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MINIREVIEW

Antisense peptide nucleic acids as a potential anti-infective agent

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Antibiotics have long been used for anti-infective control of bacterial infections, growth promotion in husbandry, and prophylactic protection against plant pathogens. However, their inappropriate use results in the emergence and spread of multiple drug resistance (MDR) especially among various bacterial populations, which limits further administration of conventional antibiotics. Therefore, the demand for novel anti-infective approaches against MDR diseases becomes increasing in recent years. The peptide nucleic acid (PNA)-based technology has been proposed as one of novel anti-infective and/or therapeutic strategies. By definition, PNA is an artificially synthesized nucleic acid mimic structurally similar to DNA or RNA in nature and linked one another via an unnatural pseudo-peptide backbone, rendering to its stability in diverse host conditions. It can bind DNA or RNA strands complimentarily with high affinity and sequence specificity, which induces the target-specific gene silencing by inhibiting transcription and/or translation. Based on these unique properties, PNA has been widely applied for molecular diagnosis as well as considered as a potential anti-infective agent. In this review, we discuss the general features of PNAs and their application to various bacterial pathogens as new anti-infective or antimicrobial agents.

***Keywords*:** peptide nucleic acid, anti-infective agent, antimicrobials, antibiotic resistance

Introduction

Public concerns toward the risk of infectious diseases have been continuously growing because the number of disease outbreaks in humans have increased from 1980 to 2010 (Smith *et al.*, 2014). Not surprisingly, it was also reported that approximately 25% of annual deaths are caused by infectious diseases in worldwide (Fauci *et al.*, 2005). Although the discovery of penicillin and other antibiotics has greatly contributed to the treatment of infectious diseases (Fleming, 1929; Aldridge, 1999), their inappropriate usage resulted in the emergence and rapid spread of antibiotic-resistant bacteria (Clatworthy *et al.*, 2007). Diseases by antibiotic-resistant bacteria cannot be treated using conventional antibiotics, which has been documented all around the world. Therefore, researchers continue to search for novel anti-bacterial or anti-infective strategies that can overcome therapeutic limitations of conventional antibiotic treatment. Among these strategies, development of a new class of antibiotics is the most feasible solution. For example, Kim *et al.* (2018) have recently discovered a new class of synthetic retinoid antibiotics that were highly effective on methicillin-resistant *Staphylococcus aureus* (MRSA) as well as bacterial persisters with highly resistance to antibiotics (Kim *et al.*, 2018). However, discovery or development of novel antibiotics become more difficult, more expensive, and more time consuming over time. To combat continuously evolving antibiotic-resistant bacteria, it is necessary to develop alternative therapeutic or anti-infective strategies that can replace conventional antibiotics.

Recently, bacteriophages have been considered as an antibiotic alternative for biocontrol of some pathogenic bacteria such as *Salmonella* spp. (Atterbury *et al.*, 2007; Hungaro *et al.*, 2013), *Campylobacter* spp. (Loc Carrillo *et al.*, 2005; Connerton *et al.*, 2011), and *Escherichia coli* O157:H7 (Rozema *et al.*, 2009; Carter *et al.*, 2012). However, they have relatively narrow-spectrum target specificity, compared to conventional antibiotics (Pirisi, 2000; Keen, 2012). Moreover, it is difficult to deliver them effectively to target tissues or organs during a host infection. Therefore, recent studies have focused on developing novel antibiotic alternatives such as non-specific immunomodulators (Lillehoj and Lee, 2012), anti-virulence agents (Totsika, 2016), and antibiotic adjuvants (Gill *et al.*, 2015). Peptide nucleic acid (PNA) has been proposed as one of such candidates.

PNA and its applications

As shown in Fig. 1, PNA is an artificially synthesized nucleic acid mimic that is structurally similar to DNA or RNA, with its backbone composed of repeating *N*-(2-aminoethyl)-glycine units linked by unnatural pseudo-peptide bonds (Nielsen *et al.*, 1991; Nielsen and Egholm, 1999). Unlike DNA and RNA, it does not possess any sugar (pentose) moieties or phosphate residues. Because of these structural differences, PNAs are electrically neutral so that they generate no charge repulsion to DNA (or RNA) strands (Nielsen and Egholm, 1999). As a

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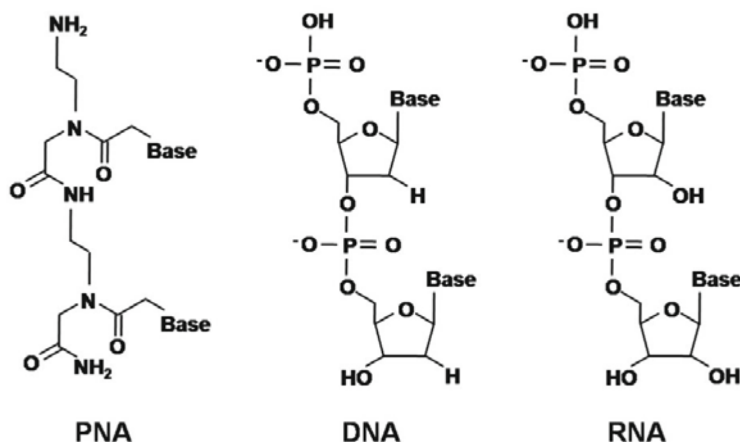


Fig. 1. **Chemical structures of PNA, DNA, and RNA.**

result, the PNA::DNA complex can bind much stronger than the DNA::DNA (or RNA) complex. In addition to high affinity and sequence specificity to negatively charged complementary DNAs or RNAs (Nielsen, 2010; Malcher *et al.*, 2014), PNA is known to be little toxic *in vivo* as well as very stable chemically and biologically (Lundin *et al.*, 2006).

As a large hydrophilic molecule, PNA is hardy to deliver into intracellular space because it cannot cross lipid membranes easily. This is one of the basic challenges for its clinical applications. To overcome this problem, PNA must be conjugated with a bacterial penetration peptide (BPP), a short amino acid motif that can penetrate bacterial cell wall effectively. Several BPPs have been discovered for different bacterial species. For example, a BPP motif, -VLTNENPFSDP-, can effectively deliver PNAs into *Staphylococcus aureus* whereas another motif, -YKKSNNPFSD-, is most efficient on *Bacillus subtilis* and *E. coli* (Rajarao *et al.*, 2002). Both -KFFKFFKFFK- and -CFFKDEL motifs have also been demonstrated for *E. coli* (Good *et al.*, 2001; Rajarao *et al.*, 2002).

Based on their unique features as above, PNAs have been applied in various fields such as bio-imaging, detection of single nucleotide polymorphism, microarray, and therapeutic agents (Table 1) because they are easily designed and modified in accordance with research purposes (Malcher *et al.*, 2014). For example, a previous study demonstrated that PNA is modified and applied for fluorescence *in situ* hybridization to quantify and visualize heterogeneous biofilm populations (Almeida *et al.*, 2011). Zhao *et al.* (2016) have also reported a highly sensitive and rapid method for detection of KRAS mutations of colorectal cancer using PNA-based real time PCR clamping (Zhao *et al.*, 2016). Based on the facts that PNAs can bind their target genes and inhibit the expression of these genes (Dean, 2000), several studies showed that PNAs are useful for treating some bacterial infectious diseases and/or cancers (Nekhotiaeva *et al.*, 2004; Kulyte *et al.*, 2005; Faccini *et al.*, 2008; Bai *et al.*, 2012; Thomas *et al.*, 2013).

Molecular mechanisms of PNA-mediated gene silencing

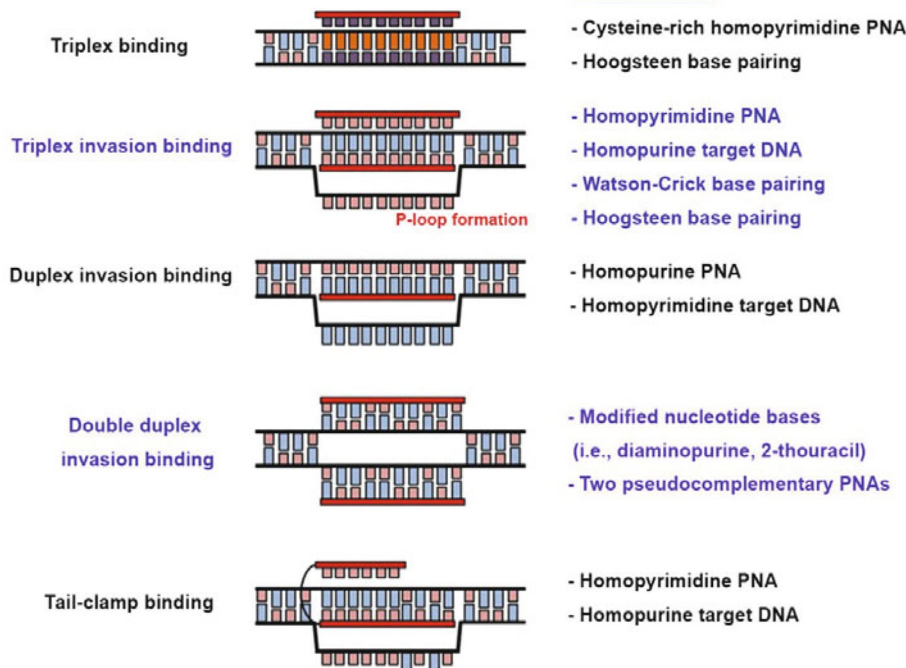
PNA-mediated gene silencing can be explained by either antigene or antisense activities (Nielsen, 2010; Malcher *et al.*, 2014). Among them, the antigene activity by PNAs is to inhibit the transcription initiation or elongation of target gene(s) by complementarily binding of PNAs to the target DNA sequences (Hanvey *et al.*, 1992; Nielsen *et al.*, 1994b). The antisense activity is to block the translation of target gene(s) by interfering the mRNA target sequences. Several types of possible PNA::DNA complexes have been reported depending on their sequence compositions (% of pyrimidine and purine bases), the number of PNA molecules binding to the target DNA sequence, and the structural features of PNAs (Fig. 2). As shown in Fig. 2A, the complexes are formed by triplex binding, triplex invasion binding, duplex invasion

Table 1. **PNA applications in molecular biology and medical fields**

Applications	Purposes	References
Bio-imaging	*In vivo* - Detection of cancer cells (breast cancer, prostate cancer, and pancreatic cancer) *In vitro* - Detection of bacteria (from water, patient's blood) - Cancer diagnosis	Heckl *et al.* (2003), Tian *et al.* (2004, 2005, 2007), Marciniak *et al.* (2005), Wilks and Keevil (2006), Chakrabarti *et al.* (2007), Forrest *et al.* (2008)
SNP assay	SNP genotyping (basal cell carcinoma, lung cancer, neurodegenerative disease, etc.)	Gaylord *et al.* (2005), Nagai *et al.* (2005), Rockenbauer *et al.* (2005)
Microarray	- Detection of genetically modified organisms (GMOs) - Detection of hepatitis B virus (HBV) and human papilloma virus (HPV)	Chen *et al.* (2005), Germini *et al.* (2005), Choi *et al.* (2009)
Gene therapeutic agents	- Antiviral therapy (HIV-1) - Anticancer therapy (squamous cell carcinoma, lung cancer, etc.) - Antibacterial therapy (*Escherichia coli*, *Bacillus subtilis*, *Mycobacterium smegmatis*, etc.) - Antiparasitic therapy (*Plasmodium falciparum*)	Riguet *et al.* (2004), Kulyte *et al.* (2005), Faccini *et al.* (2008), Hatamoto *et al.* (2009), Thomas *et al.* (2013), Kolevzon *et al.* (2014)

binding, double duplex invasion binding, or tail clamp binding between PNAs and DNAs.

Triplex binding of PNA::DNA occurs when a cytosine-rich homo-pyrimidine PNA molecule binds to the complementary DNA sequence via Hoogsteen base paring (Wittung *et al.*, 1997). This type of complex is known to be formed in the major grooves of the DNA double strand where displacement of non-complementary DNA is not necessary (Knauert and Glazer, 2001). Similarly, triplex invasion binding of PNA::DNA is formed when a homo-pyrimidine PNA binds to a homo-purine DNA sequence. For example, one PNA molecule forms a PNA::DNA complex by binding to the DNA duplex target at the major groove via Hoogsteen base paring, while the other PNA molecule binds to the other side via Watson-Crick base paring (resulting in a PNA::DNA::PNA complex by P-loop formation; Fig. 2A) (Nielsen *et al.*, 1994a). Although this type of complex has a very slow binding kinetic, it is very stable and maintained for a long period of time (Kosaganov *et al.*, 2000). In contrast, duplex invasion binding of PNA::DNA is formed when a homo-purine PNA molecule binds to a homo-pyrimidine DNA target sequence, which is accompanied with the displacement of non-complementary DNAs (Nielsen and Christensen, 1996). Thus, this type of complex has been recommended for PNAs to contain the purine-pyrimidine sequences to improve its binding efficiency (Nielsen and Christensen, 1996). Double duplex invasion binding of PNA::DNA occurs when two pseudo-complementary PNA molecules (i.e., di-aminopurine and 2-thiouracil) bind to a target DNA sequence simultaneously (Fig. 2A; Lohse *et al.*, 1999). Among these various PNA::DNA complexes, the most unusual binding structure is tail-clamp binding (Fig. 2A). This type of complex is formed when the PNA molecule with a short homo-pyrimidine triplex clamp domain (hexamer) and a duplex-forming tail domain (decamer) bind to



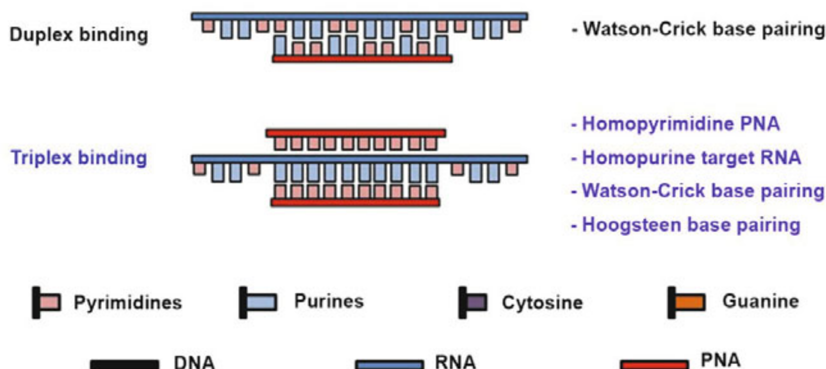


Fig. 2. **Binding patterns and characteristics of the PNA-nucleic acid complexes.**

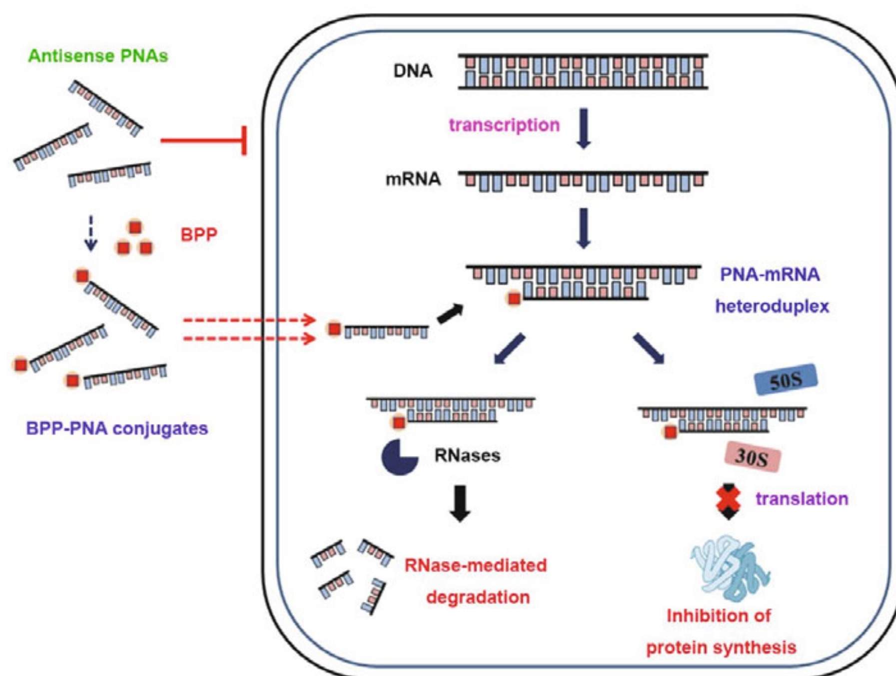


Fig. 3. **Antisense activity of the BPP-conjugated PNAs.**

target DNAs (Bentin *et al.*, 2003). Because of the unique structural characteristics of tail-clamp PNAs, they have been applied for inhibition of targeted mRNA translation, visual detection of virus, and targeting of micro-RNAs (miRNAs) (Knudsen and Nielsen, 1996; Schleifman *et al.*, 2011; Kaihatsu *et al.*, 2013; Ghidini *et al.*, 2016).

Similar to DNA, PNAs can bind RNAs to form a PNA::RNA complex. As shown in Fig. 2B, the PNA::RNA complex is formed by duplex or triplex binding. Duplex binding occurs when a single PNA molecule binds to a single RNA strand. Triplex binding occurs when one PNA molecule forms the PNA::RNA complex via Hoogsteen base paring, and the other PNA binds to other side via Watson-Crick base paring, ultimately resulting in a PNA::RNA::PNA complex (Almarsson and Bruice, 1993; Brown *et al.*, 1994; Li *et al.*, 2010). By doing these interactions, PNAs can block the mRNA translation and suppress the synthesis of the target proteins, which is referred to as the antisense activity by PNAs (Fig. 3). A previous study demonstrated that the antisense activity by PNAs depends on the position of target DNA sequences. For example, the highest antisense activity was demonstrated when PNAs were targeted to the translation initiation region such as the Shine-Dalgarno sequence (Dryselius *et al.*, 2003).

Application of PNAs as an anti-infective agent

It is well known that RNA interference (RNAi) via either short interfering RNAs (siRNAs) or miRNAs requires the protein complex to induce degradation or cleavage of the

Table 2. **Clinical application in bacterial diseases**

Target bacteria	Target gene	Function	Bacterial penetrating peptide	Application (*In vitro*/*In vivo*)	References
Escherichia coli	23S rRNA	Bacterial translation and growth	$(KFF)_3K$	*In vitro*	Xue-Wen *et al.* (2007)
ESBLs-*E. coli*	*rpoD*	RNA polymerase sigma factor	$(KFF)_3K$, $(RXR)_4XB$	*In vitro*, *In vivo**	Bai *et al.* (2012)
MDR-*E. coli*	*rpoD*	RNA polymerase sigma factor	$(KFF)_3K$, $(RXR)_4XB$	*In vitro*	Bai *et al.* (2012)
MDR-*Salmonella enterica*	*rpoD*	RNA polymerase sigma factor	$(KFF)_3K$, $(RXR)_4XB$	*In vitro*	Bai *et al.* (2012)
ESBLs-*Klebsiella pneumoniae*	*rpoD*	RNA polymerase sigma factor	$(KFF)_3K$, $(RXR)_4XB$	*In vitro*	Bai *et al.* (2012)
Campylobacter jejuni	*cmeABC*	Multidrug efflux transporter	$(KFF)_3K$	*In vitro*	Jeon and Zhang (2009), Oh *et al.* (2014)
MDR-*Shigella flexneri*	*rpoD*	RNA polymerase sigma factor	$(KFF)_3K$, $(RXR)_4XB$	*In vitro*, *In vivo**	Bai *et al.* (2012)
S. aureus	*fmhB*	Cell wall biosynthesis	$(KFF)_3K$	*In vitro*	Nekhotiaeva *et al.* (2004)
	gyrA	DNA replication	$(KFF)_3K$	*In vitro*	Nekhotiaeva *et al.* (2004)
MRSA	*mecA*	Penicillin-binding protein (PBP2a)	$(KFF)_3K$	*In vitro*	Goh *et al.* (2015)
Streptococcus pyogenes	*gyrA*	DNA gyrase	$(KFF)_3K$	*In vitro*	Patenge *et al.* (2013)
Mycobacterium smegmatis	*inhA*	Mycolic acid biosynthesis	$(KFF)_3K$	*In vitro*	Kulyte *et al.* (2005)

* *In vivo* application of PNA in BALB/c mice.
ESBL, Extended-spectrum beta-lactamases; MDR, Multidrug-resistant; MRSA, Methicillin-resistant *S. aureus*.

target mRNAs (Watts and Corey, 2012). Unlike siRNAs or miRNAs, however, PNAs can induce RNAi without the aid of other factors since they physically interfere mRNA translation (Fig. 3). For this reason, they have been applied to inhibit bacterial essential genes required for bacterial survival. Both *in vitro* and *in vivo* anti-bacterial activities have been demonstrated against various bacterial pathogens, including Gram-positive (Nekhotiaeva *et al.*, 2004; Patenge *et al.*, 2013) and -negative bacteria (Xue-Wen *et al.*, 2007; Bai *et al.*, 2012), as well as *Mycobacterium* species (Kulyte *et al.*, 2005) (Table 2). Recently, Patenge *et al.* (2013) demonstrated that HIV-1 Tat peptide-conjugated PNAs specific for the essential gyrase A gene (*gyrA*) can inhibit the growth of *Streptococcus pyogenes*, an exclusively human pathogen that causes necrotizing fasciitis (flesh-eating disease) or streptococcal toxic shock syndrome (Patenge *et al.*, 2013). Interestingly, the PNA conjugates displayed antimicrobial synergistic effects with the gyrase-targeting antibiotics such as levofloxacin and novobiocin.

Several studies have focused on the synergistic effect of PNAs in combination with conventional antibiotics. For example, a previous work showed that antibiotic resistance of MRSA to oxacillin is greatly reduced by applying the BPP-conjugated anti-*mecA* PNAs that can target the penicillin-binding protein (PBP2a) gene (Goh *et al.*, 2015). Similarly, growth of antibiotic-resistant bacteria was effectively suppressed by ciprofloxacin and erythromycin after the co-treatment of PNAs able to interfere the CmeABC efflux pump-encoding genes in *Campylobacter jejuni* (Jeon and Zhang, 2009; Mu *et al.*, 2013; Oh *et al.*, 2014) (Table 2). More recently, Castillo *et al.* (2018) demonstrated the adjuvant effect of mRNA targeted PNAs in *E. coli* O157:H7 (Castillo *et al.*, 2018). Using the standard checkerboard assay with the essential acyl carrier protein (AcpP)-targeting PNAs (namely, anti-*acpP* PNA), they found two novel synergistic combinations of antibiotics; anti-*acpP* PNA with polymyxin B and anti-*acpP* PNA with trimethoprim. These imply that mRNA targeted PNAs can maximize the bactericidal effects of certain antibiotics by suppressing the expression of target genes functionally associated with antibiotic resistance. Interestingly, it should be noted that antisense PNA molecules can specifically downregulate both a stably expressed transgene as well as an endogenous essential gene in *Plasmodium falciparum*, the causative agent of malaria in humans, resulting in a significant reduction in viability of the pathogenic parasites. Therefore, PNA can be applied for designing novel anti-infective strategies against various microbial infectious diseases by viruses, bacteria, or parasites.

Safety and stability

In order to apply PNAs as a therapeutic agent, drug selectivity on bacterial pathogens is one of the key aspects because PNAs must not interfere with any other host genes and/or cellular processes. Previous studies showed that both siRNAs and miRNAs with the antisense effects activate the host immune system through the toll-like receptors, and thus promote cytokine induction (Sioud and Sorensen, 2003; Kariko *et al.*, 2004; Fabbri *et al.*, 2012). However, it has been reported that PNA does not induce any immune responses during a host infection because it is not recognized by intracellular proteins (Demidov *et al.*, 1994).

As shown in Fig. 1, its unusual backbone structure seems to allow high stability of PNA although the *in vivo* safety and stability of PNAs need to be more intensively demonstrated. A previous study revealed that the administered PNAs can last for several hundred days under appropriate physiological circumstances (Kosaganov *et al.*, 2000). Furthermore, PNAs are not degraded by host nucleases and proteases during administration (Demidov *et al.*, 1993; Nielsen and Egholm, 1999).

Future directions

PNA is considered as one of the most promising candidates to treat various antibiotic-resistant infectious diseases. However, the underlying technology is premature to discuss its application to humans, as there are unresolved issues that are necessary for being addressed to reduce the potential risks. As mentioned above, its high bio-stability within host cells is a strong advantage (Demidov *et al.*, 1994). On the other hand, however, it is likely problematic because its biological half-life, *in vivo* disposal and the potential side-effects on host cells have not been yet addressed, which require further investigation in future. Improving the delivery efficiency of PNA should also be addressed in order to maximize the biological significance of PNA-derived therapeutics. Although $(KFF)_3K$ is the most commonly used BPP for a wide range of bacteria (Vaara and Porro, 1996), only the limited number of BPPs are currently available. Thus, discovery of novel BPPs with various functions of either wide- or narrow-spectrums are being needed.

Since antibiotics generally have a broad-spectrum antibacterial activity, they may affect both pathogenic bacteria and the normal microflora. In contrast, the highly-sequence specific PNAs are capable of selectively killing pathogenic bacteria, but not the normal microflora; a previous work demonstrated the species-specific killing using the bactericidal PNAs conjugated with BPPs (Mondhe *et al.*, 2014). In addition, as certain bacteria can acquire antibiotic resistance via one or more point mutations within the gene sequences responsible for antibiotic resistance (Jaktaji and Mohiti, 2010; Johnning *et al.*, 2015), targeting of such DNA regions may allow for PNA to distinguish between antibiotic-sensitive and -resistant bacteria. These unique features of PNAs can be useful to develop novel therapeutic agents against severe diseases caused by MDR bacteria without disturbing the bacterial community in a host, which suggests PNAs as one of the strong candidates to MDR diseases.

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