Cleavage of RNA oligonucleotides by aminoglycosides†‡

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A number of aminoglycoside antibiotics, and in particular neomycin B, are demonstrated to promote strand cleavage of RNA oligonucleotides (minimised HIV-1 TAR element and prokaryotic ribosomal A-site), by binding and causing sufficient distortion to the RNA backbone to render it more susceptible to intramolecular transesterification.

Ribonucleic acids (RNA) are central to replication and continuation of life. They are involved in genetic storage (retro-viruses),[1] 'enzymatic' activity (ribozymes),[2] protein synthesis (ribosome),[3,4] gene regulation (RNAi, riboswitches),[5,6] and the transferal of genetic information (mRNA). This functional biomolecule has been shown to be a viable drug target,[1,6,7] and as a consequence, understanding its interactions with small molecules is vitally important.

Aminoglycosides are a particularly well-studied class of RNA-binding molecules, which are essentially *pseudo*-oligosaccharides incorporating numerous amine groups within their structure (Fig. 1).[8] These molecules are best known for their antibiotic properties, which derive from their ability to selectively bind to bacterial ribosomal RNA (rRNA), disrupting protein biosynthesis.[3,9] In addition to their ability to bind rRNA,[3,10] aminoglycosides are also capable of interacting with other RNA targets.[11,12] Examples include various portions of the HIV RNA genome, such as the trans-activation response (TAR) element,[13–16] the rev-response element (RRE),[17] and the dimerisation initiation site (DIS).[18,19]

Although it is generally assumed that aminoglycosides interact with their RNA targets in a reversible, non-covalent and non-destructive fashion, a handful of examples illustrate other effects. Tor *et al.* have reported that neomycin B hydrolyses the phosphate ester bonds in a di-ribonucleoside monophosphate (ApA).[20] $tRNA^{Phe}$ was also found to be susceptible to aminoglycoside-mediated cleavage.[21] More recently, Pandey and coworkers have demonstrated that a neamine moiety (rings I and II in neomycin B, see Fig. 1) attached to a peptide nucleic acid (PNA) sequence designed to target the HIV-1 TAR RNA, was able to induce RNA strand cleavage.[22]

Inspired by these findings, we set out to further explore the hydrolytic potential of aminoglycosides. Here, we demonstrate that neomycin B, kanamycin B and some other aminoglycosides not only bind strongly to short constructs of the HIV-1 TAR element and prokaryotic rRNA decoding A-site (Fig. 2), but are also capable of carrying out the site-specific cleavage of these motifs at significant rates under mild conditions. We postulate that a number of aminoglycosides promote cleavage of the target RNA by altering the RNA's conformation upon binding, rendering certain phosphodiester bonds more susceptible to an intramolecular transesterification reaction.

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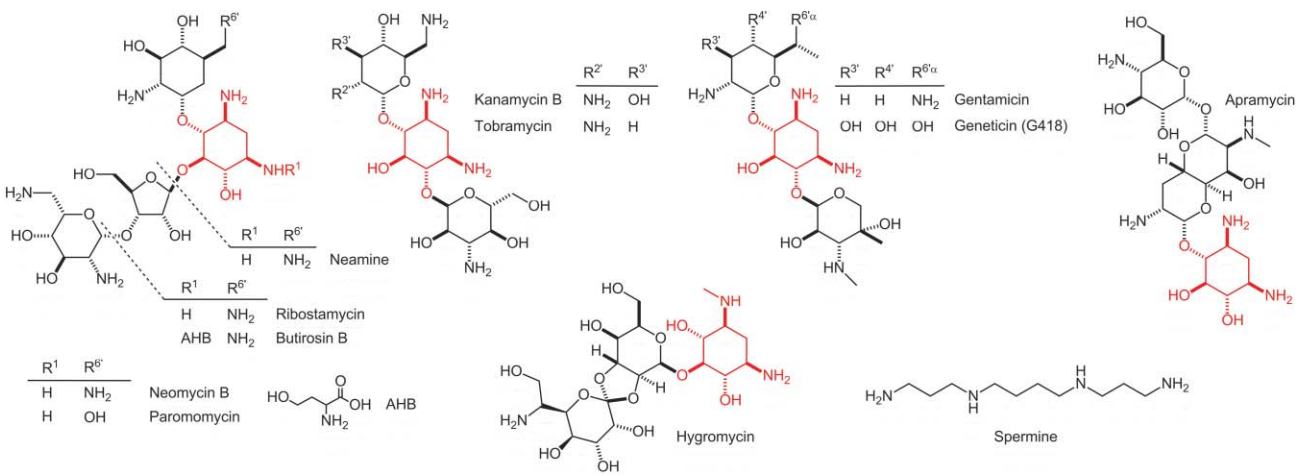

Fig. 1 Structures of aminoglycosides containing the 2-deoxystremptamine (2-DOS) core (highlighted in red) used in the screening study.

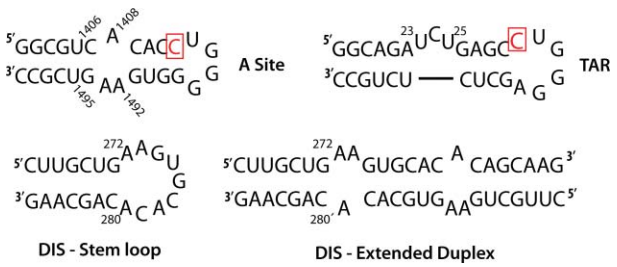

Fig. 2 The structures of the three RNA constructs used in this study: the minimised prokaryotic ribosomal A-site, HIV-1 TAR element, and HIV-1 DIS (stem-loop and extended duplex forms). Highlighted in red are the observed sites of aminoglycoside-induced cleavage.

To probe the phenomenon of aminoglycoside-induced RNA cleavage, neomycin B was initially incubated with ^{32}P end-labelled TAR and A-site constructs. To exclude metal-mediated hydrolysis and radical-induced strand cleavage, control experiments included the addition of 10 mM EDTA as a metal chelator and 10% DMSO (v/v) as a radical scavenger. As shown in Fig. 3, a single major cleavage product was observed in all cases. For the neomycin B–TAR reaction, the extent of the major strand cleavage after 12 hours of reaction was found to be 4.5%, while for the neomycin–A-site reaction, a lower amount of strand scission (ca. 1%) was observed (Fig. 3; see also Table S1 in the ESI‡). The extent of oligonucleotide cleavage increased with increasing neomycin B concentration, clearly highlighting that cleavage is directly related to the antibiotic. Strand scission was observed even in the presence of DMSO or EDTA, suggesting that the reaction does not proceed through an oxidative or metal ion-promoted mechanism.

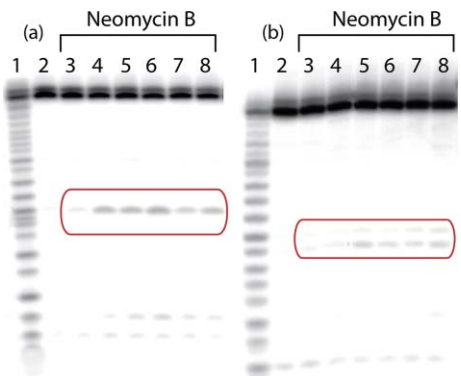

Fig. 3 PAGE analysis of ^{32}P end-labelled TAR (a) and A-site (b) after incubation with neomycin B, showing a single major cleavage product in each case (highlighted in red). Conditions: 1 μM ^{32}P end-labelled TAR or A-site, 20 mM MOPS (pH 6.5 @ 21 °C), 100 mM NaCl, 12 h reaction time for TAR, 24 h reaction time for A-site, 21 °C. lane 1: alkaline hydrolysis ladder; lane 2: RNA only; lane 3: 1 μM neomycin B; lane 4: 7.5 μM neomycin B; lane 5: 15 μM neomycin B; lane 6: 25 μM neomycin B; lane 7: 25 μM neomycin B, 10 mM EDTA (control); lane 8: 25 μM neomycin B, 10% v/v DMSO (control).

To evaluate the time dependency of the cleavage reaction, ^{32}P end-labelled TAR was reacted under *pseudo*-first-order conditions by using neomycin B in large excess compared to the RNA. Samples were quenched at specific time intervals and analysed by PAGE. The *pseudo*-first-order rate constant of 6.1(3) × 10^{-5} s^{-1} (corresponding to a second-order rate constant of 1.2(6) $M^{-1}s^{-1}$) extracted from this experiment indicates that cleavage of the TAR construct by neomycin B occurs at a much more rapid rate than the background cleavage of the TAR in buffer. For this control experiment, the phosphorimage of the gels showed that cleavage after 72 hours is barely observable (see Fig. S4 in the ESI‡) under the same conditions. Significantly, the first-order rate constant is well over two orders of magnitude faster than the cleavage of di-ribonucleotide (ApA) by neomycin B, for which a rate constant of 2.7×10^{-7} s^{-1} was measured at a pH of 8.0 and temperature of 50 °C (as compared to this study, which was performed at a pH of 6.5 and temperature of 21 °C).[20] Moreover, the current experiments were performed using a much smaller concentration of neomycin B (50 μM, as compared to 0.3 M in the earlier study). Assuming a first-order dependence of the cleavage rates on neomycin B concentration, this indicates that neomycin B is more than one million times more efficient at inducing cleavage of TAR than that of ApA.

To ascertain the exact location of the scission point for the reaction of the aminoglycosides with both the TAR and A-site constructs, RNase sequencing experiments were performed. PAGE analysis of degradation by RNase T1 (3′→G) and RNase A (3′→C/U), for both RNA targets, located the cleavage point as the stem loop junction of the RNA strands, between C and U (Fig. 1). This was confirmed for the TAR sequence *via* detailed chemical sequencing experiments (see Fig. S1 and S2 in the ESI‡). Co-migration of the cleavage band with the alkaline hydrolysis generated band suggests the formation of a 2′-3′-cyclic phosphodiester, supporting a hydrolytic mechanism (see Fig. S2 in the ESI‡). Of note here is that the main cleavage point observed for both RNA constructs is not at the proposed high affinity binding site. A solution NMR structure proposed by Faber *et al.*[23] showed neomycin B binding at the triplet nucleotide (^{23}U^{24}C^{25}U) bulge, and a crystal structure has shown that neomycin binds at the doublet adenosine bulge in the A-site.[24] However, the observed site of cleavage is located at the beginning of the stem-loop on both constructs, suggesting the cleavage may not be a direct chemical reaction induced by the aminoglycoside. This is also confirmed by the fact that in the background measurements of RNA degradation, the site of cleavage for the TAR construct is observed at the same region, albeit at a substantially slower rate (see Table 1).

To examine the pH dependence of neomycin B-induced RNA cleavage, a series of cleavage reactions were carried out, buffered over a range of different pHs (Fig. 4). The greatest amount of

Table 1 Observed rate constants for the cleavage of the TAR by neomycin B, kanamycin B and the flexible polyamine, spermine. Conditions: 1 μM TAR (spiked with ^{32}P end-labelled TAR), 50 μM aminoglycoside/polyamine, 20 mM MOPS (pH 6.5 @ reaction temp), 100 mM NaCl. Reaction monitored over a period of 72 h by PAGE. (Standard error indicated by parentheses)

	k_{obs} (× 10^5 s^{-1}) (pH 6.5)
T = 21 °C (background)	N/O[a]
T = 37 °C (background)	0.41(3)
Neomycin B	6.1(3)
Kanamycin B	1.7(1)
Spermine	0.35(3)

[a] N/O: not observed (reaction too slow to obtain rate constant).

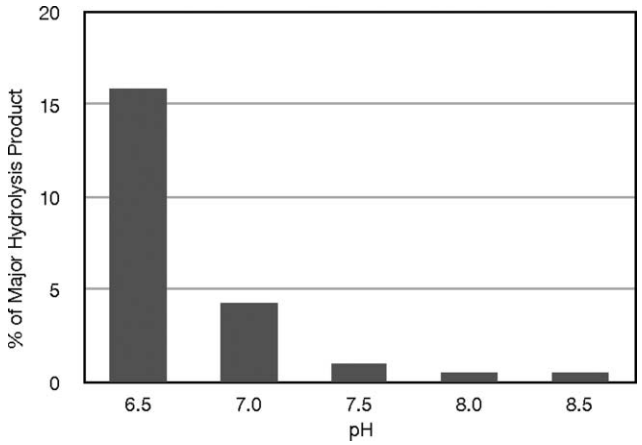

Fig. 4 Relative phosphorimage density counts (%) for the major TAR cleavage product as a function of pH. Reaction conditions: 1 μM TAR (spiked with ^{32}P end-labelled TAR), 25 μM neomycin B, 20 mM MOPS (pH 6.5, 7.0, 7.5 or 8.0 @ 21 °C) or 20 mM HEPES (pH 8.5 @ 21 °C), 100 mM NaCl, 24 h reaction time, 21 °C.

cleavage was observed at pH 6.5, the lowest pH tested. The extent of cleavage was three-fold lower at pH 7.0, and decreased to negligible levels at pH 8 and above. These results correlate with the higher degree of protonation of neomycin B, and the greater affinity of the aminoglycoside for the RNA targets, at lower pH, as discussed below.

Ligand binding to the two RNA targets was probed using fluorescence-based assays. For this purpose, we employed the TAR stem loop construct of Marino and co-workers,[13] in which cytosine-24 is substituted by a fluorescent 2-aminopurine (2AP), as well as a fluorescent construct of the A-site described by Tor and co-workers,[25] which contains a furan-decorated uracil (U^{Fu}).§ The emissive properties of both modified nucleobases are highly sensitive to their microenvironment, which has previously been shown to allow the accurate measurement of the strength of interaction between aminoglycosides and the two RNA constructs.

Table 2 summarises the EC_{50} and K_a values measured for neomycin B binding to the two RNA constructs at varying pH using the assays described. The binding results for the interaction of neomycin B with the A-site are comparable to those obtained in previous studies,[25] and indicate that this interaction is not as favourable as the neomycin B–TAR interaction at low pH. The EC_{50} values for neomycin B binding to the TAR element were found to vary strongly with pH, suggesting that binding is dependent on the protonation state of the ligands. This is consistent with the fact that neomycin B has a pK_{a1} of 6.2 (N3),[15,26] such that it would predominately be protonated (6+ overall charge)

Table 2 EC_{50} and K_a values for the binding of neomycin B to the A-site (1 μM) and TAR (2 μM) constructs, as determined by fluorescence titrimetry (standard deviations given in parentheses)

	A-site-1406(U^{Fu})		TAR-24(2AP)	
pH	[EC_{50}] (μM)	K_a ($\times 10^5$ M^{-1})	[EC_{50}] (μM)	K_a ($\times 10^5$ M^{-1})
6.5	1.06(2)	4.7(1)	0.3(1)	16(5)
7.0	0.81(1)	6.2(1)	0.65(2)	7.7(2)
7.5	1.4(5)	3(1)	1.55(2)	3.2(1)
8.0	2.2(1)	2.3(1)	4.5(5)	1.1(2)

at the lowest pH value tested, leading to enhanced RNA affinity. This binding result also lends insight into the pH dependence of the strand scission reaction, indicating that cleavage could be driven by the affinity of the aminoglycoside for its RNA target. The binding of neomycin B to the A-site was found to be much less sensitive to pH, suggesting that the strength of the interaction might be more dictated by shape complementarity in this instance, rather than only electrostatics (protonation state of the ligand).[12] We note, however, that there is a slight increase in the binding of neomycin to the A-site at pH 7.

The crucial link between binding and strand cleavage was borne out by the results of a screening experiment (Fig. 5), in which a wide range of different aminoglycosides (Fig. 1) were reacted with the TAR construct for a fixed time period. RNA cleavage was most pronounced for the aminoglycosides that have been shown to bind strongly to TAR,[27] namely those belonging to the neomycin family (butirosin, paramomycin and neomycin B), containing 4 amino-sugar rings (3 in the case of butirosin), and kanamycin B and tobramycin from the kanamycin family. Further experiments revealed the rate of cleavage of TAR by kanamycin B at pH 6.5 to be three-fold lower than for neomycin B (Table 1; see also Fig. S3 in the ESI‡). This can be directly attributed to the respective K_a values for the binding of neomycin B and kanamycin B, which indicate that kanamycin B binds to the TAR more weakly.[27]

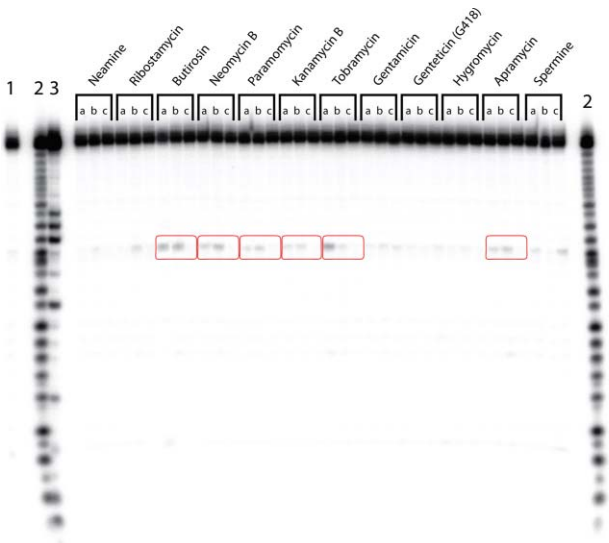

Fig. 5 PAGE gel analysis of ^{32}P end-labelled TAR after incubation with various aminoglycosides. Conditions: 1 μM TAR (spiked with ^{32}P end-labelled TAR), 50 μM aminoglycoside, 20 mM MOPS (pH 6.5 @ 21 °C), 100 mM NaCl, 5% v/v DMSO (control reactions only), 5 mM EDTA (control reactions only), 24 h reaction time, 21 °C. Lane 1: TAR in buffer only; lane 2: alkaline hydrolysis ladder; lane 3: T1 RNase digestion of TAR (denaturing conditions); lane a: aminoglycoside/TAR; lane b: aminoglycoside/TAR/DMSO; lane c: aminoglycoside/TAR/EDTA.

We hypothesise that the enhanced cleavage of the TAR and A-site constructs might be due to the aminoglycosides inducing conformational strain on certain phosphate diester bonds within the RNA structures, in a manner reminiscent of the Hammerhead Ribozyme,[28] rendering them more susceptible to internal transesterification by the 2′-OH in the ribose backbone. Previous work with TAR has shown that it is a flexible RNA substrate, able to

adjust its structure in order to accommodate ligand binding.[14] In this study, evidence for RNA conformational changes was obtained from circular dichroism (CD) titrations (Fig. 6), which showed that the CD spectrum of TAR changes upon the addition of increasing amounts of neomycin B. It is conceivable, however, that secondary binding at high aminoglycoside concentrations is contributing to the observed cleavage.

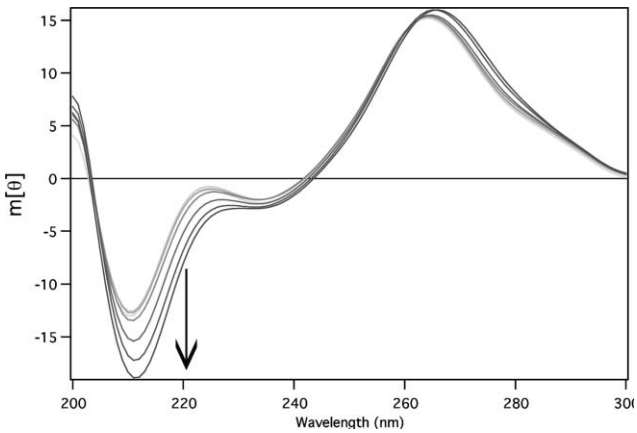

Fig. 6 CD titrations for neomycin B binding to the TAR construct. Lightest shade is the lowest concentration of aminoglycoside and darkest is the highest. The arrow shows the increasing intensity of the band at 213 nm with increasing aminoglycoside concentration. Conditions: 200 μM TAR, 0–190 μM neomycin B, 10 mM MOPS (pH 6.5 @ 21 °C), 100 mM NaCl, 21 °C.

Evidence to support our hypothesis of strain-induced cleavage is provided by the fact that (i) spermine, a flexible linear tetraamine that would not be expected to distort RNA upon binding, was found to cleave the TAR target at a significantly slower rate than that achieved by the aminoglycosides (Table 1 and Fig. 5), and (ii) another important RNA motif, the HIV-1 DIS, which has been shown to form a more 'rigid' extended duplex that is more discriminating to potential small molecule binding,[18] showed no evidence of cleavage upon incubation with neomycin B or kanamycin B (see Fig. S4 in the ESI‡).

In summary, we have shown that upon binding to certain folded RNA constructs, some aminoglycosides have the ability to exploit Achilles' heels in RNA structures and promote strand cleavage. This is most pronounced with a TAR construct, whose structure presumably distorts in such a way that significant strain is placed upon a phosphate ester linkage, making inline transesterification more facile. The extent of cleavage was found to be greatest at lower pH, coincident with the strongest aminoglycoside binding. Taken together with previous observations, these results suggest that certain effects of aminoglycosides on RNA targets may be due to their ability to facilitate strand cleavage. While speculative at this point, this is a testable hypothesis that can fuel further experimentation.

Notes and references

§ 2AP labelled A-site has been shown not to be as useful for the detection of neomycin B for this particular RNA construct, see reference 25.

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