Aminoglycoside interactions and impacts on the eukaryotic ribosome

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Aminoglycosides are chemically diverse, broad-spectrum antibiotics that target functional centers within the bacterial ribosome to impact all four principle stages (initiation, elongation, termination, and recycling) of the translation mechanism. The propensity of aminoglycosides to induce miscoding errors that suppress the termination of protein synthesis supports their potential as therapeutic interventions in human diseases associated with premature termination codons (PTCs). However, the sites of interaction of aminoglycosides with the eukaryotic ribosome and their modes of action in eukaryotic translation remain largely unexplored. Here, we use the combination of X-ray crystallography and single-molecule FRET analysis to reveal the interactions of distinct classes of aminoglycosides with the 80S eukaryotic ribosome. Crystal structures of the 80S ribosome in complex with paromomycin, gentamicin (G418), gentamicin, and TC007, solved at 3.3- to 3.7-Å resolution, reveal multiple aminoglycoside-binding sites within the large and small subunits, wherein the 6′-hydroxyl substituent in ring I serves as a key determinant of binding to the canonical eukaryotic ribosomal decoding center. Multivalent binding interactions with the human ribosome are also evidenced through their capacity to affect large-scale conformational dynamics within the pretranslocation complex that contribute to multiple aspects of the translation mechanism. The distinct impacts of the aminoglycosides examined suggest that their chemical composition and distinct modes of interaction with the ribosome influence PTC read-through efficiency. These findings provide structural and functional insights into aminoglycoside-induced impacts on the eukaryotic ribosome and implicate pleiotropic mechanisms of action beyond decoding.

Aminoglycosides are well known as antibiotics that target the bacterial ribosome. However, they also impact the eukaryotic translation mechanism to promote read-through of premature termination codons (PTCs) in mRNA. Aminoglycosides are therefore considered as potential therapies for PTC-associated human diseases. Here, we performed a comprehensive study of the mechanism of action of aminoglycosides in eukaryotes by applying a combination of structural and functional approaches. Our findings reveal complex interactions of aminoglycosides with eukaryotic 80S ribosome caused by their multiple binding sites, which lead to inhibition of intersubunit movement within the human ribosome that impact nearly every aspect of protein synthesis.

Significance

Aminoglycosides are broad-spectrum, bactericidal antibiotics of critical importance to the treatment of life-threatening infections. Despite their proven clinical utility, these therapeutics can lead to potential toxic side effects, including ototoxicity and nephrotoxicity, and an increased prevalence of resistance (1, 2). These activities have, in part, been distilled to the capacity of the 2-DOS ring to engage chemical features within the deep and narrow major groove of the 16S rRNA secondary structure (7). Structural insights into the mechanisms of 2-DOS aminoglycoside action were first obtained through chemical footprinting methods (8) and later using RNA fragments of the bacterial ribosome (7, 9) and isolated Thermus thermophilus 30S ribosomal subunits (10–12). These investigations revealed that aminoglycosides interact within the major groove of a conserved, asymmetric internal loop within the helix 44 (h44) decoding center of 16S rRNA within the small subunit ribosome to affect the decoding mechanism.

Structural investigations using isolated 30S ribosome subunits led to the hypothesis that the universally conserved A1492, A1493, and G530 residues within the h44 decoding center actively “monitor” the interaction between the tRNA anticodon and the mRNA codon (12). To do so, A1492/A1493 must extrude from the helical axis of h44 to “recognize” the codon–anticodon helix through A-minor groove interactions. This local conformational change then couples to global conformational changes in the ribosome (domain closure) that enable tRNA accommodation. It was also suggested that the inability of mismatched near-cognate tRNA to form proper A-minor groove interactions prevents domain closure, thereby favoring tRNA rejection (11). The binding of paromomycin and neomycin to helix 44 in the crystals of isolated 30S subunits also extrudes both decoding nucleotides A1492 and A1493, leading to the hypothesis that stabilization of extrahelical A1492/A1493

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Aminoglycosides target the decoding center of T. thermophilus Homo sapiens (29, 30). Consequently, moiety in ring I, have all been shown, and amikacin, which possess, during protein synthesis (25, 26). Paromomycin and, with much less efficiency, neomycin were also recognized as being effective suppressors of nonsense mutations (27). Since that time, gentamicin, G418, tobramycin, and amikacin, which possess either a 6′-OH or 6′-NH₂ moiety in ring I, have all been shown to induce suppression of premature termination codons (PTCs) (28). Mutations that introduce PTCs are understood to be causative in ∼11% of the >5,000 human genetic diseases identified to date, including sporadic cancers arising from mutations in tumor-suppressor genes such as TP53 (29, 30). Consequently, aminoglycosides are regarded as potential therapies for the treatment of human disease.

The application of aminoglycosides for suppression therapies has been limited in practice by their toxicities and their low efficiencies of stop-codon read-through (31, 32). Despite these shortcomings, aminoglycosides have been enrolled in clinical trials for the treatment of cystic fibrosis (33) and Duchenne muscular dystrophy (34) and have shown therapeutic potential for the treatment of dystrophic epidermolysis bullosa (35) and Werner syndrome (36) as well as specific cancers (37). The neomycin derivative TC007 has also been tested in the context of spinal muscular atrophy (SMA) in both human fibroblasts (32) and mouse models of disease (38).

As the molecular basis of aminoglycoside action against eukaryotic ribosomes is currently lacking, we have examined the
interactions of aminoglycosides with 80S eukaryotic ribosomes using X-ray crystallography and single-molecule FRET (smFRET) imaging. X-ray structural analyses of *Saccharomyces cerevisiae* 80S ribosomes in complex with paromomycin, G418, gentamicin, and TC007 reveal that aminoglycosides interact at multiple sites within both 18S and 28S rRNA. Multivalent aminoglycoside–ribosome interactions were further corroborated by the impact of aminoglycosides on the spontaneous dynamics within the human ribosome and their propensity to promote errors in RNA transcription. These investigations further revealed that TC007 exhibited distinct modes of interaction with and miscoding and structural impacts on the eukaryotic ribosome at ~20-fold lower concentrations than observed for G418, paromomycin, or gentamicin. These findings are consistent with the efficacy profiles of TC007 in human cells and with this compound’s unique capacity to promote PTC readthrough.

**Results**

**Aminoglycoside Binding to the Eukaryotic h44 Decoding Site.** To investigate the binding mode of aminoglycosides with low affinity to the 80S ribosome, we first sought alternative experimental conditions for *S. cerevisiae* 80S ribosome crystal treatment. Osmium hexamine, which is normally present in this procedure, binds tRNA, including the aminoglycoside-binding site within the h44 decoding region (39). Hence, we examined the binding of aminoglycosides that exhibit low affinities for the 80S ribosome, we removed osmium hexamine and instead treated crystals of the ribosome in the presence of high concentrations (up to 4 mM) of aminoglycosides (*Materials and Methods*).

Under these conditions, we obtained a structure of the 4,6-linked aminoglycoside G418 bound to the *S. cerevisiae* 80S ribosome at 3.7-Å resolution (Table S1). A similar structure had been previously solved in the presence of osmium hexamine [Protein Data Bank (PDB) ID code 4U4O (24), where it was shown that G418 can compete with osmium hexamine for h44 binding. In both structures, G418 was found to exhibit canonical binding to the h44 decoding site with ring I of the antibiotic stacked on nucleotide A1754 (G1491 in bacterial numbering), displacing the conserved nucleotides A1755 (A1492) and A1756 (A1493) away from the helical axis (Fig. S2). There, the 6′-OH group of ring I is positioned within hydrogen bonding distance (2.7 Å) of the N2 atom of G1645 (A1408) (Fig. S2). Hence, an electronegative group of ring III also makes sequence-specific contacts with O4 of U1758 (U1495) and N7 of G1642 (G1405) (Fig. 1D and Fig. S3C). This noncanonical pose, which appears to be enforced by repulsion between the N1 and N2 atoms of G1645 (A1408) and the NH2-group in the 6′ position of ring I, rationalizes gentamicin’s capacity to compete with paromomycin binding while being unable to support miscoding.

**Interactions of TC007 with the 80S Ribosome and Implications for PTC Read-Through.** SMA, a leading genetic cause of infantile death, is an autosomal recessive disease for which there is currently no cure (42). SMA is associated with the loss of full-length SMN protein (43). Initial screens for small molecule-mediated suppression therapies for potential treatment of SMA led to the discovery of TC007, a three-ring 4,5-linked aminoglycoside bearing a 6′-NH2 group on ring I (Fig. S1) (32). TC007 testing in fibroblasts from SMA patients and in SMA mouse models showed good toxicity profiles and prolonged the lifespan of SMA mice while partially lessening the severity of disease (44).

To gain insight into TC007 interactions with the 80S ribosome, we solved the X-ray structure of the 80S–TC007 complex at 3.7-Å resolution (I/σ = 0.98, CC1/2 = 36.1) (Table S1). Consistent with a steric clash between the 6′-NH2 and the G1645 (A1408) residue (24), TC007 was not observed to bind the small subunit decoding center, despite being present at 4 mM concentration. Accordingly, no electron density was evidenced for the disordered nucleobases of residues A1755 (A1492) or A1756 (A1493). However, positive electron density was observed for TC007 (TC007-1) immediately below the canonical decoding site and above intersubunit bridge B3, spanning between h44 of the small subunit and Helix 71 (H71) of the large subunit at the intersubunit space (Fig. 2A and Fig. S44). A second TC007 molecule (TC007-2) was also evidenced between Helix 68 (H68) and Helix 70 (H70) in the large ribosomal subunit, proximal to the highly conserved H69 element of intersubunit bridge B2. This binding site is close to (within ~10 Å) but is distinct from the noncanonical aminoglycoside-binding site observed within the H69 major groove in the bacterial ribosome (3, 20, 21) and distorts the H69 conformation (Fig. 2A and Fig. S4B).

**Interactions of TC007 with Bacterial 70S Ribosome.** To shed light on the selectivity of TC007, we solved the structure of the bacterial 70S ribosome from *T. thermophila* cocryrstallized in complex with three tRNAs, mRNA, and TC007 at 2.95 Å (I/σ = 1.38,
CC_{1/2} = 47.1) (Table S1), where the drug was at 50-fold excess over the ribosome (Materials and Methods). As expected, we observed strong positive electron density in the h44 decoding site, where rings I and II were observed to overlap with those of paromomycin (Fig. S4C). In contrast to ring III of paromomycin, which interacts with the Hoogsteen face of G1491 (Escherichia coli numbering), the O7 atom of ring III of TC007 is positioned within hydrogen bonding distance of N4 of C1407. When TC007 was soaked into preformed crystals of the same 70S–tRNA–mRNA complex (500 μM for 24 h), we were able to obtain a structure at 3.05 Å resolution (I/σ1 = 1.25, CC_{1/2} = 39.9) (Table S1) in which TC007 was bound in an identical position within h44 (TC007–h44) (Fig. S4D). As for neomycin, gentamicin, and paromomycin binding to H69 within the Escherichia coli 70S ribosome (3, 20, 21), TC007 was observed to make an array of hydrogen bonding contacts with bases lining the H69 major groove, including sequence-specific contacts with residues G1921 and G1922 (Fig. S4 B and E). Remarkably, the orientation of TC007 is different from that in a previously reported 70S–neomycin structure (21): The position of ring II is similar in the two antibiotics, but the positions of rings I and III are swapped. As for gentamicin, intersubunit bridging contacts present in both paromomycin and neomycin structures were not observed due to the absence of ring IV. As H69 is sterically accessible within the eukaryotic 80S ribosome, we attribute the observed differences in TC007 binding to Ψ2264 (G1921) and C2265 (G1922) substitutions present in the 80S ribosome (Fig. S4 B and F), which likely disrupt the drug’s capacity to pack tightly against the floor of the major groove. These findings confirm that the TC007-binding sites in the T. thermophila 70S ribosome are distinct from those found in the 80S ribosome of S. cerevisiae (Fig. S4B).

**Aminoglycosides Inhibit Intersubunit Rotation of the Eukaryotic Ribosome.** To assess the impact of aminoglycosides on the dynamics of subunit rotation within functional 80S ribosome complexes, we performed smFRET imaging on surface-immobilized human 80S pretranslocation (PRE) complexes in the absence and presence of paromomycin, G418, gentamicin, and TC007 (45). As previously reported (45), in the absence of drug, human PRE complexes predominantly exhibited a mixture of lower-FRET (H2: ∼0.2 and H1: ∼0.4) hybrid state configurations in which the 3′-CCA end of deacylated tRNA occupies the large subunit E-site and the 3′-CCA end of peptidyl-tRNA occupies either the aminosyrl (A)- or P-site, respectively (A/A and P/E; A/P and P/E) (Fig. 3 A and B, Left). The addition of paromomycin to the 80S PRE complex first lowered the average FRET value of the hybrid-state tRNA configurations, suggesting a relative stabilization of the H2 hybrid state in which peptidyl-tRNA returns to its classic (A/A) position while deacylated tRNA remains in its hybrid (P/E) state (46). This impact was maximized at a drug concentration of ∼10 μM. Above this concentration, we observed the appearance of an intermediate-FRET (∼0.55) state, followed by stabilization of a high-FRET, classic (C) PRE complex conformation (Fig. 3B). The estimated EC_{50} of this effect was ∼35 μM. These data are consistent with a multivalent impact of paromomycin binding to the 80S PRE complex, which first stabilizes peptidyl-tRNA in its classic position within the A-site, followed by a shift of deacylated P-site tRNA from its hybrid to its classic position. By contrast, G418, which also bears a ring 6′-OH substituent, only stabilized at an intermediate-FRET state (∼0.55) (Fig. 3C). Notably, consistent with the concentration range used to inhibit mammalian cell culture growth (47), the EC_{50} of this impact was ∼2 mM. By analogy to bacterial systems, the intermediate-FRET state may reflect a reversal of subunit rotation from the P/E hybrid state, which promotes a chimeric, intersubunit hybrid configuration of deacylated P-site tRNA associated with the global inhibition of translation factor binding (20, 21).

Analogous investigations of gentamicin, which contains a 6′-NH₂ group on ring I, also led to intermediate-FRET (∼0.55) state stabilization, but the EC_{50} of its impact was approximately an order of magnitude lower (∼100 μM) than that of G418 (Fig. 3D). TC007, which also has a 6′-NH₂ group on ring I, exhibited impacts on the 80S PRE complex that shared characteristics of G418 and gentamicin as well as paromomycin, in which drug binding was bimodal in nature. At concentrations below 2 μM, TC007 predominantly promoted a lower-FRET PRE complex conformation, in line with H2 hybrid state stabilization (A/A; P/E). Higher TC007 concentrations increasingly stabilized an intermediate-FRET (∼0.55) state, similar to that evidenced in the presence of subsaturating paromomycin and saturating G418 and gentamicin concentrations (Fig. 3E). At 10 μM, both H2 and intermediate-FRET PRE complex conformations persisted. Above
Aminoglycoside-induced changes in human 80S PRE complex formation. Consistent with a high-fidelity decoding mechanism, near-cognate ternary complexes were efficiently rejected from the A-site in the absence of drug so that little or no PRE complex formed (Figs. 4 A and B, Left). By contrast, increasing levels of A-site miscoding were observed as a function of paromomycin, G418, gentamicin, and TC007 concentration. In each case, the extent of miscoding correlated with the concentrations of drug, where the EC₅₀ of miscoding closely mirrored that observed for drug binding to the cognate PRE complex (Figs. 3 and 4). Strikingly, the distribution of FRET values exhibited by precomplexes bearing near-cognate tRNA at the A-site were distinct from those bearing cognate tRNAs; the near-cognate complexes were found to exhibit multiple distinct FRET states (compare far right panels in Figs. 3 and 4). Inspection of individual FRET trajectories of PRE complexes bearing near-cognate tRNA in the A-site showed that the FRET states observed were in dynamic exchange. These findings suggest that the nature of the codon–anticodon pair in the A-site influences the ribosome’s interaction with antibiotics and tRNA in a manner that affects both the conformation and dynamics of the PRE complex.

### Secondary Binding Sites: Hot Spots Targeted in the Eukaryotic 80S Ribosome by Aminoglycosides

Aminoglycosides are positively charged and are well known to bind a diverse range of RNA molecules, including catalytic RNAs such as ribonuclease P, self-splicing introns, and ribozymes (48–50). Crystal structures of the isolated small ribosomal subunit as well as intact 70S ribosomes from bacteria have all shown evidence of multiple aminoglycoside-binding sites (3, 13, 14, 20, 21). The positively charged anticancer drug cisplatin also has several binding sites in the 70S ribosome (51).

As high aminoglycoside concentrations were used in each of our crystallographic investigations under conditions of relatively low ionic strength (~130 mM salt concentration) and neutral pH (7.0–7.5), secondary binding sites were observed for each of the aminoglycosides examined. While it is difficult to link the binding sites observed with the impairment of specific ribosome functions due to the presence of numerous copies of rRNA genes in the genome, we note that several of the secondary aminoglycoside-binding sites map to key functional centers of the ribosome (Fig. 5A). These findings are consistent with the known properties of aminoglycosides to show diverse impacts on the bacterial translation mechanism (20, 52).

The peptide exit tunnel, which spans the peptidyl-transferase center to the solvent side of the ribosome, is subjected to translation regulation by small molecules and peptides that induce translational stalling (53). We find that gentamicin, G418, and TC007 each bind to the peptide exit tunnel (Fig. 5B). Paromomycin was not found in the exit tunnel, suggesting that only aminoglycosides with a maximum of three rings can be accommodated within this pocket. Superposition with the structure of the 50S ribosome subunit from *Haloarcula marismortui* complexed with erythromycin shows that aminoglycosides and erythromycin bind opposite sides of the exit tunnel wall (Fig. SS4) (54). Interestingly, although each drug interacts with the same 2SS rRNA residues, the orientations of G418 and gentamicin within the exit tunnel, which are very similar, are distinct from TC007 (Fig. 5B). These distinctions likely reflect the unique ring topologies of the 4,6-linked (G418 and gentamicin) and 4,5-linked (TC007) aminoglycosides. Alignment of each of these structures with that of the *Escherichia coli* ribosome containing the SecM stalling peptide (55) suggests that aminoglycoside occupancy within the exit tunnel wall is unlikely to be sufficient to

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**Fig. 3.** Aminoglycoside-induced changes in human 80S PRE complex formation. (A) Schematic showing classic (C) and hybrid (H2 and H1) states of the human PRE ribosome complex. Large (60S) and small (40S) subunits (unrotated, gray; rotated, pink), tRNAs (orange), and sites of donor (Cy3, green) and acceptor (Cy5, red) fluorophore labeling on tRNA in the P- and A-sites, respectively, are indicated. (B–E) Population FRET histograms showing the impact of paromomycin (B), G418 (C), gentamicin (D), and TC007 (E) on the equilibrium distribution of FRET states exhibited by the 80S PRE complex. The concentration of antibiotic is indicated; n, number of single-molecule observations made in each experiment.

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**Aminoglycoside-Induced Errors in tRNA Selection on the Eukaryotic Ribosome.** To evaluate the impact of aminoglycosides on aminoacylating tRNA (aa-tRNA) miscoding at the A-site, we examined the extent of aa-tRNA misincorporation of Phe-tRNA_Phe into surface-immobilized 80S initiation complexes (ICs) containing (Cy3)Met-tRNA_Met in the P-site, in which the A-site codon was changed from the cognate UUU codon to the near-cognate UCU codon. To provide adequate time for miscoding, the eEF1A(GTP)-(Cy5)Phe-tRNA_Phe ternary complex (20 nM) was incubated with surface-immobilized ribosome complexes for 2 min (Materials and Methods) followed by buffer exchange to remove unbound ternary complexes. Using this approach, we could specifically examine the extent of PRE complex formation in the absence of convoluting signals arising from transient ternary complex binding events. This method allowed us to directly compare the rate of PRE complex formation with that of conformational changes in the ribosome. We observed that the PRE complex formation rate decreases as the concentration of each antibiotic increases (Fig. 3A). This observation suggests that aminoglycosides reduce the rate of PRE complex formation by inhibiting the conformational changes that lead to the formation of the PRE complex. Moreover, the extent of PRE complex formation also decreases as the concentration of each antibiotic increases (Fig. 3A). This observation suggests that aminoglycosides reduce the rate of PRE complex formation by inhibiting the conformational changes that lead to the formation of the PRE complex. Moreover, the extent of PRE complex formation also decreases as the concentration of each antibiotic increases (Fig. 3A).
Aminoglycoside-induced miscoding during tRNA selection on the human ribosome. (A) Schematic showing the process of tRNA selection, in which the ternary complex of eEF1A (blue), GTP, and aa-tRNA (orange) enters the A-site of the 80S ribosome. The process of tRNA selection proceeds through the A/T state in which codon–anticodon pairing on the small subunit occurs while the 3′-aminoacylated CCA-end of tRNA remains bound to eEF1A. GTP hydrolysis by eEF1A facilitates the aa-tRNA release from eEF1A and the accommodation of its 3′CCA end into the large subunit A-site on the classically (C) configured (unrotated) PRE complex. Peptidyl- and aminoacyl-tRNAs in the P- and A-sites, respectively, then undergo peptide bond formation (PBF), enabling deacylated P-site tRNA and peptidyl-tRNA to achieve hybrid (H2 and H1) states. Large (60S) and small (40S) subunits (unrotated, gray; rotated, pink), tRNAs (orange), and sites of donor (Cy3, green) and acceptor (Cy5, red) fluorophore labeling on tRNA in the P- and A-sites, respectively, are indicated. (B) Population FRET histograms showing that aminoglycoside-induced errors in tRNA selection lead to the accumulation of PROF ribosome complexes bearing near-cognate tRNA in the A-site. The concentration of antibiotic is indicated; n, number of single-molecule observations made in each experiment.

Fig. 4. Aminoglycoside-induced miscoding during tRNA selection on the human ribosome. (A) Schematic showing the process of tRNA selection, in which the ternary complex of eEF1A (blue), GTP, and aa-tRNA (orange) enters the A-site of the 80S ribosome. The process of tRNA selection proceeds through the A/T state in which codon–anticodon pairing on the small subunit occurs while the 3′-aminoacylated CCA-end of tRNA remains bound to eEF1A. GTP hydrolysis by eEF1A facilitates the aa-tRNA release from eEF1A and the accommodation of its 3′CCA end into the large subunit A-site on the classically (C) configured (unrotated) PRE complex. Peptidyl- and aminoacyl-tRNAs in the P- and A-sites, respectively, then undergo peptide bond formation (PBF), enabling deacylated P-site tRNA and peptidyl-tRNA to achieve hybrid (H2 and H1) states. Large (60S) and small (40S) subunits (unrotated, gray; rotated, pink), tRNAs (orange), and sites of donor (Cy3, green) and acceptor (Cy5, red) fluorophore labeling on tRNA in the P- and A-sites, respectively, are indicated. (B) Population FRET histograms showing that aminoglycoside-induced errors in tRNA selection lead to the accumulation of PRE ribosome complexes bearing near-cognate tRNA in the A-site. The concentration of antibiotic is indicated; n, number of single-molecule observations made in each experiment.

Discussion

The structural and functional insights into aminoglycoside interactions with the 80S eukaryotic ribosome obtained through the present investigations serve as a foundation for exploring the molecular mechanisms of aminoglycoside action in eukaryotes. Although the impairment of mitochondrial translation is considered one of the main causes of side effects produced by aminoglycosides in eukaryotic cells (63, 64), emerging evidence...
Fig. 5. Overview of the secondary binding sites of aminoglycosides in 80S ribosome. (A) Binding sites of gentamicin (GENT), G418, TC007, and paromomycin (PAR) in the 80S ribosome from *S. cerevisiae*. All structures were aligned either on 18S rRNA or on 28S rRNA, for small and large subunits, respectively, in the 80S–gentamicin structure. The ribosome is colored light green; elements of the binding pockets of aminoglycosides are colored light orange; gentamicin is colored green; G418 is colored yellow; TC007 is colored magenta; and paromomycin is colored violet. (B) Binding of GENT-7, G418-2, and TC007-3 to the peptide exit tunnel of the 80S ribosome. G418 is colored yellow; gentamicin is colored light green; TC007 is colored magenta; the large ribosomal subunit is colored blue; and the eukaryote-specific protein eL37 is colored green. Similar poses are observed for G418-2 and GENT-7, but the orientation of TC007-3 is different. Oxygen atoms are colored red, and nitrogen atoms are colored blue. Oxygen atoms are colored red, and nitrogen atoms are colored blue. (C) Binding sites of PAR-2, GENT-2, and G418-3 in the E-site of the small ribosomal subunit overlapping the position of the mRNA. Structures of the 80S ribosome in complex with paromomycin, gentamicin, and G418 were locally aligned on the structure of the 70S ribosome from *T. thermophilus* in complex with tRNAs and mRNA (PDB ID code 5EL6). mRNA is colored red; elements of the 70S ribosome are omitted for clarity. The 40S subunit is colored orange; paromomycin is colored violet; the universal protein uS11 is colored yellow; gentamicin is colored light green; and other color-coding is as in A. (D) Interactions of PAR-3 with the elements of the intersubunit bridge B2c. Contacts made between paromomycin and G984 of the 40S subunit, A2152 of the 60S subunit, and Asp176 of uL2 are marked with dashed lines. The 40S subunit is colored light pink; paromomycin is colored violet; the 60S subunit is colored blue; and the universal protein L2 (uL2) is colored blue. Oxygen atoms are colored red, and nitrogen atoms are colored blue. (E) GENT-4 and GENT-5 stabilize particular conformations of bridge B2c. The 80S–paromomycin structure was aligned on the 80S–gentamicin structure based on the 28S rRNA. The alignment demonstrates that rearrangement of the bridge B2c would be blocked by gentamicin due to a clash with h24 of the 40S subunit in the 80S–paromomycin structure (colored in pink). 80S–gentamicin contacts are marked with dashed lines. The 40S subunit from 80S–gentamicin structure is colored lime; other color-coding is as in A–C. (F) GENT-5 targets bridge B4 formed by the universal protein uS15 protein and H34 of the large subunit. uS15 is shown in yellow; other color-coding is as in D. Glutamine 142, which interacts with GENT-6, is depicted as spheres. (G) Interactions of GENT-8 and TC007-4 with the elements of the ribosomal P-stalk. Different conformation of the helices 42 and 97 of the P-stalk are shown in blue for 80S–GENT and 80S–TC007 structures and in cyan for the 80S-apo structure (PDB ID code 4V88). H42 in the apo conformation would clash with GENT-8. Arg62 in the uL6 protein approaching TC007 is shown as spheres. Color-coding is as in A–E.

suggests that aminoglycosides also exert effects on cytosolic ribosomes to alter translation elongation and termination in a manner that induces read-through of PTCs. These findings have led to their consideration as potent drugs to treat human diseases caused by PTCs. Aminoglycosides also operate against eukaryotic human pathogens, including *Leishmania* and *Trypanosoma* families, due to sequence variations in their canonical h44 decoding sites. The concentrations of aminoglycosides required for eukaryotic cell growth inhibition and the EC_{50} values measured for the inhibition of eukaryotic translation by distinct aminoglycosides in vitro generally correlate with their affinities for the canonical h44 decoding region within the small subunit A-site. As illustrated by crystal structures of the 80S ribosome with gentamicin and TC007, which adopt noncanonical poses in the vicinity of the h44 decoding site, the eukaryotic-specific G1645 residue (equivalent to A1408 in bacteria) within h44 tends to preclude the binding of aminoglycosides containing a 6′-NH_{2} substituent in the ring I (Figs. 1D and 2A). Paromomycin and G418, which contain a 6′-OH substituent, can achieve canonical interactions with the h44 decoding site, but the absence of Watson–Crick base pairing between C1646 and A1754 impedes drug-binding interactions that hinge on ring I interactions with the floor of the aminoglycoside-binding site (Fig. 1B and Fig. S1). Human mitochondrial ribosomes contain an adenosine at the 1408 position (bacterial numbering), making it a good target for aminoglycosides with both 6′-OH and 6′-NH_{2} substituents in ring I. However, aminoglycoside affinity to mitochondrial ribosomes is likely to be attenuated by two consecutive noncanonical base pairs [C1494–A1555 (A1410–U1490) and C1493–C1556 (C1409–G1491)] that are likely to strongly disrupt ring I interactions (Fig. S8). Consistent with this notion, the reestablishment of
Watson–Crick interactions in the floor of the aminoglycoside-binding site by the naturally occurring mutations A1555G or C1494T leads to aminoglycoside hypersusceptibility in humans (69, 70).

Our smFRET experiments indicate that each of the aminoglycosides tested increases the error rate of A-site decoding (Fig. 4). However, the behaviors of the miscoded 80S precomplexes bearing near-cognate tRNA in the A-site are unique for each drug. These findings suggest that aminoglycosides may promote PTC read-through by distinct mechanisms. Aminoglycosides containing 6′-OH substituent in ring I likely induce miscoding and PTC read-through due to residual binding to the canonical binding site in h44 of the eukaryotic ribosome. In this case, near-cognate or noncognate tRNA may efficiently accommodate at the stop codon-programmed A-site to compete with the termination factors. Aminoglycosides containing a 6′-NH₂ substituent in ring I, including gentamicin and TC007, do not bind h44 in a canonical fashion. Their impacts on PTC read-through may entail alternative mechanisms, including intersubunit rotation effects that are anticipated to hamper RF1 interactions with the classically configured ribosome (Fig. S9) (71).

The propensity of eukaryotic ribosomes to adopt rotated states and the impact of aminoglycosides in enforcing closer interactors (higher FRET) between deacylated and peptidyl-tRNA within the 80S human PRE complex suggest that aminoglycoside binding to intersubunit regions of the ribosome (bridges B2 and B4 for gentamicin and bridge B3 for TC007) facilitate conformational changes in the PRE 80S ribosome complexes that shift peptidyl tRNA toward the P-site. Such impacts may relate to the stabilization of partially rotated ribosome configurations that move deacylated tRNA toward the A-site (20, 21).

The nature of the observed aminoglycoside interactions with the eukaryotic ribosome hint at potentially multiple modes of action on the translation mechanism. These insights also provide an important framework for understanding the diversity of aminoglycoside interaction sites and drug-binding modes with the 80S ribosome. The combined perspectives afforded by X-ray crystallography and direct imaging of aminoglycoside impacts on functional ribosome complexes using smFRET has the potential to facilitate the design of new antibiotic derivatives and may be particularly suited for the identification of compounds capable of mediating efficient PTC read-through. Such efforts will be greatly aided by in-depth functional investigations of a diversity of functional ribosome complexes relevant to termination. In this regard, the present findings suggest that nonspecific impacts on decoding may be reduced by avoiding aminoglycoside scaffolds bearing a ring I 6′-OH moiety, which exhibit generally higher affinity for h44 decoding site, and instead focusing on aminoglycosides, other compounds, or mixtures of compounds that give rise to stop codon-specific miscoding in the absence of dominant-negative downstream impacts.

**Materials and Methods**

**Yeast and Bacterial Ribosome Purification, Crystallization, and Crystal Treatment.** Ribosomes from *S. cerevisiae* were purified and crystallized as described (39). The crystal treatment procedure was modified based on the procedure described previously (39). Briefly, crystals were transferred to the solution containing 80 mM Tris-acetate (pH 7.0), 70 mM KSCN, 10 mM Mg(OAc)₂, 20% (vol/vol) glycerol, 5% (wt/vol) PEG 20,000, 6.5 mM spermidine, 7.5 mM NH₄OAc, 1.4 mM N,N′-bis-(3-D-glucaminamidopropyl) deoxycholamide (Deoxy Big Chap), 2 mM DTT, and stepwise increasing concentrations of PEG 4000, PEG 3350, or PEG 2000 MME up to 20%. The crystals were incubated for 1 h or 4 h and were flash-frozen in a stream of liquid nitrogen.

**Data Collection, Crystal Structure Determination, and Analysis.** Data collection were collected at 90 K using 0.05° oscillation on beamline PROXIMA I at the Soleil synchrotron (Saint-Aubin, France) equipped with a status EM detector (Dectris) or on the PXL beamline at the Swiss Lightsource synchrotron (Villigen, Switzerland) equipped with an Eiger 16M detector (Dectris). Two to eight crystals were used for each dataset. Data were reduced and scaled using the XDS suite (74). Coordinates of vacant 80S ribosome from *S. cerevisiae* from PDB 4V8B were used to determine structures of 80S–paromomycin, 80S–gentamicin, and 80S–TC007. Phenix software was used for structure refinement and another with several iterations of refinement, and validation (75, 76). P-stalk elements were disordered and were removed from the structures except for P-stalk RNA in the 80S–paromomycin structure and P-stalk RNA and protein L12 in the 80S–gentamicin structure. Due to weak electron density, protein S31 was removed from the 80S–paromomycin structure, and a few structural elements were remodeled, in particular, amino acids 103–113 of protein UL16 and residues 8980–8985 of RNA. An unbiased difference electron density map (Fcal−Fobs) was used to locate the binding sites of aminoglycosides. Ligand fitting was performed in Coot (77). Geometry restraints for antibiotics were generated with the help of Grade web server (Global Phasing, grade.globalphasing.org/cgi-bin/grade/server.cgi). Peaks of positive electron density maps were inspected manually to add magnesium ions, with coordinated water molecules often replacing osmium hexamine molecules. Manual correction of calculations followed by several iterations of refinement and validation. A set of atomic coordinates, B-factors (one isotropic B-factor per residue), and occupancies (one occupancy value per ligand and individual occupancies for magnesium ions). Real-space refinement in Phenix was applied to fit rotamer outliers. Finally, translation–liberation–screw-rotation (TLS) refinement was performed with two TLS groups. For structure determination of 70S–rRNA–mRNA–TC007 complexes, coordinates of the 70S ribosome from PDB 4V8B and tRNAs and mRNAs coordinates from PDB 4V6F were used for two rounds of rigid-body refinement. The electron density maps were inspected manually, and the molecules of TC007 were localized in the peaks of positive electron density. Secondary binding sites of TC007 (four in total) were located on the periphery of the ribosome far from the functional centers. Additionally, one binding site of TC007 to the intersubunit region was detected. Here TC007 interacts with the low part of h44 of the 50S subunit and the junction of helices 62 and 64 in the 25S rRNA of the 50S subunit between intersubunit bridges B5 and B6. Geometry restraints for TC007 were generated with the help of Grade web server (Global Phasing, grade.globalphasing.org).

Manual modeling was done in Coot and was followed by several rounds of reciprocal space refinement of atomic coordinates and B-factors. Crystallographic statistics are reported in Table S1. All figures were generated using PyMOL 1.5 (https://pymol.org/2) (Schrödinger). Local structure alignments were performed in Coot (77). Ribosomal proteins are named throughout the paper according to the newly established nomenclature (78). Atomic coordinated and structure factors for structures...
of 80S–polymycin, 80S–gentamicin, 80S–TC007, 80S–geneticin (G418), 705- tRNAs-mRNA–TC007 (cocrystallization), and 70S–tRNAs–mRNA–TC007 (soaking–) have been deposited in the Protein Data Bank (https://www.rcsb.org/pdb/home/home.do) under ID codes 5NDV (80S–polymycin), 5OBM (80S–geneticin), 5NDW (80S–TC007), 5NDG (80S–geneticin (G418)), 5NDJ (70S–tRNA– mRNA–TC007 cocrystallized), and 5NDJ (70–tRNA–mRNA–TC007 soaked).

Preparation of Native and Fluorescently Labeled tRNAs. E. coli tRNA<sup>Met</sup> and tRNA<sup>U</sup> were purified as previously described (79, 80). Aminoacylation and fluorescent labeling of tRNAs (tRNA<sup>Met</sup> at 4′U and tRNA<sup>U</sup> at acp<sup>U</sup> UG7 positions) were performed following established protocols.

In Vitro Reconstitution of 80S ICs. As previously described (45), 80S ICs were assembled following a procedure that bypasses the need for exogenous initiation factors (81). Purified 40S subunits were mixed with an equal volume of 80S association buffer (30 mM Hepes (pH 7.5), 5 mM MgCl<sub>2</sub>, 50 mM NaCl, 2 mM spermidine, 5 mM putrescine, 1 mM DTT) and then were heat activated at 42 °C for 5 min. Fourfold excess of mRNA with the sequence 5′-CAU AAA AACU UACA CCC UUA GAG GGA CAA UCG AUG UUU UUU UUU UUU UUU UUU-3′ (Dharmacon) (henceforth referred to as “MFF mRNA”) and 5′-CAU AAA AACU UACA CCC UUA GAG GGA CAA UCG AUG UUC UCUC UUC UCUC UUC-3′ (henceforth referred to as “MFF near-cognate mRNA”) was added, heated to 37 °C for 10 min and subse- quently cooled to 4 °C. Then 80S complexes were formed in this mixture, a twofold excess of fluorescently labeled Met-tRNA<sup>Met</sup> (prepared as described in ref. 82) was added, and the reaction was heated and cooled as above. At this time, equimolar amounts of 60S subunits were heat activated at 42 °C for 5 min. The 60S subunits were then added to the mixture of 40S/tRNA/mRNA. After an additional heating and cooling cycle, the MgCl<sub>2</sub> concentration of the reaction was raised to 15 mM before it was cooled on ice for 5 min. It was then loaded onto a 10–30% sucrose gradient in 80S association buffer and was ultracentrifuged in a Beckman SW41 rotor at 35,000 rpm for 1.5 h at 4 °C before fractionation. The peak corresponding to 80S complexes was collected and aliquoted before storage in liquid nitrogen. To achieve surface immobilization, the mRNA was hybridized to a double-stranded, biotinylated DNA oligonucleotide (sequence: 1′:5-GTA AGT ATT AGG TTC CCC CTT TTT TTT TTT TTT TTT TT TTT TTT TT TTT TTT TTT CTT CTT TTT TTT TTT TTT) and 2′:5-GTA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA-3′ before its mixture with the 40S subunit.

Formation of the eEF1A(GTP)–aa-tRNA Ternary Complex. aa-tRNAs (tRNA<sup>Phe</sup>, tRNA<sup>Met</sup>) were first generated as previously described (82) and were mixed with 1 mM GTP, 6 mM phosphoethanolpyruvate, 12 units pyruvate kinase, and 12 units/ml myokinase. A twofold excess of eEF1A isolated from rabbit reticulocyte lysate (45), which bears 100% sequence identity with human eEF1A, was then added, and the mixture was incubated at 37 °C for 5 min to form the ternary complex.

Single-Molecule Fluorescence Imaging. Complexes were surface-immobilized via the biotin–streptavidin interaction in PEG-passivated quartz chambers. All imaging experiments were performed in Hepes (KOH)-Polyimix buffer (pH 7.5) containing 5 mM MgCl<sub>2</sub>, 50 mM NaCl, 2 mM spermidine, and 5 mM putrescine, as well as an oxygen scavenging system (2 mM methyl-2-cococaycte, 50 mM protocatechue acid, 50 mM protocatechue 3,4-dioxygenase) together with a mixture of solution additives (1 mM Trolox, 1 mM cyclooctatetraene, 1 mM nitrobenzyl-alcohol) (83) to reduce photobleaching. As previously described (82), single-molecule fluorescence imaging was performed using a custom prism-based total internal reflection fluorescence microscope. Cy3 fluorophores were il- luminated with an Opus S32-nm solid-state laser (Laser Quantum), and fluo- rescence emissions from Cy3 and Cy5 fluorophores were collected using a 60×, 1.27 NA Plan-Apo water immersion objective (Nikon) and were spectrally separated using a MultiCam-LS device (Cairns) equipped with a T635px- UF2 dichroic mirror (Chroma) and imaged onto ORCA-Flash 4.0 v2 sCMOS cameras (Hamamatsu). Data were acquired at 40-ms time resolution using custom software implemented in LaVIEW (National Instruments).

Single-Molecule tRNA Selection Assay. As previously described (45), the process of tRNA selection on the ribosome was performed by stopped-flow injection of a 20-nM solution of ternary complex [eEF1A(GTP)–aa-tRNA<sub>Met</sub> containing (Cy3)Phe-tRNA<sup>Met</sup> into surface-immobilized ribosome complexes containing (Cy3)Met-tRNA<sub>Met</sub> in the P-site. Here, 80S ICs were formed with the near-cognate UCU mRNA codon in the A-site, and the period of incubation with the ternary complex was extended from 30 to 5 min. To prevent spurious, nonenzymatic binding of deacylated tRNA<sup>Met</sup> to the E-site, tRNA selec- tion experiments were performed in the presence of 500 μM cyclohexamide. Subsequent steady-state imaging of PRE complexes was performed following buffer exchange into a solution lacking the ternary complex.

Analysis of smFRET Data. Analysis of single-molecule fluorescence data was performed using the SPARTAN analysis software package MATLAB (84). Single-molecule fluorescence traces were extracted from wide-field movies and were corrected for background, spectral crosstalk, and unequal apparent brightness (85). FRET trajectories were calculated as I<sub>FRET</sub> = I<sub>E</sub>/(I<sub>E</sub> + I<sub>D</sub>), where I<sub>E</sub> and I<sub>D</sub> are the acceptor and donor fluorescence intensities at each frame, respectively. Traces were selected for further analysis according to the following criteria: (i) single step photoswitching event with a signal-to-background noise ratio >8; (ii) fewer than four donor blinking events; and (iii) >0.12 FRET efficiency for at least 50 frames (2 s). FRET histograms were calculated from the first 50 frames of all individual molecules passing the aforementioned criteria from each dataset with bin sizes of 0.03.

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