Antibiotic resistance is a global public health concern that impacts to varying extents on the efficacy of all licensed antibacterial agents. Resistance limits therapeutic options and drives clinicians to use newer and more expensive agents. In extreme cases, multiresistance leaves no treatment options. A continuing supply of new antibiotics offers an obvious way to overcome resistance, but the pipeline of agents in development by the pharmaceutical industry is very limited; the number of companies investing in new antibacterials is falling, partly due to intense media and political interest and impacts on personal health and costs to health infrastructures. Bacteria have developed resistance to all licensed antibacterial agents, and their ability to become resistant to unlicensed agents is often demonstrated during the development process. Conventional approaches to antimicrobial development, involving modification of existing agents or production of synthetic derivatives, are unlikely to deliver the range or type of drugs that will be needed to meet all future requirements. Although many companies are seeking novel targets, further radical approaches to both antimicrobial design and the reversal of resistance are now urgently required. In this article, we discuss ‘antisense’ (or ‘antigene’) strategies to inhibit resistance mechanisms at the genetic level. These offer an innovative approach to a global problem and could be used to restore the efficacy of clinically proven agents. Moreover, this strategy has the potential to overcome critical resistances, not only in the so-called ‘superbugs’ (methicillin-resistant Staphylococcus aureus, glycopeptide-resistant enterococci and multidrug-resistant strains of Acinetobacter baumannii, and Pseudomonas aeruginosa), but in resistant strains of any bacterial species.

Keywords: resistance inhibitors/modulators, oligonucleotides, modified nucleic acids, bacteriophage, delivery systems

Antibiotic resistance is a global public health concern that impacts to varying extents on the efficacy of all licensed antibacterial agents. Resistance limits therapeutic options and drives clinicians to use newer and more expensive agents. In extreme cases, multiresistance leaves no treatment options. A continuing supply of new antibiotics offers an obvious way to overcome resistance, but the pipeline of agents in development by the pharmaceutical industry is very limited; the number of companies investing in new antibacterials is falling, partly due to mergers, but also because of the costs involved and the return on investment relative to the development of other therapeutic classes of drugs.1,2 There is a pressing need to develop and evaluate novel alternative strategies for combating a worsening clinical situation, to overcome resistance and reduce the morbidity and mortality associated with infections caused by antibiotic-resistant bacteria.

One strategy would be to use ‘antisense’ or ‘antigene’ agents to inhibit resistance mechanisms at the nucleic acid level. Strictly, ‘antisense’ and ‘antigene’ (hereafter referred to collectively as antisense) oligonucleotides bind mRNA to prevent translation or bind DNA to prevent gene transcription, respectively. Interrupting expression of resistance genes in this manner could restore susceptibility to key antibiotics, which would be co-administered with the antisense compound. This would extend the lifespan of existing antibiotics, which offer clinically proven therapies, and are often cheaper, more effective or less toxic than the alternatives. Antisense molecules that bind complementary mRNA sequences are a well-established means of modifying gene expression in mammalian systems.3 Indeed, the manipulation of eukaryotic RNA processing pathways with small interfering RNAs (siRNAs) has revolutionized research in mammalian cell biology, with libraries of custom-made molecules spanning entire genomes now commercially available. Antisense strategies have been used therapeutically in the treatment of human genetic disorders, such as muscular dystrophy and familial hypercholesterolaemia,4 or viral diseases (see http://www.avibio.com and http://www.isispharm.com), with some clinical trials ongoing and with a small number of agents already licensed for clinical use.5

However, antisense strategies have been considered as antibacterial agents by relatively few researchers, and usually as bactericidal agents targeting essential genes.5–10 A complementary,
but equally valid, research path involves the use of antisense oligonucleotides to target the genes responsible for antibiotic resistance. There is limited proof-of-principle evidence for resistance modulation by antisense agents; the approach has been applied successfully in vitro to reverse, for example, amikacin resistance,\textsuperscript{11,12} chloromycetin resistance\textsuperscript{13} and multidrug efflux in \textit{Escherichia coli},\textsuperscript{14} and glycopeptide resistance in enterococci.\textsuperscript{15}

Developing resistance inhibitors is a sound, well-validated strategy, which complements the development of directly antibacterial agents. For example, \(\beta\)-lactamase inhibitors, such as clavulanic acid, tazobactam and sulbactam, are widely used clinically to restore the susceptibility of bacteria to co-administered \(\beta\)-lactam antibiotics. The economic and clinical value of this rationale is demonstrated by efforts to market new combinations (for example, cefixime/clavulanate; http://www.ranbaxy.com) or to develop novel \(\beta\)-lactamase inhibitors (for example, NXL104; http://www.novexel.com).\textsuperscript{16} Beyond \(\beta\)-lactamases, efflux pump inhibitors offer a tantalizing and much-explored route whereby bacterial susceptibility could be restored simultaneously to multiple antibiotic classes.\textsuperscript{17} The principle of using antisense therapeutics as modulators of bacterial resistance is broadly applicable and could be used to overcome resistance, potentially, in any pathogenic species. Furthermore, in contrast to agents targeting essential genes, it may be possible to target only antibiotic-resistant bacteria, limiting disruption of the normal flora, particularly if the antisense allows the co-administration of a narrow-spectrum agent. Toxicity would also be anticipated to be minimal because: (i) antibiotic resistance genes have virtually no homology to human genes, and (ii) humans are continually exposed to bacterial nucleic acids. However, many obstacles must be overcome if these innovative technologies are to be harnessed to reduce the burden of antibiotic resistance for the benefit of patients.

Inhibition of prokaryotic protein synthesis by antisense molecules may occur by at least four mechanisms, most of which rely on the activity of intracellular nucleases. The degradation of RNA:mRNA duplexes by dsRNA-specific RNAses is one example of a natural means of transcriptional regulation controlling membrane transport in \textit{E. coli}\textsuperscript{18} and virulence in \textit{Staphylococcus aureus}.\textsuperscript{19} When antisense RNA is artificially generated from transfected plasmid vectors, short-lived inhibition of bacterial gene expression can also be observed.\textsuperscript{20} The introduction of antisense oligonucleotides able to form stable DNA:RNA interactions leads to degradation of the resulting heterodimers through the activity of RNase H. If external guide sequences are coupled to the oligonucleotide, degradation occurs via the action of RNase A. Alternatively, undegraded oligonucleotide:RNA heteroduplexes are able to inhibit translation by a steric block of ribosomal maturation and polypeptide elongation processes (Figure 1). However, the steric block is not efficient in the coding region of the genes and is restricted to RNA sites where translation is initiated or where other RNA processing events occur. There are myriad chemistries for modified nucleic acids that could be developed as antisense therapeutics, including phosphorothioate oligonucleotides (PS-ODNs), locked nucleic acids (LNAs), 2'-O-methyloligoribonucleotides (2'OMes), phosphorodiamidate morpholino oligonucleotides (PMOs) and peptide nucleic acids (PNAs).\textsuperscript{21–24} All offer advantages of increased nuclease stability and markedly increased antisense activity, but at hugely inflated synthetic cost.\textsuperscript{25} 2'OMes, LNAs, PMOs and PNAs all bind more tightly to RNA than oligonucleotides or PS-ODNs and therefore can be used at shorter lengths and at lower concentrations when used in the steric block mode.\textsuperscript{26}

Although conceptually simple, translating the antisense approach into tangible therapeutic agents is hampered by two formidable obstacles. First, how can the most appropriate antisense molecule be identified? Secondly, how can it be delivered: (i) to the site of infection in the patient, and (ii) to its site of action within the bacterial cell? Clinically useful antibacterial antisense agents require research far beyond proving their efficacy in \textit{in vitro} cell-free translation systems, or after electroporation of recombinant DNA into antibiotic-resistant bacterial cells.

![Figure 1. Mechanisms of ‘knock down’ of gene expression by various antisense molecules](adapted from Figure 1 in Rasmussen et al. with permission from the authors).\textsuperscript{38}
Another potential hurdle could be how the regulatory authorities would handle such agents; early consideration may serve to encourage those seeking to develop this type of approach.

Design of the antisense oligonucleotide is crucial but, in contrast to work in eukaryotes, very little has been undertaken to design oligonucleotides for the inhibition of bacterial genes. A search of the AOBase database (http://www.bioit.org.cn/ao/aobase/), which catalogues sequences known to be effective, returns results for only a single bacterial gene; *E. coli* K12 23S rRNA. In order to form stable DNA:RNA heteroduplexes, antisense molecules must bind accessible regions of the target mRNA. However, as very few crystal structures are available, sites are usually selected using bioinformatic algorithms that predict the secondary structure of the molecule using calculations of minimal free energy. Although these algorithms (for example mfold) have been useful in modelling structural RNAs, they have been less successful when applied to mRNAs. This shortcoming may be attributable to the existence of multiple heterogeneous secondary structures within a given population of mRNA molecules. Other approaches, such as random screening of oligonucleotide libraries or scanning arrays, represent little more than costly ‘fishing’ exercises with limited chances of success. ‘Sfold’ (http://sfold.wadsworth.org/) offers improved mRNA structural prediction, and has parameters specific for prokaryotic RNA. Sfold-designed oligonucleotides have been evaluated in cell-free expression experiments and shown to be highly potent inhibitors of the *E. coli lacZ* gene.

Importantly, Sfold assesses the maximum length of antisense sequences, which must be considered to reduce the costs associated with modified nucleic acids.

Following identification, candidate sequences have to be delivered into bacterial cells. Aside from the pharmacokinetic and pharmacodynamic parameters that would need to be addressed in the development of an antisense drug, the molecules will have to traverse the bacterial membrane(s) and resist degradation by intracellular nucleases to exert their effect. Stability of the antisense message can be improved by using modified nucleic acids, but these molecules are too large to enter the cell by passive diffusion. An unmodified 10-mer oligonucleotide is 2-3 kDa, and the various chemical modifications outlined above add further to this size. In short, antisense therapeutics are likely to be considerably larger than vancomycin, for example, and therefore require a delivery system. A schematic diagram of the processes involved in the delivery of an antisense molecule targeting a β-lactamase is shown in Figure 2.

Entry into bacterial cells can be improved by attaching the antisense agent to a cell-permeabilizing peptide (CPP) carrier, which enables uptake through peptide permeases in the cytoplasmic membrane. Such peptides can be attached readily to PNAs or PMOs, which are electrically neutral nucleic acid analogues, and may then facilitate better delivery than when coupled to unmodified oligonucleotides. Indeed, it is peptide-PMO conjugates that are being taken to clinical trials for other disease indications, including Duchenne muscular

![Figure 2](image-url)

**Figure 2.** Schematic to illustrate modulation of a resistance mechanism (in this case, β-lactamase production) using an antisense strategy. 1: mRNA encoding a β-lactamase is translated into a functional polypeptide by the bacterial ribosome and is transported across the cytoplasmic membrane by type II secretion. 2: β-Lactams traverse the bacterial outer membrane via specific porins. 3: β-Lactams are bound to β-lactamases in the periplasm and hydrolysed. 4: The peptide component of a CPP-antisense oligonucleotide conjugate causes local disruption of the outer membrane allowing entry to the periplasm. The conjugate traverses the cytoplasmic membrane aided by peptide permeases and enters the cytoplasm. 5: The antisense oligonucleotide then binds to the complementary sequence on the mRNA and inhibits translation of the β-lactamase. 6: β-Lactams are not hydrolysed in the periplasm, and are able to bind to peptidoglycan-synthesizing enzymes (the PBPs), leading to cell lysis.
dystrophy (see, for example, http://www.avibio.com). Many CPP carriers are known, but few have been evaluated, in part due to the complete lack of data on the appropriate antisense molecule with which to couple them, and the lack of any standardized way of quantifying their efficiency. A PMO directed against a critical target (acpP, encoding acyl carrier protein, which is involved in fatty acid biosynthesis) and linked to the decapptide KFFKFKFFKFK had activity in a mouse model of E. coli bacteraemia and peritonitis. However, this peptide has considerable haemolytic activity and so is unlikely to be a strong candidate for clinical development. Other variants, such as RFFRFRFFRXB (X is 6-aminohexanoic acid and B is β-alanine), look significantly better. The ability of any CPP considered for clinical development to transport antisense agents across bacterial membranes would have to be assessed not just in E. coli, but also in relatively ‘impermeable’ genera, such as Acinetobacter and Pseudomonas.

Delivery of antisense molecules packaged in bacteriophage has not been widely considered, but offers the conceptual advantage over CPP-oligonucleotide conjugates of tailored delivery only to the bacterial species of interest. Such specificity might be expected to reduce collateral damage to commensal species coincidently carrying the targeted resistance gene, and thus potentially to reduce the speed of development of resistance by reducing non-species-specific selection pressure. Liposomes might also be considered as delivery vehicles.

It would be shortsighted to assume that antisense agents will be ‘immune’ to the emergence of resistance; rather, its emergence is an evolutionary inevitability. However, by giving careful consideration to the plethora of potential strategies available, and by pursuing those considered to pose the least risk for the selection of resistant variants, we could develop antisense agents as both effective antibacterial agents and resistance inhibitors. It may well be possible to design synergistically acting antisense agents, targeting either more than one resistance gene, or a resistance gene(s) in combination with a critical bacterial target. It is exciting to consider a future where clinicians treating infections caused by an antibiotic-resistant bacterial strain might be able to co-administer a molecular inhibitor of the critical resistance gene together with the relevant antibiotic, such as a mecA inhibitor plus fluoroquinolone for methicillin-resistant S. aureus. Such an approach would be of enormous benefit to patients and healthcare systems. The concept should attract funding and the developmental powers of the pharmaceutical industry which could find new niches for long-abandoned generic drugs or agents still under patent but now compromised by resistance.

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