Supporting Information

for

Dramatic alterations in neomycin-B protonation state upon binding to a 23-mer RNA aptamer: a simple NMR analysis of the protonation equilibrium that accompanies aminoglycoside recognition

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CONTENTS:

Experimental Section

Figures S1 to S8
Experimental Section

Neomycin-B, Paromomycin, Tobramycin, Kanamycin-B and Amikacin were purchased from Sigma-Aldrich.

Amberlite IRA-400 resin in its OH form was placed in a column of 2.5 cm in diameter and washed with 500 mL of water. Solutions of 200 mM aminoglycoside (Neomycin-B, Paromomycin, Kanamycin-B or Tobramycin) were loaded onto the column. The aminoglycosides were eluted at a flow rate of 1 mL/min and 15 mL fractions were collected. After checking the pH of each fraction they were pooled, lyophilized and stored. Amikacin was purchased in its free base form.

NMR experiments were acquired in a Varian Unity 500 MHz spectrometer at 40 º C. The aminoglycoside pKa values were derived by recording 2D $^1$H-$^{13}$C HSQC experiments of a series of samples at different pH values (ten to twelve different pH values per aminoglycoside). Typically, a data matrix of 512 * 4K points was employed to digitize a spectral width of 3500 Hz. 32 scans were used per increment with a relaxation delay of 1 s. Prior to Fourier transformation, zero filling was performed to expand the data to 1K*8K. Baseline correction was applied in both dimensions. In order to analyze the effect of different anions on the aminoglycoside pKa values, SO$_4$Na$_2$, NaCl, HPO$_4$Na$_2$ or CF$_3$CO$_2$H were added to the aminoglycoside solution at the desired final concentration before pH adjustment. This solution was then employed to prepare the different NMR samples whose pH was adjusted by addition of either HCl or NaOH in D$_2$O. All the pH measurements of the NMR samples were performed in a Oakton (Eutech Instruments) pH-meter. Coupled $^1$H-$^{13}$C HSQC experiments were also performed at 40 º C at the different pH values.

The 23-mer RNA oligonucleotide used in this study was purchased in its PAGE-purified sodium salt form from Dharmacon Research (Lafayette CO). This RNA fragment was dialyzed against 5 mM phosphate at pH 6.0. NMR samples were prepared at 2 mM RNA in 5 mM phosphate pH 6.1. In order to form the neomycin-B/aptamer complex a neomycin-B stock solution was
gradually added to the RNA aptamer and complex formation was followed by recording the imino proton spectra. Coupled H-C\textsuperscript{13} HSQC experiments at the different pH values were performed at 25°C in a Brucker AMX-600.

The geometry optimization and NMR proton-carbon spin-spin coupling constants of monosaccharides have been obtained with the Gaussian03 program using density functional theory (DFT) with Lee–Young–Parr (B3LYP) correlation functional and the 6-31+G* basis set. Geometry optimizations were obtained with the gradient optimization routine, the convergence criteria were set to 1.10\textsuperscript{-6}.
Figure S1.- a) HSQC experiment corresponding to neomycin-B. The chemical shift variations observed for the HC-NH$_2$ functions present in this aminoglycoside, as a function of pH are also shown. b) Typical profiles obtained from the analysis of pH titration experiments. For protons H1 and H3 of the 2-DOS moiety (unit II) two different transitions are apparent, reflecting the sequential change in the protonation state of the two amino groups present in this ring. Therefore, the two pKa values could be derived independently from each individual titration curve. In contrast, for the rest of the amino groups a unique transition was detected.
Figure S2.- Chemical shift variations observed for protons 3 of unit 2 (3-II, lower panel) and 2 of unit IV (2-IV, upper panel) during the pH titration experiments. pKa values were derived both in absence and presence of different ionic compounds (TFA, NaCl, Sodium Phosphate and Na$_2$SO$_4$) at 500 mM concentration. Fitted curves are represented as dotted lines.
Figure S3.- $J_{C-H}$ increments computed for aminosugar and aminocyclitol fragments present in different aminoglycosides. In all cases $\Delta J = J_{(protonated)} - J_{(non \text{ protonated})}$ is shown.
Figure S4. - HC-NH region corresponding to HSQC experiments measured for neomycin-B at different pH values. It can be observed that the proposed heteronuclear couplings are 10-11 Hz larger for the charged fragments.
Figure S5.- It could be argued that different factors apart from the charged/uncharged state of the amino functions might have an influence on the proposed J values. Thus, in principle, the formation of a strong salt-bridge between the aminoglycoside positive charges and a phosphate groups within an RNA receptor might also affect the heteronuclear $^1$H-$^{13}$C coupling. To check this possibility we synthesized, as a model, the neomycin-B derivative shown below. This compound is characterized by the presence of a strong intramolecular salt-bridge between NH$_3^+$-2 and the carboxylate present on the ribose unit, at neutral pH. In fact, the presence of this interaction is reflected in the unusual pKa values of these two functions (1.9 and 8.4 for the carboxylate and amino respectively). The $^1$J$_{CH}$ for position 2 of unit I was measured at 3 pH values corresponding the presence COOH/NH$_3^+$, COO$^-$/NH$_3^+$, and COO$^-$/NH$_2$ contacts between rings I and III. It can be observed that the COO$^-$/NH$_3^+$ polar interaction at neutral pH has not measurable effect on the J$_{CH}$ coupling constant, which is mainly sensitive to the charged/uncharged state of the amino group.
Figure S6—NMR structure corresponding to the neomycin-B/aptamer complex. Interestingly, the amino group at position 3 of unit II, whose pKa is altered in more than 3.6 units is not involved in a direct interaction with a charged group within the binding pocket, according to this data. Instead it forms two hydrogen bonds with a guanine base. This fact, together with the strongly negative electrostatic potential of the binding pocket its sufficient to cause such a large change in basicity.
Figure S7.- The change in the protonation state of neomycin-B that accompanies its non-specific recognition by DNA was tested employing two different 16-mer DNA duplexes (TA)$_{16}$ and (CG)$_{16}$. To determine the fraction of the drug complexed at different pH values, NMR diffusion (DOSY) measurements were previously performed. According to these data for the (CG)$_{16}$ duplex, the drug remains almost fully bound up to pH 7.5. At larger pH values the increase in the diffusion coefficient reveals the dissociation of the complex.
Figure S8.- HC-NH₂/NH₃⁺ region corresponding to coupled HSQC experiments of the neomycin-B/DNA complexes. It can be observed that, in contrast with the behavior observed for the RNA aptamer, in this case the amino group at position 2 of ring II remains mainly non-protonated in the complexed state. Therefore the non-specific electrostatic interaction between the antibiotic and both DNA fragments is not sufficient to promote the protonation of this function at pH > 7.0.