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Resistance to Linezolid

22

Eleni Ntokou and Birte Vester

1 Introduction

Linezolid is an antimicrobial agent that binds to the bacterial ribosome and thereby inhibits protein synthesis. Soon after its release as a clinical drug, it became clear that bacteria could become resistant to linezolid. The resistance mechanisms are mainly causing alteration of the drug target site, but probably efflux might also play a role. The resistance is still rare in surveillance studies, but outbreaks of resistant clones from hospitals have been observed. So far the main mechanisms of resistance are occurrence of mutations in ribosomal genes or obtaining plasmids with a gene coding for a methyltransferase providing resistance. The most obvious way to avoid resistance may be development of derivatives of linezolid overcoming the known resistance mechanisms.

2 Linezolid and Its Derivatives

Linezolid belongs to the oxazolidinones, a synthetic drug class, and is one of few new drugs on the market for antibiotics in many years. The history of the discovery of linezolid has already been extensively reviewed [1–4]. Oxazolidinones were primarily identified and patented by E. I. du Pont de Nemours & Company (DuPont) in 1978 [5]. DUP-105 and DUP-721 were developed as first lead compounds of oxazolidinone antibacterials and showed activity against Gram-positive bacteria, but the project was terminated due to lethal toxicity in animal models [4, 6]. Later, scientists at Upjohn Laboratories started a project in order to modify the original compound and produce new oxazolidinones, with better antibacterial activities and higher safety levels. Among a series of oxazolidinones, PNU-100766 (Linezolid) and PNU-100592 (Eperezolid) showed oral efficacy, good water solubility, and good activity against Gram-positive bacteria. Both of them were further evaluated by phase 1 clinical trials but only linezolid proceeded to phase 2 clinical trials due to its superior bioavailability. Linezolid was approved by FDA in 2000 and marketed as Zyvox™ [4, 7]. Linezolid has been employed for treating diseases caused by Gram-positive bacteria [8, 9], which include streptococci, vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), some Gram-negative anaerobic species, and *Mycobacterium tuberculosis* [10–12].

Linezolid (Fig. 22.1a) is proven to be a highly effective drug and a good alternative for the treatment of difficult infections being able to be administered either intravenously or orally. However, it does have some liabilities and can cause adverse effects such as interaction with serotonergic agents that could lead to serotonin syndrome in patients with depression, and production of reversible thrombocytopenia and bone marrow suppression when given for prolonged periods of time [13, 14]. The biggest issue raised by the use of linezolid in clinical practice, soon after it was available on the market, was the appearance of linezolid-resistant strains of *S. aureus* and enterococci [15, 16]. The mechanisms that confer this resistance will be described in following sections of this chapter. However, development of derivatives of linezolid to overcome this issue is currently underway (Fig. 22.1) [17].

The most important linezolid derivative is currently tedizolid (Fig. 22.1b) (formerly torezolid), which was under clinical development by Cubist pharmaceuticals for the treatment of serious Gram-positive infections. Tedizolid phosphate (TR-701) is an inactive prodrug that is chemically converted by serum phosphatases to the active form tedizolid (TR-700) [18]. Tedizolid phosphate was approved by the FDA (20/06/2014) with the commercial name Sivextro™. Sivextro is indicated for the treatment of acute bacterial skin and skin structure infections (ABSSSI). It is active against Gram-positive organisms, including staphylococci, enterococci, streptococci, and certain anaerobes [19, 20].

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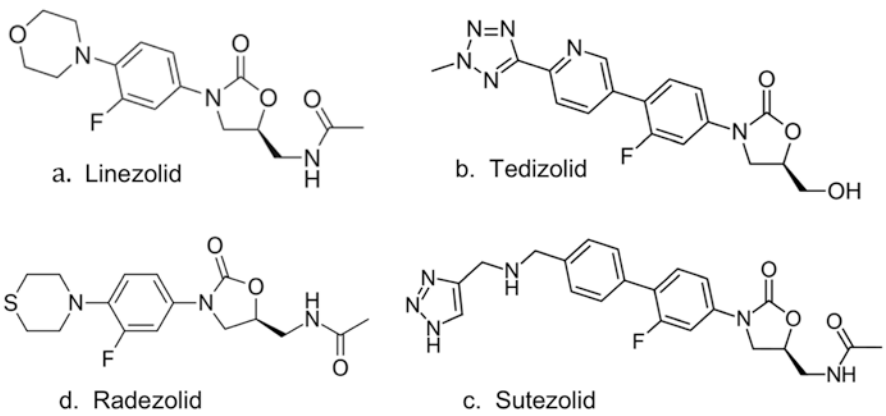

Fig. 22.1 Chemical structural formula for linezolid (**a**) and three derivatives (**b**)–(**d**)

Tedizolid demonstrates a greater potency than linezolid, at least fourfold for all bacteria tested [20]. Of particular interest, were the tested linezolid-resistant *S. aureus* strains, which possess mutations in chromosomal genes encoding ribosomal rRNA and proteins, or carrying the horizontally transferable *cfr* gene. Methylation of A2503 of 23S rRNA by the Cfr methyltransferase confers resistance to linezolid but not to tedizolid because of structural differences between the two drugs [21]. Initial studies have also shown that tedizolid may not have the negative effects on serotonergic agents and thrombocytopenia as linezolid show [22, 23].

Other derivatives under investigation are radezolid and sutezolid (Fig. 22.1c, d). Radezolid is a unique oxazolidinone because it has activity against fastidious Gram-negative bacteria like *H. influenzae* and *M. catarrhalis,* as well as against Gram-positive bacteria, including MRSA, linezolid-resistant staphylococci and enterococci [24]. Radezolid has completed two phase 2 clinical trials to date: the first in community-acquired pneumonia (CAP) and the second trial in complicated skin and skin structure infections (cSSSI) [2]. To date, phase III trials have not been initiated [25]. It is unclear at this point, based upon published literature, whether radezolid has any advantages over linezolid. Sutezolid is a linezolid derivative with superior bactericidal activity against *M. tuberculosis* as demonstrated by a Phase 2 clinical study [26].

Because linezolid resistance has started to arise by various mechanisms, in various bacteria, the development of new derivatives seems to be the next step in the battle against isolates resistant to this class. The derivatives mentioned earlier in this section demonstrate higher potency and lower resistance rates compared to linezolid. Due to their properties, they could potentially compensate at occasions where linezolid-resistant isolates arise. They will probably not yet replace linezolid in clinical use, as it is still a widely used antibiotic with relatively low incidence of resistance.

3 Mechanism of Action of Linezolid

Early studies of the effect of oxazolidinones pointed to inhibition of protein synthesis in growing bacteria [27] and suggested an effect on synthesis initiation, which was also supported by later studies [28, 29]. Studies of the effect on peptidyl transferase using puromycin reactions reported contradicting results that might be due to the relative unnatural conditions of these assays. Other studies demonstrated frame-shifting and nonsense suppression [30] and effect on fMet-tRNA binding and translocation [31].

The fact that linezolid binds to the peptidyl transferase center (PTC) of the bacterial ribosome (illustrated in Fig. 22.2) was first indicated by mutations in 23S ribosomal RNA conferring resistance [32], 23S mutagenesis studies, and cross-linking studies [33, 34]. The site was finally confirmed and defined in 2008 by crystal structures of linezolid bound to the 50S ribosomal subunit from the archaeon *Haloarcula marismortui* [35] and from the bacterium *Deinococcus radiodurans* [36]. The site is in the bottom of the cleft of the 50S ribosomal subunit where the 3′-ends of aminoacyl-tRNA and peptidyl-tRNA are positioned for peptide transfer (Fig. 22.2b), and is highly conserved in all bacteria. The same site in the ribosome binds other antibiotics such as chloramphenicol, clindamycin, tiamulin, and streptogramin A, several of which are characterized as peptidyl transferase inhibitors. It seems like the size and the environment of the PTC facilitates binding of a range of antibiotics, which at binding interfere with the peptide transfer process. They can either disturb the positioning of aminoacyl-tRNA or peptidyl-tRNA for peptide transfer or directly block some movements required during peptide transfer. How the effect will show up in various assays to elucidate the specific mechanism will also depend on their exact competition with the components of the peptide synthesis apparatus. A very

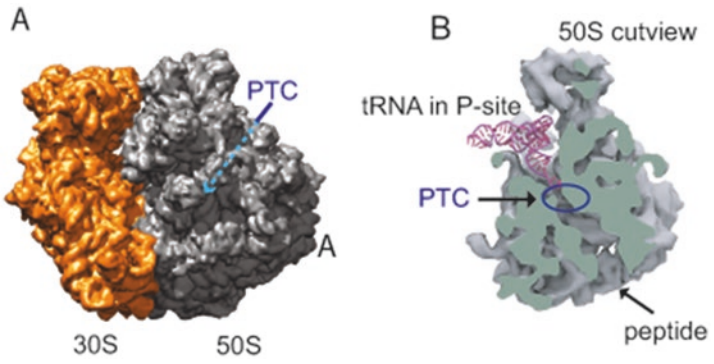

Fig. 22.2 (**a**) A model of the two ribosomal subunits in bacteria (based on PDB: 4YBB). The *arrow* points to the peptidyl transferase center in the middle of 50S where the amino acids are added together and where linezolid binds. (**b**) A cut-view of the 50S subunit (based on PDB: 2 J00, 2 J01, 2 J02, 2 J03), again showing the PTC area in the *blue circle*

recent study of ribosome function in a linezolid-resistant *Staphylococcus epidermidis* mutant showed a functional and structural adaptation of ribosomes. The study reported an increased peptidyl transferase activity, as measured by puromycin reactivity, as well as an enhanced growth rate in the presence of linezolid [37]. Even though the very exact step of inhibition has not been determined for oxazolidinones and maybe will never be completely elucidated, as more than one step might be involved, it can be concluded that the general effect of linezolid is inhibition of protein synthesis by binding to the peptidyl transferase center of the bacterial ribosome and affecting some step directly related to the peptidyl transferase reaction.

4 Mechanisms of Resistance

Several ways of resistance to linezolid have been published. The very well investigated and proven ones are mutations in 23S rRNA in the peptidyl transferase area of the ribosome, and methylation of 23S rRNA nucleotide A2503. The less proven but highly indicative ones are mutations in the ribosomal protein L3 and efflux. In addition, mutations in ribosomal protein L4 have been connected with reduced linezolid susceptibility but the extent of this correlation remains to be elucidated. Finally, fitness cost in relation to resistance seems to be an issue. The following section will review the present knowledge of this field.

4.1 Resistance Caused by 23S rRNA Mutations

Although early laboratory investigations suggested that resistance to linezolid might be slow to emerge [32, 38], as almost all bacteria have multiple copies of the 23S rRNA gene, linezolid-resistant strains soon appeared [15, 39]. The first linezolid-resistant strains were associated with mutations in domain V of the 23S rRNA genes, mainly G2576U transversion. Over time various mutations have been identified in domain V of 23S rRNA (Fig. 22.3) and they remain the predominant mutations conferring linezolid resistance [55]. The G2576U transversion is the most prevalent mutation in linezolid-resistant clinical isolates, including *S. aureus*, coagulase negative staphylococci (CoNS), viridans group streptococci, *Enterococcus faecium*, and *Enterococcus faecalis* [56, 57]. The first reported linezolid-resistant enterococcal isolates were obtained from patients treated with linezolid as part of the *Linezolid Compassionate Use Program* (1999). They had the G2576U mutation in multiple operons of the 23S rRNA genes and with MICs correlating to the number of mutated operons [58].

The first clinical isolate of linezolid-resistant *S. aureus*, with a G2576U mutation, was reported in 2001 [15]. Later, this isolate was found to contain five copies of the 23S rRNA gene, all of which were mutated at position 2576 [59] and again a clear correlation between the number of mutated rRNA operons and the linezolid MIC was established [40, 60, 61]. Most reports of the G2576U mutation in clinical isolates is associated with some form of increased or prolonged linezolid treatment, and it has been shown that the duration of linezolid exposure and dose can affect the number of mutated rRNA operons and thus linezolid resistance [62]. Mutant gene-dosage effects have also been seen in laboratory-derived linezolid-resistant *S. aureus* mutants and in clinical isolates of linezolid-resistant enterococci [40, 41]. A report from 2011 demonstrated that the G2576U mutation was retained in a *Staphylococcus haemolyticus* isolate even after 30 serial passages in antibiotic-free medium [42], although some studies have documented reversion of the G2576U mutation in the absence of linezolid pressure [41, 63]. Therefore prolonged linezolid usage should be judicious and minimized in clinical settings.

The linezolid-binding site at the PTC comprises conserved nucleotides (G2061, A2451, C2452, A2503, U2504, G2505, U2506, and U2585), which interact directly with linezolid, see Fig. 22.3 [35, 36]. Laboratory derived strains selected for linezolid resistance show mutations in either nucleotides at the proximity of the binding pocket (2061, 2452, 2503, 2504, and 2505) or at nucleotides further away from it (2032, 2062, 2192 2447, 2453, 2499, 2500, 2576, 2571, 2572, 2608, and 2612) [32, 36, 38, 43–48, 64–67]. The degree of linezolid resistance is not a simple function of the

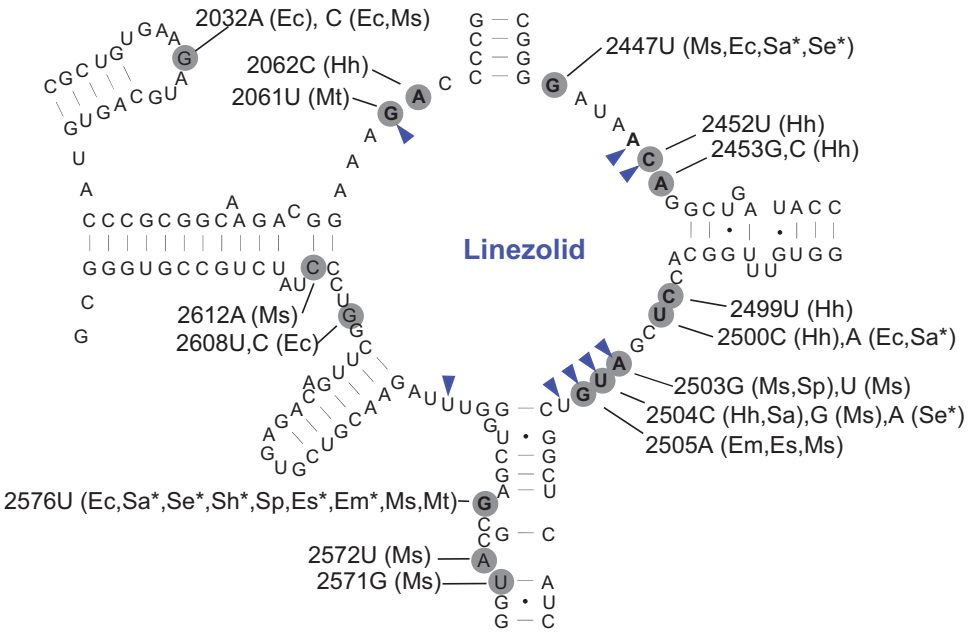

Fig. 22.3 A secondary structure model of the peptidyl transferase loop of domain V of 23S rRNA (*E. coli* sequence and numbering). *Blue triangles*: nucleotides that form the linezolid-binding pocket, *gray circles*: mutations that confer linezolid resistance with bold type for nucleotides where mutations have a considerable effect on linezolid MIC and regular type for mutations with a small to moderate effect. Organisms: *E. coli* (Ec), *S. aureus* (Sa), *S. epidermis* (Se), *S. haemolyticus* (Sh), *S. pneumoniae* (Sp), *E. faecalis* (Es), *E. faecium* (Em), *Mycobacterium smegmatis* (Ms), *M. tuberculosis* (Mt), and *Halobacterium halobium* (Hh) [32, 38, 40–54]. Asterisks indicate mutations found in clinical isolates

nucleotide-linezolid distance and distal nucleotides that do not interact with linezolid directly, as G2576U and G2447U can confer significantly high resistance [3].

Acquired resistance to linezolid has been observed in various clinical isolates of Gram-positive cocci. A methicillin-resistant *S. aureus* (MRSA) bloodstream isolate, derived from a patient exposed to a prolonged course of linezolid, developed resistance and had a U2500A mutation in the 23S rRNA and a loss of a single copy of the gene in the most resistant isolates [41]. Various clinical strains of *S. aureus, S. epidermidis, E. faecium, E. faecalis* that are highly resistant to linezolid show a variety of 23S rRNA mutations including G2447U [52], A2503G [45], U2504C [45], U2504A [51], and G2505A [68], despite of evidence of fitness cost associated with some of these mutations [60]. Some additional mutations of the 23S rRNA operons have been reported at positions G2603U [69–71] and C2534U [51, 52] but direct relationship between these mutations and linezolid resistance is not yet established.

Up to date, G2576U is the most common mutation found in clinical isolates [72]. In addition, the U2500A and G2447U mutations have been reported in linezolid-resistant clinical isolates of staphylococci and these mutations have also been shown to confer linezolid resistance in in vitro selected mutants of *E. coli* and *Mycobacterium smegmatis* [38, 46].

4.2 Resistance Caused by Alterations in 23S rRNA Modification

Ribosomal RNA is intrinsically modified with methyl groups and pseudouridine residues, and these modifications are clustered at functional centers on the ribosome. Methylations can also be an acquired trait, and it is well established that RNA modifications placed at or near an antibiotic-binding site can affect drug binding to the ribosome [73]. Resistance generally occurs either by the inactivation of an indigenous methyltransferase or the acquisition of an antibiotic resistance methyltransferase.

Some housekeeping modifications at the PTC are shown to affect linezolid susceptibility. The pseudouridylation of 23S rRNA nucleotide 2504 confers reduced susceptibility to linezolid, clindamycin, and tiamulin, suggesting that this modification may have evolved as an intrinsic resistance mechanism to protect bacteria from PTC-binding antibiotics [74]. Inactivation of the methyltransferase targeting G2445 in 23S rRNA results in decreased susceptibility to linezolid in *Streptococcus pneumoniae* [43, 75]. Likewise, a mutation inactivating the methyltransferase RlmN that methylates 23S rRNA at the C2 position of A2503 also results in slightly lowered linezolid susceptibility in *S. aureus* [76, 77]. None of these mechanisms of linezolid resistance or reduced susceptibility has yet been shown to be of clinical importance, either because of nonoccurrence or not being revealed yet. This is in contrast to the only known transferable form of linezolid resistance conferred by the multi-resistance gene *cfr* that has been found in many clinical strains, especially in *Staphylococcus*. Cfr encodes an rRNA methyltransferase [78] that adds a methyl group at the C8 position of the 23S rRNA nucleotide A2503 [79], a position interacting directly with linezolid and where mutations have shown to result in resistance (see Fig. 22.3). The methylation confers some resistance to linezolid as well as resistance to five other classes of antibiotics that bind at overlapping nonidentical sites at the PTC [80, 81]. A direct interference of the methylation with drug binding is supported by the X-ray

structures of linezolid bound to the *Deinococcus radiodurans* and *H. marismortui* 50S subunits [35, 36].

The *cfr* gene was originally discovered on multiresistance plasmids isolated during surveillance studies of florfenicol resistance in *Staphylococcus* spp. of animal origin [82, 83]. In 2005, the first *cfr*-positive clinical strain of a methicillin-resistant *S. aureus* was reported from a patient briefly treated with linezolid [84]. The strain had *cfr* on the chromosome together with the *ermB* gene on a transposable genetic element and the co-expression of these two rRNA methyltransferase genes conferred resistance to all clinically relevant antibiotics that target the large ribosomal subunit [81]. Since then a large number of staphylococcal clinical isolates containing *cfr* in different genetic contexts have been found around the world [85–90]. In some instances, a connection between the resistant isolates and prior linezolid treatment can be documented (i.e., see section on clinical linezolid-resistant strains below). The *cfr* gene has also been identified in other pathogenic bacteria, both Gram-positive and Gram-negative, often from animals and with no relation to linezolid treatment. The presence of *cfr* on mobile genetic elements such as plasmids and transposons in different geographical locations strongly suggests that it can be disseminated within the microbial community and spread among pathogenic bacteria, thus conferring resistance to linezolid without prior exposure to the drug.

4.3 Linezolid Resistance and a Conceivable Relationship to Mutations in Ribosomal Proteins L3

Mutations in the ribosomal L3 protein have recently received attention as a linezolid resistance determinant. The main part of ribosomal protein L3 is positioned on the surface of the large ribosomal subunit, but a loop extends into the PTC near the linezolid-binding site. Bacterial L3 mutations have been associated with resistance to linezolid, tiamulin/valnemulin, and anisomycin, that all bind to overlapping sites at the PTC [3]. The first L3 resistance mutation in bacteria was detected by selection with tiamulin, and its role in resistance was verified by transfection and plasmid-coded mutant L3 expression [91]. Since then, a number of studies have associated L3 mutations with linezolid resistance in various staphylococci and few other clinical relevant pathogens. A selection of some of these is displayed in Table 22.1. As evident in the table, most of the L3 mutations are present together with one or two other resistance determinants, namely 23S rRNA mutations and the *cfr* gene. Unfortunately, most of the studies presenting L3 mutations do not provide evidence that the L3 mutations are the direct cause of resistance. Seemingly, only Cfr and the 23S rRNA mutations give a medium to high resistance and it might be that the appearance of the L3 mutations are merely a selection to adopt to changes in the 23S rRNA (see section discussing fitness cost below). Nevertheless, the positions of most of the L3 mutations are relatively close to the linezolid binding in the ribosome with the closest being at a distance of approximately 7 Å [3]. Also, the relation between decreased susceptibility to the pleuromutilins retapamulin and tiamulin and L3 mutations in the same region [46, 98, 105, 106] supports the relation between L3 mutations and linezolid resistance, as pleuromutilins and linezolid bind at overlapping sites in the PTC but are otherwise very different [80]. There are also reports about L3 mutations that have been detected in linezolid susceptible strains and are therefore not considered relevant to linezolid resistance (e.g., L101V that is positioned far from the PTC [100]). At the moment, it is difficult to establish exactly which L3 mutations do have a relation to reduced linezolid susceptibility, although the circumstantial evidence point to the part of the L3 protein nearest to PTC with some variations between species. One study of in vitro development of linezolid resistance in *M. tuberculosis*, as well as findings in clinical isolates, does provide strong evidence for the involvement of an L3 C154R mutation in linezolid resistance [103]. This is also supported by another finding concerning the same L3 mutation plus a neighboring mutation in clinical samples of *M. tuberculosis* [104].

4.4 Other Aspects of Linezolid Resistance: Fitness Cost, Cross-resistance, and Enhancement of Growth

In addition to reports about L3 mutations there are also reports about L4 mutations related to linezolid resistance [3]. Part of the ribosomal protein L4 is also placed relatively close to the PTC, but in the tunnel through which nascent peptides exit the ribosome [3]. Again, most studies do not prove a relationship between L4 mutations and resistance effects, except for a surveillance study of *S. pneumoniae* with a six-nucleotide deletion in the L4 gene (ΔW65-R66) in one strain and a neighboring six-nucleotide deletion (ΔK68-G69) in another strain [107]. These deletions caused a slightly reduced susceptibility to linezolid, as evident by transformations, and were associated with a fitness cost [107]. The amino acid deletions are located in the same region as mutations known to be involved in macrolide resistance [108], and as macrolide antibiotics bind to a site neighboring, but not directly overlapping, the linezolid-binding site, we imagine the effect of these deletions is probably caused by an allosteric mechanism. In general, the L4 mutations presented in relation to linezolid resistance do not present a

Table 22.1 A selection of mutations in L3 that have been associated with linezolid resistance in staphylococci and *Mycobacterium tuberculosis*

L3 mutations	Organism	Remarks[a]	Reference
ΔF127-H146	*S. aureus*	In vitro selected mutant	[92]
Q136H/H146Δ	*S. aureus*	L4-G69A/T70P/G71S	[93]
G137A/L94V[b]	*S. epidermidis*	2576 T	[55]
G139R	*S. aureus*	T, 2576 T	[94]
G139R/M156T	*S. hominis*	T, 2576 T	[95]
ΔS145	*S. aureus*		[96]
ΔS145/H146Y	*S. aureus*	*cfr*	[97]
H146R/M156T/L101V[b]	*S. epidermidis*	T, 2215A, 2576 T, L4-ins70G	[98]
H146Q/V154L/A157R/L101V[b]	*S. epidermidis*	T, L4- ins70G/[c]	[98]
H146Q/L94V[b]	*S. epidermidis*	L4-$_{71}$GGR$_{72}$/[c]	[55]
H146Q/V154L	*S. epidermidis*	2319U, L4-$_{71}$GGR$_{72}$	[93]
H146Q/V154L/A157R	*S. epidermidis*	C2534T, L4-$_{71}$GGR$_{72}$	[99]
F147L	*S. epidermidis*	*cfr*	[93]
F147L/L94V[b]	*S. epidermidis*	L4[c]	[55]
F147L/L94V[b]	*S. epidermidis*	*cfr*, L4-G71D/[c]	[55]
F147I/L101V[b]	*S. epidermidis*	T, 2576 T	[98]
F147I	*S. hominis*	T, 2576 T	[95]
F147L/A157R/L101V[b]	*S. epidermidis*	*cfr*, L4[c]	[100]
F147L/A157R/L101V[b]	*S. epidermidis*	L4-K68R/[c]	[100]
G152D	*S. aureus*	In vitro selected mutant, 2447 T	[92]
G152D	*S. aureus*	T	[94]
G152D	*S. haemolyticus*	*cfr*	[87]
G152D/D159Y/L101V[b]	*S. epidermidis*	T, 2504A/2534 T	[51]
G152D	*S. epidermidis*	90 % T, +/− cfr, +/− 2576 T	[90]
G152D/D159E/A160P/L94V[b]	*S. epidermidis*	T, 2504A, 2530A, 2631U	[101]
G155R	*S. aureus*	In vitro selected mutant	[92]
G155R/M169L	*S. aureus*	In vitro selected mutant	[92]
M156T	*S. haemolyticus*	T, *cfr*, 2576 T	[88]
A157R	*S. epidermidis*	2447 T	[96]
S158Y/D159Y/L101V[b]	*S. epidermidis*	*cfr*	[102]
S158F/D159Y	*S. cohnii*	*cfr*, L4-N20S/A133T/V155I	[102]
Y158F	*S. cohnii*	*cfr*	[87]
ΔM169-G174	*S. aureus*	*cfr*	[97]
C154R	*M. tuberculosis*	In vitro selected mutant	[103]
C154R	*M. tuberculosis*	+/− 2061 T	[104]
H155R	*M. tuberculosis*		[104]

All isolates are clinical except from the ones depicted as "In vitro selected mutant". Information about treatment with linezolid was omitted for the strains from reference [55], because of inadequate data. The L3 positions are according to the various organisms and can thus correspond to similar positions although they have different numbering

[a]Selected additional information: treatment with linezolid (T), contain *cfr* gene (*cfr*), potential additional resistance determinants (xxxxN refers to 23S rRNA positions corresponding to *E. coli* 23S rRNA, L4-…. indicate additional mutations)

[b]L3 mutations that are considered strain markers and not relevant for antibiotic resistance are only included when found together with other mutations

[c]L4-N158S, which is not expected to influence linezolid resistance

consistent pattern and it is not definitively established which changes, if any, contribute directly to linezolid resistance.

Another potentially important resistance determinant is the presence of efflux pumps. Linezolid is not well suited for fighting Gram-negative pathogenic bacteria because they are intrinsically resistant due to efflux pumps that force linezolid out of the cell faster than it can accumulate [109, 110]. For example, a remarkably high linezolid MIC at 256 μg/mL (a 102-fold increase) was seen after cloning of a putative multidrug efflux pump from a *Vibrio cholerae* to a plasmid in a hypersensitive *E. coli* [111]. It is thus not surprising that changes in efflux in Gram-positive bacteria may influence the effect of linezolid. It has been shown that *S. aureus* possesses a gene for a major facilitator superfamily type multidrug efflux pump named LmrS that is capable of extruding linezolid [112]. Linezolid resistance caused by mutations

increasing the expression of ATP-binding cassette (ABC) transporter genes has been observed in *S. pneumoniae* [43, 75]. The mutations were found by genome sequencing of a linezolid-resistant strain and the effect was analyzed by gene disruption experiments [43]. A follow-up study involving stepwise increase of resistance by genome transformation supported the role of a specific mutation that increased expression of an ABC transporter as a resistance determinant [75]. However, not surprisingly, such changes may come with a cost in growth rate. Future experiments might reveal if efflux is a significant factor in linezolid resistance or not. As a general lesson from research on antibiotic resistance, starting to look might greatly enhance the insight.

It is one thing for bacteria to obtain a resistance determinant but another thing to sustain it and to avoid being outgrown by nonresistant neighbors. The maintenance and spread of resistance genes is related to their fitness cost. Expression of the linezolid resistance determinant Cfr in a laboratory strain had only a small effect on growth rate [113]. Such low fitness cost is troublesome as it suggests that cells can maintain a gene even in the absence of antibiotic selection. Competition experiments showed that cells with an inactivated *rlmN* gene (i.e., showing slightly lowered linezolid susceptibility, as mentioned above) outcompeted *S. aureus* wild-type cells under linezolid selection [77]. The fitness cost of resistance mutations varies, and is also dependent on the specific organism. A decrease in growth rates for 23S rRNA mutations at the PTC is expected because many of the nucleotides are phylogenetically conserved and are considered functionally important. For example, the single mutations in the PTC area of 23S rRNA in *M. smegmatis* that have the most significant effects on linezolid resistance show either a moderate (A2503G/U and G2447U) or a large (U2504G and G2576U) decrease in growth rate, where the G2576U mutation with the largest resistance effect results in a threefold slower growth [48, 50, 66]. This is consistent with the fact that although both the G2447U and G2576U mutations lead to 32-fold increases in linezolid MIC values, only the G2447U mutation was isolated by selection in the presence of linezolid [38, 48]. The G2576U mutation has also been studied extensively in *S. aureus*, where a progressive decrease in growth rate is observed with each additional 23S rRNA gene copy harboring the mutation [60]. However, the ability of the mutation to persist in one copy in the absence of antibiotic selection and the rapid reemergence of multiple mutated copies upon reexposure to linezolid suggests that a single copy has a minimal fitness cost [114]. Such a resistance mutation may be accompanied by other mutations that compensate for deleterious effects or act synergistically to enhance resistance. An example is the stepwise genome transformation study mentioned above [75], where linezolid resistance by G2576U in 23S rRNA comes with a fitness cost that can be counteracted by an L3 mutation at position Y137H in *S. pneumoniae*. The study shows that the L3 mutation alone does not confer reduced susceptibility to linezolid. The mutation corresponds to the L3 F147L mutation in *S. epidermis* that has been related to linezolid resistance in several studies (Table 22.1). It remains to be established how many of the mutations in Table 22.1 are true resistance determinants and how many are compensatory "fitness cost" mutations or just random mutations without any phenotypic effect. A possibly related matter has recently been published concerning linezolid-resistant *S. epidermis* strains that grow better in the presence of linezolid than in the absence, and which contained mutations at positions U2504A and C2534U in 23S rRNA together with L3 mutations G152D and D159Y [51]. Also, a synergistic effect of linezolid resistance determinants has been verified in *S. epidermis* with *cfr* plus C2534U in 23S rRNA (in two of six alleles) plus mutations in L3 and L4 [99]. Possible synergistic effects have also been reported for other PTC antibiotics in other bacteria such as *M. smegmatis* [48] and *Brachyspira* spp. [115, 116], indicating interplay between multiple mutations in relation to resistance, accommodation of mutations, and fitness cost. More specific information about the effects of the single and combined mutations is needed to elucidate their detailed interactions.

It was anticipated that purely synthetic compounds like linezolid would not show cross-resistance, but maybe cross-resistance is more a matter of sharing binding sites than being chemically similar. The efflux pumps that expel linezolid also work on other compounds [110, 112]. The methylation performed by Cfr provides linezolid resistance as well as resistance to five other classes of antibiotics [80, 81]. Examples of cross-resistance between PTC antibiotics resulting from 23S rRNA mutations have been observed [48, 66, 116, 117], although no straightforward relationship between overlapping binding sites and cross-resistance was found. There is a correlation between linezolid and chloramphenicol resistance for the single G2447U, A2503G, U2504G, G2505A, and G2576U mutations in *M. smegmatis* [48, 66]. However, this correlation does not apply for G2032A-U2504G and C2055A-U2504G double mutations and no relationship between linezolid, clindamycin, and valnemulin resistance could be observed [48, 66]. In addition, cross-resistance between linezolid and tiamulin has been documented for the G2447U and U2500A mutations in *E. coli* and the G2576U mutation in *E. coli* and *S. aureus* [46]. The different sets of specific bacteria, mutations and antibiotics reported in the literature preclude simple and common conclusion, and more information is needed.

5 Linezolid Resistance Among Clinical Isolates

As already mentioned, linezolid has a broad spectrum of activity against various Gram-positive clinical strains including *S. aureus,* CoNS, *E. faecalis, E. faecium, S. pneumoniae,* viridans group and other streptococci, β-hemolytic streptococci and other rarely isolated Gram-positive human pathogens [118]. It is also widely used to treat infections from multidrug-resistant (MDR) clinical isolates such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) [119–125].

Clinical isolates with resistance to linezolid were first documented in 1999 and included two isolates from 2/169 patients (1.2 %) receiving linezolid treatment for enterococcal infections [14]. Both of the patients received linezolid for a long period of time in order to treat bacteraemia associated with intravascular devices. The first report of a clinical isolate of methicillin-resistant *S. aureus* with linezolid resistance was reported in 2001 and was isolated from an 85-year-old man who had received prior linezolid treatment [15]. The resistance was due to G2576U mutations in the V domain of the 23S rRNA [15]. The first report of *cfr* as a resistance determinant in a clinical staphylococcal isolate was in 2008 from the USA through the surveillance program LEADER [86].

Documented resistance to linezolid appears to be sporadic and can occur in outbreaks [118, 126–131]. In most cases of sporadic clinical isolates exhibiting resistance to linezolid, the resistance was associated with prior linezolid therapy [39, 63, 132–134] although there have been reports of rapid emergence of resistance after short-term treatment [135], or resistance not related to prior treatment with linezolid [136, 137].

Due to the widespread use of linezolid for treating nosocomial infections by MDR staphylococcal and enterococcal clinical isolates, a need immerged to monitor the spectrum and potency of linezolid and for that two surveillance programs have been established. The original surveillance program for linezolid was ZAPS (Zyvox Activity and Potency Surveillance) [129, 138–140] and was renamed ZAAPS, enrolling medical centers in Latin America (LATAM), Asia Pacific (APAC), and Europe [127, 130, 131, 141, 142]. The second surveillance program is the LEADER surveillance program and it has monitored linezolid activity, spectrum, and resistance rates in the USA since 2004 [121, 143–147]. The most recent results from the LEADER surveillance program are from 2011, and monitored 7303 Gram-positive clinical isolates from 60 medical centers. It shows that resistance to linezolid is particularly rare in clinical MRSA (≤0.2 %) and CoNS (≤1.2 %) [148]. Linezolid was one of the most active agents among 1160 enterococcal strains (66 % *E. faecalis*, 30.6 % *E. faecium*) with a susceptibility rate of 99.7 %. The most important finding in this surveillance program was a nonsusceptible viridans group streptococcus, *Streptococcus sanguinis* (MIC >8 μg/mL), that was encountered for the first time in this program [148]. In the same manner, the latest ZAAPS Program report tested linezolid and comparators against 7972 Gram-positive clinical isolates from 73 medical centers (33 countries) from five continents, in order to summarize its activity and spectrum. Resistance to linezolid occurred in ≤0.1 % of strains of *S. aureus*, ≤0.9 % of CoNS, and ≤0.3 % of enterococcal strains [93]. Although the results from the two surveillance programs appear to be encouraging, concerns are lately raised by the appearance of linezolid-resistant clinical isolates in multiple studies around the world. Enterococcal clinical isolates resistant to linezolid due to L3 mutations and *S. cohnii* clinical isolates resistant to linezolid harboring the *cfr* and the 23S rRNA mutation G2576U were documented from a multicenter study in China [149]. A study conducted on clinical isolates of CoNS from two hospitals in China reports the emergence of *cfr*-harboring CoNS [150]. Emergence of linezolid-resistant *S. aureus* from cystic fibrosis (CF) patients was documented in Ohio with isolates having L3 mutations or the 23S rRNA mutation G2576U, raising serious concerns for CF patients [94]. Linezolid-resistant clinical isolates of *E. faecium* were isolated in Ontario, Canada, from 2010 to 2012 in a study that documents the first appearance of *cfr* in a clinical isolate of *E. faecium* [151]. A linezolid-resistant *S. pneumoniae* isolate with a linezolid MIC at 4 μg/mL was encountered for the first time in the LEADER Program results for 2010, and molecular characterization indicated that this strain had wild-type 23S rRNA and L22 ribosomal protein DNA sequences but had mutations in the ribosomal protein L4: Q67K and G69V [152].

Concerns also rise by studies that document the dissemination of the *cfr* gene among linezolid-resistant clinical isolates of various species [87, 150, 151, 153–155]. In a recent study from China, linezolid-resistant staphylococcal clinical isolates had the *cfr* gene located on a plasmid segment identical to a sequenced 14 kb *cfr*-carrying segment, from the plasmid pSS-02 [87]. This plasmid was originally identified in staphylococci isolated from pigs. This finding indicates that closely related—if not identical—plasmids carrying the *cfr* gene can be exchanged between CoNS from animals and methicillin-resistant CoNS (MRCoNS) from humans and that these MRCoNS can be involved in severe infections in humans [87].

6 Clinical Significance of Linezolid Resistance and Concluding Remarks

Linezolid remains highly active against most staphylococci, and its value in treating serious infections caused by MRSA has been well documented. Its availability as an oral formulation makes it desirable for outpatient treatment [128]. However, up to a quarter of patients prescribed the oral formulation of linezolid are non-adherent with therapy [156].

Among patients treated with linezolid for extended periods, resistance rates may be significantly elevated as compared with data reported in surveillance studies. Clinicians should remain aware that linezolid resistance may arise following prolonged treatment with linezolid and of the possibility of linezolid-resistant staphylococci (LRS) in patients that have not been previously treated with linezolid, given the high incidence of LRSA carrying *cfr* [128]. As an example, cystic fibrosis patients with respiratory tract infections caused by *S. aureus* have LRSA rates of up to 11 %, related to the number and length of linezolid treatments [94]. In addition, linezolid resistance may be underreported based on technical complications in the interpretation of both MIC and disc diffusion results [157]. Compared with the Clinical and Laboratory Standards Institute broth microdilution reference method, one study demonstrated 8/15 (53.3 %) LRS were falsely reported susceptible by disc diffusion and 6/15 (40.0 %) by Etest [157].

Treatment options for linezolid-resistant isolates are limited, so susceptibility testing for linezolid resistance should be considered prior to using linezolid for serious infections. In addition, judicious use of linezolid, accurate identification of resistance, and application of strict infection control measures are essential to the preservation of linezolid as a therapeutic agent. Also, it is very important to clearly identify all linezolid resistance determinants. It is obvious that linezolid resistance may occur both as transmissible element (*cfr* gene) and as acquired ribosomal mutations and probably as efflux changes caused by mutations. It is possible that development of derivatives of linezolid can overcome some of the resistance determinant and there seems to be steps in this direction.

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