Supplementary Information for

The cyclic octapeptide antibiotic argyrin B inhibits translation by trapping EF-G on the ribosome during translocation

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This PDF file includes:

- Materials and methods
- Figures S1 to S11
- Tables S1
- SI References
Materials and Methods

**In vitro transcription-translation assays.** To assess translation inhibition by argyrins, an *E. coli* cell-free lysate-based translation system (RTS100 *E. coli* HY; biotechrabbit) was employed for firefly luciferase expression, as previously described for other translation inhibiting antibiotics (1, 2). Briefly, 5 µl reactions were prepared according to manufacturer protocol, mixed with 0.5 µl argyrin at the indicated concentrations. Since the argyrin stocks were dissolved in DMSO, a final concentration of 3% DMSO was used for all reactions to normalize for the amount of DMSO present at the highest argyrin concentration tested (50 µM). The reactions were incubated for 30 min at 32°C at 600 rpm and was stopped by adding 5 µl of Kanamycin (50 µg/µl) to each reaction tube. The stopped reactions were transferred to a black 96-well chimney flat-bottom microtiter plate and mixed with 40 µl of Fluc substrate (Promega). Fluorescence was measured using a TECAN infinite 200Pro plate reader. Samples were normalized relative to reactions without antibiotic.

**Evaluation of the in vitro synergistic activity of argyrin B and fusidic acid**

The *in vitro* synergy between argyrin B and fusidic acid was measured as described previously (3). Briefly, the individual IC50 values of each single compound were first determined using the *in vitro* translation assay employing the *E. coli* cell-free lysate-based translation system (RTS100 *E. coli* HY; biotechrabbit) and Fluc reporter gene amplified by PCR and purified (QIAquick Nucleotide Removal Kit; QIAGEN) as before (1, 2). Briefly, 5 µl reactions were prepared according to manufacturer protocol, mixed with 0.5 µl of each test compound at the indicated concentrations. The reactions were incubated for 30 min at 32°C at 600 rpm and was stopped by adding 5 µl of Kanamycin (50 µg/µl) to each reaction tube. Then, 5 µL of stopped reactions were transferred to a black 96-well chimney flat-bottom microtiter plate and mixed with 40 µl of Fluc substrate (Promega). Fluorescence was measured using a TECAN infinite 200Pro plate reader. Samples were normalized relative to reactions without antibiotic. Subsequently, the same titration was repeated, but now in presence of the test synergistic partner compound (Appendix SI, Fig. S1A-F). The partner compound (0.5 µL) was added at a fixed final concentration that corresponded to its previously established IC50. The IC50 of each compound alone was then compared with the IC50 measured in the presence of the partner compound (Appendix SI, Fig. S1G-H). Since the *in vivo* (4) and *in vitro* (5) synergy between virginiamycin M1 (VrgM1) and virginiamycin S1 (VrgS1) is well-documented, this combination was used as positive control for synergy. In addition, we considered the use of the erythromycin (Ery) as a negative control since macrolides such as erythromycin have a different mechanism of action, bind to the ribosome (and not EF-G), and the binding site is located far from ArgB-EF-G (6). Moreover, Ery and ArgB display IC50 values within the same order of magnitude. Collectively, the results reveal that the combination of VrgM1 and S1 leads to a 6-fold increase in inhibitory activity compared to when these compounds are used separately, whereas no significant increase was observed for all other combinations of compounds tested (Appendix SI, Fig. S1H).

**Ribosome purification for co-sedimentation assays and the EF-G-ArgB-70S cryo-EM studies.** Ribosomes were purified as previously described (7). In short, *E. coli* BL21 (2 L in LB media) was incubated at 37°C and harvested 5,000 x g and 4°C for 25 min in mid exponential phase. Cell pellets were resuspended in 20 mL of buffer A (25 mM HEPES-KOH, pH 7.4, 20 mM
Mg(OAc)$_2$, 100 mM KOAc, 1 mM DTT) and disrupted utilising a microfluidizer. The crude cell lysate was centrifuged at 21,000 x g for 20 min at 4 °C. Subsequently, the cleared lysate was layered on a high salt (800 mM KCl) 25% sucrose cushion in buffer A and centrifuged for 17.5 h at 39,000 rpm and 4°C in a Ti45 rotor (Beckmann coulter). The obtained ribosome pellet was resuspended in buffer A. 120 OD$_{260}$ of the purified ribosomes were layered onto six 10-40% sucrose density gradients in buffer A and centrifuged for 3.5 h at 30,000 rpm and 4°C using a SW32 rotor (Beckmann coulter). Ribosome profiles were recorded at a wavelength of 254 nm using a gradient station (Biocomp). The 70S peaks were collected and subsequently pelleted for 14.5 h at 43,000 rpm and 4°C using a Ti45 rotor. The obtained pellets were resuspended in buffer A and following A$_{260}$ concentration measurements was stored at -80°C.

Co-sedimentation assays. Co-sedimentation assays were performed as previously described (8). In short, 0.4 μM of purified E. coli 70S were mixed in buffer A (25 mM HEPES-KOH, pH 7.4, 20 mM Mg(OAc)$_2$, 100 mM KOAc, 1 mM DTT) with 1 μM EF-G, 500 µM GTP and 50 μM of either argyrin B or fusidic acid. Reactions were incubated for 15 min at 37 °C and subsequently loaded on a 10% (w/v) sucrose cushion in buffer A and centrifuged for 35 min at 70,000 rpm in a TLA100 rotor at 4 °C. The pre-centrifugation reaction (R) and supernatant (S), as well as 10 pmol of the pellet (P), were applied to an 18% SDS-PAGE and stained with instant blue (Expedeon).

Data collection and single particle reconstruction for the EF-G-ArgB-70S cryo-EM studies. For grid formation, 3.5 μl of ArgB–EF-G–70S complex (4.5 A$_{260}$/ml formed as described for the binding assays i.e. with 1 μM EF-G, 500 μM GTP and 50 μM ArgB) was applied to 2 nm precoated Quantifoil R3/3 holey carbon supported grids and vitrified using a Vitrobot Mark IV (FEI company). Data collection was performed using a FEI Titan Krios transmission electron microscope equipped with a Falcon II direct electron detector at 300 kV with a dose of 2.5 e/Å$^2$ per frame, using a pixel size of 1.084 Å and a defocus range of -0.5 to -3.5 μm, resulting in 7,891 micrographs. The first ten frames (28 e/Å$^2$ in total) were aligned using MotionCor2 (9). Power spectra, defocus values, astigmatism and estimation of micrograph resolution were determined by CTFFIND4 software (10). A resolution cut-off of 3.5 Å and defocus cut-off of 30,000 were applied. Following further visual inspection of micrographs for thon-rings and ice quality, 3,466 micrographs were used for automated particle picking by GAUTOMATCH (http://www.mrc-lmb.cam.ac.uk/kzhang). 610,946 particles were extracted using RELION 3.0 (11) and subjected to 2D-classification using 130 classes. 557,436 particles were focus-sorted with a soft-mask around EF-G into four classes for 300 rounds. Class II to IV contained density for EF-G, whereas class I appeared to contain a vacant 70S ribosome and was not further pursued. Contrast transfer function (CTF) and beam tilt refinement with subsequent Bayesian polishing was performed on class II to IV. Using the gold standard criterion (Fourier shell correlation of 0.143), post-processed maps showed an improved resolution by roughly 0.5 Å, resulting in average resolution of 3 Å for class II to IV. Class IV was subjected multi-body refinement with three masks, consisting of large subunit with EF-G, small subunit body and platform, and small subunit head. Resolutions were improved to 2.76 Å, 2.77 Å and 3 Å, respectively, reaching 2.6 Å in the large subunit core. The refined maps were sharpened by applying an automatically determined B factor using RELION 3.0 (11). Local resolution and
maps filtered according to local resolution were computed using SPHIRE 1.3 (12). For detailed information on data collection parameters see Fig. S5 and Table S1.

Modelling for the EF-G-ArgB-70S complex. The INT2-FA-translocation intermediate structure (PDB-ID 7N2C), which was based on PDB-ID 4YBB (13), was used as a starting model (14), fit into the EM map and refined through iterative rounds of manual model building in Coot (v.0.9.4.1) (15, 16), and real-space refinement using PHENIX (v.1.19-4092) (17, 18). A 3D atomic model for Argyrin B was generated de novo from the chemical 2D structure (PubChem CID 9940787) using the Chemdraw-integrated Chem3D software. Restraints for Argyrin B were generated using PHENIX eLBOW (19). Molecular models were manually adjusted and refined using Coot (15, 16). The atomic models were combined and refined using real space-refined with secondary structure restraints calculated by PHENIX (v1.14) (17, 18). For detailed information on model-building statistics see Table S1.

Buffers and reagents for smFRET and EF-G-ArgB-INT2 structure determination. All experiments were carried out in polymix buffer (50 mM Tris-OAc (pH 7.5), 100 mM KCl, 5 mM NH₄OAc, 0.5 mM Ca(OAc)₂, 5 mM Mg(OAc)₂, 6 mM 2-mercaptoethanol, 0.1 mM EDTA, 5 mM putrescine and 1 mM spermidine) (20). A cocktail of triplet-state quenchers (1 mM Trolox, 1 mM nitrobenzyl alcohol and 1 mM cyclooctatetraene) and an enzymatic oxygen scavenging system (protocatechuic acid (PCA)/protocatechuate-3,4-dioxygenase) were used for smFRET experiments. Fusidic acid sodium salt and GTP were from Sigma-Aldrich. GTP was further purified using a Mono Q 5/50 GL anion exchange column (GE Healthcare Life Sciences). Pyruvate kinase, myokinase and phosphoenolpyruvate (PEP) were purchased from Sigma-Aldrich. All other standard reagents were purchased from Sigma-Aldrich or VWR. Cryo-EM and smFRET complexes contain MFK mRNA (Biotin-5′-CAA CCU AAA ACU UAC ACA CCC UUA GAG GGA CAA UCG AUG UUC AAA GUC UUC AAA GUC AUC-3′).

smFRET experiments. 70S ribosomes labelled with LD550 on uL1 and LD650 on uS13, programmed with 5’-biotinylated mRNA, containing P-site bound fMet-tRNA^{Met} and displaying the codon UUC in the A site were immobilized on passivated coverslips as described previously (21-24). The ribosomes were then incubated for 2 minutes with EF-Tu ternary complex containing Phe-tRNA^{Phe}, leading to stoichiometric formation of PRE ribosomes containing A-site fMet-Phe-tRNA^{Phe}, P-site tRNA^{Met}. To initiate translocation, 5 µM EF-G with 1 mM GTP, with or without 10 µM ArgB or 10 µM FA, was delivered to the flow cell by stopped-flow injection. All smFRET experiments were carried out at 25°C and ribosomes, tRNA, EF-Tu and EF-Ts were prepared as previously described (23, 24). The time-evolution of the FRET signal was then recorded using a home-built total internal reflection-based fluorescence microscope (25) at ~0.1 kW/cm² laser (532 nm) illumination at a time resolution of 40 or 400 ms. Donor and acceptor fluorescence intensities were extracted from the recorded movies and FRET efficiency traces were calculated using the SPARTAN software implemented in MatlabR2015b (25). FRET traces were selected for further analysis according to the following criteria: a single catastrophic photobleaching event, at least 8:1 signal/background-noise ratio and 6:1 signal/signal-noise ratio, less than four donor-fluorophore blinking events, a correlation coefficient between donor and acceptor <0.5. To accurately estimate
the FRET efficiencies of the major states occupied by the ribosome during translocation histograms were constructed using the first 50 frames of all FRET efficiency traces for each condition. Three gaussian functions were then fit to each histogram to estimate the mean FRET intensity of the PRE, intermediate and POST (either EF-G-bound in the presence of the drugs or unbound in their absence) states.

**Purification of reagents for EF-G-ArgB-INT2 cryo-EM structure determination.** Wild-type ribosomal subunits were purified from *E. coli* MRE600 for cryo-EM experiments as previously described (20). EF-Tu/EF-Ts (22) and EF-G (21) were purified as previously described. *E. coli* tRNA<sub>Met</sub> and tRNA<sub>Phe</sub> were purified as previously described (23). To prepare Phe-tRNA<sub>Phe</sub> ternary complex, phenylalanine (1 mM), PheRS (0.2 μM), pyruvate kinase (0.6 μM), myokinase (0.6 μM), PEP (0.4 mM), GTP (1 mM) and tRNA<sub>Phe</sub> (1.6 μM) were combined in charging buffer (50 mM Tris pH 8, 10 mM KCl, 100 mM NH₄Cl, 10 mM MgCl₂, 1 mM DTT, 2.5 mM ATP and 0.5 mM EDTA) before addition of EF-Tu–EF-Ts (8 μM). The resulting mixture was incubated for 10 min at 37°C to aminoacylate the tRNA and form a ternary complex. Successful aminoacylation was confirmed by fast protein liquid chromatography (FPLC). Initiation complexes containing fMet-tRNA<sub>Met</sub> in the P site were thawed and diluted in polymix buffer with 1 mM GTP for a final concentration of 1 μM ribosomes. For preparation of the translocation intermediate sample, ArgB (10 μM) was added to the dilution buffer. The initiation complexes were incubated with Phe-tRNA<sub>Phe</sub> ternary complex (1 μM final) for around 5–10 s before the solution was applied to cryo-EM grids.

**Cryo-EM grid preparation for EF-G-ArgB-INT2 structure.** Cryo-EM grids were prepared using a Vitrobot Mark IV plunge-freezing device (Thermo Fisher Scientific). For each experiment, 3 μl of sample was applied to UltrAuFoil R 1.2/1.3 gold 300 mesh grids that had been glow-discharged (Ar/O₂) for 20 s using a Solarus II Plasma Cleaning system (Gatan). Grids immediately blotted in the Vitrobot chamber (4°C, 100%, 4.5-5.5 s blot time; -5 blot force) and plunged into liquid ethane.

**Data collection and single particle reconstruction for EF-G-ArgB-INT2 cryo-EM studies.** Cryo-EM data for the translocation intermediate structure were collected using a Titan Krios G3i (Thermo Fisher Scientific) transmission electron microscope equipped with a K3 direct electron detector and post column GIF (energy filter). K3 gain references were acquired just before data collection. Data collection was performed using an image shift protocol (nine images were collected with one defocus measurement per nine holes). Movies were recorded at defocus values from −0.5 μm to −2.0 μm at a magnification of 130,000×, which corresponds to the pixel size of 0.648 Å per pixel at the specimen level (super-resolution 0.324 Å per pixel). During the 2-s exposure, 70 frames (0.03 s per frame, dose/frame 1 e⁻ per frame per Å²) were collected with a total dose of around 70 e⁻ per Å². Motion correction was performed on raw super-resolution movie stacks and binned twofold using MotionCor2 software (9). CTF parameters were determined using CTFFind4.1 (10) and refined later in Relion 4.0 (26). All pre-processing and particle sorting were conducted in Relion 4.0 (26). Sharpened and locally filtered maps were used to aid in model building. Pixel size was calibrated against high resolution 70S crystal structure PDB-ID 4YBB. Cryo-EM density map values
were normalized to mean = 0 and standard deviation (σ) = 1 in UCSF Chimera (27) using the vop scale function. For detailed information on data collection parameters see Fig. S5 and Table S1.

**Modelling for EF-G-ArgB-INT2 complex.** The INT2-FA-translocation intermediate structure (PDB-ID 7N2C), which was based on PDB-ID 4YBB (13), was used as a starting model (14), fit into the EM map and refined through iterative rounds of manual model building in Coot (v.0.9.4.1) (15, 16), and real-space refinement using PHENIX (v.1.19-4092) (17, 18). mRNA nucleotide 37 corresponds to the +1 position. Restraints for refinement were generated using phenix.elbow (19). Models were validated using phenix.validation_cryoem (28) with the built-in MolProbity (29) scoring. See Table S1 for more information.

**Figure preparation.** Figures showing atomic models and cryo-EM map densities were generated using either UCSF Chimera (27), PyMOL Molecular Graphic Systems (Schrödinger), UCSF ChimeraX (30). Sharpened maps from Relion 4.0 (26). Post-processing was used for figure images, threshold σ = 4, unless otherwise stated. Angle and distance measurements were performed in UCSF Chimera using the Fit in Map and the Distance tools. All figures were prepared using structures and models aligned on the large subunit core, unless otherwise noted. The large subunit core used was simulated 3 Å density of high-resolution ribosome crystal structure PDB-ID 4YBB (13) in UCSF Chimera using the molmap command with the following mobile elements omitted: uL5, uL6, uL9, uL10, uL120, uL31, H34 (709–723), A-site finger (ASF; H38; 866–906), the L11 stalk (1045–1112), H69 (1908–1925), the L1 stalk (2093–2198), H83/84 (2297–2318), the SRL (2651–2667) and 5S. Cryo-EM map density was coloured using the Colour Zone tool with a 3-Å radius. Inhibition graphs were prepared using Graphpad Prism and Microsoft Excel. Figures were assembled with Adobe Illustrator.
**Fig. S1** *In vitro* study on the synergy of Argyrin B and fusidic acid. (A-F) Effect of the titration of each compound alone and in presence of a test synergy partner compound on the *in vitro* translation for (A, E) ArgB, (B) FA, (C) VrgM1, (D) VrgS1 and (F) Ery. Each histogram represents the residual luminescence from the translation of the fluc reporter gene, where the absolute signal in absence of the test compound is considered 100%; the error bars represent the standard deviation from the mean for at least three independent experiments. (G) Comparison of the calculated IC₅₀ of the combinations of compounds tested (A-F). The combinations VrgM1-VrgS1 are considered as positive control for synergy, while the combinations ArgB-Ery are considered as negative control, as explained in the Methods. Synergy is evident in VrgM1-VrgS1 combinations from the significant shifts in the IC₅₀ values of the compounds alone and the compounds combined; this shift is not significant in ArgB-FA combinations, whose IC₅₀ values have overlapping error bars, as it is for the negative control ArgB-Ery combinations, meaning that no synergy is observed between ArgB and FA. (H) Fold changes of the IC₅₀ values of each combination, calculated as the ratio between the IC₅₀ of the compound alone and the IC₅₀ of the compound in presence of the partner. Numerical values are shown on top of each histogram depicting them. The fold change is significantly greater than 1 only for both VrgM1-VrgS1 combinations.
Fig. S2. Cryo-EM Processing of the 70S-EF-G-ArgB complex. (A) From 610,946 2D classified particles, 557,434 were used for initial reconstruction. Subsequently, a focus mask of EF-G was applied for sorting into four classes. Class I-III contained EF-G, whereas Class IV (19.85%) was an empty 70S. Following undecimated refinement were classes containing EF-G subjected to two rounds of CTF-refinement and Bayesian polishing. Multibody refinement of class I using masks for 50S-EF-G, 30S-body/platform, and 30S-head significantly improved final resolution. (B and C) FSC curves of (B) refined classes I-IV: EF-G containing classes CTF-refined and subjected to Bayesian polishing (blue curves), and (C) multibody refined bodies of class I (green curves). (D-F) Local resolution of locally filtered multibody refined maps in (D) overview and (E) transverse section, and (F) of EF-G from Multibody refined class IV, viewed from the platform (top) and the ribosome interacting side (bottom).
**Fig. S3. G-domain conformation of EF-G-ArgB on the ribosome.** (A) Cryo-EM density (surface, coloured) with fitted model of EF-G as seen from the large subunit GTPase activating centre and Sarcin-ricin loop (SRL; top) and zoom-in of the GTP-binding pocket, with nucleotide cryo-EM density shown in mesh (bottom). (B and C) Cryo-EM density (mesh, coloured) and molecular model for (B) the EF-G-ArgB-70S complex, coloured as in (A), or (C) ribosome-bound *E. coli* EF-G (PDB-ID 4V9O, grey) in the presence of non-hydrolysable GTP analogue GDPCP (31).
**Fig. S4.** Argyrin B stalls translating ribosomes in a state highly similar to that stalled by the unrelated antibiotic fusidic acid. (A) Population FRET histogram showing the time-evolution of the FRET signal between donor-labeled uS13 and acceptor-labeled uL1 at 400 ms time-resolution after delivery of EF-G to the microscope flow cell performed in the absence of drugs (left), or the presence of 10 µM ArgB (middle) or 400 µM fusidic acid (FA; right). N represents the number of observed molecules. The dashed black lines indicate the position of the pre-translocation (PRE), intermediate (INT), drug-bound EF-G post-translocation state (POST-EF-G-Drug) and drug-free EF-G post-translocation state (POST). (B) Population FRET histogram showing the time-evolution of the FRET signal at 40 ms time-resolution after delivery of EF-G to the microscope flow cell performed with 10 µM ArgB and 10 µM EF-G (left) or 10 µM ArgB and 50 µM EF-G (right). N represents the number of observed molecules.
Fig. S5. Cryo-EM Processing of the EF-G-ArgB-INT2 complex. (A) Data processing for the EF-G-ArgB-INT2 complex structure was conducted in RELION 4.0 (26). Particles from two datasets were refined separately for initial reconstructions. Subsequently, a focus ligand mask containing EF-G, P-site tRNA, and E-site tRNA was applied for sorting each reconstruction into ten classes. Class X of dataset 1 and Class VIII of dataset 2 contained EF-G. Classes containing EF-G were combined, refined, and subjected to another round of classification with a ligand mask into five classes. The EF-G-containing class was refined and subjected to a final round of unmasked classification into eight classes. Class II containing two tRNAs and EF-G was refined (undecimated) followed by CTF-refinement, Bayesian polishing, and Post Processing. (B) FSC curve of the final EF-G containing class CTF-refined and subjected to Bayesian polishing. (C and D) Local resolution of refined map viewing (C) overview and (D) transverse section. Threshold $\sigma = 3$, central protuberance (CP). (E) Local resolution of EF-G in refined map, domains I-V (DI-V), threshold $\sigma = 4$. 
Fig. S6. Comparison of free and ribosome-bound ArgB-EF-G structures. (A) Alignment of ArgB molecule in the EF-G-ArgB-INT2 complex (coloured) with ArgB bound to *P. aeruginosa* EF-G (PDB-ID 4FN5, grey, top) (32). Superimposition of the binding site of ArgB and the interaction with domains III and V, which is conserved in both *P. aeruginosa* and *E. coli* EF-G (bottom). Labelled with *E. coli* numbering. (B) Global alignment of EF-G from the EF-G-ArgB-INT2 complex (coloured) and from the EF-G-ArgB-70S complex (grey, top). Superimposition of the binding site of ArgB (bottom).
Fig. S7. Location of mutations of EF-G conferring resistance to Argyrin B. (A) Table of identified EF-G mutations and level of ArgB resistance in wild-type (WT) or hypersensitive *P. aeruginosa* strains as measured by mean inhibitory concentration (MIC) (32, 33). *E. coli* numbering in parentheses. (B and C) Location of ArgB resistance mutations (yellow) positioned (B) proximal and (C) distal to the ArgB binding site, mapped onto EF-G from the EF-G-ArgB-INT2 complex, labelled with *E. coli* numbering.
Fig. S8. Model of argyrins A-D within the EF-G binding site. (A) Binding site of ArgB (purple) between domains III (pink) and V (orange) of the EF-G-ArgB-INT2 complex. (B-D) Molecular models for (B) ArgA (light blue), (C) ArgC (green) and (D) ArgD (light purple) generated based on ArgB from (A) by removal or addition of R₁-R₃ moieties as appropriate.
Fig. S9. Ribosome conformation in the translocation intermediate stalled by Argyrin B. (A and B) Overview of small subunit (30S) body rotation (white arrow, left and middle) and head swivel (purple arrow, right) of the EF-G-ArgB-INT2 structure as compared to EF-G-FA-INT2 and POST structures (PDB IDs 7N2C and 7N31, respectively) (14) when aligned to (A) the large (50S) subunit core and (B) the 30S body. (C) Approximate distance between the C-terminus of uS13 (green sphere) and residue 202 of uL1 (red sphere) in the EF-G-ArgB-INT2 (light coloured) and EF-G-FA-INT2 (dark coloured, PDB-ID 7N2C) structures.
Fig. S10. G-domain conformation of EF-G(ArgB) on the ribosome. (A) Global alignment of EF-G from the EF-G-ArgB-INT2 complex (coloured by domain, solid) to EF-G from the EF-G-ArgB-70S complex (transparent, left) as viewed from the large subunit GTPase activating centre and Sarcin-ricin loop (SRL, grey). EF-G overlay from panel (A), highlighting the nucleotide binding pocket, with cryo-EM density from the EF-G-ArgB-INT2 complex shown in mesh representation (right), threshold σ = 3.5. (B) Nucleotide binding pocket of the EF-G-ArgB-INT2 complex showing molecular model and cryo-EM density at different σ threshold levels. (C) G-domain alignment of EF-G from the EF-G-ArgB-INT2 complex to EF-G from an early translocation intermediate structure visualized with a structured switch-I element before P_i release (PDB-ID 7N2V, grey, transparent) (14) as viewed from the large subunit GTPase activating centre and SRL. (D) Cryo-EM density and molecular model of EF-G from the EF-G-ArgB-INT2 complex at low contour (σ = 2) showing the absence of cryo-EM density. Panels B-D coloured as in (A).
Fig. S11. Comparison of ArgB binding site on EF-G with Sordarin binding site on eEF2. Global alignment (top) and of EF-G from the EF-G-ArgB-INT2 complex (coloured by domain) to S. cerevisiae eEF2 (grey) bound to Sordarin (yellow) and zoom-in of drug binding site between domains III and V of EF-G/eEF2.
### Table S1. Cryo-EM data collection, refinement and validation statistics

| Data collection and processing | EF-G-ArgB-Eco70S | EF-G-ArgB-Eco70S-INT2 |
|-------------------------------|------------------|------------------------|
| Magnification                | 129,151          | 130,000                |
| Voltage (kV)                 | 300              | 300                    |
| Electron exposure (e⁻/Å²)    | 28               | 70                     |
| Defocus range (µm)           | -0.5 to -3.5     | -0.5 to -2.0           |
| Pixel size (Å)               | 1.084            | 0.6485                 |
| Final pixel size (Å)         | 1.084            | 1.054                  |
| Initial particle images (no.)| 610,946          | 2,851,902              |
| Final particle images (no.)  | 153,360          | 35,220                 |
| Map resolution (Å)           | 2.9              | 2.6                    |
| FSC threshold                | 0.143            | 0.143                  |
| Map resolution range (Å)     | 2.6-7            | 2.3-7                  |

| Refinement                    | EF-G-ArgB-Eco70S | EF-G-ArgB-Eco70S-INT2 |
|-------------------------------|------------------|------------------------|
| Initial model used (PDB code) | 7N2C             | 7N2C                   |
| Model resolution (Å)          | 2.9              | 2.6                    |
| FSC threshold                 | 0.143            | 0.143                  |
| Map sharpening B factor (Å²)  | -58.06, -71.48, -77.46 | -30                   |
| Model composition             |                  |                        |
| Non-hydrogen atoms            | 150,861          | 154,476                |
| Protein residues              | 6,521            | 6,781                  |
| Ligands                       | 306              | 403                    |
| B factors (Å²)                |                  |                        |
| Protein                       | 33.09            | 75.22                  |
| Ligand                        | 79.59            | 38.15                  |
| R.m.s. deviations             |                  |                        |
| Bond lengths (Å)              | 0.004            | 0.008                  |
| Bond angles (°)               | 0.682            | 1.096                  |
| Validation                    |                  |                        |
| MolProbity score              | 1.74             | 1.58                   |
| Clashscore                    | 9.16             | 3.51                   |
| Poor rotamers (%)             | 0.04             | 2.08                   |
| Ramachandran plot             |                  |                        |
| Favored (%)                   | 96.26            | 96.85                  |
| Allowed (%)                   | 3.63             | 3.08                   |
| Disallowed (%)                | 0.11             | 0.08                   |
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