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REVIEW ARTICLE

The A–Z of bacterial translation inhibitors

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Abstract

Protein synthesis is one of the major targets in the cell for antibiotics. This review endeavors to provide a comprehensive "post-ribosome structure" A–Z of the huge diversity of antibiotics that target the bacterial translation apparatus, with an emphasis on correlating the vast wealth of biochemical data with more recently available ribosome structures, in order to understand function. The binding site, mechanism of action, and modes of resistance for 26 different classes of protein synthesis inhibitors are presented, ranging from ABT-773 to Zyvox. In addition to improving our understanding of the process of translation, insight into the mechanism of action of antibiotics is essential to the development of novel and more effective antimicrobial agents to combat emerging bacterial resistance to many clinically-relevant drugs.

Keywords: *Antibiotics; inhibitors; protein synthesis; resistance; ribosome; translation*

Protein synthesis at a glance

The translation machinery ensures accurate conversion of the genetic information of the messenger RNA (mRNA) into the corresponding polypeptide sequence. The ribosome provides the platform on which the mRNA can be recognized or "decoded" by transfer RNAs (tRNAs). tRNAs connect the RNA and protein worlds since at one end of the molecule they have an anticodon that is complementary to a codon specifying a particular amino acid, and at the other end, the specific amino acid is linked to the 3′ terminal CCA-end of the tRNA by an ester bond. There are three main tRNA binding sites on the ribosome, the A-, P- and E-sites. The A-site is where the correct *aminoacyl-tRNA* (aa-tRNA) is selected on the basis of the mRNA codon displayed at this site, and therefore the A-site is often referred to as the decoding site. Prior to peptide bond formation, the P-site carries the *peptidyl-tRNA*, i.e. this is the tRNA bearing the nascent polypeptide chain. The E-site binds exclusively deacylated or uncharged tRNAs, i.e. those tRNAs that have transferred the amino acid and are ready to *exit* from the ribosome.

Protein synthesis can be divided into four distinct phases: initiation, elongation, termination and recycling (Figure 1). The role of the initiation phase is to position the ribosome correctly on the mRNA so that protein synthesis initiates at the right place in the correct reading frame. The result is a 70S ribosome programmed with the start codon of the mRNA and the initiator-tRNA located at the P-site of the ribosome, a so-called 70S initiation complex (Figure 1). The elongation phase involves the movement of tRNAs in a cyclic fashion through the three tRNA binding sites (A → P → E) on the ribosome, where the number of cycles is dictated by the length of the polypeptide being synthesized. The first step in the cycle involves binding of the aa-tRNA to the A-site, which is facilitated by a protein factor EF-Tu. EF-Tu hydrolyzes GTP and dissociates from the ribosome, allowing the A-tRNA to accommodate on the large subunit (Figure 1). Peptide-bond formation proceeds, transferring the entire polypeptide chain from the peptidyl-tRNA in the P-site to the aminoacyl moiety of the A-site tRNA. Now the ribosome has a peptidyl-tRNA at the A-site and a deacylated-tRNA at the P-site. This ribosomal state is highly dynamic and the tRNAs move back and forth into so-called A/P (A/P denotes that the tRNA is in the A-site on the 30S and P-site on the 50S) and P/E hybrid states. Next, translocation of the tRNAs occurs, a process that is catalyzed by a second elongation factor, EF-G. Binding

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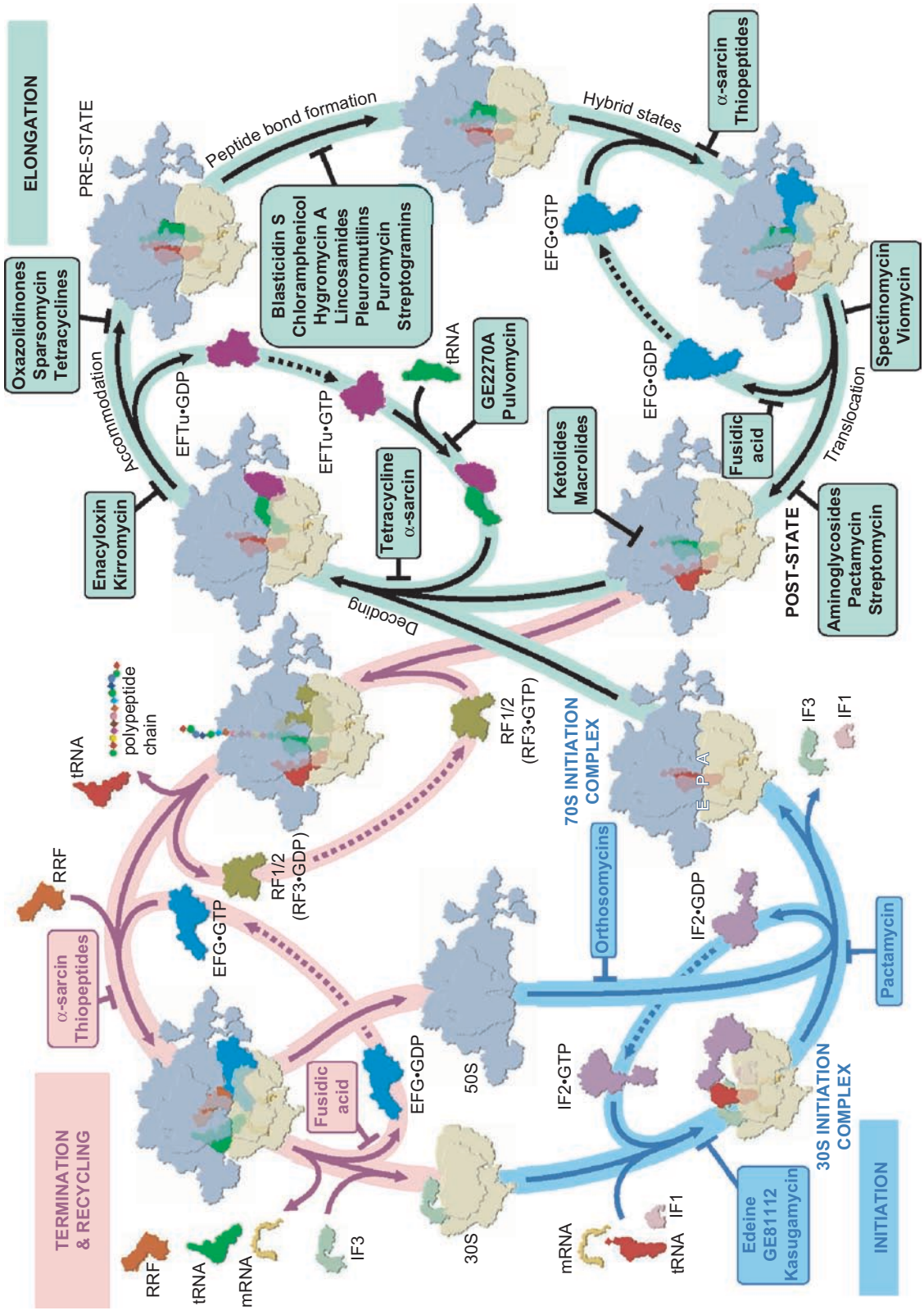

Figure 1. Sites of antibiotic action during protein synthesis. Schematic showing the sites of antibiotic action for the different stages of protein synthesis.

of EF-G to the ribosome locks the tRNAs in hybrid states and the subsequent translocation reaction shifts the peptidyl-tRNA from the A/P hybrid state to the P-site and the deacylated tRNA from the P/E to the E-site – the outcome being that the A-site is free to bind the next incoming aa-tRNA. Thus, in the course of an elongation cycle the ribosome can be thought to oscillate between two functional states: the pre- (PRE) and post-translocation (POST) states (Figure 1). When a stop signal in the mRNA enters the A-site the ribosome is then channeled into termination and recycling phases. The stop signals are recognized by protein termination factors, RF1 and RF2, which function to hydrolyze the peptidyl-tRNA bond and release the translated polypeptide chain from the ribosome. RF1 and RF2 are recycled from the ribosome by a third release factor RF3, in a GTP dependent fashion. The post-termination ribosome complexes are then split into subunits by the concerted action of EF-G and the ribosome recycling factor RRF and the components recycled for the next round of translation (Figure 1).

Protein synthesis inhibitors

As illustrated in Figure 1, protein synthesis is a major target for antibiotics. In fact, antibiotics have been identified that inhibit almost every step of translation, although with differing degrees of specificity. Large numbers of excellent reviews addressing specific classes of antibiotics (which will be specified in the relevant sections) are available; however this review article intends to provide a comprehensive overview of the large diversity of translation inhibitors that exist. This review does not intend to reiterate the huge wealth of biochemical data presented in the book chapters of Vasquez (1979) and Gale *et al.* (1981), but rather attempts to correlate the wealth of biochemical data (reviewed by Spahn and Prescott, 1996) with the more recently available ribosome structures, in order to understand antibiotic function.

The turn of the century saw the first crystal structures of antibiotics bound to the small (Brodersen *et al.*, 2000; Carter *et al.*, 2000) and large subunit (Schlünzen *et al.*, 2001). Since then most classes of antibiotics that target the ribosome have been visualized in complex with a ribosomal particle (see Table 1 and Figure 2). These structures have revealed that antibiotics predominantly target the functional centers of the ribosome, namely the tRNA-mRNA pathway on the small subunit (Figure 2A, B), peptidyl-transferase center (PTC) and adjacent exit tunnel on the large subunit (Figure 2C, D). However, there are exceptions, such as the orthosomycins (evernimicin and avilamycin) and thiopeptides (thiostrepton and micrococcin) which bind to distinct sites on the large subunit and interfere with translation factor binding. Generally, antibiotics targeting the ribosome interact predominantly, if not exclusively, with ribosomal RNA (rRNA), which reflects the fact that the functional centers of the ribosome are rich in RNA. Exceptions include the thiopeptides, streptomycins and spectinomycins, where ribosomal proteins (r-proteins) L11, S12 and S5 contribute to their respective binding sites.

Because of the importance of these functional centers for protein synthesis, the ribosomal components comprising the drug binding sites are usually highly conserved between bacteria, archaea and eukarya. This conservation explains how some compounds, such as edeine and sparsomycin, can act as universal inhibitors of translation. However the majority of the antibiotics discussed here are either bacterial- or prokaryotic-specific inhibitors, raising the question as to how kingdom specificity is attained. In some cases, the antibiotics do in fact bind to the ribosomes and inhibit translation *in vitro* across different kingdoms or species (for example, Gram-positive versus Gram-negative bacteria); however the ineffectiveness of the antibiotic *in vivo* is related to ribosome-unrelated aspects, such as poor cellular uptake. Although efflux is one of the major resistance mechanisms utilized by bacteria, a variety of other mechanisms are known, ranging from drug inactivation by modification, degradation or complex formation, to target-dilution and target-bypassing (reviewed by Walsh, 2000; Wilson, 2004). However, in terms of the ribosome, target specificity appears to be conferred by the regions surrounding the drug binding sites. This outer layer of less conserved components may also contribute to differential binding and inhibition of antibiotics across distinct bacterial species. Many examples where antibiotic resistance results from mutations or alterations in nucleotides or ribosomal proteins that do not directly interact with the drug, but rather affect nucleotides in the drug-binding site indirectly, are discussed in the following sections.

Most antibiotics that target the ribosome are bacteriostatic, which means that they inhibit growth, but do not kill the bacteria (Kohanski *et al.*, 2007 and references therein). One exception is the aminoglycoside family, which induces cell death and is therefore considered as a bactericidal class of antibiotics (reviewed by Davis, 1987). It should be noted however that some bacteriostatic antibiotics exhibit bactericidal activity when incubated at high concentrations for extended time periods (Bakker-Woudenberg *et al.*, 2005). Nevertheless, the aminoglycosides induce cell death at dramatically lower concentrations and incubation times than other translation inhibitors (see for example Bakker-Woudenberg *et al.*, 2005). The bactericidal effect of aminoglycosides is thought to result from misreading and misfolding of membrane proteins, which leads to oxidative stress and cell death (Kohanski *et al.*, 2007; 2008). This is somewhat surprising since bacterial strains are viable that

Table 1. Summary of available structures of antibiotics in complex with their targets.

Antibiotic	Class	Species[1]	Target	PDB ID	Reference
ABT-773	Ketolide	D.r	50S	1NWX	Schlünzen *et al.*, 2003
Anisomycin	PTF inhibitor	H.m	50S	1K73	Hansen *et al.*, 2003
Anisomycin	PTF inhibitor	H.m	50S	3CC4	Blaha *et al.*, 2008
Azithromycin	Azalide	D.r	50S	1NWY	Schlünzen *et al.*, 2003
Azithromycin	Azalide	H.m	50S	1M1K	Hansen *et al.*, 2002a
Azithromycin	Azalide	H.m	50S-G2099A	1YHQ	Tu *et al.*, 2005
Blasticidin S	PTF inhibitor	H.m	50S	1KC8	Hansen *et al.*, 2003
Carbomycin A	Macrolide	H.m	50S	1K8A	Hansen *et al.*, 2002a
Chloramphenicol	PTF inhibitor	D.r	50S	1K01	Schlünzen *et al.*, 2001
Chloramphenicol	PTF inhibitor	H.m	50S	1NJI	Hansen *et al.*, 2003
Clarithromycin	Macrolide	D.r	50S	1K00	Schlünzen *et al.*, 2001
Clindamycin	Lincosamide	D.r	50S	1JZX	Schlünzen *et al.*, 2001
Clindamycin	Lincosamide	H.m.	50S-G2099A	1YJN	Tu *et al.*, 2005
Dalfopristin (+ Quinupristin)	Streptogramins A (+ B)	D.r	50S	1SM1	Harms *et al.*, 2004
Edeine A	Edeine	T.t	30S	1I95	Pioletti *et al.*, 2001
Enacyloxin IIa	Enacyloxin	T.a	EF-Tu	2BVN	Parmeggaini and Nissen, 2006
Enacyloxin IIa	Enacyloxin	T.a	EF-Tu-tRNA	1OB5	Parmeggaini and Nissen, 2006
Erythromycin	Macrolide	D.r	50S	1JZY	Schlünzen *et al.*, 2001
Erythromycin	Macrolide	H.m	50S	1YI2	Tu *et al.*, 2005
Fusidic acid	Fusidic acid	E.c	EF-G-70S	2BCW. 1JQS	Agrawal *et al.*, 2001
GE-2270A	Thiopeptide	T.t	EF-Tu	2C77	Parmeggaini *et al.*, 2006
GE-2270A	Thiopeptide	E.c	EF-Tu	1D8T	Heffron and Jurnak, 2000
Gentamicin	Aminoglycoside	E.c	50S (70S)	2QBA-C/9	Borovinskaya *et al.*, 2007a
Gentamicin (+ RRF)	Aminoglycoside	E.c	50S (70S)	2QBH-K	Borovinskaya *et al.*, 2007a
Hygromycin B	Aminoglycoside	T.t	30S	1HNZ	Brodersen *et al.*, 2000
Hygromycin B	Aminoglycoside	E.c	30S (70S)	3DF1-4	Borovinskaya *et al.*, 2008
Kasugamycin	Kasugamycin	E.c	30S (70S)	1VS5-8	Schuwirth *et al.*, 2006
Kasugamycin	Kasugamycin	T.t	30S	2HHH	Schlünzen *et al.*, 2006
Kirromycin	Kirromycin	E.c	EF-Tu-70S	3FIC, 3FIN	Schuette *et al.*, 2009
Kirromycin	Kirromycin	E.c	EF-Tu-tRNA	1OB2	Not published
Kirromycin (Aurodox)	Kirromycin	E.c	EF-Tu	1HA3	Vogeley *et al.*, 2001
Linezolid + CC-Puro	Oxazolidinone	H.m	50S	3CPW	Ippolito *et al.*, 2008
Linezolid	Oxazolidinone	D.r	50S	3DLL	Wilson *et al.*, 2008
Micrococcin	Thiopeptide	D.r	50S	2ZJQ	Harms *et al.*, 2008
Neomycin	Aminoglycoside	E.c	50S (70S)	2QAL-O	Borovinskaya *et al.*, 2007a
Nosiheptide	Thiopeptide	D.r	50S	2ZJP	Harms *et al.*, 2008
Pactamycin	Pactamycin	T.t	30S	1HNX	Brodersen *et al.*, 2000
Paromomycin	Aminoglycoside	T.t	30S	1IBK, 1FJG[3]	Ogle *et al.*, 2001; Carter *et al.*, 2000
Paromomycin (+ ASL^{Phe}/U_6-mRNA)	Aminoglycoside	T.t	30S	1IBL	Ogle *et al.*, 2001
Paromomycin (+ ASL^{leu2}/U_6-mRNA)	Aminoglycoside	T.t	30S	1N32	Ogle *et al.*, 2002
Paromomycin (+ ASL^{Ser}/U_6-mRNA)	Aminoglycoside	T.t	30S	1N33	Ogle *et al.*, 2002
Pulvomycin	EF-Tu inhibitor	T.t	EF-Tu	2C78	Parmeggaini *et al.*, 2006
Puromycin[4a]	PTF inhibitor	D.r	50S	1NJ0	Bashan *et al.*, 2003
Puromycin[4b]	PTF inhibitor	H.m	50S	1FG0	Nissen *et al.*, 2000
Puromycin[4c]	PTF inhibitor	H.m	50S	1FFZ	Nissen *et al.*, 2000
Puromycin[4d]	PTF inhibitor	H.m	50S	1KQS	Schmeing *et al.*, 2002
Quinipristin	PTF inhibitor	H.m	50S-G2099A	1YJW	Tu *et al.*, 2005
Retapamulin	Pleuromutilin	D.r	50S	2OGO	Davidovich *et al.*, 2007
Roxithromycin	Macrolide	D.r	50S	1JZZ	Schlünzen *et al.*, 2001
SB-571519	Pleuromutilin	D.r	50S	2OGM	Davidovich *et al.*, 2007

Table 1. continued on next page.

Table 1. Continued.

Antibiotic	Class	Species[1]	Target	PDB ID	Reference
SB-280080	Pleuromutilin	D.r	50S	2OGN	Davidovich *et al.*, 2007
Sordarin	EF2 inhibitor	yeast	EF2	1N0U,	Jorgensen *et al.*, 2003
Sparsomycin	PTF inhibitor	D.r	50S	1NJN	Bashan *et al.*, 2003
Sparsomycin + ASM	PTF inhibitor	D.r	50S	1NJM	Bashan *et al.*, 2003
Sparsomycin + CCA-pcb	PTF inhibitor	H.m	50S	1M90	Hansen *et al.*, 2002b; Hansen *et al.*, 2002a
Spectinomycin	Translocation inhibitor	T.t	30S	1FJG[3]	Carter *et al.*, 2000
Spectinomycin (+ neomycin)	Translocation inhibitor	E.c	30S (70S)	2QOU/W/Y/2	Borovinskaya *et al.*, 2007b
Spiramycin	Macrolide	H.m	50S	1KD1	Hansen *et al.*, 2002a
Streptomycin	Aminoglycoside	T.t	30S	1FJG[3]	Carter *et al.*, 2000
Telithromycin	Ketolide	D.r	50S	1P9X	Berisio *et al.*, 2003a
Tetracycline	Tetracycline	T.t	30S	1HNW	Brodersen *et al.*, 2000
Tetracycline	Tetracycline	T.t	30S	1I97	Pioletti *et al.*, 2001
Thiostrepton	Thiopeptide	D.r	50S	3CF5	Harms *et al.*, 2008
Tiamulin	Pleuromutilin	D.r	50S	1XBP	Schlünzen *et al.*, 2004
Tiamulin	Pleuromutilin	H.m	50S	3G4S	Gurel *et al.*, 2009
Troleandromycin	Macrolide	D.r	50S	1OND	Berisio *et al.*, 2003b
Tylosin	Macrolide	H.m	50S	1K9M	Hansen *et al.*, 2002a
Virginiamycin M + S	Streptogramins A + B	H.m	50S	1YIT	Tu *et al.*, 2005

[1] T.t, T.a, D.r and H.m correspond with the thermophilic bacteria *Thermus thermophilus* or *T. aquaticus*, the radiation resistant eubacterium *Deinnoccus radiodurans* and the archaebacterium *Haloarcula marismortui*, respectively.

[2] Protein data bank (pdb) files for each antibiotic complex can be downloaded at http://www.rcsb.org/pdb/ and easily viewed with rasmol (http://www.bernstein-plus-sons.com/software/rasmol_2.7.7/ (Sayle and Milner-White, 1995)), swiss-pdb-viewer (http://www.expasy.ch/spdbv/ (Guex and Peitsch, 1997), VMD (http://www.ks.uiuc.edu/Research/vmd/ (Humphrey et al., 1996)) or PyMOL (http://www.pymol.org/).

[3] These antibiotics have the same pdb number since their structures were determined at the same time by soaking crystals in a solution containing a mixture of all three antibiotics, streptomycin, paromomycin and spectinomycin.

[4a] Puromycin is in the form of ACC-puromycin; [4b] puromycin attached to a 13 bp minihelix and thus mimics a tyrosyl-tRNA acceptor stem; [4c] puromycin in the form of an analog of A-site aa-tRNA and P-site peptidyl-tRNA covalently linked by the tetrahedral carbonyl carbon intermediate during peptide bond formation (Yarus inhibitor); [4d] the products of the PTF reaction where the A-site has CCA and the P-site contains puromycin in the form of CC-puromycin-phenylalanine-caproic acid-biotin.

harbor mutations of the ribosomal proteins S4 and S5, which cause comparable levels of misincorporation as aminoglycosides (Zimmermann *et al.*, 1971).

A large number of antibiotics have been proposed to act as inhibitors of ribosomal subunit assembly in addition to their well characterized role as protein synthesis inhibitors (reviewed by Champney, 2006). Recently, there has been a suggestion that the assembly defects are secondary effects that result from inhibition of translation instead of direct effects on assembly (Siibak *et al.*, 2009). The argument is that when translation is inhibited then production of ribosomal proteins is prevented whereas transcription continues, leading to an excess of rRNA over ribosomal proteins. This non-physiological imbalance between rRNA and r-proteins has been proposed to lead to the observed defects in ribosomal assembly, at least in the presence of the antibiotics erythromycin and chloramphenicol (Siibak *et al.*, 2009).

Translation initiation inhibitors

Canonical translation initiation by bacterial ribosomes operates through a pre-initiation complex, consisting of the small 30S ribosomal subunit, mRNA, the initiator fMet-tRNA and three initiation factors, IF1, IF2 and IF3 (Figure 1). Association of the pre-initiation complex with the large ribosomal subunit releases the remaining IFs, leaving the initiator tRNA at the P-site base-paired with the start codon. The A-site is free and ready for binding of the next aminoacyl-tRNA dependent upon the codon displayed at the A-site (reviewed by Gualerzi and Pon, 1990) (Figure 1). There are at least five classes of antibiotics that are commonly referred to as translation initiation inhibitors and these are discussed here: kasugamycin, edeine, pactamycin, and GE81112 which bind to the 30S subunit (Figure 2A, B), and the orthosomycins (evernimicin and avilamycin) that bind to the 50S subunit (see Figure 4 later on).

Kasugamycin prevents translation of canonical mRNAs

Kasugamycin (Ksg) is an antibiotic produced by *Streptomyces kasugiensis* that exhibits activity against a wide variety of microorganisms, but has low toxicity against plants, humans, fish and animals. Ksg is used commercially as the fungicide Kasumin® to control

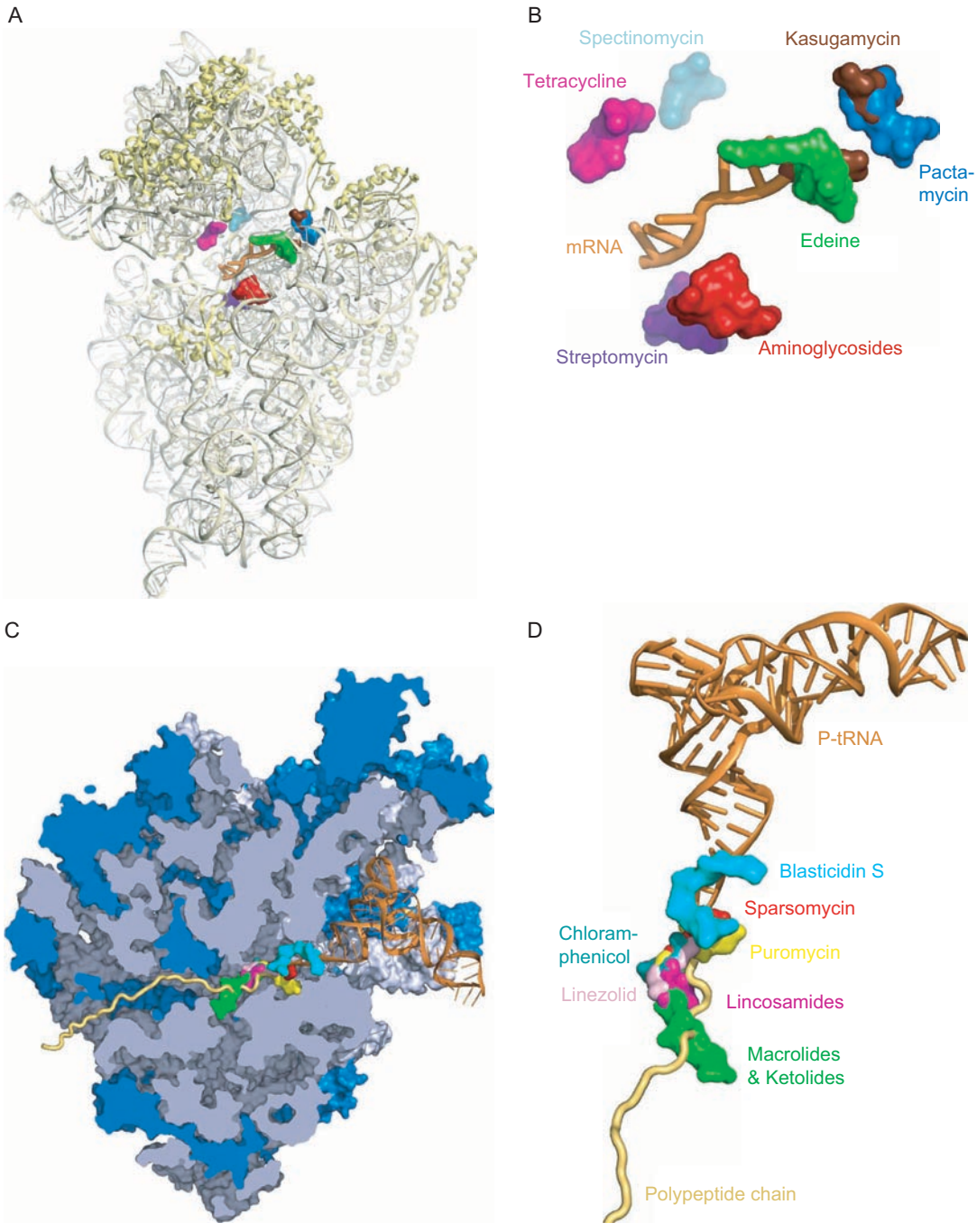

Figure 2. Binding positions of antibiotics on the ribosome. (A, B) Superimposition of binding sites of antibiotics (colored) that target the 30S subunit (yellow). The position of the mRNA (orange) in the A- and P-site is shown for reference. (C, D) Superimposition of binding sites of antibiotics (colored) that target the large subunit (rRNA, grey; ribosomal proteins, blue). (C) represents a transverse section of the subunit to reveal the ribosomal tunnel. The position of the P-tRNA (orange) and the path of the nascent polypeptide chain (tan) are shown for reference.

rice blast infestation. The primary binding site of kasugamycin (Ksg1) is at the top of helix 44 (h44; small "h" denotes helix of 16S rRNA) on the 30S subunit (Figure 3A, B), where the drug forms hydrogen bonding interactions from the D-inositol (ring I) to nucleotide G926 (*E. coli* numbering is used throughout this review) of h28 (Schlünzen *et al.*, 2006; Schuwirth *et al.*, 2006), as well as from the tail of Ksg to A792 and A794 of h23 (Figures 3B, 3C). This binding site is in agreement with earlier studies demonstrating that binding of Ksg to ribosomes protects these same bases from chemical probing (Woodcock *et al.*, 1991). A secondary binding

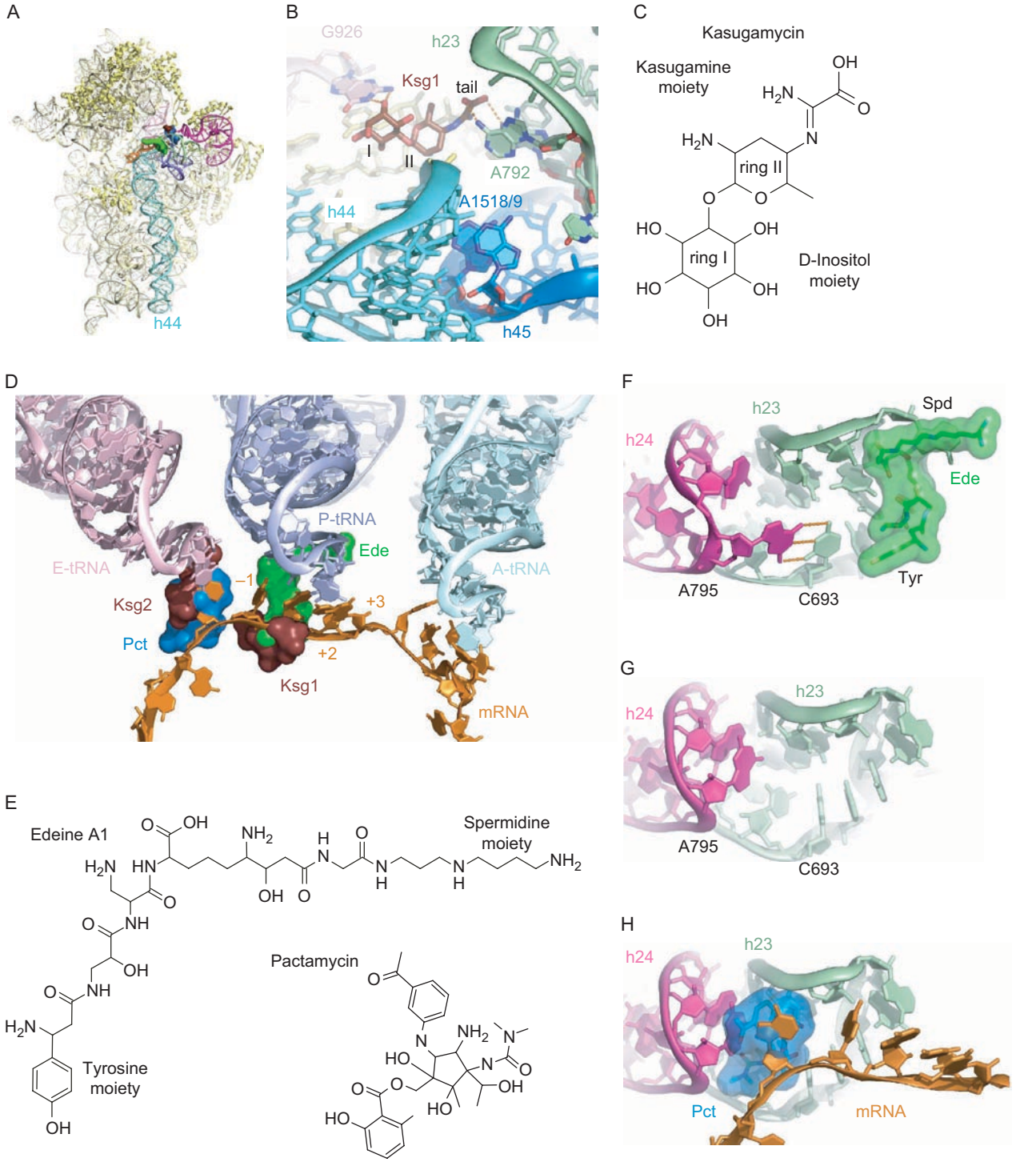

Figure 3. Initiation inhibitors that bind to the small subunit. (A) Superimposition of the binding sites of kasugamycin (burgundy), edeine (green), and pactamycin (blue) on the 30S subunit. Helices 23 (pale green), 24 (magenta), 28 (pink), 44 (cyan) and 45 (blue) are colored for reference. (B) The primary kasugamycin (Ksg) binding site showing possible hydrogen bonds between ring I of Ksg and G926 (pink) as well as the tail of Ksg and A792/A794 in helix 23 (h23, pale green). (C) Chemical structure of kasugamycin, showing D-inositiol and kasugamine moieties. (D) Relative location of mRNA (orange), A- (cyan), P- (pale blue) and E-tRNAs (pink) to the binding positions of edeine (Ede, green), pactamycin (Pct, blue) and kasugamycin (Ksg1/2, burgundy). (E) Chemical structures of edeine (isomer A1), with spermidine and tyrosine moieties highlighted, and pactamycin. (F–H) Conformations of h23 and h24 in the (F) presence of edeine (green), (G) native 30S structure, or (H) bound with pactamycin (blue). In (F), dashed lines indicate hydrogen bonding induced between C693 in h23 and A795 in h24 upon edeine binding. In (H), the relative position of the mRNA (orange) is also indicated.

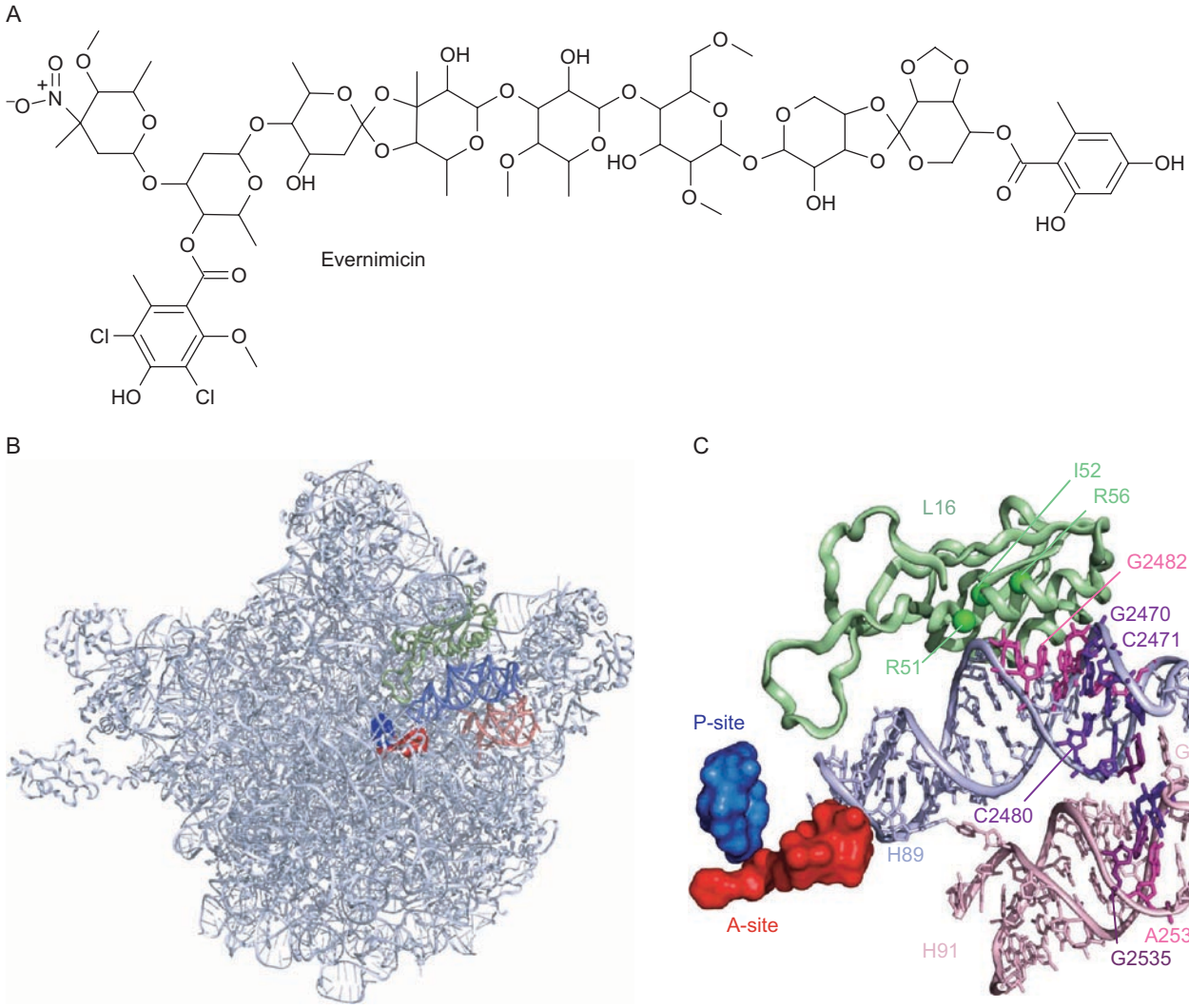

Figure 4. Orthosomycins bind to a distinct site on the large ribosomal subunit. (A) Chemical structure of evernimicin. (B) Overview of the large ribosomal subunit (light blue), with the regions associated with binding of the orthosomycins evernimicin and avilamycin highlighted, namely ribosomal protein L16 (green), 23S rRNA helices H89 (blue) and H91 (pink). The positions of CCA-ends of A and P-site tRNAs are shown in red and dark blue, respectively. Chemical structure of evernimicin. (B) Overview of the C) Zoom of (B) with positions in L16 (green spheres) and H89/91 (pink) associated with resistance. Nucleotides protected from chemical attack by orthosomycins are shown in magenta, with dark purple indicating both resistance and protection sites.

site (Ksg2) was also found in *T. thermophilus* located in the E-site (Figure 3D). The relevance of this site is unclear, especially since mutations at A794 or G926 in the primary site are alone sufficient to confer high level Ksg resistance (Vila-Sanjurjo *et al.*, 1999). Nevertheless, resistance to Ksg can arise from alterations outside the primary binding site: disruption of the *ksgA* gene, which encodes a methytransferase responsible for methylation of two universally conserved adenine residues, A1518 and A1519, located in h45 near the 5′ end of 16S rRNA, leads to low-level resistance (reviewed by Gale *et al.*, 1981). Additionally, modest Ksg resistance is also observed when A1519 (but not A1518) is mutated (Vila-Sanjurjo *et al.*, 1999). Although A1518 and A1519 do not directly comprise the Ksg binding site, h45 makes intimate contact with h24 and h44, which in turn directly contact the drug (Figure 3B). Therefore, it seems likely that conformational changes within h45 would indirectly influence drug binding. Indeed, methylation has been shown to dramatically influence the conformation of RNA mimics of h45 (Rife and Moore, 1998) and may even lead to completely alternative conformations for the h45 stem-loop (Micura *et al.*, 2001). Moreover, deletion of *ksgA* leads to assembly defects, indicating the importance of KsgA and methylation for efficient small subunit biogenesis (Connolly *et al.*, 2008).

Ksg1 and Ksg2 are located within the path of the mRNA, with the primary site overlapping the position of the first nucleotide (+1) of the P-site codon and the

last nucleotide (–1) of the E-site codon and Ksg2 being located in the E-site (Figure 3D). This led to the suggestion that Ksg inhibits the binding of the initiator fMet-tRNA^{fMet} to the prospective P-site on the 30S subunit indirectly, i.e. via perturbing the path of the mRNA (Schlünzen *et al.*, 2006) (Figure 1). Curiously, however, Ksg does not inhibit translation of leaderless mRNAs (i.e. mRNAs that have a short or absent 5′ untranslated region, and thus initiate translation directly at the 5′ AUG) at concentrations where translation of canonical mRNAs is abolished (Chin *et al.*, 1993; Moll and Bläsi, 2002). This observation was consistent with the suggestion that translation of leaderless mRNAs utilizes a 70S-type initiation mode, rather than passing through a 30S pre-initiation complex as occurs for canonical mRNAs (Balakin *et al.*, 1992; Moll *et al.*, 2004). Translation of leaderless mRNAs in the presence of Ksg *in vivo* and *in vitro* leads to the formation of 61S particles, which contain the full complement of 50S ribosomal proteins, but are missing specific 30S ribosomal proteins (Kaberdina *et al.*, 2009). These particles do not appear to derive from an alternative (or defective) assembly pathway, but rather result from the spontaneous loss (or release) of specific proteins from mature 70S ribosomes (Kaberdina *et al.*, 2009). The ability of an antibiotic to reprogram the ribosome to preferentially translate a specific subset of (leaderless) mRNAs may have implications for prebiotic translation regulation (Davies, 1990), with the reduced ribosomal particle possibly providing us with a novel glimpse of a translational relic.

Edeine prevents binding of initiator-tRNA to the small subunit

The edeine class of antibiotics are produced by the bacterium *Bacillus brevis* Vm4 and display activity against both Gram-positive and Gram-negative bacteria, as well as yeast, moulds and *Mycoplasma* sp. (reviewed by Gale *et al.*, 1981). The edeines are pentapeptide amides; the active isomer α of edeine A (Ede) has an N-terminal β-tyrosine (β-Tyr) residue linked to a C-terminal spermidine-like (Spd) moiety by glycine and three non-protein amino acids (Figure 3E). Ede binds to a single site on the 30S subunit located on the solvent side of the platform, spanning between h23, h44 and h45 (Pioletti *et al.*, 2001) (Figure 3A). Binding of Ede induces base-pair formation between G693 and C795 at the tips of h23 and h24, respectively (Pioletti *et al.*, 2001) (compare Figures 3F and 3G), in agreement with the observation that Ede protects these same two bases from chemical probing (Woodcock *et al.*, 1991). G693 and C795 are also protected from chemical probing when tRNA-mRNA is bound to the ribosomal P-site (Moazed and Noller, 1987b). On the basis of the available crystal structures of 70S ribosomes bound with tRNAs at A-, P- and E-sites (Yusupov *et al.*, 2001; Selmer *et al.*, 2006), the protection of G693 and C795 is most likely due to mRNA in the E-site, rather than direct protection by the presence of the P-tRNA. Consistently, Ede has been shown *in vitro* to specifically inhibit mRNA-directed binding of aminoacyl-tRNAs to both bacterial 30S subunits and 70S ribosomes, whereas binding of aminoacyl-tRNAs to the P-site of 70S ribosomes in the absence of mRNA remains unaffected (Dinos *et al.*, 2004). Although the spermidine-like moiety of Ede overlaps to some extent with the position of the anticodon stem loop of a P-tRNA (Figure 3D), the available data support a more indirect mode of inhibition for Ede: Formation of the C795-G693 base-pair resulting from Ede binding would perturb the path of the mRNA, preventing the correct positioning of the start codon at the P-site and thus precluding the binding of the initiator-tRNA. Indeed, the predominant contacts between the P-tRNA and the 30S subunit are due to codon–anticodon interactions (Yusupov *et al.*, 2001; Schäfer *et al.*, 2002), suggesting that the mechanism of action of Ede is to prevent a stable interaction between start codon of the mRNA and anticodon of the initiator-tRNA at the P-site (Figure 1). Further support for the P-site specificity of Ede inhibition comes from the observation that Ede inhibits the binding of the encephalomyocarditis virus internal ribosome entry site (IRES) to the ribosome, but not that of the cricket paralysis virus IRES, since the former initiates out of the P-site using Met-tRNAi and eIF2-GTP, whereas the latter initiates directly out of the A-site (Wilson *et al.*, 2000). Nevertheless, Ede appears to exert an influence on the A-site as well, since Ede can induce translational misreading at levels approaching those of the classic misreading antibiotic streptomycin (Dinos *et al.*, 2004). The ability of Ede bound in the E-site to induce misincorporation at the A-site, supports a link between E-site and translational fidelity (Geigenmüller and Nierhaus, 1990; reviewed by Wilson and Nierhaus, 2006).

Pactamycin inhibits the first translocation reaction

Pactamycin (Pct, Figure 3E) was isolated from a fermentation broth of *Streptomyces pactum* var *pactum* as a potential anti-tumor agent (Bhuyan, 1967). Pct was subsequently shown to be equally effective against intact cells of both bacteria and eukaryotes, thus limiting its clinical use as an antimicrobial agent. Pct has been reported to be an inhibitor of translation initiation as well as elongation. In eukaryotes, initiation appears to be more sensitive to the drug than elongation, and the action of Pct on initiation depends on the presence of initiation factors. Pct permits binding of the initiator-tRNA to the small subunit, but either prevents joining of the large subunit, or allows subunit joining but

the resulting initiation complex is non-functional for elongation (reviewed by Gale *et al.*, 1981). Under other conditions, di- and tripeptidyl-tRNAs accumulate, indicating that Pct inhibits an early elongation step. Indeed, in bacteria, Pct stabilizes polysomes *in vivo* and *in vitro* (Gale *et al.*, 1981). Pct binds to the small 30S (or 40S) subunit, as well as an empty 70S (or 80S) ribosome, but not to the 50S subunit, nor to mRNA-containing 70S ribosomes (Gale *et al.*, 1981). The crystal structure of the 30S subunit in complex with Pct reveals a single binding site located between h23 and h24 (Figure 3A) (Brodersen *et al.*, 2000). Bound in this position, Pct mimics mRNA in the E-site, leading to the proposal that Pct disrupts the path of the mRNA through the ribosome (Brodersen *et al.*, 2000) (Figure 3H). Indeed, a systematic study analyzing the effect of Pct on each step of bacterial initiation and elongation (Dinos *et al.*, 2004) could find no inhibitory effect of Pct during the initiation stage of translation, subunit association nor on A-site binding, instead, translocation was shown to be inhibited (Figure 1). Curiously, the translocation inhibition appeared to be markedly influenced by the type of mRNA-tRNA complex, such that translocation of Met-tRNA, Val-tRNA or Lys-tRNA was dramatically inhibited yet when Phe-tRNA was present, little or no inhibition was observed (Dinos *et al.*, 2004). Consistently, Pct had no effect on poly(U)-dependent poly(Phe) synthesis, but severely inhibited poly(A)-dependent poly(Lys) (Dinos *et al.*, 2004). The IC_{50}s for translocation inhibition and poly(Lys) formation were < 0.5 µM and ~ 3 µM, respectively, indicating that the concentrations are physiologically relevant (Dinos *et al.*, 2004). Therefore pactamycin should be considered as both an initiation and elongation inhibitor.

The orthosomycins evernimicin and avilamycin inhibit IF2-dependent subunit joining

The orthosomycins, such as evernimicin (Evn) and avilamycin (Avn), are oligosaccharide antibiotics (Figure 4A) that exhibit excellent activity against a broad range of Gram-positive bacteria both *in vivo* and *in vitro*. Evn was isolated from *Micromonospora carbonaceae* and developed by Schering-Plough under the name Ziracin for the treatment of serious infections caused by vancomycin-resistant *Enterococci faecalis* and methicillin-resistant *Staphylococcus aureus* (MRSA). In 2000, phase III clinical trials on Evn were discontinued and subsequent studies revealed that Ziracin caused anomalies of the external genitalia in F1 female rats and decreased reproductive performance (Poulet *et al.*, 2005). Avilamycin is produced by cultures of the organism *Streptomyces viridochromogenes* strain NRRL 2860, and is used as a growth promoter in animal feeding, which limits its clinical usefulness. Mutations in ribosomal protein L16 (Aarestrup and Jensen, 2000; Adrian *et al.*, 2000b; McNicholas *et al.*, 2001; Zarazaga *et al.*, 2002) as well as in helix 89 (H89; large "H" denotes helix of 23S rRNA) and H91 of the 23S rRNA (Adrian *et al.*, 2000a; Belova *et al.*, 2001; Kofoed and Vester, 2002) can confer resistance to Evn and Avn, suggesting that both antibiotics bind to overlapping sites on the large ribosomal subunit (Figures 4B, 4C). The binding site of orthosomycins at the base of the ribosomal stalk, is far (~50 Å) from the PTC (and tunnel) – the "hotspots" for antibiotic interference on the 50S subunit (Figures 4B, 4C). Indeed, the orthosomycins are the only known antibiotics to interact with this region of the ribosome, thus explaining the previous observations that bacteria resistant to numerous other antibiotics exhibit no cross-resistance to Evn or Avn, and other classes of ribosomal antibiotics do not compete with Evn for ribosome binding (McNicholas *et al.*, 2000). Chemical footprinting experiments have identified multiple nucleotides, for example A2482 in H89 and A2534 in H91 that are protected by the presence of Avn and Evn (Belova *et al.*, 2001; Kofoed and Vester, 2002) (Figure 4C). Moreover, position G2470 is methylated by the plasmid-borne methyltransferase, EmtA, which confers Avn and Evn resistance (Mann *et al.*, 2001). Although the locations of H89, H91 and L16 are in close proximity to one another (Figure 4C), it is unlikely that all three comprise the drug binding site. Most probably, the orthosomycins span between the minor groove of H89 and loop region of H91, whereas mutations in L16 confer resistance indirectly via perturbation of the 23S rRNA. This is consistent with the fact that mutations in *rplP* (L16 gene) confer relatively low level resistance (MIC < 12 µg ml^{-1}), whereas higher level resistance (MIC > 256 µg ml^{-1}) is obtained by EmtA-mediated methylation or rRNA mutations (Belova *et al.*, 2001; Mann *et al.*, 2001). As a hint to the mechanism of inhibition of the orthosomycins, it is noteworthy that H89 and H91 are also associated with initiation factor IF2 function, namely, that IF2 protects nucleotides A1476 and A2478 in H89 from chemical probing (La Teana *et al.*, 2001), and mutations that perturb the interaction between H89 and H91, such as the C2475-G2529 base-pair, lead to defects in IF2-dependent initiation complex formation (Burakovskii *et al.*, 2007). More direct evidence is the observation that Evn inhibits 70S initiation complex formation in an IF2-dependent manner *in vitro*, and incubation of bacterial cells with Evn reduces the amount of 70S ribosomes (Belova *et al.*, 2001). Collectively, these data suggest that the mechanism of action of orthosomycins is to prevent the association of the pre-initiation complex with the large subunit, by perturbing the accommodation of IF2 on the 50S subunit during subunit joining (Figure 1).

GE81112 binds to the 30S subunit to inhibit initiator–tRNA binding

GE81112 represent a family of tetrapeptide antibiotics isolated from fermentation of *Streptomyces* sp. DSMZ 14386 (Brandi *et al.*, 2006b; 2006c). GE81112 antibiotics are specific inhibitors of protein synthesis *in vivo* and *in vitro*, inhibiting translation of canonical and leaderless mRNAs in *E. coli* and archaea, but having no effect on translation in yeast. The IC_{50} (1 μM) of GE81112 for inhibition of 30S initiation complex formation is the same as for inhibition of translation initiation, suggesting that the drug is specific for the initiation phase (Brandi *et al.*, 2006b). Indeed, GE81112 is far more effective at inhibiting 30S complex formation than the classical translation initiation inhibitors, such as kasugamycin, edeine or pactamycin (Brandi *et al.*, 2006b). GE81112 inhibits the binding of fMet-tRNA (and also AcPhe-tRNA) to the P-site of mRNA programmed 30S subunits (Figure 1), but in contrast to edeine also inhibits binding in the absence of mRNA. This suggests that the binding site of GE81112, at least partially, overlaps the position of the P-tRNA (Brandi *et al.*, 2006b), rather than exhibiting the inhibitory effect indirectly through perturbation of the mRNA. Footprinting experiments indicate that GE81112 protects G693 (and to a lesser extent G700) in h23 from kethoxal modification, whereas the reactivity of C795 in h24 to the chemical agent DMS increases (Brandi *et al.*, 2006b). These two nucleotides/helices are located in the platform region of the small subunit, and comprise the binding site of other translation inhibitors such as kasugamycin, edeine and pactamycin (Figure 3). It is interesting that such chemically diverse translation inhibitors utilize a limited region of the ribosome to exert their inhibitory effects (Figure 2A, 3D) – clearly, the platform, in particular h23/h24, represents a functional hotspot for translation initiation inhibitors.

Inhibitors of the elongation cycle

The elongation cycle can be thought of as the heart of protein synthesis and as such is the prime target of the majority of antibiotics targeting translation (Figure 1). For convenience, the elongation inhibitors have been divided into specific categories; however it is recognized that some antibiotics could be classified into more than one place, for example, streptomycin and the aminoglycosides are discussed here with respect to their ability to influence translational fidelity, but these antibiotics are also characterized as translocation inhibitors. It should also be pointed out that some antibiotics (good examples being the thiopeptides, such as thiostrepton, and the ribotoxins, such as α-sarcin) that inhibit the action of the elongation factors EF-G and EF-Tu, also affect other translational GTPases, such as initiation factor IF2 and release factor RF3. Similarly, because EF-G participates in ribosome recycling in addition to its role during elongation, antibiotics that inhibit the translocation function of EF-G (for example, viomycin and fusidic acid), have also been shown to inhibit recycling (Hirokawa *et al.*, 2002). Nevertheless, these antibiotics are still categorized as elongation inhibitors simply because in an actively growing cell the majority of ribosomes are going spend most of time in the elongation phase of translation, and therefore the antibiotics are more likely to trap the ribosome in an elongating state. Since there are no specific inhibitors of termination and recycling, the antibiotics that target these steps are discussed here in the context of translation elongation.

Antibiotic action on tRNA delivery to the ribosomal A-site

The first step of the elongation cycle involves A-site occupation. Binding of tRNA to the A-site can be separated into two consecutive steps: (i) an initial step involving the binding of the ternary complex aa-tRNA•EF-Tu•GTP to the ribosome, during which the tRNA is kinked to allow codon–anticodon interaction, while the CCA-end of the tRNA remains bound to EF-Tu (A/T-tRNA state); and (ii) a second step involving the hydrolysis of GTP to GDP by EF-Tu and dissociation of EF-Tu•GDP from the ribosome, which in turn releases the CCA-end of the tRNA enabling it to move from the A/T state into the A-site on the 50S subunit, i.e. A/A state. This process is termed tRNA accommodation. We consider here seven different antibiotics that use different modes to inhibit or impair delivery of the tRNA to the A-site of the ribosome. The tetracyclines prevent the stable binding of the ternary complex aa-tRNA•EF-Tu•GTP to the ribosome by directly overlapping with the anticodon stem-loop of the tRNA. In contrast, the kirromycins (and most likely the enacycloxins) trap the aa-tRNA•EF-Tu complex on the ribosome, whereas pulvomycin and GE2770A prevent ternary complex formation. The streptomycin and aminoglycoside families induce translational misreading by influencing the relative stabilities of near-cognate versus cognate tRNA at the decoding site.

Tetracycline inhibits accommodation of the A-tRNA

Tetracyclines fall into two groups: (i) atypical tetracyclines, such as chelocardin, 6-thiatetracycline and anhydrotetracycline, which are poor protein synthesis inhibitors and probably target the cytoplasmic membrane; and (ii) typical tetracyclines, such as tetracycline (Tet), which inhibit protein synthesis and are the topic of this section. Tet is produced from *Streptomyces aureofaciens* and has been used to treat a variety of bacterial infections since its introduction into medicine

around the 1950s (chlortetracycline, 1948; tetracycline, 1953). The primary binding site of tetracycline (Tet1) is located on the head of the 30S subunit (Figure 5A), where the drug interacts with the irregular minor groove of h34 and the loop of h31 (Figures 5A, 5B) (Brodersen *et al.*, 2000; Pioletti *et al.*, 2001). The binding site of Tet1 overlaps slightly with the anticodon-stem-loop of an A-tRNA (Figures 5A, 5C), consistent with the observation that Tet prevents stable binding of the ternary complex aa-tRNA•EF-Tu•GTP to the A-site of the ribosome (Figure 1), and, upon dissociation of EF-Tu•GDP, aa-tRNAs that managed to be delivered to the decoding site on the 30S subunit, cannot accommodate on the 50S subunit and are lost from the ribosome (Blanchard *et al.*, 2004). The broad spectrum of Tet action is consistent with the mode of interaction of Tet1 with the ribosome, namely that Tet1 interacts only with the sugar-phosphate backbone of nucleotides in h31 and h34, and therefore the contacts are sequence independent. Mutations that give rise to Tet resistance have been reported at position 1058 in h34, adjacent to Tet1 binding site, or result from deletion of three bases, 965–967 in h31, directly in the Tet1 binding site (Ross *et al.*, 1998; Gerrits *et al.*, 2002; Trieber and Taylor, 2002). Collectively, these results suggest that secondary Tet binding sites (Tet2, Figure 5A, as well as other three other Tet binding sites observed by Pioletti *et al.* (2001)) are not relevant for the inhibitory mechanism of the tetracyclines. There are a variety of mechanisms that give rise to Tet resistance (Chopra and Roberts, 2001), but one that is unique to tetracycline is the use of the so-called ribosome protection proteins (RPPs), such as Tet(O) or Tet(M) (reviewed in Connell *et al.*, 2003). RPPs are GTPases that bind to the ribosome analogously to EF-G and confer resistance to Tet by chasing Tet1 from the ribosome (Connell *et al.*, 2002). Indeed, the increasing incidence of bacterial resistance to the tetracyclines has led to a decline in their medical usage, which in turn has led to a new drive to find novel Tet derivatives, such as the recently developed third generation glycylcyclines (Chopra, 2002). The glycylcyclines, such as tigecycline marketed by Wyeth as Tygacil®, contain extensions at the C9 position (Figures 5D, 5E), and display enhanced antimicrobial activity, even being effective against some bacterial strains bearing *tet* resistance genes (Rasmussen *et al.*, 1994; Bergeron *et al.*, 1996), which is likely to result in part from the higher affinity for the ribosome of glycylcyclines compared to tetracyclines (Bauer *et al.*, 2004).

The kirromycins and enacyloxins trap EF-Tu on the ribosome

The ternary complex aa-tRNA•EF-Tu•GTP (Figure 6A, (Nissen *et al.*, 1995) binds initially to the A-site

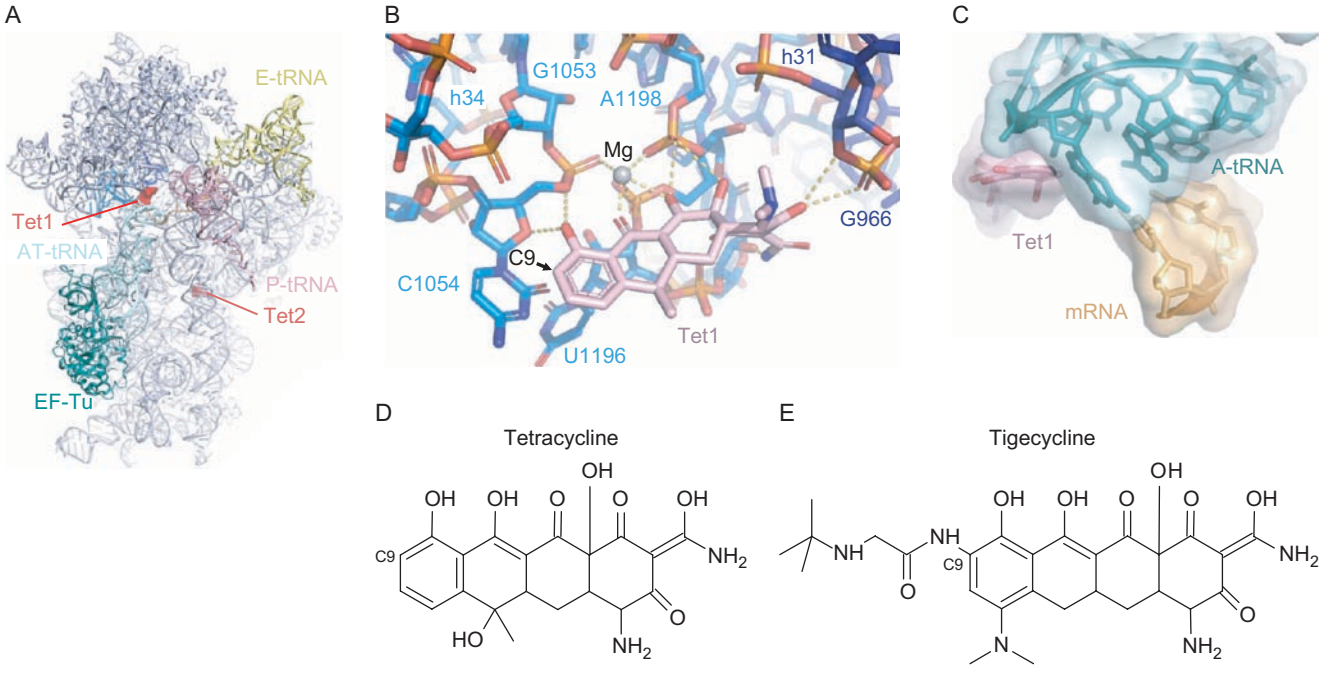

Figure 5. Tetracyclines inhibit delivery of the tRNA to the ribosomal A-site. (A) Overview of the primary (Tet1) and secondary (Tet2) tetracycline binding sites on the 30S subunit, relative to P-tRNA (pink), E-tRNA (yellow) and (AT-tRNA) bound to EF-Tu (teal). The mRNA is shown in orange and helices 31 and 34 are colored dark and light blue, respectively. (B) In the primary binding site, the charged or polar face of Tet can form hydrogen bonds (dashed lines) exclusively to the backbone of nucleotides in h34 (light blue) and h31 (dark blue) of the 16S rRNA. (C) Relative position of the primary binding site (Tet1, pink) to the A-site codon of the mRNA (orange) and anticodon of the tRNA (teal). (D, E) Chemical structures of (D) tetracycline and (E) tigecycline, with C9 positions indicated.

such that the aa-tRNA is kinked to allow simultaneously codon–anticodon interaction while remaining bound to EF-Tu (A/T state see in Figure 5A) (Stark *et al.*, 1997; Schuette *et al.*, 2009; Villa *et al.*, 2009). Correct recognition of the codon–anticodon pairing sends a signal, possibly through the tRNA, that leads to ribosome-stimulated EF-Tu-dependent GTP-hydrolysis. Cleavage of GTP causes EF-Tu to undergo a conformational change, such that domain I (the GTP binding domain) is rotated relative to domains II and III by up to 40 Å (compare Figures 6B and 6C) (Parmeggiani and Nissen, 2006). The aa-tRNA is released by EF-Tu and allowed to accommodate on the ribosome and the low affinity EF-Tu•GDP dissociates from the ribosome (Figure 1). Kirromycin and aurodox (N-methyl kirromycin) are members of a large family of compounds produced by the actinomycetes that stall the ternary complex on the ribosome, not by preventing hydrolysis of GTP to GDP, but by preventing the conformational changes in EF-Tu that are associated with GTP hydrolysis (reviewed by Hilgenfeld *et al.*, 2000; Parmeggiani and Nissen, 2006).

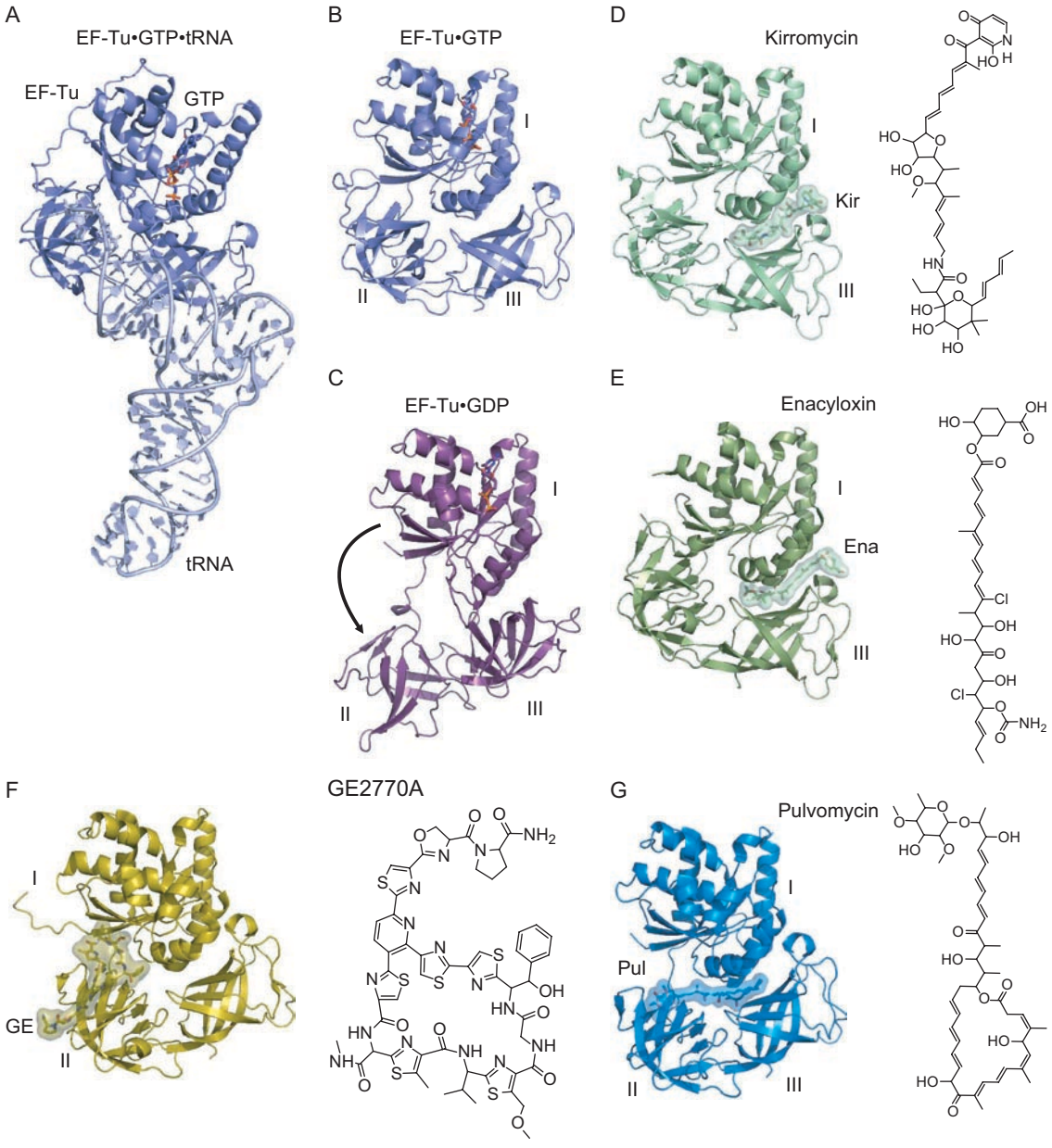

Figure 6. The binding site of GE2270A-like thiopeptides on EF-Tu. (A–G) Crystal structures of (A) EF-Tu•GTP•tRNA, (B) EF-Tu•GTP, (C) EF-Tu•GDP, (D) EF-Tu•kirromycin (E) EF-Tu•enacyloxin, (F) EF-Tu•GE2770A and (G) EF-Tu•pulvomycin. Chemical structures of (D) kirromycin, (E) enacyloxin IIa, (F) GE2270A and (G) pulvomycin are also included. The arrows in (C) indicate that relative movement of domains II and III with respect to domain I that occurs upon GTP hydrolysis.

Indeed, the ability of kirromycins to lock EF-Tu in a high-affinity state on the ribosome was utilized to visualize the stalled ternary complex-bound ribosomes by cryo-electron microscopy (Stark *et al.*, 1997), with ever-increasing resolution (Valle *et al.*, 2002; 2003; Schuette *et al.*, 2009; Villa *et al.*, 2009). The binding site of kirromycin (aurodox) is located between domains I and III of EF-Tu (Figure 6D), consistent with the many resistance mutations that locate to this interface (Wilson, 2004). The kirromycin-bound EF-Tu conformation, both on (Figure 5A) and off (Figure 6D) the ribosome, resembles closely EF-Tu•GTP (Figure 6B), suggesting that the drug probably locks domains I and III together, thereby preventing conformational switching. Mutations that confer kirromycin resistance either alter the kirromycin binding site to prevent binding of the drug to EF-Tu•GTP or allow kirromycin to bind, but decrease the affinity such that the drug dissociates from the ternary complex following GTP hydrolysis. Enacyloxin IIa is a linear polyenic acid produced by *Fratueria* W-315 (Figure 6E), which displays activity against both Gram-positive and Gram-negative bacteria (Watanabe *et al.*, 1982; 1992). Enacyloxin has a very similar binding site to kirromycin, located between domains I and III of EF-Tu (Figure 6E) (Parmeggiani *et al.*, 2006), and therefore most likely utilizes a similar mechanism of inhibition as kirromycin. Indeed, mutations that give rise to enacyloxin resistance were originally identified as kirromycin resistance mutations.

GE2270A and pulvomycin inhibit ternary complex formation

Both GE2270A and pulvomycin prevent the binding of the aa-tRNA to EF-Tu, i.e. they prevent ternary complex formation (Figure 1) (reviewed by Hogg *et al.*, 2002; Parmeggiani and Nissen, 2006), yet the antibiotics are structurally unrelated; GE2770A is a member of the cyclic thiazolyl peptide family (Figure 6F) and is thus more closely related in structural terms to the thiostreptons and microccocins, which are also inhibitors of protein synthesis, but act by binding directly to the ribosome. In contrast, pulvomycin bears some resemblance to the kirromycins (compare Figures 6G and 6D). Both GE2270A and pulvomycin have distinct (but overlapping) binding sites on EF-Tu (compare Figures 6F and 6G). GE2770A binds within a cleft of domain II, where ionic interactions with R223 and E259 contribute to the strong affinity of this antibiotic. In agreement, mutations at these two residues are associated with resistance to this antibiotic – however, it should be noted that these residues are not invariant throughout the prokaryotes, suggesting that some organisms may be naturally resistant to GE2270A, a good example being the producer of GE2770A, *Planobispora rosea*. Comparison of kirromycin•EF-Tu•GTP with the EF-Tu•GTP•tRNA suggests that the binding position of GE2770A would sterically clash with that of the amino-acyl moiety of the aa-tRNA (compare Figures 6A and 6F). This in itself explains the inhibitory action of GE2770A to prevent ternary complex formation, but in addition the binding position of GE2770A at the interface of domain II would prevent tight association with domain I, an interaction necessary to fully adopt the GTP conformation. Therefore, GE2770A can be thought as having a dual action in preventing ternary complex formation. Pulvomycin on the other hand has a binding site that encompasses all three domains of EF-Tu and resistance mutations are located at the junction between domains II and III. A striking difference between pulvomycin and GE2770A relates to their resistance phenotype; pulvomycin sensitivity was found to be dominant to resistance (Zeef *et al.*, 1994), whereas in contrast GE2770A resistance was dominant over sensitivity (Landini *et al.*, 1992; Mohrle *et al.*, 1997). This suggests that in the case of pulvomycin an additional mechanism must be operating other than simply limiting the availability of active ternary complex.

Antibiotics affecting the fidelity of translation

The fidelity of gene expression depends upon accurate transcription of the mRNA as well as accurate charging of the tRNAs with their corresponding amino acids. These two processes have been estimated to occur with an accuracy in the order of 10^{-4}–10^{-5}, whereas estimates of translational accuracy are in the range of 1×10^{-3}–10^{-4} misincorporations (see Zaher and Green, 2009a and references therein). This effectively means that for every 1000–10,000 correct amino acids introduced into a nascent polypeptide chain, it is the ribosome that is responsible for the incorporation of one single erroneous amino acid. Since the average protein in *E. coli* is ~300 amino acids, this intrinsically low rate of misincorporation ensures that almost every protein produced by the ribosome is functionally active. In contrast, the aminoglycoside and streptomycin families of antibiotics are well known for their ability to stimulate misreading, increasing the misincorporation levels by up to ~1 in 10^2. Both types of antibiotics induce misreading using distinct mechanisms: aminoglycosides, such as paromomycin, stabilize the binding of near-cognate tRNAs as well as promote their accommodation on the ribosome, whereas in contrast, streptomycins reduce the rate at which cognate tRNAs are selected and slightly enhance near-cognate tRNA binding (Karimi and Ehrenberg, 1994; Pape *et al.*, 2000). However, it should be noted that aminoglycosides are also potent translocation

inhibitors (Figure 1) (Cabanas *et al.*, 1978; Misumi *et al.*, 1978), as well as promoting back-translocation (Shoji *et al.*, 2006; Borovinskaya *et al.*, 2007b; Szaflarski *et al.*, 2008), which alone is enough to explain their potent inhibitory activity. In contrast, streptomycin has been shown to stabilize the A-tRNA (Peske *et al.*, 2004) and increase the rate of back-translocation (Shoji *et al.*, 2006), however no effect on translocation has been observed (Peske *et al.*, 2004). Indeed, there are strains with mutations in ribosomal protein S4 that impair the accuracy of protein synthesis to a similar extent as streptomycins or aminoglycosides without affecting cell viability.

Aminoglycosides are translocation inhibitors that induce misreading

Aminoglycosides that target the ribosome contain a 2-deoxystreptamine (2-DOS/ring II) group with a distinct patterns of sugar substitutions depending on the member. For example, the paromomycins and neomycins contain sugars at the C4 and C5 positions (Figures 7A, 7B), whereas gentamycin and kanamycin are 4,6 di-substituted (Figures 7C, 7D). The 4,6 di-substituted aminoglycosides are clinically preferred, with the predominant examples being gentamycin (Garamycin® introduced in the mid 1960s by Schering-Plough) and two kanamycin derivatives, tobramycin (Nebcin® marketed Eli Lilly and Company) and amikacin (marketed under the name Amikin® by Bristol-Myers Squibb). All aminoglycosides bind within an internal loop in h44 of the 30S subunit, which comprises the decoding site (Figure 7E) (reviewed by Ogle *et al.*, 2003). This has been observed biochemically by chemical probing (Moazed and Noller, 1987b; 1989; Woodcock *et al.*, 1991) as well as structurally in complexes of aminoglycosides bound to small RNA fragments mimicking h44 (Fourmy *et al.*,

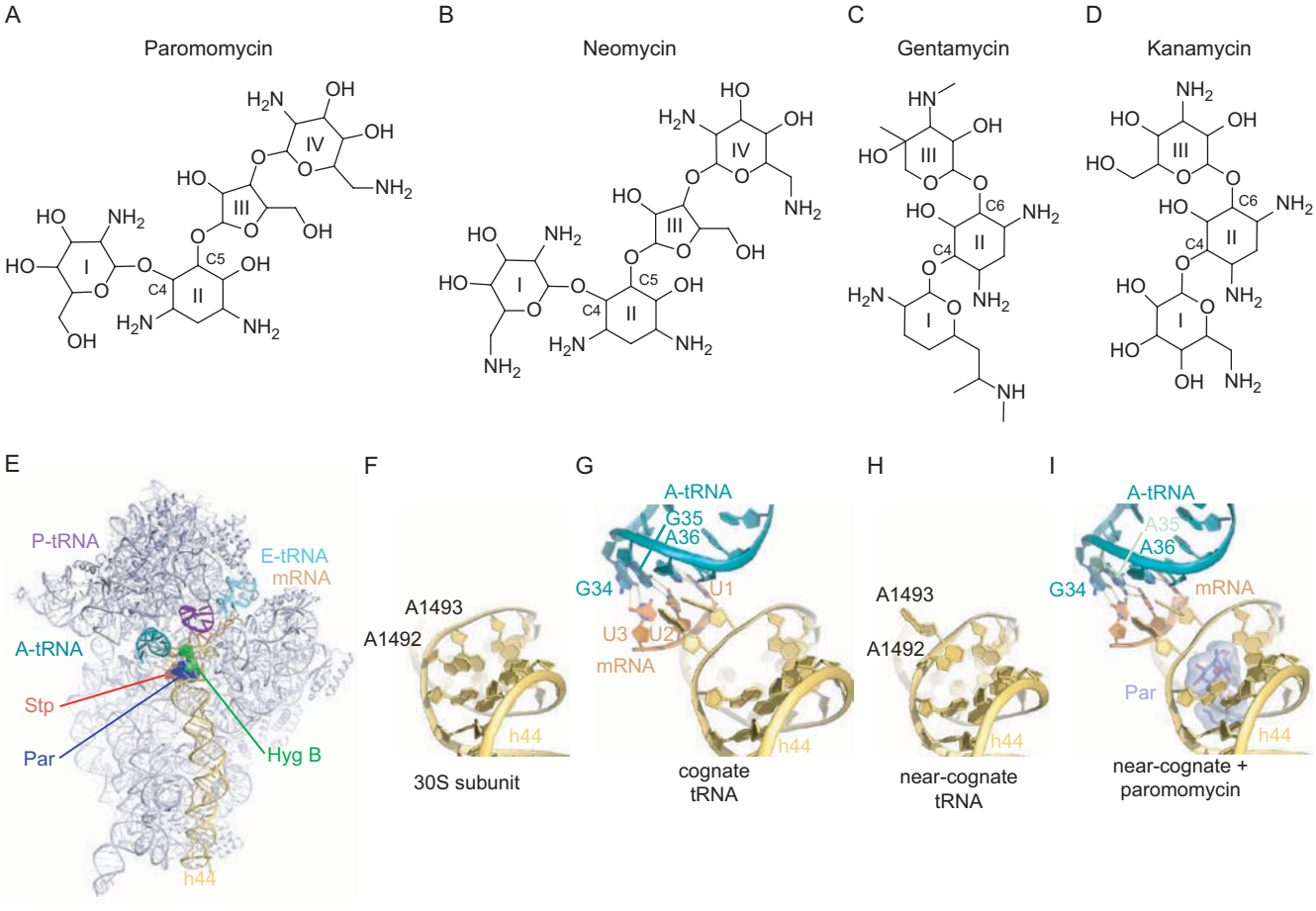

Figure 7. Aminoglycoside antibiotics induce translational misreading. (A–D) Chemical structures of aminogylcoside antibiotics (A) paromomycin, (B) neomycin, (C) gentamycin and (D) kanamycin. (E) Overview of the streptomycin (Stp, red) and aminoglycoside paromomycin (Par, blue) binding site on the 30S subunit. Helix 44 (h44, yellow), ribosomal protein S12 (light green) and the relative positions of mRNA (orange), A- (teal), P- (purple) and E-tRNA (cyan) are shown for reference. (F–I) Conformation of A1492 and A1493 in h44 in (F) the native 30S subunit, (G) the presence of mRNA (5′-UUU codon in A site) and cognate A-tRNA (anticodon 5′-GGA), (H) the presence of mRNA (5′-UUU codon in A site) and near-cognate A-tRNA (anticodon 5′-G**A**A), (I) the presence of paromomycin (Par, blue), mRNA (5′-UUU codon in A site) and near-cognate A-tRNA (anticodon 5′-G**A**A).

1996; 1998; Vicens and Westhof, 2001; Lynch *et al.*, 2003) as well as to 30S subunit (Carter *et al.*, 2000; Ogle *et al.*, 2001; 2002) or 70S ribosome (Selmer *et al.*, 2006). Furthermore, mutations within h44 confer aminoglycoside resistance and organisms that produce aminoglycosides protect themselves by having methylases that specifically modify either the drug or the 16S rRNA at nucleotides, such as G1405(N7) and A1408(N1) in helix 44 (reviewed by Pfister *et al.*, 2003; Vicens and Westhof, 2003).

During decoding, the ribosome monitors the codon–anticodon interaction to ensure that the A-tRNA is cognate to the mRNA. This monitoring involves two universally conserved nucleotides of the 16S rRNA, A1492 and A1493 (Figure 7F), which flip-out of helix 44 in the 30S subunit to analyze the minor groove of the codon–anticodon duplex (Figure 7G) (Ogle *et al.*, 2001). Presumably the energy required to flip-out A1492 and A1493 during decoding is compensated for by additional A-minor interactions established with the codon-anticodon duplex, thus stabilizing this "flipped-out" conformation (Ogle *et al.*, 2001). In the presence of near-cognate tRNA these compensatory interactions are obviously insufficient to stabilize the flipped out A1492 and A1493 and thus the near-cognate tRNA dissociates (Figure 7H). However, in the presence of paromomycin (Par), the uncompensated loss of energy is absorbed by Par since the drug has already induced A1492 and A1493 to flip-out and stabilized them in this open conformation (Figure 7I). The outcome being that a near-cognate tRNA becomes fully accommodated into the A-site, which results in mis-incorporation of an amino acid (reviewed by Ogle *et al.*, 2003). An additional binding site for aminoglycosides, such as gentamicin and neomycin, has been observed in H69 of the large ribosomal subunit, where it has been proposed to inhibit the action of the ribosome recycline factor (RRF) during the ribosome recycling phase of translation (Borovinskaya *et al.*, 2007a). However, it remains to be determined whether this secondary binding site plays a role in the inhibitory activity of aminoglycosides.

Hygromycin B (Hyg B) is an atypical aminoglycoside produced by *Streptomyces hygroscopicus* that is structurally and functionally unique compared to other aminoglycosides. Although Hyg B also binds in h44 of the 30S subunit, the location is slightly displaced towards the top of h44 when compared to the position of, for example, Par (Figure 7E) (Brodersen *et al.*, 2000; Borovinskaya *et al.*, 2008). Hyg B has only a modest effect on translational fidelity, consistent with the observation that binding of Hyg B does not induce the same conformational changes in A1492 and A1493 as seen for Par (Brodersen *et al.*, 2000; Borovinskaya *et al.*, 2008). Like Par, Hyg B is a powerful translocation inhibitor (Cabanas *et al.*, 1978; Hausner *et al.*, 1988; Peske *et al.*, 2004), but unlike Par, Hyg B also inhibits back-translocation (Borovinskaya *et al.*, 2008). Hyg B directly contacts the mRNA in the P-site (Figure 7E) and places A1493 in a position to interact with the A-site codon, suggesting that Hyg B inhibits translocation through confinement of the mRNA in the A- and P-sites (Borovinskaya *et al.*, 2008).

The interplay between streptomycin, S4, S5 and S12 for translational fidelity

Streptomycin (Stp) is structurally related to the aminoglycoside family of antibiotics (Figure 8A) and exhibits the same classical hallmark, i.e. Stp induces translational misreading (Kurland *et al.*, 1996). Stp binds to a distinct site on the ribosome (Figure 7E) and therefore mediates its inhibitory and misreading effects by an unrelated mechanism to aminoglycosides. Unlike aminoglycosides that bind in h44, Stp has a single binding site on the 30S subunit that connects helices from all four different domains of the 16S rRNA, namely h1, h18, h27 and h44, and makes interactions with r-protein S12 (Figure 8B). Selection of the correct or cognate tRNA by the ribosome results in domain closure of the 30S subunit, where the head and platform close in on the A-tRNA (Ogle *et al.*, 2002) (arrowed in Figure 8C). The domain closure induced by Stp binding (Ogle *et al.*, 2002) has been proposed to reduce the rate of GTPase activation for cognate tRNAs, while slightly enhancing the rate for near-cognate tRNAs, such that the overall rates are similar and rate-limiting (Gromadski and Rodnina, 2004), thus explaining the high translational misreading that Stp induces.

A number of the mutations in the r-protein S12 that confer resistance to, and in some cases even dependence on, Stp map within the loop of S12 that directly contacts the drug (Figure 8B) (reviewed by Kurland *et al.*, 1996). Of these mutations, only position Lys42 directly interacts with Stp (Figure 8B), suggesting that the other mutations confer resistance indirectly by altering in the loop conformation. Mutations in other r-proteins, mainly S4 and S5, were found to reverse the Stp-dependent phenotype of the S12 mutations (Kurland *et al.*, 1996) (Figure 8C, 8D). With the exception of the K42R resistance mutant, which does not alter translation accuracy, ribosomes bearing the S12 mutations are hyper-accurate in tRNA selection, i.e. they restrict errors. In contrast, the S4 or S5 mutants are error-prone and termed ribosomal ambiguity mutants (*ram*). Transition into the closed form involves (i) disruption of multiple interactions at the interface between S4 and S5 and (ii) establishment of salt bridge interactions between S12 and either h44 or h27 of the 16S rRNA. Mutations in S4 and S5 that promote formation of the *ram* state have been proposed to partially induce the closed state, although recent data suggests that this does not necessarily correlate with disruptions at the S4-S5 interface (Vallabhaneni and

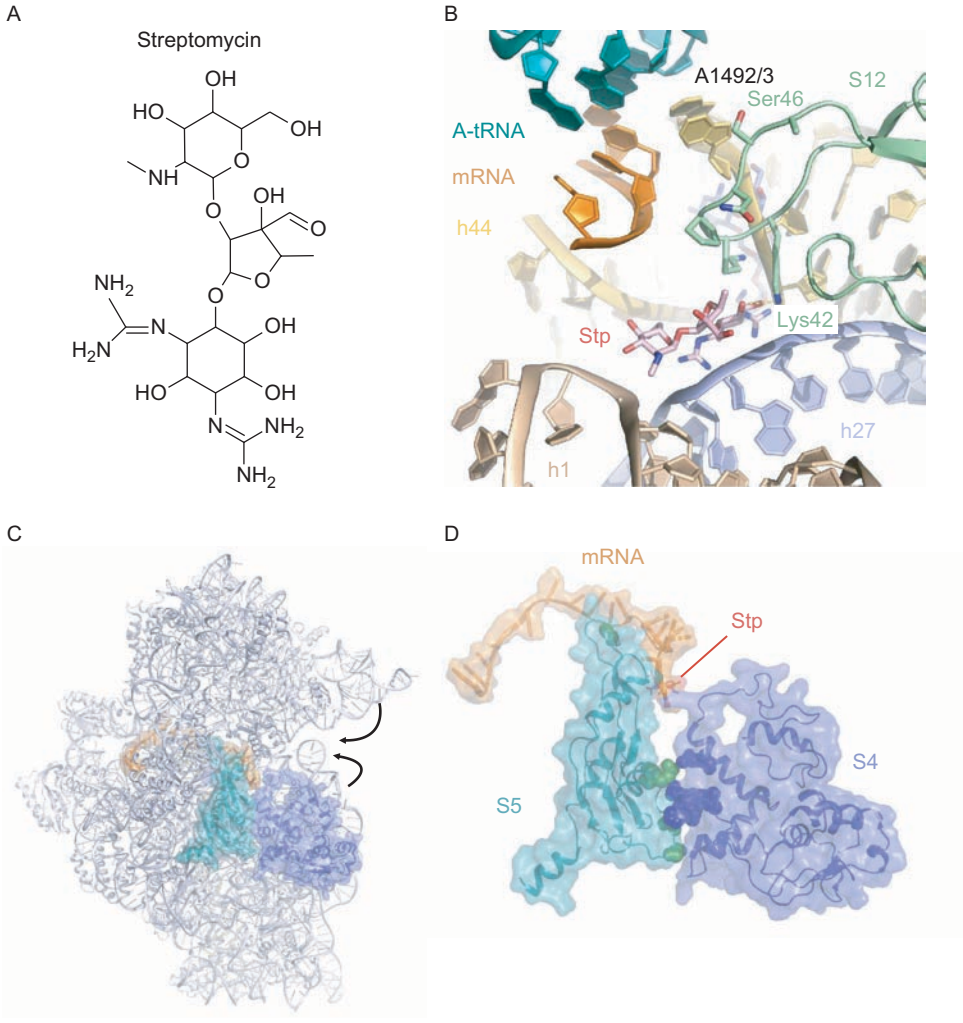

Figure 8. Streptomycin and translational fidelity. (A) Chemical structure of streptomycin. (B) Detailed view of the streptomycin binding site. Streptomycin (red) interacts exclusively with sugar-phosphate backbone of the 16S rRNA and in doing so locks together all four of the 16S rRNA domains, namely, the 5′domain (h1, tan), the central domain (530 loop, not shown), 3′ major domain (h27, light blue) and 3′ minor domain (h44, yellow). Lysine 41 of S12 interacts with ring I of streptomycin. (C) Solvent view of the 30S subunit, with ribosomal proteins S4 (blue), S5 (teal) and mRNA (orange) highlighted. (D) Zoom of the interface between S4 and S5, with *ram* mutations indicated by space-filling, and relative location of streptomycin (Stp, red).

Farabaugh, 2009). In contrast, mutations in S12 that block salt-bridge formation may destabilize the closed form and thus confer resistance (or in some cases even dependence on the drug). The antagonistic effects of the S4/S5 *ram* mutants to destabilize the closed form while the S12 streptomycin resistance mutants stabilize it, rationalizes the compensatory effects observed on translational fidelity.

Inhibitors of peptide bond formation and nascent chain progression

The central enzymatic function of the ribosome is peptidyl-transferase, i.e. formation of peptide bonds, the active site for which is located on the large ribosomal subunit (reviewed by Moore and Steitz, 2003; Polacek and Mankin, 2005; Beringer and Rodnina, 2007; Simonovic and Steitz, 2009). Peptide bond formation involves nucleophilic attack by the α-amino group of the aminoacyl-tRNA bound in the A-site on the carbonyl-carbon of the peptidyl-tRNA located in the P-site, which resolves to give a peptidyl-tRNA in the A-site (elongated by one amino acid) and a deacylated (or uncharged) tRNA in the P-site. Put simply, the entire nascent polypeptide chain is transferred from the P-tRNA to the incoming amino acid of the A-tRNA during each elongation cycle. This contrasts with transcription where incoming nucleotides are added to the transcribed mRNA. The importance of this distinction is that if an incorrect nucleotide is misincorporated into the mRNA, then it can simply be deleted and replaced with the correct one, whereas if an incorrect amino acid is misincorporated into the

nascent polypeptide chain, then the entire chain must be aborted and translation must restart from the beginning. Such a proofreading mechanism has been shown to exist in bacteria, where termination release factors recognize and bind to the ribosome (even though a sense and not a stop codon is displayed at the A-site!) bearing the misincorporated aa-tRNAs, hydrolyzing and releasing the error-containing nascent polypeptide chain from the ribosome (Zaher and Green, 2009a; 2009b). As the nascent polypeptide chain is being elongated, it passes through a tunnel in the large ribosomal subunit. For many years this was thought to be a passive process, but accumulating evidence suggests that some nascent polypeptide chains can interact with the components of the tunnel wall (Lu and Deutsch, 2008), which can have implications for initial protein folding events (Lu and Deutsch, 2005; Kosolapov and Deutsch, 2009) as well as translation regulation (reviewed by Tenson and Ehrenberg, 2002).

This review covers ten distinct classes of antibiotics that bind at the PTC and/or in the ribosomal tunnel. A number of these antibiotics bind (i) within the A-site of the PTC, such as the puromycins, chloramphenicols, anisomycins, oxazolidinones, sparsomycins, lincomycins, and some macrolides; (ii) exclusively at the P-site, such as blasticidin S; (iii) cover both the A- and P-site, for example, the pleuromutilins and streptogramin A; or (iv) within the ribosomal tunnel adjacent to the PTC, such as the macrolides and streptogramin B. A naïve view of the mode of inhibition of most of these antibiotics that bind at the PTC would be that they simply prevent the placement of the aminoacylated CCA-end (CCA-aa) of the tRNA at the respective site by steric overlap, and by doing so act as peptidyltransferase inhibitors. However, the ribosome is very dynamic and the substrates (aa-tRNAs) vary dramatically in their chemical properties, hinting that certain antibiotics may favor specific functional states of the ribosome, as has been proposed for the oxazolidinones. With respect to the macrolide antibiotics, their general mode of action has been proposed to involve preventing elongation of the nascent polypeptide chain by blocking its passage through the tunnel. In most cases, this leads to a drop-off of the peptidyl-tRNA from the ribosome (Tenson *et al.*, 2003) (reviewed by Gaynor and Mankin, 2003; Mankin, 2008).

Antibiotics binding at the A-site of the peptidyltransferase center

Puromycin mimics the 3′ end of an aminoacyl tRNA. Puromycin (Puro) is a structural mimic of the 3′-end of aminoacyl-tRNA, with the most important exception being that the aminoacyl residue is linked to the ribose via an amide bridge rather than an ester bond (Figure 9A). Analogous to an aa-tRNA, Puro binds to the A-site region of the PTC (Figure 9B), and undergoes peptidyl transfer accepting the nascent polypeptide chain from the P-tRNA and covalently linking it to the drug. Subsequently, the peptidyl-Puro dissociates from the ribosome due to its low affinity, being bound at the A-site only *via* the 3′-terminal adenine, rather than with complete tRNA or CCA-end. Furthermore, should the peptidyl-Puro be transferred to the P-site by rebinding, translation cannot continue because Puro has an amide bridge that cannot be cleaved by the incoming aa-tRNA. Puromycin is produced by *Streptomyces alboniger* and inhibits growth across all three kingdoms. For this reason, the drug is not used clinically, but has been an important tool for studying the peptidyltransferase reaction. In fact, the classical definitions of A- and P-site are based on the inability or ability, respectively, of aa- or peptidyl-tRNAs to react with Puro. A series of CC-Puro analogs mimicking substrates (Figure 9C) (Hansen *et al.*, 2002b), transition intermediates (Nissen *et al.*, 2000) as well as post-peptide bond formation products (Schmeing *et al.*, 2002) have been visualized bound to the ribosome (Table 1), providing an atomic understanding of the mechanism of peptide bond formation (Schmeing *et al.*, 2005a; 2005b) (reviewed by Polacek and Mankin, 2005; Steitz, 2008; Simonovic and Steitz, 2009).

Hygromycin A binds at the A-site of the PTC. Hygromycin A (HygA) is a secondary metabolite produced by the soil bacterium *Streptomyces hygroscopicus* NRRL 2388 (Pittenger *et al.*, 1953). Although it was identified in the 1950s, it was not until 30 years later that HygA was shown to target the ribosome by inhibiting the peptidyltransferase reaction as well as blocking the binding of chloramphenicol to the ribosome (Guerrero and Modolell, 1980). More recently, chemical footprinting studies have shown that HygA competes for binding with 16-membered macrolide antibiotics bearing disaccharides attached the C5 position of the lactone ring (Poulsen *et al.*, 2000). Structure–activity relationships have indicated that the aminocyclitol moiety at one end of the molecule is essential for biological activity, whereas the 5-dehydrofucofuranose can be chemically substituted by an allyl group (Hayashi *et al.*, 1997).

Chloramphenicol binds the PTC and displays substrate-specific inhibition. Chloramphenicol (Cam) was originally isolated from *Streptomyces venezuele* and displays a broad-spectrum activity, inhibiting a wide range of Gram-positive and Gram-negative bacteria, but not translation in eukaryotes. Cam binds in the A-site of the PTC of a bacterial ribosome (Schlünzen *et al.*, 2001), in a position overlapping the methylated-tyrosyl-moeity of Puro (Schmeing *et al.*, 2002) (Figure 9C) or more generally the aminoacyl moiety of an aa-tRNA. This is consistent with biochemistry showing that Cam interferes with the puromycin reaction as well as the binding of small tRNA fragments to the A-site of the PTC (Celma *et al.*, 1971), but stimulates

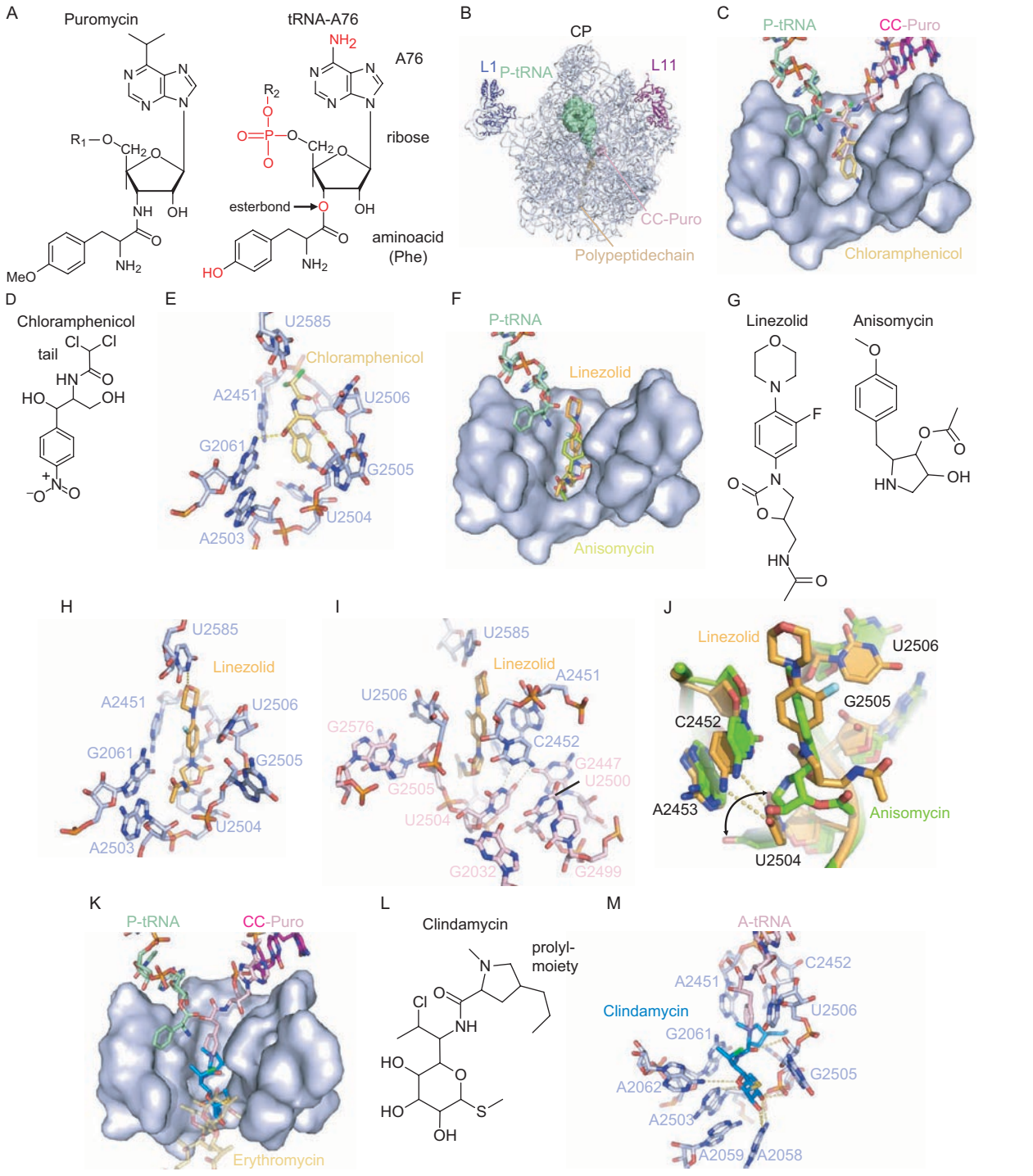

Figure 9. Puromycin, chloramphenicol, linezolid, anisomycin and clindamycin bind at the A-site of the PTC. (A) Comparison of structures of puromycin with the terminal adenine (A76) aminoacylated with phenylalanine. Differences between puromycin and the physiological tRNA substrate are indicated in red on the tRNA. (B) Overview of the large subunit with P-tRNA (green), CC-puromycin (CC-Puro, pink), polypeptide chain (tan), central protuberance (CP), and ribosomal proteins L1 (blue) and L11 (magenta) indicated for reference. (C, F, K) Surface representation (blue) of the peptidyltransferase center (PTC) with position of (C) chloramphenicol and CC-Puro (CC, magenta; Puro, pink), (F) linezolid (orange) and anisomycin (green) relative to the CCA-end of P-tRNA (green), and (K) tiamulin (blue), erythromycin (tan) and CC-puro. (D, G, H, L) Chemical structures of (D) chloramphenicol, (G) linezolid and anisomycin and (L) clindamycin with prolyl moiety indicated. (E, H and M) Nucleotides of the PTC involved in forming the binding pocket for (E) chloramphenicol, (H) linezolid and (M) clindamycin. (I) Location of nucleobases (pink) associated with linezolid resistance. (J) Comparison of the position of 23S rRNA nucleotide U2504 in the anisomycin-archaeal (green) versus the linezolid–bacterial large ribosomal complexes. Arrow indicates the shifted position of U2504 and the dashed lines indicate potential hydrogen bonding of U2504 with C2452 in the linezolid-50S structure.

the binding of tRNA fragments to the P-site (Ulbrich *et al.*, 1978). The dichloroacetamido tail of Cam is in a position to directly contact the ribose of the A76 of a P-tRNA (Figures 9C, 9D), which can also explain the enhancement that the presence of a P-tRNA on the ribosome has for Cam binding. Cam is a classic elongation inhibitor, in the sense that addition of the drug to growing bacterial cells blocks the ribosomes on the mRNA and protects the peptidyl-tRNA from hydrolysis, enabling the visualization of polysomes on sucrose gradients. There is some indication that the ability of Cam to inhibit the peptidyltransferase reaction is dependent on the nature of the substrates, such that tRNAs bearing bulky aromatic side chains are less prone to inhibition than tRNAs bearing smaller or charged amino acids, such as glycine or lysine (Pestka, 1969a; 1969b; Rheinberger and Nierhaus, 1990). Indeed, the *in vitro* synthesis of poly(U)-dependent poly(Phe) is poorly inhibited by Cam, whereas Cam inhibits poly(A)-dependent poly(Lys) significantly better. This may suggest that tRNAs bearing the large aromatic residues such as phenylalanine are able to more effectively displace Cam from binding site. Cam has also been shown to influence translational accuracy, promoting stop codon readthrough and framshifting, but unlike the aminoglycosides, does not induce misincorporation (Thompson *et al.*, 2002; 2004).

Seven highly conserved nucleotides comprise the Cam binding site (Figure 9E). Cam can potentially form hydrogen bond interactions with the base of G2061 and the ribose of G2505 (Figure 9E), although additional Mg^{2+} ion mediated contacts have been proposed (Schlünzen *et al.*, 2001). Cam alters the protection pattern of multiple nucleotides within the PTC, including A2451, G2505 and U2506, but also nucleotides that are located in the ribosomal tunnel, such as A2058 and A2059 (Moazed and Noller, 1987a; Rodriguez-Fonseca *et al.*, 1995). However, mutations that confer resistance to Cam generally map directly in the binding site, e.g. 2451, 2503 and 2504, or are located relatively close by, such as 2447 and 2452. There are also mutations, e.g. G2505C and G2583U/C that result in hypersensitivity towards Cam (Saarma and Remme, 1992). Cam does not bind at the A-site of the PTC on an archaeal ribosome (even at high concentrations (20 mM) of the drug), but a second site was observed in the ribosomal tunnel overlapping the macrolide binding site (Hansen *et al.*, 2003). Cam has also been reported to enhance the premature release of short oligopeptidyl tRNAs *in vitro* (Rheinberger and Nierhaus, 1990), reminiscent of the macrolide antibiotics, suggesting that a second site may also exist in bacteria. Indeed, early experiments detected two binding sites for Cam on the large subunit, one with high affinity and one with low affinity (Contreras and Vazquez, 1977). In the bacterial Cam-50S structure (Schlünzen *et al.*, 2001), the antibiotic was soaked at 100 µM and therefore the second site may not have been observed due to its lower affinity. Nevertheless, it seems unlikely that this secondary site is relevant for inhibition given that most of the resistance mutations cluster around the primary site at the A-site of the PTC. Indeed, methylation of A2503 by the methyltransferase Cfr is sufficient to confer resistance to Cam in *E. coli* (Kehrenberg *et al.*, 2005).

Oxazolidinones such as linezolid inhibit A-tRNA accommodation. The oxazolidinones are a class of synthetic antibiotics that act against a wide spectrum of Gram-positive and anaerobic bacteria as well as exhibiting activity against multi-drug resistant Gram-positive bacteria, such as MRSA. The oxazolidinone linezolid was approved for human use in 2000, and is now marketed by Pfizer under the name Zyvox®. Crystal structures of linezolid bound the bacterial (Wilson *et al.*, 2008) and archaeal large subunits (Ippolito *et al.*, 2008) reveal that linezolid binds to the A-site of the PTC (Figure 9F), in a position overlapping the binding sites of anisomycin (Figure 9F), chloramphenicol as well as the aminoacyl moiety of an A-site bound tRNA (Figure 9C). In these structures, the aromatic ring of linezolid, anisomycin (Figure 9G) and chloramphenicol (Figure 9D) are similarly located, in good agreement with the observation that oxazolidinones compete with chloramphenicols, lincosamides and puromycins for ribosome binding (Lin *et al.*, 1997; Skripkin *et al.*, 2008). An elegant series of *in vivo* crosslinking experiments revealed that I^{125} labeled-oxazolidinones crosslink to components of the PTC (Colca *et al.*, 2003) and led to model for the binding position of linezolid at the A-site of PTC (Leach *et al.*, 2007), which is in excellent agreement with the subsequent crystal structures (Figure 9H). Strains selected for linezolid resistance have mutations in the 23S rRNA that map directly to the linezolid binding site, such as A2451, C2452, U2504, and G2505, but also many mutations are located more distally (Figure 9I) (Kloss *et al.*, 1999; Prystowsky *et al.*, 2001; Sander *et al.*, 2002; Lobritz *et al.*, 2003; Beringer *et al.*, 2005), such as the G2576U mutation that has been identified in the few clinical isolates that display linezolid resistance (Prystowsky *et al.*, 2001; Saager *et al.*, 2008). Consistently, a dramatic reduction in crosslinking efficiency was observed *in vivo* when strains of *Staphylococcus aureus* bearing the mutation G2576U or G2447U were used (Colca *et al.*, 2003). It should be noted that there seems to be a unique species-specific pattern of resistance to oxazolidinones, since there is little overlap in the reported mutation sites across different species, such as archaea and bacteria (Mankin, 2001; Sander *et al.*, 2002). This may reflect slight differences in binding site, and most likely relates to the difference in fitness costs associated with mutations in different organisms,

for example the G2447U mutation in *Mycobacterium smegmatis* is lethal in *E. coli* (Sander *et al.*, 2002 and references therein). Novel oxazolidinones that establish additional interactions with the ribosome, such as ranbezolid (Das *et al.*, 2005; Kalia *et al.*, 2009) and Rx-01 (Franceschi and Duffy, 2006; Skripkin *et al.*, 2008) are being developed, which display improved inhibitory activities compared to linezolid (Das *et al.*, 2005; Lawrence *et al.*, 2008), and even act against linezolid-resistant strains (Lawrence *et al.*, 2008; Skripkin *et al.*, 2008).

Despite the good characterization of the oxazolidinone binding site at the A-site of the PTC, the exact mechanism of action of oxazolidinones is still unclear. The oxazolidinones have a tendency to bind non-specifically *in vitro* (Matassova *et al.*, 1999), which has led in the past to some confusion with respect to the binding site of the oxazolidinones (discussed by Mankin, 2001). Moreover, there have been conflicting reports as to the ability of oxazolidinones to inhibit the puromycin reaction (Lin *et al.*, 1997; Burghardt *et al.*, 1998; Kloss *et al.*, 1999; Aoki *et al.*, 2002; Sander *et al.*, 2002), and many proposals that oxazolidinones exert their influence through the positioning or accommodation of initiator fMet-tRNA on the ribosome, suggesting that the site of action of oxazolidinones may in fact be the initiation phase of translation (Eustice *et al.*, 1988; Shinabarger *et al.*, 1997; Burghardt *et al.*, 1998; Swaney *et al.*, 1998). However, the non-physiologically high drug concentrations required to inhibit these reactions suggests that the oxazolidinones target a particular functional state of the ribosome that has yet to be identified. In this regard, oxazolidinones cross-link *in vivo* not only to ribosomal components, but also to a small tRNA-sized RNA, as well as to a ribosomal translation factor called LepA (Colca *et al.*, 2003). LepA binds to POST state ribosomes inducing a back-translocation to the PRE state (Qin *et al.*, 2006). Cryo-EM reveals that during back-translocation LepA stabilizes a tRNA state (Connell *et al.*, 2008), reminiscent of that observed during tRNA accommodation at the A-site (Sanbonmatsu *et al.*, 2005). This finding led to the suggestion that LepA may recognize and bind to the functional state induced by the oxazolidinone, namely a partially accommodated A-tRNA (Wilson *et al.*, 2008). However, it is unclear whether this functional state occurs directly after initiation or during translation elongation. The perturbation of A-tRNA could also explain the loss of translational fidelity observed in the presence of oxazolidinones (Thompson *et al.*, 2002). However, it needs to be made clear that a Δ*lepA* strain has an identical MIC for oxazolidinones as the wild-type strain (Colca *et al.*, 2003), indicating that the binding of LepA is a consequence, rather than a cause, of the functional state induced by the oxazolidinones. Clearly, further work needs to be done to directly show which functional state of the ribosome the oxazolidinones target and whether A-tRNA accommodation is directly affected.

Anisomycin resistance results from subtle changes within the PTC. Anisomycins bind in the A-site of the PTC in a position overlapping that of the oxazolidinone linezolid (Figure 9F) (Hansen *et al.*, 2003; Blaha *et al.*, 2008), as well as chlorampenicol and puromycin (Figure 9C). Structurally, anisomycin shares similarities with both puromycin and chloramphenicol (compare Figures 9A, 9D and 9G), however anisomycin binds to archaeal (Hansen *et al.*, 2003; Blaha *et al.*, 2008), yeast and human ribosomes, but not to bacterial ribosomes (Barbacid and Vazquez, 1974). In agreement with the A-site location of anisomycin, the drug has been shown to inhibit the puromycin reaction on yeast ribosomes (Barbacid and Vazquez, 1974; Ioannou *et al.*, 1997), but it is harder to rationalize the report that anisomycin can also inhibit binding of tRNA to the P-site (Carrasco and Vazquez, 1972). The crystal structure of anisomycin bound to an archaeal large ribosomal subunit shows that the drug makes extensive interactions with C2452, the aromatic methoxylphenyl ring of anisomycin stacks upon C2452 and the N3 of the pyrrolidine sugar can also form a hydrogen bond with C2452 (Figure 9J). This is consistent with the weak protection of A2451-C2452 from chemical probing (Rodriguez-Fonseca *et al.*, 1995), and the observation that mutation of C2452U confers resistance to anisomycin in archaea (Hummel and Boeck, 1987a; Blaha *et al.*, 2008). Other mutations conferring resistance to anisomycin are located in nucleotides adjacent to the binding site, such as G2447A/C, G2576U, C2499U, U2500A/C (Hummel and Boeck, 1987a; Blaha *et al.*, 2008), which exhibit an overlap with linezolid resistance mutations (Figure 9I). An exhaustive analysis, involving the structures of 11 different anisomycin-resistant archaeal large subunits, revealed that these 23S rRNA mutations result in subtle changes within the PTC that either interfere with specific interactions between drug and target, or stabilize alternative conformations that can be reversed by substrate binding (Blaha *et al.*, 2008). Importantly, this study allowed the visualization of alterations within the drug binding site resulting from conformational changes that are propagated through the rRNA from an outlying resistance mutation (Blaha *et al.*, 2008). Mutations in ribosomal protein L3 also confer resistance to anisomycin (Fried and Warner, 1981; Meskauskas *et al.*, 2005), which is also likely to be indirect, since L3 does not comprise the anisomycin binding site. Interestingly, the L3 anisomycin-resistance mutations have been shown to promote binding of the aa-tRNA to the A-site (Meskauskas *et al.*, 2005; Meskauskas and Dinman, 2007). Comparison of bacterial and archaeal

ribosomes clearly indicates that U2504 is a major factor in determining the specificity of anisomycin: In bacterial ribosomes U2504 base-pairs with C2452, and clashes with the position of the anisomycin, whereas in archaea, U2504 is rotated by 90° to allow drug binding (Figure 9J) (Blaha *et al.*, 2008; Wilson *et al.*, 2008).

Lincosamides overlap in binding site with macrolides and chloramphenicols. Two lincosamides are used clinically: lincomycin, which is naturally produced by several species of actinomycetes (such as *Streptomyces lincolnensis*, *S. espinosus* and *Actinomyces roseolus*), and clindamycin, a semi-synthetic derivative of lincomycin (reviewed by Spizek *et al.*, 2004). The lincosamides are excellent inhibitors of Gram-positive bacteria, but not Gram-negative, with clindamycin also affecting protozoa, such as *Pneumocystis carinii*, but not inhibiting archaea, nor eukaryotic systems, such as rabbit reticulocyte (Gale *et al.*, 1981; Spizek *et al.*, 2004). Lincosamides bind within the A-site of the PTC (Schlünzen *et al.*, 2001; Tu *et al.*, 2005) (Figure 9K). Specifically, the prolyl-moiety of clindamycin (Figure 9L) overlaps the aminoacyl-moiety of A-tRNA (Figure 9K) and the binding site of chloramphenicol (Figure 9C), whereas the sugar moiety extents into the ribosomal tunnel and overlaps the binding position of the macrolide antibiotics, such as erythromycin (Figure 9K) (Schlünzen *et al.*, 2001; Tu *et al.*, 2005). Consistently, lincomycin competes with both chloramphenicol and erythromycin for binding to *E. coli* ribosomes (Fernandez-Munoz *et al.*, 1971). The majority of the interactions involve hydrogen bonds from hydroxyl groups on the sugar-moiety of clindamycin with nucleotides of the PTC, such as the nucleobases of A2058 and A2062, as well as the backbones of G2505 and A2503 (Figure 9M). Lincomycin (and clindamycin) protects A2058 (clindamycin also A2059), G2061, A2451 and G2505 from chemical modification (Douthwaite, 1992) – all nucleotides that comprise the drug binding site (Figure 9M). The overlap in binding site with chloramphenicols and macrolides is also illustrated by the lincosamide resistance mutations: the Cfr methyltransferase that modifies A2503 of the 23S rRNA confers resistance to chloramphenicol as well as clindamycin (Kehrenberg *et al.*, 2005), and the most prevalent resistance mechanism is the so-called MLS_B resistance, where modification of the 23S rRNA nucleotides A2058 and/or A2059 confers multi-drug resistance to macrolides, lincosamides and streptogramin B antibiotics (Poehlsgaard *et al.*, 2005; reviewed in Poehlsgaard and Douthwaite, 2003). In contrast to macrolides such as erythromycin, lincosamides, such as lincomycin, have been shown to directly inhibit the transfer of fMet or AcPhe to puromycin (Kallia-Raftopoulos and Kalpaxis, 1999; Kouvela *et al.*, 2006), which is in agreement with the overlapping binding position of these two drugs (Figure 9K, 9M). Some influence of lincosamides on binding of tRNA fragments to the P-site has also been reported (Celma *et al.*, 1970); this needs to be investigated further.

Antibiotics that inhibit P-tRNA binding at the peptidyltransferase center

Blasticidin S mimics the CC of the CCA-end of a P-tRNA. Blasticidin S is a nucleoside derivative, like puromycin, and was discovered as a metabolite of *Streptomyces griseochromogenes* (Takeuchi *et al.*, 1958). However, unlike puromycin, which mimics an aminoacylated-terminal adenosine (A76) of an A-tRNA (Figure 10A), two molecules of blasticidin S bind to the ribosome and interact with nucleotides G2251 and G2252 of the P-loop at the PTC in a manner analogous to the C74 and C75 of a P-tRNA (compare Figures 10B and 10C) (Hansen et al., 2003). Specifically, blasticidin S is composed of a cytosine base and a pyranose sugar with an N-methylguanidine tail (Figure 10D). The cytosine nucleobase forms Watson-Crick base-pairing with the P-loop nucleotides, whereas the N-methylguanidine tail of one blasticidin S molecule interacts with nucleotide U2438 (not shown) and A2439 (Figure 10C). In *E. coli*, A2439 is protected from chemical attack by blasticidin S (Rodriguez-Fonseca *et al.*, 1995) and removal or alteration of the tail of blasticidin S dramatically decreases the effect of the drug on protein synthesis (Lichtenthaler and Trummlitz, 1974). Moreover, mutation of U2438C confers blasticidin S resistance in *Halobacterium halobium* (Porse *et al.*, 1995). However, the major blasticidin S resistance mechanisms that have been reported are non-ribosomal and relate to modification of the drug. For example, *Streptoverticillum* sp., which is a blasticidin S producer, protects itself by producing an acetyl-transferase encoded by the *bls* gene, whereas *Aspergillus terrues* for example contains a deaminase that converts blasticidin S into the non-toxic deaminohydroxy form (Yamaguchi *et al.*, 1975). Blasticidin S displays activity against all three kingdoms (see Rodriguez-Fonseca *et al.*, 1995 and references therein), and was the first introduced into agriculture as a microbial fungicide to control rice blast caused by *Pyricularia oryzae* (Takeuchi *et al.*, 1958). Blasticidin S has been reported to inhibit the peptidyltransferase reaction of both bacterial 70S and eukaryotic 80S ribosomes (Suhadolnik, 1970; Vasquez, 1979), with recent evidence suggesting that blasticidin S binds transiently at the A-site of the PTC and is then slowly accommodated at the final stable binding on the P-site (Petropoulos *et al.*, 2004). Therefore, blasticidin S could both act as a translation initiation inhibitor preventing placement of the initiator tRNA at the P-site of the PTC during subunit joining, and also bind to the P-site of a post-peptide bond formation pre-translocation hybrid state ribosome when the peptidyl-tRNA is at the A-site and deacylated tRNA is in a hybrid P/E state; it therefore acts as an elongation inhibitor.

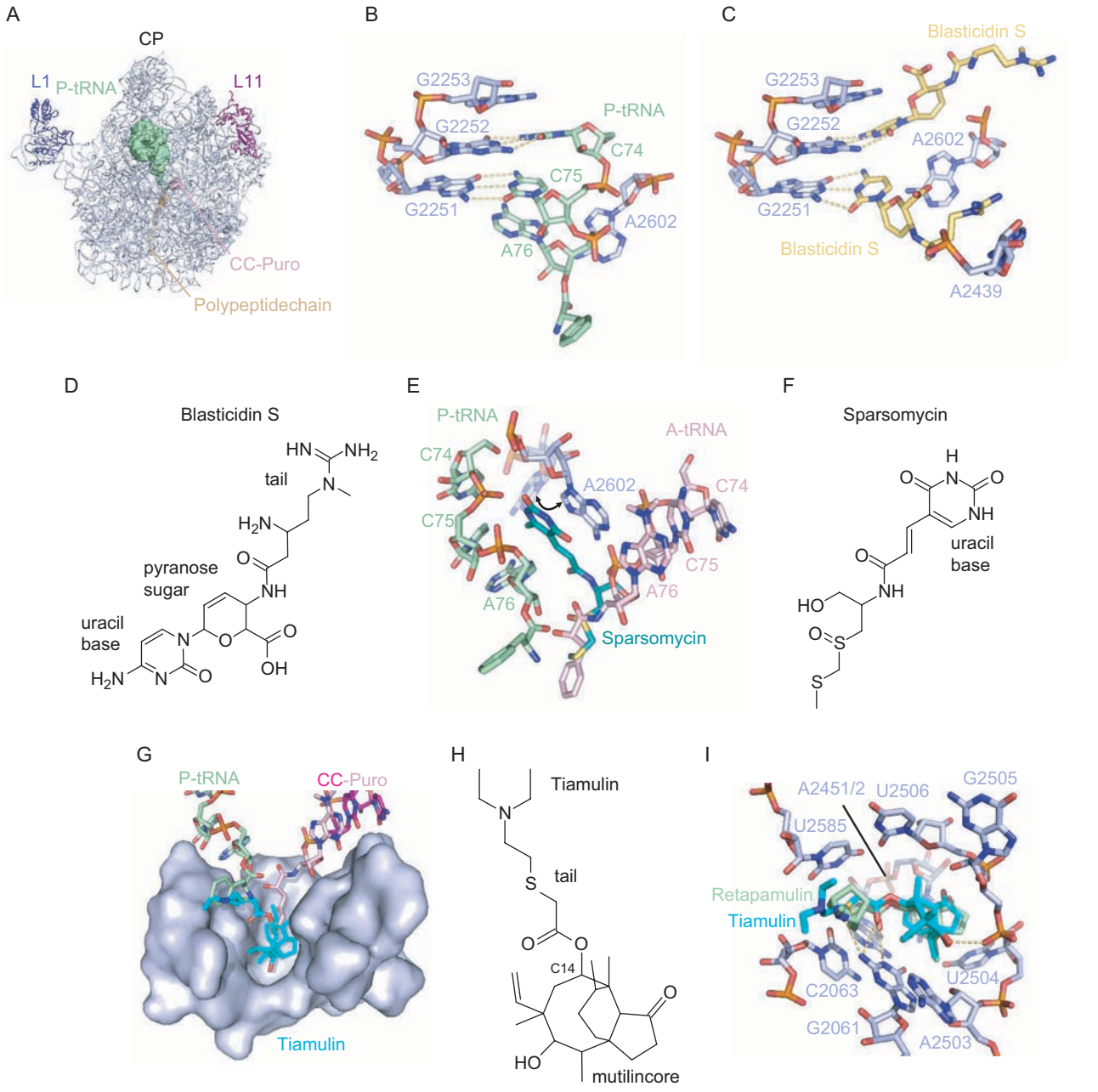

Figure 10. Blasticidin S, sparsomycin, and tiamulin inhibit tRNA binding at the PTC. (A) Overview of the large subunit with P-tRNA (green), CC-puromycin (CC-Puro, pink), polypeptide chain (tan), central protuberance (CP), and ribosomal proteins L1 (blue) and L11 (magenta) indicated for reference. (B) Hydrogen bond interactions (dashed lines) of the C74 and C75 of the P-tRNA (green) with nucleotides G2252 and G2251, respectively, of the P-loop. (C) Two molecules of blasticidin S mimic C74 and C75 to interact with nucleotides of P-loop. (D) Chemical structure of blasticidin S. (E) The uracil moiety of sparsomycin (teal) stacked between nucleotide A2602 and the CCA-end of the P-tRNA, while the tail of sparsomycin overlaps with the CCA-end of the A-tRNA (pink). (F) Chemical structure of sparsomycin, with uracil base indicated. (G) Surface representation (blue) of the PTC with position of tiamulin relative to CC-Puro (pink) and the CCA-end of P-tRNA (green). (H) Chemical structure of tiamulin with mutilin core indicated. (I) Nucleotides of the PTC involved in forming the binding pocket for tiamulin.

Sparsomycin prevents A-tRNA accommodation and enhances P-tRNA binding. Sparsomycin is a nucleoside analog of uracil produced by *Streptomyces sparsogenes*, which acts as a potent inhibitor of peptidyltransferase activity in bacteria, archaea and eukaryotes (reviewed by Lazaro *et al.*, 1991a). Sparsomycin has been shown to interfere with binding of tRNA (and CCA-end fragments) to the A-site of the PTC (Pestka, 1969b), while enhancing the affinity of peptidyl-tRNAs (especially N-acetylated aa-tRNAs and CCA-aa end fragments) to the P-site (Monro *et al.*, 1969; Lazaro *et al.*, 1991b). Sparsomycin has been crystallized in complex with CC-Puro bound

to the P-site of the large subunit (Hansen *et al.*, 2002b; 2003) (Figure 10E), revealing that the uracil moiety of sparsomycin (Figure 10F) is stacked between 23S rRNA nucleotide A2602 and the terminal CA of the P-tRNA mimic (Figure 10E). Sparsomycin has been crosslinked to A2602 (Porse *et al.*, 1999b) and mutations (C2499, C2452 and U2500) (Tan *et al.*, 1996), and modification deficiencies (Lazaro *et al.*, 1996; Rakauskaite and Dinman, 2008) that confer sparsomycin-resistance (or sensitivity) map to the PTC (these nucleotides can be seen in Figure 9I). The extensive interaction between sparsomycin and the P-tRNA provides a rationale as to why the presence of the P-tRNA enhances drug binding and *vice versa* (Lazaro *et al.*, 1991a). In contrast, the tail of sparsomycin encroaches on the binding position of the aminoacyl-moiety and terminal adenine of an A-tRNA at the PTC, suggesting that sparsomycin prevents peptide bond formation by preventing the binding and placement of the A-site substrate. Consistently, sparsomycin competes for binding of A-site inhibitors chloramphenicol and lincomycin (Lazaro *et al.*, 1991b). Ribosomes that are in a PRE state, i.e. having both A- and P-sites occupied, are not protected from the action of sparsomycin, since under these conditions sparsomycin induces translocation of the tRNAs, such that the peptidyl-tRNA is at the P-site and the A-site is blocked by the drug (Fredrick and Noller, 2003) (as seen in Figure 10E). A2602 is at the center of the rotational symmetry of the PTC where it has been proposed to play a role in guiding the CCA-ends from the A- to P-site during translocation (Agmon *et al.*, 2003). Sparsomycin appears to induce a rotation of the base of A2602, compared to the PRE state conformation (Figure 10E), which may reflect the two-step reaction mechanism of sparsomycin – a slow initial step that isomerizes slowly to adopt a more stable conformation (Ioannou *et al.*, 1997).

Pleuromutilins, such as tiamulin, overlap A- and P-sites of the PTC. Pleuromutilins are a class of protein synthesis inhibitors derived from the natural product pleuromutilin, which is produced by the basidiomycete *Pleurotus mutilus* (renamed to *Clitopilus scyphoides*) (Brooks *et al.*, 2001). Of the semi-synthetic derivatives, tiamulin and valnemulin are used almost exclusively in veterinary medicine, whereas retapamulin was recently approved for human use as a topical agent (Altabax®/Altargo®) to treat skin infections (Parish and Parish, 2008). Pleuromutilins specifically affect eubacteria, displaying excellent activity against staphylococcal, streptococcal and anaerobic Gram-positive bacteria, including MRSA. Pleauromutilins, such as tiamulin, bind at the PTC in a position overlapping both A- and P-tRNA (Figure 10G) (Schlünzen *et al.*, 2004; Davidovich *et al.*, 2007; Gurel *et al.*, 2009), chloramphenicol and puromycin (Figure 9C), consistent with the observation that pleuromutilins compete with chloramphenicol and puromycin for ribosome binding (Hogenauer, 1975). The tricyclic mutilin core (Figure 10H), common to all pleuromutilins, inserts into the A-site pocket formed by A2451, A2452, U2504 and G2505, whereas the sulfanyl-acetate substituted C14-tail, seen in tiamulin and retapamulin, can form hydrogen bond interactions with the base of G2061 (Figure 10I) (Schlunzen *et al.*, 2004; Davidovich *et al.*, 2007). Variation of the C14-tail influences the types of contacts that are made with G2061 as well as U2585 and are likely to play an important role in determining the activity profiles of the different pleuromutilin derivatives (Lolk *et al.*, 2008). Consistently, nucleotides such as G2505 and U2506 are footprinted similarly by different pleuromutilin derivatives, whereas varying effects are observed at U2584 and U2585 (Long *et al.*, 2006). Resistance to plearuomutilins results from mutations at positions 2055, 2447, 2504, 2572 and 2576 of the 23S rRNA (Pringle *et al.*, 2004; Miller *et al.*, 2008; Long *et al.*, 2009), many of which give rise to cross-resistance to other antibiotics that bind at the A-site, such as linezolid, chloramphenicol and clindamycin (reviewed by Davidovich *et al.*, 2008). Resistance is also associated with alterations in ribosomal protein L3 (Bosling *et al.*, 2003; Pringle *et al.*, 2004), which, like many of the 23S rRNA mutations, probably results from indirect perturbation of the binding site. One exception is the position U2504 that comprises the pleuromutilin binding site and is observed in different positions in bacterial and archaeal ribosomes (Figure 9J), and is thus likely to play an important role for the kingdom specificity of pleuromutilins (Davidovich *et al.*, 2008; Gurel *et al.*, 2009). It remains unclear exactly which step of translation is targeted by the pleuromutilins, but they have been shown to inhibit the peptidyltransferase reaction and prevent binding of aa-tRNAs to the A-site (Hodgin and Hogenauer, 1974), yet they do not appear to inhibit translation elongation (Dornhelm and Hogenauer, 1978). Instead, pleuromutilins have been proposed to target translation initiation, based on the observations that (i) pleuromutilins destabilize fMet-tRNA binding during initiation complex formation, and (ii) addition of pleuromutilins to intact cells causes the disappearance of polysomes while stabilizing 70S monosomes (Dornhelm and Hogenauer, 1978). The overlap of pleuromutilins with the P-site would support the inability to correctly place the initiator-tRNA at the P-site during subunit joining.

Macrolide antibiotics block the progression of the nascent polypeptide chain

Macrolides represent a large class of polyketide compounds synthesized by actinomycetes that inhibit eubacterial, but not archaeal nor eukaryotic, protein synthesis (reviewed by Gaynor and Mankin, 2003; Poehlsgaard and Douthwaite, 2003; Takashima, 2003; Katz and Ashley, 2005; Mankin, 2008). Macrolide

antibiotics bind in a position adjacent to PTC, located within the exit tunnel of the large ribosomal subunit, through which the polypeptide chain passes during translation (Figure 11A). Clinically used macrolides have a 14- (erythromycin, telithromycin), 15- (azithromycin), or 16-membered (tylosin, spiramycin and carbomycin) lactone ring, to which amino sugars are attached at various positions (Figures 11B–11E);

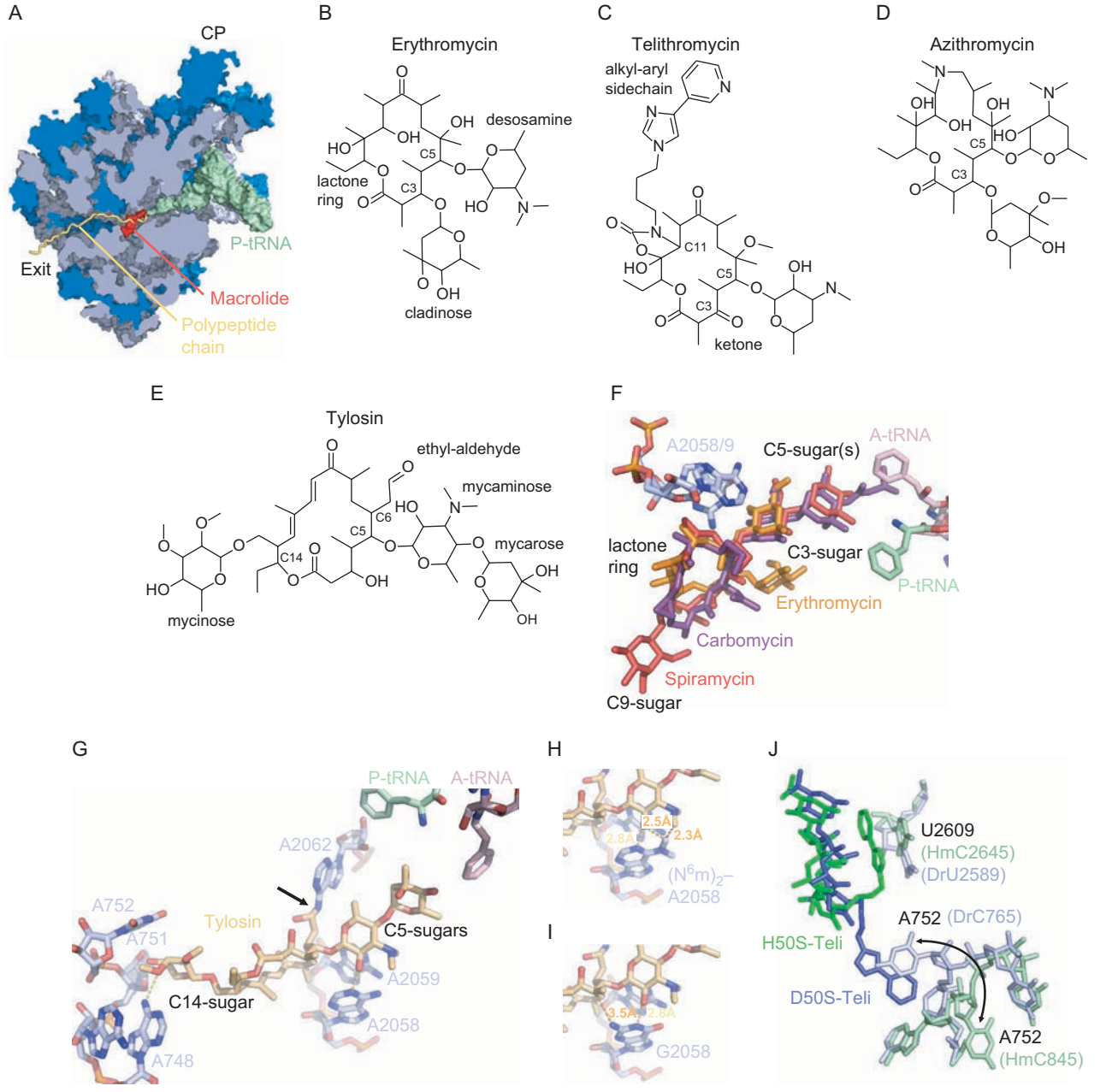

Figure 11. Macrolides bind within the tunnel of the 50S subunit. (A) Cross-section of the large ribosomal subunit (rRNA, gray; ribosomal proteins, blue) to reveal the ribosomal tunnel and the binding site of the macrolide class of antibiotics (red). A mock polypeptide chain (tan) and the P-tRNA (green) are shown for reference. (B–E) Chemical structures of (B) erythromycin, (C) telithromycin, (D) azithromycin and (E) tylosin. (F) Superimposition of the binding position of the macrolides erythromycin (orange), spiramycin (red) and carbomycin (purple) relative to A-tRNA (pink), P-tRNA (green) and 23S rRNA nucleotides A2058 and A2059 (blue). (G) Tylosin (tan) forms potential hydrogen bonds (dashed lines) from the C5 sugar with A2058 and from the C14-sugar with A748 and A751, as well as a reversible covalent bond with A2062 (arrowed). (H) Dimethylation of the N6 of A2058 confers resistance to macrolide antibiotics, possibly because the methyl groups encroach on the binding position of the C5-sugar. (I) Mutation of A2058G confers resistance to macrolide antibiotics, possibly because the N2 approaches the position of the lactone ring. (J) Comparison of the binding position of telithromycin between bacterial (D50S, blue) and archaeal (H50S, green) ribosomes. In the archaeal structure, the alkyl-aryl sidechain interacts with U2609, whereas in bacteria, the same sidechain interacts with A752 – an interaction that is not possible because of the different position of A751 in archaea (arrowed).

for example, erythromycin has a single C3- and C5-monosaccharide (Figure 11B), whereas tylosin has a C14-mycinose sugar and a C5-disaccharide (Figure 11E). Multiple structures of a variety of macrolides bound to bacterial and archaeal large subunits (Table1) reveal a common binding mode, such that the general orientation and conformation of the lactone ring and C5-sugar is similarly placed to establish interactions with A2058 and A2059 of the 23S rRNA (Figures 11F, 11G) (Schlünzen *et al.*, 2001; Hansen *et al.*, 2002a; Tu *et al.*, 2005; Wilson *et al.*, 2005). Consistently, macrolides protect A2058 and A2059 from chemical attack (Moazed and Noller, 1987a; Rodriguez-Fonseca *et al.*, 1995; Poulsen *et al.*, 2000), and N6 dimethylation of A2058, as well as mutation of A2058/9, reduce the affinity of the drug for the ribosome and confer resistance to macrolides (Lai and Weisblum, 1971; Sigmund and Morgan, 1982; Skinner *et al.*, 1983; Douthwaite *et al.*, 2000; Vester and Douthwaite, 2001). Dimethylation of A2058 is carried out by Erm-type methyltransferases and would lead to steric clashes with the C5-monosaccharide (Figure 11H), whereas G2058 would encroach on the binding position of the lactone ring (Figure 11I). In archaea and eukaryotes, guanine naturally occurs at position 2058, and mutation of G2058A in archaea restores binding of erythromycin to the ribosome (Tu *et al.*, 2005). However, mutation G2058A was not sufficient to make yeast susceptible to erythromycin, suggesting that other factors contribute to resistance in eukaryotes (Bommakanti *et al.*, 2008). An important factor in determining the binding affinity of macrolides is the sugar moieties, since they make a sizeable contribution (50–66%) to the interaction surface with the ribosome (Hansen *et al.*, 2002a). Tylosin, for example, forms additional interactions from the C14-sugar to nucleotides A748–A752, located deeper in the tunnel (Figure 11G). The importance of these additional interactions is illustrated by the fact that methylation of G748 by RimA(II), which is utilized by the drug-producing strain *Streptomyces fradiae*, confers resistance to tylosin (Douthwaite *et al.*, 2004). Additionally, macrolides with a C6-ethyl-aldehyde such as tylosin (Figure 11E) form a reversible covalent bond with the N6 of nucleobase A2062 (Hansen *et al.*, 2002a) (arrowed in Figure 11G), which is likely to contribute significantly to the binding affinity. Resistance to macrolides also arises from alterations in ribosomal proteins L4 and L22 (Chittum and Champney, 1994; Wittmann *et al.*, 1973), neither of which directly contact the macrolide drugs, but rather induce conformational changes within 23S rRNA nucleotides that comprise the macrolide binding site (Gregory and Dahlberg, 1999).

The emergence of bacterial resistance to macrolide antibiotics has led to the development of ketolides, semi-synthetic derivatives of macrolides where the C3-sugar is replaced with a keto group (Figure 11C). The broader spectrum of activity of the ketolides, such as cethromycin (ABT-773) and telithromycin, despite the absence of the C3-cladinose, seems to be related to the presence of their additional sidechains and modifications, such as the alkyl-aryl sidechain of telithromycin (Figure 11C). Bound to the bacterial (*Deinococcus radiodurans*) large subunit (D50S), the sidechain of telithromycin penetrates deeper into the tunnel forming stacking interactions with A752 (Figure 11J) (Berisio *et al.*, 2003; Wilson *et al.*, 2005), consistent with the protection of A752 from chemical modification (Douthwaite *et al.*, 2000; Hansen *et al.*, 1999) and occurrence of ketolide-resistance mutations in this region (Xiong *et al.*, 1999). Interestingly, in contrast to the similar positions of the lactone rings, the conformation of the telithromycin sidechain bound to the archaeal (*Haloarcula marismortui*) large subunit (H50S) is dramatically different (Figure 11J). These structural differences that modulate the mode of binding of the drug to the ribosome probably stem from the low sequence conservation within this region. More generally, this suggests that antibiotic binding to ribosomes of different organisms/strains may utilize distinct binding modes, which has important implications for structure-based drug design (Wilson *et al.*, 2005). Such variation in binding modes is also illustrated by the binding of two molecules of azithromycin to *D. radiodurans* 50S ribosomes (Schlünzen *et al.*, 2003), but only one molecule to *E. coli* (Petropoulos *et al.*, 2009) and *H. marismortui* ribosomes (Hansen *et al.*, 2002a).

The binding site of macrolides is vacant on free or initiating ribosomes, but unavailable in elongating ribosomes (Tai *et al.*, 1974; Contreras and Vazquez, 1977). Translation inhibition by macrolide antibiotics restricts the synthesis to short oligopeptides that eventually fall off the ribosome as oligopeptidyl-tRNAs, in an poorly defined process called "drop-off" (Otaka and Kaji, 1975; Menninger and Otto, 1982; Tenson *et al.*, 2003). Moreover, the size of the oligopeptides synthesized appears to be related to the extent to which the macrolides occlude the tunnel, such that macrolides like spiramycin with a C5-disaccharide allow the synthesis of 2–4 amino acids, whereas smaller macrolides, such as erythromycin with a C5-monosaccharide, permits 6–8 amino acids to be polymerized before drop-off (Tenson *et al.*, 2003). Indeed, macrolides with extensive sidechains extending from the C5 position even inhibit peptidyltransferase activity to varying extents, such as carbomycin (100% inhibition), spiramycin (85%) and tylosin (~60%), whereas those with shorter sidechains, such as erythromycin, do not (Poulsen *et al.*, 2000). The strong inhibition of peptide bond formation by carbomycin is consistent with the overlap of

the isobutyrate extension of the C5-disaccharide that encroaches on the A-site (Figure 11F) and the PTC specific footprints observed by this drug (Poulsen *et al.*, 2000). The effect of macrolides in blocking egression of the nascent polypeptide chain may not be uniform for all mRNAs, but rather be dependent on the nascent polypeptide chain being synthesized (see Mankin, 2008). Indeed, induction of erythromycin-resistance genes, such as *ermC*, requires stalling during translation of an upstream leader sequence (Weisblum, 1995). The translational stalling has been shown to be dependent on the sequence of the leader peptide in the tunnel and the presence of erythromycin (reviewed by Ramu *et al.*, 2009). Moreover, the translation of some amino acid sequences even leads to removal of macrolides from their binding site in the ribosomal tunnel, and that certain polypeptide sequences confer resistance to specific macrolide members (Tripathi *et al.*, 1998; Tenson and Mankin, 2001; Lovmar *et al.*, 2006; Vimberg *et al.*, 2004). It is therefore possible that certain proteins synthesized *in vivo* will display a natural resistance to erythromycin based on the similarity of their N-terminal sequence with the erythromycin-resistance peptides (see Mankin, 2008).

Streptogramins act synergistically to enhance their antimicrobial activity

Streptogramins are produced by a variety of *Streptomyces sp* as a mixture (ratio of 7:3) of two chemically unrelated compounds, type A (S_A) and B (S_B). The streptogramins bind to adjacent sites in the 50S subunit, spanning the PTC and ribosomal tunnel (Figure 12A) (Hansen *et al.*, 2003; Harms *et al.*, 2004; Tu *et al.*, 2005), and inhibit growth of Gram-positive bacteria (reviewed by Cocito *et al.*, 1997; Mukhtar and Wright, 2005). The streptogramin combination of dalfopristin (S_A) and quinupristin (S_B) (Figures 12B, 12C) is now marketed as Synercid® (Aventis) (Lamb *et al.*, 1999) to treat skin infections, and is also active against some Gram-negative and anaerobic bacteria. S_A compounds are cyclic polyunsaturated macrolactones (Figure 12B) that interfere with binding of tRNA substrates to both A- and P-sites (Chinali *et al.*, 1984; Di Giambattista *et al.*, 1989; Cocito *et al.*, 1997), consistent with the overlap in binding position of S_A with the aminoacyl moieties of both A- and P-tRNAs (Figure 12D). Suppression of bacterial growth persists for a prolonged period subsequent to the removal of the S_A drug (Parfait and Cocito, 1980; Nyssen *et al.*,

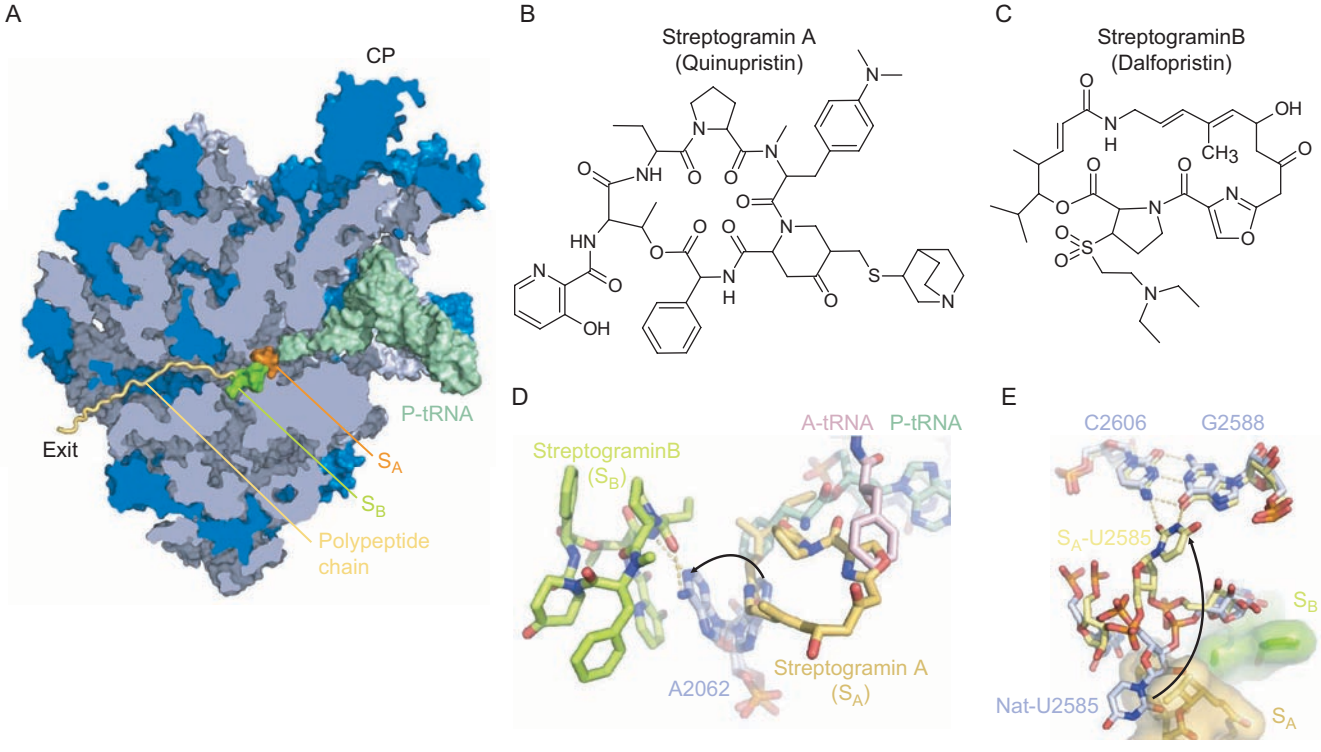

Figure 12. The synergistic action of the streptogramins. (A) Cross-section of the large ribosomal subunit to reveal the ribosomal tunnel and the binding sites of the streptogramin A (S_A, orange) and B (S_B, green) antibiotics. (B, C) Chemical structures of (B) streptogramin A quinupristin and (C) streptogramin B dalfopristin. (D) Binding of streptogramins A (yellow) leads to a shift (as arrowed) in nucleotide A2062 (blue), which stabilizes the binding of streptogramin B (green). Streptogramins A overlap the binding position of both A- (pink) and P-tRNA (pale green). (E) In bacteria, streptogramin binding leads to a repositioning (as arrowed) of nucleotide U2585, such that it can hydrogen bond (dashed lines) with C2606 and G2588.

1989), suggesting that S_A binding induces a conformational change within the PTC that is *slowly* reversible. Indeed, S_A alter the reactivity of multiple residues within the PTC (Vannuffel *et al.*, 1992; Porse and Garrett, 1999). In particular, binding of the dalfopristin (S_A) to bacterial large 50S subunit induces a dramatic shift in the position of U2585 compared to the native 50S, such that it can form hydrogen bonds to C2606 and G2588 (Figure 12E). U2585 plays an important role in the positioning of tRNA substrates and peptide bond formation (Schmeing *et al.*, 2005a; 2005b) and therefore the stabilization of U2585 in a non-productive conformation has been suggested to contribute to the post-antibiotic inhibitory effect of S_A compounds (Harms *et al.*, 2004). In contrast, the S_B compounds prevent extension of the nascent polypeptide chain and induce drop-off of short oligopeptidyl-tRNAs, analogous to the macrolides (Chinali *et al.*, 1988a; Chinali *et al.*, 1988b). The S_B compete with macrolides for ribosome binding (Parfait *et al.*, 1981; Di Giambattista *et al.*, 1986), and A2058/9 mutations confer cross-resistance to macrolides, lincosamides and streptogramin B (MLS_B) antibiotics (reviewed Cocito *et al.*, 1997; Poehlsgaard and Douthwaite, 2003). Furthermore, the overlap in the S_B and macrolide binding sites (comparing Figures 11A and 12A) is consistent with the overlap in protection of nucleotides, such as A2058 and A2059, from chemical attack (Moazed and Noller, 1987a; Vannuffel *et al.*, 1994).

A unique feature of the streptogramin A and B compounds is that they act synergistically *in vivo* and *in vitro*, such that presence of the S_A enhances the binding of the corresponding S_B compound (Parfait *et al.*, 1978). The synergistic action significantly lowers the concentrations of both antibiotics that is needed to obtain the same level of inhibition when each compound is used separately (Champney, 2001), as well as enabling the streptogramins to overcome some resistance mutations (Vannuffel *et al.*, 1992; Canu and Leclercq, 2001). Moreover, some S_A and S_B combinations can convert a bacteriostatic effect into bactericidal lethality. The basis for the synergy between S_A and S_B combinations is most likely related to a rotation of A2062 seen upon binding of S_A compounds to the PTC (arrowed in Figure 12E) (Harms *et al.*, 2004; Tu *et al.*, 2005). In the new position, A2062 can stabilize the binding of S_B compounds via hydrogen bond interactions (Figure 9H). Indeed, mutations of A2062 can also lead to streptogramin resistance (Depardieu and Courvalin, 2001). In summary, the synergistic action of streptogramins is likely to block both A- and P-sites, and therefore function during initiation or by inducing peptidyl-tRNA drop-off at an early elongation step.

Translocation inhibitors

The thiopeptide antibiotic thiostrepton perturbs translation factor accommodation on the ribosome

In addition to the thiopeptides that target elongation factor EF-Tu, such as GE2270A, another family exists that target the ribosome, the best characterized of which include thiostrepton (Thio), nosiheptide and micrococcin (reviewed by Bagley *et al.*, 2005; Nicolaou *et al.*, 2009). These antibiotics are synthesized as precursor peptides on the ribosome and then post-translationally modified to generate the active compound (Liao *et al.*, 2009; Wieland Brown *et al.*, 2009) (see Figure 13A for chemical structure of thiostrepton). The thiopeptide antibiotics are effective against Gram-positive bacteria, in particular, methicillin-resistant *Staphlococcus aureus* (MRSA), as well as against the malarial parasite *Plasmodium falciparum* (Rogers *et al.*, 1998). Although thiostrepton is already in veterinary usage, the low water solubility and poor bioavailability of this drug has so far precluded its use in human medicine. Recent success in the total synthesis of Thio (Nicolaou *et al.*, 2005a; 2005b), amongst others (reviewed by Hughes and Moody, 2007; Nicolaou *et al.*, 2009), has led to a renewed interest in this family of compounds. Moreover, the thiopeptides, such as Thio, bind to a distinct site on the large subunit (Figure 13B), compared to most other antibiotics that target the PTC (Figures 2C and 2D), and therefore cross-resistance is not an issue. Thiopeptides insert into a cleft formed by the N-terminal domain (NTD) of ribosomal protein L11 and helices 43 and 44 (H43/44) of the 23S rRNA (Figure 13C) (Harms *et al.*, 2008). Although Thio can interact with H43/44 alone, the binding affinity is enhanced dramatically by the presence of L11 (Cundliffe *et al.*, 1979; Thompson *et al.*, 1979; Xing and Draper, 1996). In the cleft, Thio interacts with nucleotides A1067 and A1095, located at the tips of H43 and H44, respectively, as well as the thiazole rings of Thio stack upon proline residues (P22 and P26) located in the NTD of L11 (Figure 13D). Consistently, mutations in A1067, A1095, or in the numerous proline residues of the L11-NTD reduce thiopeptide binding and confer drug-resistance in bacteria and archaea (Hummel and Boeck, 1987b; Thompson *et al.*, 1988; Mankin *et al.*, 1994; Rosendahl and Douthwaite, 1994; Porse *et al.*, 1998; 1999a; Cameron *et al.*, 2004). Furthermore, the producer of Thio, *Streptomyces azureus*, inhibits drug binding to its own rRNA by 2′-O-methylation of position A1067 (Thompson *et al.*, 1982). Eukaryotes are naturally resistant to Thio, most probably due to sequence differences in L11, since yeast ribosomes bearing bacterial L11 are sensitive to the drug (Garcia-Marcos *et al.*, 2007).

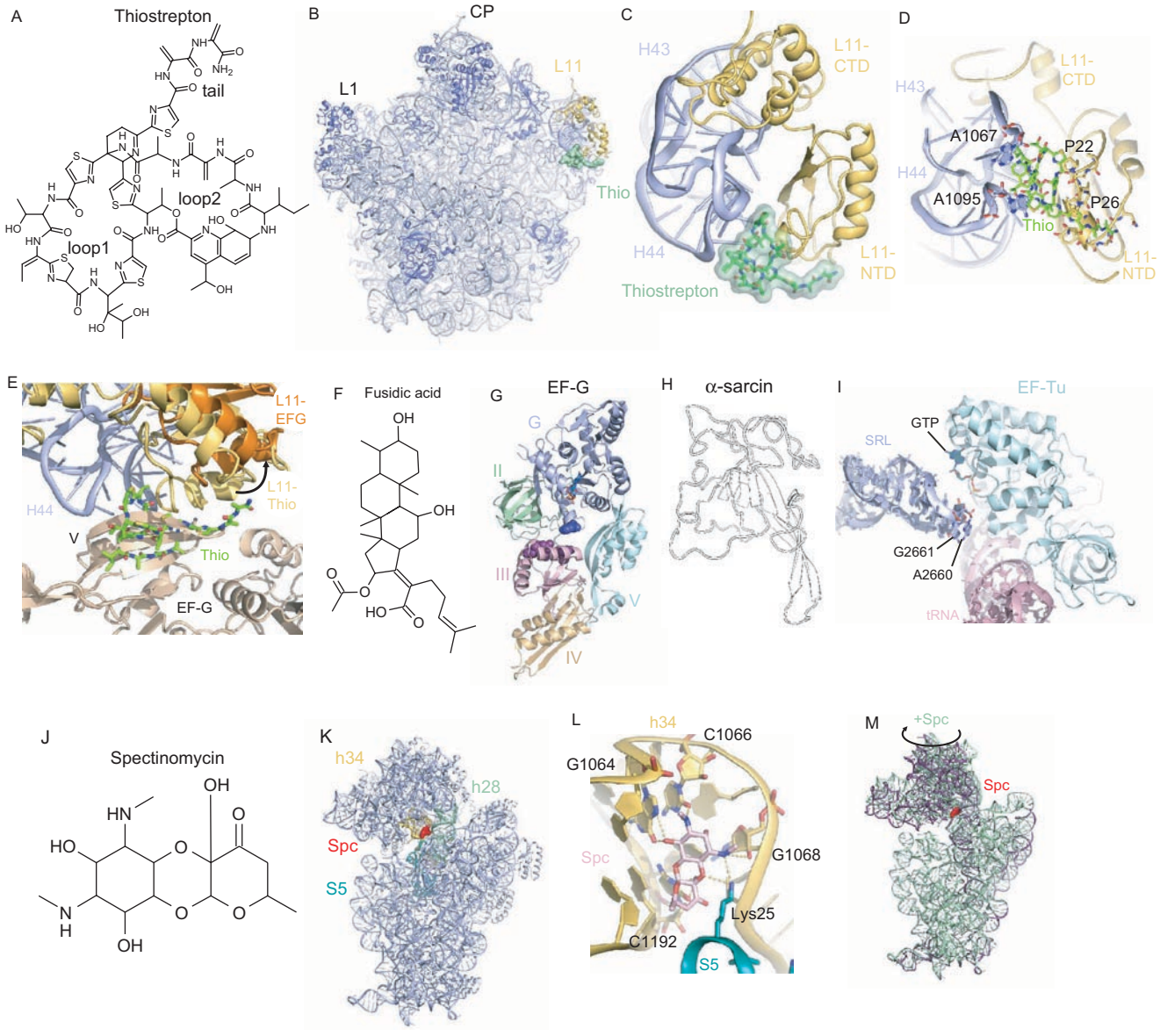

Figure 13. Thiostrepton, fusidic acid, α-sarcin and spectinomycin inhibit translocation. (A) Chemical structure of thiostrepton. (B) Overview of the binding site of thiostrepton (Thio, green) on the large ribosomal subunit, with the central protuberance (CP) and ribosomal proteins L1 and L11 (yellow) indicated. (C) Thiostrepton (green) inserts into a cleft formed by the N-terminal domain of L11 (L11-NTD, yellow) and helices 43 and 44 of the 23S rRNA. (D) Mutations of proline 22 and 26 (P22, P26) and of nucleotides A1067 and A1095, confer resistance to thiostrepton. (E) The binding position of thiostrepton (Thio, green) overlaps that of domain V of elongation factor G (EF-G, tan). Thiostrepton also prevents the opening of the cleft resulting from the movement (as arrowed) of the L11-NTD away from the rRNA that occurs when EF-G binds to the ribosome. (F) Chemical structure of fusidic acid. (G) Crystal structure of EF-G with each domain colored differently. Mutations that confer resistance to fusidic acid found in the G domain (blue) and domain III (pink) are shown as spacefill representations. (H) Structure of the ribotoxin α-sarcin. (I) α-sarcin cleaves between nucleotides A2660 and G2661 located in the sarcin-ricin loop (SRL). The relative position of EF-Tu-GTP (cyan) and tRNA (pink) when trapped on the ribosome with kirromycin are shown. (J) Chemical structure of spectinomycin. (K) Overview of the spectinomycin (Spc, red) binding site on the 30S subunit, with h34 (yellow), h28 (light green) and ribosomal protein S5 (teal) highlighted. (L) Spectinomycin (Spc, pink) forms hydrogen bond interactions (dashed lines) with nucleotides in h34 as well as with lysine 25 (Lys25) of S5. (M) Spectinomycin binds within the neck of the 30S subunit and may prevent translocation by preventing the relative rotation of the head that couples movement of tRNAs during translocation.

The Thio binding site overlaps with the binding site of translation factors, such as the initiation factor IF2 (Allen *et al.*, 2005; Myasnikov *et al.*, 2005), and elongation factors EF-Tu (Schuette *et al.*, 2009; Villa *et al.*, 2009) and EF-G (Connell *et al.*, 2007). Thio has been shown to inhibit IF2-dependent initiation complex formation (Brandi *et al.*, 2004; Grigoriadou *et al.*, 2007), delivery of the aa-tRNA the A-site of the ribosome by EF-Tu (Gonzalez *et al.*, 2007), as well as stable accommodation of EF-G on the ribosome (Seo *et al.*,

2006), which leads to inhibition of the translocation reaction (Rodnina *et al.*, 1999; Pan *et al.*, 2007). The inhibition is most likely direct since these translation factors interact/protect H43/44 from chemical attack (Marzi *et al.*, 2003; Moazed *et al.*, 1988; Moazed and Noller, 1989). Indeed, Thio overlaps with domain V of EF-G and prevents the movement of L11-NTD which opens the cleft for the insertion of domain V of EF-G during accommodation (Figure 13E) (Harms *et al.*, 2008). Together with fast kinetic and FRET studies, this suggests that Thio does not inhibit initial binding of EF-G, nor GTP hydrolysis, but instead prevents inorganic phosphate (Pi) release and translocation and multiple turnover by trapping the factor on the ribosome (Rodnina *et al.*, 1999; Seo *et al.*, 2006; Pan *et al.*, 2007).

Fusidic acid prevents dissociation of EF-G from the ribosome

Fusidic acid is a steroidal antibiotic (Figure 13F) produced by the fungus *Fusidium conccineum* that was introduced into clinical use in 1962 (Godtfredsen *et al.*, 1962a; 1962b), mainly for the treatment of *Staphylococcus aureus* infections (Whitby, 1999). Resistance to fusidic acid in *S. aureus* strains has been selected for (Laurberg *et al.*, 2000; Besier *et al.*, 2003) and mutations identified in the *fusA* gene, which encodes EF-G. High level resistance mutations cluster in two main regions of EF-G, namely at the interface between domains I (G-domain) and III (Figure 13G) (Laurberg *et al.*, 2000). Fusidic acid does not bind to free EF-G, but rather to EF-G•GTP in the complex with ribosome, indicating that the antibiotic requires a specific conformation of EF-G for binding. Cryo-EM reconstructions of fusidic acid-stalled EF-G•70S ribosome complexes have revealed the binding site and interaction mode of EF-G with the ribosome (Agrawal *et al.*, 1998; 1999; 2001), however the resolution is not sufficient to visualize the fusidic acid molecule. Biochemically, fusidic acid permits ribosome-stimulated GTP hydrolysis by EF-G, but prevents the associated conformational changes in EF-G, and thus prevents EF-G turnover by stabilizing EF-G•GDP on the ribosome (Figure 1) (Bodley *et al.*, 1969; Willie *et al.*, 1975; Ticu *et al.*, 2009), analogous to the action of kirromycin on EF-Tu. By binding at the interface of domains I and III, fusidic acid could restrict the movement of these domains relative to each other and thus prevent EF-G from adopting the low affinity GDP conformation (reviewed by Liljas *et al.*, 2000). Therefore, mutations within this region probably either facilitate the conformational changes in EF-G required for dissociation from the ribosome (despite the presence of the drug), or may simply prevent the drug from binding to EF-G (Laurberg *et al.*, 2000). However, there is a fitness cost associated with the resistance mutations, such as P406L and H457Y, and clinical fusidic-acid resistant isolates contain second-site mutations, such as S416F, that alleviate the deleterious effects from the original resistance mutation (Besier *et al.*, 2005).

At very high concentrations (2–3 mM) fusidic acid allows only single turnover GTPase (Bodley *et al.*, 1969), however at lower concentrations fusidic acid acts as slow inhibitor, allowing multiple GTPase turnover (Seo *et al.*, 2006). At all concentrations, fusidic acid does not prevent translocation of tRNAs into A- and P-sites but rather stabilizes the binding of EF-G•GDP to the ribosome and prevents EF-G turnover (Bodley *et al.*, 1969; Spiegel *et al.*, 2007; Ticu *et al.*, 2009). Because of EF-G's dual function, fusidic acid is also a potent inhibitor of ribosome recycling (Figure 1), inhibiting subunit dissociation with an IC_{50} of ~0.1–15 µM (Hirokawa *et al.*, 2005; Savelsbergh *et al.*, 2009), whereas translocation inhibition by fusidic acid is inhibited with an IC_{50} in the range of 10–200 µM (Bodley *et al.*, 1969; Okura *et al.*, 1970; Savelsbergh *et al.*, 2009).

Fusidic acid does not work on eukaryotes, but sordarin is thought to act similarly on yeast EF2 (the homologue of EF-G) and was used to assemble EF2-80S yeast ribosome complexes for cryo-EM analysis (Gomez-Lorenzo *et al.*, 2000; Spahn *et al.*, 2004). However, Sordarin binds to free EF2 and forms interactions with domains III, IV and V, but not the G domain (Jorgensen *et al.*, 2003), and sordarin resistance mutations arise in domains III, IV and V of yeast EF2 (Justice *et al.*, 1998; Shastry *et al.*, 2001). Many of these substitutions are naturally occurring in plant and mammalian EF2s, explaining why sordarin is a fungal-specific translation inhibitor.

Alpha-sarcin cleaves the SRL to prevent GTPase activation of translation factors

Ribosome-inactivating proteins (RIPs) are ribotoxins produced by bacteria, fungi and plants to damage the ribosomes of other organisms, either prokaryotic or eukaryotic (reviewed by Stirpe and Battelli, 2006; Wool *et al.*, 1992). These ubiquitous proteins can be grouped, based on their method of inactivation, into either the α-sarcin-like fungal ribonucleases (RNases, Figure 13H) or the bacterial and plant RIP family of glycosidases, for which ricin is perhaps the best known member. This review focuses on the RIPs that target helix 95 of the 23S rRNA, often termed the sarcin-ricin loop (SRL). The SRL is one of the most highly conserved regions of the ribosome and comprises a continuous irregular helix closed by a GAGA tetraloop, of which the two bases A2660 and G2661 are exposed at the tip (Figure 13I). α-sarcin is active against bacterial

ribosomes and cleaves the SRL on the 3′ side of G2661. In contrast, ricin depurinates eukaryotic ribosomes at the nucleotide corresponding to *E. coli* A2660, i.e. ricin hydrolyzes the N-glycosidic linkage between the ribose and sugar to remove the purine base. The action of α-sarcin or ricin completely abolishes protein synthesis by perturbing the function of elongation factors (Wool, 1984; Hausner *et al.*, 1987). Specifically, the SRL is thought to play an important role in stimulating the GTPase activities of translation factors (Blanchard *et al.*, 2004). In the high-resolution cryo-EM structures of EF-G•GDPNP•70S (Connell *et al.*, 2007) and kirromycin stalled EF-Tu•GDP•tRNA•70S (Schuette *et al.*, 2009; Villa *et al.*, 2009), the SRL loop contacts the G-domains of the translation factors (Figure 13I), where it has been proposed to trigger GTP-hydrolysis. Deciphering how RIP alterations in this loop perturb translation factor function will require a more detailed understanding of how the SRL triggers GTPase activation.

Spectinomycin stabilizes an intermediate state during translocation

Spectinomycin (Spc) is an aminocyclitol antibiotic closely related to the aminoglycosides (Figure 13J), produced by the bacterium *Streptomyces spectabilis* (Hanka *et al.*, 1961; Lewis and Clapp, 1961; Mason *et al.*, 1961; Hoeksema and Knight, 1975). Spc is a broad-spectrum antibiotic that is mainly used clinically as Trobicin® to treat *Neisseria gonorrhoea* infections (Willcox, 1962), but has been withdrawn in many parts of the world due to increasing resistance. Spc binds to the neck region of the 30S subunit (Figure 13K), where it interacts predominantly with the minor groove of helix 34 (Figure 13L) (Carter *et al.*, 2000; Borovinskaya *et al.*, 2007b). The two sugar moieties of Spc form hydrogen bonds to four nucleotides (G1064, C1066, G1068 and C1192) in h34 (Figure 13L), consistent with chemical protections (Moazed and Noller, 1987b) and Spc-resistance mutations (Fromm *et al.*, 1987; Svab and Maliga, 1991; Brink *et al.*, 1994; Johanson and Hughes, 1995; Kehrenberg and Schwarz, 2007) within this region. In addition, Lys25 extending from a loop of ribosomal protein S5 can potentially establish interactions with the drug (Figure 13L). Many of the mutations conferring Spc-resistance in S5 are located in the N-terminus (Funatsu *et al.*, 1971; Davies *et al.*, 1998; Kehrenberg and Schwarz, 2007) and thus most likely confer resistance indirectly. Unlike aminoglycosides, Spc does not promote back-translocation, but rather impedes it, and Spc does not induce translational misreading (Davies *et al.*, 1965). However, like aminoglycosides, Spc inhibits the translocation reaction (Figure 1) (Bilgin *et al.*, 1990; Fredrick and Noller, 2003; Peske *et al.*, 2004), but the mechanism of inhibition differs since Spc appears to stabilize an intermediate state of the ribosome that occurs during translocation (Pan *et al.*, 2007). The crystal structure of Spc bound to the *E. coli* 70S reveals that the presence of the drug rotates and locks the head in a distinct position relative to the body (Figure 13M) (Borovinskaya *et al.*, 2007b). Collectively, this suggests that Spc traps the ribosome in a translocation intermediate by preventing the rotation of the head necessary for movement of the tRNAs and mRNA into the P- and E-sites.

Viomycin blocks translocation by stabilizing hybrid state formation

Viomycin, a cyclic peptide antibiotic produced by *Streptomyces sp*, is a member of the tuberactinomycin family that are active against *Mycobacterium tuberculosis*. Viomycin was the first tuberactinomycin used to treat TB (Bartz *et al.*, 1951) until it was replaced by the structurally related capreomycin, which is less toxic but is now only used as a second-line drug (reviewed by Jain and Dixit, 2008). Viomycin stabilizes the peptidyl-tRNA at the A-site and the deacylated tRNA in a hybrid P/E state (Peske *et al.*, 2004; Shoji *et al.*, 2006; Ermolenko *et al.*, 2007; Pan *et al.*, 2007). Thus, like spectinomycin, viomycin inhibits translocation (Figure 1) (Liou and Tanaka, 1976; Modolell and Vazquez, 1977) by trapping the ribosome in an intermediate state on the translocation pathway. In addition, viomycin induces back-translocation (Shoji *et al.*, 2006) and translational misreading (Wurmbach and Nierhaus, 1983), analogous to the aminoglycosides. The binding site of viomycin is likely to overlap with that of the aminoglycosides (Figures 6A, 6E), since both drugs compete with each other for ribosome binding (Misumi *et al.*, 1978). Similar to some aminoglycosides, viomycin may have two binding sites on the ribosome, one on the small subunit and one on the large. Viomycin protects nucleotides 912–915 and A1408 in the 16S rRNA as well as U913 and G914 in the 23S rRNA from chemical attack (Moazed and Noller, 1987a). Furthermore, resistance to viomycin results from ribosomes that have mutations or alterations in either 16S or 23S rRNA (Yamada *et al.*, 1978; Maus *et al.*, 2005; Johansen *et al.*, 2006), and inactivation of the *tylA* gene, a methyltransferase that methylates C1409 in h44 of the 30S subunit and C1920 in H69 of the 50S subunit, confers resistance to viomycin and capreomycin (Johansen *et al.*, 2006). Thus it seems likely that tuberactinomycins bind in the vicinity of intersubunit bridge B2a, with one binding site in h44 of the 30S and a second within H69 of the 50S. Binding of viomycin locks B2a in a particular ratcheted conformation that stabilizes hybrid site formation. Therefore mutations

in these components are likely to either prevent drug binding or permit completion of translocation in the presence of the bound drug. Hybrid P/E-site formation also occurs during ribosome recycling and has been shown to be inhibited by viomycin (Hirokawa *et al.*, 2002).

Future perspectives

With the ever-increasing emergence of antibiotic-resistant bacteria, the development of novel and more potent antibiotics continues to be the challenge for the future. The availability of structural data for the ribosome as well as a huge array of ribosome-antibiotic complexes (Table 1) provides an excellent basis for the rational design of new inhibitors (Sutcliffe, 2005; Franceschi and Duffy, 2006). In terms of the ribosome, a number of excellent antimicrobials have been or are being developed (Sutcliffe, 2005), which encompasses the continuing improvement of known protein synthesis inhibitors, but also the use of novel approaches to make hybrid antibiotics, such as linking (i) macrolides with chloramphenicols (Wu and Su, 2001), (ii) two different types of aminoglycosides, hygromycin B and paromomycin (Vourloumis *et al.*, 2005), or (iii) linking sparsomycin with oxazolidinones (Franceschi and Duffy, 2006). However, a number of compounds that have been reported to inhibit translation need to be characterized further, e.g. TAN-1057 (Boddeker *et al.*, 2002) and the orthosomycins (Belova *et al.*, 2001) that bind to the large subunit, as well as GE82832 (Brandi *et al.*, 2006a) and the NRI compounds (Dandliker *et al.*, 2003) that appear to act on the small subunit. In addition, a number of biochemical approaches have been taken to identify new functionally important hotspots on the ribosome that do not overlap with known antibiotic binding sites (Laios *et al.*, 2004; Yassin *et al.*, 2005; Yassin and Mankin, 2007). The huge plethora of bacterial-specific factors involved in specific stress responses or ribosomal assembly (Wilson and Nierhaus, 2007) have also been suggested as potential targets for antimicrobials (Comartin and Brown, 2006; Maguire, 2009). The general trend of large pharmaceutical companies in shifting away from antimicrobial research suggests that academic institutes and small biotech companies will play a more important role in the future direction of fundamental antibiotic research.

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