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Dityromycin and GE82832 bind protein S12 and block EF-G catalyzed translocation

David Bulkley[1,*], **Letizia Brandi**[2,*], **Yury S. Polikanov**[3,4], **Attilio Fabbretti**[2], **Michael O'Connor**[5], **Claudio O. Gualerzi**[2,a], and **Thomas A. Steitz**[1,3,4,a]

[1]Department of Chemistry, Yale University, New Haven, CT 06511, USA

[2]Laboratory of Genetics, Department of Biosciences and Biotechnology, University of Camerino, 6032 (MC), Italy

[3]Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06511, USA

[4]Howard Hughes Medical Institute, New Haven, CT 06511, USA

[5]School of Biological Sciences, University of Missouri-Kansas City, Kansas City, MO 64110, USA

Summary

The translocation of messenger RNA and transfer RNA through the ribosome is catalyzed by EF-G, a universally conserved GTPase. The mechanism by which the closely related decapeptide antibiotics dityromycin and GE82832 inhibit EF-G-catalyzed translocation is elucidated in this study. Using crystallographic and biochemical experiments we demonstrate that these antibiotics bind to ribosomal protein S12 in solution as well as within the small ribosomal subunit, inducing long-range effects on the ribosomal head. The crystal structure of the antibiotic in complex with the 70S ribosome reveals that the binding involves conserved amino acid residues of S12 whose mutations result in *in vitro* and *in vivo* antibiotic resistance and loss of antibiotic binding. The data also suggest that GE82832/dityromycin inhibits EF-G-catalyzed translocation by disrupting a critical contact between EF-G and S12 that is required to stabilize the post-translocational conformation of EF-G, thereby preventing the ribosome-EF-G complex from entering a conformation productive for translocation.

Introduction

The movement of tRNA through the ribosome is catalyzed in bacteria by elongation factor G (EF-G), a universally conserved GTPase that accelerates the translocation of tRNA from the ribosomal A-site to the P-site (Katunin et al., 2002; Rodnina et al., 1997). Although spontaneous translocation occurs, its rate in the absence of EF-G is orders of magnitude slower – too slow to meet the needs of a living cell (Asatryan and Spirin, 1975; Katunin et

[a]To whom correspondence should be addressed: claudio.gualerzi@unicam.it and thomas.steitz@yale.edu.
[*]These authors contributed equally to this work

Accession Numbers

Coordinates for the complex of dityromycin with the 70S ribosome have been deposited in the PDB under accession codes 4NVU, 4NVV, 4NVW, 4NVX (dityromycin) and 4NVY, 4NVZ, 4NW0, 4NW1 (GE82832).

al., 2002; Peske et al., 2000; Southworth et al., 2002). Several small molecule inhibitors of translocation have been identified (Peske et al., 2004; Walter et al., 2012), providing insight into the workings of EF-G and trapping EF-G-bound conformations of the ribosome for use in structural studies (Gao et al., 2009a; Munro et al., 2010; Ogle et al., 2002; Stark et al., 2000). In addition, inhibitors of translocation can be used as antibiotics, provided they do not also interfere with protein synthesis in higher organisms (Stanley et al., 2010).

Dityromycin is a cyclic decapeptide antibiotic containing several modified amino acids. It was originally discovered in the 1970s (Omura et al., 1977) and structurally characterized a decade later (Teshima et al., 1988) (Fig. 1A). The closely related antibiotic GE82832 was identified as a translation inhibitor by high throughput screening of a library of natural products (Brandi et al., 2006, 2012). Despite the fact that GE82832 and dityromycin are secondary metabolites of different microorganisms, recent studies have shown that both antibiotics target the 30S ribosomal subunit, inhibit translocation and display the same microbiological and functional properties; in addition, their chemical structures are identical, except for a difference of two mass units (Brandi et al., 2012).

Biochemical studies have shown that GE82832/dityromycin blocks the EF-G-catalyzed movement of peptidyl-tRNA and mRNA from the ribosomal A-site to the P-site without preventing the ribosomal binding of the elongation factor, which remains able to stimulate GTP hydrolysis on the ribosome and to dissociate the cleaved γPi, albeit at a somewhat slower rate than in the absence of GE82832/dityromycin (Brandi et al., 2006, 2012).

While GE82832/dityromycin arrests the translocation of tRNA through the ribosome, the compound has almost no effect on the accommodation of tRNA into either the P- or A-site, either in the context of the 30S subunit or the full 70S ribosome (Brandi et al., 2006). Additional experiments showed that pre-incubation of 30S subunits with GE82832/dityromycin inactivates protein synthesis while the same treatment of 50S subunits has little effect, thereby suggesting that GE82832/dityromycin interacts almost exclusively with the 30S subunit. Furthermore, experiments of chemical and hydroxyl radical cleavage protection of 16S rRNA suggest that GE82832/dityromycin modulates the reactivity/accessibility of nucleotides located in the head of the small subunit, raising the possibility that the compound may alter the conformational dynamics of the ribosome (Brandi et al., 2006, 2012). However, despite these data and the suggestion that GE82832/dityromycin might interfere with intersubunit rotation and ratcheting (Brandi et al., 2006), the exact mechanism whereby this antibiotic inhibits translocation remains unknown.

Thus, to better understand the mechanism of action of GE82832/dityromycin, we determined the structure of its complex with the bacterial ribosome from *Thermus thermophilus*. We find that GE82832/dityromycin interacts with a region of the ribosome that has not been previously identified as an antibiotic binding site (Wilson, 2009), but has been shown to be functionally important in controlling the translocation of tRNA through the ribosome (Cukras et al., 2003; Mcmahont and Landau, 1982; Vila-Sanjurjo et al., 2007). Based on this observation, we suggest that the antibiotic acts through a previously-unknown mechanism and further outline a new region of the ribosome that can be the target of future structure-based antibiotic design efforts.

Results

After demonstrating that GE82832 is active in inhibiting translation in a *T. thermophilus* system at both 50° and 70° C (Fig. S1), the crystal structures of GE82832 and dityromycin in complex with the bacterial ribosome from *T. thermophilus* were determined and statistics for the data are shown in Table 1. The structures were solved by molecular replacement using an existing model of the 70S ribosome (Polikanov et al., 2012) in which P-site tRNA and mRNA were bound, but no other ligands were present. Coordinates for the antibiotics were withheld during refinement in PHENIX (Adams et al., 2010), and minimally biased F_{obs}-F_{calc} difference Fourier electron density maps were used to localize the antibiotics with respect to the ribosome. An atomic model of dityromycin was generated from the chemical structure of the antibiotic (Teshima et al., 1988) using the PRODRG server (Schüttelkopf and Van Aalten, 2004) and restraints based on the idealized three-dimensional geometry of dityromycin were used to refine dityromycin into our electron density maps. The final model for dityromycin, along with difference Fourier maps for GE82832 and dityromycin are shown in Figure 1.

GE82832/dityromycin interacts with ribosomal protein S12

GE82832/dityromycin binds to the shoulder of the 30S subunit and interacts exclusively with ribosomal protein S12 on the small subunit (Fig. 2A, B). No direct interaction between dityromycin and the ribosomal RNA is observed; the antibiotic is 5.5Å away from the phosphate of residue G361 and 7Å away from the phosphate of G362 (16S rRNA, *E. coli* numbering). On the ribosome, GE82832/dityromycin contacts histidine 76 of S12, probably forming a hydrogen bond between the delta nitrogen of His76 and the hydroxyl group of the epoxy hydroxy dehydroisoleucine residue of GE82832/dityromycin (Fig. 2C, D). The phenyl glycine residue of GE82832/dityromycin also appears to stack against the aromatic surface of His76, further stabilizing this contact. The second major contact is formed by the cyclic di-tyrosine moiety of GE82832/dityromycin, which stacks against the guanidinium group of arginine 30 and is supported by hydrophobic interactions with the side chains of valine 32 and valine 78 of S12 (Fig. 2E). The N,N-dimethyl valine residue of GE82832/dityromycin also comes within 3.5 Å of Arg55 of S12 (Fig 2D) The interaction of the antibiotic with His76 and Val78 is confirmed by the finding that *E. coli* ribosomes bearing mutations of residues His76 (R,G or K) or Val78D of protein S12 are resistant to inhibition of *in vitro* mRNA translation by GE82832 (Fig. 3 A). Furthermore, the same mutations confer *in vivo* resistance to GE82832, whereas both wild type *E. coli* MRE600, as well as a standard laboratory strain, MC4100, which carries an S12 substitution (K42R) conferring streptomycin resistance (Casadaban, 1976) (see Experimental Procedures) are sensitive to the antibiotic (Fig. 3 B, middle plate). However, the same S12 GE82832-resistant mutants display a streptomycin-sensitive phenotype, unlike *E. coli* MC4100 (Fig. 3 B, lower plate).

It has long been known that decoding fidelity is influenced by ribosomal protein S12 (Gorini, 1971; Strigini and Brickman,1973); thus, the evidence that GE82832/dityromycin binds to this protein prompted us to investigate a possible effect of this antibiotic on the accuracy of mRNA translation. To do so, the level of translation measured from the incorporation of a radioactive precursor in the acid-insoluble product was compared to the

level of correctly synthesized protein, as immunologically quantified by ELISA (Fabbretti et al., 2012). By contrast to what occurs in the presence of streptomycin, both tests show the same extent of inhibition in the presence of GE82832/dityromycin, indicating that this antibiotic does not induce codon misreading (Fig. S2).

The interactions observed between GE82832/dityromycin and S12 suggest that the thermodynamic stability of the protein-drug complex could be quite high. In light of this, the possibility that the antibiotic might establish an interaction with the isolated ribosomal protein was also investigated. The results obtained in this experiment clearly show that, unlike two control proteins (serum albumin and initiation factor IF3), GE82832/dityromycin co-purifies with protein S12, indicating that this isolated ribosomal protein can indeed establish a stable interaction with the antibiotic (Fig. 3C).

Previous chemical (Brandi et al., 2006) and hydroxyl radical (Brandi et al., 2012) probing experiments had shown that GE82832/dityromycin affects the accessibility of 16S rRNA bases located in the head of the 30S subunit, near protein S13, but no effect was detected near S12. Whereas the latter result would be fully compatible with the crystallographic data showing the lack of contacts between the antibiotic and 16S rRNA, whose closest phosphate group is >5 Å away (see above), the altered RNA accessibility near S13 deserved renewed attention, also in light of the role played by this protein and by the 30S head rotation in translocation (Cukras et al., 2003; Pulk and Cate, 2013; Tourigny et al., 2013; Zhou et al., 2013). Therefore, a possible role of protein S13 in the mechanism of translation inhibition by this antibiotic was investigated using ribosomes from cells bearing an *rpsM* (the gene encoding S13) null mutation (Cukras and Green, 2005). However, the *in vitro* mRNA translation test carried out with these ribosomes demonstrates that the lack of S13 does not alter their sensitivity to GE82832/dityromycin inhibition (Fig. 3A blue tracing).

Finally, *in situ* probing of the rRNA was analyzed (Fig. 4A, B) by comparing the effects of GE82832/dityromycin on the accessibility of 16S rRNA to hydroxyl radical cleavage in wt 30S subunits with its accessibility in mutated 30S subunits containing an H76 substitution that made them GE82832/dityromycin-resistant. As seen in Figure 4 A, the altered accessibility to hydroxyl radical cleavage of 16S rRNA bases in the 30S subunit head near S13 was fully confirmed in wt 30S subunits, with a clear enhancement of exposure for bases A1329-2330, A1332-1339, 1344 and C1359 and a slight protection of G1331; however, no comparable effects could be detected in the 30S subunits bearing the S12 H76 mutation. Remarkably, all the bases whose accessibilities are affected by the antibiotic are very far from S12 (Fig.4 B). Concerning the S12- proximal region of the 30S subunit, it can be seen that the antibiotic has no effect on the accessibility of bases in this region, aside from a slight increased exposure of bases 362–363 (Fig. 4 B).

Taken together, these findings could be explained either by the existence of multiple antibiotic binding sites on the 30S subunit and/or by long-range conformational changes of the subunit induced by GE82832/dityromycin binding. However, the crystallographic data are consistent with a single ribosomal binding site for the antibiotic; furthermore, although our “pull down” experiments do not allow us to rule out the existence of additional, low affinity antibiotic binding site(s) (Fig. 4D), the nature of most of the effects (increased

exposure) observed near S13 in wt 30S and the fact that no alterations of the cleavage patterns are seen with the 30S subunit bearing the H76 mutation in S12 give a clear indication that GE82832 causes a long range conformational change in the head of the subunit. The fact that no conformational rearrangements resulting from GE82832/dityromycin binding were detectable in the crystal structure can be explained by the fact that the crystal packing has probably stabilized a single conformation of the subunit.

Discussion

Protein S12 plays a critical role in bacterial translation (Cukras et al., 2003; Gregory et al., 2009; Mcmahont and Landau, 1982; Sharma et al., 2007; Vila-Sanjurjo et al., 2007; Yates, 1979; Zengel et al., 1977). Protein S12 is positioned on the shoulder of the 30S subunit, where it reaches into the decoding center and acts as a control element in tRNA selection (Yates, 1979) and the translocation of tRNA-mRNA through the ribosome (Cukras et al., 2003). S12 has also been shown to influence the inhibition of translation by several antibiotics, including streptomycin (Vila-Sanjurjo et al., 2007) and paromomycin (Sharma et al., 2007). The structural and biochemical data presented here show that S12 is the target of GE82832/dityromycin inhibition through an interaction which is distinct from that of any other ribosomal inhibitor and also pivotal in a GE82832/dityromycin-mediated conformational change of the head of the 30S subunit that might be related to the mechanism of translation inhibition.

The long distance S12-mediated effects of GE82832 on the accessibility of 16S rRNA bases of the 30S subunit head underlie the well-established dynamics of this region of the subunit (Pulk and Cate, 2013; Tourigny et al., 2013; Wang et al., 2012; Zhou et al., 2013), as well as the ability of protein S12 to act as a conformational relay for the subunit (Gregory et al., 2009; Vila-Sanjurjo et al., 2007; Zengel et al., 1977) and the functional interrelationship between S12 and S13 (Cukras et al., 2003). However, it is not possible to conclude whether these effects, which do not occur in the GE82832-resistant 30S subunits, have any causal relationship with the mechanism of translocation inhibition.

EF-G-catalyzed translocation has been shown to involve a number of conformational rearrangements of EF-G, the ribosome, and ribosome-bound tRNA-mRNA (Ermolenko and Noller, 2011; Frank et al., 2007; Munro et al., 2010; Peske et al., 2000, 2004; Ratje et al., 2010; Savelsbergh et al., 2003). In addition to biochemical evidence indicating that interactions between S12 and EF-G control translocation (Cukras et al., 2003), crystal structures of EF-G bound to the ribosome in post-translocational and ratcheted pre-translocational conformations show that EF-G interacts extensively with S12 (Feng et al., 2013; Gao et al., 2009b; Pulk and Cate, 2013; Tourigny et al., 2013; Zhou et al., 2013). Critically, domain 3 of EF-G binds to S12 in the region of His 76 (*E. coli*), and comparison of the ribosome bound to EF-G versus GE82832/dityromycin shows that both molecules interact with a similar region of S12 (Fig. 5). Thus, our structure indicates that the binding of GE82832/dityromycin sterically occludes EF-G from entering a post-translocational conformation on the ribosome by blocking the interaction between domain 3 of EF-G and protein S12 on the small ribosomal subunit.

While our crystal structure indicates that GE82832/dityromycin blocks the binding of EF-G in the post-translocational conformation, EF-G can still catalyze the hydrolysis of GTP on the ribosome, though the kinetics of phosphate release are altered (Brandi et al., 2012). Because GTP hydrolysis and phosphate release occur in steps separate from the engagement of domain 4 of EF-G with the A-site (Munro et al., 2010), the possibility that GTP hydrolysis and translocation are decoupled by GE82832/dityromycin is not unreasonable; viomycin and spectinomycin also inhibit translocation, but do not block GTP hydrolysis (Pan et al., 2007), while mutations in EF-G have also been shown to decouple translocation and GTP hydrolysis (Savelsbergh et al., 2000).

Both biochemical and structural experiments have established that S12 plays a critical role in translocation (Cukras et al., 2003; Gao et al., 2009a; Pulk and Cate, 2013; Tourigny et al., 2013; Zhou et al., 2013). Entering the post-translocational state in the presence of EF-G requires that domain 3 of EF-G engages S12. S12 also acts as a control element in translocation; removal of S12 along with proteins S11 or S13 stimulates factor-independent translocation, while removal of S12 and S5, S7, S8 or S14 blocks factor-catalyzed translocation (Cukras et al., 2003). Interestingly, removal of protein S13 does not reduce the sensitivity of *E. coli* ribosomes to GE82832/dityromycin (Fig. 3A). Overall, the results indicate that GE82832/dityromycin may inhibit translation by preventing the complex between EF-G and the ribosome from adopting a conformation that is productive for translocation. This premise is consistent with the finding that even an excess of EF-G does not relieve the inhibition (Fig. S3), a result which is consistent with GE82832/dityromycin affecting the position of EF-G on the ribosome and not its binding.

While the structure of GE82832/dityromycin in complex with the bacterial ribosome explains its activity as a translocation inhibitor, our structure would also be consistent with GE82832/dityromycin affecting the ability of EF-Tu to deliver aminoacyl-tRNA to the ribosomal A-site. The mutation of several residues of protein S12 that are distant from the decoding center have been shown to increase miscoding errors; two of these, Thr57 and Val78 (*E. coli*) (Agarwal et al., 2011), form part of the binding pocket for GE82832/dityromycin. In addition, His76 (*E. coli*), the same residue that we show to be critical for GE82832/dityromycin binding (Fig. 3 and 4), is involved in signaling EF-Tu when codon recognition has taken place (Gregory et al., 2009). However, only at high concentrations (~10 μM) does GE82832/dityromycin inhibit (~50%) the delivery of tRNA to the A-site in the absence of EF-Tu, whereas at the same concentration it has virtually no effect when EF-Tu is present (Brandi et al., 2006). While this is probably due to the fact that aa-tRNA and EF-Tu outcompete the antibiotic from its binding site, it should be noted that overall protein synthesis and translocation are inhibited at the same rate by GE82832/dityromycin (Brandi et al., 2006).

Unlike EF-G, the affinity of the ternary complex of EF-Tu with tRNA and GTP for the ribosome depends heavily on the interaction between the anticodon of the EF-TU-bound tRNA and the codon of the mRNA: the correct pairing of tRNA and mRNA in the decoding center can increase the affinity of the ternary complex for the ribosome by as much as 1000-fold as compared to a single mismatch (Gromadski and Rodnina, 2004; Pape et al., 1999; Thompson et al., 1986) and the equilibrium dissociation constant of the correct EF-Tu/

tRNA/GTP ternary complex for the ribosome is on the order of 1 nM (Harrington et al., 1993; Louie and Jurnak, 1985). In contrast, the binding of domain IV of EF-G in the decoding center does not appear to be as critical to the overall affinity of the factor for the ribosome, as evidenced by the fact that a conformationally-restrictied EF-G which is unable to translocate tRNA (and therefore likely does not bind with domain IV in the A-site) has only a slightly reduced affinity for the ribosome and remains capable of catalyzing GTP hydrolysis on the ribosome (Peske et al., 2000). Therefore, we speculate that the affinity of the EF-Tu/tRNA/GTP ternary complex for the decoding center is sufficient to outcompete GE82832/dityromycin bound to the ribosome. Since the MIC for GE82832/dityromycin is only in the low μM, it is possible that an antibiotic which bound to the same region of the ribosome with increased affinity would be capable of inhibiting the delivery of aminoacyl-tRNA by EF-Tu.

The binding site of GE82832/dityromycin also overlaps with that of ribosome recycling factor (RRF) (Gao et al., 2007). Because RRF and EF-G work together in recycling, it is unclear whether the effects of GE82832/dityromycin on RRF could be disentangled from its effects on EF-G alone, but a superposition of RRF bound to both the *E. coli* and *T. thermophilus* ribosomes shows that RRF and GE82832/dityromycin share a contact point with S12 (Borovinskaya et al., 2007; Weixlbaumer et al., 2007; Yokoyama et al., 2012). Superposition with the ratcheted *E. coli* ribosome bound to RRF shows that GE82832/dityromycin would sterically clash with domain 2 of RRF, while only a slight clash would exist between GE82832/dityromycin and RRF bound to a classical-state ribosome (Fig. S4).

While questions remain regarding the impact of GE82832/dityromycin on RRF, EF-Tu and the accommodation of tRNA, our structure provides a clear mechanism for how this antibiotic is able to inhibit the EF-G-catalyzed translocation of tRNA. When bound in the post-translocational conformation, domain 3 of EF-G makes extensive contacts with S12 in the region of His 76 (*E. coli*) (Gao et al., 2009b; Pulk and Cate, 2013; Tourigny et al., 2013; Zhou et al., 2013). A comparison of S12 bound to EF-G versus GE82832/dityromycin shows that both molecules engage a similar region of S12 and that both cannot simultaneously bind S12 (Fig. 5). Therefore, the binding of GE82832/dityromycin sterically occludes EF-G from entering a post-translocational conformation on the ribosome.

Experimental Procedures

GE82832 was originally identified, isolated and purified from *Streptosporangium cinnabarinum* strain GE82832 at Biosearch Italia spa (Gerenzano, Italy) within the European Commission project ‘‘Ribosome Inhibitors’’ (contract QLRT-2001-00892EC). Dityromycin, purified from *Streptomyces* sp. strain AM-2504 was a kind gift of Prof. O. Omura (Tokyo, Japan). 70S ribosomes from *T. thermophilus* were purified and crystallized according to previously published protocols (Bulkley et al., 2010). Prior to crystallization, dityromycin or GE82832 was added at a concentration of 100 μM and incubated at 37°C for 5 min. 15 μM mRNA with sequence GGCAAGGAGGUAAAAAUGUUC was then added and further incubated for 5 min at 37° C after which fMet-tRNA^{fMet} was added at a concentration of 8 μM, and ribosomes were further incubated for 5 min at 37°C. Ribosomes were then diluted to a final concentration of 5 μM in buffer composed of 5 mM Hepes pH 7.5, 50 mM KCl, 10

mM NH_4Cl and 10 mM $MgAc_2$ and then equilibrated via sitting drop vapor diffusion against a reservoir solution made up of 2.9% (w/v) PEG 20K, 9% (v/v) MPD, 175 mM L-arginine and 100 mM Tris-HCl pH 7.6. Immediately prior to equilibration, 3 to 4 μL of reservoir solution were added to 3 μL of ribosome-containing solution.

Crystals appeared after approximately 4 days and were harvested after 8 days. Following stabilization by gradually increasing the concentration of 2-methyl-2,4-pentanediol to 40% (v/v), crystals were equilibrated for 12 hours and flash frozen in a nitrogen cryostream at 80 K. Diffraction data were collected at the National Synchrotron Light Source on beamline X-25 using a Pilatus 6M detector. Data reduction was performed with the program XDS (Kabsch, 2010) and an initial solution was generated by molecular replacement with PHASER (McCoy et al., 2007) using the *T. thermophilus* 70S ribosome from which all ligands were removed as a search model (Polikanov et al., 2012). The solution was then refined with the PHENIX package (Adams et al., 2010).

The model for dityromycin was generated using the PRODGR server (Schüttelkopf and Van Aalten, 2004) based on the chemical structure of dityromycin (Teshima et al., 1988). Restraints from PRODRG were originally used, but modified restraints with idealized geometry were generated and used for the final refinement. The antibiotic model was placed into F_o-F_c difference Fourier maps using ribosome models that had been refined in the absence of antibiotic coordinates. Final coordinates for the antibiotic ribosome complexes are deposited in the PDB and the accession codes are listed below.

In vitro translation and miscoding tests were carried out in *E. coli* or *T. thermophilus* systems programmed with 027IF2Cp(A) mRNA as described (Brandi et al., 2007; Fabbretti et al., 2012). *In situ* probing the 16S rRNA by hydroxyl radical cleavage was carried out as described (Fabbretti et al., 2007).

E coli strains carrying mutations in S12 were constructed by recombineering, as previously described (Agarwal et al., 2011). Briefly, chromosomal DNA from a strain carrying a chloramphenicol acetyl transferase (*cat*) cassette integrated upstream of the *rpsL* gene was used as a template in a two-step, extension overlap PCR, with oligonucleotides randomized at codon positions 76 or 78. The amplified fragments carrying the mutated *rpsL* gene and *cat* cassette were electroporated into MC323 (Agarwal et al., 2011), selecting for chloramphenicol resistance. The *rpsL* mutations were identified by sequencing and the desired alleles were then transferred into strain MC41 (F^- Δ(*lac-pro*) *thi*$^-$). The *cat* cassette was removed by transient expression of plasmid-borne Flp recombinase (Datsenko and Wanner, 2000).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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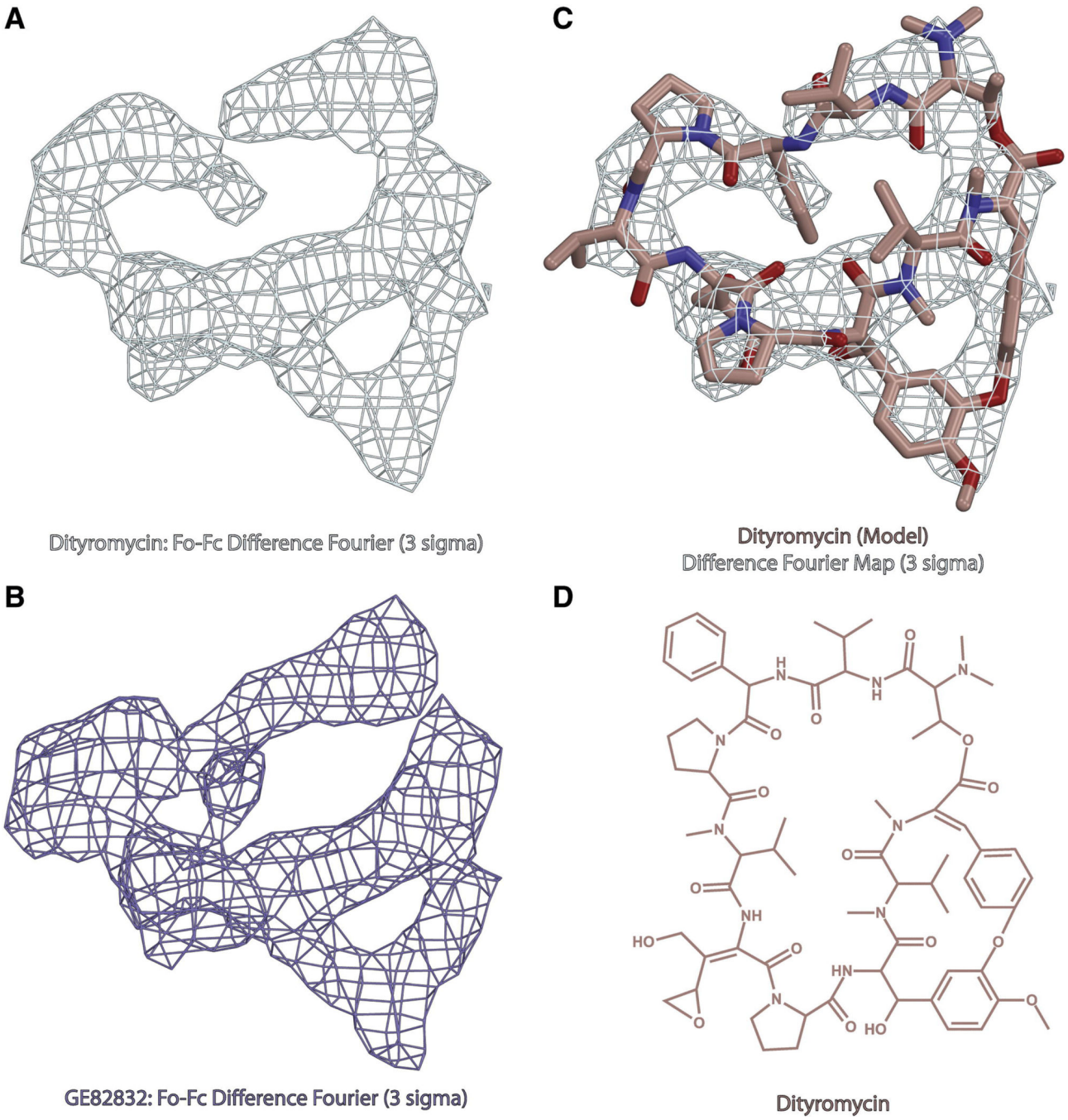

Fig. 1. Structure of dityromycin and comparison with GE82832

(A) Minimally biased F_o-F_c difference Fourier electron density map contoured at 3σ for dityromycin in complex with the bacterial ribosome from *T.thermophilus*. (B) Minimally biased F_o-F_c difference Fourier electron density map contoured at 3σ for GE82832 in complex with the bacterial ribosome from *T.thermophilus*. (C) Model of dityromycin (tan with oxygen colored red and nitrogen blue) docked into the same difference Fourier map shown in panel A. (D) Chemical structure of dityromycin, a secondary metabolite produced by *Streptomyces* strain AM-2504 (13,14). GE82832 is a secondary metabolite produced by

Streptosporangium cinnabarinum (strain GE82832) whose structure is nearly identical to that of dityromycin but for a 2 dalton mass difference likely resulting from an additional point of unsaturation.

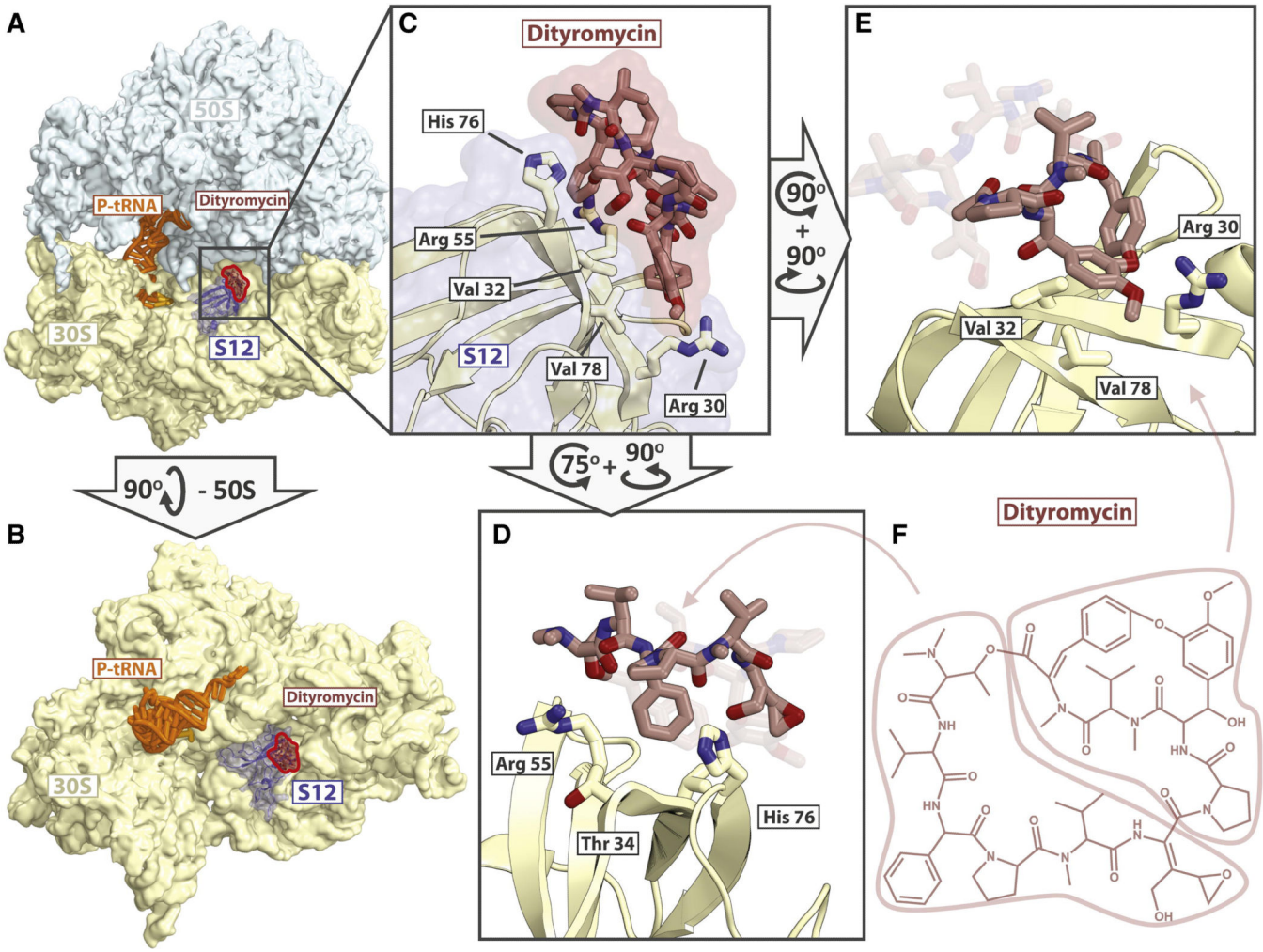

Fig. 2. Binding site of GE82832/dityromycin with the ribosome and their interactions with S12

(A) Overview of the 70S ribosome (50S light blue, 30S yellow) in complex with dityromycin (red highlight) with S12 (blue) shown. (B) Another view of the binding site of GE82832/dityromycin in which the large subunit has been removed for clarity. (C) Close up of dityromycin (tan, red highlight) bound to S12 (white, blue highlight). (D) Another view of dityromycin (tan) interacting with S12 (white), highlighting the interaction between His76 of S12 and the phenyl glycine and epoxy hydroxy dehydroisoleucine residues of dityromycin. Arg55 packs against the N,N-dimethyl valine of dityromycin, and Thr34 comes within 4 Å of the phenyl glycine residue. (E) The di-tyrosine moiety of dityromycin stacks with Arg30 and rests on a hydrophobic surface formed by Val32 and Val78. (F) Chemical structure of dityromycin, indicating the regions of the antibiotic highlighted in figures D and E.

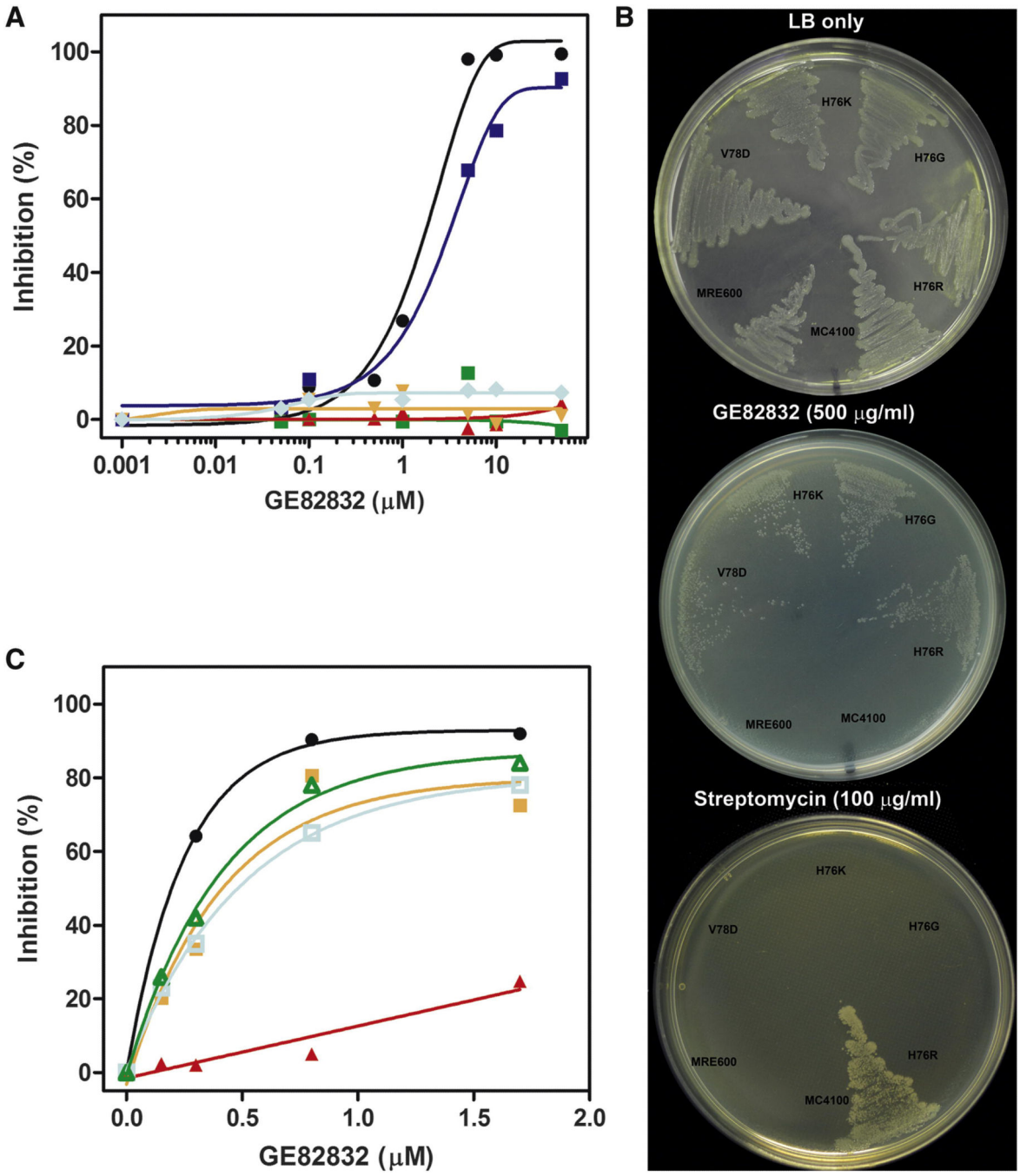

Fig. 3. Involvement of ribosomal protein S12 in GE82832/dityromycin binding

(A) Effect of S12 mutations on the *in vitro* ribosome susceptibility to GE82832/dityromycin. 027IF2Cp(A) mRNA translation was tested in the presence of the concentrations of GE82832 indicated in abscissa in an *E. coli* MRE600 translational system reconstituted with post-ribosomal supernatant and purified wt ribosomes (●) or ribosomes lacking ribosomal protein S13 (■) or carrying the following amino acid substitutions in S12: H76R (▼); H76G (▲); H76K (■); V78D (♦). The % inhibition reported in the ordinate was calculated by comparison with mRNA translation in the absence of antibiotic. (B) Effect of

S12 mutations on the *in vivo* susceptibility to GE82832 and streptomycin; Wild type *E. coli* MRE600, *E. coli* MC4100 (carrying a K42R substitution in S12 conferring streptomycin resistance) and *E. coli* MC41 carrying H76R, H76 G, H76K and Val78D mutations in S12 were plated as indicated in each case on LB-agar plates containing no antibiotics (upper plate), 500 μg/ml GE82832 (middle plate) or 100 μg/ml streptomycin (lower plate). (C) Binding of GE82832 to isolated S12. 7 μM of GE82832 were incubated for 10 min at 20 °C in 100 μl of buffer (10mM Tris-HCl pH7.1; 10 mM MgAcetate; 60mM NH_4Cl; 1mM DTT) without further additions (■) or containing 20 μM of purified ribosomal protein S12 (▲) or 20 μM of initiation factor IF3 (□) or 20 μM of bovine serum albumin (Δ). The samples were the loaded on a Amicon spin filter (MWCO 3 KDa). After 10' of centrifugation at 12K rpm, the eluate was collected and increasing aliquots tested for their capacity to inhibit 027IF2Cp(A) mRNA translation in a standard *E. coli*-based system. A sample of non-centrifuged antibiotic was also tested as a control (●).

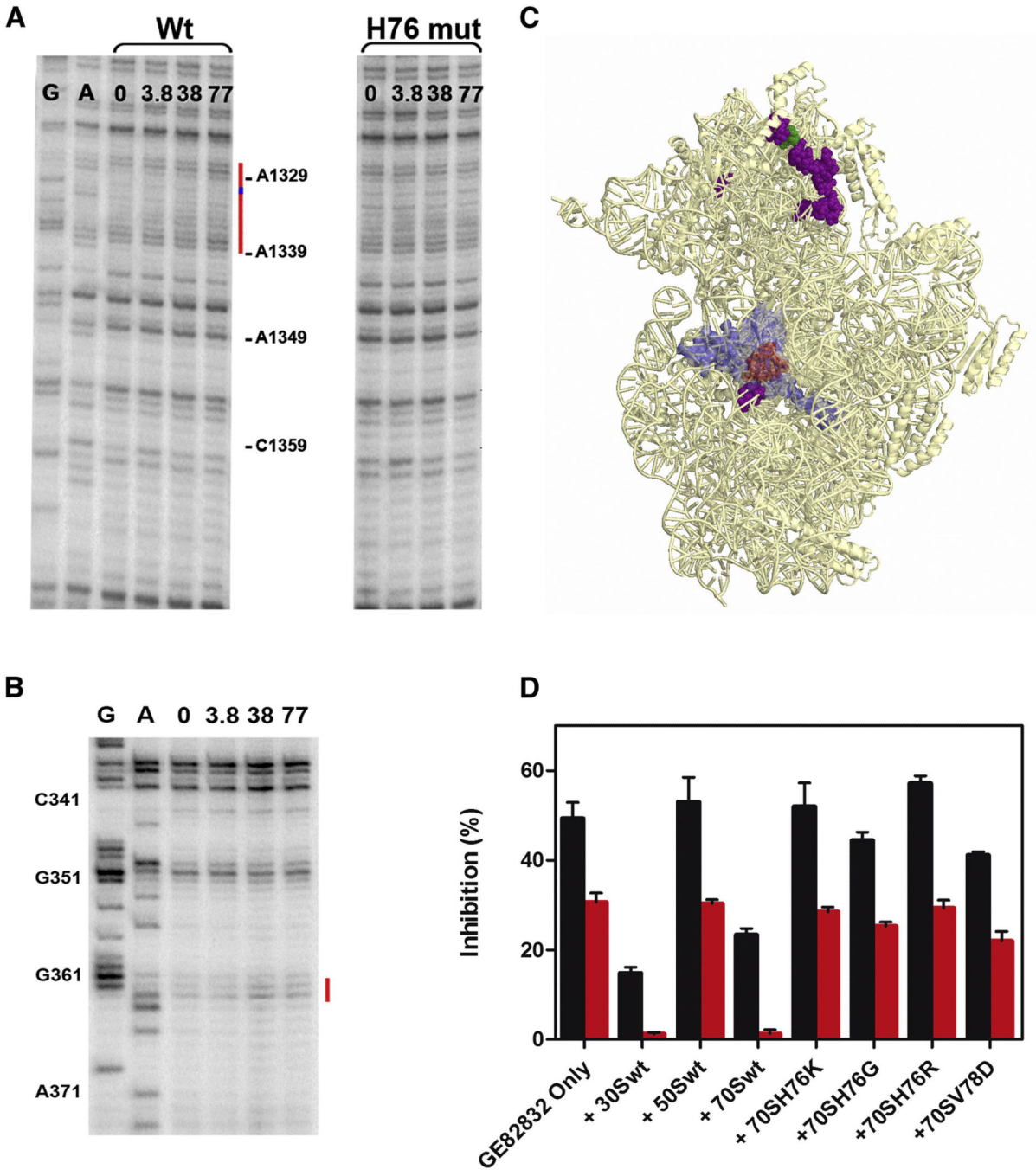

Fig. 4. Long-distance effects of GE82832 on the in situ 16S rRNA cleavage by hydroxyl radicals

Primer extension analysis of the *in situ* cleavage pattern of 16S rRNA (A) in the 1340 region (near S13) in wt 30S (left lanes) and S12 H76K mutant 30S ribosomal subunits (right lanes) and (B) in the 360 region (near S12) in wt 30S subunits in the absence (0) and in the presence of 3.8, 38 and 77 μM GE82832, as indicated above each lane. The lanes marked G and A are sequencing gels. (C) Image of the 30S ribosomal subunit in which the bases whose accessibility to cleavage is increased (purple) or decreased (green) by GE82832 are highlighted; the position of protein S12 (light blue) and of GE82832/dityromycin binding

site (light red) are also indicated. (D) Lack of GE82832 binding by ribosomes carrying His76 and Val78 substitutions. The extent (%) of inhibition of mRNA translation by eluates of Amicon spin filter (MWCO 50 KDa) on which 3 μM (red bars) or 9 μM (black bars) of GE82832 were loaded alone or together with 30 μM of wt 30S, 50S and 70S particles or 70S ribosomes carrying H76K, H76G, H76R and V78D substitutions in S12, as indicated under each histogram bar. The other experimental details are described in the legend of Fig.3.

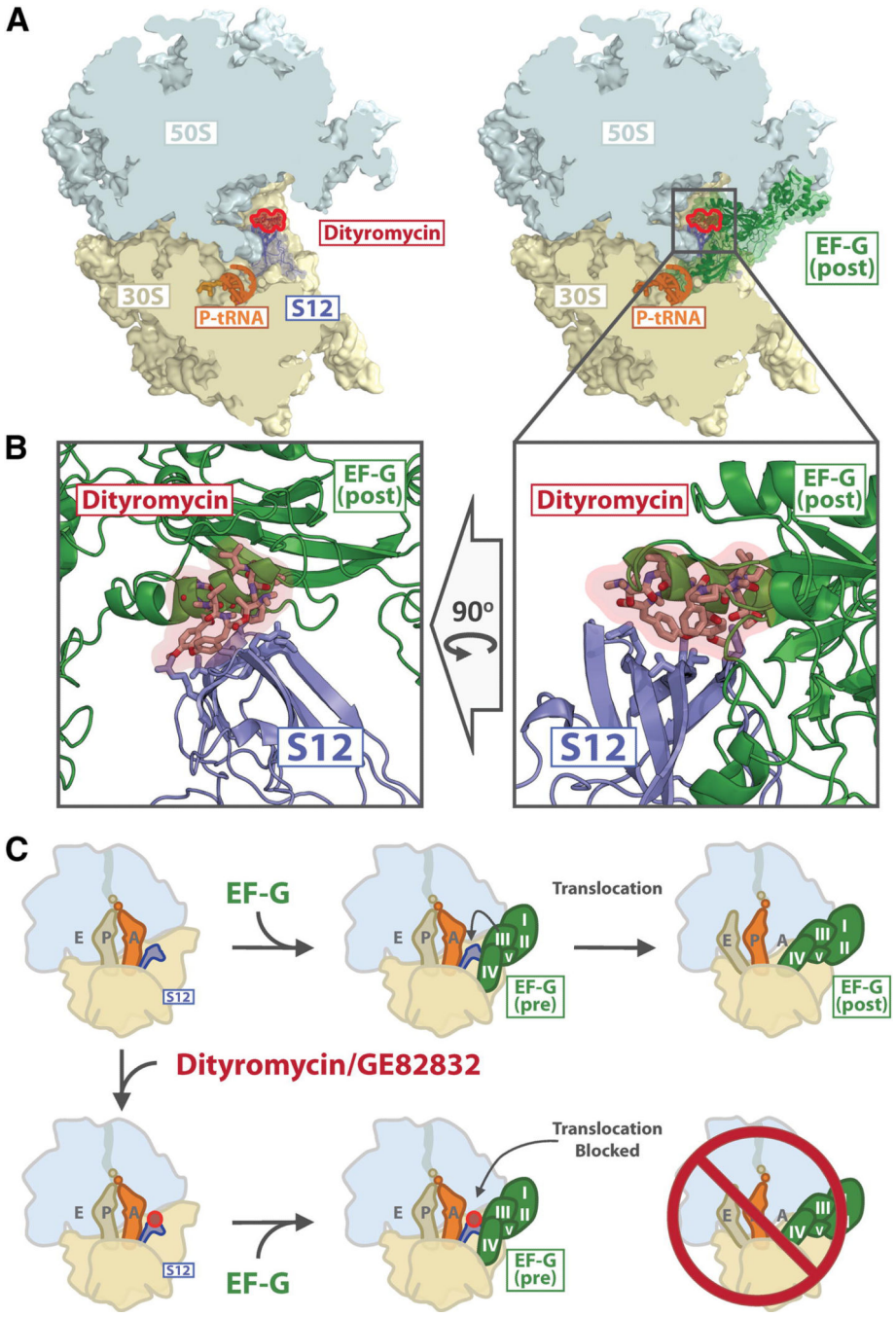

Fig. 5. Dityromycin blocks the transition from pre- to post-translocational conformations of EF-G

(A) The left panel shows a cross section of the ribosome (30S yellow, 50S light blue) with dityromycin (red) and S12 (blue) highlighted. The right panel shows the same view, but with EF-G (green) bound in the post translocational conformation (Gao et al., 2009a), PDB accession code 2WRI. (B) Close-up view of the clash between dityromycin and EF-G bound in the post translocational conformation. (C) Schematic of translocation and its inhibition by dityromycin/GE82832. The 50S (light blue) and 30S (yellow) subunits, deacylated tRNA

(light brown), peptidyl tRNA (light grey) and S12 (blue) are shown, along with EF-G (green with domains I–V indicated) and dityromycin (red).

Table 1

Data collection and refinement statistics

Crystal		70S-tRNA-Dityromycin	70S-tRNA-GE82832
Diffraction Data			
Space Group		$P2_12_12_1$	$P2_12_12_1$
Unit Cell Dimensions, Å (a × b × c)		210.08 × 449.83 × 619.65	209.68 × 450.64 × 622.54
Wavelength, Å		1.100	1.100
Resolution range (outer shell), Å		49.7–3.00 (3.08–3.00)	49.7–3.10 (3.18–3.10)
I/σI (outer shell with I/σI=1)		6.19 (1.00)	5.19 (0.96)
Resolution at which I/σI=2, Å		3.27	3.45
Wilson B-factor, $Å^2$		66.79	70.99
Completeness (outer shell), %		98.8 (99.1)	98.2 (99.2)
R_{merge}, %		19.9 (140.4)	20.5 (121.9)
CC(1/2) at which I/σI=1, %		24.0	27.5
No. of crystals used		1	1
No. of Reflections Used:	Observed	3,948,062	3,118,277
	Unique	1,143,138	1,034,964
Redundancy		3.45	3.01
Refinement			
R_{work}/R_{free}, %		20.3/25.9	20.3/25.9
No. of Non-Hydrogen Atoms			
RNA		190,374	190,375
Protein		91,181	91,173
Ions (Mg, Zn, Fe)		2,003	2,003
Waters		2,520	2,520
Ramachandran Plot			
Favored regions, %		93.43	92.25
Allowed regions, %		5.65	6.54
Outliers, %		0.92	1.21
Deviations from ideal values (RMSD)			
Bond, Å		0.010	0.009
Angle, degrees		1.515	1.466
Chirality		0.062	0.060
Planrity		0.007	0.007
Dihedral, degrees		17.113	16.907
Average B-factor (overall), $Å^2$		62.3	62.6

$R_{merge} = \Sigma |I - \langle I \rangle| / \Sigma I$, where I is the observed intensity and <I> is the average intensity from multiple measurements. $R_{work} = \Sigma |F_{obs} - F_{calc}| / \Sigma F_{obs}$. For recalculation of R_{free}, 5% of the truncated data set was excluded from the refinement.