Surface Plasmon Resonance Evaluation of Various Aminoglycoside–RNA Hairpin Interactions Reveals Low Degree of Selectivity

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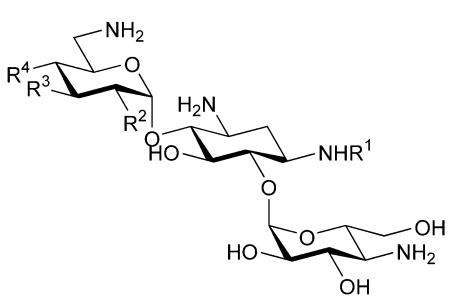
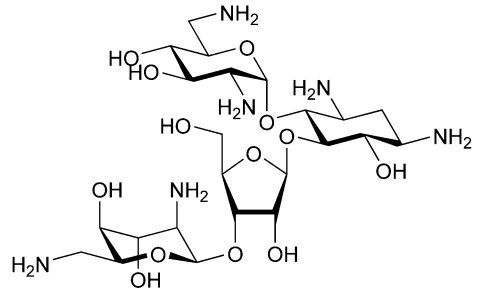
Aminoglycoside antibiotics, which are able to selectively bind to RNA, are considered to be an important lead in RNA-targeting drug discovery. In this study, surface plasmon resonance (SPR) was employed to explore the interaction of aminoglycosides with known tobramycin-binding RNA hairpins (aptamers) and an unrelated RNA hairpin. It was established that aminoglycosides have multiple interactions with RNA hairpins. Unexpectedly, the different hairpins showed comparable affinity for a set of related aminoglycosides. The observed absence of selectivity presents an extra hurdle in the discovery of novel aminoglycosides as specific drugs that target defined RNA hairpins.

Introduction

In the last decade several RNA structures besides the A-site of the 16S rRNA[1] were reported to bind aminoglycoside antibiotics. These include HIV trans-activating region,[2] HIV Rev responsive element,[3] several ribozymes[4,5,6] and parts of mRNA sequences of two oncogenic fusion proteins.[7] As a result, aminoglycosides gained attention as possible RNA targeting drugs,[8] generally applicable to targets beyond bacteria. Ultimate future drug discovery would encompass the rational design of small molecules that could selectively recognise given RNA structures.[9]



	R^2	R^3	R^4	R^1
1 Tobramycin	NH_2	H	OH	H
2 Kanamycin A	OH	OH	OH	H
3 Kanamycin B	NH_2	OH	OH	H
4 Dibekacin	NH_2	H	H	H
5 Amikacin	OH	OH	OH	$C(O)\text{-}CH(OH)CH_2CH_2NH_2$



6 Neomycin B

Scheme 1. *Various aminoglycoside antibiotics containing 2-DOS.*

The majority of aminoglycoside antibiotics are aminocyclitol 2-deoxystreptamines (2-DOS) substituted with different aminosugars (Scheme 1). Under physiological conditions, most amino groups are protonated, and the importance of electrostatic interactions of the aminoglycosides with their RNA target is generally recognised.[8] On the other hand, nonspecific binding to RNA molecules is expected to be directly proportional to the number of positively charged amino functions in the antibiotic. Consequently, the ideal aminoglycoside antibiotic would bear a low number of amino groups optimally oriented in space to allow specific and sufficiently strong binding with one particular RNA target.

With the aim of studying the RNA–aminoglycoside interaction in more detail, high-affinity RNA fragments, also called aptamers, were generated by using in vitro selection methodologies.[10] This technology, often referred to as SELEX (systematic evolution of ligands by exponential enrichment) has been applied to several aminoglycoside antibiotics including neomycin B,[11] lividomycin,[12] kanamycin A,[12] kanamycin B,[13] tobramycin[14] and streptomycin.[15] It has been assumed that the resulting aptamers have high selectivity towards the aminoglycoside they have been derived from.

The best studied aptamer is the one that binds tobramycin. The original sequence, called j6, contained 109 nucleotides. The j6 aptamer could be reduced in length to a 40-mer

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(j6f1)[16] or a 27-mer (j6sl; Figure 1),[14] while still retaining the aminoglycoside binding activity. In addition, it was stated that the 3 nucleotide bulge in j6f1 was crucial for selective recognition of the tobramycin moiety, since j6f2 was found not to bind fluorescently labelled aminoglycosides.[16]

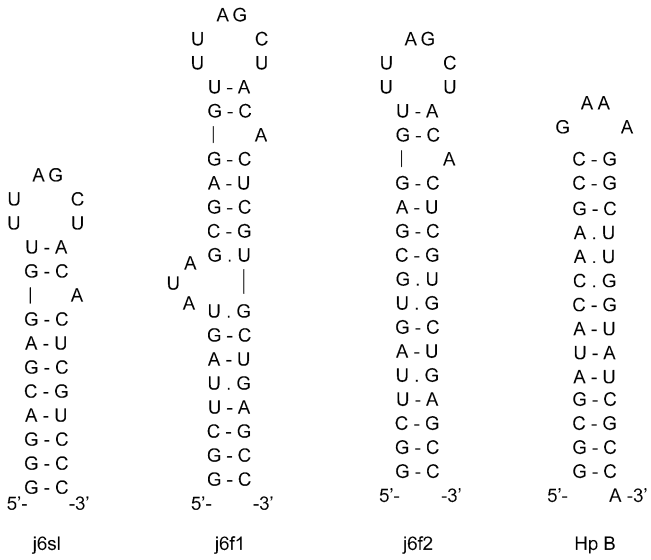

Figure 1. *Tobramycin aptamers j6sl, j6f1, j6f2 and reference Hp B.*

NMR studies demonstrated that tobramycin binds in the widened major groove of the RNA stem of j6sl and is further encapsulated by the loop structure.[17] Structure refinement revealed that on average one to three amino functions of the antibiotic interact with backbone phosphates of j6sl.[17] However, it is uncertain to what extent these contacts contribute to the specificity of complex formation.

The well-studied j6 aptamer, which was selected to bind a predestined target, that is, tobramycin, was chosen as a tool to investigate the selectivity of RNA–aminoglycoside interactions. Accordingly, we set out to examine the binding behaviour of j6sl, j6f1 and j6f2 towards a set of aminoglycosides. Besides tobramycin (**1**), four related aminoglycosides of the 4,6-substituted 2-DOS class (see compounds **2**–**5** in Scheme 1) were selected for affinity analysis. In addition, neomycin B (**6**) was chosen as a member of the 4,5-substituted 2-DOS aminoglycosides. Hairpin B (Hp B; Figure 1), comprising a structured GAAA tetraloop[18] and an A-helix stem without bulges, was designed as a reference to evaluate the specific properties described for the j6 hairpin series.

In our studies, surface plasmon resonance (SPR) was applied to determine dissociation constants between aminoglycoside antibiotics and RNA.[19] SPR is an optical phenomenon that occurs at a specific angle of the incoming light and leads to a reduction in intensity of the reflected light (see Figure 2). The SPR of the system is proportional to the mass concentration near the surface. Therefore, the binding of an analyte to a ligand immobilized on the gold surface leads to a change in resonance angle, usually converted to resonance units (RU). Originally used to monitor the interaction between biomacromolecules, advances in the sensitivity of the SPR technique resulted in its application to the detection of small molecules interacting with biomacromolecules.[20] In contrast to other methods, SPR is able to detect interactions in real time and does not require derivatization of aminoglycosides. Moreover, the response level indicates the amount of bound ligand, enabling calculation of stoichiometry.

Here we report that all hairpins used in the SPR assay show binding of multiple aminogycoside molecules. The approximate binding site size consists of four base pairs. For each hairpin, nonspecific binding in the high micromolar range was observed, whereas the first equivalent of the aminoglycoside binds with a low micromolar affinity.

The SPR analysis shows that j6sl, j6f1 and j6f2 have similar affinities for a set of related aminoglycosides. In addition, Hp B, an unrelated hairpin, exhibits comparable binding characteristics as the tobramycin aptamers j6sl, j6f1 and j6f2.

Results and Discussion

In order to immobilize the aptamer on streptavidin-coated SPR-chips, the 5′-end of the RNA was functionalized with a

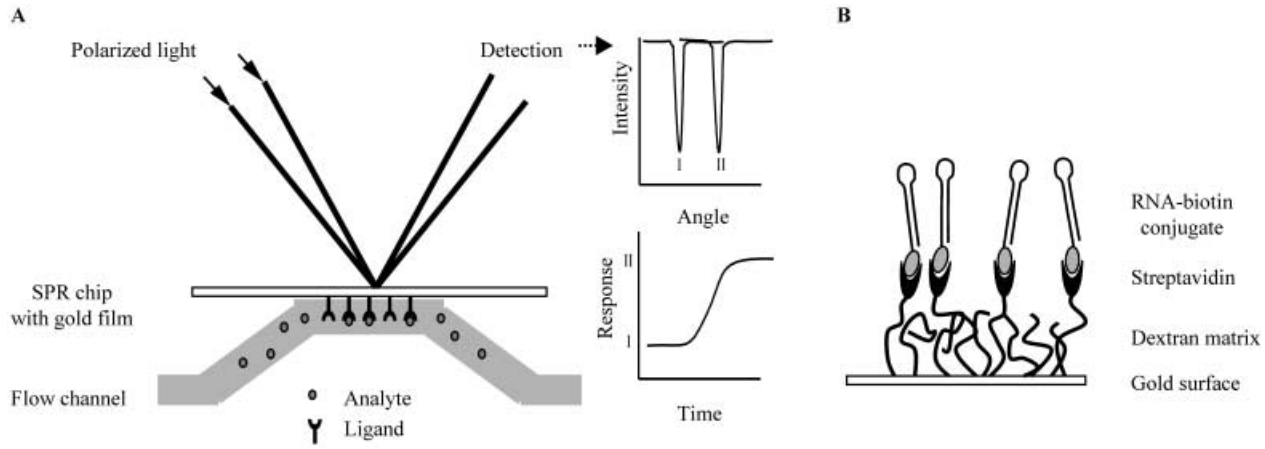

Figure 2. *Schematic representation of surface plasmon resonance.*

biotin moiety. Prior to binding experiments we verified, by NMR (Figure 3a), whether the biotinylated j6sl folded in the same manner as the nonbiotinylated j6sl sequence described in the literature.[17] Based on the 1D imino proton spectra, the biotin moiety on the 5′ end of the RNA had, as expected, no effect on the base pairing in the stem loop. In a second NMR experiment (Figure 3b), it was confirmed[17] that tobramycin indeed interacts with the binding pocket in the aptamer.

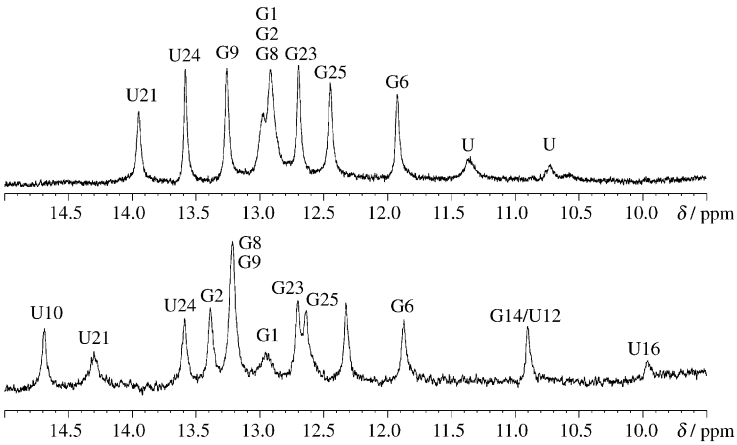

Figure 3. *1D iminoproton spectra of j6sl (0.1 mM) in 10 mM phosphate buffer pH 6.8 in the absence (upper spectrum) and presence (lower spectrum) of tobramycin. The spectra are in agreement with literature data.[17]*

After immobilization of RNA on an SPR-chip, sensorgrams (response versus time) were recorded in the presence of aminoglycoside solutions with concentrations up to 200 μM. A typical sensorgram resulting from SPR experiments is depicted in Figure 4a. It can be seen that a steady state is reached within seconds after injection of the aminoglycoside antibiotic; this indicates very fast kinetics, which is expected for small molecule–RNA interactions.[21] Accordingly, the dissociation of the aminoglycoside from the RNA takes place at the end of the injection, when the chip is flushed with buffer, thereby restoring the original SPR signal, before regeneration of the surface with 1 M NaCl takes place.

Surprisingly, the sensorgrams of neomycin B (**6**) show a different picture (Figure 4b). Below 300 nM concentrations of **6**, no significant increase in RU was observed. At higher concentrations, however, no complete dissociation took place when the chip was flushed with a buffer solution. Apparently, at higher aminoglycoside concentrations, a very strong binding to the RNA occurred, and regeneration at high salt concentration was necessary to release the antibiotic molecule from the RNA hairpin. Since the binding mode of neomycin B is clearly different, these data were not compared with the dissociation constants of other aminoglycosides.

From other sensorgrams (compounds **1**–**5**), it can be directly observed that no increase in SPR response occurs below aminoglycoside concentrations of approximately 30 nM. In most measurements, a steady-state response was only detected above concentrations of 0.1 μM. Below this concentration, no association was observed during the 180 s injection; this indicates that no interaction between the aminoglycosides and the RNA hairpins takes place. On the other hand, at concentrations higher than 2.5 μM, the response level, which is linear to the amount of bound aminoglycoside, indicates that multiple antibiotic molecules bind to the RNA hairpins. This feature complicates the calculation of the dissociation constants (K_D) when using a 1:1 binding model [Eq. (1), below]. Instead, a model with n binding sites corresponding to the number of bound aminoglycosides was used [Eq. (2), below].[22] It was found that the long RNA fragments (j6f1 and j6f2) bound up to five molecules of **1**–**4**. On the other hand, j6sl interacted with three molecules of **1**–**4**, whereas Hp B bound 3–4 equivalents of **1**–**4**. These values approximately correspond to one antibiotic molecule per four base pairs. This ratio is in agreement with a recent paper by Pilch et al., who reported an aminoglycoside binding-site size of four base pairs in a poly(rI)·poly(rC) duplex.[23] Amikacin (**5**), which is larger due to the acyl group at the 1-position of the 2-DOS moiety, was bound with lower stoichiometry.

Interestingly, the binding of multiple equivalents of aminoglycoside antibiotics was not found with fluorescence polarization,[16] due to a difference in experimental setup: the affinity of unlabelled small molecules is calculated through the displacement of a fluorescent derivative, and lower affinity binding events are not monitored.

The affinity constants $K_{D,1}$ that were calculated with Equation (2) are listed in Tables 1–4 and represent the affinities of the first equivalent aminoglycoside that binds to the RNA. Generally, it was observed that the next equivalents bind with a K_D of (at least) one order of magnitude less. Note that when the correct stoichiometry is overlooked, curves were also

Table 1. K_D *and stoichiometry (n) j6sl.*

	Aminoglycoside	K_D [μM]	n[a]
1	tobramycin	1.1 ± 0.15	3
2	kanamycin A	11 ± 1.7	3
3	kanamycin B	2.2 ± 0.14	3
4	dibekacin	0.85 ± 0.17	3
5	amikacin	33 ± 2.4	3

[a] The number of aminoglycosides bound to the RNA hairpin as determined from the SPR sensorgrams.

Table 2. K_D *and stoichiometry (n) j6f1*

	Aminoglycoside	K_D [μM]	n
1	tobramycin	1.2 ± 0.26	5
2	kanamycin A	11 ± 3.0	5
3	kanamycin B	1.8 ± 0.20	5
4	dibekacin	1.8 ± 0.43	5
5	amikacin	14 ± 1.9	4

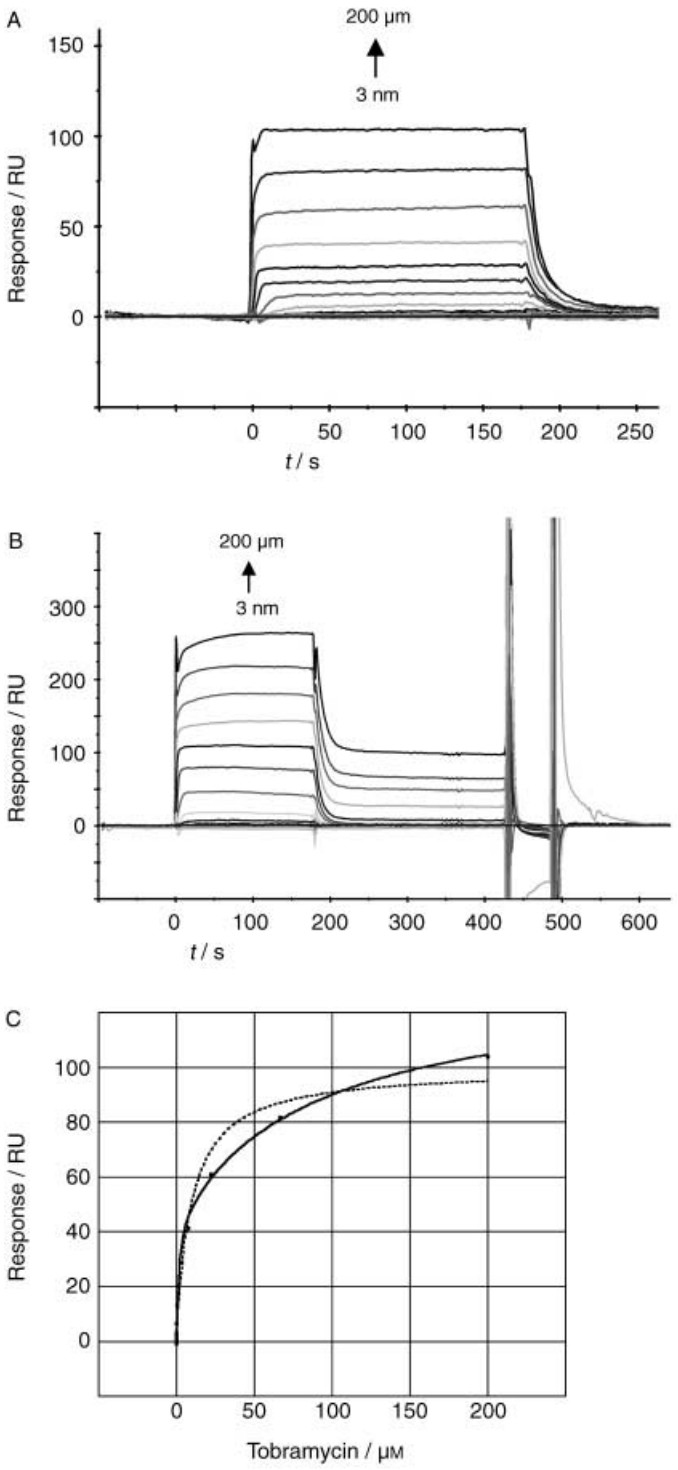

Figure 4. *Surface plasmon resonance results: A) Sensorgram of tobramycin injected on the surface loaded with j6sI: different concentrations of tobramcyin (3 nM to 200 µM) are injected from t = 0 to 180 s. B) Sensorgram of neomycin B–j6sl interaction. Regeneration takes place from t = 420 to 480 s. C) Calculation of affinity of tobramycin for j6sl by using a one-site binding model [Eq. (1)] and a multiple-site binding model [Eq. (2)] corresponding to the number of bound aminoglycoside molecules.*

Table 3. K_D *and stoichiometry (n) j6f2.*

	Aminoglycoside	K_D [µM]	*n*
1	tobramycin	0.32 ± 0.04	5
2	kanamycin A	4.1 ± 1.1	5
3	kanamycin B	0.58 ± 0.06	5
4	dibekacin	0.46 ± 0.04	5
5	amikacin	26 ± 7.0	4

Table 4. K_D *and stoichiometry (n) hp B.*

	Aminoglycoside	K_D [µM]	*n*
1	tobramycin	2.3 ± 0.09	4
2	kanamycin A	20 ± 2.2	3
3	kanamycin B	1.6 ± 0.15	4
4	dibekacin	1.5 ± 0.21	4
5	amikacin	13 ± 9.9	3

found to fit equations with lower stoichiometry, but with higher statistical errors and lower correlation constants.

The interaction of tobramycin (**1**) and the j6sl aptamer, which was subject of the reported NMR studies,[17] lies in the low-micromolar range (K_D = 1.1 µM; Table 1, entry 1), considerably higher than the reported value (K_D = 9 nM) determined by fluorescence quenching experiments with pyrene-labelled tobramycin (PYT).[14] However, we were unable to repeat this experiment due to an unexpected decrease in fluorescence unrelated to the RNA concentration.

Alternatively, fluorescence polarization experiments with carboxytetramethylrhodamine-labelled tobramycin (CRT)[16] were performed, but these did not reveal any interaction with up to 0.5 µM of j6sl (data not shown).

Note that in both the SELEX selection process and the fluorescence experiments, the tobramycin molecule is attached via its 6′-amino function by means of an amide linkage. SPR and NMR experiments, in contrast, use "free" aminoglycosides. However, the effect of this amine function on the binding event is assumed to be low, as the NMR structure analysis reveals that this particular amino group is located outside the RNA hairpin.

Given the fact that binding between unmodified tobramycin and j6sl has been unambiguously monitored by SPR, we reason that the interaction between the CRT and j6sl is probably disrupted in the polarization experiment due to the bulky fluorophore. A similar weak binding of CRT has also been observed for j6f2 by Rando et al.,[16] which in that case precluded competition experiments with unlabelled aminoglycosides. Based on the result obtained with fluorescently labelled tobramycin (CRT), it was concluded that the unlabelled aminoglycosides would also have a weak affinity and that the three-nucleotide bulge, deleted in j6f2, was essential for aminoglycoside binding.[16] SPR analysis, however, shows that j6sl, j6f1 and j6f2 have similar binding characteristics (Tables 1–3). For all three hairpins, tobramycin binds with a dissociation constant of 0.3–1.2 µM. Kanamycin B (**3**) and dibekacin (**4**), which differ in the presence or absence of a hydroxyl group, respectively,

compared to tobramycin, show affinities similar to that of tobramycin (**1**). Thus, the hydrogen bonds that are possibly formed between the hydroxyls and functional groups on the RNA[17] do not contribute to the affinity. The considerably lower affinity of kanamycin A (**2**) and amikacin (**5**) is most likely due to the absence of the C2′-amino function; this confirms the important role of that particular amino group in essential electrostatic interactions.

The similar binding of **1**, **3** and **4** shows that the j6 fragments do not display a high specificity against the aminoglycoside that was used in the SELEX selection. Another illustrative example in this regard is the outcome of a study that used SELEX technology to select RNA for kanamycin B (**3**) binding.[13] The resulting RNA aptamer, however, bound tobramycin (**1**) 15 times more strongly than the original selection tool kanamycin B.[13] This inability to detect the minor structural differences in these related antibiotics could be a result of the SELEX procedure. We suggest that an additional deselection step with other aminoglycosides might be required to find RNA fragments that discriminate between these closely related antibiotics. On the other hand, we cannot exclude the possibility that the original 109-nucleotide j6 aptamer provides additional contacts with the aminoglycosides, resulting in an increased selectivity.

Interestingly, interaction studies of the aminoglycosides with reference Hp B (see Table 4) gave some remarkable results. Hp B binds tobramycin only 2–7 times more weakly (entry 1), while its affinity for the other aminoglycosides is similar to those of the aptamers j6sl, j6f1 and j6f3. Thus, the individual aminoglycosides cannot significantly discriminate between the aptamers and RNA fragment Hp B. The largest selectivity in favour of j6f2, although not more than sevenfold, is observed for tobramycin. Similar moderate preferences for binding to j6f2 were seen for the other aminoglycosides. The observed poor selectivity between j6sl, j6f1, j6f2 and Hp B is in accordance with the fact that aminoglycosides, due to their charged nature, can bind to a wide variety of RNA hairpins with low-micromolar affinities.[8]

The absence of selectivity between different RNA hairpins brings about some serious concerns. Scientists in drug research should be aware of the critical issue of selectivity when hairpins or other small RNA fragments are used to evaluate the properties of aminoglycoside analogues.

Conclusion

In this study we used SPR to evaluate the binding of aminoglycosides to different RNA hairpins. Analysis of SPR sensorgrams showed that the SELEX generated RNA structures j6sl, j6f1 and j6f2 bind multiple equivalents of aminoglycoside antibiotics. The stoichiometry proved to be dependent on the size of the RNA hairpin, but showed an approximate binding-site size of four base pairs. The aptamers showed similar binding to different aminoglycosides. To our surprise, control Hp B showed affinities comparable to the aptamers. The lack of observed selectivity at the level of RNA hairpins could hamper the future development of aminoglycoside-based drugs aimed at specifically chosen RNA hairpin structures. Therefore, the design of RNA-targeting drugs may need another approach. As electrostatic forces are not only responsible for specific but also non-specific binding to RNA, a guided reduction of the number of amino functions can produce a possible solution for the above-mentioned problem. In this respect, it would be interesting, following a structure-based approach, to study the aminoglycoside in the targeted RNA pocket and to delete the amino functions that are not involved in direct ionic interactions (i.e. salt-bridge formation). Any loss in affinity can be compensated for by placing small lipophilic groups at positions close to base residues.

Experimental Section

Tobramycin, kanamycin A, kanamycin B, dibekacin, amikacin and neomycin B were purchased from Sigma. Biotin-labelled RNA fragments were purchased at Dharmacon Research. SPR buffer (10 mM HEPES pH 7.4, NaCl (150 mM), EDTA (3 mM), 0.005% surfactant P20) was obtained from Biacore and used as received. NMR spectra were recorded on a Bruker DMX-600 (600 MHz) at 5 °C in a sodium-phosphate buffer (10 mM, pH 6.8).

SPR measurements were conducted on a Biacore 3000 system from Biacore AB and performed as described.[19] Streptavidin coated sensor-chips (SA-chips) were obtained from Biacore and loaded with RNA fragments to approximately 800–1000 RU. An empty cell was used as a reference surface. Different samples of aminoglycosides were prepared in the appropriate buffer by serial dilution of a 200 μM stock solution. Aminoglycoside concentrations were injected for 3 min at a flow rate of 5 $\mu L\,min^{-1}$. The chip was subsequently flushed with a buffer solution for 2 min, after which regeneration of the surface took place by injection of 1 M NaCl for 1 min and buffer injection for 2 min. Steady state responses were determined from sensorgrams by using BIAevaluation. Calculation of dissociation constants by fitting the steady state responses was performed with Kaleidagraph, by using the following formulae:

$$R = R_{max}\left[\frac{c}{K_D + c}\right] \quad (1)$$

$$R = R_{max}\left[\frac{c}{K_{D,1} + c} + \frac{c}{K_{D,2} + c} + \ldots\right] \quad (2)$$

Where R = response, R_{max} = maximum response of one binding site occupied, c = concentration, $K_{D,1}$ = dissociation constant for specific binding, $K_{D,n}$ = dissociation constant for lower affinity binding.

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Keywords: aminoglycosides · antibiotics · RNA recognition · surface plasmon resonance

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