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Pleuromutilins: Potent Drugs for Resistant Bugs—Mode of Action and Resistance

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Pleuromutilins are antibiotics that selectively inhibit bacterial translation and are semisynthetic derivatives of the naturally occurring tricyclic diterpenoid pleuromutilin, which received its name from the pleuromutilin-producing fungus *Pleurotus mutilus*. Tiamulin and valnemulin are two established derivatives in veterinary medicine for oral and intramuscular administration. As these early pleuromutilin drugs were developed at a time when companies focused on major antibacterial classes, such as the β-lactams, and resistance was not regarded as an issue, interest in antibiotic research including pleuromutilins was limited. Over the last decade or so, there has been a resurgence in interest to develop this class for human use. This has resulted in a topical derivative, retapamulin, and additional derivatives in clinical development. The most advanced compound is lefamulin, which is in late-stage development for the intravenous and oral treatment of community-acquired bacterial pneumonia and acute bacterial skin infections. Overall, pleuromutilins and, in particular, lefamulin are characterized by potent activity against Gram-positive and fastidious Gram-negative pathogens as well as against mycoplasmas and intracellular organisms, such as *Chlamydia* spp. and *Legionella pneumophila*. Pleuromutilins are unaffected by resistance to other major antibiotic classes, such as macrolides, fluoroquinolones, tetracyclines, β-lactam antibiotics, and others. Furthermore, pleuromutilins display very low spontaneous mutation frequencies and slow, stepwise resistance development at sub-MIC in vitro. The potential for resistance development in clinic is predicted to be slow as confirmed by extremely low resistance rates to this class despite the use of pleuromutilins in veterinary medicine for >30 years. Although rare, resistant strains have been identified in human- and livestock-associated environments and as with any antibiotic class, require close monitoring as well as prudent use in veterinary medicine. This review focuses on the structural characteristics, mode of action, antibacterial activity, and resistance development of this potent and novel antibacterial class for systemic use in humans.

Pleuromutilins are a well-known class of antibiotics discovered in the 1950s by the isolation of the naturally occurring pleuromutilin from *Pleurotus mutilus* (now renamed *Clitophilus scyphoides*), an edible mushroom (Fig. 1) (Kavanagh et al. 1951). Semisynthetic derivatizations have led to tiamulin and valnemulin, which were introduced to veterinary medicine in 1979 and 1999, respectively, for the treatment of pulmonary and intestinal infections caused

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Pleurotus mutilus
(Clitopilus scyphoides)

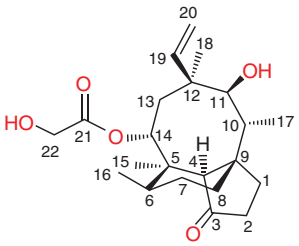

Figure 1. Structure of pleuromutilin with numbering system (Arigoni 1968). (*Left* panel from Lindsey 2006.)

by *Mycoplasma* spp., *Brachyspira* spp., and *Lawsonia intracellularis* in pigs, poultry, and, to some extent, in rabbits. Despite the use of pleuromutilins for treatment in veterinary medicine for more than three decades, resistance development has been uncommon. This can likely be attributed to several factors including the unique and highly specific mode of action of the pleuromutilins. Further, this class has not been used for enhancement of food-producing animal production (e.g., as growth promoters or for enhancement of feed efficiency) unlike the tetracyclines, penicillins, or sulfonamides (EMA 2014a,b). Even though oral valnemulin has been reported to be efficacious in the treatment of persistent or life-threatening mycoplasma infection in humans (Heilmann et al. 2001), no pleuromutilin had received marketing authorization by the end of the last century.

In the new millennium, interest in the pleuromutilin class significantly increased as evidenced by the development of new derivatives for human use. Retapamulin, a topical agent, was the first to be approved for the treatment of impetigo and infected small lacerations, abrasion or sutured wounds caused by *Staphylococcus aureus* and *Streptococcus pyogenes* (FDA 2007; EMA 2008). More recently, lefamulin, the first pleuromutilin for intravenous and oral administration, has entered into late-stage clinical development for the treatment of community-acquired bacterial pneumonia (CABP) and acute bacterial skin and skin structure infections (ABSSSIs). BC-7013 and BC-3205, which have a similar antibacterial profile but differ in ADMET properties, are in early-stage clinical development for topical and oral administration, respectively. In addition, recent research has been directed at further extending the antibacterial spectrum to include the ESKAPE pathogens (Boucher et al. 2009; Paukner et al. 2014a,b, 2015a,b,c; Strickmann et al. 2014; Wicha and Ivezic-Schoenfeld 2014; Wicha et al. 2015b).

To the question "Are pleuromutilins finally fit for human use?" (Novak 2011), which has been raised because of unjustified anecdotal concerns regarding metabolic stability, gastrointestinal side effects, cardiac safety, or intravenous tolerability, a clear response can be given: Yes. In a phase 2 study, lefamulin was well-tolerated and showed comparable efficacy to IV vancomycin in patients with ABSSSI (Prince et al. 2013). Despite challenging medicinal chemistry, a number of compounds are in the pipeline and further developments in this antibiotic class are anticipated.

PLEUROMUTILINS—MODE OF ACTION, ACTIVITY, AND RESISTANCE

Structure

The diterpenoid pleuromutilin comprises a tricyclic scaffold with unique annelation of a five-, six-, and eight-membered ring and eight stable chiral centers, as well as a glycolic ester moiety forming the side chain also regarded as an extension at position C14 (Fig. 1) (Anchel 1952; Arigoni 1962, 1968; Birch et al. 1963, 1966). Remarkable efforts have been made to achieve chemical modifications at several positions of

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the tricyclic core (Naegeli 1961; Berner et al. 1980, 1981, 1983, 1987; Brooks and Hunt 2000; Bacqué et al. 2002; Springer et al. 2003, 2008; Takadoi et al. 2007; Wang et al. 2009; Paukner et al. 2015b), as well as biotransformation (Hanson et al. 2002), and the total synthesis of this unique scaffold (Gibbons 1982; Paquette and Bullman-Page 1985; Paquette and Wiedemann 1985; Bacqué et al. 2003; Liu et al. 2011; Lotesta et al. 2011; Ruscoe et al. 2015). Most modifications, however, are primarily performed at the glycolic side chain of pleuromutilin with replacement of the terminal hydroxyl group or the entire side chain resulting in semisynthetic pleuromutilin analogs of two main types: (1) the flexible sulfanylacetyl, and (2) the rigid acylcarbamate linker type. Despite significant efforts in the field of acylcarbamate pleuromutilins by the GlaxoSmithKline group (Hunt 2000; Brooks et al. 2001; Andemichael et al. 2009), only sulfanylacetyl derivatives, mostly with one basic function at the side chain, have progressed beyond phase 1 clinical studies. The early work of the Sandoz group resulted in lipophilic orally available veterinary products, tiamulin (Egger and Reinshagen 1978) and valnemulin (Fig. 2A) (Berner and Vyplel 1987), whereas work from GlaxoSmithKline and Sandoz/Nabriva led to the lipophilic topical products retapamulin (Berry et al. 1999) and BC-7013 (Fig. 2B) (Thirring et al. 2007). Extensive modification of the C14 side chain culminated in lefamulin (Fig. 2C) (Mang et al. 2008), the first pleuromutilin with optimized physicochemical characteristics, including exceptional solubility, potent antimicrobial activity, and excellent ADMET properties including metabolic stability enabling administration by both the intravenous and oral routes.

Mode of Action

Pleuromutilins inhibit bacterial protein synthesis by binding to the central part of domain V of the 50S ribosomal subunit at the peptidyl transferase center (PTC), which prevents the correct positioning of the CCA ends of tRNAs for peptide transfer in the A- and P-site, thereby inhibiting peptide bond formation (Hogenauer 1975; Hogenauer and Ruf 1981; Hogenauer et al. 1981; Poulsen et al. 2001; Schlunzen et al. 2004; Long et al. 2006; Davidovich et al. 2007). Figure 3 shows the positioning of lefamulin in the PTC of the bacterial ribosome in relation to the positions of A- and P-site tRNA. Positioning is similar for various pleuromutilin derivatives in that the tricyclic core is located in a pocket close to the A-site, whereas the C14 side chain extends toward the P-site hindering the 3′-end tRNA A- to P-site rotary motion, as shown by various footprinting and crystallographic studies for tiamulin, valnemulin (Poulsen et al. 2001; Schlunzen et al. 2004; Long et al. 2006; Davidovich et al. 2007), retapamulin (Yan et al. 2006), lefamulin (Nabriva, unpubl.), and BC-3205 (Eyal et al. 2015). Crystallography data using ribosomal preparations from *Deinococcus radiodurans* and *S. aureus* show that the tricyclic

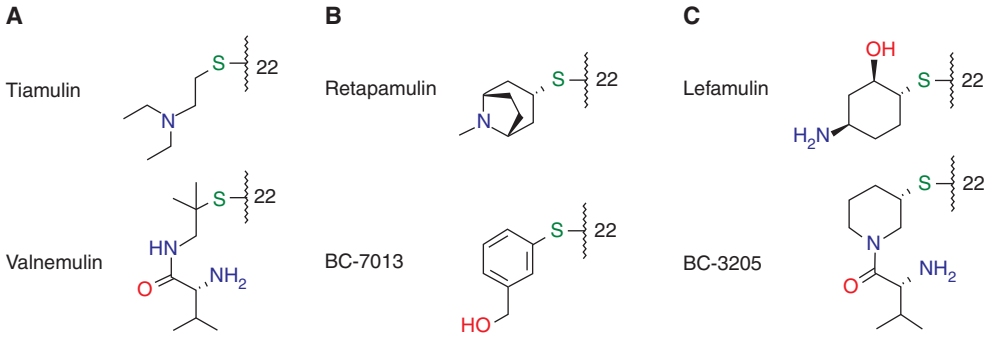

Figure 2. Structures of side chains at C22. Side chains of various pleuromutilin derivatives: (*A*) veterinary, (*B*) topical human, and (*C*) systemical human.

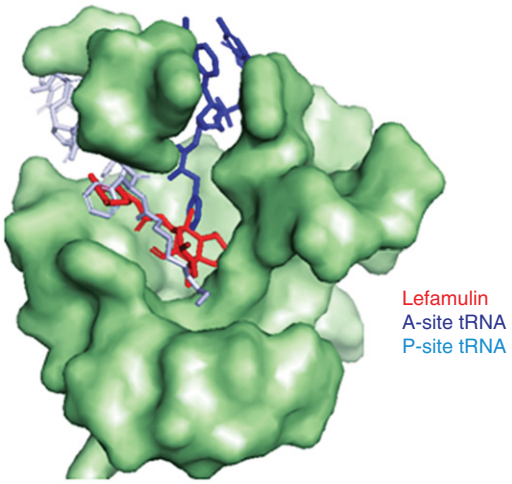

Figure 3. Lefamulin positioning in the peptidyl transferase center (PTC). PTC of the bacterial ribosome in relation to the positions of A- and P-site tRNA. Red, Lefamulin; blue, A-site tRNA; teal, P-site tRNA.

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pleuromutilin core interacts with the ribosomal nucleotides mainly through hydrophobic interactions, van der Waal forces, and hydrogen bonds with nucleotides of domain V of 23S rRNA, namely, A2503, U2504, G2505, U2506, C2452, and U2585 (Fig. 4). For the tricyclic pleuromutilin core, specific hydrogen bonds have been reported for the hydroxyl group at C11 with nucleotides G2505 or A2503 (Davidovich et al. 2007) and for the hydroxyl group at C2 (present in only few selected derivatives) with G2505. Further, hydrogen bonds have been reported for the C21 carboxyl group and the sulfur in sulfanylacetyl or acyl in acylcarbamate of the C14 extension (linker) with the nucleotide G2061, which are similar for both linker types (Schlunzen et al. 2004; Davidovich et al. 2007). In previous studies using *D. radiodurans* ribosomes, it was concluded that the rest of the C14 extension is involved only in minor hydrophobic interactions (Davidovich et al. 2007). Recent studies using *S. aureus* ribosomes, however, clearly showed additional hydrogen bonds of the C14 extensions, specifically the amino groups of BC-3205 and lefamulin, with the nucleotides U2506 (Eyal et al. 2015) and A2062 (A Yonath, Z Eyal, E Zimmerman, et al., unpubl.), respectively. Most notably, the C14 extensions of all pleuromutilins sterically interfere with the highly flexible nucleotides U2585 and U2506 causing rotational movements of these nucleotides, which consequently interact with each other by the formation of one or more additional hydrogen bonds or at least van der Waal or similar interactions. This closing of the binding pocket around pleuromutilins, also regarded as the induced-fit mechanism, tightens the binding of pleuromutilins to the ribosome (Davidovich et al. 2007; Eyal et al. 2015) and leads to the protection of these nucleotides in footprinting experiments (Schlunzen et al. 2004). Interestingly, the amino group of the C14 extension of BC-3205 forms a hydrogen bond with U2506 in *S. aureus* causing a larger shift of U2506, consequently further stabilizing BC-3205 in the binding pocket and indicating a better fit of this molecule in the pocket (Eyal et al. 2015).

It has been further hypothesized that pleuromutilins might also interfere with translation initiation or at an early point of the elongation cycle with particular sensitivity of the first peptide bond formation (Hunt 2000; Novak 2011). This is based on the fact that (1) radiolabeled tiamulin did not bind to the 50S subunit of the ribosome once elongation has begun, (2) addition of tiamulin to intact cells led to the formation of defective initiation complexes reflected

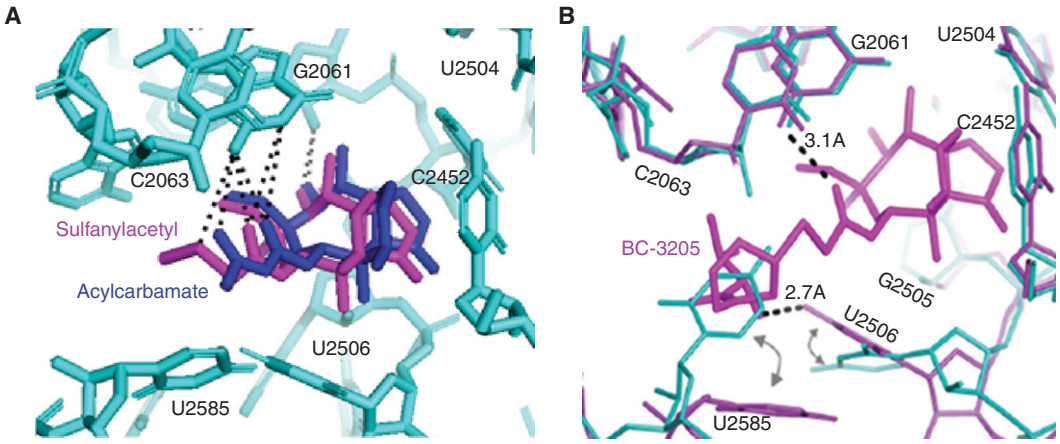

Figure 4. Interaction network. (*A*) The tricyclic pleuromutilin core and sulfanylacetyl as well as acylcarbamate linker (side chains omitted for clarity), and (*B*) the C14 side chain extension with nucleotides of the peptidyl transferase center (PTC). Hydrogen bonds are shown as dotted lines (Schlunzen et al. 2004; Davidovich et al. 2007; Eyal et al. 2015).

by the depletion of the polysome pool through blockage of reinitiation (Dornhelm and Hogenauer 1978), and (3) retapamulin partially inhibited binding of fMet-tRNA during initiation complex formation (Yan et al. 2006).

Coupled in vitro transcription/translation (TT) assays with bacterial ribosomes have shown high specificity of pleuromutilins for the inhibition of bacterial protein translation, whereas no effect on eukaryotic nonorganelle protein synthesis was observed for tiamulin and retapamulin (Yan et al. 2006). The specificity for bacterial protein synthesis was also confirmed for lefamulin with IC_{50} values of 0.51 μM and 0.31 μM in *Escherichia coli* and *S. aureus* TT-assays, respectively, whereas the IC_{50} in the eukaryotic TT-assay was with 952 μM >2000-fold higher than the IC_{50} in the bacterial system. Cycloheximide, a TT inhibitor of eukaryotic protein synthesis, and puromycin, a nonspecific inhibitor of bacterial and eukaryotic TT, were used as controls. Comparison of IC_{50} values of various pleuromutilin derivatives showed a slight trend of higher IC_{50} for *E. coli* than for *S. aureus* (Table 1; Nabriva, unpubl.). It further showed that the IC_{50} does not necessarily correlate with high antibacterial activity; for example, BC-7013, which has the highest IC_{50}, was one of the most active compounds in vitro (Biedenbach et al. 2009). Additional important factors other than translation inhibition, such as intracellular concentration, uptake, or efflux, contribute to in vitro antimicrobial activity as well (Paukner et al. 2014b).

Antibacterial Activity

The antibacterial spectrum of the pleuromutilins is characterized by potent activity against Gram-positive organisms including staphylococcal species (e.g., community-acquired methicillin-resistant *S. aureus* [CA-MRSA], hospital-acquired MRSA [HA-MRSA], vancomycin-resistant *S. aureus* [VRSA], vancomycin-intermediate *S. aureus* [VISA], heteroresistant VISA [hVISA]), streptococcal species (e.g., penicillin-resistant *Streptococcus pneumoniae* [PRSP], multidrug-resistant *S. pneumoniae* [MDR-SP]), and *Enterococcus faecium* (particularly vancomycin-resistant strains [VRE]), as well as activity against fastidious Gram-negatives, including *Haemophilus* spp., *Moraxella catarrhalis*, *Neisseria* spp., and *Legionella pneumophila* (Rittenhouse et al. 2006; Sader et al. 2012a,b; Paukner et al. 2013c). Pleuromutilins also display potent activity against mycoplasmas, ureaplasmas, chlamydia (Hannan et al. 1997), and *Brachispira hyodysenteriae* (Karlsson

Table 1. Inhibition of bacterial and eukaryotic in vitro transcription–translation by various pleuromutilin derivatives

	IC_{50} (CI95) (μM)		
Compound	*Escherichia coli*	*Staphylococcus aureus*	Eukaryotic (reticulocyte lysate system)
Pleuromutilin	0.76 (0.63–0.92)	1.73 (1.22–2.44)	ND
Tiamulin	0.50 (0.44–0.57)	0.36 (0.32–0.42)	ND
Valnemulin	0.59 (0.54–0.66)	0.38 (0.35-0.41)	ND
Retapamulin	0.69 (0.64–0.76)	0.35 (0.32–0.39)	850 (562–1287)
Lefamulin	0.51 (0.45–0.57)	0.31 (0.29–0.33)	952 (732–1238)
BC-3205	0.62 (0.56–0.68)	0.49 (0.44–0.54)	>100
BC-7013	0.74 (0.65–0.83)	0.64 (0.59–0.69)	>100
Cycloheximide	>100	>100	0.44 (0.29–0.68)
Puromycin	0.39 (0.34–0.46)	0.19 (0.16–0.23)	0.31 (0.27–0.36)

Unpublished data (Nabriva).
ND, not determined.

et al. 2001). Activity against anaerobic organisms has been seen for retapamulin, lefamulin, and BC-7013, including *Propionibacterium acnes* (Goldstein et al. 2006), *Peptostreptococcus* spp., *Prevotella* spp., *Porphyromonas* spp., *Fusobacterium* spp., and *Clostridium perfringens*, whereas pleuromutilins generally show weak activity against strains from the *Bacteroides fragilis* group (Odou et al. 2007; Paukner et al. 2013a). Pleuromutilin activity against *Clostridium difficile* varies and is dependent on the C14 side chain; retapamulin and lefamulin possess no relevant to weak activity, whereas BC-7013 has potent activity with 78% of isolates inhibited at concentrations of ≤1 μg/mL (Nabriva, unpubl.). The lack of lefamulin activity against *B. fragilis* group and Enterobacteriaceae is anticipated to result in limited disruption to the normal gastrointestinal microbiome and potentially a lower propensity to be associated with *C. difficile* infection (CDI). Further studies are warranted to assess the effect of the pleuromutilins on the gut microbiome and its impact on CDI.

No relevant activity was observed against *Enterococcus faecalis*, Enterobacteriaceae, and nonfermenting Gram-negatives, such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, although coupled in vitro transcription–translation assay results showed inhibition of the bacterial translation in these organisms and would suggest also antibacterial in vitro activity (Nabriva, unpubl.). Research at Nabriva revealed that the intrinsic resistance of Enterobacteriaceae is caused by the efflux of pleuromutilins mediated by the AcrAB-TolC efflux pump. This is supported by the fact that AcrAB-TolC deficient *E. coli* strains were susceptible to pleuromutilins (Paukner et al. 2014b) and that the minimum inhibitory concentration (MIC) values against Enterobacteriaceae were significantly reduced by the addition of efflux pump inhibitors (e.g., PAβN). Furthermore, recent new pleuromutilin derivatives partially overcome efflux and show increased activity against Enterobacteriaceae, including carbapenem-resistant isolates. These so-called "extended spectrum pleuromutilins" (ESPs) are characterized by the modification of the tricyclic pleuromutilin core at C12 (Paukner et al. 2014a,b, 2015a,b,c; Strickmann et al. 2014; Wicha and Ivezic-Schoenfeld 2014; Wicha et al. 2015b). Further investigations are needed to identify the mechanism(s) responsible for decreased susceptibility in nonfermenting organisms and *E. faecalis*.

Given that lefamulin is the first in-class IV and oral pleuromutilin antibiotic to advance into late-stage clinical development, additional information is provided on its activity. The antibacterial spectrum of lefamulin against a recent strain collection is well matched to the profile required for the empiric treatment

Table 2. Antibacterial activity of lefamulin and comparators

		MIC$_{90}$ (μg/mL)									
Species	*n*	LMU	ERY	CLI	DOX	TGC	LZD	VAN	DAP	LEV	PEN
Organisms causing predominantly SSSI and bacteremia											
Staphylococcus aureus	5527	0.12	>4	>2	0.25	0.25	1	1	0.5	>4	–
MSSA	3157	0.12	>4	≤0.25	0.25	0.25	2	1	0.5	1	–
MRSA	2370	0.25	>4	>2	1	0.25	1	1	0.5	>4	–
CoNS	878	0.12	>4	>2	2	0.25	1	2	0.5	>4	–
Enterococcus faecium	536	4	>4	–	>8	0.25	1	>16	2	>4	–
Vancomycin-nonsusceptible	304	0.25	>4	–	>8	0.25	1	>16	2	>4	–
β-Haemolytic *Streptococcus* spp.	763	0.03	>4	>2	8	0.06	1	0.5	0.25	1	0.06
Viridans group *Streptococcus* spp.	245	0.5	>4	≤0.25	>8	0.06	1	0.5	0.5	2	0.5

	n	LMU	ERY	AZI	DOX	TGC	LEV	SXT	CXM	IMI	PEN
Organisms causing predominantly RTI											
Streptococcus pneumoniae[a]	1473	0.25	>4	>4	8	0.06	1	>4	8	0.5	4
Haemophilus influenzae[b]	360	2	8	2	0.5	0.25	–	>4	2	1	–
Moraxella catarrhalis	253	0.25	0.25	≤0.25	0.25	0.25	–	≤0.5	2	≤0.12	>4
Legionella pneumophila[c]	30	0.5	0.25	0.12	–	–	0.12	–	–	–	–
Mycoplasma pneumoniae	50 (4)[d]	0.006	0.0025–0.005[f]	≤0.0003[f]	0.04–0.04[f]	–	–	–	–	–	–
Chlamydia pneumoniae	50 (2)[e]	0.04	0.04–0.16[f]	0.08–0.16[f]	0.04–0.08[f]	–	–	–	–	–	–

Data adapted from Paukner et al. (2013c) and Sader (2012b).

MRSA, Methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; CoNS, coagulase-negative *Staphylococcus* spp.; AZI, azithromycin; CLI, clindamycin; CXM, cefuroxime; DAP, daptomycin; DOX, doxycycline; ERY, erythromycin; IMI, imipenem; LEV, levofloxacin; LMU, lefamulin; LZD, linezolid; PEN, penicillin; SXT, trimethoprim-sulfamethoxazole; TGC, tigecycline; VAN, vancomycin.

[a]61.3% of *S. pneumoniae* isolates were penicillin-susceptible.

[b]23.6% of *H. influenzae* isolates were β-lactamase-positive.

[c]MICs determined by agar dilution using charcoal-supplemented BCYEα medium.

[d]LMU was tested against $n = 50$ isolates, whereas comparators were tested against $n = 4$ isolates.

[e]LMU was tested against $n = 50$ isolates, whereas comparators were tested against $n = 2$ isolates.

[f]Range of MICs for isolates tested.

of patients with CABP and ABSSSI (Table 2). Notably, lefamulin was equipotent against *S. aureus* strains that were community-acquired or healthcare-associated and its activity was not negatively influenced by the presence of Panton-Valentine leukocidin (PVL). Compared with macrolides, lincosamides, fluoroquinoloes, tetracyclines, β-lactams, linezolid, and vancomycin, lefamulin was among the most active compounds in vitro and the lefamulin activity was unaffected by resistance or multidrug resistance to these antibiotic classes (Paukner et al. 2013c). Lefamulin's activity is not adversely affected by presence of serum ($\leq$95% v/v) or lung surfactant ($\leq$1 mg/mL Survanta; 4% v/v). Lefamulin has shown high intracellular concentration in macrophages (Paukner et al. 2013b), achieves excellent penetration into human tissues including epithelial lining fluid of the lung (Zeitlinger et al. 2016), has oral bioavailability, potent in vivo efficacy in skin and pulmonary infection mouse models (Wicha et al. 2010, 2013, 2015a), and possesses a low potential for resistance development. Moreover, lefamulin has shown efficacy in patients with ABSSSI infection caused primarily by MRSA (including PVL-producing CA-MRSA) comparable to that of vancomycin (Prince et al. 2013). To date, lefamulin has been well tolerated in phase 1 and 2 clinical studies involving exposure of more than 400 subjects. Lefamulin also possesses potent activity (MIC_{90} values ≤ 2 μg/ml) against organisms causing sexually transmitted infections (STIs), including resistant *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, *Chlamydia trachomatis*, or *Haemophilus ducreyi*, warranting further evaluation of this drug for treatment of STIs (Paukner et al. 2013a).

Resistance and Cross-Resistance

The unique mode of action of pleuromutilins and the binding to highly conserved ribosomal targets implies a low probability of resistance development and lack of cross-resistance with other antibiotic classes including protein synthesis inhibitors, such as macrolides, ketolides, or fusidic acid (Yan et al. 2006). The binding sites and mode of action of pleuromutilins can be clearly differentiated from those of oxazolidinones, lincosamides, phenicols, and streptogramins; however, pleuromutilins also have partly overlapping interaction sites with these antibacterials (Schlunzen et al. 2004). Consequently, resistance mechanisms exist that can mediate cross-resistance with these antibacterials, albeit with an exceedingly low incidence.

Pleuromutilins have shown a low potential for resistance development in vitro as shown in various studies for tiamulin and valnemulin in *Brachyspira* spp. (Karlsson et al. 2001; Pringle et al. 2004), *Mycoplasma* spp. (Long et al. 2009; Li et al. 2011), *S. aureus* and *E. coli* (Miller et al. 2008), for retapamulin in *S. aureus* and *S. pyogenes* (Kosowska-Shick et al. 2006; Gentry et al. 2007), and for lefamulin in *S. aureus*, *S. pneumoniae*, and *S. pyogenes* (Paukner et al. 2012). Generally, the spontaneous mutation frequencies are low ($\leq 10^{-9}$) with no stable resistant mutants selected at four- to eightfold MIC. In multipassage experiments, resistance developed in a slow and step-wise manner with multiple mutations required to cause high-level resistance. Mutations in 23S rRNA, *rplC*, and *rplD* genes encoding the large ribosomal proteins L3 and L4, have been identified as the primary resistance mechanism in vitro. In clinical isolates, two additional resistance mechanisms have been identified: the acquisition of *vga*(A) encoded or related ATP-binding cassette (ABC)-F transporters and the acquisition of *cfr* encoding the Cfr methyltransferase. The common denominator is the alteration of the pleuromutilin target site.

Mutations in the 23S RNA gene (*rrn*) at positions G2032A, C2055A, A2058, A2058G, A2059G, G2061U, G2447A/U, C2499A, A2503U, U2504A/G, and A2572U were primarily observed in laboratory-selected *Brachyspira* spp., *Mycoplasma* spp., and in clinical isolates. Mutations in 23S rRNA have been described to confer resistance only in *Mycoplasma* spp. and *Brachyspira* spp., which only have a single copy of 23S rRNA, whereas staphylococcal and streptococcal species have multiple copies (Pringle et al. 2004; Miller et al. 2008; Long et al. 2009; Li et al. 2010; Hillen et al. 2014). Experiments with single-copy *rrn* knockout strains of *E. coli*

illustrated that "the copy number of 23S rRNA is the limiting factor in the selection of 23S rRNA mutants" (Miller et al. 2008). In *Brachyspira*, resistance is often associated with additional mutations in *rplC* (Pringle et al. 2004; Long et al. 2009; Li et al. 2010, 2011; Hidalgo et al. 2011).

Mutations and deletions in the *rplC* and *rplD* genes, although L3 and L4 do not directly interact with the pleuromutilins, can cause conformational changes in the PTC and hinder correct positioning of the pleuromutilins in the pocket formed between the nucleotides G2576 with U2506 and G2505 (Eyal et al. 2015). Mutations in *rplC* and *rplD* have been described for *Staphylococcus* spp. (Pringle et al. 2004; Kosowska-Shick et al. 2006; Gentry et al. 2007; Miller et al. 2008; Paukner et al. 2012) and together with mutations in 23S rRNA for *B. hyodysenteriae* (Hillen et al. 2014). Notably, mutations in *rplC* have also been associated with considerable loss of fitness (Gentry et al. 2007). Pleuromutilin resistance by mutational changes in *rplC* and 23S rRNA develops gradually and in a stepwise manner both in vitro and in vivo, suggesting that multiple mutations are needed to achieve high-level resistance. (Karlsson et al. 2001; Gentry et al. 2007; Miller et al. 2008; Hidalgo et al. 2011; Paukner et al. 2012).

ABC-F transporters encoded by *vga*(A) and its variants *vga*(A)v, *vga*(A)LC, *vga*(B), *vga*(C), *vga*(D), *vga*(E), and *lsa*(E) have been described to confer resistance to pleuromutilins, streptogramin A, and lincosamides in *Staphylococcus* spp., *E. faecium*, and *Erysypelothrix rhusiopathiae.* Isolates were collected almost exclusively from animal species, predominantly swine (Kadlec and Schwarz 2009; Kadlec et al. 2010; Overesch et al. 2011; Schwendener and Perreten 2011; Li et al. 2013, 2014a,b; Zhang et al. 2015). MRSA isolates collected from humans appeared to be related to animal-associated lineages of *S. aureus* such as ST398 (Lozano et al. 2012). Recent studies concluded that ABC-F transporters, which lack a transmembrane domain, likely mediate resistance by the interference of translation at the PTC and by the action as efflux transporter. This is based on the homology of *vga*(A) and variants with the ABC-F transporter EttA, which is a translation factor binding to the tRNA exit site (E-site) (Lenart et al. 2015).

Last, the rarely encountered methyltransferase Cfr, methylating the nucleotide A2503 of 23S rRNA, can confer resistance. Because of steric hindrance, binding of phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramins (PhLOPS antibiotics) is prohibited, which results in the PhLOPS-resistance phenotype. The *cfr* gene was originally identified in coagulase-negative staphylococci from animals and has been detected mostly in livestock-associated staphylococci (Kehrenberg et al. 2005, 2009; Alba et al. 2015; Feltrin et al. 2015; Moon et al. 2015) but, more recently, has also been found in a limited number of staphylococcal isolates from humans including one outbreak of a *cfr*-positive MRSA in a Spanish hospital, which was terminated by reduction of linezolid use and infection-control measures (Sanchez et al. 2010; Shore et al. 2010, 2016). Cfr was also found in nonstaphylococcal species collected, with the exception of *E. faecalis*, exclusively from livestock animals and related farm environments: one out of 1230 *E. coli* isolates collected from pigs, ducks, and chickens in China, in one *Proteus vulgaris* out of 557 nasal swabs of Chinese swine and a porcine *Bacillus* spp., as well as a *Macrococcus caseolyticus* and *Jeotgalicoccus pinnipedialis* isolate (Wang et al. 2011, 2012a,b,c,d). Cfr has also been detected in an *E. faecalis* isolate collected from a Chinese animal as well as in an animal-associated isolate from a patient in Thailand. Cfr has been located on the chromosome and on various plasmids or transferrable elements indicating the ability to spread (Locke et al. 2012; Shen et al. 2013; Li et al. 2015; Shore et al. 2016). In vitro, the *cfr*-carrying plasmid isolated from human *E. faecalis* was only transferrable by conjugation to another *E. faecalis* laboratory strain, whereas it was not transferrable to *S. aureus* or *E. faecium* (Diaz et al. 2012). Recently, the transferability of *cfr*-carrying plasmids from *S. epidermidis* to MRSA by conjugation or transduction was shown, indicating a role of *S. epidermidis* as a potential reservoir for *cfr* spread (Cafini et al. 2016).

Table 3. Cfr-positive isolates collected from human in the course of global surveillance studies

Investigation period	Number of resistant *Staphylococcus aureus* isolates per total screened isolates (%)	Number of resistant CoNS isolates per total screened isolates (%)	Country, surveillance program	References
2015	0/2434 (0%)	1/465 (0.22%)	Europe, ZAAPS	Flamm et al. 2016
2014	0/3560 (0%)	0/956 (0%)	Worldwide, ZAAPS	Mendes et al. 2016
2012	1/4077 (0.02%)	3/905 (0.33%)	Worldwide, ZAAPS	Mendes et al. 2014
2011	0/3884 (0%)	3/928 (0.32%)	Worldwide, ZAAPS	Flamm et al. 2013
2010	1/5527 (0.018%)	2/823 (0.24%)	Worldwide, lefamulin, SENTRY	Paukner et al. 2013c
2010	0/2875 (0%)	2/855 (0.23%)	Worldwide, ZAAPS	Flamm et al. 2012
2002–2009	0/5952 (0%)	2/2132 (0.09%)	Worldwide, ZAAPS	Ross et al. 2011
2007	0/3000 (0%)	0/716	Worldwide, ZAAPS	Jones et al. 2009
1999–2010	1/2215 (0.045%)	ND	Spain	Sierra et al. 2013

Note: Annual Appraisal of Potency and Spectrum (ZAAPS) Program and SENTRY Surveillance Program.
CoNS, coagulase-negative *Staphylococcus* spp.

Most importantly, it should be noted that despite the characterization of isolates resistant to pleuromutilins and the descriptions of mechanisms conferring resistance, the rate of resistance to pleuromutilins remains low. In the SENTRY surveillance program conducted with lefamulin in 2010, the total incidence of pleuromutilin resistance was 0.18% for *S. aureus* and 3.4% for coagulase-negative *Staphylococcus* spp. Among the *S. aureus* isolates, 0.018% harbored the *cfr* gene, 0.11% harbored the *vga*(A) gene, and 0.05% had mutations in *rpl*C. Among coagulase-negative *Staphylococcus* spp., the incidences for *cfr*, *vga*(A), and *rpl*D alterations were 0.11%, 2.5%, and 0.34%, respectively (Paukner et al. 2013c). The low prevalence of *cfr* is consistent with data collected in the course of linezolid-resistance monitoring (Table 3).

CONCLUDING REMARKS

In summary, pleuromutilins display potent antibacterial activity against a variety of Gram-positive, fastidious Gram-negative and atypical respiratory bacterial pathogens—a profile well suited to treat human infections, including CABP, ABSSSI, and STI. Chemical modifications have led to derivatives with optimized physicochemical properties and improved ADME properties, allowing for intravenous and oral dosing in humans; of these analogs, lefamulin is the most advanced in clinical development. The incidence of pleuromutilin-resistant bacterial isolates is low despite the use of tiamulin and valnemulin in veterinary medicine for more than 30 years. The availability of topical retapamulin in human medicine since 2007 and selection pressure for *cfr* by the use of linezolid over the past two decades does not appear to have had a major effect on the incidence of pleuromutilin-resistant bacterial isolates among organisms causing infections in humans. Nevertheless, close monitoring of resistance development to pleuromutilins is warranted, along with prudent use of oxazolidinones and veterinary pleuromutilins to maintain low resistance rates and retain the potent activity of this novel antibacterial class against pathogens that have acquired resistance to other established antibiotic classes.

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