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Linezolid *in vitro*: mechanism and antibacterial spectrum

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Oxazolidinones are prominent among the new Gram-positive antimicrobial agents now becoming available. They were discovered by DuPont Pharmaceuticals in the late 1980s but linezolid, the first analogue suitable for development, was found only when the family was re-examined by Pharmacia in the 1990s. Oxazolidinones bind to the 50S subunit of the prokaryotic ribosome, preventing formation of the initiation complex for protein synthesis. This is a novel mode of action; other protein synthesis inhibitors either block polypeptide extension or cause misreading of mRNA. Linezolid MICs vary slightly with the test method, laboratory, and significance attributed to thin hazes of bacterial survival, but all workers find that the susceptibility distributions are narrow and unimodal, with MIC values between 0.5 and 4 mg/L for streptococci, enterococci and staphylococci. Full activity is retained against Gram-positive cocci resistant to other antibiotics, including methicillin-resistant staphylococci and vancomycin-resistant enterococci. MICs are 4–8 mg/L for *Moraxella*, *Pasteurella* and *Bacteroides* spp. but other Gram-negative bacteria are resistant as a result of endogenous efflux activity. Resistance is difficult to select *in vitro* but has been reported during therapy in a few enterococcal infections and in two MRSA cases to date; the mechanism entails mutation of the 23S rRNA that forms the binding site for linezolid. Risk factors for selection of resistance include indwelling devices, undrained foci, protracted therapy and underdosage.

Introduction

Infections caused by resistant Gram-positive cocci are a worldwide threat to vulnerable hospitalized patients. Approximately 42% of *Staphylococcus aureus* isolates from bacteraemias in England and Wales are now methicillin resistant (MRSA), compared with 2% a decade ago.[1,2] This dramatic increase largely reflects the dissemination of two epidemic (EMRSA) strains, EMRSA-15 and EMRSA-16, which now account for 95% of all bacteraemic MRSA isolates in the UK.[3] Similarly, >30% of *S. aureus* isolates from bacteraemias in Spain, Portugal, Ireland, France, Italy and Greece are resistant to methicillin, with rates of 10–30% in Germany, Austria and Belgium.[4] An MRSA prevalence rate of 60% was reported in Japan, which was the first country to report vancomycin-intermediate *S. aureus*.[5] At the other extreme, the proportions of *S. aureus* bacteraemias caused by MRSA remain below 3% in Scandinavia and The Netherlands,[4] reflecting the success of rigorous infection control in preventing initial dissemination. Whether such control can reverse established dissemination is less clear.

The proportion of methicillin resistance among coagulase-negative staphylococci exceeds even that in *S. aureus*. Although much less pathogenic than *S. aureus*, these organisms are a major source of line-associated infections in, for example, haematology patients.[6] Enterococci are another group of low-grade pathogens that are increasingly problematic, with vancomycin resistance now evident in 60% of *Enterococcus faecium* infections in the USA[7] and in 20–30% of those in England and Wales.[1,8] Vancomycin-resistant *E. faecium* infections are less prevalent in most other European countries than in the UK, but have been reported even in The Netherlands, a country with an enviable record for infection control.[9] The European dissemination of vancomycin-resistant *E. faecium* may partly reflect the use (until it was banned in 1998) of avoparcin as a growth promoter for animals. Vancomycin resistance also occurs in *Enterococcus faecalis* but is less frequent than in *E. faecium*.[8] Moreover, and unlike *E. faecium, E. faecalis* is almost always susceptible to ampicillin. It is unclear why vancomycin resistance has remained so strongly associated with *E. faecium* whereas high-level gentamicin resistance has disseminated

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widely in both *E. faecalis* and *E. faecium*; both resistances are frequently encoded by transferable plasmids.

Resistance is rising among community-acquired Gram-positive pathogens as well as in the nosocomial organisms discussed above. Most importantly, penicillin non-susceptibility (i.e. resistance or intermediate resistance) is increasing in degree and prevalence among *Streptococcus pneumoniae* isolates, with many non-susceptible isolates belonging to clonal lineages that have spread between continents.[10] Members of these lineages are often resistant to macrolides and tetracyclines as well as penicillin. A growing minority of pneumococci also show resistance to the newer fluoroquinolones.[11] Penicillin non-susceptibility is still infrequent among respiratory and bloodstream pneumococci in the UK, with a prevalence of ~8–10%; moreover, most of this resistance is low level and, except in cases of meningitis, can be overcome by administering β-lactams at high dosages.[1,8] Penicillin-resistant pneumococci are much more prevalent in Southern Europe and the Far East, accounting for 30–40% of pneumococci tested in France and Spain[4] and up to 80% of those in Korea, Hong Kong and Japan.[12] In the USA (though not yet in the UK), penicillin resistance is emerging as a problem in other α-haemolytic streptococci, including the viridans streptococci that are the predominant agents of endocarditis.[3,13] Although β-haemolytic streptococci consistently remain susceptible to penicillins, they are frequently resistant to the macrolides and tetracycline, agents that would be used, for example, in penicillin-allergic patients.

Better infection control in hospitals and more prudent use of antimicrobials are vital to mitigate the impact of resistance, but ageing populations, increasing numbers of vulnerable patients and stressed health care systems suggest that these improvements will be easier to demand than to achieve. It is salutary that since 1997/8, when antibiotic resistance became a political issue in the UK, the proportion of *S. aureus* bacteraemias caused by MRSA has risen by over one-third, from 31% to 42%.

Consequently, new antimicrobial development remains vital if man is to keep ahead of resistance and, despite recent gloom, new anti-Gram-positive agents are being developed and licensed. Quinupristin/dalfopristin and linezolid have both become available in the past 2 years,[14] whilst daptomycin, oritavancin and tigecycline should follow between 2003 and 2005.

Linezolid is the first oxazolidinone to be marketed, and as such, is the first representative of a completely new systemic antimicrobial class to be launched since fosfomycin in 1972. This article outlines its mechanism of action, its *in vitro* activity and the ways in which this activity can be undermined by resistance. The potential for future oxazolidinones is briefly examined.

Discovery of the oxazolidinones

Antibacterial oxazolidinones were discovered by DuPont Pharmaceuticals in the late 1980s,[15] but the early lead analogues (DuP 105 and DuP 721) proved unsuitable for pharmaceutical development and the programme was dropped. Investigation was re-initiated by the then Upjohn Corporation in the early 1990s, leading to the delineation of a series of structure–activity relationships (Figure 1)[16] and to the synthesis of non-toxic analogues with good antibacterial activity. Pharmacokinetic evaluation was undertaken for two compounds, linezolid and eperezolid. Linezolid required twice-daily dosing whereas eperezolid required three daily doses. Consequently, only linezolid was progressed beyond Phase I development.

Mode of action

Oxazolidinones bind to the 50S subunit of the prokaryotic ribosome, preventing it from complexing with the 30S subunit, mRNA, initiation factors and formylmethionyl-tRNA (Figure 2).[17,18] The net result is to block assembly of a functional initiation complex for protein synthesis, thereby preventing translation of the mRNA. This mode of action differs from that of existing protein synthesis inhibitors such as chloramphenicol, macrolides, lincosamides and tetracyclines, which allow mRNA translation to begin but then inhibit peptide elongation. This difference may seem academic, but may be significant in two respects. First, linezolid seems particularly effective in preventing the synthesis of staphylococcal and streptococcal virulence factors (e.g. coagulase, haemolysins and protein A), perhaps because of this mode of action.[19] Second, linezolid has a target that does not overlap with those of existing protein synthesis inhibitors; consequently, its activity is unaffected by the rRNA methylases that modify the 23S rRNA so as to block the binding of macrolides, clindamycin and group B streptogramins.

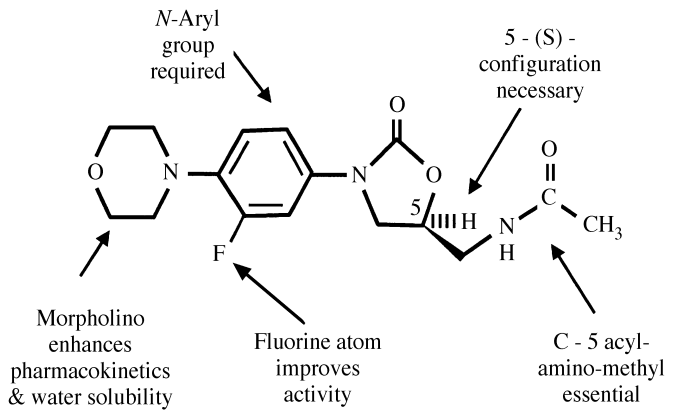

Figure 1. Structure–activity relationships leading to the development

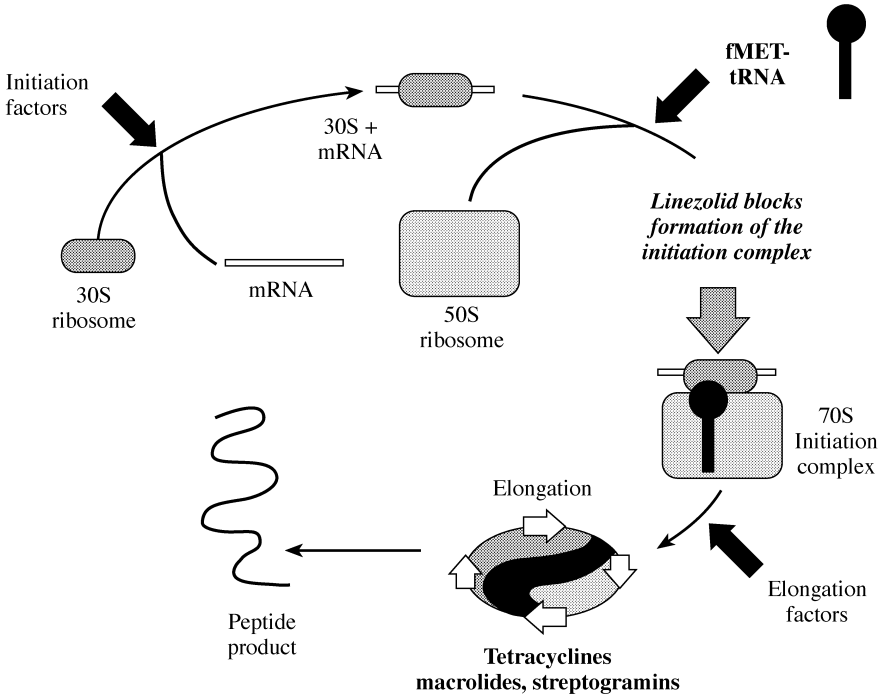

Figure 2. Mode of action of the oxazolidinones. Oxazolidinones combine with the 50S ribosomal subunit, preventing it from complexing with the 30S subunit, mRNA, initiation factors and formylmethionyl-tRNA. Consequently, no functional initiation complex is formed, and protein synthesis is halted. Most other protein synthesis inhibitors block peptide elongation. Adapted from Kloss *et al.*[18]

Preventing the initiation of protein synthesis is no more inherently lethal than prevention of peptide elongation. Consequently, linezolid, like chloramphenicol, clindamycin, macrolides and tetracyclines, is essentially bacteriostatic. The only protein synthesis inhibitors to achieve strong bactericidal activity are the aminoglycosides, which cause misreading of mRNA, leading to the manufacture of defective proteins which, among other effects, destabilize the membrane structure and cause leakage of the cell contents.

The ribosomes of *Escherichia coli* are as susceptible to linezolid as those of Gram-positive cocci but, with minor exceptions (see below), Gram-negative bacteria are oxazolidinone resistant, apparently because oxazolidinones are recognized and excreted by endogenous efflux pumps.[17]

MICs of linezolid

Numerous *in vitro* studies have shown that the MICs of linezolid for enterococci, pneumococci, staphylococci and streptococci fall between 0.5 and 4 mg/L,[8,16,20–25] with higher values recorded only for a few mutants selected during therapy (see below). Within these narrow ranges, the MIC distributions are unimodal and unskewed (Figure 3). These findings have been confirmed by studies of isolates from individual institutions and by multicentre studies in Europe and North America.[23,24] Some workers even find identical MICs for *all* members of a species.[20]

Since its mode of action is unique, it is unsurprising to find that the activity of linezolid is maintained irrespective of resistance to other drugs. Thus, linezolid is equally active against methicillin-susceptible and -resistant staphylococci, against vancomycin-susceptible enterococci and those with VanA, VanB or VanC determinants, and against pneumococci with susceptibility or resistance to penicillins and/or macrolides.[8,16,20–25]

No other available antibiotics retain such consistent activity against Gram-positive cocci. Vancomycin retains nearly universal activity against staphylococci and streptococci but has been compromised against enterococci by the spread of VanA and VanB whereas teicoplanin has been compromised against enterococci by the spread of VanA, and has inherently poor activity against some groups of coagulase-negative staphylococci.[1,7] Quinupristin/dalfopristin is active against nearly all staphylococci, streptococci and *E. faecium* isolates but it is not active against *E. faecalis*,[14] which can efflux dalfopristin. In the future, daptomycin, oritavancin and tigecycline may achieve similarly broad activity to linezolid against Gram-positive cocci, but are not yet licensed.

Most Gram-negative organisms are resistant to linezolid, but MICs of 4–8 mg/L are seen for many *Bacteroides* spp., *Moraxella catarrhalis* and *Pasteurella* spp., and MICs of ~16 mg/L (equating to low-level resistance) for most *Haemophilus influenzae*.[22] It is not known whether these organisms are deficient for efflux, are so permeable that efflux is

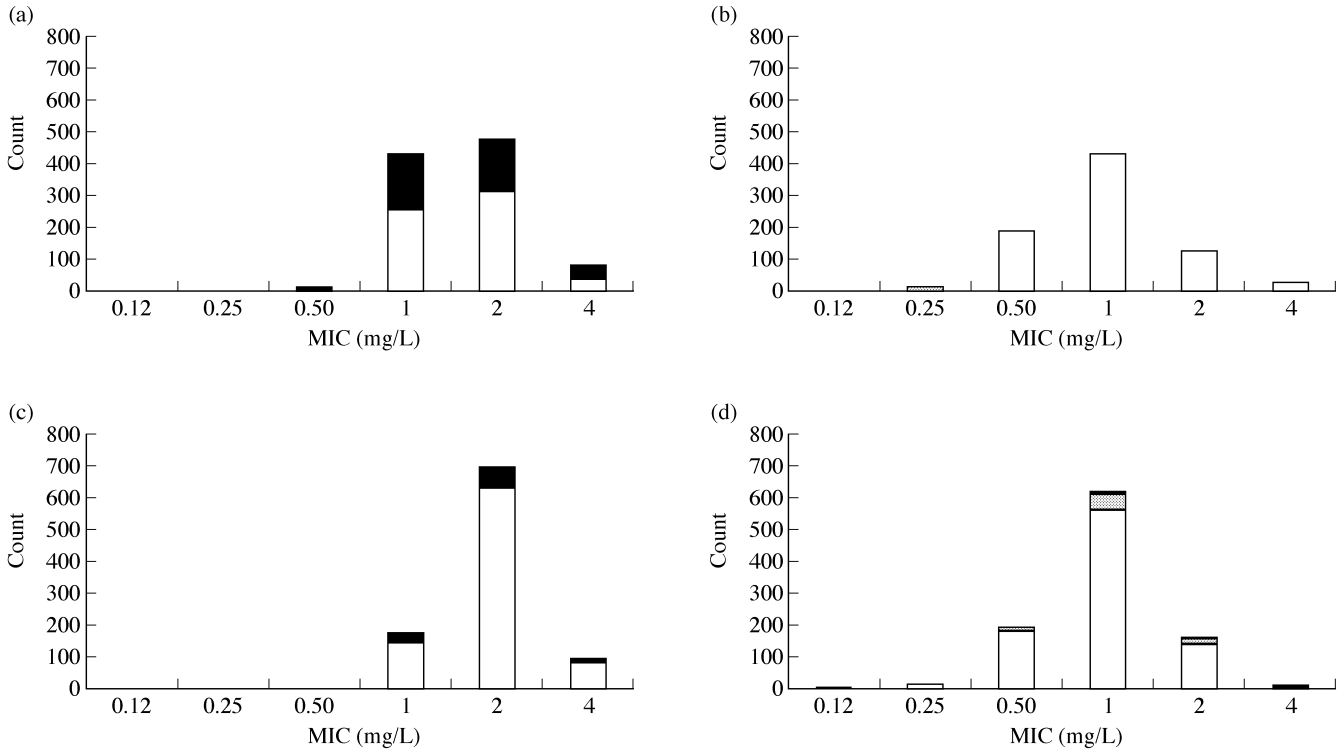

Figure 3. MIC distributions of linezolid for Gram-positive cocci in the UK, based on a survey of 25 hospitals.[8] (a) Methicillin-susceptible *S. aureus* (white) and MRSA (black); (b) coagulase-negative staphylococci; (c) *E. faecalis* (white) and *E. faecium* (black); and (d) penicillin-susceptible (white), -intermediate (grey) and -resistant (black) pneumococci. Note the narrow unimodal MIC distributions.

overwhelmed or have ribosomal targets that are unusually susceptible to linezolid.

MIC variation between studies

Although the MIC distributions of linezolid are consistently found to be narrow for Gram-positive bacteria, the actual values recorded vary slightly among workers, sometimes to the extent that the ranges found by different groups do not even overlap. For example, Wise *et al.*[25] always found MICs of 0.5–1 mg/L for enterococci isolated in the UK, whereas we consistently found MICs of 4 mg/L.[20] Both studies included isolates from multiple British hospitals, and used the same test method [British Society for Antimicrobial Chemotherapy (BSAC), agar dilution]. More generally, a review of numerous MIC studies included in the UK licensing submission indicated that research groups recording 'high' MICs for methicillin-susceptible staphylococci (i.e. 2–4 mg/L rather than 0.5–1 mg/L) also found relatively high MICs for MRSA and for enterococci (Pharmacia, data on file). Such patterns, extending across species and phenotypes, imply methodological differences between workers rather than genuine susceptibility differences between the bacteria investigated. One potential source of variation lies in how the MICs are read, since the linezolid-inhibited growth of enterococci and staphylococci commonly trails out over one to two dilutions.[20] Another potential variable involves the medium used: one study indicated that MICs for staphylococci by National Committee for Clinical Laboratory Standards (NCCLS) microbroth dilution were often 4 mg/L, whereas MICs for the same strains on Mueller–Hinton, IsoSensitest or DST agar were mostly 2 mg/L.[26] This study, and another by Gemmell,[23] found that MICs by Etest were commonly one dilution below those by classical methods, probably reflecting the manufacturer's advice to read to the point of 80% inhibition of growth. If this policy was adopted for classical MIC tests, the effect would be to reduce the linezolid MICs by one dilution or thereabouts.

Site-to-site variation in MIC would be little more than a curiosity but for the fact that some regulatory authorities recommend a breakpoint of 2 mg/L.[26] Such a value can lead to artefactual 'resistance' in those centres that routinely find MICs of 4 mg/L for staphylococci or enterococci. Care should be taken not to confuse this 'threshold' behaviour with true resistance (see below), which is associated with MICs of 16–128 mg/L. Such confusion should not arise with the breakpoint of 4 mg/L adopted by the BSAC[27] and the European Union Committee on Antibiotic Susceptibility Testing (EUCAST).

Table 1. 23S rRNA mutations conferring linezolid resistance in laboratory and clinical mutants of enterococci and staphylococci[31–32]

Species	Mutations[a] found in laboratory-selected variants	Mutations found in resistant clinical strains
S. aureus	G2447U[b] G2576U	G2576U
E. faecalis	G2576U (G2576U + G2512U + G2513U + C2610G)	G2576U
E. faecium	G2505A	G2576U

[a]Numbering is relative to the *E. coli rrlD* gene sequence (e.g. GenBank AF053964).
[b]Uracil (U) is indicated as the base present in the rRNA, but corresponds to thymidine in the encoding DNA.

Bactericidal activity and post-antibiotic effects

Linezolid is essentially bacteriostatic, achieving less than a 2 $\log_{10}$ reduction in the count of enterococci and staphylococci over 24 h when tested at 4 × MIC. One group observed a 3–4 $\log_{10}$ reduction in bacterial count over 6 h for pneumococci, and concluded that linezolid was bactericidal against these organisms;[22] another found little or no bactericidal activity for linezolid against viridans or β-haemolytic streptococci.[28] The reasons for this difference are unclear and there is no obvious reason why a compound with linezolid's mode of action should kill bacteria. Post-antibiotic effects of 1.8–3.0 h were reported for linezolid against staphylococci, enterococci and pneumococci[22] but seem academic because, following a standard 600 mg dose, the serum drug concentration remains >4 mg/L throughout the dosage interval.[29]

Emergence of resistance

No linezolid-resistant Gram-positive cocci were found in the many *in vitro* surveys performed before licensing; moreover, mutational linezolid resistance is extremely difficult to select *in vitro*.[22] When resistance was ultimately obtained by *in vitro* passage of staphylococci and enterococci, it was found to be associated with mutations to the central loop of Domain V of the 23S rRNA, which lies in the 50S ribosomal subunit (Table 1).[30] These changes presumably alter linezolid's binding site. *S. aureus* has four to seven gene copies specifying the 23S rRNA, and more than one of these must be altered for substantive resistance to arise, perhaps explaining the difficulty of selection.[30]

Despite the difficulty of *in vitro* selection, linezolid resistance can emerge during therapy, perhaps because multiple gene copies are mutated or because recombination events follow mutation of a single copy. Fifteen cases of emergent resistance in enterococci were encountered during the Phase III clinical trials, 14 with *E. faecium* and one with *E. faecalis* (Pharmacia, data on file).[31] Several subsequent cases have also been reported.[32–34] In many of these cases, the pre-therapy susceptible and post-therapy resistant isolates were confirmed to be identical by DNA fingerprinting techniques, showing that resistance had been selected in the original pathogen.[31,32] Among the resistant isolates selected in Phase III development, several were selected in the low-dosage arm of a trial where, to test anti-enterococcal dose–response behaviour, twice-daily regimens of 200 mg and 600 mg were compared. More generally, risk factors for selection include indwelling lines and devices, protracted therapy and sequestered sites of infection.[30,32,33,35] There are just two reports of emerging linezolid resistance in *S. aureus*, so far. Three linezolid-resistant MRSA isolates were obtained from a dialysis patient in Boston (USA) who had received linezolid for 4 weeks for MRSA peritonitis; strangely, these isolates had a different DNA profile from 11 linezolid-susceptible MRSA isolates obtained during the preceding 3 weeks.[36] Consequently it was unclear whether a resistant variant emerged from a second, previously undetected, strain of MRSA that was also present in the patient, or whether the patient had acquired a resistant strain from an external source. The former possibility seems the more plausible, allowing the absence of linezolid-resistant staphylococci in surveillance studies. The second patient from whom linezolid-resistant MRSA was isolated presents a clearer case. This individual received linezolid for 21 days following infection of a thoracotomy wound, with contingent empyema.[37] Although the treatment achieved an improvement, he relapsed after 3 weeks, with the MRSA now resistant to linezolid (MIC raised from 2 to 16 mg/L). The pre- and post- therapy isolates were of identical phage and PFGE type. The infection was ultimately cured with teicoplanin.

Where linezolid-resistant enterococci from patients have been investigated, they have been found to have guanosine 2576 of their 23S rRNA replaced by uracil (Table 1)—one of the mutations also recorded in laboratory mutants of *E. faecalis*.[35] In the resistant *S. aureus* isolate from Boston, all six copies of the gene were altered, whereas five out of six were altered in the London isolate.[36,37] Much, however, remains uncertain: in particular, why has resistance emerged more often in enterococci than staphylococci? Initial speculation (widely voiced though unpublished) that enterococci had fewer copies of the 23S rRNA gene has now been discounted: both staphylococci and *E. faecium* have up to seven gene copies. It remains plausible (though speculative) that internal recombination may proceed more readily in enterococci, allowing mutated gene copies to replace 'normal' ones following an initial mutation in one copy.

At a practical level, the MICs for resistant enterococci and MRSA range from 16 to 128 mg/L, and such isolates give no zone, or greatly diminished zones, in disc diffusion tests.[33] Thus, unlike vancomycin-intermediate *S. aureus*, they are easy to detect by routine methods. To minimize selection, it seems reasonable to be cautious when using linezolid in patients with the risk factors outlined earlier. If resistance is selected, stringent infection control should be mandatory, especially with *S. aureus* where previous experience has repeatedly demonstrated that a few strains with a rarely selected resistance (i.e. to methicillin) can spread widely. This advice is underscored by the recent report of linezolid-resistant enterococci spreading within a unit.[38]

The next oxazolidinones

Many further oxazolidinones have been presented at recent Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) meetings, though few of these are proceeding to clinical development. One extensively studied analogue, AZD2563,[39] was recently dropped from development by AstraZeneca, though it may be taken up by others.

Review of the analogues presented at recent ICAACs suggests several general points. First, although several (including AZD2563)[39] are two- to four-fold more active than linezolid against Gram-positive cocci, none offers a quantum leap, such as the 100-fold gain in activity between the second- and third-generation cephalosporins or between nalidixic acid and the fluoroquinolones. Secondly, none has been reported to overcome the linezolid resistance associated with mutations to the 23S rRNA. Thirdly, it is possible to alter the oxazolidinone structure so as to increase activity against haemophili and *Bacteroides* spp., but no derivatives have yet been published with activity against Enterobacteriaceae or non-fermenters. Among the compounds with greatest anti-*Haemophilus* activity are PNU-183247 (Pharmacia), a 2-aminomethyl-1,2,4-thiadiazole phenyl oxazolidinone,[40] and VRC 3599 (Versicor), a 4′-amido-3′-fluorophenyl oxazolidinone.[41] These had MICs of 0.5–2 mg/L for *H. influenzae* and 0.25–0.5 mg/L for *S. pneumoniae*, thus covering the two critical respiratory pathogens. Dramatically increased anti-anaerobe activity has been reported for RBx 7644 (Ranbaxy), with MIC_{90}s of 0.12 mg/L for the *B. fragilis* group, 0.25 mg/L for *Prevotella* spp., 0.016 mg/L for *Peptostreptococcus* spp. and 0.06 mg/L for *Clostridium difficile*, compared with 2–4 mg/L of linezolid.[42]

An alternative approach to extending the activity of oxazolidinones against Gram-negative bacteria is to prevent efflux. Inhibitors of the broad-spectrum pumps of Gram-negative bacteria (e.g. MexA-MexB-OprM of *Pseudomonas aeruginosa*) are under investigation[43,44] and should be tested in combination with oxazolidinones. This, however, is for the future. For the present, linezolid is the first representative of a new class of systemic antimicrobials to be launched for around 30 years. It has good inhibitory activity against Gram-positive cocci, with narrow, unimodal MIC distributions. With minor exceptions, Gram-negative bacteria are resistant, owing to efflux. Resistance is difficult to select and despite several reports of emergent resistance *in vivo*, there is little doubt that the compound will provide an invaluable new option against staphylococcal, enterococcal and streptococcal infections.

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