Supplementary Results

Apramycin, paromomycin, and gentamicin efficiently inhibit translation elongation

Using the intersubunit FRET signal, we assayed the effects of apramycin, paromomycin, and gentamicin on the processivity of individual ribosomes at 80 nM tRNA and 80 nM EF-G. The number of elongation (high-low-high FRET) cycles that each ribosome performed on our 12-codon mRNA is plotted as histograms. Without drugs, many ribosomes translated the entire mRNA with the distribution showing a clear peak at codon 12 (Figure S1 A). However, in the presence of 1μM aminoglycosides (Figure S1 B), this peak – representing ribosomes whose translation is limited by mRNA length – disappears, and is replaced by peaks at codons 2~4 for apramycin, 1~3 for paromomycin, and 1 for gentamicin, the strongest effect observed. Similar levels of inhibition were observed at 10 μM drug concentration (Figure S1 C), suggesting that 1 μM is at or above saturating concentration for these effects.

Paromomycin and gentamicin induce misincorporation of tRNAs

To test whether apramycin, paromomycin, and gentamicin promote misincorporation and translocation through near-cognate codons, we followed the intersubunit FRET signal of ribosomes translating an mRNA in which the coding region of three Phe codons (UUU) is followed by a stop codon (UAA), which is near-cognate to Lys (AAA), and then four more Phe codons (UUU). In the presence of Phe-tRNA^{Phe} and Lys-tRNA^{Lys}, ribosomes that do not misincorporate the near-cognate Lys-tRNA^{Lys} should perform three rounds of elongation; the fraction of ribosomes that performs more than three rounds of elongation reports on the degree of miscoding in this stop codon readthrough assay within our observation timeframe (Figure S1 D-F). Due to ribosome slippage on poly-U mRNAs and differences in the compositions of translation systems between different studies, the miscoding frequencies reported are relative and not interpreted as absolute miscoding rates.

In the absence of aminoglycosides, this miscoding frequency is 2.5%, establishing the baseline of translational fidelity for our assay. In 10 μM apramycin the miscoding frequency triples to 7.5 %. Both paromomycin and gentamicin, however, increase this miscoding frequency by an order of magnitude to 30 %. Even with only Phe-tRNA^{Phe} (non-cognate to the stop codon), paromomycin still induced 6-fold more misincorporation on 15 % of ribosomes. These data suggest that paromomycin and gentamicin severely disrupt, whereas apramycin exerts a weaker effect on tRNA selection.

Analysis of ZMW experiments with paromomycin and gentamicin

The lifetimes of tRNA binding events in the presence of aminoglycosides confirm hindered translation elongation during and after tRNA selection. When all visible events were assigned (Figure S3 D-F), the lifetimes of tRNA events are consistently longer for apramycin compared to no aminoglycosides for Phe-(Cy5)tRNA^{Phe} by 3.5-fold (7.6 s vs. 2.2 s without drugs) and Lys-(Cy2)tRNA^{Lys} by 1.7-fold (6.9 s vs. 4.1 s without drugs). Compared to no drugs, the frequencies of tRNA events in apramycin are lower for tRNA^{Phe} by 2-fold (0.027 s^{-1} vs. 0.054 s^{-1} without drugs) or similar for tRNA^{Lys} (0.031 s^{-1} vs. 0.035 s^{-1} without drugs). On average, in apramycin, tRNA^{Phe} events occupy 0.21 and tRNA^{Lys} events occupy 0.24 of each trace, approximately 2-fold increases from 0.12 and 0.13 for no aminoglycosides respectively (an occupancy of 1 means that the entire trace is covered by 1 tRNA event). The longer lifetimes and, thus, higher occupancy of tRNA events that apramycin induces are consistent with our intersubunit FRET result that apramycin blocks the EF-G dependent translocation step, which leads to longer tRNA transit times.

The presence of paromomycin affected only the event frequencies, but not the lifetime, consistent with its stronger effect on the tRNA selection step compared to translocation. The overall tRNA lifetimes for both tRNA^{Phe} (3.7 s) and tRNA^{Lys} (4.0 s) were not affected by paromomycin, whereas visible event frequencies increased by 1.5-fold (0.082 s^{-1}) for tRNA^{Phe} and 2.9-fold (0.10 s^{-1}) for tRNA^{Lys}.

Accordingly, the overall occupancy of tRNA events increased significantly by 2.5 times (0.30) for $tRNA^{Phe}$ and 3.2 times (0.41) for $tRNA^{Lys}$. Paromomycin significantly increased the number of visible tRNA events (>100 ms, the limit of reliably assigning an event), consistent with the structural prediction that paromomycin stabilizes tRNAs in the A site. In the absence of drug, sampling events are <30 ms and should be rarely observed in 30 frames per second ZMW experiments. Finally, tRNA lifetimes with gentamicin are either longer with $tRNA^{Phe}$ by 3.6-fold (8.0 s) or similar to no drugs with $tRNA^{Lys}$ (3.9 s), which could be the result of many ribosomes stalling over the first Phe codon. The tRNA frequencies in gentamicin either remained similar to no drugs for $tRNA^{Phe}$ (0.058 s^{-1}) or increased for $tRNA^{Lys}$ by 2.3-fold (0.080 s^{-1}). Overall, the changes in event frequencies were not as significant as for paromomycin. The overall event occupancy for gentamicin is also high at 3.8 times from normal (0.46) for $tRNA^{Phe}$ and 2.4 times (0.31) for $tRNA^{Lys}$. Gentamicin and paromomycin both act during the tRNA binding and selection stage, with distinct nuances as observed in the other assays.

tRNA-tRNA FRET dynamics in the presence of EF-G

In experiments without aminoglycosides and with apramycin, adding 500 nM EF-G did not noticeably changed the evolution of the FRET signal upon tRNA binding to the A site. FRET events proceeded to a high (0.8) FRET state within 100 ms of the appearance of FRET and subsequently fluctuated between high (0.8) and medium (0.5) FRET, consistent with rapid tRNA selection and accommodation, and peptide bond formation followed by classical-hybrid dynamics.

With paromomycin, the presence of 500 nM EF-G enriched moderately long-lived (1~5 s) high FRET (>0.7) events, as shown in the postsynchronized plot, perhaps as a result of EF-G trapping the A-site tRNA. As short tRNA events still dominated, the total FRET lifetime was similarly short at 1.8 s when compared to no EF-G.

Supplementary Discussion

Differences between NMR solution and X-ray crystal structures

NMR solution structure results contrast with static crystal structures of paromomycin, gentamicin and apramycin bound to a similar RNA oligonucleotide, all of which demonstrate a de-stacked conformation of A1492 and A1493 (Han et al., 2005). The crystal structure of the apramycin-RNA complex would lead to 23 NOE violations (>0.4Å), and predicts 8 strong NOEs not observed in the solution structure. Interestingly, none of the RNA-aminoglycoside solution structures are fully consistent with the exaggerated de-stacked conformation of A1492 and A1493 observed in the X-ray crystal structures. Instead, the NMR solution structures suggest a conformation of A1492 and A1493 that is biased toward the minor groove when rRNA is in complex with paromomycin and gentamicin C1A, and a conformation biased toward being stacked when in complex with apramycin (Lynch et al., 2003). The differences are reconcilable if both conformations existing in solution, albeit with different relative populations depending on the aminoglycoside. The crystal structure could be the conformation that is more conducive to crystal packing and, if the two conformations are in rapid exchange (less than 1 ms time scale, which is likely for a stacking-destacking reaction), the solution structure represents an average of the two populations (Dethoff et al., 2012). The model oligonucleotides do not fully recapitulate the situation within the full ribosome. The conformation of the apramycin-30S subunit complex was solved in the context of the codon-anticodon helix interaction in the A site. In addition, ribosomal protein S12 makes contacts to stabilize the stacked out conformation of A1492. Nonetheless, it is clear from the NMR and crystallographic studies that subtle modulations of the conformational equilibrium of A1492/A1493 by aminoglycosides give rise to their different modes of action.

Supplementary Experimental Procedures

TIRF instrumentation and analysis

We performed the intersubunit and tRNA FRET experiments on a prism-based total internal reflection fluorescence microscope as previously described(Aitken and Puglisi, 2010; Marshall et al., 2008). For FRET illumination, we used a diode-pumped solid-state 532 nm at 1 kW cm^{-2}. A Quad-View device (Photometrics) separated fluorescence emissions into four channels and two of the channels were filtered to correspond to emission spectrum of Cy3 or Cy3B and Cy5. The signal was projected onto two quadrants of a 512x512 EMCCD camera (Andor Technology). We collected videos using the MetaMorph software package (Molecular Devices) and extracted fluorescent traces using in-house MatLab (MathWorks) scripts. Intersubunit FRET videos were collected at an exposure time of 50 ms per frame for 4800 frames (240 s) and tRNA-tRNA FRET videos were collected at an exposure time of 25 ms per frame for 3600 frames (90 s). FRET states were assigned using a previously described hidden Markov model based approach (Aitken et al., 2008) and corrected visually if necessary. Lifetimes for the intersubunit FRET experiments were assigned from only productive states (eg. low FRET states that was followed by a high FRET state and vice versa) to eliminate effects from photophysical effects and molecules trapped in a long-lived non-functional state. Lifetimes for tRNA-tRNA FRET experiments were fitted from all observable non-zero FRET states that do not show abnormal photophysical effects (eg. blinking and resurrection from long-lived dark state). All lifetimes were fitted to a single-exponential distribution using maximum-likelihood parameter estimation.

ZMW instrumentation and analysis

Instrumentation and chips containing 3,000 individual ZMWs were used and prepared as described previously (Uemura et al., 2010) ZMW diameters were in the range of 120-135 nm. Lys-(Cy2)$tRNA^{Lys}$, fMet-(Cy3)$tRNA^{fMet}$ and Phe-(Cy5)$tRNA^{Phe}$ fluorescence was detected using direct excitation with lasers at 488, 532 and 632 nm. Laser power for three excitations was 0.5 $\mu W/\mu m^2$ for all ZMW experiments. Fluorescence traces were recorded at a rate of 30 frames per second for 3 minutes.

Traces were selected and analyzed using the same set of scripts as TIRF experiments and the tRNA lifetimes were fitted to single exponential distributions using the same method as described for TIRF.

Supplementary Data

Table S1. tRNA FRET lifetimes. Related to Figure 4.

mRNA	EF-G (nM)	Aminoglycoside (10 μM when present)	Lifetime (s) (Single-exponential)	*n*
MFK	-	-	10.2 ± 1.2	54
MFK	-	Apramycin	17.6 ± 1.3	136
MFK	-	Paromomycin	1.692 ± 0.066	187
MFK	-	Gentamicin	6.78 ± 0.49	66
MLK	-	Paromomycin	1.391 ± 0.056	166
MFK	500	-	9.05 ± 0.56	228
MFK	500	Apramycin	17.6 ± 1.5	126
MFK	500	Paromomycin	1.829 ± 0.069	194
MLK	500	Paromomycin	1.917 ± 0.062	283

The lifetime of each individual event was extracted and the distribution for each experiment is then fitted to a single-exponential function, errors are s.d. errors.

Table S2. NMR solution structure. Related to Figure 5.

Structure statistics and atomic root mean squared (r.m.s.) deviations

Final forcing energies (kcal mol^{-1})	
Distance restraints (612)	19.8 ± 3.1
Dihedral restraints (111)	3.1 ± 0.9
R.m.s. deviations from experimental restraints	
Distance restraints (Å)	0.025 ± 0.002
Dihedral restraints (º)	0.63 ± 0.07
R.m.s. deviation from idealized covalent geometry	
Bonds (Å)	0.003 ± 0.0001
Angles (º)	0.81 ± 0.01
Impropers (º)	0.29 ± 0.03
Heavy-atoms r.m.s. deviations for lowest 32 structures (Å)	
All heavy atom (27 nucleotides and apramycin)	2.22 ± 0.45
Binding pocket and drug (U1406-U1410, G1491-U1495 and apramycin)	1.21 ± 0.25

+ None of the final structures exhibited violations larger than 0.5 Å or diheadral violations greater than 5 degrees. Only structurally useful intraresidue and intermolecular distance restraints, involving protons separated by more than three bonds, are included

Summary on intermolecular NOEs measure for complex

NOE Atom 1		NOE Atom 2	
Residue	Atom number	Apramycin Ring	Atom number
G1405	H8	Ring 1	H21
G1405	H8	Ring 1	H3
G1405	H8	Ring 1	H4
G1405	H8	Ring 1	H5
G1405	H8	Ring 1	H6
G1405	H8	Ring 2	H2
G1405	H8	Ring 2	H1
G1405	H5	Ring 1	H6
G1491	H8	Ring 2	H7
G1491	H8	Ring 3	H2
G1491	H8	Ring 3	H62
A1492	H8	Ring 3	H4
A1492	H8	Ring 3	H7
A1492	H8	Ring 2	Me
A1493	H8	Ring 2	H31
A1493	H8	Ring 2	H32
A1493	H3'	Ring 2	H32
A1493	H3'	Ring 2	H4
A1493	H8	Ring 3	H2
A1493	H2	Ring 1	H5
G1494	H8	Ring 1	H5
U1495	H5	Ring 1	H1
U1495	H5	Ring 1	H3
U1495	H5	Ring 1	H5
U1495	H5	Ring 1	H6
U1495	H5	Ring 1	H21
U1495	H5	Ring 1	H22
U1495	H6	Ring 1	H1
U1495	H6	Ring 1	H5
U1495	H6	Ring 1	H6
U1495	H6	Ring 1	H3
U1495	H6	Ring 1	H21

Restraints for structure calculation

Base Pairing	**8**
Distance restraints	**623**
Exchangeable	169
Non-exchangeable	454
RNA-RNA	400
Base-base	28
Base-ribose	293
Internucleotide	135
Intranucleotide	158
Ribose-ribose	79
Internucleotide	47
Intranucleotide	32
RNA-apramycin	32
Base-drug	32
Ribose-drug	5
Apramycin-apramycin	22
Torsion angle restraints	**111**
Base pucker	26
B	16
e	19
x	27
Apramycin	23
Residual dipolar coupling restraints	**22**
N-H	7
C-H	15

Figure S1. Aminoglycosides effectively inhibit translation elongation and causes miscoding. Related to Figure 1.

See Figure 2 A & B for an explanation of the intersubunit FRET experiment. The number of codons decoded by each ribosome can be measured through intersubunit FRET by counting the rounds of high-low-high FRET (rotation and then counter rotation) transitions observed. On the top two rows, the distributions of the number of codons read on the 6(FK) mRNA (**A**) without drugs (n = 381), (**B**) with 1 μM of apramycin (n = 429), paromomycin (n = 264), gentamicin (n = 297), and (**C**) with 10 μM of apramycin (n = 370), paromomycin (n = 257), and gentamicin (n = 384) are plotted. Without aminoglycosides, many ribosomes translate deep into the 6(FK) mRNA; upon the addition of aminoglycosides, the distributions become skewed towards peaking around codons 1~4. The extant of inhibition is similar for both 1 and 10 μM drug concentrations. On the bottom two rows, the number of codons read on the 3F mRNA for (**D**) no aminoglycosides (n = 306) and (**E**) 10 μM apramycin (n = 243), 10 μM paromomycin (n = 227), and 10 μM gentamicin (n = 255) are plotted. No aminoglycosides and apramycin do not show significant increases in ribosomes cycling past codon 3 while paromomycin and gentamicin greatly increases the fractions past codon 3. Miscoding through the stop codon can be quantified by counting ribosomes reading more than 3 codons (elongation cycles) as shown in (**F**). Apramycin induces a noticeable (2 fold) but moderate increase in miscoding. Paromomycin and gentamicin increase miscoding by an order of magnitude. Paromomycin significantly increases the fraction of miscoding ribosomes even without near-cognate tRNA (-Phe).

Figure S2. Further reduced elongation dynamics at 10 μM aminoglycosides and partial rescue of unlocked ribosome lifetime in apramycin at increased EF-G concentration. Related to Figure 2.

At higher aminoglycoside concentrations, the ribosomal state lifetimes affected by each aminoglycoside intensified when compared to the 1 μM experiments (Figure 2). A secondary effect with paromomycin appears at the rotated lifetime over the first codon and was not seen at 1 μM. From left to right, the conditions are no aminoglycosides (**A**, *n* = 381), 10 μM apramycin (**B**, *n* = 370), 10 μM paromomycin (**C**, *n* = 257), and 10 μM gentamicin (**D**, *n* = 384). Increasing EF-G concentration from 80 nM to 160 nM partially rescued the rotated state lifetime in apramycin, suggesting a possible competition between the mechanism of apramycin and of EF-G during translocation. The conditions are (**E**, same experiment as **A**) 80 nM EF-G without apramycin, (**F**, same experiment as **B**) 80 nM EF-G with 10 μM apramycin, and (**G**, *n* = 198) 160 nM EF-G with 10 μM apramycin. Error bars are s.d. errors.

Figure S3. Analysis of tRNA binding in with aminoglycosides for tRNA transit experiments in ZMW with dye-labeled tRNAs. Related to Figure 3.

(**A**) We used single-molecule detection in zero-mode waveguides (ZMWs), nanophotonic wells approximately 100-150 nm in diameter created by depositing a metal cladding on a glass substrate. (**B**) Individual biomolecules immobilized within each well (~3000 wells per chip) can be illuminated and visualized through the glass interface, while the incident light cannot propagate far beyond the glass-well interface to excite other molecules in solution. Thus, ZMWs permit single-molecule experiments to be performed at near-physiological concentrations of fluorescent ligands (~1 μM), without significant deterioration of the signal to noise ratio. (**C**) The number of codon read with well-behaved tRNA binding events ZMW experiments can be extracted by counting the number of pulses in the correct color sequence. The results for no aminoglycosides (n = 117) and for 10 μM apramycin (n = 165) are plotted. As paromomycin and gentamicin induces too many short binding events with the wrong sequence, such an analysis was not possible for them. (**D-F**) Using labeled Phe-(Cy5)tRNA^{Phe} and Lys-(Cy2)tRNA^{Lys}, the lifetimes (**D**, fitted to single exponential functions, error bars are s.d. error) and frequencies (**E**) of all visible tRNA events (> 100 ms) can be characterized in ZMW tRNA transit experiments. Normally, true tRNA sampling events (~30 ms) are not visible in these experiments. The average per-molecule occupancy of each tRNA from individual traces (**F**) gives an indication of the extant each aminoglycoside locks tRNA into the A site. An occupancy of 1 indicates that the entire trace is occupied by a single tRNA event. For all panels from left to right, the conditions are no aminoglycosides (n = 117), 10 μM apramycin (n = 165), 10 μM paromomycin (n = 155), and 10 μM gentamicin (n = 188). See also Figure S3 for additional analysis of ZMW experiments.

Figure S4. Additional tRNA-tRNA FRET experiments between the A and P sites and example traces with aminoglycosides. Related to Figure 4.

The traces from tRNA-tRNA FRET experiments are postsynchronized to have the first appearance of FRET to be $t = 0$. The condition for each panel is listed in the title. The addition of 500 nM EF-G does not significantly change the behavior of tRNA-tRNA FRET without aminoglycosides and with apramycin (**A**, $n = 228$ and **B**, $n = 126$). On the other hand, EF-G stabilizes the long-lived events with limited classical/hybrid fluctuations of the A and P-site tRNAs in paromomycin (**C**, $n = 194$). When the Phe codon (UUU) on the MFK mRNA is changed to the near-cognate Leu (CUU) on the MLK mRNA (**D**, $n = 166$), the overall tRNA-tRNA FRET behaviors remain remarkably similar. As with the cognate MFK mRNA, addition of EF-G to the MLK mRNA with paromomycin (**E**, $n = 283$) enriched long-lived high FRET events. Two example traces that are representative of the effects of aminoglycosides seen in tRNA-tRNA FRET experiments are shown at the bottom. Both traces are from experiments with 10 μM paromomycin with Cy3-labeled (green) fMet-tRNA^{fMet}, and Cy5-labeled (red) Phe-tRNA^{Phe}. The bottom FRET panel to each trace is calculated by $I_{Cy5} / (I_{Cy3} + I_{Cy5})$, where I_{Cy3} and I_{Cy5} are the fluorescence intensity of Cy3 and Cy5 respectively. (**F**) shows the short medium to high FRET events observed with paromomycin and gentamicin and (**G**) shows traces with stable high FRET events that have limited exchanges between the hybrid and classical conformations of the A and P-site tRNAs.

Figure S5. NMR structure determination of apramycin in a model of the 30S A site. Related to Figure 5.

The 5 lowest-energy structures of the solution NMR structure of an RNA model of the 30S decoding site are plotted. Apramycin is highlighted in red.

Figure S6. MFF tripeptide formation in the presence and absence of drugs. Related to Figure 6.

Pre-initiated 70S ribosomes (0.5 μM final concentration), bound to mRNA encoding MetPhePheSTOP, containing f[^{3}H]Met-tRNA^{fMet} (0.75 μM final total concentration) bound to P site, were rapidly mixed with pre-formed Phe-tRNA^{Phe}:EF-Tu:GTP ternary complex (2 μM final) and EF-G (1 μM final) in the absence or presence of each type of drug. Fractions of remaining f[^{3}H]Met-tRNA^{fMet} and emerging f[^{3}H]MetPhePhe-tRNA^{Phe} were in each case monitored over time.

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