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CONTENTS

Combating antimicrobial resistance through gene silencing

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ABSTRACT: In the past few decades there have been indiscriminate uses of antibiotics due to which there have been increased antibiotic resistance in bacteria. Due to the constant use of conventional antibiotics, they have become ineffective against the superbugs. This has become a global emerging problem and several labs are working to adapt novel strategies to combat these superbugs. One of the promising candidates is gene silencing which involves binding of antisense oligonucleotides (ASOs) with the corresponding antibiotic resistant gene sequence inside bacterial cell. In this review, the gene silencing and carrier molecules involved in the process have been discussed in detail.

Key words: Antibiotic resistance, gene silencing, multiple drug resistance, peptide nucleic acids, phosphorodiamidate morpholino oligomers

Worldwide, infectious diseases are the leading cause of death out of which 25% mortality are caused by communicable disease (Fauci and Marston, 2014). The indiscriminate use of antibiotics have given rise to various forms of drug resistant microorganisms like multi-drug resistant *Mycobacterium tuberculosis* (MDR-TB), carbapenem resistant *Enterobacteriaceae* (CRE), methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Staphylococcus aureus* (VRSA) (English and Gaur, 2010; Gaash, 2008; Levy, 2002; Lozano *et al.*, 2012). Drug resistance mechanism in microbes involve alteration of drug targets, inactivation/ modification of antimicrobial drugs and reduction of drug accumulation by expelling drugs across the cell membranes. These all mechanisms constitutively lead to resistance against antimicrobial drugs (Tenover, 2006). This emerging problem can be solved either by discovery of new effective antibiotics against these resistant bacteria or by development of novel strategies to inhibit the gene transcription and translation inside bacterial cell. The first approach may take lot of time for discovery of new drug to combat antibiotic resistance. However, the second approach can be developed quicker than first. In second approach, the inhibition of gene transcription can be achieved by gene silencing which involves binding of antisense oligonucleotides (ASOs) with the corresponding sequence inside bacterial cell.

PNAs and PMOs

With the advent of synthetic biology, options available are synthetic RNA silencers like peptide nucleic acids (PNAs) and Phosphorodiamidate morpholino oligomers (PMO) (Dryselius *et al.*, 2003; Eriksson *et al.*, 2002; Good *et al.*, 2001; Good and Nielsen, 1998b; Mellbye *et al.*, 2009; Nekhotiaeva *et al.*, 2004; Nikravesh *et al.*, 2007; Tan *et al.*, 2005). PMOs are DNA prototypes which inhibit m-RNA expression. These are synthesized by using four bases conforming a sequence complementary (anti-sense) to a specific region of m-RNA. However, they are different to DNA in the chemical structure that links the bases. Unlike DNA, in PMO, ribose is replaced with a morpholine group and the phosphodiester is replaced by phosphorodiamidate. Due to these changes, the antisense PMOs become resistant to degradation by RNAse, neutral in charge and retains the molecular structure capable of binding to the nucleic acid's

complementary strand (Stein *et al.*, 1997; Summerton *et al.*, 1997; Summerton and Weller, 1997). Another type of DNA prototypes are PNAs which are antisense DNA and has been shown to inhibit the bacterial gene expression both in pure culture and *in vitro* (Good and Nielsen, 1998a, 1998b) . Also, it has been observed that the PNA entry in *E. coli* was inefficient due to the outer membrane of this bacterium (Good *et al.*, 2000). However, the entry has been improved largely by conjugating the PNA to cationic peptides (Rustici *et al.*, 1993; Vaara and Porro, 1996). The anti sense oligonucleotides are subjected to degradation by cellular RNAase (Felgner *et al.*, 1997; D.-Q. Xu *et al.*, 2009). Hence, effective carriers are required for the effective delivery of the oligonucleotides inside the bacterial cell. Moreover, antimicrobial activity of the carrier molecule can be an added benefit and desirable to combat antimicrobial resistance.

Carriers

Carriers used for delivery of ASOs are gelatin, chitosan, cell penetrating peptides and nanoparticles. These carrier molecules are of various types like polymer based antimicrobial delivery systems, peptides and other RNAi nanoantibiotics for the delivery of antimicrobial RNAs. Various types of natural and synthetic polymers are reported to deliver nucleic acid with their self antimicrobial activity (Carmona-Ribeiro and de Melo Carrasco, 2013; Mintzer and Simanek, 2009). Out of these polymers, chitosan and gelatin are notable polymers for antimicrobial RNA delivery (Dang and Leong, 2006). Another class of carriers having antimicrobial property is peptide (Wong *et al.*, 2014; Yeaman and Yount, 2003) and has been used for treatment of various drug resistant microbes (Debnath *et al.*, 2010; Henriques *et al.*, 2006; Yeaman and Bayer, 2006). Other nanoantibiotic systems like silver, gold, zinc, aluminium, titanium dioxide and copper also exhibit antimicrobial property. Therefore, the above activities of molecules show that combination of ASO and antimicrobial carrier particles will develop the most effective antimicrobial therapy. This comprises of combination of cationic polymers/ cationic peptides as si-RNA complexing along with inorganic nanoparticles can effectively eradicate Multidrug-resistant microorganisms (MDRMOS) (X. Xu and Zhou, 2009)

Gelatin

Gelatin is derivative of type I collagen and comprises of glycine and proline rich repeating units (Kim *et al.*, 2007; Lee *et al.*, 2009; Liu *et al.*, 2004; OU *et al.*, 2002; Sang *et al.*, 2010). Gelatin is a denatured hydrophilic protein. Both gelatin and chitosan have been shown to combine with nucleic acids (Matsumoto *et al.*, 2006; Nezhadi *et al.*, 2009) and have been found to destroy the cell membrane of *Staphylococcus aureus* (Bruschi *et al.*, 2006; Doktycz *et al.*, 2003).

Chitosan

Chitosan is a chitin product and possess excellent antimicrobial activity (Kim *et al.*, 2007). It's mode of action comprises of its activity to damage the bacterial cell membrane via strong electrostatic interactions between cationic amines on the chitosan and the anionic phosphoryl groups of the bacterial membranes (Chung *et al.*, 2004; Lee *et al.*, 2009; Liu *et al.*, 2004). Use of chitosan for delivery of si-RNA inside eukaryotic cells is well documented (Shahnaz *et al.*, 2012; Techaarpornkul *et al.*, 2010). Chitosan has also been used as a carrier particle by conjugation with peptide and adsorbing si-RNA to the chitosan-peptide complex for delivery inside the bacterial cell (Katas *et al.*, 2012). Similarly, successful attempt has been made by conjugating peptide with chitosan using a PEG linker followed by successful transfection inside the cells (Malhotra *et al.*, 2013).

Peptides

Cationic peptides are the main peptides used for delivery of RNAs inside bacterial cells eg. Stearylated melittin cationic peptide has been demonstrated to be an efficient mode for gene delivery (Zhang *et al.*, 2013). These antimicrobial peptides create pores in the bacterial membranes which results into leakage of metabolites and ions.

It also depolarizes the cell membrane and interferes with membrane coupled respiration (Debnath *et al.*, 2010; Devocelle, 2012). It has been seen that the peptide KFCKFC-KFC-KC has better penetrating capacity inside bacterial cell (Geller *et al.*, 2003; Good *et al.*, 2001). Moreover, addition of N-[3]-maleimidobutyryl-oxysuccinimide ester (GMBS) as a non-cleavable linker enhances the penetrating capacity of the peptide conjugated PMOs (Geller *et al.*, 2003) . Conjugation of peptide to 5' end and 3' end of PNA/PMO has better penetration capability than conjugation at 3'end alone (Geller *et al.*, 2003). In earlier studies (Bendifallah *et al.*, 2006; Sazani *et al.*, 2002), PNAs conjugated to peptides have been shown to penetrate well inside the mammalian cells. In a study by Tilley *et al.*, 2006, PMO-peptide conjugate has been shown to have a strong bactericidal activity in mouse model against *Escherichia coli* (Tilley *et al.*, 2007), *Acinetobacter* (Geller *et al.*, 2013) and *Bacillus anthracis* (Panchal *et al.*, 2012). The PMO-peptide conjugates have also been shown to have antiviral activity (Burrer *et al.*, 2007). *In vitro* studies targetting bacterial specific genes using PMO conjugated peptides have been tried (Wesolowski *et al.*, 2011).

Nanoparticles

Nanoparticles are also known to have antimicrobial activity. Gold nanoparticles, silver nanoparticles and carbon nanoparticles can be used as antimicrobials.

Gold nanoparticles

Gold nanoparticles have application in the diagnosis of antiviral antibodies (Jain *et al.*, 2018). In an intriguing work, Patel and colleagues (Patel *et al.*, 2008) have successfully conjugated gold nanoparticles with peptides and oligonucleotides followed by the transfection of complex inside the cell culture. Gold nanoparticles exhibit antimicrobial activity due to strong electrostatic attraction to the anionic bilayer of microbial membrane (Huh and Kwon, 2011; Zhao and Jiang, 2013). In another study, TiO_2 after conjugation with the oligonucleotides was successfully delivered inside the cells (Paunesku *et al.*, 2003).

Silver nanoparticles

Silver nanoparticles are very effective against bacteria, viruses and eukaryotic microorganisms (Huh and Kwon, 2011). Silver nanoparticles attack the respiratory chain and cell division in microbes, at the same time releases silver ions that augment antibacterial activity (Huh and Kwon, 2011). Zinc oxide nanoparticles are found to be effective against food borne pathogens such as *E. coli* (Huh and Kwon, 2011). Titanium dioxide nanoparticles produces bactericidal reactive oxygen species (ROS) such as free hydroxyl radicals and peroxides (Paunesku *et al.*, 2003).

Carbon nanoparticles

Carbon nanoparticles such as fullerenes are antimicrobial due to lipid peroxidation in prokaryotic cell membrane (Corredor *et al.*, 2013; Huh and Kwon, 2011; Sigwalt *et al.,* 2011). For the efficient delivery of antimicrobial RNAs, antimicrobial inorganic and carbon nanoparticles need to be surface functionalized or covalently linked with cationic polymers (peptides) in order to complex nucleic acids or directly conjugate with RNA, respectively (Paunesku *et al.*, 2003; Pissuwan *et al.*, 2011; Sigwalt *et al.*, 2011). Thus, the above contributions of researchers shows that these carrier particles have potential to cross the cell wall barrier of cell and thus deliver the assignment (oligonucleotides) inside the bacterial cell which subsequently silences the translation of m-RNA coding antibiotic resistant gene.

National status

In India, most of the research on antimicrobial resistance (AMR) is based on testing of plant extracts/pure compounds for their antimicrobial activity against antimicrobial resistant strains (Khan *et al.*, 2009; Srivastava *et al.*, 2014). The antibacterial effect of herbal extracts and their compounds against drug resistant strains have been reviewed in detail (Dhama *et al.*, 2014). However, in recent studies anti- herbal resistance has been reported in bacterial isolates (Singh *et al.*, 2013;

Vadhana *et al.*, 2015). Therefore, another alternative to combat antimicrobial resistance can be switching to synthetic biology regime. Not much work has been performed in Indian scenario for the development of antimicrobials using synthetic biology, and thus there is an intense need to encourage synthetic biology work in India, for combating antimicrobial resistance. There are few studies based on the testing for antimicrobial activity of nanoparticles (Kar *et al.*, 2016; Negi *et al.*, 2013). Negi and colleagues, 2013 tested the antibacterial activity of silver oxide nanoparticles against both gram positive and gram negative bacteria. Similarly, Kar and colleagues 2016 evaluated the bactericidal effect of silver nanoparticles and capsaicin against multidrug resistance and extended spectrum beta-lactamase producing *Escherichia coli* of bovine and poultry origin. Bhattacharya *et al.*, 2017 found that a synthetic molecule was able to activate the activity of fluoroquinolones against multidrug-resistant *Acinetobacter baumannii* by efflux inhibition (Bhattacharyya *et al.*, 2017). Mishra *et al.*, 2013 successfully isolated a peptide from bovine milk and were found to have bactericidal and antifungal activity (Mishra *et al.*, 2013). The details of peptides with their cell penetrating ability to kill the *Mycobacterium* bacilli have been reviewed well (Padhi *et al.*, 2014). A series of 24 peptides has been isolated from human milk and these peptides were found to have antioxidant, antibacterial and growth stimulating activity (Mandal *et al.*, 2014). Another approach to keep check on bacterial growth is developing quorum sensing inhibitors, the details of which have been reviewed by Bhardwaj and colleagues (Bhardwaj *et al.*, 2013). Gene silencing is an intriguing field in which by targeting the essential genes for bacterial metabolism/antibiotic resistance, bactericidal effect can be achieved. In this regard, Choudhary *et al.*, 2015 used CRISPRi approach to knock down multiple target genes of Mycobacterium (Choudhary *et al.*, 2015).

CONCLUSION

Due to the increased antimicrobial resistance, the biggest challenge for mankind is discovery of novel approaches to combat AMR. In this context, synthetic biology and nanoantibiotics have played a pivotal role to combat this hurdle. However, the challenging task is the effective delivery of these synthetic molecules inside the bacteria. Both Gram positive and Gram-negative bacteria have different cell wall structure, which is the biggest hurdle for synthetic molecules and nano-antibiotics to come across. Therefore, to overcome this hurdle these synthetic molecules can be conjugated with the carrier particles which will help to cross the cell wall barrier of both Gram positive and Gram-negative bacteria.

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