INTRODUCTION

Discovery of new drugs can be extremely helped by the thermodynamic measurements of the binding interactions with biological targets by assisting high-throughput screening of chemical libraries, by accelerating the lead optimization process, also for the fundamental understanding of the drug-target mechanism (Ladbury et al., 2010). In this context, increasing improvements in the accuracy and sensitivity of instrumentation are permitting that isothermal titration calorimetry (ITC) becomes the technique of choice when full thermodynamic profile is valuable (Holdgate, 2007). Commonly used for proteins, until recently, ITC was applied to the study of nucleic acids, and in particular, of RNA-drug complexes (Pilch et al., 2003; Feig, 2007). An evident advantage of the technique is the simultaneous determination of the thermodynamic binding constant ($K_b$) closely related to the free energy variation ($\Delta G$), the enthalpy ($\Delta H$) and the entropy ($\Delta S$) variations and also the binding stoichiometry (N) from a single well-designed experiment (Ladbury and Doyle, 2004). It should be mentioned, however, that other common techniques are able to measure the ratio between the bound and free species concentrations and, then, to provide the stoichiometry and the binding constant of the studied interaction, but $\Delta H$ quantity cannot be directly measured. Thus, ITC seems to be the best experimental approach to obtain a reliable and complete thermodynamic description of the interaction of interest.

The vast knowledge acquired on RNA biochemistry, particularly, the elucidation of the ribosome structure and the gene decoding at atomic level (Carter et al., 2000; Wimberly et al., 2000) has fuelled the interest on RNA-based therapies (Kole et al., 2012). Similar to proteins, RNA can fold into a broad range of different structures, which can be targeted by small molecules (Aboul-ela, 2010). In this sense, the aminoglycosides such as Paromomycin and Neomycin (Figure 1) are the paradigm of therapeutically useful RNA ligands (Herrmann, 2005). Aminoglycosides, typically formed by an aminocyclitol unit (2-deoxystreptamine in Paromomycin and Neomycin, Figure 1) attached to one or more amino sugars via glycosidic linkages, are a class of broad-spectrum antibiotics against aerobic gram-negative bacteria, which exert their activity by binding to ribosomal RNA (rRNA). X-ray crystallography (Carter et al., 2000; Vicens and Westhof, 2001; François et al., 2005) and NMR (Lynch et al., 2003) studies provided a very precise picture of the molecular binding mechanisms of aminoglycosides. These antimicrobials target the A-site within bacterial 16S rRNA of the small ribosome subunit, by binding to a three-adenine internal loop, involved in the correct deciphering of the mRNA. Upon binding, aminoglycosides provoke the structural rearrangement of the site, fact that eventually forge the ribosomal proofreading mechanism and lead to miscoding and inhibition of protein synthesis (Kaul et al., 2006; Kondo and Westhof, 2008).

The clinical use of aminoglycosides had been depreciated by toxicity, target promiscuity and the appearance of resistance mechanisms, but the alarming decrease in the activity of the current antibiotic repertoire has renewed the interest for their chemical analogues (Hainrichson et al., 2008). Among many other derivatives, aminoglycoside–oligonucleotide conjugates have recently been considered as specific ligands of bacterial...
and viral RNA (Riguet et al., 2005; Hyun et al., 2006; Charles et al., 2007; Kiviniemi and Virta, 2011) because of the additional chemical recognition properties conferred by oligonucleotide strands. Here, we decided to contribute to this trend by developing novel aminoglycoside–oligonucleotide conjugates (Alguacil et al., 2010) and gaining insight on how these analogues could act as specific RNA binders. We hypothesized that aminoglycosides derivatized with dinucleotide or diPNA moieties could improve the target selectivity because their pending nucleobase units could procure additional interactions with the RNA nucleobases close to the aminoglycoside binding site by canonical or non-canonical hydrogen bonding, or by procuring complex interactions as those observed in RNA tertiary motifs. As a first step, we intended to study the interaction of these aminoglycoside conjugates with the validated target of aminoglycosides, that is, the A-site ribosomal RNA. To this aim, inspired by the pioneer work of Pilch and colleague (Kaul and Pilch, 2002; Kaul et al., 2003; Pilch et al., 2003; Kaul et al., 2005), here we present the results of ITC experiments on the interaction of the aminoglycoside Paromomycin and Neomycin, as well as the Neomycin-conjugates depicted in Figure 1 with surrogates of bacterial (RNAEC) and human cytoplasmic rRNA (RNAHS). These two 27-mer hairpin oligonucleotides (Figure 2) were designed by the Puglisi group (Fourmy et al., 1996; Lynch and Puglisi, 2001; Lynch et al., 2003) to mimic the aminoglycoside binding sites in bacterial and human rRNA, respectively. The consensual bacterial target (RNAEC) preferred by antibiotic aminoglycosides contains an asymmetric internal loop formed by three adenines (A1408, A1492 and A1493, according to the numbering of Escherichia coli rRNA sequence, Figure 2). Instead, in the eukaryotic A-site, one of the adenines (A1408) is replaced by a guanine (G1408). Structural studies showed that this single nucleobase change (also present in some resistant bacteria) explain why human ribosomes are less sensitive to deleterious effects of aminoglycosides because they reduces the affinity of aminoglycosides for rRNA (Lynch and Puglisi, 2001; Kondo et al., 2006). Herein, we determined comparative affinities and thermodynamic values of our new analogues for the bacterial versus the human target, as a first step to assess their antibiotic activity and reduced toxicity on humans.

**MATERIALS AND METHODS**

**Instruments**

Titrations were performed by means of an isothermic titration microcalorimeter MicroCal VP-ITC (MicroCal, LLC, Northampton, MA, USA) equipped with a 1.4047 ml cell. A vacuum system ThermoVac, Microcal Inc. (MicroCal, LLC, Northampton, MA, USA) was used to degas the solutions. pH was measured with a Crison micro-pH 2002 potentiometer (Crison Instruments, Alella, Spain) equipped by a Crison 5014 combination electrode with a precision of ±0.1 mV (±0.002 pH units). The electrode system was standardized with ordinary aqueous buffers of pH 4.01 and 7.00.

**Figure 1.** Structure of aminoglycosides (Paromomycin and Neomycin), Neomycin-dinucleotide (Neomycin-TT and Neomycin-AA) and -diPNA (Neomycin-tt and Neomycin-aa) conjugates.

**Figure 2.** Oligoribonucleotides mimicking the (a) Escherichia coli (bacterial) A-site ribosomal RNA (rRNA) (RNAEC) and (b) human cytoplasmic A-site rRNA (RNAHS). The nucleotides of the internal loop are shown in bold and numbered according to the sequence of bacterial 16S rRNA.
Chemicals

The two oligoribonucleotides mimicking the bacterial (RNAEC) and human cytoplasm (RNAHS) A-site rRNA (Fourmy et al., 1996; Kaul et al., 2003; Kaul et al., 2005) (Figure 2) were prepared by solid-phase synthesis and conveniently purified by semipreparative high-performance liquid chromatography (HPLC). The compound purity has been tested by HPLC before use. Paromomycin sulfate and Neomycin trisulfate (>98%) were from Sigma-Aldrich (St. Louis, MO, USA) and used as received. Neomycin-dinucleotide (Neomycin-TT and Neomycin-AA) and -diPNA (Neomycin-tt and Neomycin-aa) conjugates (Figure 1) were synthesized in-house as described previously (Alguacil et al., 2010).

Working solutions

A mixture of sodium cacodylate 10 mM, ethylenediaminetetraacetic acid (EDTA) 0.1 mM and NaCl 150 mM adjusted at pH 5.5 and human cytoplasm (RNAHS) A-site rRNA (Fourmy et al., 1996) and 200 or 300 μM for the ligands. In the case of the RNAEC titrations, the concentration was 20 μM for the RNA and 500 μM for the ligands.

Isothermal titration calorimetry measurements

The RNA solutions were heated at 90 °C in a sand bath and cooled down slowly, that is, the sample achieves the thermal equilibrium with the ambient temperature by spontaneous losing heat process until room temperature (about 20 °C). Both titrant and titrated solution were deoxygenated before use. Successive volumes of 10 μl (0.5 μl s⁻¹) of ligand solution (aminoglycosides, dinucleotide- or diPNA-conjugates) were added to the titration cell filled with the target solution (RNAEC or RNAHS). At least three independent titrations were carried out for each ligand-target combination. Background titrations consisting in identical titrant solutions with the reaction cell filled just with the buffer solution were performed to determine the background heat, due to the ligand dilution and the syringe rotation. In all instances, the working temperature was 25 ± 0.2 °C. The obtained data were analysed through the Origin 7.0 software supplied by Microcal. The ITC data were collected automatically and analysed to obtain the N, ΔH, Kapp, ΔG and ΔS values associated to the interaction. All the data have been fitted with Origin and Setphat/Nitpic software. No significant differences in final results have been observed using these algorithms and, then, data shown in Tables 2 and 3 are those from Origin (two binding sites mode in all instances except for Neomycin-aa/RNAHS for which the sequential binding site mode has been used).

RESULTS AND DISCUSSION

It is well-known that ITC measurements are strongly unspecific because any chemical process is able to generate or consume an amount of heat. Very often, several concomitant reactions are involved in interactions with biological interest and all of them can significantly contribute to the measured heat (Zhang et al., 2000; Garrido et al., 2011). Particularly, the gain or loss of protons in the frame of the global process could be relevant in the final result. Then, to obtain biologically meaningful quantities, the experimental conditions of measurements should be as close as possible to the biological environment when the interaction of interest will be carried out.

Pilch and colleague determined the acidity constants of protonated amino groups present in Paromomycin and Neomycin and demonstrated that all of them are essentially protonated at pH 5.5 (Kaul et al., 2003). Therefore, the authors proposed sodium cacodylate (pH = 5.5) as the buffer agent for ITC titrations of RNA with Paromomycin because of the absence of ligand proton exchange and, also, the very low buffer dissociation heat (Goldberg et al., 2002; Kaul et al., 2003). Moreover, to avoid the effect of the eventual presence of metal ions traces a complexing agent, EDTA was added to the buffer solution and, also, the ionic strength was adjusted to the physiological ionic concentration with NaCl. Working in this way, the derived

Table 1. Binding constants of the interaction of aminoglycosides with A-site rRNAs

| Technique     | Paromomycin | Neomycin |
|---------------|-------------|----------|
|               | Bacterial   | Human    | Bacterial | Human |
| Fluorescence  | 6.06 × 10⁵a | 4.55 × 10⁵a | 1.89 × 10⁶a | 3.85 × 10⁶a |
| Fluorescence  | 2.10 × 10⁶b | 3.90 × 10⁶b | 3.00 × 10⁶b | 6.00 × 10⁶b |
| UV-melting curves | 2.50 × 10⁷c | —         | 2.60 × 10⁷c | —         |
| SPR           | 5.00 × 10⁶d | —         | 5.26 × 10⁶d | —         |
| ITC           | 3.70 × 10⁷e | 2.40 × 10⁷e | —         | —         |
| ITC           | 4.34 × 10⁶f | —         | 4.76 × 10⁶f | —         |
| ITC           | 1.27 × 10⁵g | —         | 1.23 × 10⁶g | —         |

ITC, isothermal titration calorimetry; SPR, surface plasmon resonance

1Experimental conditions: 150 mM Na⁺, pH 7.5 (Ryu and Rando, 2001).
2Experimental conditions: 100 mM Na⁺, pH 7.5 (Kaul et al., 2005; Kaul et al., 2006).
3Experimental conditions: 150 mM Na⁺, pH 5.5 (Goldberg et al., 2002).
4Experimental conditions: 100 mM K⁺, 2 mM Mg²⁺, pH 7.0 (Ennifar et al., 2013).
Figure 3. Isothermal titration calorimetry curves for the interactions of Paromomycin, Neomycin and Neomycin derivatives with bacterial ribosomal RNA.
binding parameters should be as close as possible to those of the pure aminoglycoside-RNA interactions. Because Paromomycin and Neomycin differ only in the 6′ substituent (OH and NH₃⁺, respectively), they are able to illustrate the effect of the global charge of the ligand in the binding behaviour with RNA (Figure 1).

As a preliminary reference, Table 1 summarizes the literature binding constants referred to Paromomycin and Neomycin interactions with both RNAEC and RNASH that were obtained with different experimental techniques and working conditions. Overall, it is noted that affinities of both aminoglycosides are higher for the bacterial RNAEC than for the human target, and Neomycin shows the higher binding constants for the two tested targets. Paromomycin, Neomycin and the Neomycin-conjugates depicted in Figure 1 have been selected for this study. The buffer recommended by Pilch and colleague (Kaul et al., 2003) has been also used in this work for all studied aminoglycosides and its diPNA conjugates it is +6, and it is +4 for Neomycin-dinucleotide conjugates.

Aminoglycosides-bacterial ribosomal RNA interactions

Results achieved for Paromomycin and Neomycin are summarized in Figure 3 and Table 2 agree with those from literature obtained in the same experimental conditions (Table 1; Kaul et al., 2005). Both compounds show two main interaction events. Results were consistent regardless of the fitting algorithm we used to produce the mathematical adjustment of the ITC curves. Here, we followed the interpretation that Pilch et al. (Kaul et al., 2003; Kaul et al., 2005) produced on ITC profiles of aminoglycoside-RNA complexes. According to their reasoning, the ITC profiles were consistent with the existence of at least two equilibria. The first and steepest phase that rendered the greatest equilibrium binding constant was consequently assigned to the specific aminoglycoside A-site interaction that had been identified in the structural studies. The second binding equilibrium was attributed to weaker nonspecific interactions and were not further explored and no more clues about their nature were offered in any of the published papers on the subject.

Based on these precedents, only this first event with the highest Kᵦ and a stoichiometry (Nᵦ) of approximately 1, which relates to the specific binding of aminoglycosides to the RNA bulge site, was used for comparing affinities. The second drug interaction event, with an stoichiometry (Nᵦ) of approximately 2–3 and an affinity constant (Kᵦ) two orders of magnitude lower, can be ascribed to unspecific binding of aminoglycosides to RNA. Secondary binding events can be originated in the fact that aminoglycosides and our conjugates, being polycationic molecules, tend to interact unspecifically with anionic targets as RNAs were. Overall, that could probably be the reason that these secondary binding interactions were not further analysed in Pilch’s papers.

Table 2. Binding parameters of the studied ligands with RNAEC

| Compound     | RNAEC | Neomycin | Neomycin-TT | Neomycin-AA | Neomycin-tt | Neomycin-aa |
|--------------|-------|----------|-------------|-------------|-------------|-------------|
| Kᵦ (M⁻¹)    |       |          |             |             |             |             |
| ΔG₁ (kcal·mol⁻¹) |       |          |             |             |             |             |
| ΔH₁ (kcal·mol⁻¹) |       |          |             |             |             |             |
| -TΔS₁ (kcal·mol⁻¹) |       |          |             |             |             |             |
| Nᵦ           |       |          |             |             |             |             |
| Kᵦ (M⁻¹)    |       |          |             |             |             |             |
| ΔG₂ (kcal·mol⁻¹) |       |          |             |             |             |             |
| ΔH₂ (kcal·mol⁻¹) |       |          |             |             |             |             |
| -TΔS₂ (kcal·mol⁻¹) |       |          |             |             |             |             |

RNAEC, bacterial ribosomal RNA; EDTA, ethylenediaminetetra-acetic acid.
Experimental conditions: 10 mM sodium cacodylate, 0.1 mM EDTA, 150 mM NaCl, pH 5.5, 25 ± 0.2 °C.

![Figure 4](image_url)
Figure 5. Isothermal titration curves for the interactions of Paromomycin, Neomycin and Neomycin derivatives with human cytoplasmic ribosomal RNA.

Table 3. Binding parameters of the studied ligands with RNA_{HS}

|          | Paromomycin$^a$ | Neomycin$^a$ | Neomycin-tta$^b$ | Neomycin-aa$^{a,b}$ |
|----------|-----------------|--------------|-------------------|---------------------|
| $N_1$    | $1.5 \pm 0.2$   | $1.29 \pm 0.03$ | $1.2 \pm 0.1$     | $-1.2 \pm 0.3$      |
| $K_{b1}$ (M$^{-1}$) | $(5.2 \pm 2.1) \times 10^6$ | $(1.7 \pm 0.2) \times 10^7$ | $(7.3 \pm 3.0) \times 10^6$ | $(3.7 \pm 1.8) \times 10^6$ |
| $\Delta G_1$ (kcal·mol$^{-1}$) | $-9.1 \pm 0.2$ | $-9.87 \pm 0.06$ | $-9.3 \pm 0.3$ | $-8.9 \pm 0.3$ |
| $\Delta H_1$ (kcal·mol$^{-1}$) | $-0.6 \pm 0.3$ | $-2.67 \pm 0.03$ | $-4.9 \pm 0.1$ | $-7.28 \pm 0.05$ |
| $-T\Delta S_1$ (kcal·mol$^{-1}$) | $-8.5 \pm 0.5$ | $-7.20 \pm 0.08$ | $-4.4 \pm 0.3$ | $-1.6 \pm 0.3$ |
| $N_2$    | $2.3 \pm 0.7$   | $2.1 \pm 0.1$  | $2.8 \pm 0.1$     | $-1.2 \pm 0.3$      |
| $K_{b2}$ (M$^{-1}$) | $(8.2 \pm 1.6) \times 10^5$ | $(1.7 \pm 0.2) \times 10^5$ | $(1.07 \pm 0.08) \times 10^5$ | $(9.5 \pm 2.4) \times 10^5$ |
| $\Delta G_2$ (kcal·mol$^{-1}$) | $-6.7 \pm 0.2$ | $-7.12 \pm 0.08$ | $-6.86 \pm 0.05$ | $-8.1 \pm 0.2$ |
| $\Delta H_2$ (kcal·mol$^{-1}$) | $-2.2 \pm 0.3$ | $-5.3 \pm 0.2$  | $-5.0 \pm 0.2$ | $-4.04 \pm 0.06$ |
| $-T\Delta S_2$ (kcal·mol$^{-1}$) | $-4.5 \pm 0.4$ | $-1.9 \pm 0.3$  | $-1.8 \pm 0.3$ | $-4.1 \pm 0.2$ |

RNA_{HS}, human cytoplasmic ribosomal RNA; EDTA, ethylenediaminetetra-acetic acid.

$^a$Experimental conditions: 10 mM sodium cacodylate, 0.1 mM EDTA, 150 mM NaCl, pH 5.5, 25 ± 0.2 °C.

$^b$In this case, curves could be only adjusted to a sequential binding mode, up to a total of three calorimetric events.
(Kaul et al., 2003; Kaul et al., 2005). Being beyond our aim, the interpretation of these secondary and complex interactions, burdened by low enthalpies and binding constants two order of magnitude lower than those of the first interaction, was thus not intended.

The complete thermodynamic signatures for the first binding event are depicted in Figure 4. The breakdown of the overall binding affinity into its constituents values of enthalpy and entropy provides useful guidelines for deducing structure-activity relationships (Chaires, 2008; Ladbury et al., 2010). A glance on the relative magnitudes of the enthalpic, ΔH, and entropic, TΔS, terms associated to the first interaction events shows the preponderance of the entropic one. This result seems to contravene with the substantial binding interactions that are established between the natural aminoglycosides and the bacterial A-site rRNA as shown by the diffraction X-ray (François et al., 2005) and NMR (Fourmy et al., 1996) studies. Nevertheless, this trend was characteristic for minor groove binders of nucleic acids (François et al., 2005). Thus, the entropy driven process could be related to unstacking and the consequent displacement of adenines A1492 and A1493 into the helix minor groove when the aminoglycoside binds into the RNA bulge. Moreover, the dependence of the binding affinities on the ionic strength (Kaul and Pilch, 2002) suggests that the electrostatic interactions play a significant role. Thus, because they produce the release of counterions from the RNA, there is an increase of the net entropy variation. Finally, it should be pointed out that target and ligand desolvation processes also alter the organized water network around both entities resulting in a significant entropic gain.

Values gathered in Table 2 point out Neomycin as the most effective natural aminoglycoside, as it binds to the RNA with higher affinity than Paromomycin (approximately 10-fold in this study and sevenfold according to Kaul et al., 2006). The enhanced binding affinity of Neomycin with respect to Paromomycin is clearly related to the presence of a 6′-amino instead of a hydroxyl group, which results in a more favourable enthalpy.

With respect to conjugates, the diPNA-containing (Neomycin-tt and Neomycin-aa) show K_b1 values of the same order than the natural aminoglycoside Paromomycin but one order of magnitude lower than that of Neomycin (Table 2). As diPNA-conjugates contain the same number of amino groups than Neomycin, their lower affinity should be attributed to the global effect of the polynucleotide chain. It is well-known that the formation of new bonds, mainly hydrogen bonds but also van der Waals or polar interactions, contributes to the ΔH term. Thus, an increase of enthalpy points out an increment in the number and/or strength of the ligand-target interactions. When comparing the first event thermodynamic quantities, the diPNA-conjugates enthalpic term is similar to that for Neomycin, being higher for Neomycin-aa than for Neomycin-tt. By contrast, the enthalpic term of the dinucleotide-conjugates (Neomycin-AA and Neomycin-TT) are lower than for neomycin, although their entropic terms are similar.

Consequently, dinucleotide-conjugates show a lower affinity than the diPNA-conjugates. Low affinity of dinucleotide-conjugates could be attributed either to their lower positive charge with respect Neomycin and diPNA-conjugates or that the array of the polar groups does not favour the interaction with RNA.

**Aminoglycosides-human cytoplasmic ribosomal RNA interactions**

The behaviour of ligands with respect the RNA_HS, a eukaryote target, has been also studied to evaluate the selectivity, that is, the ratio between the affinities of each ligand with both prokaryote and eukaryote targets. This is a key question for estimating the potential activity of the compounds as antibiotics because the effectiveness as antimicrobial agents is clearly related to the specificity of the rRNA-targeting molecules for the bacterial versus human ribosomes (Kondo et al., 2007). The two natural aminoglycosides as well as the diPNA-conjugates with higher affinity for the bacterial target (Neomycin-tt and Neomycin-aa) have been considered in this part of work. Note that K_b1 for Paromomycin-RNA_EC complex in the experimental conditions reported before (Table 1) is consistent with that obtained in our laboratory (Table 3).

The titration curves depicted in Figure 5 show also, at least, two interaction steps with RNA_HS but the shape of the ITC curves strongly differs from those obtained with the prokaryote RNA_EC. As expected, the affinity of the aminoglycosides and the diPNA-conjugates for the human target is at least one order lower than for the prokaryote target, confirming what was reported for aminoglycosides and their analogues (Kaul et al., 2005; Kondo et al., 2007). For the natural aminoglycosides, Paromomycin and Neomycin, the first event involves lower enthalpic contribution than the second despite that the associated binding constant is higher (K_b1 > K_b2). Neomycin shows the highest target affinity, K_b1 value, miming the observed behaviour with the prokaryote RNA_EC. diPNA-conjugates show similar K_b values, close to that of Paromomycin, but an order lower than Neomycin, similarly to what was observed in bacterial RNA complexes. Neomycin-tt origins successive binding steps of decreasing associated ΔH values. Only the first two events, the most significant ones, are included in Table 3. Finally, Neomycin-aa shows, at least, three interaction steps. This third event was not observed in the other studied systems, and it could not be attributed a single physical

![Figure 6](wileyonlinelibrary.com/journal/jmr)
meaning. A plausible explanation was the precipitation of RNA as a result of the saturation by positively charged aminoglycosides (a peak broadening was observed after the second binding event), but any other process could also be possible. The very low RNA$_{HS}$ concentration (20 μM) prevented to visualize any precipitation process. Then, Neomycin-aa binds RNA$_{HS}$ in a different way than other tested aminoglycosides showing a significantly higher enthalpy variation. The complete thermodynamic signatures of all studied first interaction events are shown in Figure 6.

**Comparison of aminoglycosides and conjugates interactions with eukaryote and prokaryote targets**

The selectivity of aminoglycosides and conjugates for the bacterial versus the human RNA was estimated by comparing the thermodynamic values obtained in the two sets of experiments (Tables 2 and 3). Due to the fact that binding interactions were studied on RNA surrogates of wild ribosomal RNA, the thermodynamic values assigned to the second interaction were not studied on RNA surrogates of wild ribosomal RNA, the thermodynamic values obtained in the two sets of experiments (Table 3 versus the human RNA was estimated by comparing the binding constants of the studied ligands with the two targets and gives information on the selectivity of the ligands. Thus, all the tested aminoglycosides show a higher preference for the prokaryote target being that of Neomycin the highest one. Interestingly, the selectivities of both diPNA conjugates are similar but significantly higher than for Paromomycin. In some way, this result supports the working hypothesis that the derivatization of aminoglycosides with nucleobase units can improve their selectivity by procuring additional interactions with RNA targets. The binding constants of Paromomycin and both Neomycin derivatives with each target are similar but Neomycin-aa shows a binding process mainly because of the enthalpic term, whereas the remaining ligands were governed by the entropy. Probably, this fact is explained by a different interaction mode of the Neomycin-aa derivative, which originates in the distinctive binding properties of the pendant diPNA. Notably, Neomycin-aa, which shows a slightly better selectivity than Neomycin-tt, is also the aminoglycoside analogue with the highest enthalpic contribution to binding. This appears to corroborate the convenience that the selection of drug candidates should be guided not only by ΔG values, but also considering the ΔH/(TΔS) ratios because a higher enthalpic term guarantees a better selectivity (Kondo et al., 2007; Ladbury et al., 2010; Núñez et al., 2012).

**CONCLUSIONS**

Here, the comparative thermodynamic analysis by ITC of the binding interaction of natural aminoglycosides and chemical analogues with A-site rRNA surrogates has permitted to select Neomycin-diPNA conjugates as potential lead compounds for antimicrobial activity. Although at a preliminary stage, this result seems to corroborate that it is possible to fine-tune the binding of aminoglycosides to their biological targets by incorporation of ancillary appendages. Work is in progress to further extend the set of aminoglycoside conjugates and to assess their potential antimicrobial activity.

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