Scanning conformational space with a library of stereo- and regiochemically diverse aminoglycoside derivatives: the discovery of new ligands for RNA hairpin sequences†

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Received 21st December 2006, Accepted 8th February 2007
First published as an Advance Article on the web 27th February 2007
DOI: 10.1039/b618683a

A library of stereo- and regiochemically diverse aminoglycoside derivatives was screened at 1 μM using surface plasmon resonance (SPR) against RNA hairpin models of the bacterial A-site, and the HIV viral TAR and RRE sequences. In order to double the stereochemical diversity of the library, the compounds were screened against both enantiomers of each of these sequences. Remarkably, this initial screen suggested that the same four aminoglycoside derivatives bound most tightly to all three of the RNAs, suggesting that these compounds were good RNA binders which, nonetheless, discriminated poorly between the RNA sequences. The interactions between selected isomeric aminoglycoside derivatives and the RNA hairpins were then studied in more detail using an SPR assay. Three isomeric tight-binding aminoglycoside derivatives, which had been identified from the initial screen, were found to bind more tightly to the RNA hairpins (with K_D values in the range 0.23 to 4.7 μM) than a fourth isomeric derivative (which had K_D values in the range 6.0 to 30 μM). The magnitude of the tightest RNA–aminoglycoside interactions stemmed, in large part, from remarkably slow dissociation of the aminoglycosides from the RNA targets. The three tight-binding aminoglycoside derivatives were found, however, to discriminate rather poorly between alternative RNA sequences with, at best, around a twenty-fold difference in affinity for alternative RNA hairpin sequences. Within the aminoglycoside derivative library studied, high affinity for an RNA target was not accompanied by good discrimination between alternative RNA sequences.

Introduction

The aminoglycoside antibiotics (such as **1–5**) interfere with protein synthesis by interacting with 16S rRNA within the 30S subunit of the bacterial ribosome, thereby inhibiting translation and causing miscoding.[1] The structural basis of recognition of aminoglycosides by the prokaryotic A site has been determined.[2] In addition, the aminoglycosides bind to a range of other RNA sequences: aminoglycosides inhibit the splicing of group I introns,[3] and can disrupt key protein–RNA recognition events, for example, the formation of the RRE–Rev[4] and TAR–Tat[5] complexes required in the life cycle of the HIV virus.

The binding between aminoglycosides and their RNA targets is, however, often not particularly specific. In one study, the specificity of aminoglycoside–RNA hairpin interactions was investigated in detail:[6] the affinity of five aminoglycosides—tobramycin, kanamycin A, kanamycin B, dibekacin and amikacin—for four RNA hairpins—three variants of an aptamer selected to bind tobramycin, j6, and an A-helix stem without bulges, HpB‡—was measured. Several equivalents of the aminoglycosides were found to bind to each equivalent of the RNA hairpins. However, the first equivalent of each aminoglycoside was found to bind similarly tightly to all four hairpins; in fact, the binding affinity depended, in large part, only on the charge of the aminoglycosides, with an approximate 10-fold increase[7,8] in (non-specific) electrostatic interaction associated with each additional (protonated) amine. Thus, the hydrogen bonds that are formed between the hydroxyl groups on the aminoglycosides and the RNA do not contribute significantly to the affinity: the aminoglycosides studied did not, therefore, display significant RNA sequence selectivity, and were unable to discriminate between the three aptamers and the RNA fragment HpB.

Rather low specificity is also observed in the interactions between the aminoglycosides and RNA hairpin models of the prokaryotic A-site.[7] For example, kanamycin B and tobramycin, which both have a 4,6-disubstituted 2-deoxystreptamine ring, have low (2- to 4-fold) specificity for the A-site sequence relative to its U1495A variant (see Fig. 1 for the sequences of these RNAs). In contrast, neomycin B and paromomycin, which both have a 4,5-disubstituted 2-deoxystreptamine ring, bind up to 20 times

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† Electronic supplementary information (ESI) available: Data on the preliminary screening of the aminoglycoside derivative library, kinetic parameters for binding of aminoglycoside derivatives to RNA sequences, experimental procedures, and mass spectra of RNA sequences. See DOI: 10.1039/b618683a

‡ Hairpin B, HpB, comprising a structured GAAA tetraloop and an A-helix stem without bulges, was designed as a reference sequence to evaluate the properties of the j6 hairpin series. The sequence of HpB was 5′-GGCGAUACCAAGCCGAAAGGCUUGGUAUCGCCA-3′ (ref. 6).

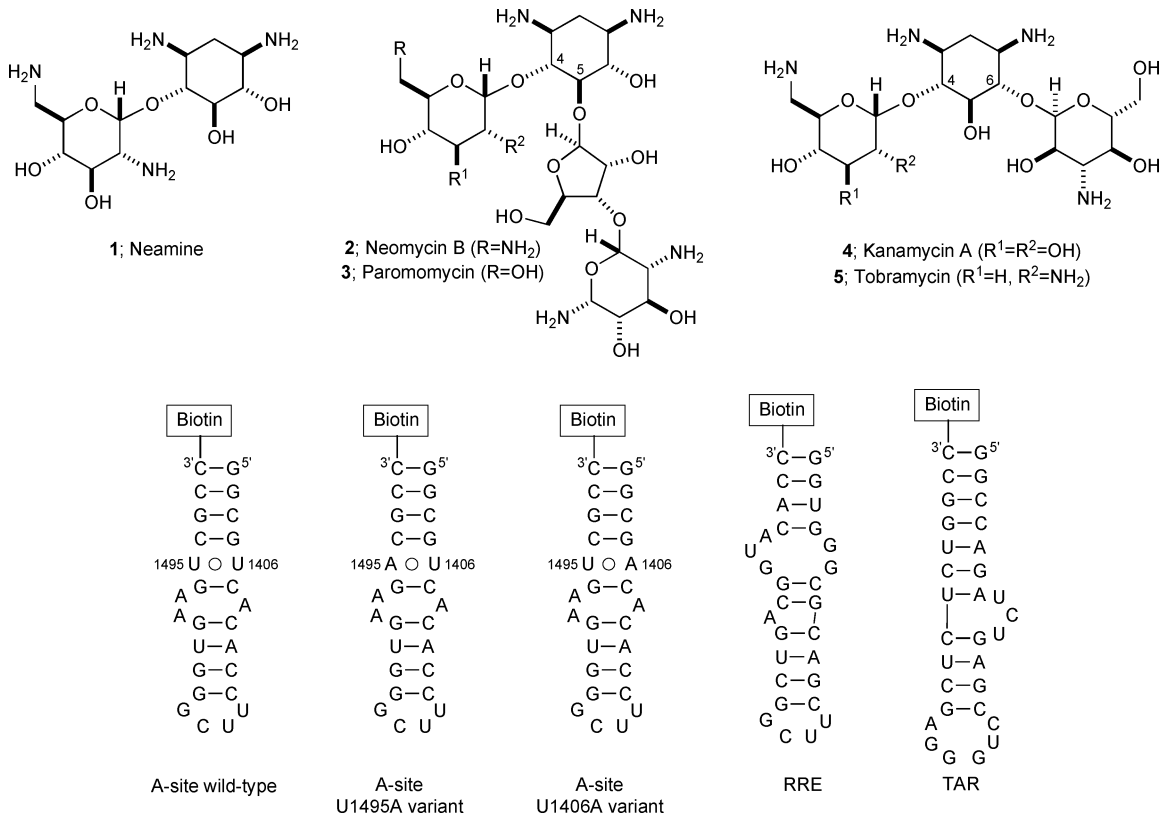

Fig. 1 RNA hairpins used in this study. Both enantiomers of the A-site, TAR and RRE RNAs were prepared by solid-phase oligonucleotide synthesis.

more tightly to the A-site sequence than to its U1495A variant. In *E. coli*, the U1495A mutation confers low levels of resistance to neomycin, tobramycin and gentamicin, and a high level of resistance to paromomycin.[9]

Although aminoglycosides generally bind with similar affinities to structurally diverse RNA targets, nucleobase–aminoglycoside conjugates have been discovered with selectivity for the TAR sequence over the A-site sequence.[10] In addition, a cyclic aminoglycoside derivative has been shown to recognise the TAR sequence by interacting simultaneously with the bulge residues required for Tat binding, and the A35 residue of the hexanucleotide loop.[11]

We have screened a library of aminoglycoside derivatives[12]§ (see Fig. 2 and 3) against RNA hairpin models of the prokaryotic A-site and the viral RNA sequences RRE and TAR. Similar models of RNA binding sites have previously been used to understand the structure and function of complexes of the aminoglycosides and larger RNA targets found *in vivo*.[7,13c,16–18] Within the library, the configuration and regiochemistry of the aminoglycoside derivatives were widely varied, allowing regions of conformational space, which are not available to the natural products, to be explored. In addition, we effectively doubled the stereochemical diversity of the library by measuring the affinity of each aminoglycoside derivative for *both* enantiomers of each RNA hairpin; in this way, the affinities of both enantiomers of each aminoglycoside derivative could be inferred without doubling the synthetic effort required. The pattern of amino groups can modulate the structure, dynamics and function of natural aminoglycosides.[19]

The library was designed such that many of the library members were isomeric, having the same number of amino groups. It was hoped that the comparison of largely isomeric compounds would allow compounds which recognised each RNA target specifically to be identified. Previously, libraries of stereo- and regioisomeric ligands, probing large areas of conformational space, have been used to identify unnatural ligands for macromolecular targets;[20a] for example, two compounds with higher affinity for a bacterial lectin than its natural ligand were identified from a 1300 member library of acylated amino di- and tri-saccharides.[20b]

Screen of the library of stereo- and regiochemically diverse aminoglycoside derivatives

The library of stereo- and regiochemically diverse aminoglycoside derivatives was screened against models of the bacterial ribosomal A-site, and the viral TAR and RRE sequences (see Fig. 1).[7,13c,16,17] The compounds were also screened against the enantiomeric RNA sequences to allow the affinities of the enantiomeric compounds for the natural targets to be inferred indirectly.

Surface plasmon resonance (SPR) was used to screen for binding of each aminoglycoside to each of the RNA targets.[7,13c,16,17] The biotinylated RNAs were immobilised on streptavidin-coated sensor chips, and a regeneration buffer was injected to remove any unbound RNA. The affinity of kanamycin A, a 4,6-disubstituted aminoglycoside with four amino groups, for a range of RNA targets has been determined previously by surface plasmon

§ A wide range of aminoglycoside derivatives have previously been prepared in which the substitution[8,13] of one or more carbohydrates[14] has been varied systematically. In addition, orthogonally protected sugar diamino acids have been exploited as building blocks in the synthesis of linear and branched aminoglycoside derivatives.[15]

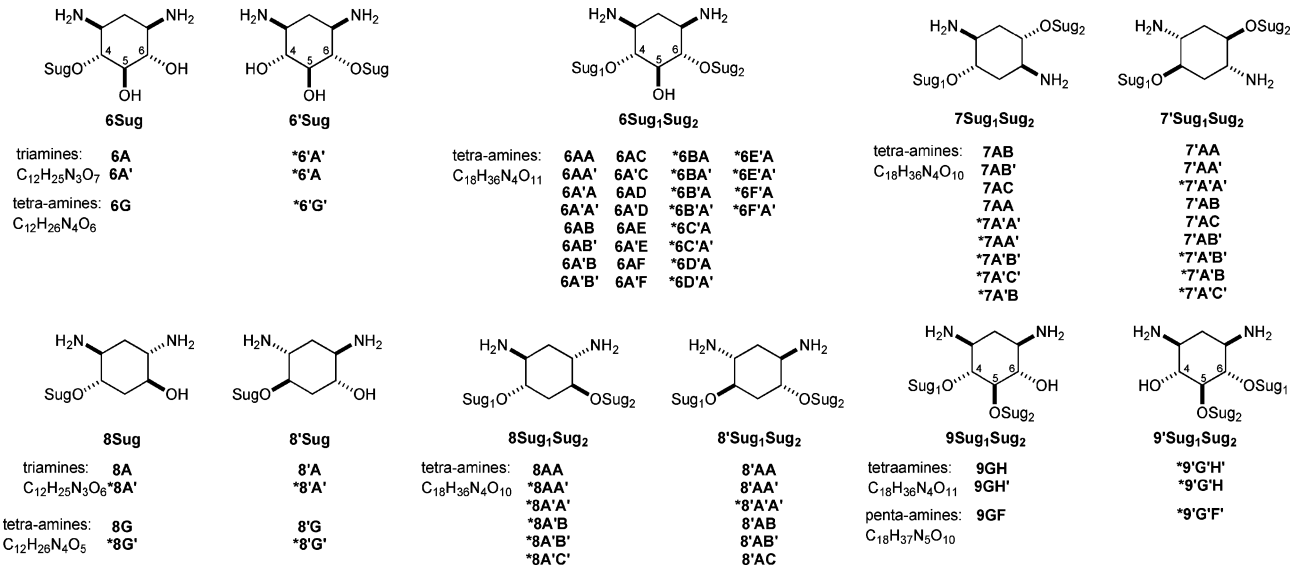

Fig. 2 Aminoglycoside derivatives screened in this study. The compounds indicated with an asterisk were not prepared; instead, the enantiomeric compounds were screened against enantiomeric RNA targets. See Fig. 3 for the range of modified glycosyl substituents A–G and A′–G′ explored.

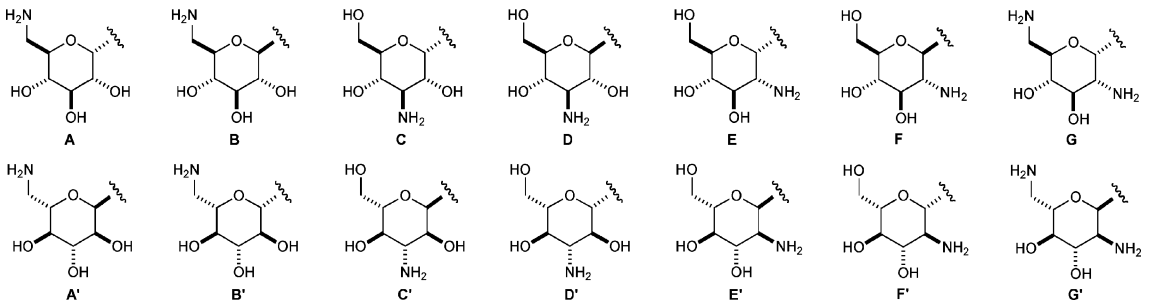

Fig. 3 Variations in the sugar substituents.

resonance (K_D = 18 μM for the A-site;[7] K_D in the range 4–11 μM for three variants of the aptamer j6;[6] $K_D = 20 \pm 2$ μM for the hairpin HpB[6]). An aim of our study was to identify ligands which were similar in structure to kanamycin A, but which, nonetheless, bound more tightly to the RNA hairpins. Most of the aminoglycoside library members were tetra-amines, and, therefore, it was hoped that compounds exhibiting higher specific binding to RNA, rather than simply increased electrostatic interaction, would be identified. The ligands were screened at 1 μM, and the relative increases in resonance units (RU) in the association phase were determined. The results of this screen are summarised in Fig. 4 and Table S1 (Supporting Information).†

Most of the compounds screened against the RNA targets resulted in small increases in RU. However, between four and six of the compounds, depending on the RNA target, resulted in increases in RU which were at least 20% of R_{max}, the maximum response observed when one binding site is occupied. The affinities of the compounds for each of the RNA targets are strongly correlated:¶ compounds which bind strongly to one of the RNA sequences generally also bind strongly to the other two RNA sequences (see Fig. 5). Remarkably, for each of the RNAs, the four compounds which resulted in the largest relative response were the same: **6A′C** and **6G** were ranked first and second for all three targets, and **6A′F** and **6A′D** were ranked either third or fourth. The compounds with the highest relative responses (**6A′C**, **6G**, **6A′F** and **6A′D**) are all tetra-amines; their higher apparent affinity for RNA, relative to isomeric tetra-amines within the library, cannot simply stem, therefore, from increased electrostatic interaction. However, the preliminary screen suggested that none of these compounds were able to discriminate effectively between the A-site, TAR and RRE sequences.

The relationship between the activity of the compounds **6Sug$_1$Sug$_2$**, and the identity of each of the glycosyl substituents **Sug$_1$** and **Sug$_2$**, is summarised in Fig. 6. Within this series, the three compounds with the highest affinity for the three RNA targets (**6A′C**, **6A′D** and **6A′F**) all have an L-α-6-amino-6-deoxyglucopyranosyl **Sug$_1$** substituent, A′ (see Fig. 6, panel A); however, presence of this substituent is not sufficient for high activity. The dependence of activity on the nature of the **Sug$_2$** substituent is less clear (Fig. 6, panel B): however, the compounds with a D-α-6-amino-6-deoxyglucopyranosyl **Sug$_2$** substituent, A, are generally found in a cluster which is distinct from the least active compounds.‖ Figures which summarise the relationships

¶ The correlation coefficients for the affinities of the compounds for pairs of RNA sequences are: 0.96 (TAR and RRE), 0.92 (TAR and A-site) and 0.88 (RRE and A-site).

‖ The identity of the **Sug$_1$** substituent has little effect on the activity of the compounds in the series **6Sug$_1$A**.

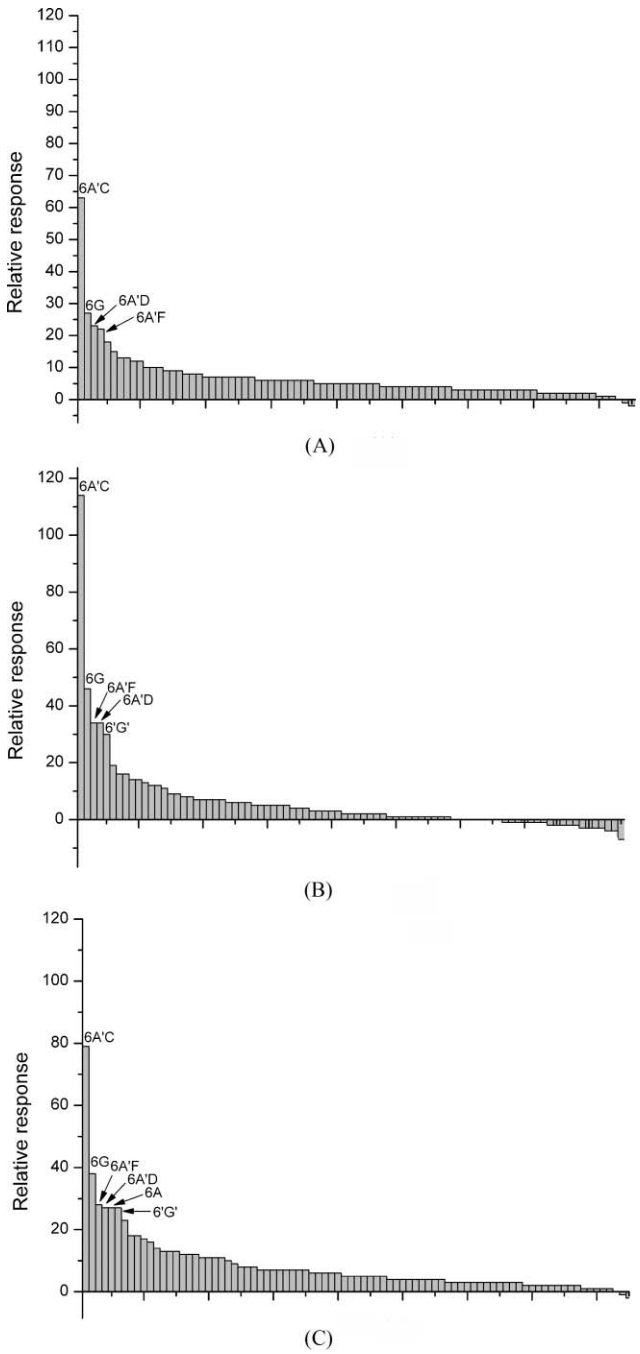

Fig. 4 Relative change in resonance units (RU) observed in the association phase in the presence of 1 μM of each aminoglycoside derivative. The data have been normalised to account for differences in the immobilisation levels and relative molecular masses of the RNA molecules and the aminoglycosides. Data for many compounds have been obtained by screening the enantiomeric compound against enantiomeric RNA (see Table S1, Supplementary Information†). A response of 100% refers to an increase in RU of R_{max}, the maximum response observed when one binding site is occupied. Panel A: A-site sequence; panel B: TAR sequence; panel C: RRE sequence.

between activity and the regiochemistry and configuration of the aminoglycoside derivatives are provided as Supplementary Material.†

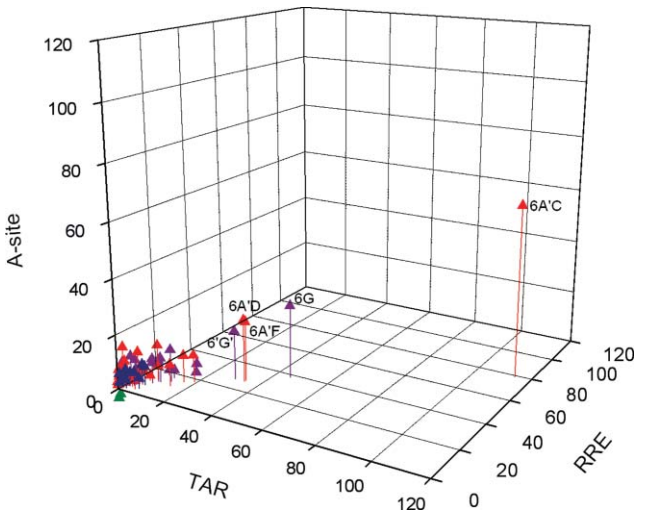

Fig. 5 Relative change in resonance units (RU) observed in the presence of 1 μM of each aminoglycoside derivative (see Fig. 4). The aminoglycoside class is indicated by colour: **6Sug$_1$Sug$_2$** (red); **7Sug$_1$Sug$_2$** (blue); **8Sug$_1$Sug$_2$** (green) and other derivatives (purple). The affinity of the aminoglycoside derivatives for the three target RNA sequences (RRE, TAR and the bacterial A-site) are strongly correlated. For each of the targets, the four compounds which resulted in the largest relative change in RU in the association phase were the same (**6A′C**, **6G**, **6A′F** and **6A′D**).

Determination of apparent dissociation constants for aminoglycoside derivatives

Apparent dissociation constants were determined for selected pairs of aminoglycoside derivatives and RNA sequences (Table 1). The binding of the aminoglycoside derivatives was monitored at four concentrations (0.3 μM, 0.9 μM, 1.8 μM and 3.0 μM) using surface plasmon resonance. Previous studies have shown that multiple equivalents of aminoglycoside derivatives bind to similar RNA sequences;[6,21] for example, three equivalents of a range of aminoglycosides bind to a 27-mer variant of an RNA aptamer.[6] The experimental data were, therefore, fitted to a model with three aminoglycoside binding sites (see Table S2, Supplementary Information†). The dissociation constants, K_D, representing the binding of the first equivalent of each aminoglycoside to the RNA sequences are shown in Table 1. In general, it was found that subsequent equivalents of aminoglycoside analogues bound with (at least) one order of magnitude less affinity.

In addition to the RNA sequences used in the initial screen, two variants in which the U·U non-canonical base pair was replaced by an A:U or a U:A Watson–Crick base pair, U1495A and U1406A, were also investigated. In *E. coli*, these mutations confer bacterial resistance to aminoglycoside antibiotics:[9,22] the U1406A mutation confers resistance to aminoglycosides with a 4,6-disubstituted 2-deoxystreptamine ring, and the U1495A mutation confers low to moderate levels of resistance to neomycin and tobramycin and a high level of resistance to paromomycin. SPR studies have also shown that the affinity of aminoglycosides for the A-site depends critically on the presence of the non-canonical U·U base pair.** [7]

** Paromomycin binds about eight-fold more tightly to the U1406A variant than to the A-site sequence. Neomycin and paromomycin bind between ten- and twenty-fold less tightly to the U1494A variant than to the A-site sequence.

Table 1 Apparent dissociation constants for the binding of the first equivalent of aminoglycoside derivatives to RNA sequences

	A site	A site U1495A variant	A site U1406A variant	TAR	RRE
Compound	K_D[a]/μM	K_D[a]/μM	K_D[a]/μM	K_D[a]/μM	K_D[a]/μM
6A′B	29 ± 1	6.0 ± 1.5	14 ± 1	—[b]	30 ± 13
6A′C	0.23 ± 0.2	—[b]	0.32 ± 0.1	0.27 ± 0.03	2.7 ± 0.5
6A′D	—[b]	—[b]	—[b]	0.26 ± 0.03	0.27 ± 0.04
6A′F	4.7 ± 0.1	2.8 ± 0.1	3.9 ± 0.1	0.26 ± 0.07	—[b]

[a] Experimental data were fitted to a three binding site model. The apparent dissociation constant, $K_{D,a}$, representing the binding of the first equivalent of the aminoglycoside derivative to the RNA is provided here. [b] Not determined.

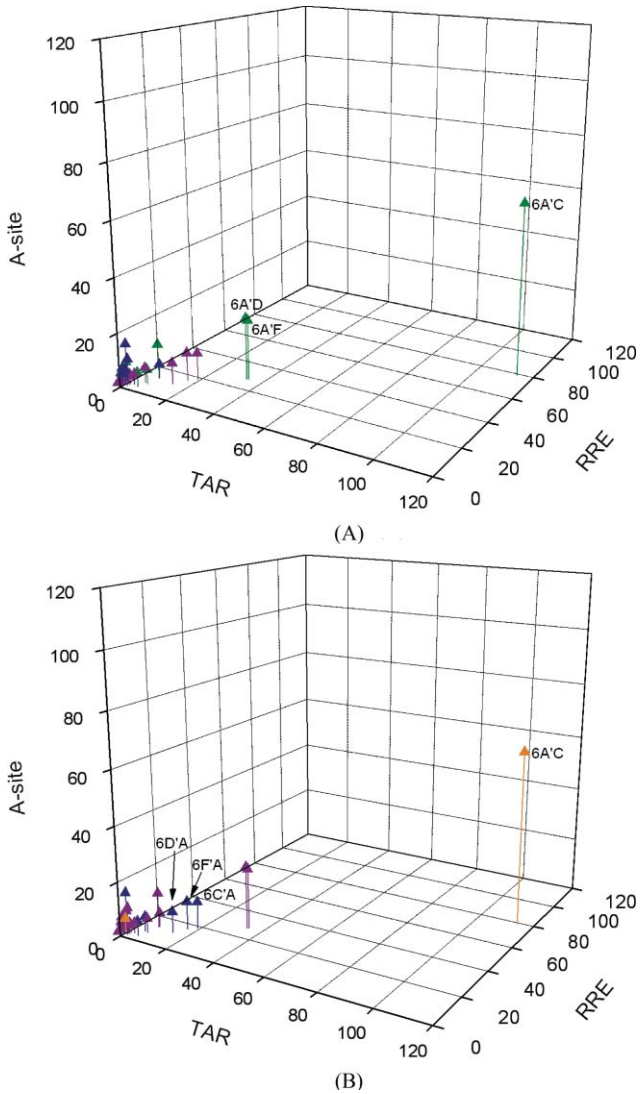

Fig. 6 Relationship between the relative change in resonance units (RU) observed in the association phase in the presence of 1 μM of the aminoglycoside derivatives **6Sug$_1$Sug$_2$** (see Fig. 4) and the nature of the **Sug$_1$** and **Sug$_2$** substituents. The compounds are coloured according to the nature of the glycosyl substituents. Panel A: dependence of activity on the nature of **Sug$_1$**: A (blue), A′ (green) and other substituents (purple). Panel B: dependence of activity on the nature of **Sug$_2$**: A (blue), C (orange) and other substituents (purple).

The aminoglycoside derivative **6A′B** was used as a negative control (Table 1). The aminoglycoside **6A′B** bound to the RNA hairpins with K_D in the range 6–30 μM, that is, with significantly lower affinity than the compounds **6A′C**, **6A′D** and **6A′F** which were identified from the screen (Table 1). The affinity of the aminoglycoside **6A′B** for the RNA hairpins was similar to that of kanamycin A for RNA hairpin models of the bacterial A-site (K_D = 18 μM),[7] its U1495A (K_D = 33 μM) and U1406A (K_D = 28 μM) variants,[7] and four other RNA hairpins (K_D = 4–20 μM).[6]

The aminoglycosides **6A′C**, **6A′D** and **6A′F** were found to bind significantly more tightly to the RNA hairpins than their isomer **6A′B** *i.e.* with up to 120-fold higher affinity (Table 1). The aminoglycoside derivative **6A′C** bound to the RNA hairpin model of the bacterial A-site with K_D = 0.23 μM and the model of the TAR sequence with K_D = 0.27 μM. The tetra-amine **6A′D** was found to bind to both the TAR and RRE sequences, and its isomer **6A′F** to the TAR sequence, with similar affinity *i.e.* with K_D values in the range 0.2–0.3 μM. In all cases, the highest affinity RNA–aminoglycoside interactions stem, in large part, from remarkably slow "off" rates in the range 0.4 to 1.0 × 10^{-4} s^{-1} (see Fig. 7 and Table S2, Supplementary Information†).

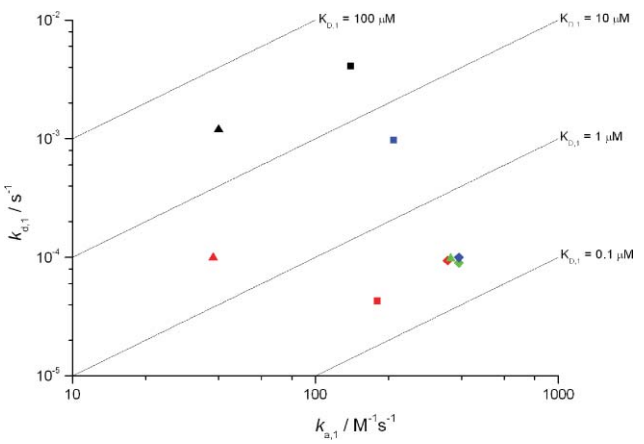

Fig. 7 Kinetic parameters for the binding of selected aminoglycosides to RNA sequences: A-site (square), TAR (diamond) and RRE (triangle). The aminoglycosides are indicated by colour: **6A′B** (black), **6A′C** (red), **6A′D** (green) and **6A′F** (blue).

Although the aminoglycoside derivatives **6A′C**, **6A′D** and **6A′F** bind tightly to the RNA hairpins, they discriminate rather poorly between the alternative RNA sequences. The aminoglycoside **6A′C** bound similarly tightly to the A-site model, its U1406A variant, and the TAR site model; **6A′C** did, however, bind to the RRE model sequence with approximately 10-fold lower affinity. The aminoglycoside **6A′D** discriminated poorly between the TAR and

the RRE sequences. However, the aminoglycoside derivative **6A′F** bound to the TAR sequence more than 10-fold more tightly than to the A-site and its variants. Although the aminoglycoside derivatives **6A′C**, **6A′D** and **6A′F** bind more tightly to the RNA hairpins than isomeric derivatives, their sequence specificity is rather low.

Summary

An SPR screen was used to identify tight-binding ligands for the bacterial A-site, and the viral TAR and RRE sequences from a library of stereo- and regiochemically diverse aminoglycoside derivatives. Remarkably, this initial screen suggested that the same four aminoglycoside derivatives bound most tightly to all three of the RNAs, suggesting that these compounds discriminated poorly between the RNA sequences.

The interactions between selected isomeric aminoglycoside derivatives and the RNA hairpins were studied in more detail using surface plasmon resonance. Experimental data obtained at four aminoglycoside concentrations was fitted to a three-site binding model, and dissociation constants were obtained. The aminoglycoside derivatives **6A′C**, **6A′D** and **6A′F**, which were identified from the screen, were found to bind more tightly to the RNA hairpins than their isomeric derivative **6A′B** (Table 1); indeed, **6A′C** had about 120-fold higher affinity than **6A′B** for the RNA model of the bacterial A-site. The magnitude of the tightest RNA–aminoglycoside interactions stemmed, in large part, from remarkably slow dissociation of the aminoglycosides from the RNA targets. Aminoglycosides possessing unnatural sugar configurations may be poor substrates for aminoglycoside-modifying enzymes which confer antibiotic resistance.[23]

The aminoglycoside derivatives **6A′C**, **6A′D** and **6A′F**, however, discriminate rather poorly between alternative RNA sequences. The aminoglycoside **6A′C** bound similarly tightly to the A-site model, its U1406A variant, and the TAR site model (with K_D values in the range 0.23–0.32 μM), though it bound to the RRE model sequence with approximately 10-fold lower affinity. The aminoglycoside **6A′D** discriminated poorly between the TAR and the RRE sequences. The aminoglycoside derivative **6A′F** bound to the TAR sequence more than 10-fold more tightly than to the A-site and its variants. Although the aminoglycoside derivatives **6A′C**, **6A′D** and **6A′F** bind more tightly to the RNA hairpins than isomeric derivatives, their sequence specificity is rather low.

In this study, we showed that the affinity of aminoglycoside derivatives for RNA sequences does not stem entirely from their electrostatic interaction with a macromolecular target: within our library of isomeric aminoglycosides, ligands with the same number of amino groups had widely differing affinities for RNA. However, within our library, high affinity was not accompanied by good sequence discrimination: even the tightest binding aminoglycoside derivatives discovered were found to discriminate poorly between alternative RNA sequences.

Acknowledgements

We thank the Wellcome Trust, BBSRC and EPSRC for funding.

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