Structural Basis of the Action of Pulvomycin and GE2270 A on Elongation Factor Tu[†,‡]

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ABSTRACT: Pulvomycin inhibits protein synthesis by preventing the formation of the ternary complex between elongation factor Tu (EF-Tu)·GTP and aa-tRNA. In this work, the crystal structure of *Thermus thermophilus* EF-Tu·pulvomycin in complex with the GTP analogue guanylyl imino diphosphate (GDPNP) at 1.4 Å resolution reveals an antibiotic binding site extending from the domain 1−3 interface to domain 2, overlapping the domain 1−2−3 junction. Pulvomycin binding interferes with the binding of the 3′-aminoacyl group, the acceptor stem, and 5′ end of tRNA. Only part of pulvomycin overlaps the binding site of GE2270 A, a domain 2-bound antibiotic of a structure unrelated to pulvomycin, which also hinders aa-tRNA binding. The structure of the *T. thermophilus* EF-Tu·GDPNP·GE2270 A complex at 1.6 Å resolution shows that GE2270 A interferes with the binding of the 3′-aminoacyl group and part of the acceptor stem of aa-tRNA but not with the 5′ end. Both compounds, pulvomycin more markedly, hinder the correct positioning of domain 1 over domains 2 and 3 that characterizes the active form of EF-Tu, while they affect the domain 1 switch regions that control the EF-Tu·GDP/GTP transitions in different ways. This work reveals how two antibiotics with different structures and binding modes can employ a similar mechanism of action.

Elongation factor Tu (EF-Tu),[1] an essential component of bacterial protein synthesis, is a GTPase that in the active GTP-bound form delivers aa-tRNA to the mRNA-programmed ribosome (for a review, see refs *1* and *2*). Crystallographic analysis has shown a folding of EF-Tu into three distinct domains that in EF-Tu in complex with the GTP-analogue guanylyl imino diphosphate (GDPNP) display a compact organization with extensive interfaces (Figure 1A). In EF-Tu·GDP, the domain organization is less compact and the interfaces are loosened with a central hole now formed between the domains (Figure 1B). In *Thermus thermophilus* (*Tt*) EF-Tu·GDPNP, the nucleotide binding domain 1 includes amino acid residues 1−211 and domains 2 and 3 include residues 212−311 and 312−405, respectively (*3*). EF-Tu is an important target for inhibitors of protein

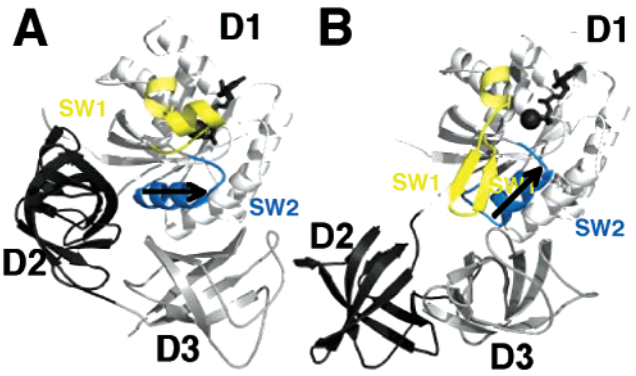

FIGURE 1: Three-dimensional structure of the two basic forms of EF-Tu. The compact GDPNP(GTP)-bound form (A) (PDB entry 1EXM, 1EFT) and the GDP-bound form (B) (PDB entry 1EFC, 1Tui) with reduced interfaces and an opening between the domain 1 (labeled D1) and the domain 2 (D2) and domain 3 (D3) unit. The switch (SW) 1 and 2 regions of EF-Tu·GDPNP (A) and EF-Tu·GDP (B) are depicted in yellow and blue, respectively. The arrow indicates the angles of the switch 2 region in EF-Tu·GDPNP and EF-Tu·GDP.

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[‡] Atomic coordinates and structure factor amplitudes of the EF-Tu·GDPNP·pulvomycin (entry code 2C78) and EF-Tu·GDPNP·GE2270 A (entry code 2C77) complexes have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http:www.rcsb.org/).

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[1] Abbreviations: EF-Tu, elongation factor Tu; EF-Ts, elongation factor Ts; D1, D2, and D3, EF-Tu domains 1, 2, and 3, respectively; mc, main chain; *Ec*, *Escherichia coli*; *Tt*, *Thermus thermophilus*; *Ta*, *Thermus aquaticus*, pulvo, pulvomycin; GEA, GE2270 A; GDPNP or GNP, guanylyl imino diphosphate; PEP, phosphoenolpyruvate; PK, pyruvate kinase; DTT, dithiothreitol; PEG, poly(ethylene glycol).

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synthesis. In fact, many antibiotics interfere with the multistep process of protein synthesis not only by binding to the ribosome but also by binding to translation factors (*4*). Four structurally unrelated families of antibiotics, comprising analogues of about 30 members, act on EF-Tu. Thr prototype members are kirromycin, enacyloxin IIa, pulvomycin, and GE2270 A (for a review, see refs *1* and *2*). These biological compounds affect the regulation of the EF-Tu conformation and/or inhibit its interaction with ligands. They have played a fundamental role in defining EF-Tu functions and encoding genes and ligands. Functionally, they can be divided in two groups. Kirromycin and enacyloxin IIa hinder the release of EF-Tu·GDP from the ribosome, thus, its recycling and peptide bond formation, while pulvomycin and GE2270 A both hinder the formation of the ternary complex between EF-Tu·GTP and aa-tRNA (*5*−*8*). Specific differences between the pulvomycin and GE2270 A complexes concern several of the EF-Tu characteristics, such as the nucleotide interaction, intrinsic GTPase activity, and electrophoretic behavior. Notably, both pulvomycin and GE2270 A can bind to EF-Tu simultaneously with the nucleotide exchange factor Ts, unlike kirromycin and enacyloxin IIa. Pulvomycin stimulates the release of GDP by EF-Ts, indicating a cooperative effect on the nucleotide exchange reaction (*8*).

To date, of these two antibiotics, only the crystal structure of GE2270 A bound to *Escherichia coli* (*Ec*) EF-Tu·GDP (the "off" state) has been elucidated at 2.35 Å resolution (*9*). In this model, the antibiotic binding site was located on domain 2, interfering with amino acid residues that in the active "on" state of EF-Tu, complexed with the GTP analogue GDPNP, are part of the binding site for aa-tRNA. Because the GDP-bound conformation of EF-Tu is unable to bind aa-tRNA, in this work, we set out to resolve the crystal structures of EF-Tu bound with either pulvomycin or GE2270 A in complex with GDPNP for a most accurate comparison of the 3D structures of the two complexes. The models obtained, at 1.4 Å resolution for the pulvomycin complex and at 1.6 Å resolution for the GE2270 A complex, have defined the binding sites of the two antibiotics and their stereochemical configuration. They reveal their interference with aa-tRNA and overall and specific changes in the EF-Tu domains, disclosing the structural background of their influence on EF-Tu functions. This work accurately reveals how two antibiotics of totally different structures and with different binding modes and sites can employ a similar mechanism of action.

MATERIALS AND METHODS

Crystallization and Data Collection. Purification of EF-Tu(*Tt*) HB8 overproduced in *E. coli* was carried out essentially as described (*10*). To eliminate most proteins of the host cell, a heating step was carried out at 65 °C for 10 min, followed by ion-exchange chromatography on Q-Sepharose and gel filtration on Sephacryl H 100. The purified EF-Tu·GDP displayed a single band on SDS−PAGE and was stored at −81 °C. Pulvomycin was obtained from *Streptoverticillium netropsis* and purified as reported (*8*). GE2270 A produced from *Planobispora rosea* was a gift of Dr. Enrico Selva (Biosearch, Gerenzano, Italy). Both antibiotics are highly insoluble in water and were dissolved in methanol. The extinction coefficients (ϵ) in methanol at 320 nm for pulvomycin and 310 nm for GE2270 A are 74 591 and 34 158 M^{-1} cm^{-1}, respectively (*11*, *12*). Stock solutions (10 mM) in methanol at −81 °C were stable for at least 1 year. EF-Tu·GDPNP was obtained by incubating EF-Tu·GDP (0.5−1.0 mg) in 20 mM Tris-HCl at pH 8.0, 200 mM $(NH_4)_2SO_4$, a 2.5 molar excess of GDPNP, 2 mM dithiothreitol (DTT), 0.5 mM NaN_3, and 2 units/mg EF-Tu of calf intestinal alkaline phosphatase (molecular biology degree, Roche, Mannheim, Germany). Incubation was for 60 min at 30 °C in the absence of $MgCl_2$ and for a further 30 min in the presence of 0.5 mM $MgCl_2$. After the concentration to 12−15 mg/mL using Centricon YM 30 ultra-spinfilters, EF-Tu·GDPNP was stored at −81 °C in 20 mM Tris-HCl at pH 7.6, 20 mM NaCl, 5 mM $MgCl_2$, 0.5 mM NaN_3, and 2 mM DTT. The presence of alkaline phosphatase was found not to affect the crystallization of the EF-Tu·GDPNP·antibiotic complexes. The crystallization solution contained 4.0−4.5 mg/mL of EF-Tu·GDPNP in 20 mM Tris-HCl buffer (pH 7.6), 20 mM citric acid disodium salt, a 2-fold molar excess of GDPNP, 5 mM $MgCl_2$, 2 mM DTT, 1% glycerol, and 0.5 mM NaN_3. The mixture was slightly acidic (pH $\sim$ 6.0). Pulvomycin or GE2270 A was added as the last component in a 3−5 molar excess over EF-Tu. The very slight turbidity was removed by centrifugation. Sitting drops of 2−5 μL of the clear supernatant were mixed with a 0.4−0.5 volume equivalent from the reservoir solution [0.5 mL of 100 mM Tris-HCl at pH 7.6, 5−8% glycerol, and 19−23% poly(ethylene glycol) (PEG) 6000 as a precipitant]. The trays with the samples were kept at 19 °C. Hexagonal crystals of the pulvomycin complex (space group, $P6_5$) appeared within 24−36 h. Growth was completed within 1−2 weeks to a maximum size of 1.0 × 0.4 × 0.4 mm. Orthorhombic crystals of EF-Tu·GDPNP·GE2270 A (space group, $P2_12_12_1$) appeared within 1−2 days as oblong rods, forming clusters. Growth up to 2.5 × 0.2 × 0.2 mm was completed within 1−2 weeks. EF-Tu·GDPNP·pulvomycin and EF-Tu·GDPNP·GE2270 A crystals were mounted directly from the cryoprotecting mother liquor and flash-cooled in a 100 K cryostream (Oxford Cryosystems, U.K.). X-ray crystallographic data extending to 1.4 and 1.6 Å resolution were collected from single crystals at EMBL beamline X11 at DESY. Diffraction data were integrated and scaled using the HKL package (*13*), and structure factor amplitudes were calculated using TRUNCATE of the CCP4 package (*14*).

Structure Determination and Refinement. The structure of EF-Tu·GDPNP·pulvomycin was determined by molecular replacement at 6−3.5 Å resolution using AmoRe (*15*), and search models of individual domains were derived from the structure of *T. thermophilus* EF-Tu·GDPNP (*3*). Model-derived phases were refined and extended by density modification (DM) at 43−1.4 Å resolution as implemented in CNS (*16*). The resulting DM map showed unbiased electron density with atomic features for missing parts or incorrectly modeled regions of the model, for the antibiotic and ordered solvent molecules. A lead structure of pulvomycin was modeled by minimization using the AM1 parametrization supplied with the CHEMDRAW package, and initial parameter files were obtained from the HIC-UP server (*17*). The DM map at 1.4 Å resolution allowed for the assignment of absolute configuration of chiral centers in pulvomycin, and model building was performed using the O program (*18*). Model refinement was carried out in CNS using the maximum likelihood target function, and the free

Table 1: Data Collection and Refinement Statistics of *T. thermophilus* EF-Tu·GDPNP Complexes with Pulvomycin and GE2270 A

complex	EF-Tu·GDPNP·pulvomycin	EF-Tu·GDPNP·GE2270 A
space group	$P6_5$	$P2_12_12_1$
cell dimensions (Å)	$a = b = 86.69$, $c = 105.51$	$a = 43.32$, $b = 94.58$, $c = 104.38$
mosaicity (deg)	0.23	0.30
wavelength (Å)	0.8133	0.8133
resolution (Å)	43−1.4 (1.42−1.40)	46−1.6 (1.63−1.60)
R_{sym}[a]	0.057 (0.489)[b]	0.063 (0.245)[b]
$I/\sigma I$	39.4 (2.5)	33.3 (4.3)
unique reflections	88 023	57 292
redundancy	4.3 (3.3)	4.6 (2.9)
completeness (%)	99.8 (99.0)	99.7 (96.5)
B value, Wilson plot (Å^2)	15.1	13.5
R/R_{free}[c]	0.196/0.214 (0.262/0.295)	0.187/0.217 (0.229/0.232)
residues	9−405, Mg·GDPNP, pulvomycin, 327 water	2−405, Mg·GDPNP, GE2270 A, PEG, 377 water
Ramachandran (%)[d]	92.8/0.6	92.9/0.3
rmsd bond (Å)	0.0071	0.0078
rmsd angle (deg)	1.47	1.46
average B value (Å^2)	17.5 (protein) 25.1 (solvent)	14.5 (protein) 23.0 (solvent)

[a] $R_{sym} = \sum_h\sum_i|I_i(h) - \langle I(h)\rangle|/\sum_h\sum_i I_i(h)$, where $I_i(h)$ is the ith measurement. [b] Values in parentheses here and below refer to the outer resolution bin as indicated for the two data sets. [c] $R = \sum_h||F(h)_{obs}| - |F(h)_{calcd}||/\sum_h|F(h)_{obs}|$, and R_{free} is the R factor calculated for a subset of ~1700 reflections excluded from the refinement throughout. [d] Percentages of amino acid residues occupying the most favored and disallowed regions, respectively, of the Ramachandran plot according to PROCHECK (*39*).

R factor was calculated on a subset of 1761 randomly selected reflections excluded from refinement throughout. The σ_A-weighted $2F_o - F_c$ and $F_o - F_c$ electron-density maps were used in later rounds of model building. A total of 327 water molecules were identified at appropriate positions for interactions with the protein. The final model comprises amino acid residues 9−405 as well as complete models of GDPNP·Mg^{2+} and pulvomycin. The final R factor and free R factor derived from the model are 0.196 and 0.214, respectively, at 43−1.4 Å resolution (all data).

The EF-Tu·GDPNP·GE2270 A structure was solved by molecular replacement at 6−3.2 Å resolution using the EF-Tu·GDPNP·pulvomycin structure as a search model. Model-derived phases were extended and refined at 46−1.6 Å resolution as described for the pulvomycin complex, producing an unbiased electron-density map for model building. The EF-Tu·GDPNP·GE2270 A model was refined at 46−1.6 Å resolution using CNS and with a set of 1702 randomly selected reflections excluded throughout for the calculation of the free R factor. The GE2270 A starting model and associated geometrical parameters were derived from the EF-Tu·GDP·GE2270 A complex (PDB entry 1D8T) using the HIC-UP server, and it was fitted to the map with few modifications. An R to S interchange of the C_β atom configuration of the phenylserine moiety was evident from the 1.6 Å resolution map. The N-terminal end of EF-Tu was traced as an extended peptide interacting with a neighboring molecule in the crystal packing. The final model comprises amino acid residues 2−405, full models of GE2270 A and GDPNP·Mg^{2+}, a diethylene glycol fragment of a PEG molecule, and 377 water molecules, yielding an R factor and a free R factor of 0.187 and 0.217, respectively. Table 1 shows data statistics of the crystallographic analysis of the two antibiotic complexes. The figures were prepared using PyMol (*19*).

RESULTS

Binding Site of Pulvomycin. Pulvomycin is active against Gram-positive and Gram-negative bacteria with a specificity different from GE2270 A (*20*). It induces a stable, anomalous EF-Tu·GTP complex unable to bind aa-tRNA and destabilizes EF-Tu·GDP (*6*, *7*). It affects the electrophoretic migration of EF-Tu(*Ec*) and EF-Tu(*Tt*) in a comparable manner (parts A and B of Figure 2). Notably, EF-Tu(*Tt* or *Ec*)·GDP·

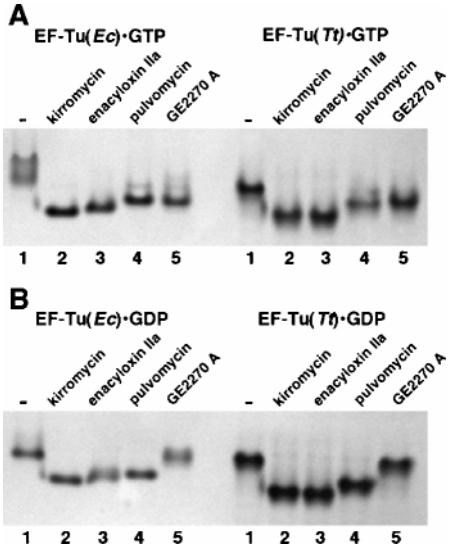

FIGURE 2: Electrophoretic patterns illustrating the effects of the four EF-Tu-specific antibiotics on EF-Tu·GTP (A) and EF-Tu·GDP (B). Left, *E. coli* EF-Tu; right, *T. thermophilus* EF-Tu. Lanes: control EF-Tu minus antibiotics (lane 1), plus 50 μM kirromycin (lane 2), enacyloxin IIa (lane 3), pulvomycin (lane 4), and GE2270 A (lane 5). The reaction mixture contained 28 pmol of EF-Tu(*Ec*)·GTP or 55 pmol of EF-Tu(*Tt*)·GDP or 20 pmol of EF-Tu(*Ec*)·GDP or 60 pmol of EF-Tu(*Tt*)·GDP. EF-Tu·GTP was obtained as described (*6*). The reaction mixtures were preincubated for 30 min at 0 °C in 10 μL of standard buffer (50 mM Tris-HCl at pH 7.6, 60 mM NH_4Cl, 10 mM $MgCl_2$, and 1 mM DTT) in the absence or in the presence of 50 μM of the antibiotics. Electrophoretic conditions were as reported (*6*). The buffer in the upper compartment contained either 20 μM GTP plus 1 mM phosphoenolpyruvate (A) and 0.5 mg/mL pyruvate kinase or 20 μM GDP (B). NB, the double band on the control lane of EF-Tu(Ec)·GTP is the result of association/dissociation effects of the nucleotide and/or aggregation phenomena.

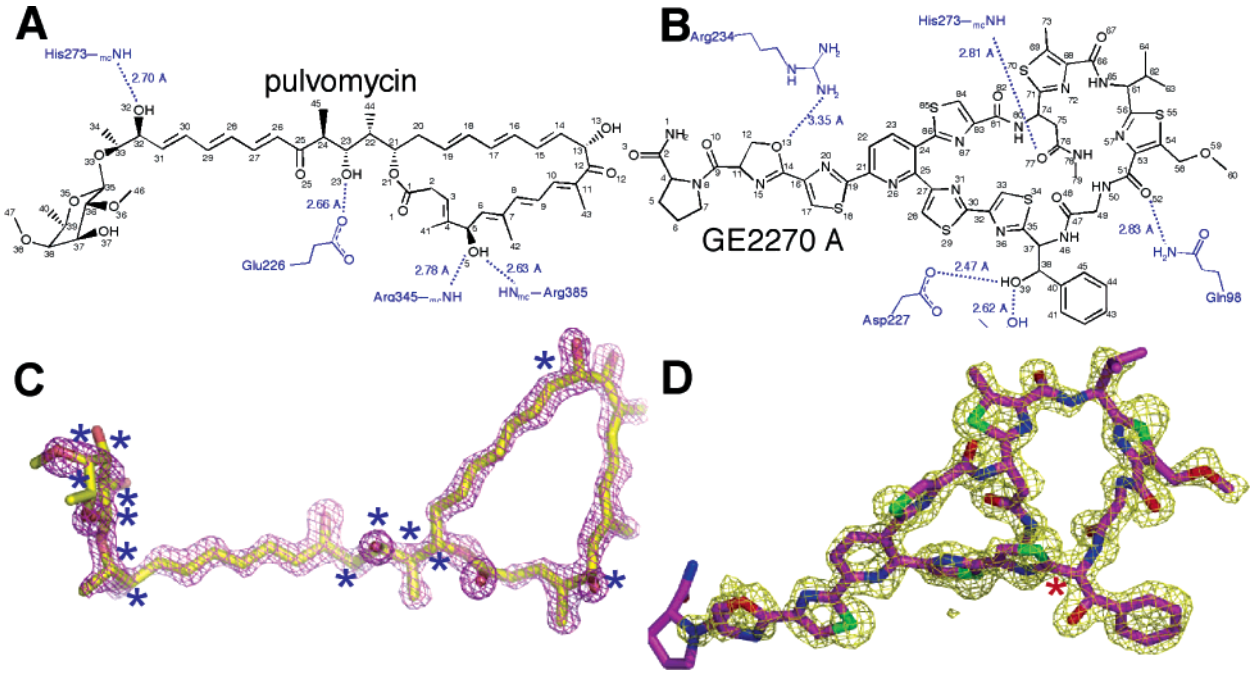

FIGURE 3: Chemical structure and electron-density map of pulvomycin and GE2270 A. Chemical structure of pulvomycin (A) and GE2270 A (B) also showing the hydrogen bonds (dotted blue lines) with the EF-Tu binding site. (C) Unbiased electron-density map of pulvomycin obtained by density modification at 1.4 Å resolution of molecular-replacement phases. The map is displayed at a 2.0σ contour level. The structure of the antibiotic including the absolute configuration of the chiral centers (marked by blue asterisks) is clearly resolved. (D) Unbiased electron-density map of GE2270 A obtained by density modification at 1.6 Å resolution of molecular-replacement phases. The chirality of the phenylseryl group (marked by a red asterisk) is evident from the electron-density map.

pulvomycin migrates much faster than the GE2270 A counterpart, unlike EF-Tu(*Tt* or *Ec*)·GDPNP in complex with pulvomycin and GE2270 A, whose migration velocity is comparable. This predicts more divergent EF-Tu·GDP conformations, an aspect as yet structurally unverified. The structure of the pulvomycin molecule consists of a 22-membered lactone ring with a side chain formed by two trienones, one triene, and a terminal sugar (*11*) (Figure 3A). Figure 3C illustrates the electron-density map of the absolute configuration of pulvomycin. The pulvomycin binding site involves the three domains of EF-Tu and extends from the domain 1−3 interface to domain 2 (parts A and B of Figure 4). Its lactone ring binds at the domain 1−3 interface proximal to the domain 1−2−3 junction (parts A and B of Figure 4). The linear side chain overlaps the three-domain junction contacting both domains 1 and 2, and its terminal hexose moiety binds to domain 2. The accommodation of the antibiotic widens and reorients the domain 1−2 interface and the domain 1−2−3 junction, affecting residues such as Ile93, Thr94, Ala97, Gln98, and Leu122 (Figure 4B, see later parts A and B of Figure 6). Thus, it binds by an induced-fit mechanism. The interaction with EF-Tu includes four hydrogen bonds (two each with domains 2 and 3) and extensive van der Waals and hydrophobic interactions, as shown in Figure 3A, the stereodiagram in Figure 4B, and Table 2. At <3.8 Å, pulvomycin contacts 23 residues (7 from domain 1, 7 from domain 2, and 9 from domain 3) (Table 2). The amino acid residues contacted at <3.8 Å are interconnected by 11 hydrogen bonds (Table 2 and Table S1 in the Supporting Information). Figure 4A also illustrates the amino acid residues whose substitution induces resistance to the antibiotic.

Binding of GE2270 A to EF-Tu·GDPNP as Compared to That of Pulvomycin and the Binding of GE2270 A to EF-Tu(Ec)·GDP. GE2270 A, a thiazolyl peptide antibiotic acting against Gram-positive and some Gram-negative bacteria, consists of a peptide ring containing thiazoles and one pyridine connected to a side chain of three modified amino acids (Figure 3B**)** (*20*−*22*). Figure 3D illustrates the electron-density map of the EF-Tu·GDPNP-bound GE2270 A. Similar to pulvomycin, it induces a stable, anomalous EF-Tu·GTP complex unable to bind aa-tRNA but differently from pulvomycin does not destabilize EF-Tu·GDP (*6*, *7*). The crystal structure of EF-Tu(*Tt*)·GDPNP·GE2270 A shows that the antibiotic is bound to domain 2 (parts A and B of Figure 5). The upper part of the thiazolyl ring that is located at the domain 1−2 interface interacts with domain 1, widening the interface (Figure 5A, see also parts B and C of Figure 6). Arg234(*223*) Nη1 and Glu271(*259*) Oϵ1 form a salt bridge over the GE2270 A side chain, as has already been described for EF-Tu(*Ec*)·GDP·GE2270 A (*9*) (parts A and B of Figure 6). The number in italics following in parentheses the *T. thermophilus* numbering refers to the homologous EF-Tu(*Ec*) amino acid residue to facilitate the comparison with EF-Tu(*Ec*). The penetration of the side chain of GE2270 A into domain 2 is very likely a consequence of an induced-fit mechanism. In the pulvomycin complex, Glu226 makes a hydrogen bond to the antibiotic very much like the neighboring Asp227 does in the GE2270 A complex where this residue points away from the antibiotic (cf. Figures 4B and 5B). GE2270 A is connected to EF-Tu by five hydrogen bonds (one with domain 1 and four with domain 2) as well as extensive van der Waals and hydrophobic interactions (Figure 3B, the stereodiagram in Figure 5B, and Table 2). Of the EF-Tu amino acids forming hydrogen bonds with GE2270 A and pulvomycin, only one is in common (His273), and this is an interaction with the main chain (mc) (Figure 5B and Table 2). At <3.8 Å, GE2270 A contacts 7 amino

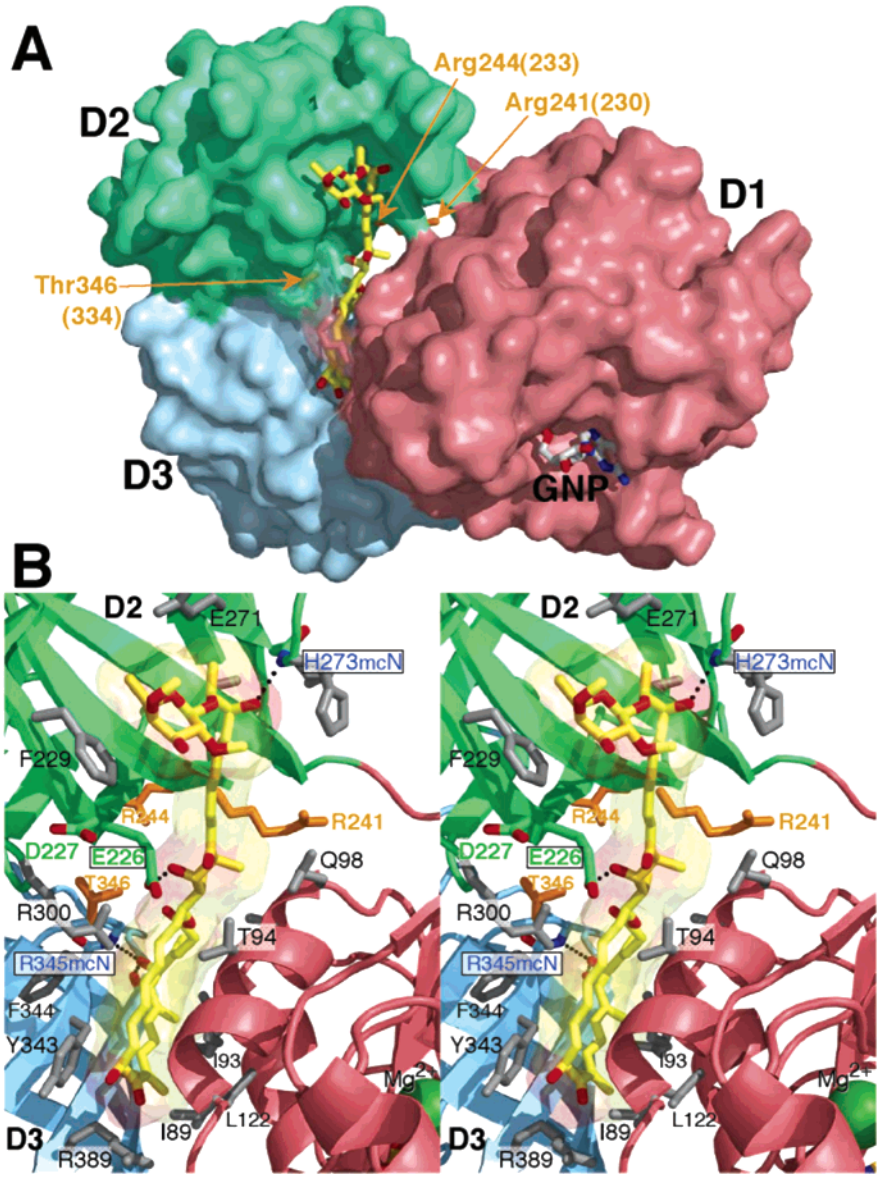

FIGURE 4: Overview of the EF-Tu·GDPNP(GNP) complex with bound pulvomycin and stereodiagram of the pulvomycin binding site on EF-Tu. (A) In the space-filling representation, the domains D1, D2, and D3 are shown in red, green, and blue, respectively, while the antibiotic molecule is represented as a stick skeleton; carbons are colored yellow, and oxygens are colored red. Most amino acid residues known thus far, of which the substitution induces pulvomycin resistance, are indicated in orange. (B) Stereodiagram of the pulvomycin binding site on EF-Tu. The color scheme is red for domain 1, green for domain 2, and blue for domain 3. The antibiotic is shown as a stick skeleton surrounded by a transparent molecular surface in yellow. Selected side chains and stretches of the EF-Tu backbone (mc) that make hydrogen bonds are shown as sticks. Hydrogen bonds are represented by dotted lines. Black-framed labels show amino acid residues forming hydrogen bonds with the antibiotic; unframed black labels indicate amino acid residues involved in hydrophobic contacts. Amino acid residues, whose substitution induces pulvomycin resistance, are labeled in orange.

acid residues from domain 1 and 14 from domain 2 but none from domain 3 (Table 2). Of these 21 amino acid residues, 2 from domain 1 (Thr94 and Gln98) and 5 from domain 2 (Glu226, Phe229, Glu271, His273, and Val286) also contact pulvomycin. Therefore, only part of the pulvomycin side chain overlaps the GE2270 A thiazolyl ring (Figure 5C, see also Figure 6C). In the GE2270 A complex, the contacting residues at <3.8 Å are interconnected by 12 hydrogen bonds versus 11 hydrogen bonds in the pulvomycin complex (Table 2 and Table S1 in the Supporting Information). In Figure 5A, the amino acid residues whose substitution induces resistance to GE2270 A are indicated.

A comparison of EF-Tu(*Tt*)·GDPNP·GE2270 A with EF-Tu(*Ec*)·GDP·GE2270 A (Figure 5D) shows that the binding site of the antibiotic is essentially the same except that in EF-Tu·GDPNP·GE2270 A the upper part of the thiazolyl ring is located at the domain 1−2 interface, whereas in EF-Tu·GDP·GE2270 A, it is exposed to the solvent, as a consequence of the conformational changes induced on EF-Tu by GDP (see arrow in Figure 5D). Therefore, in EF-Tu·GDP·GE2270 A, the hydrogen bond Gln98ϵ2−GEA O52 as well as any contact at <3.8 Å of the antibiotic with domain 1 is missing. Although the total contacts between GE2270 A and EF-Tu·GDPNP or EF-Tu·GDP are the same, in the former complex, only 14 of the 21 contacts concern domain 2. A similar situation is found for the hydrogen bonds interconnecting the amino acid residues at <3.8 Å from the atoms of the antibiotic, which is also the same in both

Table 2: Properties of the EF-Tu Binding Sites of Pulvomycin and GE2270 A: Connections of the Antibiotic[a]

	Tt EF-Tu·GDPNP·pulvomycin	*Tt* EF-Tu·GDPNP·GE2270 A	*Ec* EF-Tu·GDP·GE2270 A[a]
buried surface (Å^2)[b]	1751	1935	1493
contacts (<3.8 Å)	23	21	21
domain 1[c,d]	Ile89, Ile93, **Thr94**, Ala97, **Gln98**, Leu122, Gln125	Asn13, His67, Val68, Glu69, Ser78, **Thr94**, **Gln98**	
domain 2[d,e]	**Glu226**, **Phe229**, Gly240, Arg241, **Glu271**, Met272, **His273**, **Val286**, Arg300	**Glu226**, Asp227, **Phe229**, Arg234, Thr239, Thr268, Gly269, **Glu271, His273**, Arg274, Asn285, **Val286**, Gly287, Leu289	**Glu226**, Asp227, **Phe229**, Gly233, Arg234, Val237, Thr239, Gly240, Arg241, Thr268, Gly269, Val270, **Glu271**, Met272, **His273**, Arg274, Thr276, Asn285, **Val286**, Gly287, Leu289
domain 3	Tyr343, Phe344, Arg345, Thr346, Arg385, Ala387, Arg389		
protein−antibiotic hydrogen bonds (<3.5 Å)	4	5	4
atoms[f,g]	Glu226 Oε2−O23 **His273 N**−O32 Arg345 N−O5 Arg385 O−O5	Gln98 Nε2−O52 Asp227 Oδ1−O39 Thr239 Oγ−O39 Arg234 Nη1−O13 **His273 N**−O77	Asp227 Oδ2−O39 Gly233 O−N1 **His273 N**−O77 Asn285 Oδ1−O48
protein hydrogen bonds[h]	11	12	12

[a] Residues in the table refer to the equivalent residues of *T. thermophilus* of the following *E. coli* residues (*Tt residue numbers in parentheses*): Glu215(*226*), Asp216(*227*), Phe218(*229*), Gly222(*233*), Arg223(*234*), Val226(*237*), Thr228(*239*), Gly229(*240*), Arg230(*241*), Thr256(*268*), Gly257(*269*), Val258(*270*), Glu259(*271*), Met260(*272*), Phe261(*His273*), Arg262(*274*), Leu264(*276*), Asn273(*285*), Val274(*286*), Gly275(*287*), and Leu277(*289*). [b] Buried surface areas are calculated using a 1.4 Å probe radius and the algorithm of Lee and Richards (*40*). [c] Amino acid residues of the EF-Tu domains: domain 1, 1−211; domain 2, 212−311; domain 3, 312−405. [d] In bold are common amino acid residues of EF-Tu·GDPNP·pulvomycin, EF-Tu·GDPNP·GE2270 A, and EF-Tu·GDP·GE2270 A. [e] Underlined are common amino acid residues of EF-Tu·GDPNP·GE2270 A and EF-Tu·GDP·GE2270. [f] In bold are common amino acid residues of EF-Tu·GDPNP·pulvomycin, EF-Tu·GDPNP·GE2270 A, and EF-Tu·GDP·GE2270. [g] Underlined are common amino acid residues of EF-Tu·GDPNP and EF-Tu·GDP·GE2270 A. [h] Hydrogen bonds between protein atoms within a 3.8 Å radius of the bound antibiotic.

complexes, but in the case of EF-Tu·GDPNP, only 9 of the total 12 hydrogen bonds are located in domain 2, while the remaining 3 are situated in domain 1 (Table 2 and Table S1 in the Supporting Information). This difference in contacts with domain 2 is very likely due to (i) for the most part, the different orientation of the GE2270 A terminal side chain in the two complexes (Figure 5D), (ii) subtle variations in the positions of the other components of the antibiotic, and (iii) the slightly diverging primary structure of domain 2(*Tt*) and domain 2(*Ec*). The protein surface buried by the antibiotic is somewhat larger (∼10%) in EF-Tu·GDPNP·GE2270 A than in EF-Tu·GDPNP·pulvomycin but markedly smaller (∼30%) in EF-Tu·GDP·GE2270 A (Table 2).

Overall Changes in the EF-Tu Domains. Superimposition of the mc's of domain 3 from the antibiotic-free and the pulvomycin-bound EF-Tu·GDPNP complexes shows that domain 1 is displaced backward relative to domain 2 because of a steric clash with the antibiotic. As a consequence, the distance between the domain 1 structural elements and domain 2 is markedly increased (up to ∼10 Å). The displacement of domain 1 resembles that of a rigid-body movement, because the relationships between its secondary elements remain essentially the same as in the antibiotic-free conformation. A similar situation is found in the GE2270 A complex, where the insertion of the upper portion of the thiazolyl ring into the domain 1−2 interface widens this interface, preventing the domain 1 from completing its "rolling" movement over domains 2 and 3 when switching from the GDP- to the GTP-bound conformation. This effect is similar to that of pulvomycin even if the backward displacement of domain 1 relative to domains 2 and 3 is somewhat less accentuated (cf. parts A and C of Figure 6, see also Figures 4A and 5A). In both complexes, domain 2 and 3 appear to have the same relative orientation.

Specific Changes in the Switch Regions. As shown in parts A−C of Figure 7, both switches that act as pivots of the "on" /"off" transitions of EF-Tu are selectively affected by the binding of pulvomycin. In the switch 1 region, the side chain of Arg59, a residue involved in the ribosome-induced EF-Tu GTPase activity (*23*), is more distant from loop L4 (residues 82−88) than in the absence of the antibiotic and forms a hydrogen bond with Glu55 instead (Figure 7A). The side chain of Ile61(*60*), one of the two wings of the "hydrophobic gate" shielding the γ phosphate and the nearby inline water molecule from the His85(*84*) side chain (*3*) is rotated by ∼90° (Figure 7B) and so is the side chain of Ile89(*Val88*) from the switch 2 region. In contrast, the side chain of His85(*84*), a residue crucial for the active conformation of the nucleotide binding pocket (*24*), points away from the γ phosphate of GDPNP, as is found in the antibiotic-free conformation. One should however mention that in EF-Tu·GDPNP·pulvomycin His85(*84*) is involved in a crystal contact that could somewhat affect the orientation of its side chain. Some more modest perturbations were also found in the GE2270 A complex. The side chain of Arg59(*58*) is only slightly more distant from loop L4 (residues 82−88) than in the antibiotic-free conformation but still forms a hydrogen bond with Glu55(*54*) (Figure 7A). The side chain of His85(*84*), which in the GE2270 A complex is not involved in any crystal contact, is turned toward the γ phosphate of GDPNP, while the position of

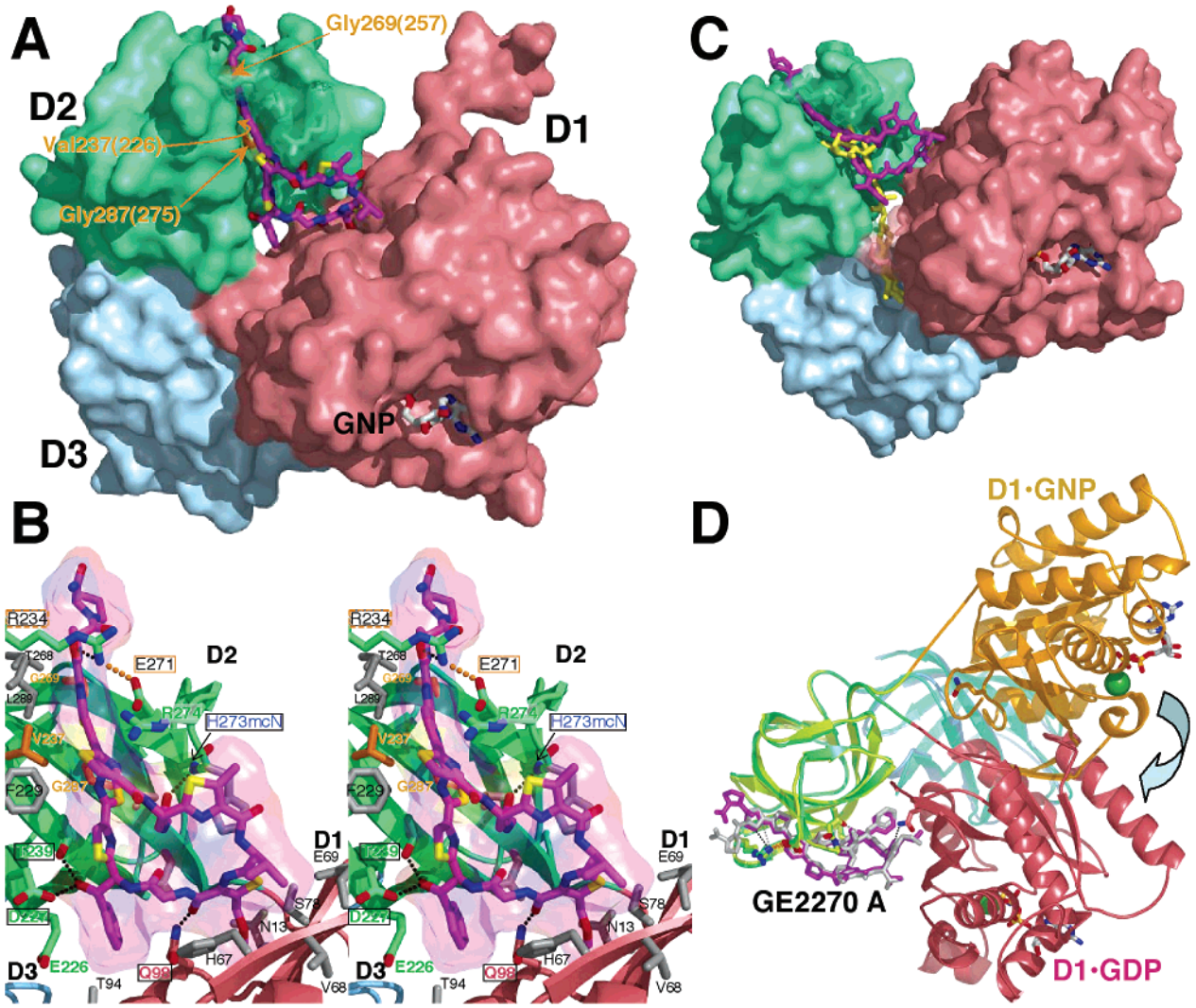

FIGURE 5: Overview of the EF-Tu·GDPNP(GNP) complex with bound GE2270 A (A), stereodiagram of the GE2270 A binding site (B), and overlap between pulvomycin and GE2270 A (C) and between EF-Tu·GDPNP·GE2270 A and EF-Tu·GDP·GE2270 A (D). (A) In the space-filling representation, the domains D1, D2, and D3 are shown in red, green, and blue, respectively, while the antibiotic molecule is represented as a stick skeleton; carbons are colored purple, oxygens red, nitrogens blue, and sulphurs yellow. Most amino acid residues known thus far, whose substitution induces pulvomycin resistance, are indicated in orange. (B) Stereodiagram of the positioning on EF-Tu of GE2270 A. The color scheme of the domains and of the antibiotic is the same as in A. The antibiotic is shown as a stick skeleton surrounded by a transparent molecular surface in purple. Selected side chains and stretches of the EF-Tu backbone (mc) that make hydrogen bonds are shown as sticks. Hydrogen bonds are represented by dotted lines, and the salt bridge over the side chain of GE2270 A [Glu271(*259*)−Arg234(*223*)] is represented as an orange dotted line. Black-framed labels show amino acid residues forming hydrogen bonds with the antibiotic; unframed black labels indicate amino acid residues involved in hydrophobic contacts. Amino acid residues, whose substitution induces pulvomycin resistance, are labeled in orange. (C) Overlap of pulvomycin and GE2270 A. The colors of D1, D2, and D3 in the space filling is the same as in A. Positions of D1 and D3 were derived from the EF-Tu·GDPNP·pulvomycin complex, and positions of D2 were derived from the EF-Tu·GDPNP·GE2270 A complex. The antibiotic molecule is represented as a stick skeleton in yellow in pulvomycin and purple in GE2270 A. (D) Overlap of EF-Tu(*Tt*)·GDPNP·GE2270 A and EF-Tu(*Ec*)·GDP·GE2270 A. The domains D1, D2, and D3 are shown in red, deep green, and blue, respectively, in EF-Tu·GDPNP·GE2270 A and in orange, light green, and blue, respectively, in EF-Tu·GDP·GE2270 A. The antibiotic molecule is represented as a stick skeleton in purple in the EF-Tu·GDPNP complex and in light gray in the EF-Tu·GDP complex. The green sphere indicates the magnesium ion. Note the dramatic displacement of domain 1 from the GDP- to the GDPNP-bound conformation (see blue arrow) leading to its contact with the thiazolyl ring of the antibiotic and the formation of the domain 1−2 interface.

the Ile61 and Ile89 side chains is virtually unchanged (Figure 7C). In both complexes, some differences are also found in the location of the water molecules (cf. parts B and C of Figure 7). These results show that the influence of the two antibiotics on the switch regions is restricted to some specific effects.

Interference with aa-tRNA. In the ternary complex Phe-tRNA^{Phe}·EF-Tu [*Thermus aquaticus* (*Ta*)]·GDPNP, the CCA-Phe enters a cleft between domains 1 and 2 below helix A″, ending in a pocket lined by His67, Glu226−Phe229, Thr239, Glu271−Arg274, and Asn285−Gly287 (*25*). The 5′ end of tRNA is tightly bound at the domain 1−2−3 junction, where a pocket is formed by the C-terminal part of helix B from domain 1 (Lys90 and Asn91), two loops of domain 2 (Asp227−Val228 and Arg295 and Glu-299−Arg300), and one β strand of domain 3. The 5′ phosphate forms a salt bridge with Lys90 and Arg300, and the ribose interacts with Asn64 and Asp90. The superimposition of the mc's of the domain 3 from Phe-tRNA^{Phe}·EF-Tu(*Ta*)·GDPNP and EF-Tu(*Tt*)·GDPNP·pulvomycin shows that the binding of the

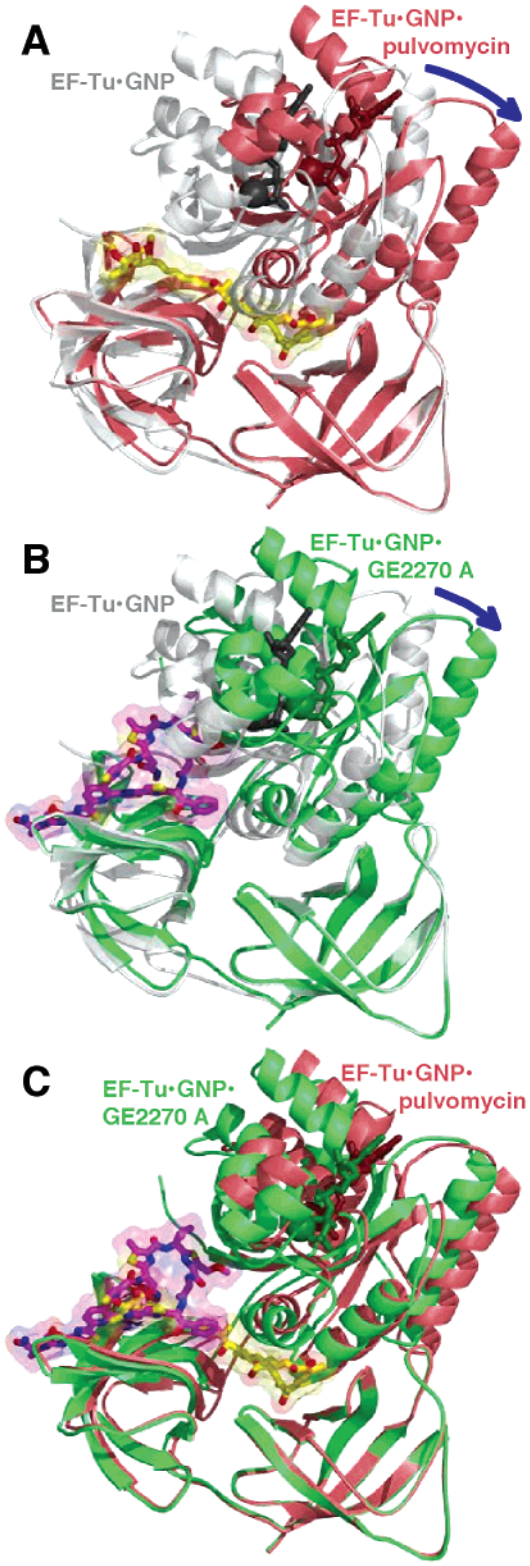

FIGURE 6: Pulvomycin and GE2270 A alter the position of domain 1 relative to domains 2 and 3. (A) EF-Tu·GDPNP(GNP)·pulvomycin (red) versus EF-Tu·GDPNP (gray). (B) EF-Tu·GDPNP·GE2270 A (green) vs EF-Tu·GDPNP (gray). (C) Displacement of domain 1 in the pulvomycin complex (red) as compared to that of the GE2270 A complex (green). The comparisons were carried out by superimposition of the mc's of the respective domain 3. Note (i) the marked backward displacement of the domain 1 in the two EF-Tu·antibiotic complexes and (ii) the more pronounced displacement of the domain 1 of the EF-Tu·pulvomycin complex as compared to the domain 1 of the GE2270 A complex.

antibiotic interferes with that of the 3′-aminoacyl group, acceptor stem, and 5′ end of Phe-tRNA^{Phe} (Figure 8A), interacting with residues such as Glu226, Phe229, Glu271, His273, Arg300, Arg345, and Thr346 (not shown). GE2270 A binding also interferes with the aa-tRNA binding by steric hindrance. Similar to pulvomycin, it affects the 3′-aminoacyl group and part of the acceptor stem of aa-tRNA (Figure 8B), interacting with residues such as Glu226, Asp227, Phe 229, Arg234, Thr239, His273, and Arg274 (not shown), but differently from pulvomycin, it does not interfere with the 5′-end binding. Figure 8 also shows that the relationships between the secondary elements of domain 1 in the two antibiotic complexes essentially correspond to those of the domain 1 of the antibiotic-free EF-Tu as part of the ternary complex, except for the shift induced by the binding of the two antibiotics in the domain interfaces. These results show that the inhibition by pulvomycin and GE2270 A of the formation of a stable complex between EF-Tu·GTP and aa-tRNA is based on simple steric hindrance.

DISCUSSION

This work reports the first structural analysis of crystals of EF-Tu(*Tt*)·GDPNP·pulvomycin and EF-Tu(Tt)·GDPNP·GE2270 A, disclosing the mechanism by which the two antibiotics inhibit complex formation between the active state of EF-Tu, the EF-Tu·GTP(GDPNP) complex, and aa-tRNA. The use of crystals from the same EF-Tu species has allowed for an accurate comparison between the two complexes at the highest resolution obtained until now for EF-Tu complexes. Large differences were observed between the binding sites of the two antibiotics. The lactone ring of pulvomycin is situated at the domain 1−3 interface; its side chain overlaps the three-domain junction, binding to domain 2. Therefore, pulvomycin interacts with all three domains of EF-Tu. GE2270 A binding is centered on domain 2 and only in EF-Tu·GDPNP interacts with domain 1, because the upper part of the thiazolyl ring is located at the domain 1−2 interface. Only part of the pulvomycin side chain overlaps the binding site of GE2270 A, with its sugar interfering with the binding site of the thiazolyl ring on domain 2 (Figures 5C and 6C). Of the more than 20 EF-Tu amino acid residues in contact with either antibiotic at <3.8 Å, only 7 are in common. It is doubtful that the salt bridge Arg234−Glu271 formed over the side chain of GE2270 A is a major factor for the tight binding to EF-Tu, as was suggested (*9*), because pulvomycin binds to EF-Tu as tightly as GE2270 A, with both compounds being only released by the denaturation of the protein. The extensive hydrophobic interactions are most likely responsible for their tight binding to EF-Tu. The amino acid substitutions thus far found to induce resistance to the two antibiotics are located in the area of the respective binding sites (see Figures 4 and 5): for pulvomycin, at the three-domain junction (R230C, R230V/233F, R233C/S, and T334A; refs *26*−*28*); for GE2270 A or its analogue amythiamicin, on domain 2 (V226A, G257S, and G275A; refs *29* and *30*). The elucidation of the binding sites for the two antibiotics supports a strict correlation between the antibiotic binding site and the position of mutations inducing resistance to the antibiotic. Our models suggest positions in domain 1 that could influence the EF-Tu sensitivity to the two antibiotics and which are worth testing by mutagenesis. These are the amino acid residues, individually or in

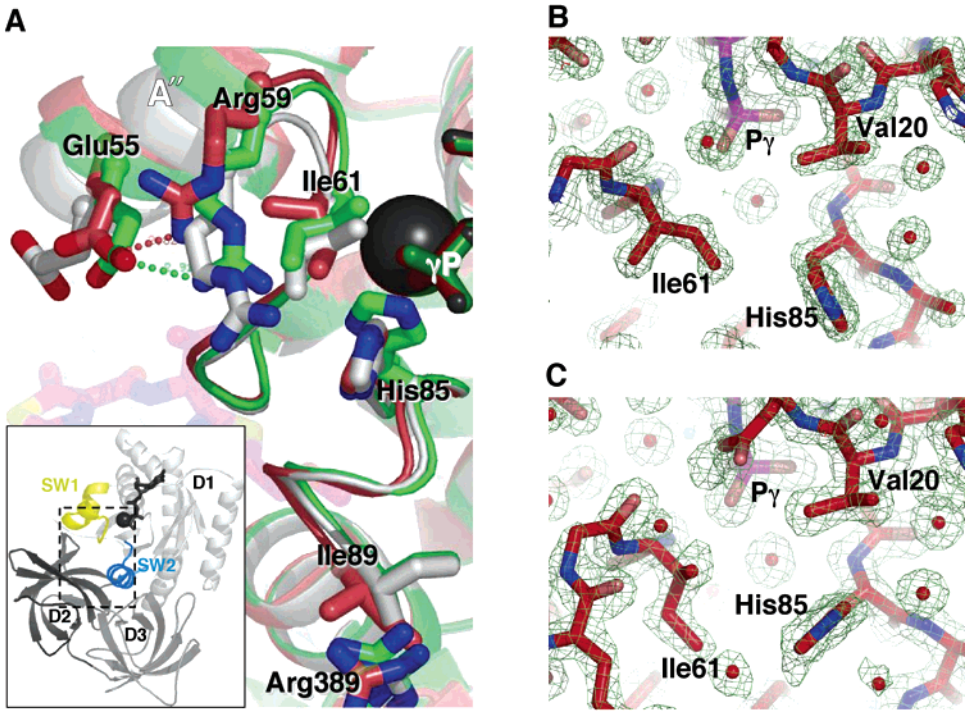

FIGURE 7: Specific effects of pulvomycin and GE2270 A on the switch 1 and 2 regions of EF-Tu. (A) Changes in the orientation of the side chain of Arg59(*58*), Ile62(*61*), His85(*84*), and Ile89(*Val88*) from the switch 1 and 2 regions induced by the binding of pulvomycin and GE2270 A. Residues from EF-Tu·GDPNP·pulvomycin are colored with carbon atoms in red; residues from EF-Tu·GDPNP·GE2270 A are colored with carbon atoms in green; and residues from the control native EF-Tu·GDPNP are colored with carbon atoms in gray. The black sphere indicates the nucleotide-coordinated magnesium ion. Note (i) the formation of a hydrogen bond between Arg59 and Glu55 in the two antibiotic complexes and the greater distance of Arg59 from the loop L4 (residues 82−88) from the switch 2 region; (ii) the different rotamer of Ile61, forming one of the two wings of the hydrophobic gate, in EF-Tu·GDPNP·pulvomycin as compared to EF-Tu·GDPNP·GE2270 A and antibiotic-free EF-Tu·GDPNP; (iii) the His85(*84*) side chain points away from the γ phosphate of GDPNP in the absence of the antibiotic and in the pulvomycin complex, whereas in the GE2270 A complex, it is turned toward the γ phosphate of GDPNP; and (iv) the different rotamer of Ile89 from the switch 2 region in the pulvomycin complex. The inset shows the location of the illustrated area on the EF-Tu molecule. (B) Final σ_A-weighted $2F_{obs} - F_{calcd}$ electron-density map showing details around the GTP analogue and the Ile61 residue in the pulvomycin complex. (C) Same region in the GE2270 A complex showing differences at the detailed level, including a different side-chain rotamer of Ile61.

combination, that form hydrogen bonds with critical structural components of the two compounds, among them, in the case of pulvomycin, Thr94, Glu98, Glu226, His273, the combination Arg345/Arg385 (interacting with the lactone ring), and Glu226/His273 (interacting with the side chain of the antibiotic). Mutation of Ile93 centering the lactone ring would also be interesting. In the case of GE2270 A, Thr94, Gln98, Glu226, and His 273 and their combinations could also be potential candidates for mutagenesis. In principle, any substitution perturbing the interfaces domains 1−2 and 1−3 and the three-domain junction could potentially affect the sensitivity to pulvomycin. The same holds for the domain 1−2 interface in the case of GE2270 A.

Despite their completely different structures and binding sites, the similarity between the actions of the two antibiotics is rationalized by the observation that both compounds prevent the aa-tRNA binding to EF-Tu by steric hindrance. Their interference affects the 3′-amino-acylated group and acceptor-stem binding site on domains 1 and 2 and, in the case of pulvomycin, also the 5′ end on domains 2 and 3. Accordingly, a number of amino acid residues interacting with the amino-acylated 3′ end of tRNA, such as Phe229, Glu271, and His273, are in contact with both antibiotics (Table 2). The GE2270 A steric hindrance, although more limited than that of pulvomycin, is sufficient for a full inhibition of stable ternary complex formation (*6*, *7*). Previously, the superimposition of domain 2 of the EF-Tu(*Ec*)·GDP·GE2270 A complex on domain 2 of the EF-Tu(*Ta*)·GDPNP·Phe-tRNA complex had already suggested a direct interference between this antibiotic and the binding site of the aminoacyl group of tRNA (*9*), despite the inability of EF-Tu·GDP to bind aa-tRNA. This conclusion has now been proven by our work.

The reason for the binding of the EF-Tu-targeted antibiotics to the domain 1−2 and 1−3 interfaces relates to the regulatory functions of these areas that contact the nucleotide binding pocket via the helix B/switch 2 region and the interconnected switch 1 region. Because the function of these switches is to amplify the signal of the nucleotide to domains 2 and 3 (*3*, *31*), the binding of ligands to these interfaces interferes with the switch mechanisms, altering the relative positions of the domains and the regulation of their activities.

It was interesting to analyze our 3D models with respect to other EF-Tu functions such as the GTPase activity and the formation of a high-affinity EF-Tu·GTP complex. Pulvomycin is known to enhance the intrinsic GTPase activity of EF-Tu (*32*) ~10-fold less strongly than kirromycin (0.25 versus 2.5−3 mol of GTP hydrolyzed (mol of EF-Tu)$^{-1}$ min^{-1} in the presence of high concentrations of LiCl), with both activities showing the same behavior with respect to the nature and concentration of monovalent cations (*8*, *33*, *34*). From the 3D model, this effect of pulvomycin could

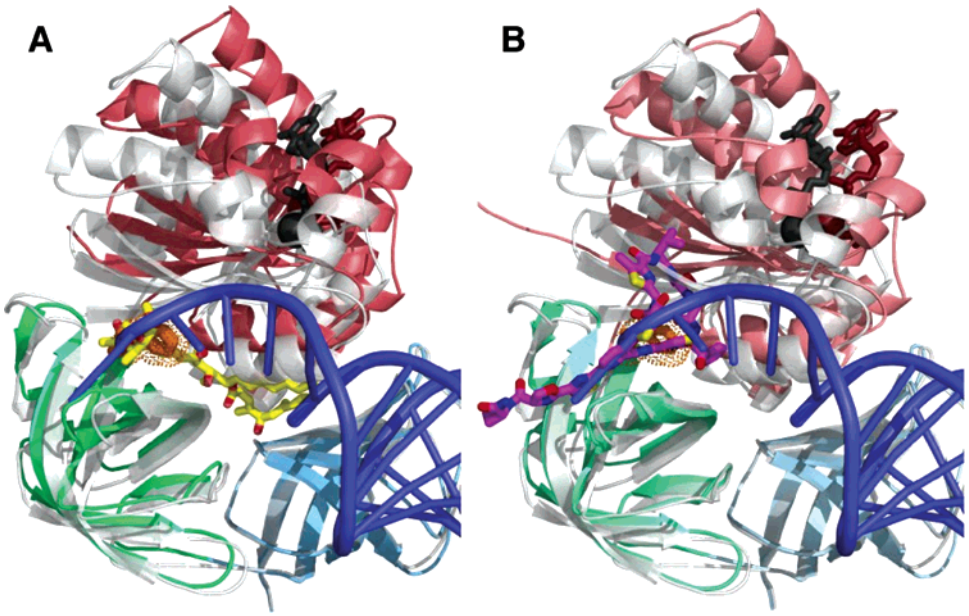

FIGURE 8: Steric hindrance between pulvomycin or GE2270 A and aa-tRNA on EF-Tu. EF-Tu(*Tt*)·GDPNP·pulvomycin and EF-Tu(*Tt*)·GDPNP·GE2270 A are colored red, green, and blue for domains 1, 2, and 3, respectively. EF-Tu in the superimposed ternary complex EF-Tu(*Ta*)·GDPNP·Phe-tRNA^{Phe} is all white. The domain 3 mc of EF-Tu(*Tt*)·GDPNP·pulvomycin (A) or EF-Tu(*Tt*)·GDPNP·GE2270 A (B) was aligned with that of the ternary complex EF-Tu(*Ta*)·GDPNP·Phe-tRNA^{Phe}, where tRNA is depicted in blue and the phenylalanyl residue is depicted in orange. In both EF-Tu complexes, the overlapping of the antibiotic binding site and the amino-acylated acceptor stem of Phe-tRNA^{Phe} is evident. In the pulvomycin complex, the antibiotic also interacts with residues of domain 3 involved in binding the 5′ end of Phe-tRNA^{Phe}.

depend upon the binding of its lactone ring at the domain 1−3 interface, where it contacts several amino acid residues of the binding sites of kirromycin and enacyloxin IIa, with the latter antibiotic also enhancing the intrinsic GTPase activity of EF-Tu (*33*). How the selective effects induced by pulvomycin on the EF-Tu switch regions could influence the cleavage of the γ phosphate is not clear. One can speculate that perturbation of the domain 1−3 interface can affect via the switch regions the microenvironment of the nucleotide binding pocket. Local changes of pH and cation concentrations could influence the stability of the γ phosphate (*8*, *34*, *35*) and be determinant for the level of the GTPase activity in a substrate-assisted GTP hydrolysis (*36*). The observation that GE2270 A induces little, if any, stimulation of the intrinsic GTPase activity of EF-Tu (*8*) appears to support a specific function of the pulvomycin-induced effects. The fact that, in contrast to GE2270 A, pulvomycin does not affect the orientation of the His85(*84*) side chain further disproves the participation of this amino acid residue in a nucleophilic attack on the γ phosphate, as was proposed (*37*). Such a mechanism has already been judged highly improbable from site-directed mutagenesis results (*1*, *24*, *38*).

As a common feature, all of the EF-Tu-specific antibiotics stabilize an anomalous GTP-bound state associated with a unique positioning of domain 1. Kirromycin and enacyloxin IIa (*33*, *37*) binding at the domain 1−3 interface displaces part of domain 1 beyond its GTP-bound "on" state by altering the positions of helices B and C. Pulvomycin and GE2270 A prevent the completion of the antibiotic-free "on" conformation by widening the domain 1−2 interface. The GTP binding affinity of all these complexes is 2−3 orders of magnitude higher than for the antibiotic-free EF-Tu complex (for a review, see refs *1* and *2*) This high-affinity EF-Tu·GTP complex could represent a stabilized precursor of the low-affinity GTP complex found in physiological conditions. Also in this case, microenvironmental effects could be responsible for the stabilization of these complexes.

For the future, it would be interesting to elucidate the 3D structure of EF-Tu·GDP·pulvomycin, of which the behavior in PAGE suggests a conformation sharing common features with EF-Tu·GDPNP·pulvomycin, differently from the GE2270 A counterpart. The finding that pulvomycin interacts intimately with all three domains of EF-Tu could be the reason for inhibiting extensive changes in the relative positions of the domains in the "on"/"off" transitions. Another important aspect was revealed by the finding that the binding of pulvomycin to EF-Tu is compatible with the binding of EF-Ts and their effect on the nucleotide exchange is additive (*8*). These features suggest that pulvomycin stabilizes a late intermediate in the nucleotide exchange reaction, where GTP binding is improved and GDP binding is diminished in agreement with its effect on the GTP and GDP binding constants (*8*). Elucidation of the structure of EF-Tu·EF-Ts·pulvomycin together with that of EF-Tu·GDP·pulvomycin could be important, because it may contribute to the disclosure of new structural aspects of the mechanism of the nucleotide release.

In conclusion, the inhibition of the elongation process by pulvomycin and GE2270 A is based on steric hindrance of the aa-tRNA binding to EF-Tu. The associated anomalous positioning of domain 1 locks GTP and generates in the case of pulvomycin an intrinsic GTPase activity, sharing properties with that induced by kirromycin. The fact that pulvomycin and GE2270 A, two chemically very different molecules with only partially overlapping binding sites, have the same physiological effect opens avenues for designing new drugs taking features from both of these antibiotics, possibly resulting in better activity and selectivity.

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SUPPORTING INFORMATION AVAILABLE

Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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