Supplementary Material

Cleavage of RNA Oligonucleotides by Aminoglycosides

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Table of Contents:

Experimental:

Materials

All chemicals were of reagent grade quality or better, obtained from commercial suppliers and used without further purification. Solvents were used as received or dried over 4 Å molecular sieves or dried following literature procedures.[1] Water was distilled prior to use, except for all biochemical reactions, where autoclaved water was used. NTPs and Phage T7 polymerase and RNase Inhibitor (Ribolock) were purchased from Fermentas, T4 Kinase from New England Biolabs and Alkaline phosphatase from Roche Biochemicals. γ-^{32}P-ATP (4500 Ci mmol^{-1}) was acquired from MP Biochemicals. Custom RNA oligonucleotides (including the 2AP labelled TAR) were procured from Dharmacon and were deprotected using the company

supplied procedure, purified by PAGE gels and desalted on a Waters Corporation C-18 Sep-Pak column. DNA oligonucleotides were purchased from DNA Technologies Inc. and were purified by PAGE gels and desalted in the same manner as the RNA oligonucleotides. The A-Site RNA construct containing the furan-modified uracil (A-Site-1406(U^{Fu})) was prepared according to a published procedure.[2]

Purification of aminoglycosides

The aminoglycosides used in this study were prepared in the "free base" form by using DOWEX MONOSPHERE 550A (OH) anion exchange resin. A general procedure involved loading *ca.* 5 mL of aqueous aminoglycoside sulfate (or aminoglycoside TFA salt) (~50 mg mL^{-1}) onto the resin column that had been equilibrated with water. The aminoglycoside was then eluted with 1.0 M NH_4OH. Fractions containing the free base (identified by ninhydrin staining on thin layer chromatography plates) were combined and lyophilized.

Instrumentation

MALDI-TOF spectra were collected on a PE Biosystems Voyager-DE STR MALDI-TOF spectrometer in positive-ion, delayed-extraction mode using CHCA (a-cyanohydroxycinnamic acid) as the matrix. Polyacrylamide gels containing radiolabeled RNA were analysed using a Molecular Dynamics Storm 840 phosphorimager (Amersham Pharmacia Biotech). Steady-state fluorescence experiments were carried out in a microfluorescence cell with a path length of 1.0 cm (Hellma GmH & Co KG, Mullenheim, Germany) on a Horiba Jobin Yvon FluoroMax-3 spectrophotometer. Circular Dichroism (CD) measurements were taken on an AVIV Model 215 Circular Dichroism Spectrophotometer, using a 0.1 cm path length quartz CD cell.

Fluorescence assays

For the binding experiments, 50 μM solutions of the fluorescent RNA constructs in 20mM HEPES buffer (pH 7.5 @ 25°C) and 100mM NaCl were flash annealed by heating at 90°C for 3 min, and then cooled directly on ice. Fluorescence measurements were made at 21°C. For the 2AP construct, the excitation and emission slit widths were 10 nm and 15 nm, respectively, and the excitation wavelength was 320 nm. For the U^{Fu} construct, the excitation and emission slit widths were 12 nm and 15 nm, respectively, and the excitation wavelength was 322 nm.

In a typical experiment, the emission spectrum of a 120 μL solution of the buffer (20 mM HEPES or MOPS, 100 mM NaCl, 0.5 mM EDTA was measured in the absence of both the RNA and aminoglycoside. This spectrum was used as the blank (for which only Raman scatter was observed) and subsequently subtracted from all following spectra. Following this blank determination, 5 μL of a 50 μM solution of the refolded RNA (5 μL of a 25 μM solution was used for A-Site RNA construct) was added to the solution and thoroughly mixed, yielding a final RNA concentration of 2 μM (1 μM for the A-Site construct). The fluorescence spectrum was recorded once again, followed by 1 μL additions of aliquots of increasing concentrations of aqueous aminoglycoside solution, coupled each time with a measurement of the fluorescence spectrum. The change in total volume of the solution was never more than 7%. EC_{50} values were determined in a similar fashion to that previously reported.[3] the following buffers were used for titrations carried out at different pH values: MOPS for pH 6.5, 7.0 and 7.5, and HEPES for 7.5 and 8.0. EC_{50} values for binding were determined by fitting the fluorescence data to equation 1, where y is the fluorescence intensity, x is the concentration of aminoglycoside and n is the sigmoidal coefficient:

$$y = y_{\min} + \frac{y_{\max}}{1 + e^{\frac{[EC_{50}]-x}{n}}}$$ Equation 1

In addition, association equilibrium constants, K_a, were calculated as the reciprocal of the concentration of aminoglycoside at 50% saturation (at which point the concentration of "free" and complexed forms of RNA construct are equal), according to equation 2, where [Adduct], is the concentration of the RNA/aminoglycoside complex, and [AG] is the concentration of the aminoglyocoside:

$$K_a = \frac{[Adduct]}{[RNA][AG]} = \frac{1}{[AG]}(M^{-1})$$ Equation 2

Circular dichroism titrations

The TAR RNA construct was firstly flash annealed (heated at 90°C for 3 min, and then cooled directly on ice). A blank CD spectrum of the buffer solution (10 mM MOPS (pH 6.5 @ 25°C), 100 mM NaCl) was recorded, which was subtracted from all subsequent spectra. The RNA was added to the cuvette, resulting in a final RNA concentration of 20 μM and the CD spectrum re-recorded. Subsequently, 1 μL aliquots of an aminoglycoside stock solution were added, with a spectrum being recorded after each addition. Spectra were measured twice and averaged. No CD signal was observed for the neomycin B.

Cleavage experiments

The ^{32}P end-labelled RNA transcripts were refolded by flash annealing (as per the fluorescence assays). A solution was prepared containing 20 mM MOPS buffer (pH 6.5 @ 21°C), 100 mM NaCl, 1 μM RNA, and varying concentrations of Neomycin B, to a total reaction volume of 20 μL. Control reactions containing RNA in the reaction buffer, or 10 mM EDTA and aminoglycoside, or 2 μL of DMSO and aminoglycoside were also prepared and incubated along with the other reaction mixtures. The

resulting solutions were incubated at 21°C for 1 or 2 days, 10 μL of loading buffer (7 M urea in 10 mM Tris.HCl (pH 8.0), 100 mM EDTA and 0.05% w/v bromophenol blue) added, and an aliquot loaded directly onto a 20 % denaturing PAGE gel. The gel was then visualised by phosphorimaging and analysed using the ImageQuant™ software package (Amersham Pharmacia Biotech, 2000).

pH dependence of cleavage reactions

Solutions (20 μL total reaction volume containing 25 μM aminoglycoside and freshly annealed TAR (spiked with ^{32}P end-labelled TAR; 6,000 cpm) in 20 mM MOPS buffer (pH 6.5, 7.0 or 7.5 @ 21°C) or HEPES buffer (pH 8.0 @ 21°C) were reacted for a period of 24 h. After this time, 10 μL of loading buffer was added to each reaction (7 M urea in 10 mM Tris.HCl (pH 8.0), 100 mM EDTA and 0.05% w/v bromophenol blue, 10% (v/v) glycerol), and then loaded directly onto a 20% denaturing PAGE gel. The gel was imaged and analysed as in the previous experiments.

Time dependence of cleavage reactions

Each of the reactions was prepared as described in the pH dependence experiments. Individual reactions were quenched at varying times by the addition of 10 μL of loading buffer (7 M urea in 10 mM Tris.HCl (pH 8.0), 100 mM EDTA and 0.05% w/v bromophenol blue, 10% (v/v) glycerol) and stored at -20°C until all the reactions had completed. Each reaction mixture was loaded onto a 20% denaturing PAGE gel. The gel was then imaged by phosphorimaging and analysed using the ImageQuant™ software package (Amersham Pharmacia Biotech, 2000). The phosphorus density counts of the main cleavage band were plotted against the time and a single exponential equation was fitted through the data, using the Igor Pro™ software package, in order to determine the *pseudo*-first order rate constants for the reactions.

Transcription RNA constructs

A single-strand DNA template was annealed to an 18-mer T7 polymerase consensus promotor in TE Buffer (10 mM Tris.HCl, 1mM EDTA, 100 mM NaCl, pH 7.8), by heating a 1:1 solution (5 μM) at 90°C for 2min and cooling the resulting solution slowly to room temperature. The transcription reaction was performed in 40 mM Tris.HCl (pH 7.9 @ 25°C), 15 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 2 mM spermidine, 2 mM ATP, 2 mM CTP, 2 mM UTP, 3 mM GTP, 0.5 U μL^{-1} ribolock, 300 nM of annealed DNA template, H_2O and 2.5 U μL^{-1} of T7 polymerase were combined to a total volume of 250 μL. This solution was kept at 37°C for 4 h and subsequently quenched with 80 μL of loading buffer (7 M Urea in 10 mM Tris.HCl (pH 8.0), 100 mM EDTA and 0.05% w/v bromophenol blue) and loaded directly onto a 20% denaturing polyacrylamide gel. The product was visualised by UV-shadowing, the gel section containing product was cut-out and extracted with 0.5 M sodium acetate. The resultant RNA oligo was desalted using a Waters Corporation C-18 Sep-Pak column and quantified by UV-Vis spectrophometry (TAR, ε_{260} = 269,000 $M^{-1}cm^{-1}$, A-Site ε_{260} = 254,000 $M^{-1}cm^{-1}$).

Alkaline phosphatase removal of the 5′ triphosphate

The transcribed RNA (40 μM) was reacted with 90 U of calf intestine alkaline phosphatase in alkaline phosphatase buffer (100 mM Tris.HCl, pH 8.5 @ 37°C and 10 mM $MgCl_2$) at a total volume of 400 μL. This solution was heated at 37°C for 3 h and then heated at 65°C for 10 min to fully denature the enzyme. The solution then was extracted with 25:24:1 phenol:chlorform:iso-amyl Alcohol. To the aqueous layer was added 20 μL of 3 M sodium acetate and the dephosphorylated oligo was precipitated by addition of EtOH. The pellet was resuspended in 70% EtOH and reprecipitated. Removal of the 5′ triphosphate was confirmed by MALDI-TOF mass spectrometry

TAR; calcd [M+H]; m/z = 9286.36, found; m/z = 9288.63, A-Site; calcd [M+H]; m/z = 8679.46, found; m/z = 8675.59.

^{32}P end-labelling of TAR RNA using T4 Kinase

Dephosphorylated RNA (300 pmol) was reacted with Phage T4 polynucleotide kinase (80 U) in kinase buffer (70 mM Tris.HCl (pH 7.6 @ 25^{o}C), 10 mM $MgCl_2$, 5 mM DTT) containing 40 μCi of γ-^{32}P-ATP (4500 Ci mmol^{-1}), 1 mg mL^{-1} bovine serum albumin in a total reaction volume of 30 μL. The solution was heated at 37^{o}C for 1.5 h, quenched with 20 μL loading buffer and loaded directly onto a 20% denaturing PAGE gel. The gel was imaged by autoradiography allowing location of the product band, which was cut out and extracted off the gel by 0.5 M NH_4OAc (2 × 3 mL at 55^{o}C for 30 min). The resulting solution was desalted by a Waters C-18 Sep-Pak column. The product was then resuspended in 100 μL of H_2O yielding a 200 k cpm μL^{-1} solution of end-labelled RNA.

Sequencing reactions

Enzymatic digestions:

1 μM of RNA (spiked with ^{32}P end-labelled RNA (6000 cpm)) was added to a solution containing 10 mM Tris.HCl (pH 7.5 @ 37°C, 100 mM KCl, and 10 mM $MgCl_2$. This solution was equilibrated to 21°C, at which point RNase T1 (4U) or RNase A (0.032 ng) was added to the mixture, to a total reaction volume of 10 μL. After the reaction had incubated at 21°C for 15 min, 10 μL of loading buffer (7 M urea in 10 mM Tris.HCl (pH 8.0), 100 mM EDTA and 0.05% w/v bromophenol blue) was added to the mixture and the resulting solution heated at 90°C for 2 min, before being cooled on ice. These enzymatic digestions were subjected to 20% denaturing PAGE electrophoresis, alongside incubated mixtures of neomycin B with the RNA targets.

Chemical digestions:

A reaction: To a solution (total volume 20 μL) containing ^{32}P end-labelled TAR (80,000 cpm), 0.4 μg μL^{-1} of yeast carrier RNA, 200 mM HEPES (pH 7.8 @ 21°C), 100 mM KCl, and 10 mM $MgCl_2$, was added 0.5 μL of diethylpyrocarbonate. The resulting solution was incubated at 21°C for 45 min, after which point 4 μL of 3 M sodium acetate was added and the RNA was precipitated with ethanol, pelleted, washed with 70% (v/v) ethanol, pelleted again, and dried.

The pellet was redissolved in 20 μL of 1 M Tris.HCl (pH 8.0 @ 21°C) and 20 μL of 0.2 M $NaBH_4$ added. The solution was then incubated in the dark at 0°C for 30min. 5 μL of sodium acetate (3 M) was added to the mixture and the RNA precipitated with ethanol, pelleted, then washed with 70% (v/v) ethanol, pelleted again, and dried.

The pellet was resuspended in 40 μL of 1 M aniline acetate (pH 4) and incubated at 60^{o}C for 20 min. The solution was then cooled on ice and 5 μL of 3 M sodium acetate was added and the RNA precipitated with ethanol, pelleted, then washed with 70% (v/v) ethanol, pelleted again, and dried.

The resulting pellet was finally dissolved in 20 μL of loading buffer (7 M urea in 10 mM Tris.HCl (pH 8.0), 100 mM EDTA and 0.05% w/v bromophenol blue, 10 % (v/v) glycerol) and 20 μL of H_2O.

G reaction: The G sequencing reaction was identical to the A reaction, except in the first step 0.5 μL of dimethylsulphate was added, and the first incubation time was only 2 min.

U reaction: ^{32}P end-labelled TAR (80,000 cpm) and 0.25 μg μL^{-1} of yeast carrier RNA, were reacted in a 1:1 (v/v) solution of hydrazine and water (total reaction volume 20 μL). The solution was incubated at 2^{o}C for 5 min, after which point 5 μL of 3 M sodium acetate was added, and the RNA precipitated with ethanol, pelleted, washed with 70% (v/v) ethanol, pelleted again, and dried.

The pellet was resuspended in 40 μL of 1 M aniline acetate (pH 4) and incubated at 60^{o}C for 20 min. The solution was then cooled on ice and 5 μL of 3 M sodium acetate added. The RNA was precipitated with ethanol, pelleted, washed with 70% (v/v) ethanol, pelleted again, and dried.

The resulting pellet was finally dissolved in 20 μL of loading buffer (7 M urea in 10 mM Tris.HCl (pH 8.0), 100 mM EDTA and 0.05% w/v bromophenol blue, 10% (v/v) glycerol) and 20 μL of H_2O.

Tables and Figures

Table S1. Relative phosphor image density counts (%) for major cleavage product observed in the reaction of ^{32}P end-labelled TAR and A-site RNA constructs with varying concentrations of neomycin B. Conditions: 1 μM RNA (spiked with ^{32}P end-labelled RNA), 20 mM MOPS (pH 6.5 @ 21°C), 100 mM NaCl, 12 h reaction time for TAR, 24 h for A-site, 21°C.

	[Neomycin B]					
	1 μM	7.5 μM	15 μM	25 μM	25 μM + 10 mM EDTA	25 μM + DMSO 5% v/v
Cleavage band (%) (TAR)	**1.3**	**2.2**	**2.4**	**2.5**	**1.6**	**4.5**
Cleavage band (%) (A-site)	**0.31**	**0.67**	**0.72**	**0.69**	**0.49**	**1.05**

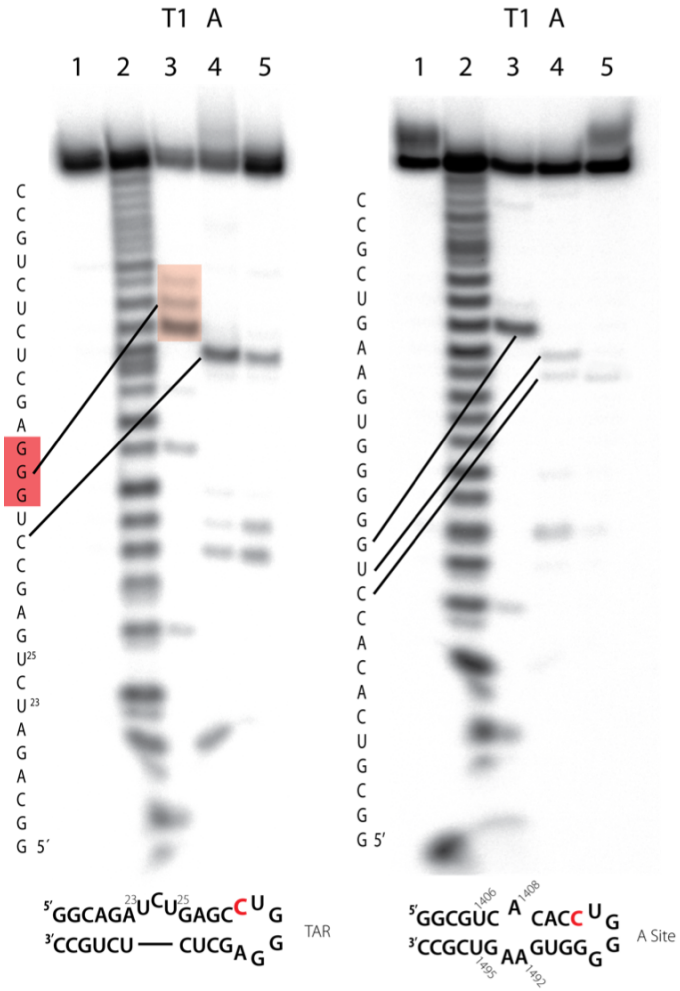

Figure S1. Phosphorimages of PAGE gels for RNase sequencing of ^{32}P end-labelled TAR and A-site constructs, and determination of the points of RNA scission by neomycin B. Lane 1: RNA only; Lane 2: alkaline hydrolysis ladder, Lane 3: RNase T1 digestion of RNA; Lane 4: RNase A digestion of RNA; Lane 5: RNA after incubation with neomycin B. Conditions: 1 μM RNA (spiked with ^{32}P end-labelled RNA), 25 μM neomycin B, 20 mM MOPS (pH 6.5 @ 21°C), 100 mM NaCl, 12 h reaction time for TAR, 24 h for A-site, 21°C. See page 9 for full details of RNase digestions.

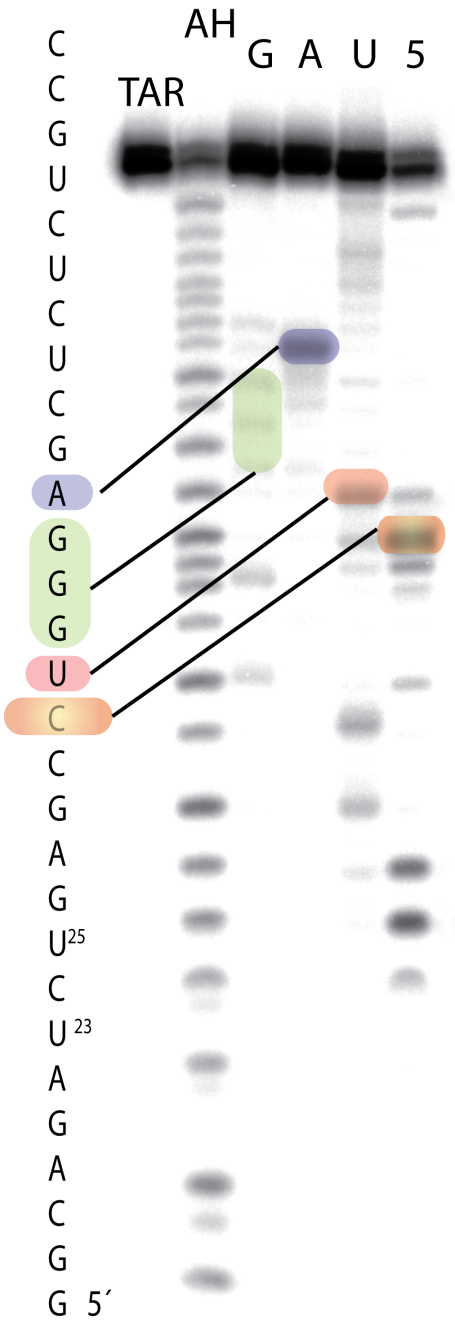

Figure S2. Phosphorimage of PAGE gel for chemical sequencing of ^{32}P end-labelled TAR and determination of the point of TAR scission by neomycin B. Lane 1: TAR only; Lane 2: alkaline hydrolysis ladder; Lane 3: G sequencing lane; Lane 4: A sequencing lane; Lane 5: U sequencing lane; Lane 6: TAR after incubation with neomycin B. Conditions: 20 mM MOPS (pH 6.5 @ 21°C), 100 mM NaCl, 1 μM TAR (spiked with ^{32}P end-labelled TAR), 25 μM aminoglycoside, 24 h reaction time, 21°C. See page 9 for full details of chemical sequencing reactions.

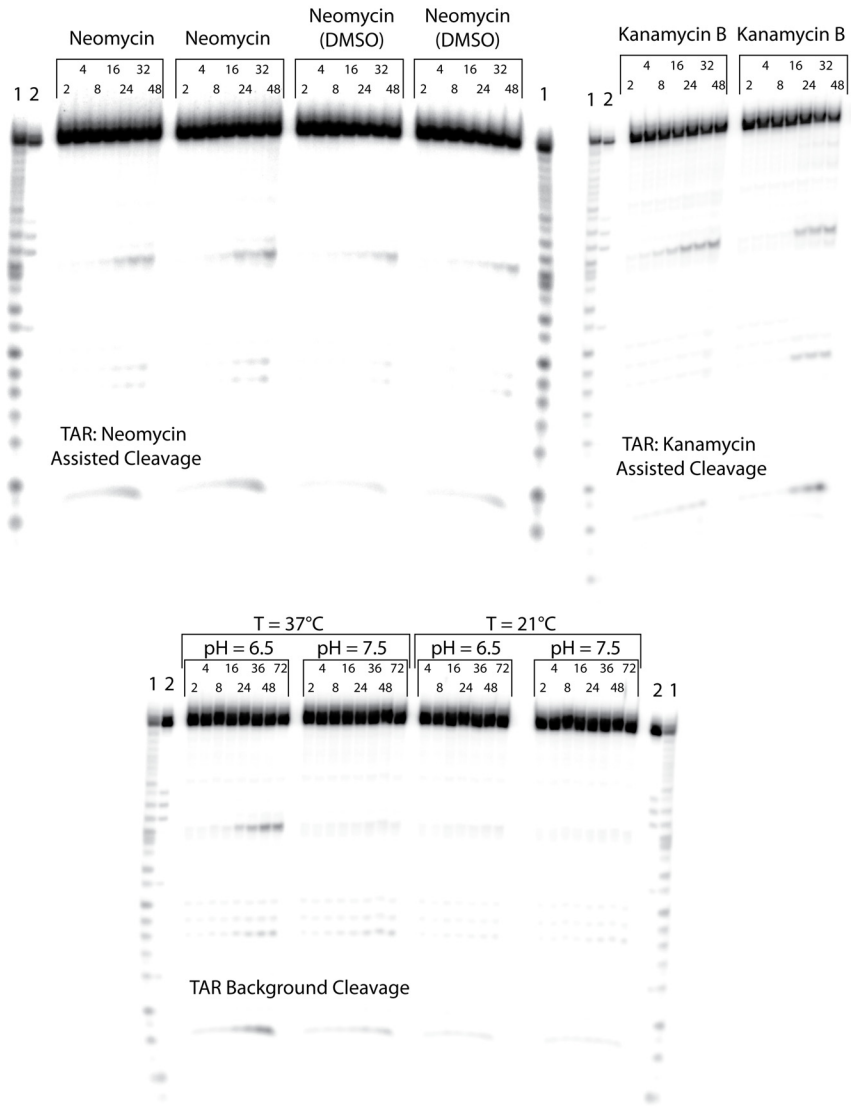

Figure S3. Phosphorimages of PAGE gels showing time dependence of TAR cleavage by neomycin B and kanamycin B, as well as background cleavage of TAR. Lane 1: alkaline hydrolysis ladder; Lane 2: T1 RNase digestion of TAR (denaturing conditions); other lanes: aminoglycoside/TAR reaction mixture after indicated time period (h). Conditions: 1 μM TAR (spiked with ^{32}P end-labelled TAR), 50 μM aminoglycoside, 20 mM MOPS (pH 6.5 or 7.5 @ reaction temp), 100 mM NaCl, 5% v/v DMSO (control reactions only), 21°C for reactions with aminoglycosides.

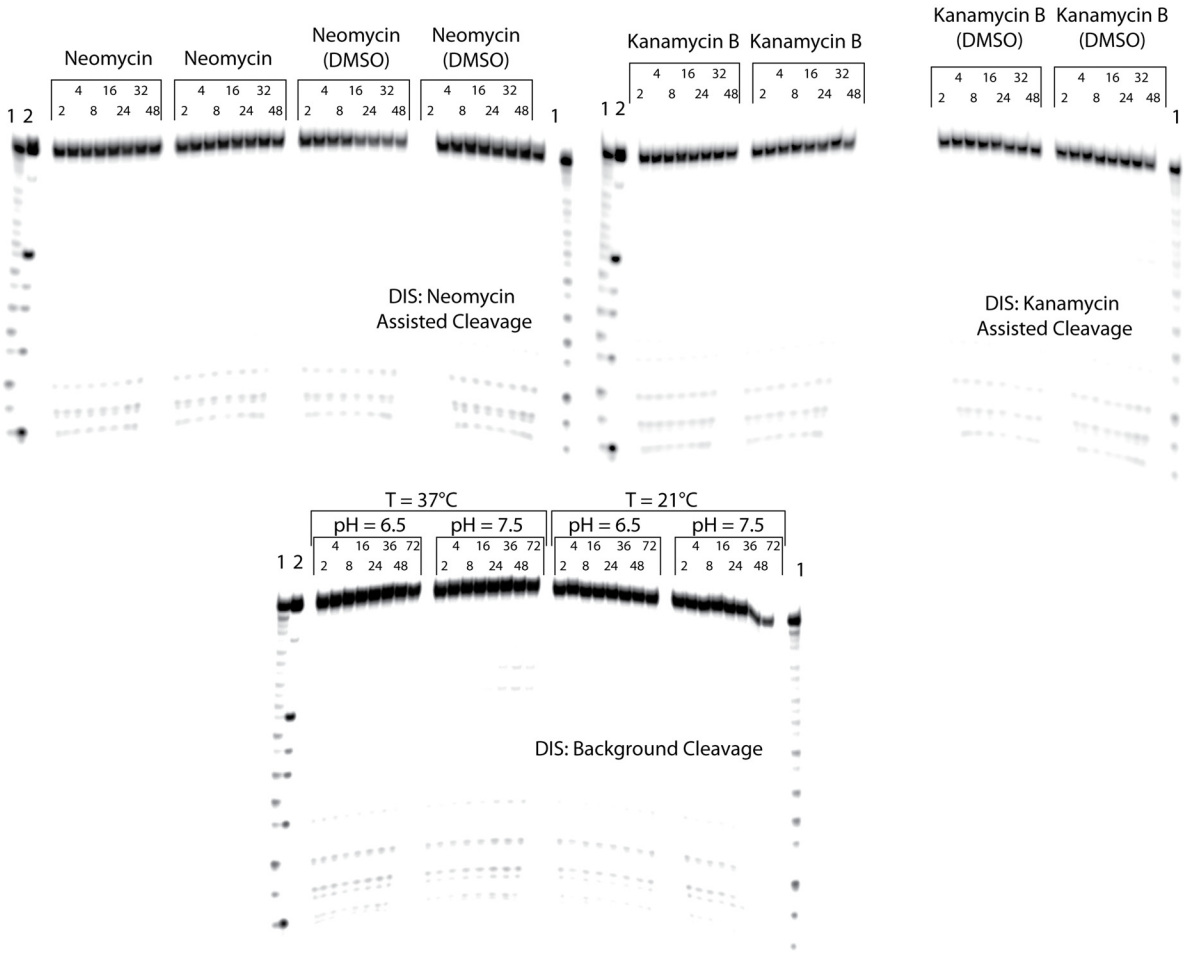

Figure S4. Phosphorimages of PAGE gels showing time dependence of DIS cleavage by neomycin B and kanamycin B, as well as background cleavage of DIS. Lane 1: alkaline hydrolysis ladder; Lane 2: T1 RNase digestion of DIS (denaturing conditions); other lanes: aminoglycoside/DIS reaction mixture after indicated time period (h). Conditions: 1 μM DIS (spiked with ^{32}P end-labelled DIS), 50 μM aminoglycoside, 20 mM MOPS (pH 6.5 or 7.5 @ reaction temp), 100 mM NaCl, 5% v/v DMSO (control reactions only), 21°C for reactions with aminoglycosides.

References:

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