Supporting Information

Fabbretti et al. 10.1073/pnas.1521156113

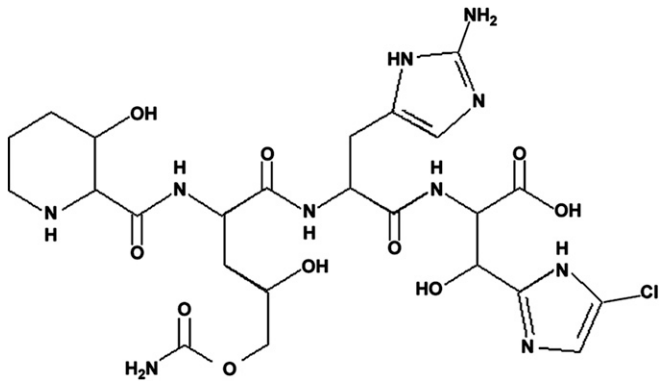

Fig. S1. Structure of GE81112. Structure of variant B of GE81112 (658 Da) as determined by NMR spectroscopy. The molecule consists of four amino acids: 3-hydroxypipecolic acid, 2-amino-5-[(aminocarbonyl)oxy]-4-hydroxypentanoic acid, 5-amino-histidine, and 5-chloro-2-imidazolylserine (10).

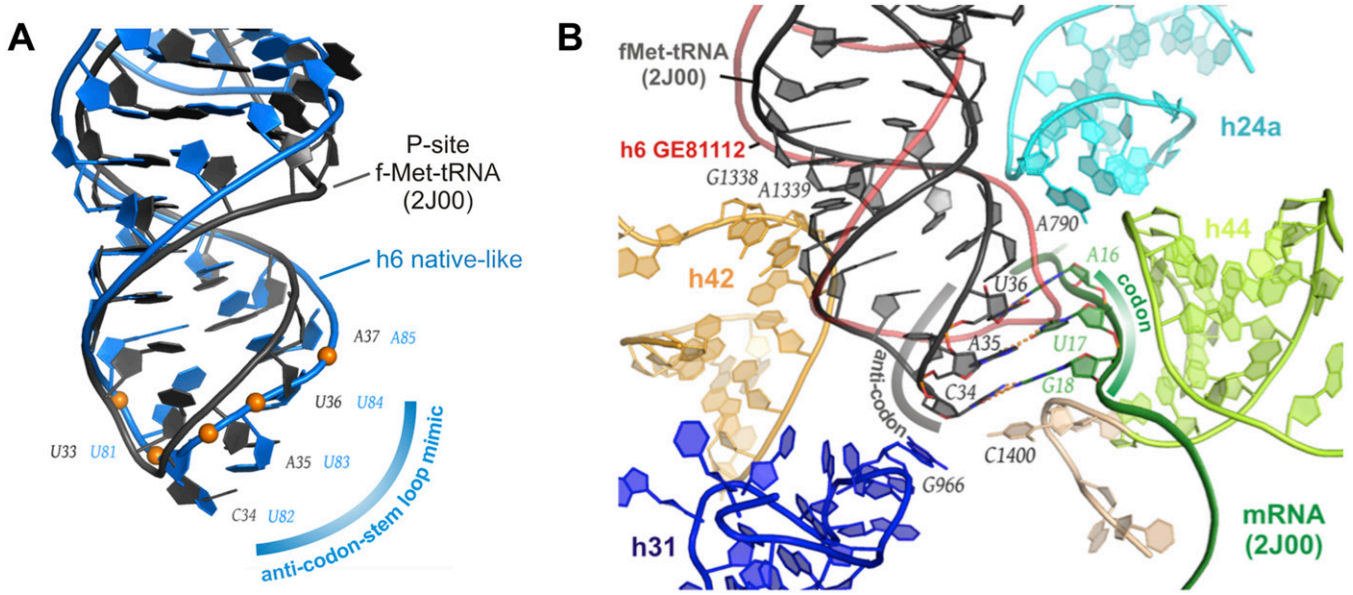

Fig. S2. Comparison and alignment of P-site tRNAs and ASL. (*A*) In 30S crystals h6 of one subunit is inserted into the P-site of a symmetry-related subunit mimicking the ASL of the P-site–bound tRNA. The figure in the panel presents the structure alignment of initiator fMet–tRNA as seen in the 70S structure (PDB ID code 2J00) and the ribosomal spur (h6) as seen in the 30S crystals in the absence of GE81112 (PDB ID code 2ZM6). The structures were aligned using 16S rRNA residues surrounding the P-tRNA. (*B*) The alignment between the tip of the ASL mimic distorted in the presence of GE81112 with the initiator P-site–bound tRNA as seen in the crystal structure of the 70S ribosome (PDB ID code 2J00) illustrates how the conformational change precludes codon–anticodon interaction. The residues involved in the canonical codon–anticodon interaction are labeled in green (A16–G18) and gray (C34–U36). The distorted ASL mimic (h6) as seen in the presence GE81112 is colored red. The structural alignment was preformed using 16S rRNA elements surrounding the P-tRNA.

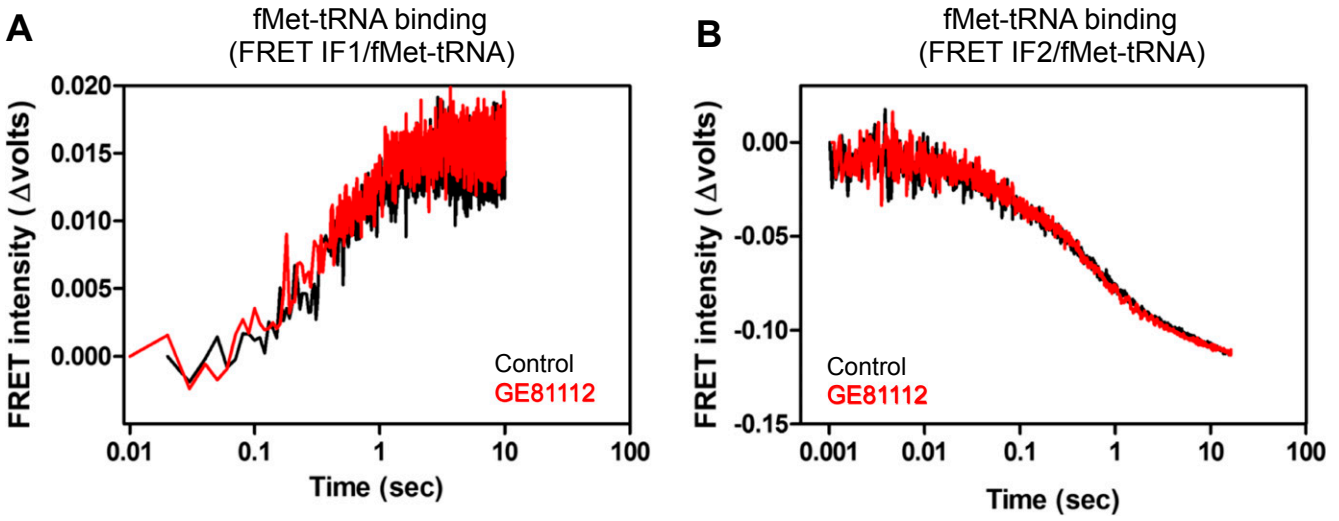

Fig. S3. GE81112 does not affect the kinetics of 30S preIC formation. Binding kinetics of fMet–tRNA to the 30S ribosomal subunit in the absence (black tracing) or presence (red tracing) of 100 μM GE81112 measured by a fluorescence stop-flow apparatus making use of two types of observables, namely the FRET signals generated by the proximity of $fMet–tRNA_{8fluo}$ acting as a donor and $IF1_{Alexa555}$ as acceptor (*A*) and the quenching of $IF2_{757Alexa488}$ fluorescence by the approaching $fMet–tRNA_{8QSY35}$ (*B*).

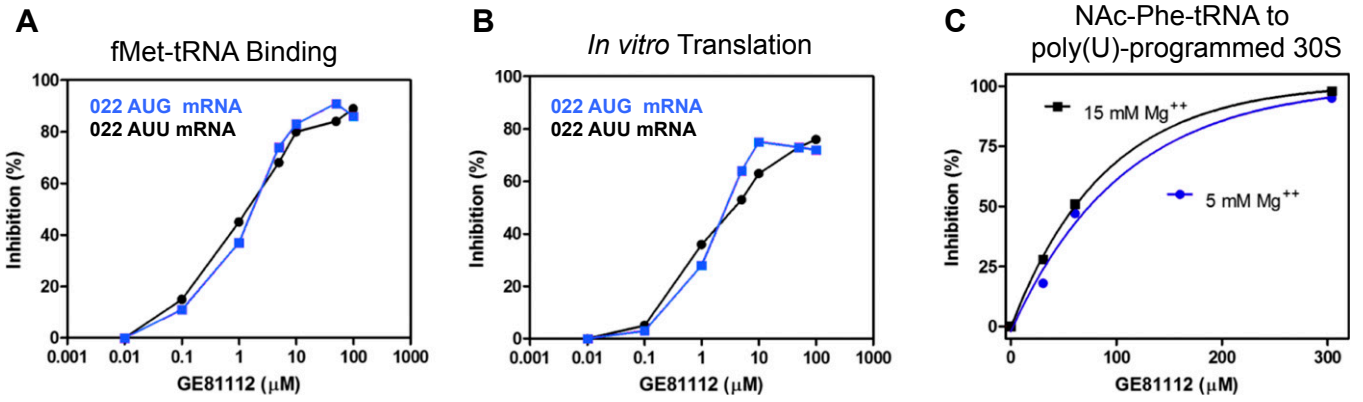

Fig. S4. Inhibition of translation initiation by GE81112 does not depend on the nature of the initiation codon or the nature of the aminoacyl-tRNA and template. (*A*) GE81112 concentration-dependent inhibition of [^{35}S]fMet–tRNA binding to 30S ribosomal subunits programmed with 022AUGmRNA (■) or 022AUU mRNA(●) measured by filtration through nitrocellulose filters as described previously (25). (*B*) In vitro translation of an *E. coli* cell-free system programmed with 022AUGmRNA (■) or 022AUUmRNA (●). (*C*) Binding of NAc[^{3}H]–Phe–tRNA to poly(U)-programmed 30S ribosomal subunits to form a pseudoinitiation complex. The reaction was carried out at 5 mM (●) or 15 mM (■) of magnesium acetate (26).

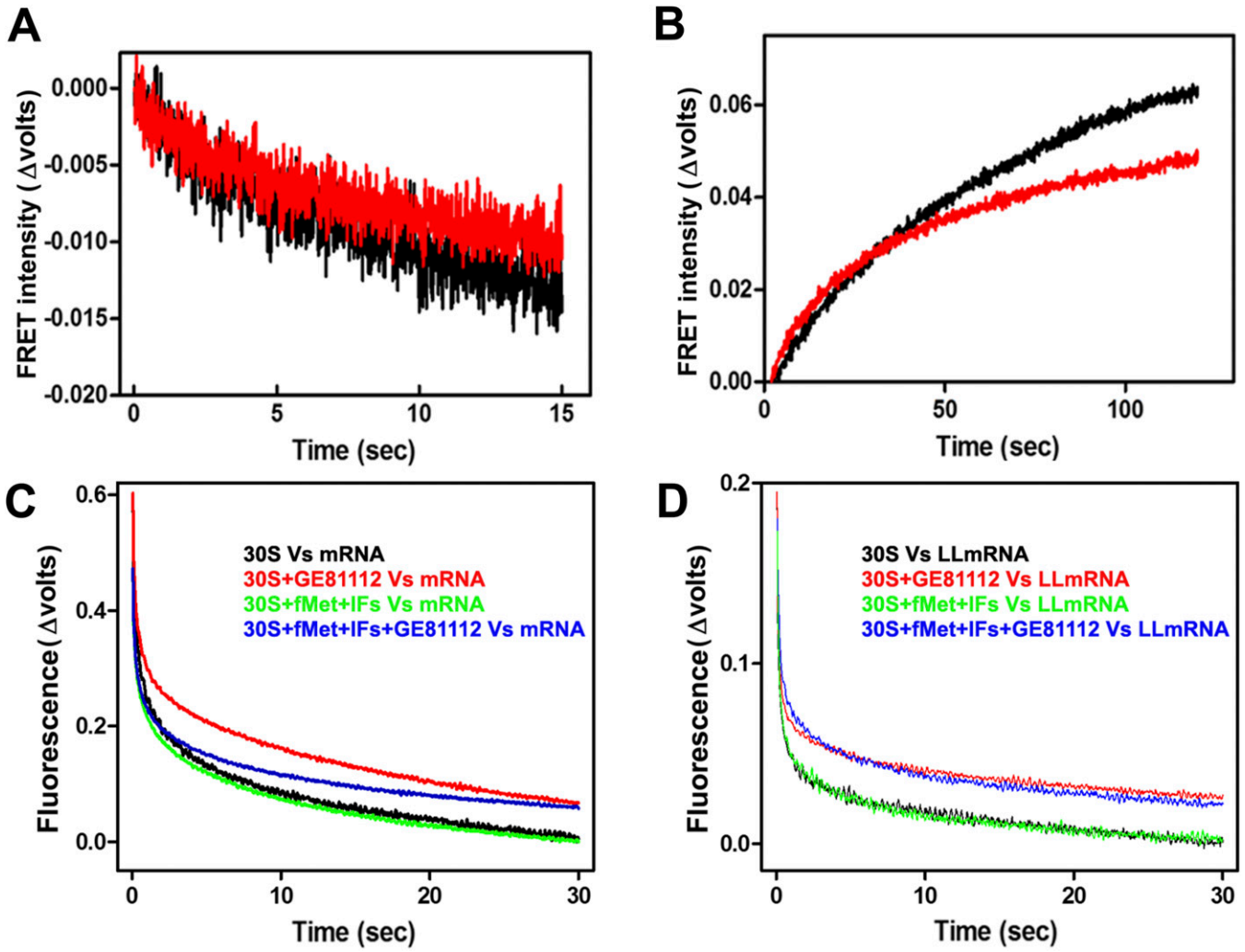

Fig. S5. Effect of GE81112 on the rate of exchange between 30S-bound and free IF1 and IF2 and on the kinetics of mRNA binding to the 30S subunit. (*A* and *B*) Two types of complexes were prepared in the presence (red tracings) and absence (black tracings) of GE81112. One complex contained fluorescently labeled IF1 $_{4\text{-ALEXA555}}$ and fluorescein-labeled fMet–tRNA; IF1 dissociation is monitored by the reduction of the FRET signal between the two fluorescent ligands upon the addition of a 10-fold excess of nonfluorescent IF1 (*A*). The other complex contained fluorescently labeled IF2 $_{757\text{-Alexa488}}$ and fMet–RNA$_{8\text{-QSY35}}$ (*B*). Upon the addition of a 10-fold excess of nonfluorescent factor, the dissociation of IF1 (*A*) causes a decrease in fluorescence intensity (because of the loss of the FRET signal), whereas IF2 dissociation (*B*) causes an increase in fluorescence because of diminished quenching. (*C* and *D*) Fluorescence stopped-flow binding kinetics of 3′ fluorescein semithiocarbazide-labeled (24) leadered (*C*) and leaderless (*D*) mRNA to 30S subunits (black and red tracings) or to 30S subunits containing all components of the initiation complex except for the mRNA (green and blue tracings) in the absence (black and green tracings) or in the presence (red and blue tracings) of 100 μM GE81112.

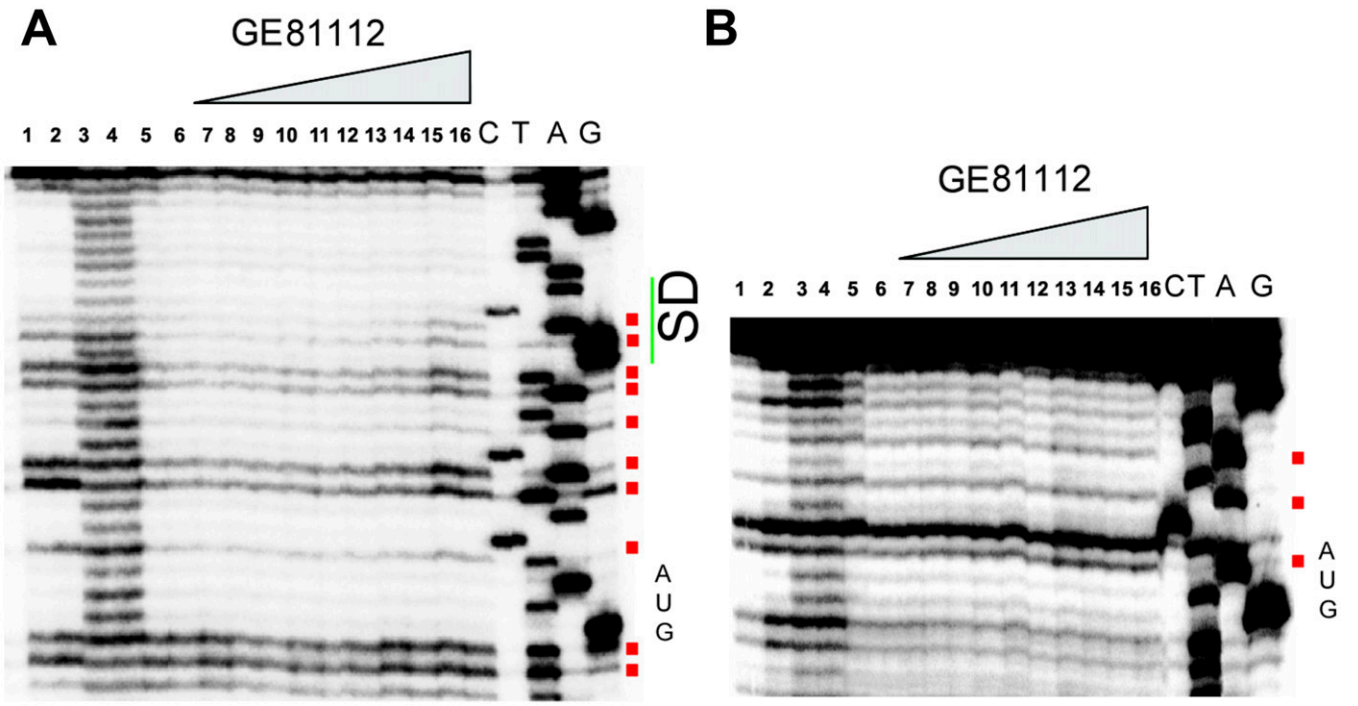

Fig. S6. GE81112 alters the mRNA position on the 30S subunit. Primer extension analysis of the 022 mRNA (*A*) and 003 mRNA (*B*) sites cleaved by hydroxyl radicals. Uncleaved mRNA (lanes 1 and 2), mRNA cleaved in the unbound form (lanes 3 and 4) and within a complete 30SIC assembled in the absence (lanes 5 and 6) or in the presence of 0.5 μM (lanes 7 and 8), 1.5 μM (lanes 9 and 10), 12 μM (lanes 11 and 12), 25 μM (lanes 13 and14), and 50 μM (lanes 15 and 16) of GE81112. Lanes C, T, A, and G are the sequencing ladders. Bases becoming more exposed in the presence of the antibiotic are indicated by red squares. The positions of the initiation triplet AUG and of the SD sequence (in the case of 022 mRNA) are indicated on the right side of the gels.

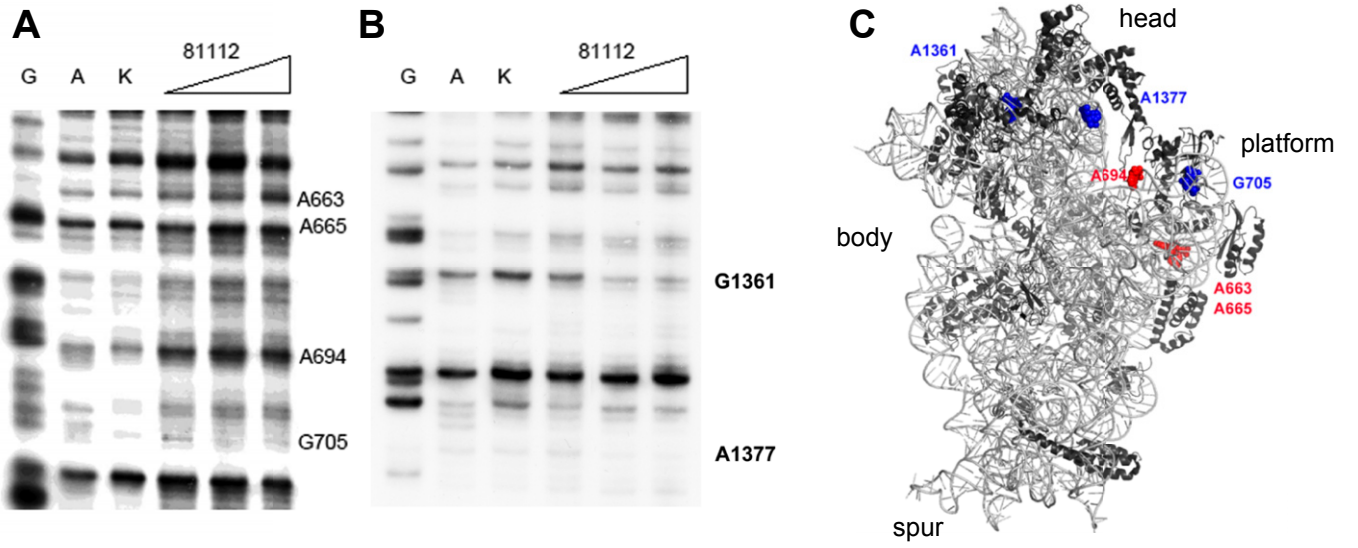

Fig. S7. Effect of GE81112 on the in situ accessibility of 16S rRNA to hydroxyl radical cleavage. Primer extension analysis of the 16S rRNA cleavage sites generated by hydroxyl radicals within the 30S subunit (9) in the presence of increasing concentrations of GE81112 (10–100 μM; increasing concentrations indicated with a triangle). (*A* and *B*) Analyses of the 16S rRNA residues 650–710 and 1,340–1,380, respectively. The G and A lanes contain the sequencing reactions. Bases whose accessibility to the cleavage is affected by GE81112 are indicated on the right side of the gels. (*C*) The positions of 16S rRNA bases becoming more (red) or less (blue) exposed to cleavage in the presence of GE81112 are indicated within the in the 3D 30S structure.

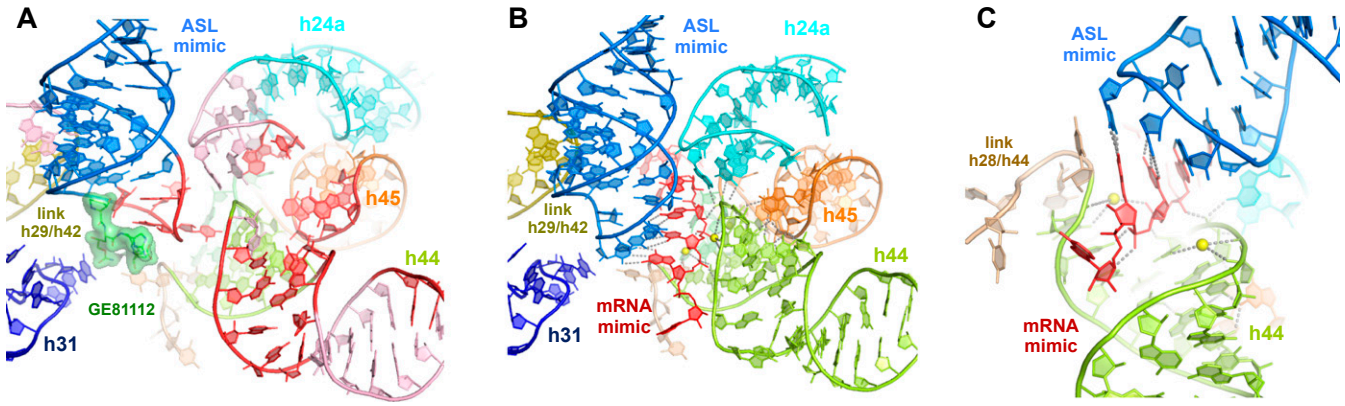

Fig. S8. Comparison of h44/h45/h24a in the engaged and disengaged conformation. The overall structure of the P-site region encompassing h44 (green), h45 (orange), h24a (cyan), the ASL (blue), and the mRNA (yellow) acquires a different conformation in the presence (*A*) or absence (*B* and *C*) of GE81112. The drug stabilizes the ASL tip in an altered conformation so that the mRNA cannot be positioned properly, and P-site codon–anticodon interaction is hampered. The architecture of the adjacent helices h44, h45, and h24a is altered, reflecting the formation of a different hydrogen bonding network that in the absence of GE8112 involves the mRNA and two additional Mg^{2+} ions (yellow spheres in *B* and *C*). In the two complexes, the h44/h45/h24a conformation is either disengaged (*A*) or engaged (*B* and *C*) (also see also Fig. 2 *B* and *C*). (*C*) A close-up and rotated view of *B* illustrating the potential hydrogen bond network formed by the tip of h44, the mRNA codon, and the ASL. This network could contribute to the stability of the h44 residues that are part of the h44/h45/h24a interface. The mRNA sequence is 5′-AGAAAGGAGGGUUUGGAAUGAACGAGC-3′. The residues most affected by the presence of GE81112 are colored in red (rms value higher than 2 Å) or pink ($1 \leq \text{rmsd} < 2$ Å).

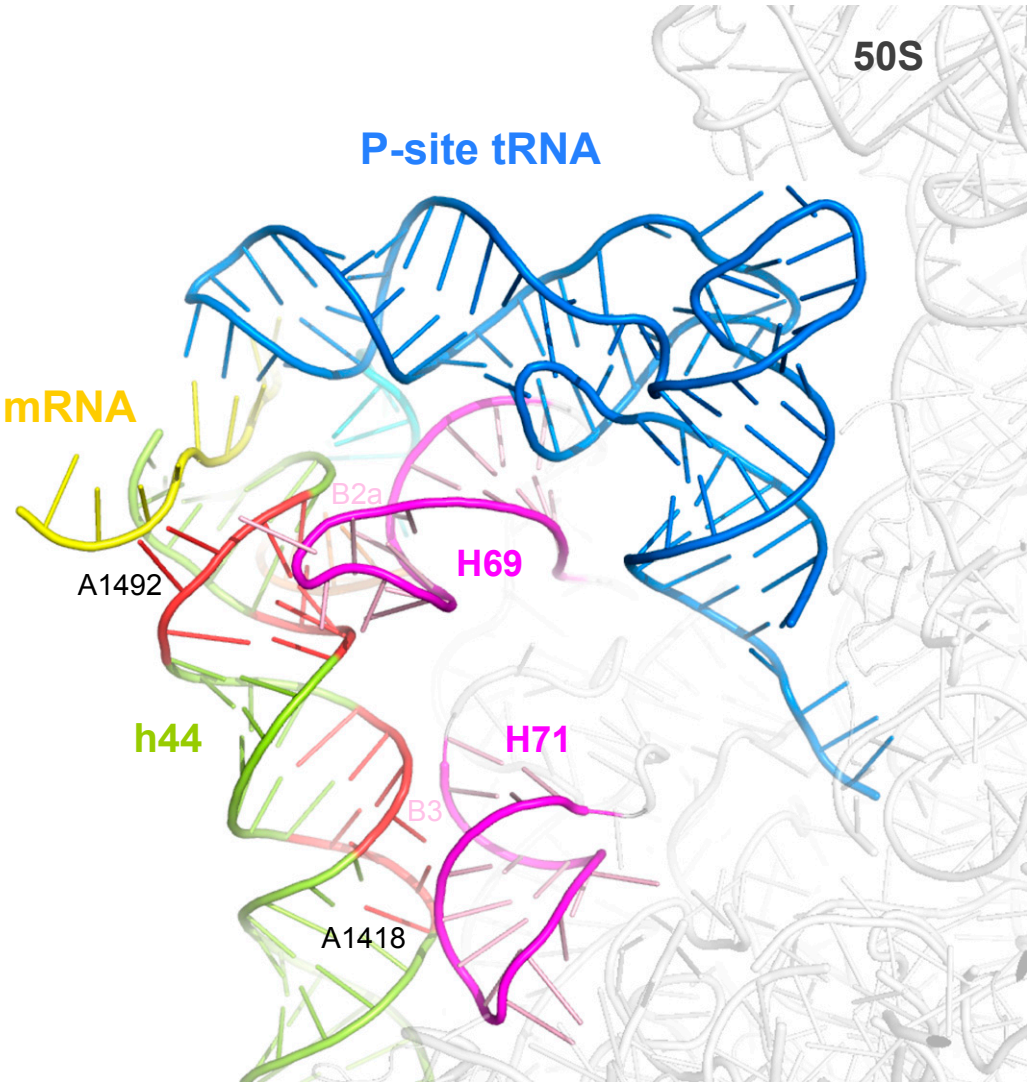

Fig. S9. The h44 nucleotides affected by GE81112 take part in the formation of bridges B2a and B3. Representative structure of the subunit interface of the 70S ribosome [PDB ID codes 2J00 (30S, including tRNAs and mRNA) and 2J01 (50S)] is shown in cartoon representation; the mRNA is colored in yellow, the P-site tRNA in blue, H69 and H79 of the 50S subunit in purple, h44 of the 30S subunit in green, and its nucleotides (1406–1409, 1491–1495, and 1417–1419) affected by GE81112 and involved in the switch to the disengaged conformation are in red. These nucleotides are either close to or directly involved in the formation of two intersubunit bridges (B2a and B3) that control and stabilize the interaction between the 30S and 50S subunits. In particular, the conformation of the h44 nucleotides (1409–1410 and 1495–1496) involved in the formation of B2a may have a significant role in the formation of a stable 70SIC, because the tip of H69 on the 50S, which participates in the formation of this bridge, is completely disordered in the free 50S subunit (e.g., PDB ID codes 3CC2 and 2ZJR) (34, 35), but it acquires a stable conformation by induced fit upon binding to the 30S subunit and interaction with h44. In the disengaged conformation, h44 in this region has the highest conformational distortion (see also Fig. 2 and Fig. S8), suggesting that this conformation in the 30SIC would hamper the formation of the bridge B2a, but the formation of this bridge would be favored by the engaged conformation, which is the only one observed in the 70S ribosome, and on the 30S in the presence of a proper accommodation of the P-site ligands. H69 also interacts with the P-site tRNA (blue), further suggesting that in the presence of an incorrect positioning of the mRNA and nonproper codon–anticodon interaction in the P site, the stability of the P-site tRNA would be compromised and possibly explaining why, under these conditions, the fMet tRNA dissociates from the complex. This hypothesis finds support from fMet–tRNA binding experiments in the presence of streptomycin, which induces the disengaged conformation of h44/h45/h24a and causes the release of fMet–tRNA from the 70S ribosome (36–39). Similarly, in the absence of methylation of 1518 and 1519, which also causes the stabilization of the disengaged conformation, the association between 30S and 50S is weakened (39).