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Review

Silencing antibiotic resistance with antisense oligonucleotides

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Abstract: Antisense technologies consist of the utilization of oligonucleotides or oligonucleotide analogs to interfere with undesirable biological processes, commonly through inhibition of expression of selected genes. This field holds a lot of promise for the treatment of a very diverse group of diseases including viral and bacterial infections, genetic disorders, and cancer. To date, drugs approved for utilization in clinics or in clinical trials target diseases other than bacterial infections. Although several groups and companies are working on different strategies, the application of antisense technologies to prokaryotes still lags with respect to those that target other human diseases. In those cases where the focus is on bacterial pathogens, a subset of the research is dedicated to produce antisense compounds that silence or reduce expression of antibiotic resistance genes. Therefore, these compounds will be adjuvants administered with the antibiotic to which they reduce resistance levels. A varied group of oligonucleotide analogs like phosphorothioate or phosphorodiamidate morpholino residues, as well as peptide nucleic acids, locked nucleic acids and bridge nucleic acids, the latter two in gapmer configuration, have been utilized to reduce resistance levels. The major mechanisms of inhibition include eliciting cleavage of the target mRNA by the host's RNase H or RNase P, and steric hindrance. The different approaches targeted resistance to β-lactams including carbapenems, aminoglycosides, chloramphenicol, macrolides, and fluoroquinolones.

Keywords: antisense, antibiotic resistance, RNase P, RNase H, nucleotide analogs

1. Introduction

It has now been over 40 years since the pioneering research published by Zamenick and Stephenson, where the authors showed that addition of a 13-mer oligonucleotide complementary to the repeated sequences located at the ends of the genome inhibited the replication of the Rous sarcoma virus in infected chicken embryo fibroblast cells [1]. These experiments were the origin of the antisense technologies, i.e., the utilization of oligonucleotides or oligonucleotide analogs to interfere with undesirable biological

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processes, commonly (but not exclusively), through inhibition of expression of selected genes. Since these pioneering experiments took place, a wide variety of strategies were designed to achieve therapeutic effects for the treatment of diverse diseases like viral and bacterial infections, genetic disorders, or cancer. Challenging problems such as toxicity, non-specific effects, or inability to penetrate the target were evident during the early years of antisense research, and progress was slow. However, steady progress was made, and in 1998 FDA approved fomivirsen (Vitravene™), an antisense drug indicated for cytomegalovirus retinitis treatment [2]. Following this breakthrough, a large number of antisense drugs were approved by the Food and Drug Administration (FDA) or European Medicines Agency (EMA) (Table 1, reproduced from [3]) and numerous others are at different stages of development [3-7].

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Table 1. Antisense medicines approved by FDA or EMA

Drug	Chemistry	Route	Target	Indication	Year (FDA approval)	Year (EMA approval)	Designation	Company
Fomivirsen (Vitravene™)	PS	Intravitreal	CMV mRNA	CMV infection	1998	-	-	Ionis
Mipomersen (Kynamro™)	2′-O-MOE, PS, 5-methyl cytosine	Subcutaneous	apo-B-100 mRNA	HoFH	2013	-	Orphan	Genzyme
Nusinersen (Spinraza®)	2′-O-MOE, PS, 5-methyl cytosine	Intrathecal	pre-mRNA	SMA	2016	2017	Orphan	Biogen
Patisiran (Onpattro®)	siRNA	Intravenous	TTR mRNA	hATTR	2018	2018	Orphan	Alnylam
Inotersen (Tegsedi®)	2′-O-MOE, PS	Subcutaneous	TTR mRNA	hATTR	2018	2018	Orphan	Ionis
Eteplirsen (Exondys 51®)	PMO	Intravenous	exon 51	DMD	2016	2018	Orphan	Sarepta
Golodirsen (Vyondys 53™)	PMO	Intravenous	DMD pre-mRNA	DMD	2019		Orphan	Sarepta
Givosiran (Givlaari®)	siRNA	Subcutaneous	ALS1 mRNA	AHP	2019	2020	Orphan	Alnylam
Milasen	2′-O-MOE, PS, 5-methyl cytosine	Intrathecal	Intron 6 spice acceptor cryptic site	CLN7	*2018		Orphan	Boston Children's Hospital

*is a personalized medicine developed for a single patient.
Reproduced from Dhuri et al [3].

A quick revision of the drugs approved for utilization in clinics and those in clinical trials shows that most compounds being evaluated do not target bacterial pathogens,

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and although several groups and companies are working on different strategies [6,8-11], the application of antisense technologies to prokaryotes still lags with respect to those that target other human diseases.

There is a wide variety of antisense mechanisms of inhibition exploited to design therapies for bacterial infections [6-8,10,12-15]. In most of these attempts, the antisense compounds target essential genes such that reducing their expression leads to bacterial death or weakening [6-8,10,12,14,15]. A less common strategy consists of designing antisense compounds that inhibit expression of resistance genes. Therefore, in combination with the appropriate antibiotic, they would act as adjuvants facilitating a successful treatment.

The most common mechanisms of antisense oligonucleotide inhibition of gene expression are through degradation of the target mRNA by eliciting endogenous RNases like RNase H or RNase P, or interference with transcription or translation by steric hindrance (Figure 1) [3,7,8,16]. Thorough descriptions of different antisense mechanisms of inhibition of gene expression can be found in several excellent recent reviews [3,7-9,17-19]. In this article we briefly summarize representative examples of the utilization of antisense strategies to counter antibiotic resistance. Although we subdivided the article in sections based on antisense mechanisms, they should be taken with caution because in numerous cases they have not been proved.

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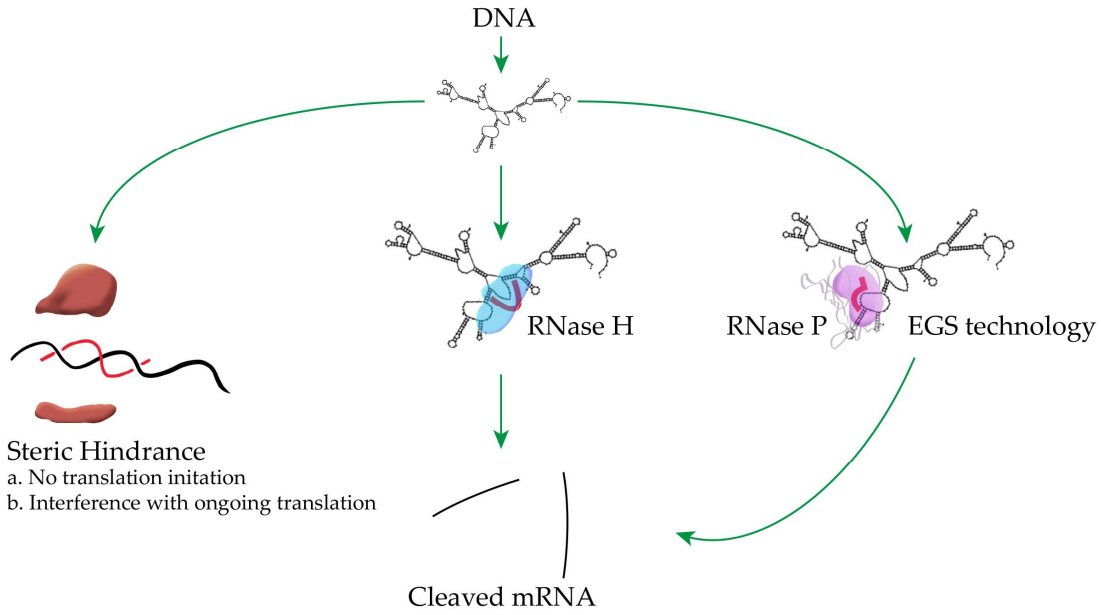

Figure 1. Representative mechanisms of inhibition of gene expression by antisense oligonucleotides. Left: the antisense compound binds the mRNA at the translation initiation region preventing initiation of translation or at any location of the mRNA, in which case it will interfere with the progress of the ribosome. The figure shows only the binding at the translation initiation region. Center: an antisense oligodeoxynucleotide or analog compatible with activation of RNase H upon binding to RNA binds a single-stranded region of the target mRNA, which becomes substrate for cleavage by RNase H. Right: EGS technology, an antisense oligodeoxynucleotide or analog compatible with activation of RNase P binds a single-stranded region of the target mRNA and forms the structure required to become a substrate of RNase P.

2. EGS technology

EGS technology (External Guide Sequences technology) was intensely researched to design antisense molecules to override resistance. The general mechanism of antisense action in this case is of the type of target cleavage. EGS technology takes advantage of the bacterial host's RNAse P. This is a ribozyme present in all organisms that plays many functions, but it was first identified by its catalytic activity that mediates

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digestion of the immature tRNAs 5′-end termini leading to formation of the mature tRNAs [20,21]. The RNA moiety of the enzyme is the catalytic subunit, and, in bacteria, there is usually one cofactor protein [21-23]. The holoenzyme recognizes structural properties in specific regions of the immature tRNA to catalyze the endonucleolytic cleavage [24-26]. A breakthrough for the utilization of RNase P as the basis for an antisense strategy occurred after the finding that most of the immature tRNA molecule could be removed without losing the ability to serve as substrate as long as the key regions and structure are preserved. Furthermore, bimolecular complexes with the appropriate structure, independently of the nucleotides sequence, were also substrates for RNase P [24-26]. These characteristics led to the idea that an oligonucleotide complementary to a target mRNA, provided that after interacting the resulting structure was appropriate, could recruit RNase P to cleave the latter reducing the level of expression of the cognate gene [24,27-29]. Oligonucleotides with these properties are known as external guide sequences (EGSs). The earliest work that showed the possibility that EGSs could be used as adjuvants to obliterate resistance followed the proof-of-concept experiments in which selected EGSs reduced expression of β-galactosidase and alkaline phosphatase in *Escherichia coli* [30]. The sequences of EGSs complementary to *bla*$_{TEM}$ (β-lactamase) and *cat* (chloramphenicol acetyl transferase) were cloned as part of a DNA fragment consisting of a T7 promoter, the EGS sequence, a core hammerhead sequence, and a T7 terminator (T7p-EGS-HH-T7t). *E. coli*

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BL21(DE3) cells carrying these recombinant clones express an RNA fragment containing the EGS and the core hammerhead ribozyme, which directs a self-endonucleolytic cleavage that releases the EGS into the cytosol [31]. Addition of isopropyl β-d-1-thiogalactopyranoside to the cultures of cell harboring *bla*TEM or *cat* resulted in a significant reduction of resistance to ampicillin or chloramphenicol [31]. Few years later Gao et al confirmed the results of conversion to susceptibility to chloramphenicol in four *E. coli* strains harboring *cat* [32]. This early approach was also applied to reduce expression of the *aac(6′)-Ib* gene, which codes for an acetyltransferase that inactivates amikacin and other clinically important aminoglycosides [33,34]. The *aac(6′)-Ib* mRNA was mapped using RNase H mapping and the sites selected as single stranded were used as targets for selection of several EGSs [35]. The most active EGSs were cloned to a DNA arrangement as that described above and upon introduction into *E. coli* cells harboring *aac(6′)-Ib* the levels of resistance were reduced dramatically [35]. In parallel with these advances, it was necessary to explore the activity of antisense oligomers constructed with non-hydrolyzable analogs to prevent degradation. An update on nucleotide analogues has recently been published [36]. The EGSs active in reducing expression of *aac(6′)-Ib* were tested when they were synthesized using nuclease resistant analogs [37]. Oligomers containing locked nucleic acids (LNAs) (Figure 2) and deoxyribonucleotides in gapmer configuration acted as effective EGSs. Exogenous administration of the locked nucleic acids/deoxyribonucleotides EGSs to the

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permeable mutant *E. coli* AS19 strain harboring *aac(6′)-Ib* resulted in a reduction of levels of amikacin resistance [37]. Comparison of gapmers with different configurations, i.e., with different numbers of analogs at the ends and deoxyribonucleotides in the central region, showed large variations in activity. Furthermore, it was somewhat surprising that similar gapmers in which the LNAs were replaced by the related 2′-4′-bridged nucleic acid-NC residues (Figure 2) did not elicit RNase P-mediated degradation of the target mRNA [38]. Other nuclease resistant analogs were incapable of eliciting digestion of the target mRNA [37].

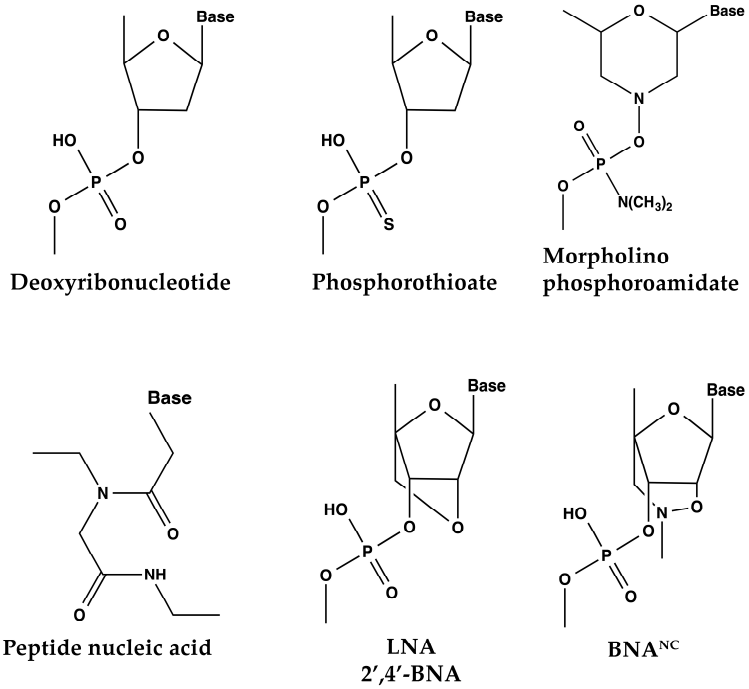

Figure 2. Chemical structures of nucleotide analogs mentioned in the text.

A forward step towards advancing beyond the proof-of-concept stage was the conjugation of EGSs to cell permeabilizer peptides that facilitate uptake of the oligomers

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by the target bacterial cells. Cell permeabilizer peptides had been used for delivery of oligonucleotides to diverse cells [39-41], but their conjugation to locked nucleic acids had been challenging due to the charged nature of these analogs. The active LNA/deoxyribonucleotides gapmers were conjugated to the $(RXR)_4XB$ (R, arginine; X, 6 aminohexanoic acid; B, beta-alanine) peptide, a tried cell permeabilizer peptide for delivery of oligonucleotides to bacterial cells [42,43]. When these compounds were added to cultures of *A. baumannii* strains that harbor *aac(6')-Ib* they produced a reduction in the levels of resistance to amikacin [38,44]. Further studies using a gapmer composed of locked nucleic acids flanking deoxyribonucleotides conjugated to different CPPs showed that these compounds that behaved as active EGSs eliciting cleavage of the *aac(6')-Ib* mRNA in vitro (Figure 3). However, they had modest activities in cellula when tested in different bacteria (Table 2). Six CPPs were evaluated, $(RXR)_4XB$ (CPP1), GRKKRRQRRRPPQ (CPP2), $(RFF)_3RXB$ (CPP3), GWTLNSAGYLLGKINLKALAALAKKIL (CPP4), AAVALLPAVLLALLP (CPP5), and TRQARRNRRRRWRERQR (CPP6). The conjugates were tested at 1 μM concentration on *K. pneumoniae* JHCK1 [45], *A. baumannii* A155 [46], and *E. coli* TOP10 (pNW1) [47] cultures containing 25 μg/ml amikacin. In the case of *K. pneumoniae* JHCK1 only the conjugate to CPP4 produced significant inhibition of growth (28%). *A. baumannii* A155 was inhibited by addition of CPP1 and CPP6 (34.2% and 34.6%, respectively), and *E. coli*

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TOP10 (pNW1) was inhibited by addition of CPP1, CPP2, CPP5, and CPP6 (40.1%, 21.6%, 42.1%, and 37.6%, respectively).

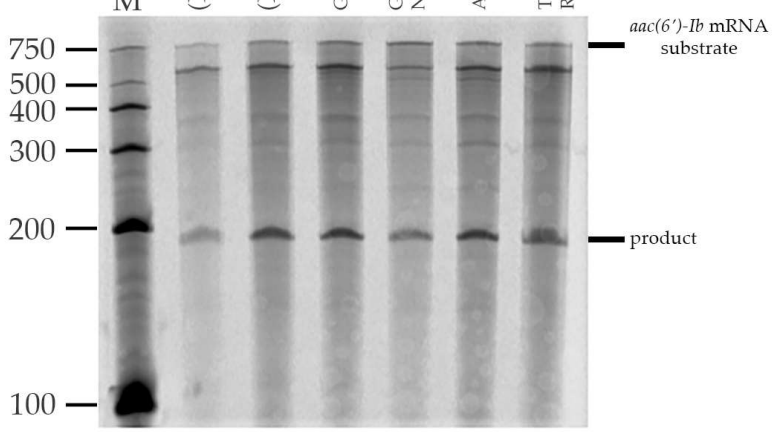

Figure 3. *In vitro* RNase P cleavage of *aac(6')-Ib* mRNA. The reactions were carried out preincubating 5'-end-labeled *aac(6')-Ib* mRNA and the CPP conjugated EGSs at 25°C for 30 min in a volume of 3 µl. The mixture was then added to 7 µl of a solution containing RNase P holoenzyme (2.5 pmol of M1 RNA and 70 pmol of C5 protein), 20 mM HEPES-KOH (pH 8.0), 400 mM ammonium acetate, 10 mM magnesium acetate, and 5% glycerol that had been preincubated at 37°C for 15 min. The mix was incubated at 37°C for 60 minutes and the reaction was stopped by adding of 1 volume of phenol-chloroform followed by ethanol precipitation. The pellet was dissolved in 10 µl DEPC-treated water, mixed with 10 µl of 2 x gel loading buffer, and analyzed by 5% denaturing TBE-PAGE along with labeled RNA Century Marker (M). The running position of the substrate and labeled product are indicated to the right. The extra band is a secondary product that has been observed before and remains to be identified. The sequence of the EGS that was conjugated to the different CPPs was CGATAATGAGATCGACCA, the underlined nucleotides are locked nucleic acids, the central residues are deoxyribonucleotides. Synthesis of the compounds was done at BioSynthesis,

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Inhibition of the *cat* gene was also observed using EGSs conjugated to a cell permeabilizer peptide, in which the oligonucleotide moiety was constructed with phosphorodiamidate morpholino residues (Figure 2) [48]. Two EGSs conjugated to cell permeabilizer peptides, with sequences targeting different locations of the *cat* mRNA were tested in experiments where *E. coli* cells in rich medium containing chloramphenicol were mixed with the compounds. After four hours, the surviving cells were determined, and the results showed potent activity by each of the antisense compounds and a higher efficiency when both compounds were used together [48]. An EGS targeting the *bla*$_{TEM}$ conjugated to a CPP was also effective in inhibiting resistance to ampicillin in *E. coli* cells after mixing the compound was mixed awith the cells suspended in culture medium [49].

2. RNase H

Degradation of an mRNA target by RNase H is an important mechanism utilized to silence genes through antisense oligomers. RNase H is an enzyme present in all living organisms that is characterized by its endonucleolytic cleavage of RNA when it is in duplex with DNA [50]. Despite this section being called "RNase H" it must be clarified that most examples described in it have the potential to act through activation of this enzyme, but confirmation is lacking. Oligodeoxynucleotides or analogs compatible with the enzymatic activity are used to direct cleavage of the target mRNA. White al. [51]

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utilized antisense phosphorothioate oligodeoxynucleotides (Figure 2), which are known to induce RNase H degradation of RNA in duplex [52], to reduce the resistance to norfloxacin mediated by the expression of the multiple antibiotic resistance (*mar*) locus in *E. coli*. The *mar* locus includes two divergent transcriptional units, *marC* and *marRAB*, that are transcribed from a central regulatory region occupied by the *marO*, a regulatory locus [53]. An antisense phosphorothioate oligonucleotide that reduced expression of the transcriptional activator MarA was associated with the increase in susceptibility to norfloxacin. However, this work required the introduction of the antisense compounds by chemical transformation or electroporation and the mechanism of inhibition was not confirmed to be through activation of RNase H. Thus, steric hindrance remains a possibility [51]. Similar successes and limitations in the understanding of the mechanism of action were observed in work carried out to inhibit expression of the *aac(6')-Ib* gene using oligodeoxynucleotides and internalizing them into the bacterial cells through electroporation [47].

Inhibition of multiple drug resistance was achieved by antisense inhibition of expression of the *Pseudomonas aeruginosa* MexAB-OprM efflux pump [54]. Accumulation of the components of this pump are associated with resistance to numerous antibiotics such as β-lactams, macrolides, quinolones, tetracycline, and chloramphenicol [55,56]. MexAB-OprM belongs to the resistance–nodulation–cell division (RND) superfamily, it is one of many efflux pumps that can be harbored by *P. aeruginosa* [57]. MexAB-OprM

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includes MexB, an inner membrane transporter, OprM, an outer membrane protein, and MexA, which connects MexB with OprM. The structure of this pump and a model mechanisms of drug efflux were recently proposed [56]. A phosphorothioate oligodeoxynucleotide antisense to the *oprM* mRNA was encapsulated in anionic liposomes as a delivery for internalization inside the bacterial cells [54]. Several clinical *P. aeruginosa* strains were tested and addition of the antisense resulted in a reduction of resistance levels to piperacillin, ciprofloxacin, levofloxacin, cefoperazone, imipenem, and amikacin in all cases. Unfortunately, the mechanism of inhibition has not yet been confirmed to be through cleavage of the target mRNA by RNase H. It is noteworthy that this work describes a novel anion liposome composition for encapsulation and delivery of oligonucleotides inside the bacterial cells. Liposomes were used scarcely in the past with these purposes, but new advances, including conjugation of the oligonucleotide molecule to the lipid, may increase their utilization in the future [58-60]. In particular, an oligonucleotide conjugated to a lipid moiety has recently proved effective in reducing the resistance level to ceftriaxone in *E. coli* cells harboring $bla_{\text{CTX-M-15}}$ [60].

3. Steric hindrance

Besides EGS technology, inhibition of resistance to ampicillin mediated by bla_{TEM} was also explored using other antisense techniques. One of the earliest attempts at inhibition

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of expression of *bla*TEM utilized oligodeoxynucleotides covalently linked to a 9-aminoacridine derivative also proved capable of inhibiting the expression of *bla*TEM. The stability of the complexes between the complementary sequence of the oligonucleotide and the target was increased due to intercalation of the 9-aminoacridine derivative [61]. In this case inhibition of gene expression occurs by interference with transcription initiation when the antisense compound binds to the transcribed strand in the open complex formed by *E. coli* RNA polymerase with the promoter [61]. Another approach consisted of a photoactivatable oligonucleotide containing psoralen monoadducts. *E. coli* harboring *bla*TEM became more susceptible to ampicillin after exposure to a photoactivated antisense 9-mer oligonucleotide bound to a psoralen 4',5'-monoadduct [62]. Inhibition of expression of *bla*TEM was also achieved using 15-mer peptide nucleic acid (PNA) oligomers (Figure 2) [63]. PNAs are oligodeoxynucleotide analogs in which the deoxyribose phosphodiester of DNA was replaced by a pseudo-peptide backbone. As a consequence, unlike DNA and RNA, which are negatively charged, PNA is a neutral compound [64]. PNA binds DNA and RNA with very high affinity, can be conjugated to CPPs, and has shown low toxicity [65,66]. PNA can interfere with gene expression preventing transcription by binding to a DNA target or translation by binding to the target mRNA [67]. The pioneering work by Good and Nielsen where an antisense PNA targeting the *bla*TEM gene reversed resistance to ampicillin utilized the PNA oligomers without any helper for internalization inside the cells. As a

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consequence, these authors carried out the assays using the permeable mutant *E. coli* AS19 [63].

Resistance to oxacillin was also reversed by the utilization of PNA antisense oligonucleotides in the methicillin-resistant *Staphylococcus aureus* and methicillin-resistant *Staphylococcus pseudintermedius* [68]. The antisense compounds were conjugated to three different permeabilizer peptides, KFFKFFKFFK, MINWKLRLKNK, and YGRKKRRQRRR but no significant differences were found in reversion of resistance. Interestingly, measurement of the mRNA levels showed a low but significant reduction in cells treated with the antisense. This could have been a somewhat unexpected observation because PNA analogs are known no to induce RNase H cleavage. However, the phenotypic effect, i.e., reduction in resistance levels, was large suggesting that the main mechanism of inhibition is mainly interference protein translation [68-70].

Carbapenems are among the antibiotics of last resort for treatment of multidrug infections. Therefore, all efforts must be made to limit development and dissemination of resistance. The most common mechanism of resistance to carbapenems in the clinics is the presence of carbapenemases [71,72]. Other means by which bacteria resist these antibiotics are the action of efflux pumps, modification in expression or synthesis of new penicillin binding proteins, and reduction or inactivation of expression of porins. In an effort to inhibit expression of the New Delhi metallo-β-lactamase (NDM-1), Sully

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et al [73] designed antisense phosphorodiamidate morpholino oligomers conjugated to the permeabilizer peptide $(RXR)_4XB$. The antisense sequence was complementary to the translation initiation region of *bla*$_{NDM-1}$. The phosphorodiamidate morpholino are nucleotide analogs known to interfere with gene expression by steric hindrance. These analogs do not elicit RNase H degradation of the target mRNA. Addition of this compound to cultures of *E. coli* strains harboring *bla*$_{NDM-1}$ produced inhibition of expression of the gene and susceptibility to meropenem [73].

PNA antisense compounds were also used to interfere with translation of the CmeA protein. This is the periplasmic component of the *Campylobacter jejuni* CmeABC efflux pump, which belongs to the RND superfamily. The PNA antisense was conjugated to a cell permeabilizer peptide, KFFKFFKFFK, and the inhibition of CmeA expression was confirmed by immunoblot analysis. This antisense compound, when added at 2 μM, was associated with a reduction of 8- and 4-fold in the *C. jejuni* MICs of ciprofloxacin and erythromycin, respectively [74].

4. Final Remarks

Bacterial infections are a leading cause of death, compromised health, and disability worldwide [75]. Outbreaks of bacterial infection continue to occur, and the etiologic agents are commonly resistant to multiple antibiotics [76,77]. Furthermore, the increase in the number of antibiotic resistant bacterial pathogens not only affects our ability to

treat infectious diseases and impose an economic burden on the health system, but also complicates medical procedures that depend on prevention of infection such as surgery, treatment of cancer and other chronic diseases, organ transplants, dental work, and care for premature infants [78-81]. Despite the dire situation, researchers responded to the call for action by diverse health organizations. New strategies are being developed to continue to produce new therapies to keep pace with the accelerated rise of antibiotic resistance [82]. One of the options explored to generate new therapies that can overcome multidrug resistance is utilizing diverse antisense oligonucleotides. This technologies' versatility permits us to envision the generation of antisense drugs that either have antibiotic activity or that disable resistance to certain antimicrobials and act as adjuvants. The high diversity of the chemistries of the nucleotide analogs used to synthesize the complementary oligonucleotide in conjunction with the different modes of action and the variety of internalization methods offer a field full of possibilities for these compounds to join the armamentarium to fight multidrug resistance.

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