755.
9 D. Fourmy, M. I. Recht, S. C. Blanchard and J. D. Puglisi, *Science*, 1996, **274**, 1367; M. I. Recht, D. Fourmy, S. C. Blanchard, K. D. Dahlquist and J. D. Puglisi, *J. Mol. Biol.*, 1996, **262**, 421.
10 (*a*) B. N. Trawick, A. T. Daniher and J. K. Bashkin, *Chem Rev.*, 1998, **98**, 939 and references cited therein; (*b*) J. K. Bashkin, E. I. Frovola and U. Sampath, *J. Am. Chem. Soc.*, 1994, **116**, 5981.
11 E. L. Hegg, K. A. Deal, L. L. Kiessling and J. N. Burstyn, *Inorg. Chem.*, 1997, **36**, 1715.
12 D. Magda, M. Wright, S. Crofts, A. Lin and J. Sessler, *J. Am. Chem. Soc.*, 1997, **119**, 6947; R. Haener, J. Hall, A. Pfuetzer and D. Huesken, *Pure Appl. Chem.*, 1998, **70**, 111; H. Inoue, T. Furukawa, M. Shimuzu, T. Tamura, M. Matsui and E. Ohtsuka, *Chem. Commun.*, 1999, 45.
13 S. Hanessian and G. Patil, *Tetrahedron Lett.*, 1978, 1031 and 1035.
14 A. Sreedhara and J. A. Cowan, *Chem. Commun.*, 1998, 1737.
15 M. G. Wallis, U. von Ahsen, R. Schroeder, and M. Famulok, *Chem. Biol.*, 1995, **2**, 543.
16 J. A. Cowan, D. Wang, K. Natarajan and T. Ohyama, manuscript submitted.
17 C. S. Chow and F. M. Bogdan, *Chem. Rev.*, 1997, **97**, 1489.
18 J. F. Milligan and O. C. Uhlenbeck, *Methods Enzymol.*, 1989, **180**, 51.

Communication 9/01140D