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Antisense Antibacterials: From Proof-Of-Concept to Therapeutic Perspectives

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1. Introduction

Recent years have witnessed several gram-negative bacteria (GNB) species and a few gram-positive bacteria (especially the *Staphylococcus aureus*) posing overwhelming threats to the healthcare-associated infections as a series of frightening superbugs (Engel, 2010; Peleg & Hooper, 2010). It is primarily due to the fact that incidence of multidrug resistance (MDR) or pan-drug resistance (PDR) bacteria have been escalating in a manner of global dimension, frequent prevalence and alarming magnitude. The predominate resistance issues are those related to GNB species, including *Enterobacteriaceae* (Deshpande & et al, 2010), *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Theses circulating isolates have created big problems for treatment of nosocomial infection because they carry highly transmissible elements encoding multiple resistance genes, e.g. extended-spectrum beta-lactamases (ESBLs) that inactivates different classes of first-line antibiotics (Bush, 2010; Engel, 2010), metallo-beta lactamase that hydrolyzes penicillins, cephalosporins and carbapenems, efflux pumps that decrease bacterial transporting ability to almost all antibiotics and natural antimicrobial products (Pages & et al, 2010), and promoters that ensure the transcription of these genes.

Traditional antimicrobial drugs target only a few cellular processes and are derived from a few distinct chemical classes. Despite that genetic screens to identify new drug targets and classic searching for new chemical leads with diverse structures (Moellering, Jr., 2011), the constant need of new broad-spectrum antimicrobial agents has rarely been met (Cattoir & Daurel, 2010). Meanwhile, antibacterial strategies that favor in offering timely therapeutic countermeasures are urgently required for possible outbreaks of new super bug infections. One promising strategy is antisense antibacterial, which can contribute to both aspects of the problem. It is generally described as RNA silencing in bacteria using synthetic nucleic acid oligomer mimetics to specifically inhibit essential gene expression and achieve gene-specific antibacterial effects. First proposed in 1991, RNA targeting in bacterial has been made more flexible by 20 years of technology refinement, circumventing major problems of target selection/validation and efficient delivery (Bai & et al, 2010). Antisense antibacterials have been developed by constructing sequence-designed synthetic RNA silencers using new

chemical classes, e.g. nucleic acid mimics peptide nucleic acid (PNA) and phosphorodiamidate morpholino (PMO), that conjugated with cell penetrating peptide (CPP) in multiple functional ways (Geller, 2005; Hatamoto & et al, 2010). And their potent bactericidal effects have been displayed in a variety of pathogens by targeting several growth essential genes *in vitro* and *in vivo* (Bai & et al, 2010). Advantage of RNA silencing is unique in having the potential to selectively kill target pathogens with species and even strain specificity. Of particular interest are possibilities to tailor the antibacterial spectrum, aid the use of conventional antibiotics by potentiating their activity, and reverse resistance. Further, antisense antibacteirals may present an unusual opportunity for developing broad-spectrum therapeutics against upgrading infections caused by multi-drug or pan-drug resistant pathogenic species, where many successful compounds have failed. This review will describe the characteristics of the antisense antibacteiral strategy (including antisense mechanism, basic chemistry involved in nucleic acid analogs, their anti-infection applications *in vitro* and *in vivo*, and preliminary studies on pharmacokinetics and toxicity), and focus on the major determinants of target accessibility and CPP-mediated delivery in the general context of antisense antibacterials. We will also highlight the promising targets and delivery strategies that favor the possible development of broad-spectrum nucleic acid-based therapeutic molecules and provide overall information of their potentials as functional component of systemic broad-spectrum antisense antibacterial agents.

2. Antisense antibacterials: 20 years of technology refinement

Antisense antibacterial strategy is revolutionary for silencing essential genes at mRNA level by antisense oligodeoxyribonucleotides (AS-ODNs) for realization of bacterial cell death or restoration of susceptibility. Significant technology advances in aspects of microbial genomics (Monaghan & Barrett, 2006), structural modification of oligonucleotides and efficient delivery systems have fundamentally promoted the transformation of antisense antibacterials from concept to future therapeutic "antisense antibiotics".

2.1 Mechanism of action and chemistry

AS-ODNs are designed to bind the target mRNA to prevent translation or bind DNA to prevent gene transcription respectively. And once bound to the target, AS-ODNs modulate its function through a variety of post binding events. Meanwhile, AS-ODNs based on the three generations of modified structures, have overcome the biological disadvantages of RNA and DNA, and shown great potency in gene expression inhibition with apparently high degree of fidelity and exquisite specificity both *in vitro* and *in vivo*.

2.1.1 Antisense mechanism

Most of the reported work on antisense drugs has been accomplished in eukaryotic systems and their mechanisms have been well explored (Houseley & Tollervey, 2009). AS-ODNs bind to the target RNA by well-characterized Watson-Crick base pairing mechanism. The effect of gene silencing or "knock down" that happens after the binding can be broadly categorized as cleavage-dependent or occupancy-only mechanism (Figure 1). Cleavage-dependent mechanism includes degradation of RNA:mRNA duplexes by double-strand RNA (dsRNA)-specific RNAases (a natural means of transcriptional regulation),

degradation of stable DNA:RNA heterodimers through the activity of RNAse H, and degradation via the action of RNase P (only if external guide sequences are coupled to the oligonucleotide). Occupancy-only mechanism, also known as translation arrest, features as that AS-ODN:RNA heteroduplexes inhibit translation by steric blocking the ribosomal maturation and polypeptide elongation process. Antisense antibacterials function on the base of above antisense mechanisms, whereas the specific mechanism is dependent on the structural chemistry and design of the modified oligonucleotides (Bennett & Swayze, 2010).

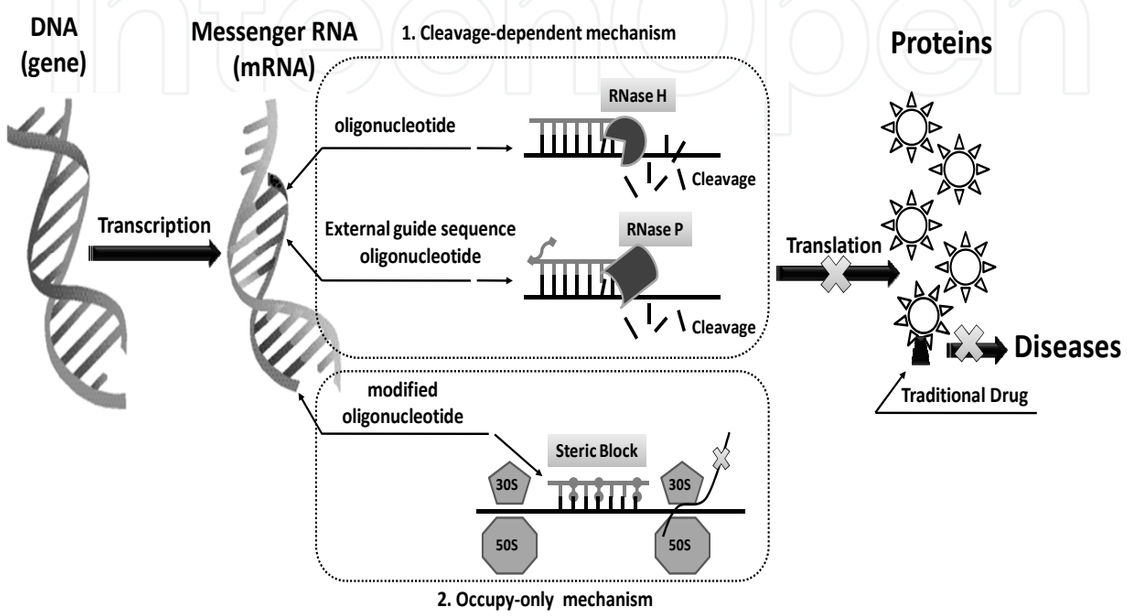

Fig. 1. Different antisense mechanisms: antisense oligodeoxyribonucleotides (AS-ODNs) are known to interact and block the function of the mRNA. Different antisense mechanisms shown include the nondegradative mechanisms (e.g., inhibition of translation) and mechanisms that promote degradation of the RNA (e.g., RNase H mediated cleavage and external guided sequence mediated RNase P cleavage).

2.1.2 Nucleic acid chemistry: structure and binding

Unmodified DNA/RNA is susceptible to nucleases attack and degradation. Furthermore, their poor pharmacokinetics properties (including weak binding to plasma proteins, rapid filter by kidney and excretion into urine, and et al) make them undesirable and unacceptable therapeutic agents for systemic administration. In order to increase their nuclease stability and intrinsic affinity to complementary target RNAs, many efforts have been made to the structural modification of DNA or RNA (Kurreck, 2003). Key modifications concentrate on the backbone, phosphodiester bond, and sugar ring, giving births to three generations of nucleic acid anologs. Representative oligonucleotide derivatives include phosphorothioate oligodeoxyribonucleotides (PS-ODNs), 2′-O-methyloligoribonucleotides (2′-OMes), 2′-O-methoxyethyl oligonucleotides (2′-MOE), locked nucleic acids (LNAs), phosphorodiamidate morpholino oligonucleotides (PMOs), thiophosphoroamidate oligonucleotides and peptide nucleic acids (PNAs) (Figure 2).

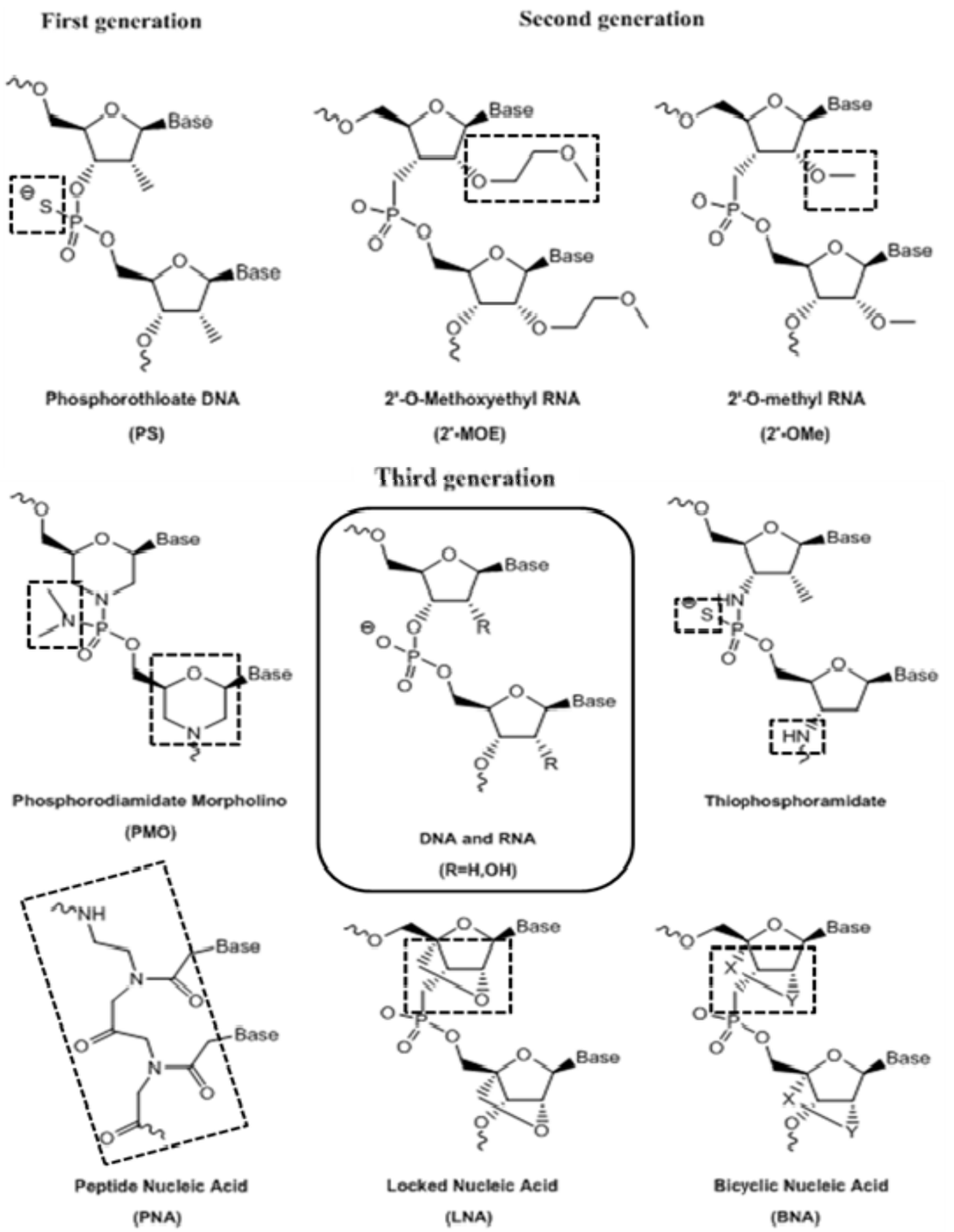

Fig. 2. Representative modified antisense oligodeoxyribonuleotides. Replacement in structure compared with DNA/RNA is highlighted by dashed rectangle. First generation of modified form shown includes only PS-ODNs. Second generation mainly includes 2'-OMes and 2'-MOE. Third generation includes a series of DNA/RNA analogs, e.g., LNA, PNA, BNA, PMO and thiophosphoroamidate oligonucleotides.

Like DNA or RNA, PS-ODNs, LNA and thiophosphoroamidate oligonucleotides are negatively charged. Other modified oligonucleotides like PNA and PMO are electric neutral, showing little repulsion during hybridization to target DNA or RNA. 2'-OMes, 2'-MOE, LNAs, PMOs and PNAs all bind to RNA more tightly than unmodified oligonucleotides or PS-ODNs. Therefore, they can be used at shorter lengths and lower concentrations for exerting specific and potent RNA silencing effect. Meanwhile, PNA and PMO have provided substantially better specificity to the same target sequence than DNA, phosphorothioate DNA, and 2'-O-methyl RNA, either at low concentration of 50 nM or at high concentration of 3.5 μM (Deere & et al, 2005). Furthermore, it is acknowledged that only PS-ODNs activate RNase H to degrade mRNA in eukaryotic cells, whereas the other modified oligonucleotides show direct translation arrest effect. The same results have also been confirmed for gene manipulation by antisense strategy in bacteria.

2.2 Antisense antibacterial strategy

The hypothesis that any gene can be antisense inhibited is quite tantalizing. Therefore, antisense oligomers have been studied as bacterial growth inhibitors for developing new types of antibiotics. In 1991, Rahman et al firstly observed the inhibited protein synthesis and colony formation in normal *E. coli* by using PEG 1000 attached methylcarbamate DNAs targeting the start codon sequence of prokaryotic 16S rRNA (Rahman & et al, 1991). Ever since, the potential of gene specific modified AS-ODNs as biomedically useful antibiotics has been well accepted and further explored. The present-day antisense antibacterial strategy has overcome major obstacles that hampered this innovative approach developing into clinically applicable therapeutics: (i) target validation and (ii) efficient delivery system. Meanwhile, modified AS-ODNs, e.g. PNA and PMO, have accepted thorough preclinical and clinical evaluation on the aspects pharmaceutical properties as promising antisense antibiotics.

2.2.1 Inherent advantages

Compared to human genome, bacterial genome is much less complicated and homogenous. Unlike eukaryotic cells, the double-strand DNA (dsDNA) of bacterial cells locates in nucleoid. And in this low electron density zone, there is no nucleic membrane to strictly separate biochemical reactions into different time and space level. DNA replication, RNA transcription and protein synthesis in bacteria are processed in cytoplasm, which allows exogenous AS-ODNs to interfere with genes and/or RNAs more readily. Meanwhile, RNA interfering (RNAi) mechanism found in eukaryotic cells has not been reported so far in bacteria. Bacteria themselves use antisense as a natural mechanism to inhibit specific gene expression, therefore, antisense technology suits better as an effective gene modulating tool in bacteria.

2.2.2 Target identification and validation

A key objective for discovery of new antisene antimicrobial agents is to determine the genes essential for survival of the pathogenic organisms. In paticular, the main critireas for measuring the quality of a candidate gene as a good target include vitality of the gene and its targeting accessability for antisense oligomers. Compared to gene knockout technique,

antisense approach itself has been proved to be an effective tool for target validation in bacteria, with controllable sensitivity, larger breadth of applicability and more realistically mimic effect of a therapeutic inhibitor (Wright, 2009).

2.2.2.1 Target site selection and design of AS-ODNs

Theoretically, antisense antibacterials as modulators of bacterial essential genes can be used to alter biological state or behavior in potentially any pathogenic species. Their growth inhibitory activity relies on sequence-specific inhibition of gene expression, which offers the potential for high specificity in immediate bacteriocidal or bacteriostatic therapeutic consequences (Rasmussen & et al, 2007). However, the fundamental requirements for potent antisense activity include sufficient concentration of antisense agent at the most sensitive targeting site, an ability to hybridize to the target mRNA sequence, the capacity of the ODN/mRNA duplex to interfere with gene expression, and sufficient biological stability of the antisense agent.

Antisense inhibitors must bind accessible regions of the target mRNA so that stable ODN:RNA(DNA) heteroduplexes or triplex (as for PNA) can be formed to elicit antisense effect. In order to obtain the antisense sequence with best potency and efficacy, researchers normally follow a comparatively fixed procedure in design (Shao & et al, 2006). Generally, possible targeting regions are those nucleotide sequences free of any double strand (e.g. hairpin) in secondary structure, which are determined by RNA secondary structure softwares (Ding & Lawrence, 2003) within full sequences. Notably, most previous studies have demonstrated that the start codon region of the mRNA (see Table 1&2) is the most effective region for RNase H independent antisense inhibition, because this region initiates the translation and includes the Shine-Dalgarno (SD) sequence (Dryselius & et al, 2003). However, a few studies also have confirmed that specific AS-ODNs complementary to sites beyond the start codon region receive equal positive results in *in vitro* efficacy test (e.g. antisense targeting of *rpoD* by PNA in methicillin resistant *Staphloccous aureus*, Bai & et al, 2012a). Then, bioinformatic algorithms are used to calculate the DNA: ODN binding parameters (e.g. minimal free energy and melting temperature, et al) with setted lengths for AS-ODN. According to the combind data, rational analysis was performed to confirm 3-10 different targeting sites/sequences with highest binding affinity and stability for sequence-specific antisense inactivation of target genes. AS-ODNs complementary to the chosen target sites are synthesized. The length of AS-ODN is predominantly determined by their chemical properties. In principle, nucleic acid analogs with stronger affinity to target RNA require shorter lengths. Customarily, policy that 14-30 monomers for PS-ODNs and 8-16 monomers for PNA, LNA and PMO have been adopted for potent inhibition and achieving better hits. In order to improve the uptake of AS-ODNs by the cell, AS-ODNs with attached membrane permeabilizing peptides have been developed (elaborated in 2.2.3).

Presently, *in vitro* modified minimal inhibitory concentration (MIC) and minimal bactericidal inhibitory concentration (MBC) tests of peptide-ODN conjugates are well-acknowledged methods to preliminarily confirm the antibacterial effect of AS-ODNs. Targeted gene vitality and accessability are determined by comparing MIC and MBC values. AS-ODN that shows the lowest MIC value indicates the most sensitive targeting site for antisense inhibition, whereas the MBC value suggests if the antisense antibacteial effect is bacteriocidal. Target specificity is generally evlauated at the same time by testing

the antibacterial activity of designed control AS-ODNs (e.g. AS-ODN with mismacted or scrambled nucleotide sequences) and pepides. Further, RT-PCR and western blotting can be used to observe the reduction of mRNA and protein product of the particularly targeted gene. Collectively, with regard to target selection, researcher are dedicated to identify an ideal essential gene that is with small nucleotide content and upmost stringency but effective region of coding sequences for potent antisense ihibition (Goh & et al, 2009). Meanwhile, the antisene property of AS-ODN itself should also be taken into consideration.

2.2.2.2 Validated targets in bacteria

2.2.2.2.1 Targeting essential genes

Essential genes that regulate or control bacterial growth, proliferation, virulence and synthesis of important living-dependent substances are candidates attracting the majority of research enthusiasms. Validated essential genes among different bacterial species include *fbpA/ fbpB/fbpC* (Harth & et al, 2002, 2007) and *glnA1* (Harth & et al, 2000) in *Mycobacterium tuberculosis*, *gyrA/ompA* in *Klebsiella pneumonia* (Kurupati & et al, 2007), *inhA* in *Mycobacterium smegmatis* (Kulyte & et al, 2005), *oxyR/ahpC* in *Mycobacterium avium* (Shimizu & et al, 2003), *NPT/EhErd2* in *Entamoeba histolytica* (Stock & et al, 2000, 2001), *gtfB* in *Streptococcus mutans* (Guo & et al, 2006), *fmhB/ gyrA/hmrB* (Nekhotiaeva & et al, 2004a) and *fabI* (Ji & et al, 2004) in *Staphylococcus aureus*, *23S rRNA* (Xue-Wen & et al, 2007), 16S rRNA plus *lacZ/bla* (Good & Nielsen, 1998), and RNAse P (Gruegelsiepe & et al, 2006) in *Escherichia coli,* and *acpP* in *Burkholderia cepacia* (Greenberg & et al, 2010), *Escherichia coli* (Deere & et al, 2005b; Geller & et al, 2003a, 2003b, 2005; Mellbye & et al, 2009, 2010; Tan & et al, 2005; Tilley & et al, 2007) as well as *Salmonella enterica serovar Typhimurium* (Mitev & et al, 2009; Tilley & et al, 2006).

Target[a]		AS-ODN[b]	Test organism[c]	Efficacy identified[d]	Delivery Method[e]	References
23S rRNA	P.T. center	PNA	*E. coli* AS19 (permeable membrane)	*in vitro*/ IC_{50} > 20 μM (duplex)	–	Good & Nielsen, 1998
				in vitro/ IC_{50} = 5 μM (triplex)		
	domain II		*E. coli* Dh5α	*in vitro*/MIC = 10 μM	CPP=$(KFF)_3K$	Xue-Wen et al,2007
	α-sarcin loop		*E. coli* AS19	*in vitro*/IC_{50} = 2 μM	–	Good & Nielsen, 1998
			E. coli K12 (wild-type)	*in vitro*/MIC* = 5 μM	–	Good et al, 2001
				in vitro/MIC* = 0.7 μM	CPP=$(KFF)_3K$	
				in vitro/MIC = 3 μM		
16S r*RNA*	preceding the start codon region	MDNA	*E. coli* lacking outer cell wall	*in vitro* / inhibit protein synthesis and colony formation	–	Rahman MA et al, 1991
			normal *E. coli*		PEG attached	
	mRNA binding site	PNA	*E. coli* AS19	*in vitro* / IC_{50} > 20 μM	–	Good & Nielsen, 1998
			E. coli K12	*in vitro*/MIC=10 μM	CPP=$(KFF)_3K$	Hatamoto et al, 2010

Table 1. Continued

Target[a]		AS-ODN[b]	Test organism[c]	Efficacy identified[d]	Delivery Method[e]	References
acpP (start codon region)	SD site -24 to -13 nt	PNA	*E. coli* K12	*in vitro* / MIC= 1.5 µM	CPP=$(KFF)_3K$	Dryselius et al, 2003
	-9 to 3 nt					
	-5 to 5 nt		*E. coli*	*in vitro* / MIC=0.2* or 1 µM		Good et al, 2001
			E. coli SM101 (defective membrane)	*in vivo*/100% rescued mice at a single i.p. dose of > 5 nmol		Tan et al, 2005
			E. coli K12	*in vivo*/60% rescued mice at a single i.v. dose of 100 nmol		
			E. coli K12	*in vivo*/ MIC=0.8µM, post antibiotic effect duration 11.7h		Nikravesh et al , 2007
	6 to16 nt	PMO	*E. coli* AS19	*in vitro* luciferase system/ most potent inhibition	–	Deere et al , 2005
			E. coli SM105 (normal membrane)	*in vitro* / EC = 20µM *in vivo*/sustanied post-infectin reduction in cfu at single i.p. dose of 76 nmol	–	Geller et al, 2005
			E. coli W3110 (ATCC27325)	*in vitro* / IC_{50} CPP_1 = 9.5µM IC_{50} CPP_2 = 10.8µM IC_{50} CPP_3 = 3.6µM	$CPP_1=(KFF)_3KXB$ $CPP_2=RTRTRFLR RTXB$ $CPP_3=(RFF)_3XB$ $CPP_4=(RXX)_3B$	Tilley et al, 2006
			EPEC (E. coli E2348.69)	Ex vivo cocultured Caco-2 culture / IC_{50} CPP_2= 5.3µM IC_{50} CPP_3= 0.5µM		
			S. enterica (ATCC29629)	Ex vivo cocultured Caco-2 culture / IC_{50} CPP_2= IC_{50} CPP_3= 0.5µM		
			E. coli W3110 (ATCC27325)	*in vivo* /100% 48h-after survival in mice at i.p. injection of 2 treatments with 30µg or 300µg conjugate	$CPP_3=(RFF)_3XB$	Tilley et al, 2007
			E. coli W3110 (ATCC27325)	*in vitro* /MIC from 0.625 to > 80µM	19 synthetic CPPs	Mellbye, 2009
				in vivo /100% 48h-after survival in mice at i.p. injection of 2 treatments with 30µg CPP_2-PMO	$CPP_1=(RX)_6B$ $CPP_2=(RXR)_4XB$ $CPP_3=(RFR)_4XB$	
		PMO	*S. enterica* LT1	*in vivo* /MIC = 1.25µM	CPP=$(RXR)_4XB$	Mitev et al, 2009
		3+Pip-PMO		*in vivo* /MIC = 0.625µM intracellular infected macrophage/99% decrease in intracellular bacteria at 3µM		
		Pip-PMO	*E. coli* W3110 (ATCC27325)	*in vivo* /MIC3+ = 0.3 µM *in vivo* /100% 48h-after survival in mice at i.p. injection of 2 treatments with 5 or 15 mg/Kg CPP-PMO		Mellbye et al, 2010
		Gux-PMO		*in vivo* /MIC5+ = 0.6 µM		
	4 to 14 nt	PMO	14 *B. cepacia strains* (5 clinical isolates+9 from ATCC)	*in vitro*/lowest MIC = 2.5 µM	CPP= $(RFF)_4XB$	Greenberg et al, 2010
	-5 to 6 nt			*in vitro*/lowest MIC = 2.5 µM *in vivo* /55% 30d-after survival in mice at i.p. injection of single dose of 200 µg CPP-PMO		

Table 1. Continued

Target[a]	AS-ODN[b]	Test organism[c]	Efficacy identified[d]	Delivery Method[e]	References
P15 loop of RNase P	LNA	*E. coli*	*in vitro* / bingding affinity value only	—	Gruegelsiepe et al, 2006
	PNA		*in vitro*/MIC = 5 μM	CPP=$(KFF)_3K$	
floA	PNA	*E. coli* AS19	*in vitro*/MIC = 2.5 μM	CPP=$(KFF)_3K$	Hatamoto et al, 2010
floP			*in vitro*/MIC = 6.5 μM		
glnA1	PS-ODN	*M. tuberculosis*	*in vitro* / combination of 3 PS-ODNs for transcript mRNA, EC = 10 μM	ethambutol or polymyxin B nonapeptide	Harth et al, 2000
fbpA,fbpB, fbpC			*in vitro* / combination of 4 PS-ODNs for each transcript mRNA, EC = 10 μM	—	Harth et al, 2002
	5'-, 3'-HP PS-ODN		*in vitro* / combination of 3 PS-ODNs for each transcript mRNA, EC = 10 μM	—	Harth et al, 2007
inhA	PNA	*M. smegmatis*	*in vitro* / MIC < 6.5 μM	CPP=$(KFF)_3K$	Kulyté A et al, 2005
adk	PNA	*S. aureus* RN4220	*in vitro* / MIC = 15 μM	CPP=$(KFF)_3K$	Hatamoto et al, 2010
fmhB	PNA	*S. aureus* RN4220	*in vitro* / MIC = 10 μM	CPP=$(KFF)_3K$	Nekhotiaeva et al. 2004
gyrA			*in vitro* / MIC = 20 μM		
hmrB			*in vitro* / MIC = 12 μM		
fabI	UM	*S. aureus*	*in vitro* / MIC = 15 μM	—	Ji et al, 2004
	PNA	*E. coli* K12	*in vitro*/ MIC = 3 μM	CPP=$(KFF)_3K$	Hatamoto et al, 2010
fabD	PNA	*E. coli* K12	*in vitro* / MIC = 2.5 μM	CPP=$(KFF)_3K$	
gyrA	PNA	*K. pneumoniae*	*in vitro* / MIC = 20 μM	CPP=$(KFF)_3K$	Kurupati et al, 2007
ompA			*in vitro* / MIC = 40 μM		
gtfB	PS-ODN	*S. mutans*	*in vitro* / reduce biomass	—	Guo et al, 2006
oxyR/ahpC	UM	*M. avium complex*	*in vitro* / ineffective	—	Shimizu T, 2003
NPT/ EhErd2	UM	*E. histolytica*	*in vitro* / inhibited cell growth	—	Stock et al, 2001, 2000

[a] The essential genes that were targeted encode the following proteins: *acpP*, acyl carrier protein; *fabI*, enoyl-acyl carrier protein reductase; *fabD*, malonyl coenzyme A acyl carrier protein transacylase; *folP*, dihydropteroate synthase; *fmhB*, protein involved in the attachment of the first glycine to the pentaglycine interpeptide; *gyrA*, DNA gyrase subunit A; *hmrB*, ortholog of the *E. coli* acpP gene; *adk*, adenylate kinase; inhA, enoyl-acyl carrier protein reductase; *ompA*, outer membrane protein A; *gtfB*, synthesis of water-insoluble glucans; *inhA*, enoyl-(acyl carrier protein) reductase; RNase P, P15 loop of RNase P; *gyrA*, DNA gyrase subunit A; *oxyR*, oxidative stress regulatory protein; *ahpC*, alkyl hydroperoxide reductase subunit C; *glnA1*, glutamine synthetase; *fbpA,fbpB, fbpC*, 30/32-kDa mycolyl transferase protein complex; *NPT*, Neomycin phosphorotransferase; *EhErd2*, marker of the Golgi system; *LacZ/bla*, beta-galactosidase/beta-lactamase; P.T. indicates peptidyl transferase; SD, Shine-Dalgarno; nt, nucleotide.

[b] UM, unmodfied; MDNA, ethylcarbamate DNA; Pip-PMO and n+Gux-PMO, cations (piperazine or N-(6-guanidinohexanoyl)piperazine) attached to the phosphorodiamidate linkages;

[c] EPEC, enteropathogenic *E. coli*.

[d] Minimal inhibitory concentrations (MIC) were tested in Mueller–Hinton broth, except in case marked with an asterisk(*), in which the MIC values were determined in LB broth at 10% of the normal strength; IC_{50} values are the concentrations that caused a 50% inhibition of cell growth relative to control cultures that lacked AS-ODN; EC values are the concentrations that caused significant decrease in cell grwoth relative to control cultures that lacked AS-ODN; PAE, post antibiotic effect; i.p. indicates intraperitoneal and i.v. indicates intravenous.

[e] CPP, cell penetrating peptide; PEG, Polyethylene glycol; "—", no delivery method used. For synthetic peptides, X is 6-aminohexanoic acid and B is beta-alanine.

Table 1. Examples of AS-ODNs targeting essential genes in antibacterial therapy.

2.2.2.2.2 Targeting resistance mechanism

Developing resistance inhibitors in traditional antibiotic industry is a sound, well-validated strategy for tackling resistance problems, because they postpone the "expire date" of on-market antibiotcs and expand their application. The economic and clinical value of this rationale is well recognized and demonstrated by offering new combinations to clincal practice. Thus, a few studies focused on interrupting the expression of genes involved in resistant mechanism by antisense approach, aiming to restore bacterial susceptibility to key antibiotics in clinical practice.

Target	Encoding Proteins	AS-ODN[a]	Test organism	Efficacy identified	Delivery Method[b]	References
oprM	outer membrane efflux protein	PS-ODN	*P. aeruginosa*	*in vitro*	liposome	Wang et al, 2010
mecA	penicillin-binding protein 2 prime	PS-ODN	*S. aureus*	*in vitro* & *in vivo*	liposome	Meng J et al, 2006, 2009
cmeA	CmeABC multidrug efflux transporter	PNA	*C. jejun*	*in vitro*	CPP	Jeon B et al, 2009
aac(6′)-Ib	aminoglycoside 6'-N-acetyltransferase type Ib, mediate amikacin resistance	UM	*E. coli*	EGS mediated RNaseP leavage / *in vitro*	—	Soler Bistué AJ et al, 2007
				in vitro	EP	Sarno R et al, 2003
metS/ murB	methionyl-tRNA synthetase /UDP-N-acetylenolpyruvoylglucosamine reductase	UM	*B. anthracis*	*in vitro*	—	Kedar GC et al, 2007
act	chloromycetin acetyl transferase	UM	*E. coli*	EGS mediated RNaseP leavage / *in vitro*	—	Gao MY et al, 2005
				in vitro	—	Chen H et al, 1997
vanA	class A (VanA) glycopeptide-resistant related protein	UM	*E. faecalis*	*in vitro*	—	Torres VC et al, 2001
marOR AB	multiple antibiotic resistance operon	PS-ODN	*E. coli*	*in vitro*	HS/EP	White DG et al, 1997
LacZ/bla	β-galactosidaze/ β-l actamase	PNA	*E. coli* AS19 (permeable membrane)	*in vitro*	—	Good & Nielsen, 1997

[a] UM, unmodfied.
[b] CPP, cell penetrating peptide; EP, electroporation; HS, heat shock; EGS, external guide sequences; "—", no delivery method used.

Table 2. Examples of AS-ODNs targeting resistance mechanism in antibacterial therapy.

First proof-of-principle evidence was given by White et al in 1997 for successful increasing the bactericidal activity of norfloxacin by antisense inhibiting the *marRAB* operon in *Escherichia coli* (White & et al, 1997). Hitherto, limited but successful trials have extended to dominating resistant genes and bacterial species with highest incidence of resistance (Table 2), e.g., antisense targeting *aac(6')-Ib* (Sarno & et al, 2003; Soler Bistue & et al, 2007), *act* (Chen & et al, 1997; Gao & et al, 2005) in *Escherichia coli*, *vanA* in *Enterococcus faecalis* (Torres & et al, 2001), *cmeA* in *Campylobacter jejun* (Jeon & Zhang, 2009), *mecA* in *Staphylococcus aureus* (Meng & et al, 2006; Meng & et al, 2009), *metS/murB* in *Bacillus anthracis* (Kedar & et al, 2007) and *oprM* in *Pseudomonas aeruginossa* (Wang & et al, 2010).

2.2.3 Efficient delivery systems

Virtually, any microbial gene could be targeted and highly organism-specific drugs could be envisioned in the development of antisense therapeutic agents. The obvious obstacle is stringent bacterial cell membrane for penetration or poor cellular uptake of AS-ODNs. An unmodified 10-mer oligonucleotide is 2-3 kDa, and various chemical modifications outlined above add further to this size. In short, AS-ODNs are likely to be considerably larger than vancomycin, therefore require efficient delivery systems. A variety of strategies exist to deliver compounds to bacterial cells in the laboratory, including electroporation, permeablilizing solvents, cationic lipid formulations (e.g. liposome), and pore-forming peptides (see Table 1&2). Although what exactly will work for AS-ODNs remains to be determined, the cell penetrating peptide (CPP) mediated delivery of AS-ODNs (especially peptide-PNA and peptide-PMO conjugates) outperformed other delivery systems in way of reaching future therapeutic applications.

2.2.3.1 Limitation in cellular uptake

Many barriers exist for the efficient transfer of genes/oligonucleotide anologs into cells, including the extracellular matrix, the endosomal/lysosomal environment, the endosomal membrane, and the nuclear envelope. Many delivery systems have been proved to serve suitably for antisense approach in eukaryotic cells regardless of their types (non-viral or viral) *vs* cell types. However, like most oligonucleotide-based strategies, the major limitation of antisense antibacterials is their poor cellular uptake due to low permeability of bacterial cell membrane to modified nucleic acids (Nekhotiaeva & et al, 2004). In particular, lipopolysaccharide outer membrane of gram-negative bacteria is a major barrier to molecule uptake (Good & et al, 2000). Meanwhile, decreased membrane permeability has been permanently observed for originally antibiotic-susceptible bacterial species after frequent exposure to multiple antibiotics present in commensal environments. Alternatively, membrane-associated energy-driven efflux in bacteria is of extremely broad substrate specificity, preventing intracellular drugs to release sustained effects.

2.2.3.2 Cell penetrating peptide (CPP) mediated delivery

Further, antisense antibacteirals may require development of delivery conditions for each bacterial species. Several strategies have been developed to improve delivery of oligonucleotides both in cultured cells and *in vivo*. So far, there is no universally applicable method for their delivery into different gram-positive and gram-negative specie, as they all present several limitations. Peptide-based strategies, representing a new and innovative concept to bypass the problem of bioavailability, have been demonstrated to improve the cellular uptake of nucleic acids both in cultured cells and *in vivo*.

Cell-penetrating peptides (CPPs) are short peptides of less than 30 amino acids that are able to penetrate cell membranes and translocate different cargoes into cells. The only common feature of these peptides appears to be that they are amphipathic and net positively charged. CPPs constitute very promising tools and have been successfully applied *in vivo* (Crombez & et al, 2008; Morris & et al, 2008). Two CPP strategies have been described to date; the first one requires chemical linkage between the drug and the carrier peptide for cellular drug internalization, and the second is based on the formation of stable complexes with drugs, depending on their chemical nature. Recently, the second strategy has the tendency to replace the first strategy for convenient delivery of DNA or AS-ODNs into eukaryotic cells,

especially considering the synthesis and cost issues. However, CPP-conjugated method is now extensively applied for antisense antimicrobial ODNs.

In order to improve cellular uptake of PNA into bacterial cells, Good L and Nielsen PE, who first established CPP conjugating to the end of PNA in chemical synthesis, have realized efficient delivery of PNA through bacterial out membrane by observing its potent bacteriocidal antisense effects at micromolar ratio (Eriksson & et al, 2002). Further evidence demonstrates that introducing spacers or linkers between PNA and CPP in the direct covalent conjugate may increase its antisense efficacy and antibacterial potency. It also has been demonstrated that the release property of the chemical bond between PNA and CPP (e.g. the more stable amide bond or the less stable disulfide bond) has no influence on the antisense efficacy of PNAs. Later chemistry inventions make possible the conjugation CPP to other oligonucleotide analogs (e.g., PS-ODNs, LNAs, PMOs) for imparting them into bacterial cells and specific intracellular targets.

Regarding CPP itself, the mechanism of cell wall penetration is controversial and still under exploration. Nonetheless, the improvement in bacterial uptake of AS-ODNs with the aid of CPP has been well-recognized, making it an indispensable delivery system before no advanced system is developed (Lebleu & et al, 2008). The "carrier peptide" KFFKFFKFFK, originally reported for efficient penetrating ability through brain blood barrier, is the first also the most extensively applied peptide sequence verified by Nielsen PE and Good L et al for successful delivery of PNA into *Escherichia coli*. Previous studies suggested that the repeated amphipathic motif with cationic residues followed by hydrophobic regions is an important structure for carrier efficiency of CPP. The more efficient peptide sequences RFFRFFRFFRXB and RXRRXRRXRRXRXB (X is 6-aminohexanoic acid and B is β-alanine), have been recently reported for improved delivery efficacy of CPP-attached PMOs. Many efficient and simple penetrating efficacy test models for CPPs have been established in eukaryotic cells, whereas standard qualification method has been developed for only a few bacterial species. Evidence shows that the efficacy of CPPs differs according to bacterial species, and the underlying mechanisms are still unclear. Inadequate information has been accumulated from sporadic studies, e.g., $(KFF)_3K$ facilitates delivery of PNAs and PMOs into *Escherichia coli*, *Salmonella enterica serovar Typhimurium, Klebsiella pneumoniae* (however much less potent) and *Staphylococcus aureus*. But it is not working for *Pseudomonas aeruginosa* membrane even at higher concentrations of conjugated PNAs. $(RFF)_3RXB$ and $(RXR)_4XB$ enables more efficient transporting of PNAs and PMOs across the membrane of *Escherichia coli*, *Salmonella enterica serovar Typhimurium, Klebsiella pneumonia*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Notably, the membrane of two gram-negative species *Acinetobacter baumanni* and *Shigella flexneri* show highest sensitivity to $(RXR)_4XB$ mediated PNA-CPP conjugates (unpublished results, and Bai & et al, 2012b). Following studies demonstrated that gram positive bacteria *Bacillus subtilis* and *Corynebacterium efficiens* exhibit increased susceptibility to CPP-PNAs, but in contrast, the gram-negative bacterium Ralstonia eutropha was not affected by addition of CPP-PNA.

2.2.3.3 Nano-material based delivery system

Nanomedicine is a growing field with a great potential for introducing new generation of targeted and personalized drugs. Membranes of eukaryotic cells and organelles, as well as the cell wall and membrane of pathogenic microorganisms, constitute a serious barrier for

the access of hydrophilic drugs to their target molecules inside the cell structures. To overcome gene delivery problems of macro-molecule like AS-ODNs, various nano-mateiral based delivery techniques, including linear polymers, dendrimers and carbon nano tubes, have been developed and further studied as delivery tool for gene therapy purposes. And some of them are definitely worthy of extended trials in the antisense antibacterial aspect, with regard to delivery efficiency and other important pharmaceutical properties (e.g. 3D size, large scale synthesis and chemical modification, solubility, bioavailability, biocompatibility, toxicity, and pharmacokinetics, et al).

2.2.3.3.1 Dendrimers as vectors

Dendrimers are new class of synthetic polymeric materials characterized by well-defined and extensively branched 3D structure (Figure 3A). They have narrow polydispersity, nanometer size range, which can allow easier passage across biological barriers (e.g. small enough to undergo extravasations through vascular endothelial tissues). Notably, affordable commercialization of different types of size-controllable and surface-functionalized dendrimers is now available, providing a high degree of versatility. Besides, the unique properties of funtinonalized dendrimers, such as uniform size, high degree of branching, water solubility, multivalency, well-defined molecular weight and available internal cavities, have made them promising biological and drug-delivery systems for traditional drug (i.e. classical organic types) and gene therapy (e.g. DNA, small interfering RNAs, AS-ODNs, IgG antibodies, etc.) applications (Ravina & et al, 2010). And their excellent pharmacological properties, such as cytotoxicity, bacteriocidal and virucidal effect, biodistribution and biopermeability, may be modulated to fit specific medicinal purposes.

The wide range of applications reported for the use of dendrimers as delivery vectors for versatile cargos in the patent and literature demonstrates the general applicability of these molecules as carrier candidate for antisense antibacterials. There have been reports on the use of poly(amidomide) based dendrimers (e.g. PAMAM) for the development of antibacterial drugs mainly by destroying the cell walls of pathogenic organisms with their cationic surface groups, leading to direct cell death. However, our research suggests that lower generation of polyamide dendrimers, such as G1.0 and G2.0 PAMAM, showed no cell-wall impairment to many bacterial species (unpublished data, Xue et al, 2010), becoming a highly active vector for bacteria-specific oligonucleotides. Thus, dendrimers are highly suitable tools in antisense drug discovery to a wide variety of bacterial receptors.

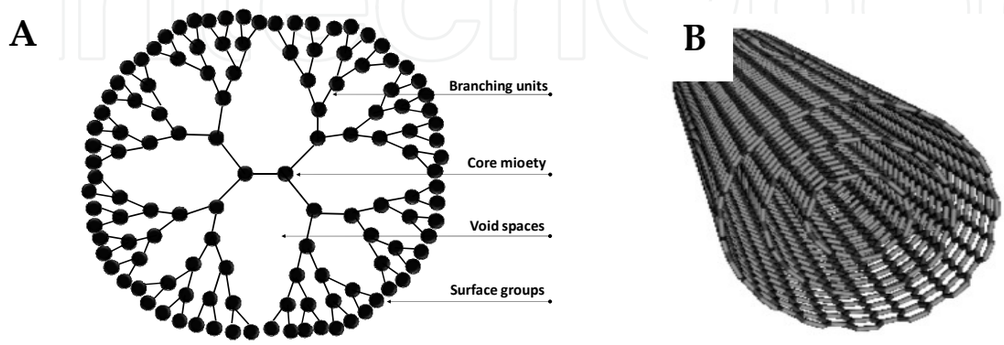

Fig. 3. (A) Schematic representation of generation 4 (G4.0) dendrimer. (B) Molecular structures of a multi-walled carbon nanotube (MWNT).

2.2.3.3.2 Funtionalized multi-walled carbon nanotubes (MWCNTs) as vectors

Synthetic inorganic gene nanocarriers have received limited attention in the transformation of bacterial cells. Amongst new generation of nano-vectors are carbon nanotubes (CNTs), a new form of carbon made-up of graphene layers rolled-up into a cylindrical from which can be produced as single or multi-walled (Figure 3B). The physico-chemical features of CNTs, such as needle-like shape, nanorange size, surface modification flexibility, and electronic properties, make them unique materials in nanoscience and nanotechnology. Multi-walled carbon nanotubes (MWCNTs) can be fabricated as biocompatible nanostructures (cylindrical bulky tubes), forming supramolecular complexes with proteins, polysaccharides and nucleic acids (Kateb & et al, 2010). These structures have been under investigation in the biomedical domain and in nanomedicine as viable and safe nanovectors for gene and drug delivery.

Research work based on nanobiotechnologies has allowed us to develop complex antigenic systems and novel delivery routes for peptides, nucleic acids and drugs covalently linked or simply adsorbed onto carbon nanotubes. In particular, Rojas-Chapana J and et al have presented a plasmid delivery system based on water dispersible multi-walled carbon nanotubes (CNTs) that can simultaneously target the bacterial surface and deliver the plasmids into *E. coli* cells via temporary nanochannels across the cell envelope (Rojas-Chapana & et al, 2005). It is the first experimental evidence that shows high potential of CNTs for nanoscale cell electroporation in bacteria. However, the study of metabolism, the toxicity and the mechanism of elimination of water-soluble carbon nanotubes in order to evaluate their impact on the health and validate the concept of CNT as new delivery system still arouse concerns in many critial ways. Recently initiated researches on hybriding the dendrimers with MWCNT has offered us new hopes (Qin & et al, 2011; Zhang & et al, 2011). Besides increased dispersility, solubility, biocompatiability and stability, (MWCNTs)-polyamidoamine (PAMAM) hybrid prepared by covalent linkage has possessed good plasmid DNA immobilization ability and efficiently delivered GFP gene into cultured HeLa cells. The surface modification of MWCNTs with PAMAM improved the transfection efficiency and simultaneously decreased cytotoxicity by about 38%, as compared with mixed acid-treated MWCNTs and pure PAMAM dendrimers. The MWCNT-PAMAM hybrid can be considered as a new carrier for the delivery of biomolecules into both mammalian and bacterial cells.

2.2.4 Other pharmaceutical properties

As far as the other properties in therapeutic application of antisense antibacterials are concerned, there should always be a systematic overview of cargo (i.e. oligonucleotides) and vector (especially the CPP, Heitz & et al, 2009). With regard to AS-ODNs, the electric neutral PNA and PMO calsses emerge and show desirable properties (especially their non-ionic backbones) as better therapeutic alternative to other antisense agents. And with regard to delivery strategies, alternative nonviral methods, such as electroporation and the use of liposomes, have been developed for delivery of antibacterial AS-ODNs. These methods have been proved to be effective *in vitro* and for research purposes, but showed limited potential for delivery *in vivo* due to toxicity, cell damage, and immunogenicity. They are also technically demanding in their application, lack tissue and cell specificity, and can deliver material to only a limited amount of cells. In view of these considerations, peptide conjugated AS-ODNs (i.e. peptide-PNAs and peptide-PMOs) offer a promising noninvasive

version of gene scilencers with potent antisense antibacterial activity, the pharmaceutical properties of which this part will mainly focus on.

2.2.4.1 PNAs, PMOs and their peptide conjugates

PNAs and PMOs (also known as morpholino) are novel classes of antisense agents that offer a better therapeutic alternative to other antisense antibacterial oligomers. They both possess a non-ionic backbone, but differed in ribose sugar replacement. For PMO, the backbone of DNA is replaced by a 6-membered morpholine moiety and the phosphorodiester intersubunit bonds with phosphorodiamidate linkages. PNA has a pseudopeptide backbone composed of (2-aminoethyl)glycine units, in which the geometry and the spacing of the bases is nearly identical to that found in a native DNA or RNA strand. The polyamide backbone of PNA has no phosphate groups, having an amino (NH_2) to carboxyl (CO_2H) orientation instead of 5' to 3' orientation as do phosphodiester backbones. Specifically, PNAs can bind to either single-stranded DNA or RNA, in which the resulting hybrid resembles the B-form of DNA, or double-stranded DNA, in which the PNA invades the DNA double stranded helix and hybridizes to the target sequence, thus displacing the second DNA strand into a 'D'loop. Although departing significantly from the sugar-phosphate backbone found in regular DNA, oligomers of both types independently (i.e. with or without delivery strategies) have been found to be remarkable steric-block ODNs for inhibiting translation and blocking mRNA activity, as demonstrated in embryos, cells and animals. Now PMOs have been taken to pre-clinical studies for treatment of cardiovascular diseases, viral diseases and genetic disorders, such as Duchenne muscular dystrophy (DMD).

Since the conjugation of CPP to negatively charged ODNs (e.g. PS-ODNs) did not result in a level of delivery into cells sufficient for biological activity, PNA- and PMO- CPP conjugates (covalently linked with or without spacers) confer on these compounds more desirable properties over the original ODN forms, as well as ribozyme and siRNA counterparts (Thompson & Patel, 2009), especially with respect to antisense antibacterials. Several CPPs have been developed for bacterial-specific transformation purposes (as mentioned in 2.2.3.2), and they can be coupled to PNA or PMO by flexible linker types (Venkatesan & Kim, 2006). No general rules have yet emerged as to optimal linkage types, since the factors affecting biological activity are often complex. Early popular labile linkers for PNA and CPP include AEEA (8-amino-3,5-dioxo-octanoic acid, a polyether spacer also known as an O-linker), and disulfide bond linkage, which were proposed to be cleavable within the reducing environment of the cell. Stable linkage such as glycine linkage, thioether linkage and thiol-maleimide linkage have also been reported for improved *in vivo* stability. The conjugation of CPPs and PMOs through a thioether (maleimide), disulfide or amide linker have previously been described. The nuclear antisense activities of the CPP–PMOs with the three linkage types were similar (Lebleu & et al, 2008). But, the amide linkage is advantageous with regard to synthetic procedures (e.g. greater yield and less steps) and *in vivo* stability.

2.2.4.2 Tissue distribution, pharmacokinetics and stability

The modified chemistry of PNAs and PMOs provides excellent resistance to nuclease and protease activity, which is the basis for the enhanced stability in plasma, tissues, cerebrospinal fluid and urine. Independently, the non-ionic character of the PNA/PMO portion of the conjugates avoids potential non-specific drug interactions with bacteiral

cellular components (except for the target RNA sequence) observed with PS-ODNs. In addition, the neutral character of PNAs and PMO chemistry not only guarantees a high safety profile but also sufficient tissue concentrations required for effective PNA or PMO oligonucleotide:RNA duplex formation, thus enhancing their affinity for the target RNA sequence and hence increasing efficacy. Rational design of conjugates (i.e. the optimal linker type and position of CPP) may eliminate CPP's stereospecific blockade that might significantly influence the antisense effect of PNA or PMO in target recognization, base matching and binging affinity. However, stability of CPPs coupled to antisense PNA or PMO may be a matter of concern. This is partially due to the fact that degradation of CPP in solution and plasma has been observed for systematic delivery of CPP-2'MOE in mouse model (Henke & et al, 2008). Another concern is the non-specificity of CPP mediated delivery of PNA or PMO when eukaryotic cells and prokaryotic cells exist in commensal environment. Although little lethal damage to cells would be done by conjugated CPP at equal molar ratio of PNA or PMO used at the highest concentrations *in vitro* and *ex vivo*, the consequences of its non-specific physical disruption to normal human cell membranes *in vivo* have not been thoroughly evaluated. Systematic study on CPP mediated antisense antibacterial therapy needs to be done if any possible candidate for clinical development is ever recommended (Zorko & Langel, 2005).

The application of unmodified PNAs as antisense therapeutics has been limited by their low solubility under physiological conditions, insufficient cellular uptake, and poor biodistribution due to rapid plasma clearance and excretion. The excelent stability of PNA-CPP conjugates has been confirmed for a 48h period at 37℃ in rat's plasma (Bai & et al, 2012b). However, there has been no report of *in vivo* tissue distribution and pharmacokinetics properties of CPP-PNA conjugates targeting bacterial genes. Nontheless, limited information from PNA-CPP conjugates targeting genes in eukaryotic cells can be refered. Jia et al have determined that PNA-CPP conjugates targeting bcl-2 mRNA showed specific tumor uptake, low uptake in blood and organs (e.g. liver and slpeen) except for kidney, as well as slower urinary clearance in Mec-1–bearing severe combined immunodeficiency (SCID) mice. Recently, Wancewicz et al have reported that conjugation of PNA (targeting murine phosphatase and tensin homolog) to short basic peptides (serve as solubility enhancers and delivery vehicles) allowed for rapidly distribution and accumulation of conjugates in liver, kidney and adipose tissue, while their rates of elimination via excretion were dramatically reduced compared to unmodified PNA.

Unlike CPP-PNA conjugates, the pharmaceutical properties of CPP-PMO conjugates have been evaluated in an extensive scope besides specific gene modulators (Amantana & et al, 2007). In general, the conjugation of CPP to PMO enhances the PMO pharmacokinetic profile, tissue uptake, and subsequent retention. Amantana et al have reported that conjugation of a PMO to the $(RXR)_4XB$ peptide increased the tissue uptake (in all organs except in brain, with greater increase being seen in liver, spleen and lungs) and retention time in these organs, while efflux of the conjugated PMO from tissues to the vascular space was slow. They have also confirmed that peptide conjugation also improved the kinetic behaviour of PMO as demonstrated by increased volume of distribution, estimated elimination half life, and area under the plasma concentration versus time curve. Youngblood et al have determined that the stabilities of CPP–PMO conjugates in cells and in human serum varied according to CPP sequences, amino acid compositions and/or linkers.

The stability of a $(RXR)_4XB$ peptide in the conjugate exhibited time- and tissue-dependent degradation, with biological stability ranked in the order of liver > heart > kidney > plasma. Meanwhile, the PMO portion of the conjugates was completely stable in cells, serum, plasma and tissues.

2.2.4.3 Toxicity

Large amount of data concerning toxicity of CPP-PNA conjugates have been collected from *ex vivo* studies (Alksne & Projan, 2000; Kurupati & et al, 2007), in which antisense peptide-PNAs cured cell cultures that were infected with bacteria in a dose-dependent manner without any noticeable toxicity to the human cells. *In vivo* inhibition of gene expression and growth have been observed for anti-*acpP* CPP-PNA conjugates in mouse intraperitoneal *E. coli* infection. However, none toxicity issues have yet been seriously addressed.

CPP-PMO conjugates targeting bacterial essential genes have been evaluated to confirm their bacteriocidal antisense effect in several animal bacteremia models, and have proven to be efficacious with an excellent safety profile (Amantana & et al, 2007) within doeses for 100% survival 48h after treatment. They have also found that survival was significantly reduced for mice treated with 2×300 mg and 2×1 mg of the 11-base AcpP peptide-PMO, indicating toxicity at these high doses. Generally, the toxicity of $(RXR)_4XB$ -PMOs is caused by $(RXR)_4XB$ while the PMO portions of the conjugates are essentially non-toxic. In particular, data from CPP-PMO conjugates targeting genes in eukaryotic cells have demonstrated that the degree of toxicity depends on the dose, dose frequency and route of administration. Collectively, mice tolerated $(RXR)_4XB$-PMOs well with repeated intraperitoneal (i.p.) or intravenous (i.v.) injection doses of ≤15 mg/kg at diverse time intervals, showing no changes in behaviour, weight and serum chemistry, and no histopathological abnormalities were detected in major organs. However, at higher doses and dosing frequency, animals experienced weight loss, despite maintaining their normal organ weights and appearances. Rats treated with a single 150 mg/kg dose appeared lethargic immediately after the injection and proceeded to lose weight, accompanied by affected kidney function. The LD_{50} of a $(RXR)_4XB$-PMO in rats was around 220–250 mg/kg.

3. Broad-spectrum antisense antibacterials

A range of functional genes in bacteria have been validated as potential targets by using unmodified PNAs or CPP conjugated ODNs. Collectively, consistent efforts on antisense targeting of a small bacterial gene *acpP* (encoding the essential fatty acid biosynthesis protein) have passed the proof-of-principle phase and have gathered plenty of positive results, especially from recent studies focusing on *in vivo* confirmation of anti-*rpoD* peptide-PMO's bactericidal effect in mice infected with several pathogenic bacteria (i.e., *Escherichia coli, Salmonella enterica serovar Typhimurium*, and *Burkholderia cepacia.* However, few reports describe promising gene targets that have potential for broad-spectrum antisense growth inhibition among different bacterial species. Indeed, the validated targets in different bacterial species show discouragingly low similarity in gene sequence and homology. Thus, identification of gene targets for broad-spectrum antisense inhibition would aid the development of new antimicrobial agents that could relieve the exacerbating therapeutic consequences caused by MDR/PDR infections.

3.1 Target accessibility

A challenging aspect of identifying essential genes in bacteria for broad-sepctrum antisense inhibition mainly involves efforts to locate the exact targeting site within a specific gene for realization of the most potent and specific antisense inhibitory effect of complementary AS-ODNs against different species (e.g. among gram-negatives, gram-positives, or both). Naturally, prequisities in target selection require searching for genes with high similarity and identidy amongst as many bacterial species as possible. As time comsuming as it is, an economical way of identifying genes that acturally fit this critea should focus on the validated targets for both traditional antibiotics and antisense antibacteirals, certainly because massive open data of their gene sequencing are available.

Antisense suppression of the above mentioned essential genes (e.g. 16S rRNA, *acpP*, *gyr*, and et al) in single bacterial species has showed potent growth inhibitory and cell death effect in a sequence-specific and dose-dependent manner. However, the issue of target accessability among different species still needs investigation and validation. (1) The ribosome has a complex structure involving rRNAs and ribosomal proteins, and therefore, inaccessibility of the target site could be one of the reasons for the ineffectiveness of the antiribosomal ODN. (2) Systematic researches *in vitro* and in animal models have demonstrated that one potential target *acpP* (encoding acyl carrier protein *AcpP*) opens limitless possibility for recommending the very first "antisense antibiotic" into market. Besides, *acpP* gene in pathogenic gram-negative species share highly homology in sequences, making itself an ideal candidate for antisense antibacterials with broad anti-gram-negative spectrum, although more candidate bacterial species are needed to confirm the accessibility of an already-validated 11-nucleotide targeting site in its start codon region of mRNA. (3) Meanwhile, newly discovered gene targets for new types of protein-targeting antibiotics, i.e. the bacterial cell division inhibitor (targeting bacterial cell division protein *FtsZ for* terminating bacterial proliferation (Boberek & et al, 2010)) and virulence inhibitor (targeting quorum sensing sensor protein *QseC* without affecting bacterial growth (Alksne & Projan, 2000)), also show promises and potential for developing specific or broad-spectrum antisense antibacterials based on their homology assessment. (4) our researches focus on validating the known target in broad-spectrum antibiotic development by antisene strategy, in which the DNA-dependent RNA polymerase (RNAP) is a candidate of great interest for it distinct advantanges (see 3.3).

3.2 Universally applicable delivery systems

Furthermore, the term "broad-spectrum" also qualifies the delivery systems for AS-ODNs. Specifically, rational design of peptide-ODN conjugates could optimize the effective AS-DONs in way of enhancing antibacterial potency and expanding antibacterial spectrum, in which CPP choice is of equal importance. The range of sensitivities observed for different bacterial species to CPPs largely determines their application potential. To our knowledge, the synthetic CPP $(RXR)_4XB$ has shown by far the most broad cell pernetrating range as an effective tool for intracellular AS-ODN delivery into many major gram-negative and gram-positive pathogens. And the transfection efficiency of the widely used CPPs $(RXR)_4XB$ and $(KFF)_3K$ appears to reflect with the features of bacterial cell walls of clinical isolates, where less potent effects were observed for $(KFF)_3K$ against species with stringent cell barrier (e.g., *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*). Collectively, our results suggested that

the peptide component of peptide-PNA conjugates may be developed for a wide range of indications to realize broad antisense antibacterial spectrum.

3.3 proof-of principle studies

Bacterial DNA-dependent RNA polymerase (RNAP) is a key enzyme in transcription regulation and gene expression. Its function requires coordination of a core enzyme (comprising five subunits α_2, β, β′ and ω) and an independent σ subunit that is reversibly recruited by core enzyme. The RNAP core enzyme is responsible for transcription elongation, and different σs are in charge of transcription initiations from promoters that express genes in diverse function. Deactivation of RNAP by any possible means leads to direct cell death, attracting much exploration for developing specific RNAP inhibitors, the most representative class of broad-spectrum antibiotics (e.g. the rifamycins) with fundamental clinical significance. The most developed σ^{70} family of σs, especially the primary σ^{70}, is essential for initiating transcription of multiple genes in exponentially growth cells , which to our knowledge has not previously been demonstrated for target validation. And most importantly, gene *rpoD* (encoding the primary σ^{70} of RNAP) is highly conserved in identity and homologous in sequence among different pathogenic gram-negative species. Such features are distinct advantages for developing broad-spectrum antisense antibacterial agents (Bai & et al, 2011).

Results from our lab (unpublished and Bai & et al, 2012b) gives the first proof-of-principle evidence for exploring and identifying bacterial RNAP σ^{70} as an antibacterial target by antisense strategy. We identified a conserved target sequence within the native *rpoD* mRNA start codon region, and a cell penetrating peptide $(RXR)_4XB$ conjugated 10-mer peptide nucleic acid was developed for potent sequence-selective bacteriocidal antisense effect against six pathogenic gram-negative species, including *Escherichia coli*, *Salmonella enterica*, *Klebsiella pneumoniae*, *Shigella flexneri*, *Citrobacter freundii*, *and Enterobacter cloacae.* It cured endothelial cell cultures from lethal infection with single or triple GNB without showing any apparent toxicity. It specifically interferes with *rpoD* mRNA, and inhibited the expression of σ^{70} in a concentration-dependent manner. Its *in vivo* antibacterial activity has also been confirmed by increased survival in bacterial infected mice.

4. Conclusion

New classes of antisense antibacterial agents (bactericidal agents or resistance inhibitors), represent an evolutionary inevitability in antibiotic industry. In the past 20 years, many essential genes have been studied as potential targets for developing bactericidal antisense agents or resistance inhibitors against clinically pathogenic bacteria. Nonetheless, much investment needs to be infused for converting this concept into real drugs. Identified targets with application potentiality or new targets under investigation should be further evaluated for posing the least risk for selection of resistant variants. Nucleic acid monomers with simple synthesis and cheap source of starting materials are more viable as antisense drugs. Versatile of delivery systems developed for eukaryotic cells, e.g., polymers, dendrimers, nanotubes, should be given considerations for AS-ODNs delivery in bacteria, if no more innovative systems than the non-invasive CPP-mediated delivery system is created. Furthermore, it is more practical and economical to develop antisense agent that targets

only multiple-resistant or pan-resistant bacteria, particularly when it allows co-administration of a narrow-spectrum antibiotic. Most importantly, "broad-spectrum" antisense antimicrobials should also be developed to meet future clinical requirements, in which target selection and validation address more attention.

We have already lagged behind our therapeutic initiatives to meet the challenges of increasing isolation of new antibiotic-resistant bacterial strains. A great many functional genes discovered in the past decade represent themselves as potential targets for developing antibacterial therapeutic agents with whole new mechanisms. Thus, innovative approaches must become a priority in antibitoitc discovery, in which antisense antibacterial strategy is absolutely a leap in our ability to effectively treat human pathogens of great concern (Woodford & Wareham, 2009). The theoretical advantage of antisense antibacterials is obvious and has been well-acknowledged by strong evidence in the long process of conquering major technological obstacles. It is our continous efforts that will make ultimate success in this glorious field.

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