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Minireview

Overcoming antimicrobial resistance by targeting resistance mechanisms

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Abstract

Three mechanisms of antimicrobial resistance predominate in bacteria: antibiotic inactivation, target site modification, and altered uptake by way of restricted entry and/or enhanced efflux. Many of these involve enzymes or transport proteins whose activity can be targeted directly in an attempt to compromise resistance and, thus, potentiate antimicrobial activity. Alternatively, novel agents unaffected by these resistance mechanisms can be developed. Given the ongoing challenge posed by antimicrobial resistance in bacteria, targeting resistance in this way may be our best hope at prolonging the antibiotic era.

Introduction

The current problem of widespread antibiotic resistance, particularly the challenge posed by difficult-to-treat multidrug resistant organisms, is the product of decades of often-inappropriate usage of antimicrobial agents (Lerner 1998; Hellinger 2000). Although resistance mechanisms certainly predated clinical usage of antimicrobial agents, the extensive use of these agents has tended to select and, thus, enrich for bacteria expressing resistance determinants or organisms that are naturally resistant (Livermore 2000). Attendant problems include increased morbidity and mortality, and cost of health care (Hellinger 2000). The need for new antimicrobials to combat multiply-resistant pathogens is driving the current focus on identifying novel drug targets, though much might be gained from targeting resistance mechanisms themselves (Coleman et al 1994; Renau et al 1998; Wright 2000). This review focuses on the major resistance mechanisms found in bacteria, as well as past and current efforts at developing approaches to overcoming these mechanisms. Strategies include developing inhibitors of resistance determinants as well as antimicrobials that resist or are unaffected by these determinants.

Impermeability

In order for antibiotics to exert their bacteriostatic or bactericidal actions on bacteria, they must access intracellular targets. In Gram-negative bacteria, this necessitates that they cross the outer membrane, a substantial permeability barrier and, thus, major determinant of antimicrobial resistance in these bacteria (Nikaido 1994; Hancock 1997a). Indeed, the outer membrane barrier likely explains, at least in part, the enhanced resistance of Gram-negative versus Gram-positive organisms to many antimicrobials (Vaara 1993a). Still, recent data indicate that the outer membrane barrier as a determinant of resistance is only significant in the context of

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additional resistance mechanisms (i.e. efflux and β-lactamases) that work synergistically with it to promote resistance (Nikaido 1994; Hancock 1997a). It is expected, therefore, that compromising this barrier by its permeabilization would be an effective approach to combating antimicrobial resistance (Hancock 1997a; Nikaido 1998). In support of this, mutational (Hancock 1984; Sukupolvi & Vaara 1989; Vaara 1993a; Vaara & Nurminen 1999) or engineered (Huang & Hancock 1996) changes that disrupt the outer membrane correlate with increased susceptibility to multiple antibiotics, including agents that poorly penetrate the outer membrane and are, thus, traditionally poor candidates for therapy of Gram-negative infections (e.g. macrolides and peptide antibiotics; see Vuorio & Vaara 1992; Vaara 1993b). A number of agents known to permeabilize the outer membrane have been described in the literature, including chelators and polycations such as polymyxins and antimicrobial peptides (Hancock & Wong 1984; Vaara 1992; Piers & Hancock 1994; Piers et al 1994; Hancock, 1997b; Wu & Hancock 1999; Zhang et al 2000). Not surprisingly, many of these potentiate the activity of clinically-relevant antimicrobials (Vaara 1992; Piers et al 1994; Vaara & Porro 1996; Scott et al 1999; Giacometti et al 2000; Li et al 2000). For example, several membrane-perturbing peptides enhanced rifampin and erythromycin susceptibility up to 300-fold in *Escherichia coli*, *Klebsiella pneuomoniae* and *Enterobacter cloacae* (Vaara & Porro 1996), and polymyxin nonapeptide enhanced *E. coli* and *Salmonella typhimurium* susceptibility to macrolides and β-lactams as much as 30-fold (Vaara 1992). Antimicrobial peptide potentiation of β-lactam, doxycycline, clarithromycin and polymyxin E activity against *E. coli* and *Pseudomonas aeruginosa* was also reported (Giacometti et al 2000). Chelators, such as EDTA and sodium hexametaphosphate, enhanced the susceptibility of, for example, *P. aeruginosa* to numerous agents, including β-lactams, fluoroquinolones, erythromycin, tetracycline and chloramphenicol (Li et al 2000).

Target site mutations/efflux

Macrolides

The macrolide–lincosamide–streptogramin B (MLS_B) group of antimicrobials is predominantly used to treat Gram-positive infections, owing to problems with their crossing of the outer membrane of most Gram-negative bacteria (Leclercq & Courvalin 1991a; Vaara 1993b). The most common mechanism of resistance to these agents involves modification of the MLS_B target site on the ribosome, specifically methylation of an adenine residue in domain V of the 23 sRNA (Leclercq & Courvalin 1991a; Weisblum 1995; Schmitz et al 2000b). This latter is carried out by a family of plasmid or transposon-encoded (Leclercq & Courvalin 1991a) *N*-methyltransferases (erythromycin resistant methylase or Erm) that use *S*-adenosylmethionine as the methyl donor (Weisblum 1995). *N*-Methyltransferase-mediated resistance can be inducible (e.g. by 14- or 15-membered ring macrolides) or expressed constitutively (Leclercq & Courvalin 1991a). Macrolide resistance due to antibiotic inactivation (Leclercq & Courvalin 1991b; Matsuoka et al 1998; Nakamura et al 2000) and efflux (Ross et al 1990; Clancy et al 1996, 1997; Tait-Kamradt et al 1997) is also known and, indeed, macrolide resistance in *Streptococcus pneumoniae* is increasingly due to efflux (Gay et al 2000), with efflux now being the predominant form of macrolide resistance in this organism in the US (Shortridge et al 1999).

Macrolide use in Gram-negative pathogens is limited to those organisms that appear to have relatively permeable outer membranes (e.g. *Hemophilus influenzae* or *Neisseria gonorrhoeae*). In these instances, an important determinant of resistance appears to be efflux via broadly specific, so called multidrug efflux systems (e.g. *H. influenzae* (Sanchez et al 1997) and *N. gonorrhoeae* (Zarantonelli et al 1999)). Moreover, in Gram-negative organisms not inherently susceptible to macrolides (e.g. *E. coli*, *P. aeruginosa*, *Burkholderia pseudomomallei*), multidrug efflux systems appear to be a contributing factor (Ma et al 1993; Aires et al 1999; Moore et al 1999; Li et al 2000).

Although targeting efflux mechanisms might make sense for *S. pneumoniae*, the overall predominance of methyltransferase-mediated resistance to macrolides has led researchers to investigate the development of inhibitors for these enzymes. Erm inhibitors (Clancy et al 1995; Hajduk et al 1999), including inhibitor peptides identified by phage display (Giannattasio & Weisblum 2000), have been described, in one instance showing potentiation of azithromycin activity in MLS_B-resistant Gram-positive and Gram-negative bacteria (Clancy et al 1995). Recently, *S*-adenosyl-L-homocysteine mimics have been reported as weak but promising inhibitors of Erm methyltransferases (Hanessian & Sgarbi 2000).

A less direct way of targeting resistance is to develop macrolides that overcome or are less impacted by existing resistance mechanisms. Ketolides, a new class of semi-synthetic erythromycin derivatives have been developed which show increased activity against a number of Gram-positive pathogens expressing efflux or methyltransferase-mediated resistance (Chu 1999; Denis et al 1999; Bemer-Melchior et al 2000; Davies et al 2000).

Although the reason for this enhanced activity is not completely clear, some ketolides fail to induce Erm methylases, which would explain their activity against so-called inducibly MLS_B-resistant strains (Agouridas et al 1998; Rosato et al 1998). A recent report of ketolides with improved binding to MLS_B-resistant ribosomes highlights yet another approach to overcoming the ribosomal modification mechanism of resistance (Douthwaite et al 2000).

Fluoroquinolones

Resistance to fluoroquinolones (FQs) has traditionally been attributed to mutations affecting the FQ targets, DNA gyrase and topoisomerase (i.e. in *gyrA* and *parC* (*grl* in *Staphylococcus aureus*)) (Hooper 2000). More recently, efflux-mediated resistance to FQs has been reported in Gram-positive (reviewed in Poole 2000a) and Gram-negative (reviewed in Poole 2000b) bacteria, as well as in the mycobacteria (Poole 2000a). Intriguingly, FQ efflux systems display broad substrate specificity, and their expression, particularly in Gram-negative pathogens, is associated with resistance to multiple clinically-relevant antimicrobials in addition to FQs (Poole 2000b). These pumps contribute to both intrinsic and acquired resistance, the latter arising from mutational hyperexpression of these chromosomally-encoded efflux systems (Poole 2000a, b).

The challenge in overcoming target site mutations is to develop agents that are unaffected or at least less impacted by alterations in DNA gyrase or topoisomerase IV. Newer FQs, such as sitafloxacin (Jones et al 2000; Schmitz et al 2000a) and clinafloxacin (Pan & Fisher 1998; Brisse et al 1999a; Jones et al 2000; Schmitz et al 2000a) are, for example, highly active against bacteria carrying individual mutations in *gyrA* or *parC* (*grl*) that adversely impact on the activity of other FQs. This appears to be due to the fact that these agents target both gyrase and topoisomerase IV equally well and, as such, mutations in both genes are required for resistance (Pan & Fisher 1998; Morrissey & George 1999; Onodera et al 2000). By contrast, most FQs target DNA gyrase or topoisomerase IV preferentially and, thus, a single mutation is sufficient to provide some resistance (Hooper 2000). As a result, agents such as sitafloxacin and clinafloxacin are not only less susceptible to target site mutations that affect other FQs, but resistance to these agents is much less common. Sitafloxacin also shows good activity against strains of *S. pneumoniae* (Jones et al 2000), *N. gonorrhoeae* (Tanaka et al 2000) and *Enterococcus faecium* (Brisse et al 1999a) harbouring mutations in both *gyrA* and *parC*, while clinafloxacin is active against *gyrA*/*parC* double mutants of *E. faecium* (Brisse et al 1999a) and several Gram-negative pathogens (Brisse et al 1999b).

Despite the importance of target site mutations in FQ resistance, efflux plays an important role (Poole 2000a, b), sometimes in conjunction with target site mutations (Celesk & Robillard 1989; Irikura et al 1989; Yoshida et al 1994; Tanaka et al 1995; Zhanel et al 1995; Everett et al 1996; Kaatz & Seo 1997; Jalal & Wretlind 1998; Ferrandiz et al 1999). Moreover, mutant studies have shown that loss of efflux actually comprises both resistance afforded by target site mutations (Lomovskaya et al 1999; Oethinger et al 2000) and the development of resistance in-vitro (Lomovskaya et al 1999). This suggests that efflux inhibition could contribute significantly to restoring antimicrobial susceptibility to target site as well as efflux mutants and to reducing the incidence of resistance.

A number of inhibitors of the FQ pump, NorA, of *S. aureus* have been described, and these enhance FQ susceptibility in-vitro (Schmitz et al 1998; Aeschlimann et al 1999a; Markham et al 1999; Stermitz et al 2000) and in-vivo (Aeschlimann et al 1999b). One of these NorA inhibitors, reserpine, also rendered *S. pneumoniae* more susceptible to FQs (Brenwald et al 1997, 1998). Moreover, reserpine treatment of *S. aureus* (Markham & Neyfakh 1996) or *S. pneumoniae* (Markham 1999) prevented emergence of FQ resistance in these organisms. Recently, the first examples of efflux pump inhibitors of the Mex efflux systems of *P. aeruginosa* have also been reported (Renau et al 1999; Lomovskaya et al 2001). These potentiate the activity of a number of antibiotics, including FQs, in-vitro (Renau et al 1999; Lomovskaya et al 2001).

Efflux-mediated FQ resistance can also be targeted by developing agents that are poor substrates for the efflux systems. The NorA efflux pump is known, for example, to contribute to resistance to a number of FQs in *S. aureus*, although over-expression of this efflux system in mutant strains has only a limited effect on the activity of many of the newer FQs (Takenouchi et al 1996; Muñoz Bellido et al 1997; Fukuda et al 1998; Ince et al 1999a, b; Pestova et al 2000). As such, efflux inhibitors such as reserpine potentiate their activity much less than older FQs such as ciprofloxacin or norfloxacin (e.g. Schmitz et al 1998). While it has been suggested that hydrophobicity explains the apparent poor export of certain FQs by NorA in *S. aureus*, NorA apparently mediating resistance to predominately hydrophilic FQs (Yoshida et al 1990; Kaatz & Seo 1995), there is not a strict correlation between hydrophobicity and improved activity in efflux-mediated resistant strains of *S. aureus*

(Takenouchi et al 1996). Nonetheless, the fact that some of the newer FQs are minimally impacted by efflux (and target site) mutations makes them particularly useful agents against FQ-resistant strains.

Tetracyclines

Resistance to tetracyclines (encoded by *tet* determinants) is generally afforded by an efflux (e.g. TetA, TetB, TetK) or a ribosomal protection (e.g. TetL, TetM) mechanism, although an example of resistance due to a tetracycline inactivating enzyme, TetX, has been reported (Roberts 1996). The *tet* genes are usually plasmid or transposon-encoded and, thus, resistance arises from acquisition of these genes from an external source. The details behind ribosomal protection are as yet unclear, making it difficult to counter this mechanism of resistance. Still, reports of agents that overcome or interfere with both ribosomal protection and efflux are known (reviewed in Levy & Nelson 1998; Sum et al 1998).

A variety of tetracycline analogues and tetracycline-like compounds have been reported as inhibitors of Tet efflux systems (Nelson et al 1993, 1994; Nelson & Levy 1999). Some inhibitors worked synergistically with doxycycline against *E. coli*, *S. aureus* and *Enterococcus faecalis* strains expressing efflux determinants of tetracycline resistance (Nelson et al 1994; Nelson & Levy 1999). In some instances the tetracycline analogues were themselves potent growth inhibitors of *S. aureus* expressing Tet efflux and ribosomal protection mechanisms (Nelson et al 1994; Nelson & Levy 1999). In addition to inhibitors, another approach to overcoming efflux-mediated tetracycline resistance has been the development of semi-synthetic tetracyclines, the glycylcyclines (Sum et al 1998), that retain activity against strains harbouring Tet efflux mechanisms. These compounds are active both in-vitro and in-vivo against Gram-positive and Gram-negative pathogens displaying the efflux-mediated resistance phenotype (Testa et al 1993; Petersen et al 1999), apparently because glycylcyclines are poor substrates for efflux (i.e. are not recognized or exported by Tet efflux proteins (Someya et al 1995)). Significantly, glycylcyclines are also active against bacteria that are tetracycline-resistant by virtue of a ribosomal protection mechanism (Testa et al 1993; Sum et al 1998; Petersen et al 1999). The observation that glycylcyclines bind more effectively than, for example, tetracycline to the tetracycline-binding site of the 70S ribosomal target (Bergeron et al 1996) likely explains the increased activity against bacteria expressing ribosomal protection mechanisms.

Vancomycin

Vancomycin targets the D-ala-D-ala termini of the UDP-*N*-acetylmuramyl-pentapeptide precursor of peptidoglycan, ultimately interfering with peptidoglycan crosslinking. Five phenotypes of vancomycin resistance have been described (VanA–E), all involving the synthesis of abnormal pentapeptide precursors possessing altered termini (e.g. D-ala-D-lac or D-ala-D-ser instead of D-ala-D-ala) with lower affinity for vancomycin (Cetinkaya et al 2000). However, efficient incorporation of the vancomycin-resistant dipeptides into the pentapeptide precursors requires the elimination of the vancomycin-binding D-ala-D-ala dipeptide, a function carried out by the *vanX* gene product (Reynolds et al 1994). As such, VanX is an attractive target for inhibiting Van-mediated resistance and, indeed, a number of VanX inhibitors have been reported (Wu & Walsh 1995, 1996; Wu et al 1995; Morrissey & George 1999). Still, there is no published evidence that these potentiate vancomycin activity against resistant strains. In light of the vancomycin inducibility of some resistance determinants, compounds that block induction have also been developed, and these do compromise Van-mediated resistance (Macielag et al 1998; Ruzin & Novick 1998). Recently, modified glycopeptides that are active against vancomycin-resistant enterococci, possibly because they bind to the altered dipeptide of resistant peptidoglycan, have been reported (Goldman & Gange 2000).

Antibiotic inactivation

β-Lactams

The predominant mechanism of resistance to β-lactams remains β-lactamases (Thomson & Smith 2000), although target site (i.e. penicillin-binding proteins) alterations, especially in the streptococci (Hakenbeck et al 1999), as well as efflux (Srikumar et al 1999; Mazzariol et al 2000) and outer membrane impermeability (e.g. Hancock & Woodruff 1988; Chen & Livermore 1993) in Gram-negative organisms, also play a role. Four molecular classes of β-lactamases are recognized, including the Class A penicillinases, Class B metallo-β-lactamases, Class C cephalosporinases and Class D oxacillinases (Thomson & Smith 2000). Because of the importance of β-lactamases vis-a-vis β-lactam resistance, the focus has traditionally been on developing agents which are stable to hydrolysis by known β-lactamases (Bush & Mobashery 1998; Livermore 1998). Cephalosporins with oxyimino-aminothiazoyl 7-acyl side chains (e.g. cefotaxime) are, for example, stable to several Class A enzymes, and the carbapenems are stable to all β-lactamases except the Class B metallo-β-lactamases

and less common Class A carbapenemases (Livermore 1998). These latter agents, especially, have proven useful in countering β-lactamase-mediated β-lactam resistance.

β-lactamase inhibitors have also been the focus of intense study (reviewed in Maiti et al 1998; Mascaretti et al 1999; Payne et al 2000; Therrien & Levesque 2000), with three, tazobactam, sulbactam and clavulanate, currently approved for use in the clinic. Unfortunately, these only work against class A β-lactamases and not the increasingly important Class C cephalosporinases (e.g. AmpC) or the Class B metallo-β-lactamases (Bush 1999). Moreover, Class A β-lactamases resistant to the currently approved inhibitors have been reported (Chaibi et al 1999). The need, therefore, for novel Class A inhibitors as well as inhibitors of the AmpC and metallo-β-lactamases is clear. Recent activity in the field has focused on developing novel structural classes of mechanism-based Class A β-lactamase inhibitors (Maiti et al 1998; Mascaretti et al 1999; Ness et al 2000), although Class A inhibitors based on β-lactamase inhibitory protein, a product of the clavulanic acid-producing soil bacterium *Streptomyces clavuligerus*, have also been proposed (Huang et al 2000). A number of metallo-β-lactamase (Walter et al 1996, 1999; Payne et al 1997a, b, 2000; Fitzgerald et al 1998; Toney et al 1998; Concha et al 2000) and AmpC (Maiti et al 1998; Mascaretti et al 1999; Babini & Livermore 2000; Buynak et al 2000a) inhibitors, as well as inhibitors of Class A nonmetallocarbapenemases (Mourey et al 1999) have been described as well. Importantly, many of the AmpC inhibitors potentiate β-lactam activity against AmpC-producing organisms (Richter et al 1996; Livermore & Chen 1997; Heinze-Krauss et al 1998; Maiti et al 1998; Mascaretti et al 1999; Powers et al 1999; Babini & Livermore 2000; Grant et al 2000), although only a few of the metallo-β-lactamase inhibitors were shown to potentiate β-lactam activity (Toney et al 1998; Payne et al 2000). Some of the more recently described β-lactamase inhibitors are broad-spectrum, being active against both Class A and Class C (e.g. AmpC) enzymes (Mascaretti et al 1999; Buynak et al 2000b; Sandanayaka & Yang 2000). While most of the Class A and newly described Class C inhibitors are themselves β-lactams (Maiti et al 1998; Mascaretti et al 1999; Buynak et al 2000a, b), a number of non-β-lactam inhibitors of Class C β-lactamases have been reported (Weston et al 1998; Powers et al 1999; Grant et al 2000). These may prove particularly useful given that they are unlikely to upregulate β-lactamase production or be cleaved by mutant β-lactamases, problems that could compromise the use of some β-lactam-derived inhibitors. The recent observation that β-lactam-derived β-lactamase inhibitors are substrates for efflux could limit their usefulness in certain organisms (Li et al 1998).

Resistance to methicillin in *S. aureus* (MRSA) is generally due to production of a low-affinity penicillin-binding protein PBP2a, which confers resistance to virtually all β-lactams (Chambers 1997). Using compounds which displace penicillin binding to PBP2a, potentiation of β-lactam activity against MRSA has been demonstrated (Renau et al 1998).

Aminoglycosides

Bacterial resistance to aminoglycosides is predominantly based on a chemical modification of the aminoglycoside, which compromises binding of the agent to its ribosomal target (Wright et al 1998; Wright 1999; Kotra et al 2000), although resistance due to altered uptake and target site (i.e. ribosomal) mutations is also known (Wright et al 1998; Kotra et al 2000). Three types of modification have been demonstrated, catalysed by *O*-phosphotransferases (APH), *O*-adenyltransferases (ANT) and *N*-acetyltransferases (AAC) (Wright et al 1998; Kotra et al 2000). The corresponding genes are generally found on mobile elements such as transposons or plasmids, although some occur on bacterial chromosomes. The aforementioned enzymes are amenable to inhibition and, indeed, inhibitors (inactivators) of APH (Roestamadji et al 1995; Daigle et al 1997; Roestamadji & Mobashery 1998) and ANT (Allen et al 1982) enzymes have been reported. Unfortunately, only one, the 7-hydroxytropolone inhibitor of an aminoglycoside-2″-*O*-adenyltransferase, actually demonstrated potentiation of aminoglycoside activity against resistant organisms expressing the corresponding transferase (Allen et al 1982). Early observations that aminoglycoside derivatives that are poor substrates for modifying enzymes are more active against resistant bacteria (e.g. gentamycin and sisomycin derivatives) (Vastola et al 1980) provided a second approach to overcoming resistance mediated by modifying enzymes. In this vein, a number of novel aminoglycosides which interact poorly with aminoglycoside-modifying enzymes (APH) have been described whose activity is unaltered in resistant versus sensitive strains (Roestamadji & Mobashery 1995). A recently described kanamycin A variant exhibits spontaneous loss of the phosphate group donated by APH, rendering it effectively resistant to APH-mediated inactivation (Haddad et al 1999). Although the compound is inherently much less potent than kanamycin A, these studies do suggest a potentially useful approach to developing enzyme-resistant aminoglycosides.

Chloramphenicol

Resistance to chloramphenicol is typically afforded by acetyltransferases (Murray & Shaw 1997) or efflux (Mine et al 1998; Arcangioli et al 1999). Despite an early report of a chloramphenicol acetyltransferase inhibitor (Miyamura et al 1979), chloramphenicol resistance mechanisms as targets have received scant attention.

Concluding remarks

Much is known vis-a-vis the mechanisms by which bacteria evade the action of antimicrobials and, thus, thwart attempts at chemotherapeutic intervention. Targeting these mechanisms specifically, in an attempt to rejuvenate antimicrobials rendered ineffective by these mechanisms, should be considered a useful complement to the current development of novel agents with novel bacterial targets. Resistance will always be a problem, and given the negative impact of, for example, the Gram-negative outer membrane barrier and efflux systems on multiple, structurally-distinct antimicrobials, it is likely that novel agents will also be adversely affected by these mechanisms.

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