**SUMMARY**

Negamycin (NEG) is a ribosome-targeting antibiotic that exhibits clinically promising activity. Its binding site and mode of action have remained unknown. We solved the structure of the *Thermus thermophilus* ribosome bound to mRNA and three tRNAs, in complex with NEG. The drug binds to both small and large ribosomal subunits at nine independent sites. Resistance mutations in the 16S rRNA unequivocally identified the binding site in the vicinity of the conserved helix 34 (h34) in the small subunit as the primary site of antibiotic action in the bacterial and, possibly, eukaryotic ribosome. At this site, NEG contacts 16S rRNA as well as the anticodon loop of the A-site tRNA. Although the NEG site of action overlaps with that of tetracycline (TET), the two antibiotics exhibit different activities: while TET sterically hinders binding of aminoaoyl-tRNA to the ribosome, NEG stabilizes its binding, thereby inhibiting translocation and stimulating miscoding.

**INTRODUCTION**

The natural peptide-like antibiotic negamycin (NEG) [2-(3,6-diamino-5-hydroxyhexanoyl)-methylhydrazino]acetic acid] (Figure 1A), which exhibits inhibitory activity against Gram-negative and Gram-positive bacterial pathogens, was originally isolated from *Streptomyces* strains (Hamada et al., 1970) more than four decades ago. Although early biochemical experiments suggested that NEG inhibits protein synthesis (Mizuno et al., 1970a, 1970b), its mode of action remained enigmatic because various in vitro studies suggested that it could inhibit steps of translation as diverse as initiation (Mizuno et al., 1970b), decoding (Mizuno et al., 1970b; Uehara et al., 1972), and termination (Uehara et al., 1974, 1976b). The putative miscoding activity of NEG has made it an appealing candidate for the treatment of human genetic disorders caused by nonsense mutations. NEG or its synthetic analogs have demonstrated promising activity in stimulating premature stop codon bypass and partially restoring expression of several disease-related genes inactivated by nonsense mutations (Allamand et al., 2008; Arakawa et al., 2003; Floquet et al., 2011; Taguchi et al., 2012). These findings have made the need to uncover the site and the mode of action of NEG even more compelling.

Lack of understanding of the mechanism of NEG action has been further exacerbated by conflicting reports regarding its binding site. In vitro studies suggested that NEG associates with an RNA fragment mimicking helix 44 of the small ribosomal subunit rRNA (Arakawa et al., 2003), which harbors the binding site of miscoding-inducing aminoglycoside antibiotics (Griffey et al., 1999; Purohit and Stern, 1994). However, ribosomes isolated from aminoglycoside-resistant bacterial strains remained sensitive to NEG (Jehara et al., 1972). Soaking crystals of the large ribosomal subunit of the archaeon *Haloarcula marismortui* with 5 mM NEG revealed that the antibiotic binds at an unexpected site that lies close to the opening of the nascent peptide exit tunnel (Schroeder et al., 2007). Unfortunately, it was impossible to confirm the functional significance of the exit tunnel binding site because of the inability to isolate and map NEG-resistance mutations and due to the difficulty of crystallizing more physiologically relevant ribosome-antibiotic complexes (Corchuelo et al., 1981; Schroeder et al., 2007; Uehara et al., 1972).

We reasoned that structural and functional studies of the antibiotic complexed to the ribosome in a functionally meaningful state could provide a more compelling and consistent view of the site and mode of NEG action. In this work, we solved the 2.7 Å resolution crystal structure of the bacterial (*Thermus thermophilus*) 70S ribosome in complex with mRNA, three tRNAs, and NEG, which shows binding of the drug to nine independent sites. Resistance mutations in 16S rRNA unequivocally identify one of them as the main site of antibiotic action. Here, NEG interacts simultaneously with 16S rRNA and the A-site bound tRNA. Strikingly, although the NEG binding site largely overlaps with that of tetracycline (TET), the two antibiotics exhibit opposite activities: while TET prevents accommodation of aminoaoyl-tRNA in the A site due to a steric clash with the anticodon, NEG likely...

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stabilizes tRNA binding, leading to an increase in the frequency of translation errors and inhibition of translocation.

RESULTS AND DISCUSSION

The Crystal Structure of NEG Bound to the Thermus thermophilus 70S Ribosome

To identify the site of action of NEG in the bacterial ribosome, we solved the crystal structure of the T. thermophilus 70S ribosome in complex with NEG, a short mRNA, and three deacylated tRNAs at 2.7 Å resolution. The complex of 70S ribosomes with mRNA and tRNAs was first crystallized, and then crystals were soaked in 250 mM solution of NEG. It should be noted that this concentration of NEG significantly exceeded its IC$_{50}$ in the cell-free translation system (Figure S1 available online); because the inhibitor’s IC$_{50}$ and dissociation constant are only loosely correlated, we used a high NEG concentration in the crystallization experiments to ensure that the drug binding site was sufficiently saturated. The structure was solved by molecular replacement using the atomic coordinates of the T. thermophilus 70S ribosome (PDB entries 4QCM and 4QCN) (Polikanov et al., 2014b). In order to localize the NEG binding site(s) within the ribosome, we calculated an unbiased difference Fourier map using the observed amplitudes from the crystal and the amplitudes and phases that were derived from a model of the ribosome without the bound antibiotic. Peaks of positive electron density resembling distinct features of the NEG chemical structure (Figure 1A) were observed at nine different locations in both copies of the ribosome in the asymmetric unit (Figures 1B, 1C, and S2; Movie S1). Four of the NEG binding sites are located on the 30S subunit (Sites 1–4), while the remaining five sites are on the 50S subunit (Sites 5–9). An atomic model of NEG was manually fitted into the difference Fourier map at each location, and their coordinates were refined. The statistics for data processing and refinement are shown in Table 1.

The multitude of binding sites likely reflects promiscuous interactions of the small, flexible, positively charged antibiotic molecule with the large, porous ribosome built on a polyanionic RNA scaffold (Voss et al., 2006). It is unlikely, therefore, that all of the observed binding sites are functionally relevant, moreover that the NEG concentration used for crystal soaking likely significantly exceeds physiologically relevant concentrations of the antibiotic. With one exception (Site 1), most of these pockets are located far from the known ribosomal functional centers. Furthermore, none of them overlap with the NEG binding site previously observed in the archaeal 50S subunit (Schroeder et al., 2007) or with the 16S RNA helix 44, which was putatively implicated as the NEG-interacting partner by RNA fragment binding studies (Arakawa et al., 2003). Some
other antibiotics bind to several ribosomal sites, of which usually only one or few represent the true targets of drug action (Borovinkskaya et al., 2007a; Brodersen et al., 2000; Pioletti et al., 2001; Schlünzen et al., 2003). Therefore, we used genetic tools to establish the site where NEG binding interferes with ribosome function.

Mapping the Site of NEG Action in the Bacterial Ribosome
To determine the true site of action of NEG in the bacterial ribosome, we selected resistant mutants using the E. coli strain SQ110DTC that, besides being hypersensitive to a large range of antibiotics, possesses only one rm allele that facilitates isolation of ribosomal mutations (Orelle et al., 2013). Plating 10^9 cells onto an agar plate containing 160 μg/ml of NEG (5-fold the minimal inhibitory concentration [MIC]) resulted in the appearance of several resistant colonies. Sequencing of the 23S rRNA gene did not reveal alterations at any position. However, in all sequenced NEG-resistant clones, mutations were found in the 16S rRNA gene. These mutations (U1052G, U1060A, and A1197U) were clustered in the middle section of helix 34 (h34) (Figure 1D). They are located in close proximity to the decoding center in the 30S ribosomal subunit, and most importantly, they are positioned immediately adjacent to the NEG Site 1 observed in our crystal structure (Figure 1C).

In order to verify that h34 mutations found in the analyzed clones were necessary and sufficient to confer NEG resistance, two of the mutations, U1060A and A1197U, were individually engineered in the 16S rRNA gene of the rrn operon. Plasmids carrying the mutations were introduced into the 16S rrn gene. These mutations (U1052G, U1060A, and A1197U) were clustered in the middle section of helix 34 (h34) (Figure 1D). They are located in close proximity to the decoding center in the 30S ribosomal subunit, and most importantly, they are positioned immediately adjacent to the NEG Site 1 observed in our crystal structure (Figure 1C).

In Site 1, the extended hydrophilic antibiotic forms multiple hydrogen bonds with the backbone of nucleotides 965, 966, 967-970, 972, and 974-976 (Orelle et al., 2013). Therefore, we focused our subsequent analysis specifically on this site.

NEG Binding to the Site of Its Action Requires Proper Configuration of the 16S RNA Backbone
In Site 1, the extended hydrophilic antibiotic forms multiple hydrogen bonds with the backbone of nucleotides 965, 966,
The terminal carboxylic moiety of NEG forms a magnesium-mediated contact with the phosphate group of nucleotide 1052. None of the contacts of the antibiotic with the ribosome directly involve rRNA bases. Rather, the drug recognizes specific sugar-phosphate backbone atoms, whose proper spatial arrangement defines the position of the drug in its binding site. Therefore, the 16S rRNA resistance mutations that were identified, all of which disrupt h34 base pairs (Figure 1D), likely interfere with drug binding by changing the geometry of the h34 backbone. Two of the E. coli mutations, U1060A and A1197U, break the same base pair in h34 (Figure 1D). It is noteworthy that in the T. thermophilus ribosome, which readily binds NEG, the U-A base pair at this location is replaced with the C-G base pair, underscoring the importance of Watson-Crick interactions rather than the identity of the nucleotide for the antibiotic binding. Disruption of the 1060–1197 base pair by NEG mutations likely perturbs hydrogen bonding of the antibiotic to the residue 1197 backbone (Figure 2E). If this view is correct, then combining these two resistance mutations in the same cell would reconstitute the base-pair and hence restore sensitivity to the drug. Indeed, the simultaneous presence of U1060A and A1197U mutations in the E. coli 16S rRNA reverted NEG sensitivity to the wild-type level (Table 2), thereby confirming that the proper structure of the h34 backbone is required for the efficient binding of the antibiotic to its site of action in the small ribosomal subunit.

NEG Interacts with the A-site tRNA

In fast-growing cells, the majority of the ribosomes are involved in active translation. Therefore, when cells are exposed to an antibiotic, the inhibitor interacts predominantly with the translating ribosomes associated with functional ligands such as mRNA, tRNAs, and translation factors. The contacts of the inhibitor with the ligands of translation can be pivotal for its inhibitory action. Thus, crystallographic structures of an antibiotic complexed to the ribosome in the functional state should more accurately reveal the mechanisms of drug action than structures of the inhibitor bound to the vacant ribosome or individual ribosomal subunits. Indeed, analysis of intermolecular contacts established by NEG in the Site 1 shows that the antibiotic forms extensive interactions with the anticodon of the A-site tRNA. The N4 secondary amine (Figure 1A) of NEG is at hydrogen-bonding distance from the nonbridging oxygen atoms of the 5’ phosphate of nucleotide 34 of the A-site tRNA (Figure 2D). In addition, the terminal carboxyl group of NEG interacts with the nonbridging oxygen of the 3’ phosphate of the same tRNA residue via a hydrated magnesium ion, further strengthening the interactions of the drug with the tRNA.
Interaction of NEG with tRNA May Inhibit Translocation

The simultaneous multiple contacts of NEG with 16S rRNA and tRNA suggest that the drug may increase the affinity of tRNA to the A site, leading to some predictable consequences. Specifically, the tighter tRNA binding is expected to interfere with translocation because clearance of the A site and relocation of the tRNA-mRNA complex will require higher activation energy. We tested this possibility by analyzing the effect of NEG on translocation using a toe-printing assay (Hartz et al., 1988). This approach allows for detection of the ribosome pausing on mRNA during in vitro translation; the pattern of transiently stalled ribosomes is indicative of the mode of antibiotic action (Orelle et al., 2013). The well-known inhibitors of translocation, viomycin (VIO) or kanamycin (KAN) (Wilson, 2009), cause the appearance of a series of evenly spaced weak toe-printing bands corresponding to ribosomes that have randomly paused during their progression through the mRNA codons (Orelle et al., 2013) (Figure 3A). The presence of NEG in the translation reaction produces a set of bands whose positions coincide with those observed for VIO or KAN (Figure 3A), indicating similarity in the modes of action of these antibiotics. NEG, which inhibits translation by preventing binding of aminoacyl-tRNA in the A site (Wilson, 2009), also generates codon-spaced toe-printing bands (Orelle et al., 2013) (Figure 3A). However, a number of the TET bands were shifted by one nucleotide relative to those of VIO, KAN—a previously described toe-printing effect associated with the lack of an A-site tRNA in the stalled ribosome (Jerinic and Joseph, 2000; Orelle et al., 2013). Thus, the results of toe-printing analysis argue that NEG exerts its action after accommodation of aminoacyl-tRNA in the A site and inhibits ribosome progression through mRNA, possibly by impeding translocation.

Of note, increasing concentration of NEG in the reaction did not increase the ribosome stalling at the earlier mRNA codons (Figure 3B). This result indicates that even at saturation NEG does not block translocation dramatically but only slows it down at each codon. Multiplication of even a weak inhibitory effect over many codons of a protein-coding gene would result in severe inhibition of protein production at a relatively low NEG concentration.

We further directly tested the effect of NEG on translocation in a model assay that monitors the EF-G-catalyzed relocation of the ribosome from the start AUG codon of the phage T4 gene 32 mRNA to the second UUU codon (Figure 3B). The presence of NEG decreased the extent of translocation at a 30 s time point corresponding to the kinetic slope of the reaction, thus confirming that binding of the antibiotic slows down progression of the ribosome along mRNA, likely due to impeded translocation.

Figure 3. Mechanism of NEG Action

(A) Toe-printing analysis of antibiotic effects upon translation of the natural gene osmC or a synthetic gene RST2. Antibiotics THS, NEG, VIO, KAN, and TET were present in the reaction at 100 μM. The antibiotic-induced toe-printing bands corresponding to ribosomes stalled at the 5’ end proximal codons of the genes are indicated by red dots for NEG, green dots for VIO and KAN, and orange dots for TET. The control antibiotic THS arrests the ribosome at the initiator codon. The initiator and subsequent codons are marked by open and filled triangles, respectively.

(B) Effect of NEG on translocation of ribosomes from the 1st (AUG) to the 2nd (UUU) codon of m291 mRNA (partial relevant sequence shown in the upper panel). Where indicated, ribosomes were incubated with NEG at 10, 100, or 1,000 μM prior to the addition of EF-G and GTP. The triangles show the toe-printing bands corresponding to the position of ribosomes at the initiator codon (white triangle, empty A site; gray triangle, N-Ac-Phe-tRNA-occupied A site) or ribosomes translocated to the second codon (black triangle). The bar graph shows the translocation efficiency (%) estimated by the ratio of the intensity of the bands indicated by the black and gray triangles. Data are represented as mean of two independent experiments ± SEM.

(C) Disk diffusion assay revealing the mis-coding activity of NEG. Cells transformed with pBR322bla-stop were plated on LB agar without (bottom) or with 25 μg/ml of ampicillin (top). In the absence of ampicillin, diffusion of antibiotics PAR, SPC, or NEG inhibits bacterial growth. In the presence of ampicillin, cell growth occurs only with mis-coding-inducing antibiotics PAR and NEG, which allow expression of functional β-lactamase, as revealed by nitrocefin staining. The inactivating nonsense mutation in the bla gene of the pBR322bla-stop plasmid is shown above the plates. See also Figure S1.
NEG May Induce Miscoding by Stabilizing Aminoacyl-tRNA Binding in the A Site

Antibiotics like paromomycin (PAR) or streptomycin (STR), which similarly to NEG may strengthen tRNA binding to the A site, promote miscoding (Karimi and Ehrenberg, 1994; Peske et al., 2004) (Figure S4). Previous data indicated that NEG might induce amino acid misincorporation in bacterial cell-free extracts (Mizuno et al., 1970b; Uehara et al., 1972). In order to verify whether NEG stimulates in vivo miscoding in bacteria, we engineered a reporter system in which a premature UGA stop codon interrupts the \textit{bla} gene in the pBR322 plasmid ("pBR322 bla-stop"), preventing expression of functional \( \beta \)-lactamase. Survival of pBR322 bla-stop-transformed cells in the presence of the \( \beta \)-lactam antibiotic ampicillin depends solely upon the ability of the translating ribosomes to bypass the premature stop codon in the \textit{bla} gene by misreading it as a sense codon. In the absence of other antibiotics, growth of the lawn of cells transformed with pBR322 bla-stop on a LB-agar plate is inhibited by 25 mg/ml ampicillin (Figure 3C). In contrast, diffusion of NEG from a disk impregnated with the drug allowed cell growth on the ampicillin plate (Figure 3C). Similarly, PAR, a translation error-inducing aminoglycoside, also promoted growth on ampicillin, while spectinomycin (SPC), which does not induce miscoding (Wilson, 2009), did not. Expression of the catalytically active \( \beta \)-lactamase in the cells surrounding the disks with NEG or PAR was confirmed by staining with nitrocefin, an indicator of \( \beta \)-lactamase activity (O’Callaghan et al., 1972). This result demonstrates substantial miscoding activity of NEG in bacterial cells, which likely stems from its ability to promote illegitimate aminoacyl-tRNA binding to the ribosomal A site programmed with stop codon. While "traditional" miscoding antibiotics (e.g., PAR or STR) stimulate tRNA binding by altering the conformation of the A site (Ogle and Ramakrishnan, 2005), NEG apparently achieves a similar effect by establishing direct interactions with the A-site tRNA. We should indicate, however, that our data are insufficient to comment on codon or tRNA specificity of NEG-induced miscoding.

The readthrough activity we observed in bacteria is reminiscent of the reported NEG ability to stimulate premature stop codon bypass in mammalian cells (Allamand et al., 2008; Arakawa et al., 2003). The alignment of the structures of the 70S ribosome in complex with NEG and the vacant yeast 80S ribosome (Ben-Shem et al., 2011) showed that the drug could form similar interactions with both bacterial 16S rRNA and eukaryotic 18S rRNA, despite their evolutionary divergence (Figure 4). Therefore, it is possible that, in both the bacterial and eukaryotic ribosomes, NEG induces stop codon readthrough by binding to the same site in the vicinity of h34 of rRNA of the small subunit.

Our structure of the ribosome-NEG functional complex offers a possible explanation for the previously reported varying miscoding capacity of NEG derivatives (Uehara et al., 1976a). The placement of the drug in Site 1 is fixed by the interaction between its positively charged \( \beta \)-amino group and the 5’ phosphate of G1197. Stereoconfiguration of the C7 carbon atom carrying this amino group is pivotal for orienting the antibiotic and its binding to tRNA. Indeed, inverting its stereo configuration dramatically diminished drug-induced miscoding (Uehara et al., 1976a). In contrast, the C9-linked hydroxyl is not involved in any major interactions, as removal of this group or its methyl-ation had no effect on miscoding (Uehara et al., 1976a). Finally,
any bulky substitutions at the terminal amino group of NEG will prevent the drug from fitting into its binding pocket. Consistently, its acylation prevented drug binding and miscoding (Uehara et al., 1976a).

**Binding in Site 1 Cannot Explain NEG Effect upon Translation Termination**

One of the activities reported previously for NEG was inhibition of translation termination (Uehara et al., 1974). We were unable to find any convincing rationale for this activity on the basis of NEG binding to Site 1. Aligning the 70S-NEG structure with those of the ribosome complexed with Class I release factors did not show any interference or specific interactions between the drug and either of the release factors (Figures S5A and S5B). However, binding of NEG to Site 5 in the large ribosomal subunit results in a steric clash with the amino acid residue Gln253 of *T. thermophilus* RF1 (Laurberg et al., 2008) (Figures S5C and S5D). Competition between NEG and RF1 for mutually exclusive binding to the 70S ribosome is also indirectly supported by the observation that ribosome crystals containing RF1 significantly lose their diffraction ability upon soaking with NEG (data not shown). This could be the result of NEG-induced disorder in 70S-RF1 crystals. Nevertheless, it should be noted that even if binding of NEG in Site 5 interferes with peptide release, this activity has a negligible contribution to the overall antibiotic effect because none of the resistance mutations were found in the vicinity of this site.

In spite of their overlapping binding sites in the ribosome, the mechanisms of TET and NEG action are quite different. NEG fits snugly between helices 34 and 31 of the 16S rRNA and the backbone of the tRNA anticodon in the A site (Figure 5C), while ribosome-bound TET clashes with the tRNA anticodon (Figure 5D). As a result of this difference, NEG likely increases the affinity of the tRNA to the ribosomal A site, while TET and its derivatives compete with aminoacyl-tRNA binding (Blanchard et al., 2004; Brodersen et al., 2000; Jenner et al., 2013). Idiosyncrasies in the contacts of NEG and TET with the ribosome prompted us to test the cross-resistance conferred by the previously known TET-resistance mechanisms and the newly discovered NEG’ mutations. The NEG’ mutations U1060A and A1197U rendered *E. coli* cells resistant to TET.
whereas combining these mutations in the same ribosome restored the wild-type sensitivity for both TET and NEG (Table 2), suggesting that the undisrupted secondary structure of h34 is required for efficient binding of both antibiotics. The TET mutation G1058C (Ross et al., 1998) also increased NEG resistance (Table 2). In contrast, TET mutation G966U (Dalladieu et al., 2002; Gerrits et al., 2002, 2003; Nonaka et al., 2005) rendered cells hypersensitive to NEG (Table 2), whereas NEG mutation U1052G increased sensitivity to TET, accentuating the difference in interactions of these chemically dissimilar antibiotics with the ribosome. A unique mechanism of TET resistance involves the action of the ribosome protection proteins (e.g., TetM), which have been suggested to promote dissociation of the drug by transient binding to the ribosome and altering the conformation of the antibiotic binding site (Dönhofer et al., 2012; Spahn et al., 2001). Expression of TetM in E. coli BL21 cells increased TET MIC by 32-fold (Mikolajka et al., 2011) (Table 2) but had no effect on NEG sensitivity (Table 2). This result suggests that the TetM-induced alteration of the TET binding pocket does not trigger release of the bound NEG, further stressing the conclusion that NEG and TET establish substantially distinct interactions with the 3OS ribosomal subunit.

The use of a ribosomal functional complex for the structural analysis made it possible to observe important interactions between NEG and tRNA, which likely stimulate tRNA binding and, as we propose, are critical for the mechanism of drug action. Our structure and the newly gained insights into the mode of action of NEG suggest that the undisrupted secondary structure of h34, or at C8 may stabilize NEG binding to the ribosome and/or tRNA, and acquiring “TET-like” activity at the expense of NEG-specific interactions (Figure 1A) will likely lead to a clash with the A-site tRNA and acquiring “TET-like” activity at the expense of NEG-specific properties. In contrast, small substitutions at the amino terminus or at C8 may stabilize NEG binding to the ribosome and/or tRNA, potentially further improving its miscoding capacity. We believe that understanding the interactions and operation of NEG in its functional site in the ribosome will provide the structural framework for future improvement of this protein synthesis inhibitor for medical purposes.

Our results also show that the affinity of the aminoacyl-tRNA to the ribosome must be carefully balanced in order to provide efficient acceptor substrate binding without compromising accuracy and speed of translocation. Perturbing this balance by decreasing aminoacyl-tRNA affinity (e.g., by TET or increasing it (e.g., by NEG) is detrimental for efficient protein synthesis.

### EXPERIMENTAL PROCEDURES

#### Crystallographic Structure Determination

T. thermophilus 70S ribosomes, unmodified tRNA\textsuperscript{Met} and tRNA\textsuperscript{Thr}\textsuperscript{I} from E. coli were purified as previously described (Lüdemann et al., 1998; Polikanov et al., 2012; Schmitt et al., 1999). Synthetic mRNA with the sequence 5'-GAG GUA AAA AUG UUC UAA-3' was obtained from IDT (Coralville). NEG was obtained from the Institute of Microbial Chemistry (Tokyo).

Ribosome-mRNA-tRNA complex was formed by programming of 5 μM 70S 70S ribosomes with 10 μM mRNA and incubation at 55 °C for 10 min, followed by addition of 20 μM P- and A-site tRNA substrates (with minor changes from Voorhees et al., 2002). Each of the last two steps was allowed to reach equilibrium for 10 min at 37°C. The final ionic conditions were as follows: 5 mM HEPES-KOH (pH 7.6), 50 mM KCl, 10 mM NH\textsubscript{4}Cl, and 10 mM MgCl\textsubscript{2},CO\textsubscript{3}.

Crystals were grown by the vapor diffusion method in sitting drops at 19 °C and stabilized as described (Polikanov et al., 2012) with NEG included only in the last stabilization buffer (250 μM NEG final concentration). Diffraction data were collected at beamline 24-ID-C at the Advanced Photon Source and beamline X25 at the Brookhaven National Laboratory. All crystals belonged to the primitive orthorhombic space group P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1}, with approximate unit cell dimensions of 210 Å × 450 Å × 620 Å and contained two copies of the 70S ribosome per asymmetric unit. The initial molecular replacement solutions were refined by rigid body refinement with the ribosome split into multiple domains, followed by positional and individual B factor refinement. The statistics for data processing and refinement are shown in Table 1.

#### Selection and Analysis of NEG- and TET-Resistant Mutants

To isolate NEG resistant mutants, E. coli SQ110DTC cells (Orelle et al., 2013) were grown overnight at 37 °C in LB medium containing selective antibiotic markers (50 μg/ml SPC and 50 μg/ml KAN). The culture was diluted to OD\textsubscript{600} = 0.05 and, upon reaching mid exponential phase (OD\textsubscript{600} = 0.5), ca. 10\textsuperscript{9} cells (≈1 OD\textsubscript{600} of the culture) were plated on a LB-agar plate containing 160 μg/ml NEG (5-fold MIC). Several colonies appeared on the plate after overnight incubation at 37 °C. 16S and 23S genes were PCR amplified from the resistant colonies and were sequenced (Orelle et al., 2013).

The T1060A and A1197T mutations were introduced in the 16S RNA gene of plasmid pAM552 using the QuikChange Lightning Multi Site mutagenesis kit (Agilent Technologies). After verifying the presence of the mutation, the mutant plasmids were introduced into E. coli SQ171 ΔtolC strain (Asai et al., 1999; Boltenbach et al., 2009), Mice was determined by microbroth dilution method.

Plasmid pAM552 was engineered as follows: a 5.6-kb DNA fragment containing E. coli rnh operon starting from the PL promoter and including both T1 and T2 terminators was PCR amplified from the plK35 plasmid (Douthwaite et al., 1989) using primers Not-PL-rnhB (CGCCGCAGGCATCTCACC TACAAACATGCG) and Asc-PL-rmB2 (CCCGGCAGCCGCGCGTGGTAGAT ATGACGACAGAAGA). The bla-orl segment of the pGE2-2 plasmid was PCR amplified using primers Not-GE2R (CCGGCGCGCGGCCGGAAAAG GGCGAAGCCGTAAAGG) and Asc-GE2X (CCGGCGGCGGGGAAAT TGTGCCGCGAACC). DNA fragments were cut with NotI and Ascl restriction enzymes, and ligated together. A unique PstI site was then introduced at the end of the bla gene by site-directed mutagenesis. This plasmid was further modified by introducing pTrc promoter in front of the bla gene, Ndel restriction enzyme at the bla gene start codon, and Trp terminator followed by the PstI site after the end of the bla gene.

#### Toe-Printing Assay

Toe-printing analysis (Hartz et al., 1988) was performed following in vitro coupled transcription-translation reactions in the PURE system (New England Biolabs) with no antibiotic or in the presence of 100 μM thiorhophen (THS), NEG, Vio, Kan, or TET. Template for cosM was amplified from genomic DNA, and overlapping PCR was used to generate RST2 (Orelle et al., 2013).

#### Translocation Assay

Translocation assay was carried out using the phage T4 gene 32 template (m291) essentially as described by Shoji et al. (2006) with minor modifications as described in Polikanov et al. (2014a).

#### In Vivo Miscoding Activity

TGA stop codon was inserted between codons 109 and 110 of the bla gene of pBR322 using the QuikChange kit (Agilent Technologies) and the primer (CA CCA GTG ACA AAG TAG CAT CTT ACG GAT GGC AT). The resulting plasmid pBR322bla-stop was transformed into E. coli TB-1 cells. Cells containing pBR322bla-stop were grown at 37°C overnight in LB medium with selective antibiotics (TET 10 μg/ml and STR 25 μg/ml). Culture was diluted to OD\textsubscript{600} = 0.05 and allowed to reach late exponential phase (OD\textsubscript{600} = 1). A total of 1.5 ml of cell culture was mixed with 13.5 ml of soft agar and poured into rectangular LB agar plate that contained 25 μg/ml ampicillin or no antibiotic. Filter discs containing PAR (7.5 μg), NEG (30 μg), or SPE...
Determining the Effect of TetM Expression on Resistance to NEG

Plasmid pET46LIC-TetM (kindly provided by Drs. Wilson and Starosta, Gene Center, University of Munich) or the empty vector pET11a were transformed into E. coli BL21(DE3) cells. Following 1 hr incubation at 37°C of the transformed culture in LB medium, ampicillin was added to a final concentration of 100 µg/ml, and incubation continued overnight. Cultures were then diluted to OD600 = 0.05, and upon reaching OD600 = 0.2, TetM expression was induced with 0.5 mM isopropyl-ß-D-1-thiogalactopyranoside, and MICs for TET and NEG were determined.

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Molecular Cell

Miscoding Antibiotic Directly Enhances tRNA Binding


Supplemental Information

Negamycin Interferes with Decoding and Translocation by Simultaneous Interaction with rRNA and tRNA

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Figure S1, related to Figure 3. Inhibitory action of NEG upon cell-free protein synthesis. (A) Inhibition of translation of the superfolder GFP protein in the PURExpress cell-free translation system assembled from the purified components (New England Biolabs). The reactions (10 μl) were assembled according to the manufacturer’s protocol and programmed with 100 ng of the PCR-amplified sfGPF template. Antibiotic was added at the indicated concentrations and the reactions were incubated in the 384-well black-wall plate with the continuous monitoring of fluorescence in the wells over 5 hr in the TECAN microplate scanner. The 3-hr time point, which corresponded to the kinetic slope of the translation reaction, was used for preparing the plot and calculating the IC$_{50}$ values. (B). Toe-printing analysis of antibiotic effects upon translation of the synthetic gene RST2. The translation reactions were supplemented with NEG at the concentrations indicated above the corresponding gel lanes. The initiator and subsequent RST2 codons are marked by open and filled triangles, respectively.
Figure S2, related to Figure 1. Difference Fourier maps of NEG binding sites in *T. thermophilus* 70S ribosome. (A, F) Overview of the structure of NEG (yellow) in complex with the *T. thermophilus* 70S ribosome. NEG binding sites within 30S subunit are shown in (A), 50S subunit – in (F). (B-E, G-K) Close-up views of all nine NEG binding sites. The refined models of NEG (yellow) are displayed in their respective electron densities before the refinement. The unbiased ($F_{\text{obs}} - F_{\text{calc}}$) difference electron density maps are contoured at ~3σ. Carbon atoms are colored yellow, nitrogens - blue, oxygens – red.
Figure S3, related to Figures 1 and 2. Schematic representation of NEG binding sites in the secondary structure diagrams of *T. thermophilus* 16S (A) and 23S (B) rRNAs (Cannone et al., 2002). The nucleotides that form direct interaction with the drug are indicated by triangles. The NEG Site 1 is highlighted in red to emphasize, that out of 9 binding sites observed in the crystal structure, Site 1 is the primary site of NEG action. (C) Schematic overview of NEG interactions on the ribosome at NEG Site 1. Nucleotides of the 16S rRNA are highlighted in light yellow, A-site tRNA is green. H-bonds and coordination bonds are shown as red dots. Non-bridging phosphate oxygens (OP) and magnesium-coordinated waters are colored red, magnesium ions – dark green.
Figure S4, related to Figure 2. Error-inducing antibiotics bound near the decoding center of the small ribosomal subunit. (A, B) Chemical structures of paromomycin (A) and streptomycin (B). (C) Overview of the superimposed binding sites of NEG (yellow), paromomycin (PAR, red), and streptomycin (STR, magenta) on the 30S subunit of the Thermus thermophilus ribosome. 30S subunit is shown in light yellow with h34 highlighted in orange, h44 highlighted in green, and protein S12 highlighted in blue. The view is from the intersubunit interface, 50S subunit is removed for clarity. 70S-NEG structure is from the current work, paromomycin is from PDB entry 2WDK (Voorhees et al., 2009), and streptomycin is from PDB entry 1FJG (Carter et al., 2000). All three structures were aligned based on the 16S rRNA. (D) Close-up view of (C). Nucleotides C518, G530, A1492, and A1493 of the
16S rRNA, which play a pivotal role during selection of cognate tRNA in the decoding center, are shown as sticks.
Figure S5, related to Figure 1. Superposition of 70S-NEG structure with the ribosome-bound Release Factor I (RF1). (A, C) Overviews and (B, D) close-up views of the structure of NEG at Site 1 (A, B) or Site 5 (C, D) on the *T. thermophilus* 70S ribosome superimposed with the structure of ribosome-bound RF1 (green) (PDB entry 3D5A (Laurberg et al., 2008)). Superposition is based on the alignment of either 16S rRNA (A, B) or 23S rRNA (C, D). The 30S subunit (light yellow) or 50S subunit (light blue) are viewed from the intersubunit interface with the counter-subunit removed for clarity. mRNA is shown in magenta and tRNAs are displayed in dark blue for the P site, and in orange for the E site. In (A), only the anticodon stems of tRNAs are shown. A site tRNA is not shown. Note,
that NEG in Site 1 on the 30S subunit does not overlap with the ribosome-bound RF1, while NEG in Site 5 on the 50S subunit clashes with residue Gln253 of RF1 domain III.
Movie S1, related to Figures 1 and 2. NEG functional site on the 70S ribosome. The movie shows: (1) locations of nine NEG binding sites in the small and large subunits of the *T. thermophilus* 70S ribosome programmed with mRNA and three tRNAs; (2) locations of NEG resistance mutations in the *E. coli* 16S rRNA; (3) close-up views of the NEG Site 1 showing details of NEG interactions with both the tRNA and the 16S rRNA in the A site of the ribosome.

