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ORIGINAL ARTICLE

Evaluation of cell-penetrating peptide–peptide nucleic acid effect in the inhibition of *cag*A in *Helicobacter pylori*

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ABSTRACT

Helicobacter pylori is the most common cause of chronic infection in human and is associated with gastritis, peptic ulcer disease, and adenocarcinoma of mucosa-associated lymphoid tissue cells. Peptide nucleic acid (PNA) is a synthetic compound, which can inhibit the production of a particular gene. This study aimed to investigate the effect of PNA on inhibiting the expression of *cag*A. After confirmation of the desired gene by polymerase chain reaction (PCR), the antisense sequence was designed against *cag*A gene. The minimum inhibitory concentrations of conjugated PNA against *H. pylori* was determined. The effect of the compound on the expression level of the *cag*A was investigated in HT29 cell culture using real-time PCR. The results showed 2 and 3 log reduction in bacterial count after 8- and 24-h treatment with 4 and 8 μM of the compound, respectively. The lowest expression level of the *cag*A gene was observed at a concentration of 8 μM after 6 h. The results of this study showed that cell-penetrating peptide antisense can be employed as effective tools for inhibiting the target gene mRNA for various purposes. Moreover, further research is necessary to assess the potency, safety, and pharmacokinetics of CPP-PNAs for clinical prevention and treatment of infections due to *H. pylori*.

KEYWORDS

Helicobacter pylori, peptide nucleic acid, antisense, real-time PCR, cell culture

INTRODUCTION

Gastric cancer is the third most common cause of cancer death in the world. Multiple epidemiological studies have documented an increasing incidence of gastric cancer with an increasing prevalence of *Helicobacter pylori* infection [1–4]. In 1994, the International Agency for Research on Cancer, World Health Organization (WHO), classified *H. pylori* as a group I carcinogen, a definite cause of cancer in humans. Additional large-scale prospective cohort studies confirmed the association between *H. pylori* infection and gastric cancer [5–8]. It is now well accepted that *H. pylori* is the single greatest risk factor for the development of gastric cancer, accounting for about 75% of all gastric cancers [9]. This microorganism is estimated to infect approximately half of the entire human population and is the most frequently occurring chronic bacterial infection in developing countries [10]. Moreover, the colonization of *H. pylori* as a bacterial pathogen in the stomach can cause a number of gastric diseases, such as peptic ulcers, chronic gastritis, and mucosa-associated lymphoid tissue (MALT) lymphoma [11, 12].

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H. pylori infection is primarily acquired during childhood and the transmission occurs through an oral–oral or fecal–oral route within families, particularly in the settings with poor sanitation. In the majority of cases, the colonized *H. pylori* persists in the stomach over the lifetime of the host unless eradicated with antibiotics [13].

In general, bacterial pathogenic factors can be divided into the factors related to colonization and factors responsible for tissue damage. *Cytotoxin-associated gene* A (*cag*A) and *vacuolating cytotoxin* A (*vac*A) are the main factors associated with *H. pylori* pathogenesis [14–18].

CagA is a surface protein with a high immune function, which is activated after penetrating gastric epithelial cells and phosphorylation. This can lead to many functional and morphological changes within the host gut cells, including apoptosis of host cells and interfering with cell signaling leading to the pathogenicity of the bacteria and the expression of the genes of *cag* pathogenicity islands (*cag* PAI) [16, 19, 20].

The presence of the *cag*A gene is associated with the development of diseases, such as duodenal ulcer, mucosal atrophy, and gastric cancer. Strains containing the *cag*A gene are more likely to be associated with apoptosis in adenocarcinoma gastric cells [21]. Initial research on 21 different species of *H. pylori* proves the relationship between the presence of *cag*A and the potentiality of multiple morphological and biochemical changes in human stomach cells.

Today, the treatment of *H. pylori* infection is facing serious challenges due to the emergence of antibacterial resistance. Researchers now argue that antibiotics alone are not enough to fight with bacteria, and there is a pressing need for new compounds. Suppressing essential virulence genes or inhibiting mRNA genes using antisense mechanisms can be considered as an interesting solution. Peptide nucleic acid (PNA) is a synthetic compound consisting of a pseudopeptide structure and a target gene complement that results in inhibitory transcriptional contractions and thus inhibition of translation as well as the affinity and specificity of these compounds in binding target genes have been proven [19–21]. PNA molecules are in fact synthetic DNA mimics with a neutrally charged chemical backbone with a higher affinity for DNA or RNA complementary sequences [22, 23]. PNA molecules that are more resistant to nucleases and proteases are usually about 13–18 bp, with the ability to penetrate the bacterial cell wall [22].

The negatively charged sugar–phosphate backbone of PNA molecules is replaced by an achiral, neutral polyamide backbone formed by repetitive units of *N*-(2-aminoethyl) glycine [17, 18]. PNA can hybridize with complementary nucleic acid targets according to the Watson–Crick base-pairing model [7].

The most important challenge in using the antisense compound as a drug has been the limitation of the penetration of bacterial cell membranes due to its selective permeability. Today, using the process of conjugating cell-penetrating peptides (CPPs) to the antisense compound as a transitional system has removed this restriction [24, 25].

Considering the importance of this bacterium and the emergence of antibiotic resistance, the aim of this study was to design antisense and intrusive peptide sequences to inhibit *cag*A virulence gene in the laboratory environment and cell culture in *H. pylori* bacteria.

MATERIALS AND METHODS

Bacterial strain

H. pylori standard strain (ATCC 43504) was used in this study. *H. pylori* strain was grown on blood agar media (43 g Columbia blood agar base (Acumedia, Svenska Labfas, Ljusne, Sweden), DL-tryptophan (0.1 g), deionised water (1 L), and horse blood (60 ml) in a humidified microaerobic environment (nitrogen 85% v/v, carbon dioxide 10% v/v, and oxygen 5% v/v) at 35 °C for 3 days.

Primer design

The sequences of primers in this study were designed by Oligo 7 [26] and Primer 3 software [27] (Table I).

DNA extraction

Genomic DNA from each isolate was prepared by vortex after suspending a loopful of colonies in 1 ml of phosphated-buffer saline, centrifuging at 14,000 × *g* for 2 min, and boiling the pellet in 1 ml of distilled water for 1 min [28]. The samples were then centrifuged at 12,000 × *g* for 4 min at 4 °C and the supernatant was then stored in sterile vials at −70 °C until use for polymerase chain reaction (PCR) templates.

Polymerase chain reaction (PCR)

To confirm the existence of the *cag*A gene, the PCR was performed using specific primers. The PCR was performed in a DNA thermal cycler (Bio-Rad, USA) in a volume of 25 µl. The PCR program consisted of an initial denaturation step at 94 °C for 4 min, 35 × 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 20 s. No template control was used as a negative control. Finally, PCR products were sequenced (Macrogen, South Korea).

CPP and antisense design

The CPP used in this study consisted of 13 amino acids, mainly arginine with a guanidine group to bind membranic carboxylic, sulfate, and phosphate groups with negative charges to facilitate peptide entry (RLRRKRRKRRKRRKRK). The peptides were designed with peptide design software including APD3 [29], Cell PPD [30], CPP pred [31], and CPP site 2.0 [32].

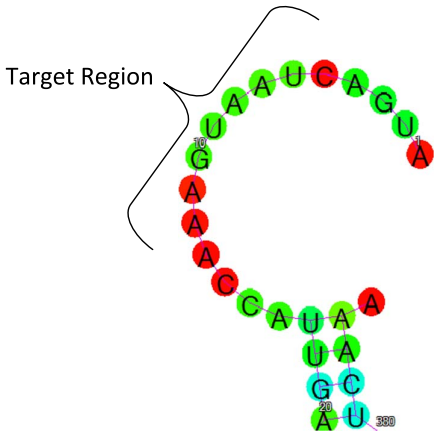
After predicting the mRNA secondary structure using m-FOLD server [33], 13 nucleotide target sequences or antisenses (TACTGATTACTTTGGT; Figure 1) were chosen as a complementary of a specific target region of *cag*A gene to construct the PNA compound. The cell-penetrating peptide–peptide nucleic acid (CPP-PNA) was synthesized by Panagene company (Daejeon, South Korea).

Table I. The sequence, product size, and melting temperature of the primers used in this study

Name	Primer sequence	Amplicon (bp)	*Tm* (°C)	Reference
*cag*A	F-5′-CAATGGTGGTCCTGGAGCTA-3′	140	60	This study
	R-5′-CCCTTTCTCACCACCTGCTA -3′			
16srRNA	F-5′-CTCATTGCGAAGGCGACCT-3′	74	60	This study
	R-5′-TCTAATCCTGTTTGCTCCCCA-3′			

Note: F: forward; R: reverse.

Figure 1. The target region studied as the secondary structure of the *cag*A gene



Minimum inhibitory concentrations (MICs)

The MIC of the CPP-PNA against *H. pylori* was determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [34]. The MIC was recorded as the lowest concentration where no turbidity was observed in the tubes. A concentration of 32 µM was prepared according to the company's protocol.

An amount of 50 µl of Brucella broth was dispensed in each well and the serial dilution was prepared starting with 50 µl of CPP-PNA with the concentration of 32 µM. An amount of 10 µl of 0.5 MacFarland was then added to each well. Brucella broth medium and the CPP-PNA were used as negative control and Brucella broth medium and the bacterium were used as a positive control. The final volume of each well was 100 µl.

The plate was incubated at 37 °C under microaerophilic conditions. The MIC result was determined after 18 h. The MICs were repeated at least twice.

Log reduction assay

H. pylori ATCC 43504 was diluted to ~105 CFU/ml in logarithmic growth phase and incubated with 4 and 8 µM of the CPP-PNA (in triplicates) at 37 °C for 24 h. Samples were collected at 0, 8, and 24 h, serially diluted, and plated onto Columbia agar plates. Plates were then incubated at 37 °C for 24 h before CFUs were determined.

Measuring *cag*A expression in CPP-PNA-treated *H. pylori*

The effect of CPP-PNA with concentrations of 1, 2, 4, and 8 µM was assessed on *cag*A expression level at 0, 2, 4, and 6 h via real-time PCR method. The sub-MIC concentration was used for RNA extraction. An amount of 100 µl of treated and untreated bacteria was then pelleted by centrifugation at 2,500 × *g* for 15 min. Total RNA was isolated using the QIAGEN RNeasy Mini kit (Qiagen, Germany). The extracted RNA was analyzed using a Nanodrop ND1000 and running on a denaturing 1.5% TAE-agarose gel (80 V for 1 h) to assess RNA concentration, quality, and integrity. The RNA was DNase-treated with Promega RNase-free DNase (at 37 °C for 1 h). Next, RNA was precipitated with 1 volume of isopropanol and 0.1 volume of 3 M NaOAc (pH 4.6). The suspension was incubated on ice for 20 min and centrifuged at high speed for 15 min at 4 °C. The RNA pellet was dried and resuspended with RNase-free MilliQ H_2O. According to the manufacturer's instructions, 500 ng–1 µg RNA was converted into cDNA using AccuPower CycleScript RT PreMix (Bioneer, Korea). Finally, quantitative real-time PCR was performed in a Rotor-Gene thermal cycler (Corbett 6000, Australia) using the SYBR Green method (AccuPower Green Star qPCR Master Mix, Bioneer, Korea). A total volume of 20 µl reaction consisting of 2 µl of cDNA, 12.5 µl of SYBR Green master mix, 4.5 µl of nuclease-free water, and 1 µl of each primer (5 pmol) was run according to the following program: an initial activation step at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 20 s *16s rRNA* was used as an internal control to normalize target gene expression measurements. Real-time PCR results were analyzed using the 2(-Delta Delta C(T)) method [35].

Cell culture infection assay

To assess the ability of CPP-PNA *in vitro*, cell culture infection assay in human HT29 cell line was performed. HT29 cells were grown in T75 flasks containing RPMI medium, 20% fetal bovine serum, 1% sodium pyruvate, 1% non-essential amino acids, and were then incubated at 37 °C.

The concentration of 2×10^7 CFU/ml of bacteria (multiplicities of infection ~1–10) was inoculated to each well

containing HT29 cells and concentrations of 1, 2, 4, and 8 μM of CPP-PNA were added to the infected cells and incubated for 4 h at 37 °C with 5% CO_2.

After washing steps, the total RNA was extracted and the expression of *cag*A was evaluated by real-time PCR.

RESULTS

Bacterial log reduction determination

Figures 2 and 3 illustrate the average number of viable bacteria after treatment and the corresponding log reduction associated with CPP-PNA (relative to the control), respectively. The results showed 2 and 3 log reduction in bacterial count at 4 and 8 μM of CPP-PNA after 18 h, and 1 and 2 log reduction in bacterial count at 4 and 8 μM of CPP-PNA after 8, respectively. Overall, a concentration-dependent bacterial reduction was observed. This reduction in the number of bacteria may be due to the peptide portion of the CPP-PNA, which causes damage to the bacterial membrane.

Figure 2. Bacterial log reduction in the presence of 4 μM of CPP-PNA at 0, 8, and 18 h. Bacterial colony counts were analyzed to assess bacterial survival ($p < 0.001$)

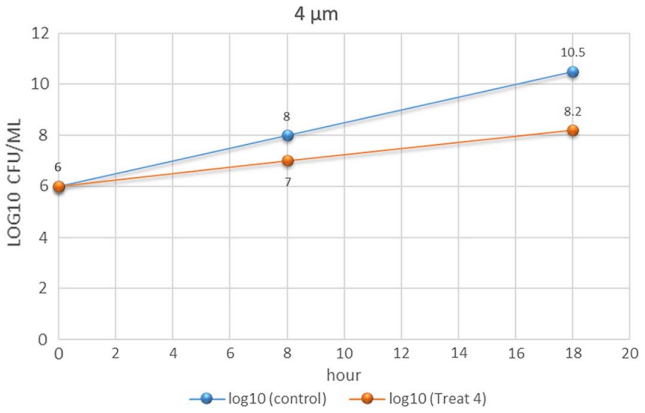
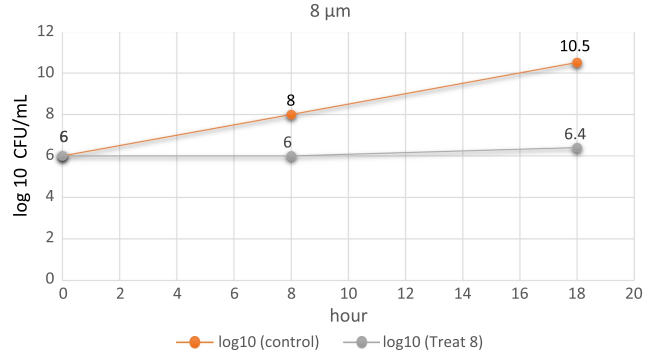


Figure 3. Bacterial log reduction in the presence of 8 μM of CPP-PNA at 0, 8, and 18 h. Bacterial colony counts were analyzed to assess bacterial survival ($p < 0.001$)



CPP-PNA inhibits cagA gene expression

In the presence of the CPP-PNA, *cag*A gene expression reduced. The results show that the treatment of *H. pylori* with CPP-PNA at different time intervals influences *cag*A gene expression. *cag*A gene expression was lower in the concentration of 8 μM of CPP-PNA, compared to the concentrations of 1, 2, and 4 μM after 6 h (Figures 4–7).

Figure 4. Gene expression analysis by real-time PCR at concentration of 1 μM of CPP-PNA at 0, 2, 4, and 6 h. Relative expression is normalized with housekeeping gene 16srRNA ($p < 0.001$ by Bonferroni test)

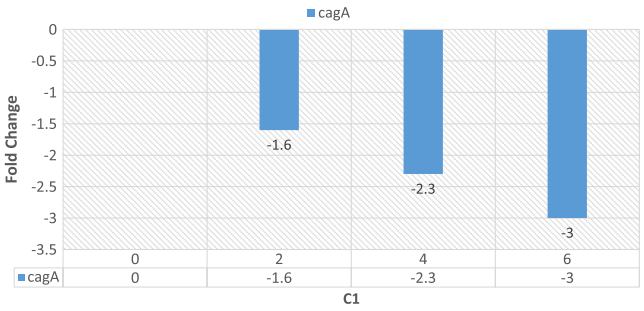


Figure 5. Gene expression analysis by real-time PCR at concentration of 2 μM of CPP-PNA at 0, 2, 4, and 6 h. Relative expression is normalized with housekeeping gene 16srRNA ($p < 0.001$ by Bonferroni test)

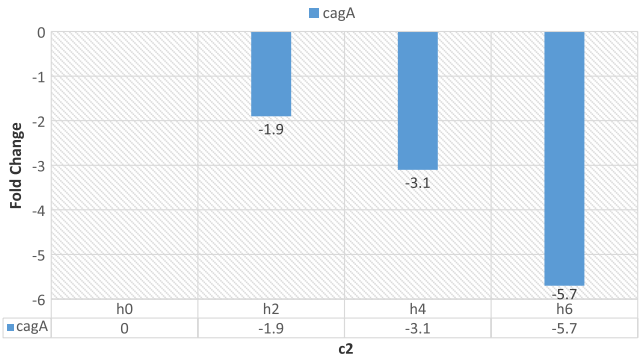


Figure 6. Gene expression analysis by real-time PCR at concentration of 4 μM of CPP-PNA at 0, 2, 4, and 6 h. Relative expression is normalized with housekeeping gene 16srRNA ($p < 0.005$ by Bonferroni test)

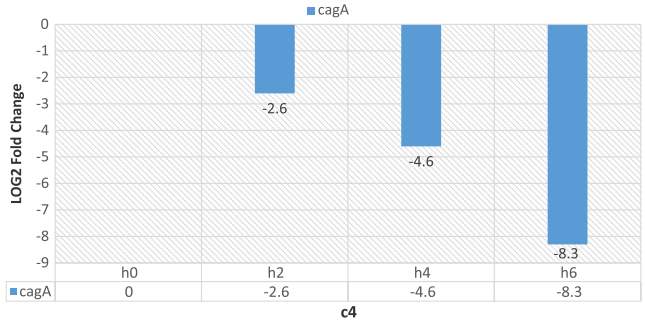
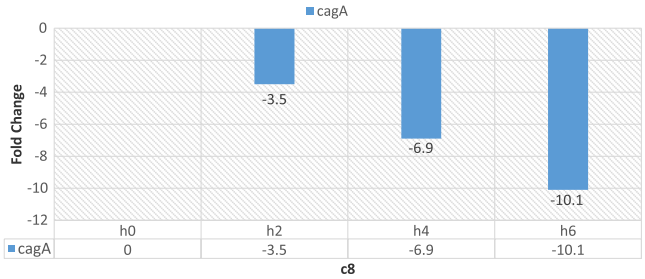


Figure 7. Gene expression analysis by real-time PCR at concentration of 8 μM of CPP-PNA at 0, 2, 4, and 6 h. Relative expression is normalized with housekeeping gene 16srRNA ($p < 0.005$ by Bonferroni test)



These results show that the CPP-PNA efficiency is directly linked with duration and concentration.

CPP-PNA inhibits *cag*A gene expression in infected cell cultures

The result of cell culture infection assay showed that CPP-PNA affects the *cag*A expression in human HT29 cell. At the concentration of 8 μM, we observed the highest decrease in *cag*A gene expression after 4 h compared to the concentrations of 1, 2, and 4 μM. After this time, about sevenfold of decrease in expression was observed (Figure 8).

DISCUSSION

The WHO categorized *H. pylori* as a group I carcinogen for gastric cancer in 1994 on the basis of observational studies [5].

Various and sporadic studies in Iran show the prevalence rate of 40%–80% for this bacterium among different populations [36–39]. In addition to high prevalence, antibiotic resistance in this bacterium is also rising similar to other bacteria [40]. Researchers today believe that antibiotics alone are not enough to cope with bacteria and that there is a pressing need for new compounds [41]. *cag* PAI is known to be a major contributor of *H. Pylori* pathogenecity [42]. *Cag*A was described as the first bacterial oncogene [42]. In this study, the *cag*A gene, which is a sample of carcinogenic cytotoxins, was targeted [42]. Suppressing essential and virulence genes, or inhibiting mRNA genes using antisense mechanisms, can be a new strategy to combat bacteria. The aim of this study was to investigate the effect of the CPP-PNA on the inhibition of *H. pylori cag*A expression. The results of this study showed at high concentrations of the CPP-PNA have a good effect on bacterial growth inhibition. Since the CPP-PNA in this study was designed against the pathogenic gene, and not the genes involved in bacterial survival, it can be concluded that the bacterial population is decreased owing to the peptide portion of CPP-PNA that in high amounts can damage the bacterial membrane, which can ultimately lead to bacterial death.

Similar results have been reported in the study of Abushahba et al. [25], in which they examined the effect of CPP on the effectiveness of antisense sequences against α subunit of RNA polymerase in *Listeria monocytogenes*. The results of their study showed that the CPP-PNA compounds rapidly kill *L. monocytogenes* within 20 min without disrupting the bacterial cell membrane. Moreover, *rpo*A gene silencing resulted in the suppression of its signaling as well as reducing the expression of other critical virulence genes (Listeriolysin O and two phospholipases *plc*A and *plc*B) in a concentration-dependent manner [25].

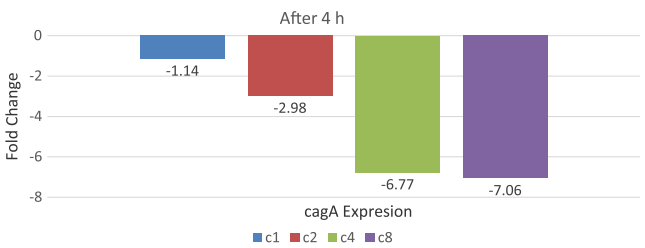
Regarding the previous studies and the role of arginine in the transport of CPPs into bacteria and cells, CPP in this study was designed to consist mainly of this amino acid, and the results showed high efficiency of the designed peptide to deliver antisense sequences. The effect of different peptides including TAT, ANT, RXR, RFR, and KFF was investigated on inhibition of the target gene in Seleem et al.'s study on *L. monocytogenes in vitro* [25]. The results of their study showed that the peptide containing arginine and lysine is more efficient than other peptides. Various studies have shown that the use of positively charged peptides is more suitable for the transmission of antisense compounds [43, 44].

A significant decrease in *cag*A gene expression was observed in the presence of CPP-PNA. This reduction is associated with an increase in the concentration of the CPP-PNA. Therefore, *cag*A gene expression was significantly lower at the concentration of 8 μM CPP-PNA compared to the concentrations of 1, 2, and 4 μM at the same time intervals.

In a study by Seleem et al. on *Salmonella enterica* in 2012, various PNAs were used to inhibit *rpo*D, *rpo*A, *eng*A, tsf, *kdt*A, and *lig*A genes. They reported that PNAs had a significant inhibitory effect on the target genes at different concentrations of 5, 10, 15, and 20 μM, and this effect showed a direct relationship with increasing concentrations [45], which is consistent with this study.

In 2013, Rajasekaran et al. [46] reported the inhibitory effect of PNAs on *Brucella suis* growth in pure culture, and in infected murine macrophages, they reported that the most effective PNA in broth culture was that targeting *pol*A at 12 μM. In contrast, in *B. suis*-infected macrophages, the most effective PNAs were those targeting *asd* and *dnaG* at 30 μM; both of these PNAs had little inhibitory effect on *B. suis* in broth culture. The *pol*A PNA that inhibited *B. suis* also

Figure 8. Gene expression analysis by real-time PCR in cell culture at concentration of 8 μM of CPP-PNA after 4 h. Relative expression is normalized with housekeeping gene 16srRNA ($p < 0.005$ by Bonferroni test)



inhibited the growth of *B. melitensis* 16M and *B. abortus* 2308 in culture media [46]. This study suggested the potential usefulness of antisense PNA constructs as novel therapeutic agents against intracellular Brucella.

In cell culture, the treatment of *H. pylori* with CPP-PNA affected the expression level of *cag*A gene after 4 h. At the concentration of 8 μM, lowest *cag*A gene expression was observed after 4 h compared to the concentrations of 1, 2, and 4 μM. These results indicate the efficacy of these compounds in cell culture.

In a study conducted on *S. enterica* in 2012 by Muhammad A. Soofi and Mohamed N. Seleem, the effect of different CPP-PNA conjugates on cell culture was investigated and infected cells were incubated for 4 h with different PNAs. Consequently, they observed that these compounds resulted in inhibition of the expression of target genes and ultimately reduced the bacterial growth [45], which was consistent with the results of this study.

CONCLUSIONS

The results of this study indicate that CPP-antisense compounds are effective tools for inhibiting the target gene mRNA and can be used for various research, therapeutic and preventive purposes, but the safety, toxicity, and immunogenicity of these compounds should be thoroughly investigated. In general, the results of this study confirm the efficacy of these compounds as auxiliary compounds or in combination with other drugs for the treatment of infectious agents and genetic defects. Moreover, due to the considerable ability of these compounds in binding mRNA and target genes compared to DNA probes, it is possible to add fluorescent tags to the antisense sequence to identify pathogenic agents such as *Mycobacterium tuberculosis* in the sputum specimen, as well as to identify infectious agents in samples with a small amount such as food and water.

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Conflict of Interest: The authors declare no competing interests.

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