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ORIGINAL ARTICLE

Therapeutic effects, immunogenicity and cytotoxicity of a cell penetrating peptide-peptide nucleic acid conjugate against *cagA* of *Helicobacter pylori* in cell culture and animal model

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ABSTRACT

Background and Objectives: *Helicobacter pylori* causes several gastrointestinal diseases, including asymptomatic gastritis, chronic peptic ulcer, duodenal ulcer, lymphoma of the mucosa-associated lymphoid tissue (MALT), and gastric adenocarcinoma. In recent years, failure to eradicate *H. pylori* infections has become an alarming problem for physicians. It is now clear that the current treatment strategies may become ineffective, necessitating the development of innovative antimicrobial compounds as alternative treatments.

Materials and Methods: In this experimental study, a cell-penetrating peptide-conjugated peptide nucleic acid (CPP-PNA) was used to target the *cagA* expression. *cagA* expression was evaluated using RT-qPCR assay after treatment by the CPP-PNA in cell culture and animal model. Additionally, immunogenicity and toxicity of the CPP-PNA were assessed in both cell culture and animal models.

Results: Our analysis showed that *cagA* mRNA levels reduced in *H. pylori*-infected HT29 cells after treatment with CPP-PNA in a dose-dependent manner. Also, *cagA* expression in bacterial RNA extracted from stomach tissue of mice treated with PNA was reduced compared to that of untreated mice. The expression of inflammatory cytokines also decreased in cells and tissue of *H. pylori*-infected mice after PNA treatment. The tested CPP-PNA showed no significant adverse effects on cell proliferation of cultured cells and no detectable toxicity and immunogenicity were observed in mice.

Conclusion: These results suggest the effectiveness of CPP-PNA in targeting CagA for various research and therapeutic purposes, offering a potential antisense therapy against *H. pylori* infections.

Keywords: *Helicobacter pylori*; *cag A*; Peptide nucleic acid; Immunogenicity; Cytotoxicity

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INTRODUCTION

Helicobacter pylori is a Gram-negative, microaerophilic gut pathogen chronically infecting approximately half of the world population (1). Numerous gastrointestinal disorders, including asymptomatic gastritis, chronic peptic ulcer, duodenal ulcer, lymphoma of the mucosa-associated lymphoid tissue (MALT), and gastric adenocarcinoma have been demonstrated to be highly associated with *H. pylori* colonization (2). In 2015, it was estimated that 780,000 of non-cardia gastric carcinoma (NCGC) cases were attributable to *H. pylori* globally that corresponded to 6.2% of worldwide cancer cases (3), implying that the worldwide prevalence of *H. pylori*-associated NCGC increased from 74.7% in 2008 to 89.0% in 2015.

Humans are the primary hosts for *H. pylori*, and once infected, a lifelong infection can occur, unless the patients receive appropriate antimicrobial therapy (4). The clinical consequences of *H. pylori* infections are determined by multiple factors, including interaction between bacterial virulence markers (mainly CagA, VacA, and BabA), host genetic predisposition (especially certain cytokine polymorphisms, such as IL-1β, IL-10, TNF-α), environmental factors (such as high-fat diet, drinking, and smoking) and the heterogeneity of *H. pylori* strains (5, 6).

Cytotoxin-associated gene A (CagA) is a well-characterized virulence factor of *H. pylori* injected into the host cells through a type IV secretion system (T4SS). The genes coding for CagA and T4SS are located on a mobile region of the chromosome, called cag pathogenicity island (cag PAI) and are detected in virulent strains of *H. pylori* (7). Many investigations have demonstrated that the strains positive for CagA are directly linked with the development of gastric ulcer, acute gastritis, and gastric cancer (8, 9). In addition, CagA might also be involved in altering host cell physiology, including the altered cytoskeleton, the formation of gastric epithelium cell pedestals, cell proliferation, and secretion of IL-8 by gastric epithelium cells (1).

The involvement of CagA in the production of pro-inflammatory cytokines has been extensively investigated. CagA has been indicated to influence cell proliferation and the upregulation of CagA in mice stimulates hyperplasia in the gastric mucosa and polyps in the glandular stomach. Moreover to inducing pro-inflammatory cytokines, infection-linked prohibition of apoptosis is one of the mechanisms by which *H. pylori* infection induces cell proliferation and the subsequent gastric cancer (10-12).

In recent years, failure to eradicate *H. pylori* infections has become an alarming problem for physicians. Failure in treatment can occur due to host, microorganism and treatment-related factors. Reportedly, *H. pylori* resistance to antimicrobial agents is widely known as the chief reason for treatment failure (13). It is now clear that the current treatment strategies may be insufficient and thus, developing innovative antimicrobial compounds is required as alternative treatments. Among the novel therapeutics, anti-virulence therapy is considered as a promising approach as instead of killing the pathogens, it deprives them from their virulence factors in order to control virulence in a pathogen-specific manner.

Opposite to antibiotic-associated treatments, anti-virulence or anti-toxin therapies do not directly influence bacterial viability and, as a result, could lead to the decreased selective pressure, thereby reducing the frequency of resistance.

Moreover, anti-virulence agents maintain the host endogenous microbiome as they target virulent factors of pathogenic bacteria (14).

Antisense silencing approach inhibits the endogenous expression of a virulence gene in a sequence-specific manner. Antisense antimicrobial agents are short (about 10-20 bases) single-stranded oligomers that bind a specific complementary mRNA of a target gene. Locked nucleic acids (LNAs), peptide nucleic acids (PNAs), and phosphorodiamidate morpholino oligomers (PMOs) are synthetic antisense antimicrobials binding the target complementary mRNA, thereby inhibiting the expression of the target gene (15, 16). Gene silencing reduces the pathogenicity of a bacterial strain by targeting virulence genes and reduces the risk of resistance evolution by targeting a non-essential gene (17). The most critical issue in the employment of the antisense compound as a therapeutic agent has been the limited penetration of bacterial cell membranes because of its selective permeability. Nowadays, the addition of conjugating cell-penetrating peptides (CPPs) to the antisense compound has surpassed this limitation (18).

Here, we used a previously designed CPP-PNA targeting the *cagA* gene to evaluate its possible effect on *H. pylori* pathogenicity both *in vitro* and *in vivo*. The results of our previous study indicated that at 8 and

24 h post treatment of *H. pylori*-infected HT29 cells with the designed CPP-PNA, bacterial count reduced for 2 and 3 log, respectively. Also, *cagA* showed the lowest expression at a concentration of 8 μM after 6 h (18).

MATERIALS AND METHODS

Bacterial strains, primers, antisense and cpp used in the study. In this study, we used *H. pylori* standard strain (ATCC 43504). Growth conditions, culture medium and biochemical and molecular confirmation is fully described in our previous article (18). Table 1 shows the primers used in this study. Primer efficiency was calculated using 10-fold dilution series of cDNA templates. Each serial dilution was used in a separate real-time reaction.

In this study, we used a previously designed CPP-PNA. Briefly a 16-nucleotide target sequence (TACTGATTACTTTGGT) complementary to a specific target region of *cagA* gene was chosen to construct the PNA compound. To improve the potency of antisense, PNA was conjugated with the cell-penetrating peptide (CPP) (18). Additionally, a Free PNA lacking CPP was synthesized and used as a negative control.

Cell Cultures and *cagA* expression in cell lines. The human colon adenocarcinoma cell line HT29-MTX-E12 was used as an epithelial model for the adherence of *H. pylori*. The HT29 cells were cultured in Roswell Park Memorial Institute (RPMI) (Gibco, Carlsbad, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Carlsbad, USA), and 4 mmol/L glutamine (Sigma-Aldrich, Germany). Cell cultures were incubated at 37°C, 5% CO_2 in a humidified atmosphere.

In all experiments, wells containing uninfected-un-

Table 1. The sequence of primers used in this study

Genes	sequence (5′→3′)	Tm	Product size (bp)	Reference
cag A	F: CAATGGTGGTCCTGGAGCTA	60	140	(18)
	R:CCCTTTCTCACCACCTGCTA	60		
Cag L	F: GGGGCATTTTGTGGGTGCTA	60	158	In this study
	R: TGGCTATGCAAAAAGCGACC	60		
16S rRNA	F: CTCATTGCGAAGGCGACCT	60	74	(19)
	R: TCTAATCCTGTTTGCTCCCCA	60		
Il-6	F: AACTCCTTCTCCACAAGCGCC	60	124	(20)
	R: GTGGGGCGGCTACATCTTT	60		
Il -8	F: GAATTGAATGGGTTTGCTAGA	54	229	(21)
	R: CACTGTGAGGTAAGATGGTGG	58		
bcl-2	F: CTGCACCTGACGCCCTTCACC	66	119	(22)
	R: CACATGACCCCACCGAACTCAAAGA	65		
caspase-3	F: GGAAGCGAATCAATGGACTCTGG	62	146	(23)
	R: GCATCGACATCTGTACCAGAC C	61		
Gapdh	F: TCACCACCATGGAGAAGGC	59	169	(23)
	R: GCATCGACATCTGTACCAGACC	61		
*Il-6**	F: GAGGATACCACTCCCAACAGACC	62	141	(24)
	R: AAGTGCATCATCGTTGTTCATACA	59		
*bcl-2**	F: GAGCGTCAACAGGGAGATGT	60	166	(22)
	R: CAGCCAGGAGAAATCAAACAG	57		
*caspase-3**	F: GTGGAACTGACGATGATATGGC	50	135	(25)
	R: CGCAAAGTGACTGGATGAACC	52		
*Gapdh**	F:GGCACAGTCAAGGCTGAGAATG	62	143	(22)
	R:ATGGTGGTGAAGACGCCAGTA	61		

* Primers used for the animal study

treated HT29 cells and wells containing infected-untreated HT29 cells were used as controls and each experiment was carried out in triplicates. The plates were incubated at 37°C for 6 h. Samples were collected at 0, 2, 4, and 6 h for RNA extraction.

Real-time PCR was carried out in a Rotor-Gene thermal cycler (Corbett 6000; Australia) with SYBR Green method using the AccuPower Green Star qPCR Master Mix (Bioneer, Korea) and specific primers (Table 1). The relative expression was normalized using *16S rRNA* as the internal reference gene. The *cagL* gene was used as a control gene to check the specificity of the designed CPP-PNA. The RT-qPCR results were analyzed via the 2(-Delta Delta C(T)) method (26). Each test was carried out in triplicates.

***In vitro* toxicity assay.** The MTT assay was used to determine the potential toxicity of the CPP-PNA. Briefly, HT29 and macrophage cells were seeded in 96-well plates (10^4 cells per well) and cultured in RPMI containing 10% FBS in 5% CO_2 at 37°C for 24 h. Then, CPP-PNA was added to the medium in a series of concentrations (1, 2, 4, and 8 µmol/l). After 48 h of incubation, 10 µl MTT solution (5 mg/ml in PBS buffer) (Invitrogen, USA) was added to each well. The plates were then incubated in 5% CO_2 at 37°C for 4 h and the media were replaced by 100 µl DMSO. The plates were then gently shaken for 10 min at room temperature to completely dissolve the precipitated crystal purple formazan. Following incubation, absorbance was measured using a microplate reader at 570 nm. In this study, as positive control and negative controls, cells lysed by 1% Triton-X-100 and untreated cells were used. Each reaction was performed in triplicates.

Apoptosis assay. The RAW 264.7 murine macrophage cell line was used to determine *H. pylori*-induced apoptosis. Macrophage cells were grown at 37°C in 5% CO_2 in L-glutamine- and glucose-containing RPMI medium (Gibco, Carlsbad, USA) supplemented with 10% heat-inactivated FBS. Flowcytometric analyses were performed using AnnexinV-FITC Apoptosis Detection Kit according to the manufacturer's instructions. In summary, cells were seeded in a 12-well plate at a density of 5×10^5 cells/well and incubated for 4-8 h, followed by the inoculation of *H. pylori* (MOI:1:10) and the addition of 1, 2, 4 and 8 µM of CPP-PNA. Free PNA, uninfected cells and untreated infected cells were used as controls. Finally, after 24 hours, samples were analyzed using a flowcytometer (CyFlow SL, PartecGermany) at 488 nm. Annexin V-FITC and PI-stained cells were considered as apoptotic and necrotic cells, respectively, while viable cells could not adsorb any of these stains. Each test was performed in triplicates.

RT-qPCR assay for expression of caspase-3 and Bcl-2. Total RNA extraction was carried out on the treated and untreated *H. pylori*-infected macrophage cells after 4 and 8 hours. The expression of *caspase-3*, and *bcl-2* were evaluated using RT-qPCR by specific designed primers (Table 1). Briefly, cDNA was synthesized from total RNA using AccuPower CycleScript RT PreMix along with random hexamers. RT-qPCR was performed using AccuPower Green Star qPCR Master Mix in a Rotor-Gene thermal cycler. *GAPDH* reference gene was used for the normalization of gene expression data. Each test was performed in triplicates and the relative quantification of the RT-qPCR transcripts were carried out using the Livak formula (26).

RT-qPCR assay for expression of IL-8 and IL-6. The effects of CPP-PNA-inhibited *cagA* were examined on IL-8 and IL-6 mRNA expression in HT29 cells after 4 and 8 hours. Accordingly, the HT29 cells were harvested and total RNA was isolated using QIAGEN RNeasy Mini kit, as described above. The RT-qPCR assays to measure IL-8 and IL-6 expression in treated and untreated cells were carried out in triplicates as described above.

The therapeutic effects of CPP-PNA in mice. Animal studies were approved by Local Research Ethics Committee of Iran University of Medical sciences, Tehran, Iran with the ethical code IR.IUMS.FMD.REC.1397.240. Using a multiple experimental group design, 50 6-8-week-old C57BL/6 mice were inoculated with *H. pylori* ATCC 43504. The *H. pylori* colonization was confirmed 8 weeks post inoculation after conducting *H. pylori* DNA test by genus-specific primers directed at conserved regions of the *16S rRNA* gene (Table 1) on freshly collected fecal pellets using PCR. Control C57BL/6 (n=10) mice were only inoculated with PBS. The fecal pellets of infected mice were tested for the presence of *H. pylori* before and after bacterial inoculation using PCR as described previously (27).

Then, the infected mice were divided into three

groups: one group (n=10) was treated with 5 mg/kg of CPP-PNA twice a day via oral administration for 8 weeks, the second group (n=10) was treated with amoxicillin, clarithromycin, metronidazole, and omeprazole via oral administration twice a day for 8 weeks, and the third group (n=10) was orally administered with PBS. After 8 weeks, mice were killed by exposure to CO_2, and gastric mucosa samples were taken from the fundic mucosa of infected mice to investigate the presence of *H. pylori* and gastric inflammation using H&E staining. Histopathological examinations were performed on biopsy samples which were ranked according to the intensity of *H. pylori* colonization as follows: severe, moderate, mild and free from infection. A histological examination was performed by two experienced pathologists blinded to the treatment given. Furthermore, the expression of *cagA IL6, caspase-3* and *Bcl-2* in the gastric mucosa were evaluated using RT-qPCR.

Immunogenicity and toxicity assays in mice. To determine the *in vivo* immunogenicity and toxicity of CPP-PNA, mice were orally treated with CPP-PNA in a concentration of 50 mg/kg (a 10-fold experimental treatment dose). An equal volume of normal saline was used as the negative control. Mice were fed in a laboratory animal facility for a week. The body weights and mortality of the mice were recorded every day. Additionally, after daily collection of mice sera, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured by blood biochemical tests. Furthermore, to test the immunogenicity of the CPP-PNA, IgG and IgM levels in mice sera were measured by ELISA. Both ELISA and biochemical tests were performed in triplicates. In addition, simultaneous to the treatment of *H. pylori*-infected mice with PNA for 8 weeks, uninfected mice were orally administered with PNA twice daily. After 8 weeks, the weight, survival rate, AST/ALT enzyme levels, as well as total IgM and IgG were measured in this treatment group and the obtained values were compared to the group which was only administered with normal saline.

Statistical analysis. Analysis of results was performed using SPSS v26 software and graph pad prism. Statistical significance was assessed using the one-way ANOVA, Repeated Measure ANOVA and Bonferroni test. Independent-samples *t*-test was used to evaluate the differences between two groups. A P-value of less than 0.05 was considered statistically significant. Data are presented as mean ± SD.

RESULTS

Inhibition of *cagA* expression in *H. pylori*- infected HT29 cells. RT-qPCR was used to examine the possibility of the reduction of *cagA* expression in *H. pylori* co-cultured with HT29 cells after treatment with antisense CPP-PNA. Our analysis showed that *cagA* expression reduced in a dose-dependent manner when treated with CPP-PNA. CPP-PNA treatment reduced *cagA* mRNA contents, whereas Free PNA treatment did not significantly affect the *cagA* expression at the same concentrations (Fig. 1A). According to the expression of *cagL* as the control gene, the studied compound did not bind these gene, indicating the specific design and efficiency of our CPP-PNA (Fig. 1B) Repeated Measure ANOVA and

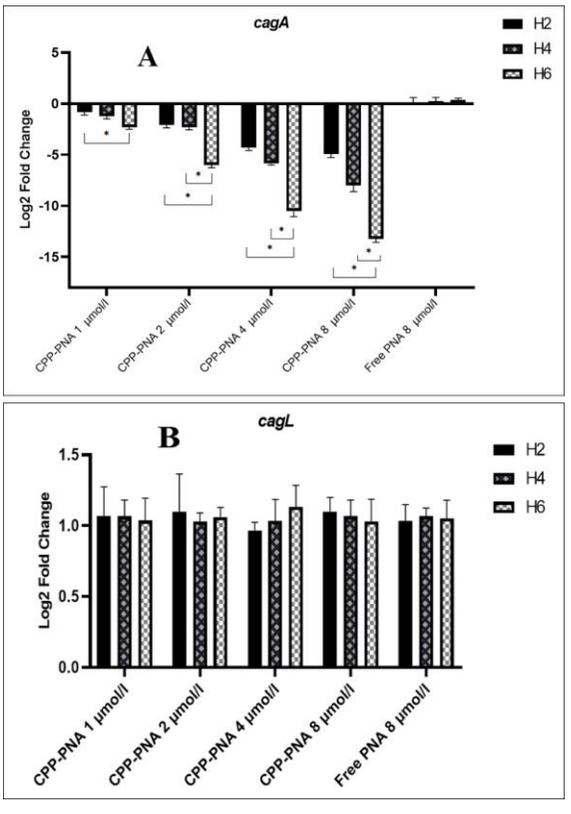

Fig. 1. Relative mRNA expression (fold-change) of *cagA* and *cagL* in CPP-PNA-treated HT29 cells compared with CPP-PNA-free HT29 (control) cells. *16S rRNA* level was used as an internal control for the normalization of mRNA expression. H2; after 2 h of treatment; H4, after 4 h of treatment; and H6, after 6 h of treatment. *:p<0.05

Bonferroni were used for data analysis ((p<0.05).

***In vitro* Cytotoxicity of the CPP-PNA.** At the concentrations of 1, 2, 4, and 8 μM, CPP-PNA did not significantly affect cell proliferation or survival in both cell lines. Accordingly, the viability of HT29 and macrophage cells treated with CPP-PNA was greater than 97% (Fig. 2). These results indicated the minimal cytotoxicity of CPP-PNA at the concentration of 8 μM or less. One Way ANOVA and Bonferroni were used for data analysis (p<0.05).

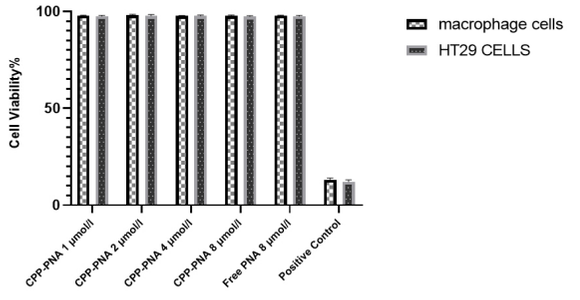

Fig. 2. Cytotoxicity of CPP-PNA. The cytotoxicity of the CPP-PNA was measured in HT29 and macrophage cells. Triton X-100 was used as positive control.

Apoptosis assay in CPP-PNA-treated cells. Our data indicated that *H. pylori*-infected cells treated with the CPP-PNA increased the apoptotic cell population in a dose-dependent manner. One Way ANOVA, Bonferroni and Independent sample t-test were used for data analysis (p value <0.05).

As shown in Fig. 3, the percentage of early apoptotic cells was 18.1%, 18.9%, 19.5%, and 20.3%, while the percentage of late apoptotic cells was 25.4%, 27.3%%, 29.8%, and 32.3% after treatment of *H. pylori*-infected cells with CPP-PNA at 1, 2, 4 and 8 μM, respectively. The percentage of early apoptotic and late apoptotic cells in *H. pylori*-infected cells treated with Free PNA was 16.1% and 27.9%, respectively.

Expression levels of caspase-3, Bcl-2, Il-6 and Il-8 after CPP-PNA treatment. RT-qPCR was used to evaluate the fold-change in expression of *caspase-3* and *bcl-2* in *H. pylori*-infected macrophages 4 and 8 hours after CPP-PNA treatment. Significantly increased *caspase-3* expression was observed in CPP-PNA-treated *H. pylori*-infected macrophages compared to CPP-PNA-free *H. pylori*-infected macrophages in a dose-dependent manner (p value<0.05). However, there was no significant fold-change in *bcl-2* expression following CPP-PNA exposure (Fig. 4).

Our data showed that the expression of *Il-6* and *Il-8* genes in HT29 cells were significantly decreased when treated with 4 and 8 μM of CPP-PNA, compared to the control. One Way ANOVA and Bonferroni were used for data analysis (p value<0.05) (Fig. 4).

Therapeutic effects of CPP-PNA administration *in vivo*. The *16S rDNA* gene amplification showed the absence of *H. pylori* in the stool of mice before inoculation whilst PCR results were positive following inoculation.

The group of mice receiving *H. pylori* ATCC 43504 suspension showed severe infection 8 weeks post inoculation as presented in Fig. 5A. As illustrated in Fig. 5B, a mild inflammation with few characteristic forms colonizing *H. pylori* was observed in the stomach tissue of the infected-mice group receiving 5 mg/kg CPP-PNA twice a day. Fig. 5C illustrates the gastric mucosa of mice free from *H. pylori* infection. The results of the expression levels of *cagA*, *caspase-3*, and *Il-6* in the gastric mucosa of mice treated with CPP-PNA, free-PNA, and antibiotics, and the untreated mice are illustrated in Fig. 6. In mice treated with antibiotics, *H. pylori* infection was totally eliminated and the expression of the aforementioned genes reduced. Mice treated with PNA were positive for *H. pylori*, however, *cagA* expression reduced 3.5 fold compared to the untreated mice.

Immunogenicity and toxicity results of CPP-PNA administration *in vivo*. The CPP-PNA fed to mice had no effect on body weight (data are not shown) and survival rate. As presented in Fig. 7A and 7B, serum ALT and AST levels in CPP-PNA treated mice did not show a significant increase compared to the normal saline-treated mice during the whole treatment. According to the serological assays, no significant difference was seen in the levels of IgG and IgM antibodies between the CPP-PNA treated and saline-treated mice (Figs. 7C and 7D).

DISCUSSION

Recently, PNAs have been considered as ideal candidates to inhibit gene expression in antisense technology and gene-based therapy. Due to the increasing rate of multi-drug resistant pathogens, it seems nec-

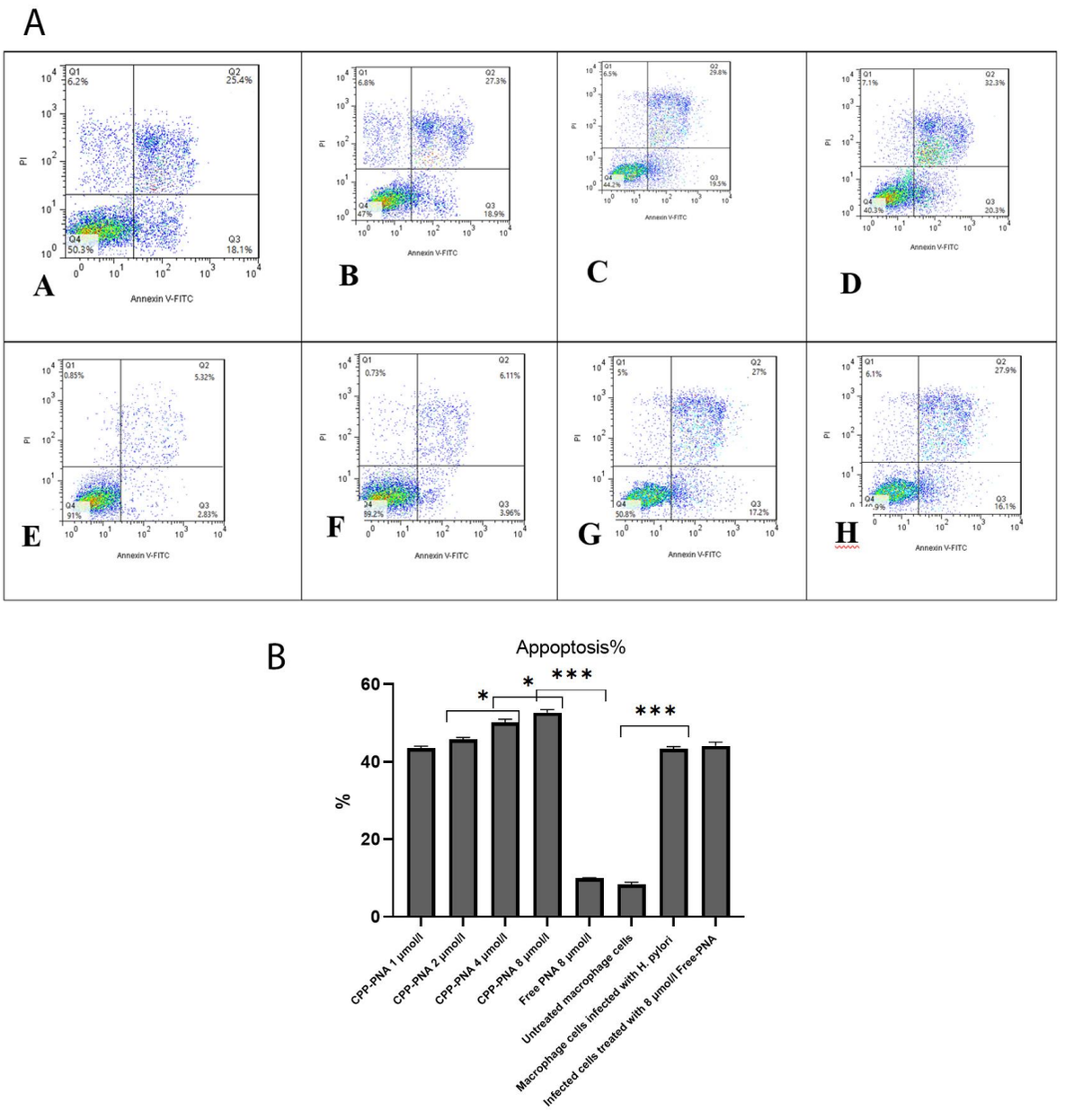

Fig. 3. A: Flowcytometry analysis of the treated *H. pylori*-infected macrophage cells with CPP-PNA at 1, 2, 4 and 8 µmol/l, Free PNA and untreated cells. (A) *H. pylori*-infected cells treated with 1 µmol/l CPP-PNA, (B) *H. pylori*-infected cells treated with 2 µmol/l CPP-PNA, (C). *H. pylori*-infected cells treated with 4 µmol/l CPP-PNA, (D) *H. pylori*-infected cells treated with 8 µmol/l CPP-PNA, (E) Control uninfected and untreated macrophage cells, (F) Uninfected macrophage cells treated with 8 µmol/l free-PNA, (G) *H. pylori*-infected cells treated with 8 µmol/l Free-PNA, (H) Macrophage cells infected with *H. pylori* without other treatments Q1—necrotic cells, Q2—late-apoptotic cells, Q3— early apoptotic cells, Q4—alive cells.
B: Column chart of the results of flowcytometry (performed in triplicates) *:$p<0.05$. ***:$p<0.001$

essary to develop novel approaches and technologies to treat a variety of infectious diseases. Considering the potential of PNA for inhibiting the expression of target genes, these agents can be used as potential antimicrobial compounds for the treatment of multi-drug resistant infections (28). Although PNAs are usually designed to target the housekeeping genes, the high similarity in these regions between the commensal flora and the pathogenic bacteria is a matter of concern in such studies. Therefore, designing

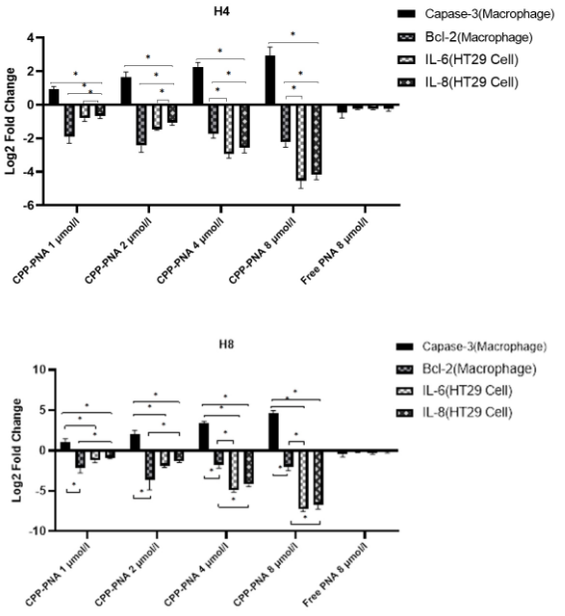

Fig. 4. Relative mRNA expression (fold-change) of *caspase-3*, *Bcl-2*, *IL-6*, and *IL-8* after 4 and 8 hours in macrophage and HT29 cells treated with different concentrations of CPP-PNA and 8 µmol/l of Free-PNA. GAPDH mRNA was used as an internal control for the normalization of mRNA expression. H4; after 4 h of treatment; H8; after 8 h of treatment. *:p<0.05

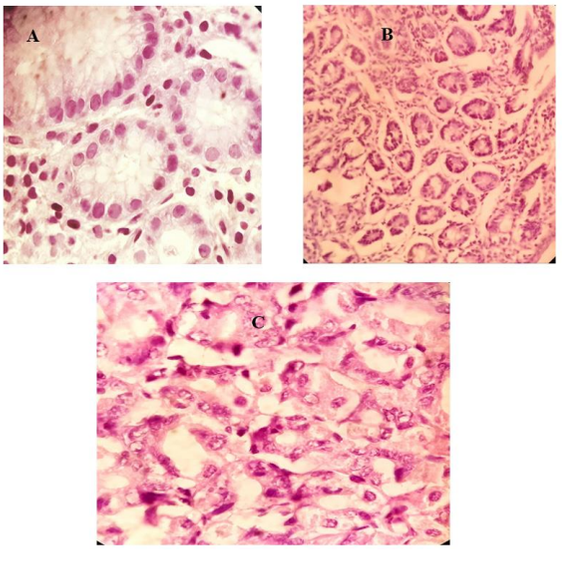

Fig. 5. Histopathological examination showing the gastric mucosa of untreated infected mice suffering from severe infection (H&E staining, ×1000x) (A), CPP-PNA-treated infected mice with mild inflammation (H&E staining, ×200×) (B), and mice free from *H. pylori* infection (H&E staining, ×1000x) (C).

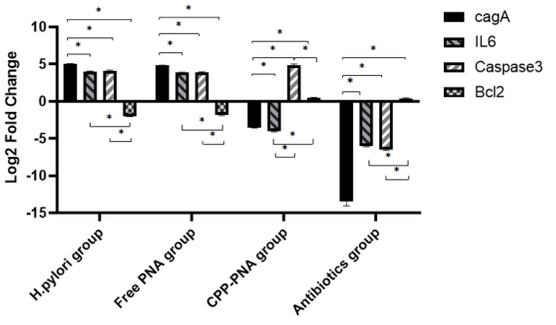

Fig. 6. Relative mRNA expression (fold-change) of *cagA*, *IL-6*, *caspase-3* and *Bcl-2* in gastric cells of mice infected with *H. pylori*, free PNA, CPP-PNA, and antibiotic compared to the untreated mice after treatment by 5 mg/kg CPP-PNA, Free-PNA or antibiotics. GAPDH mRNA was used as an internal control for the normalization of mRNA expression. *:p<0.05

PNAs for pathogen-specific virulence genes can limit the nonspecific binding of these constructs to normal bacterial flora. In the current study, we evaluated a CPP-PNA targeting *cagA* in *H. pylori*. This CPP-PNA effectively inhibited the expression of *cagA* in a dose-dependent manner and subsequently reduced the pathogenicity of CagA-producing *H. pylori* both *in vitro* and *in vivo*.

CagA is a major virulence factor of *H. pylori*, and thus, we selected the *cagA* gene as a clinically relevant target in our study. It has been previously described that the *cagA*-expressing *H. pylori* strains are closely associated with both gastric cancer and duodenal ulcer (9). Therefore, *cagA* is a potential target for anti-virulence therapies. In a previous study, we designed and synthesized a 16-nucleotide CPP-PNA that is complementary to *cagA* sequence in accordance with the critical roles of CagA protein in *H. pylori* pathogenesis (18). This antisense oligomer was used in the present study. Several studies reported the design and development of novel antisense agents targeting a single functional gene with an essential role in bacterial growth (29-31); however, our study focused on the inhibition of a virulence gene. Anti-virulence therapeutic approaches based on gene silencing strategies could specifically interfere with the ability of the pathogen to invade and multiply within the host cells and inhibit the activation of specific virulence traits required for the establishment of infection (32, 33). In fact, inhibiting the expression or activity of essential virulence factors leads to the reduced pathogenicity of bacterial cells.

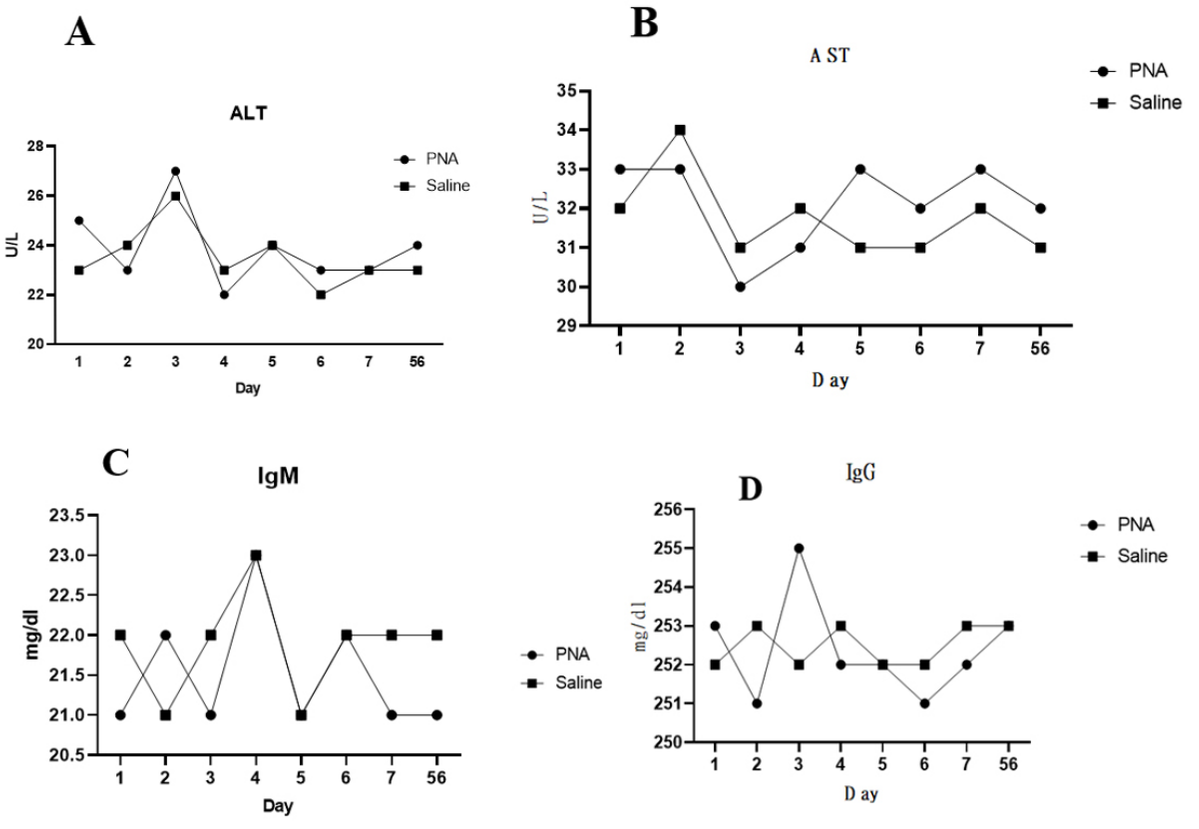

Fig. 7. Toxicity and immunogenicity of CPP-PNA in mice. ALT and AST levels were measured by blood biochemistry tests (A, B). Normal saline was used as the negative control. IgG and IgM levels were measured by ELISA (C, D).

In addition, as such strategies usually do not directly kill the microorganisms, there is apparently less evolutionary pressure to develop resistance mechanisms as compared with traditional antimicrobial therapy. It is also believed that anti-virulence therapies could allow the host immune system and the normal microbiota to inhibit bacterial colonization or prevent any established infection (17, 34). Besides, these novel therapeutic approaches could potentially be used in combination with synthetic or natural organic antimicrobials in a synergistic manner to extend the therapeutic potential of these drugs (34). Up to date, two studies have investigated the inhibition of *cagA* in *H. pylori* in order to sufficiently attenuate the infectivity of this pathogen (35, 36). In both studies, the anti-virulence compounds targeted the CagA secretion and delivery by interfering with Cag Type IV secretion system. Our study focused on blocking the expression of *cagA* using an antisense PNA as a novel anti-virulence therapeutic strategy.

Our gene expression analyses by RT-qPCR revealed a significant decrease in *cagA* expression in *H. pylori*-co-cultured with HT29 cells after treatment with CPP-PNA in a dose-dependent manner (Fig. 1). Importantly, the inhibitory rate of CPP-PNA against *cagA* was significantly detected within 6 h after treatment in all 1, 2, 4, and 8 µmol/l concentrations. This time-dependent activity may be attributable to the penetration of CPP-PNA into the bacterial cells. Of note, in this investigation, the antisense PNA was conjugated with a CPP as a cell-penetrating carrier to enhance its bioavailability. In fact, the co-administration of CPP with antisense PNA led to the effective delivery of the PNA cargo to its target in the bacterial cytoplasm as previously described by several researchers (37-39). In this study, treatment of the *H. pylori*-infected mice with CPP-PNA led to the decreased expression of *cagA*, however, this reduction was less than that observed in HT-29 cell culture, such that the *cagA* expression was reduced by 3.5 fold in the treated mice, and by 13.2 folds in the cell culture (Fig. 6). The reduced efficacy of PNA *in vivo* could be explained by the rapid clearance of PNA in the body of animals, as previously mentioned (40).

The cytotoxicity and immunogenicity of the de-

signed PNA were checked for any possible side effects associated with this construct. According to our findings, CPP-PNA did not have any noticeable inhibitory effect on cell proliferation and survival in the cultured macrophage and HT29 cells and did not increase apoptosis in macrophage cells at concentrations of 1, 2, 4, and 8 µmol/l (Fig. 2). Similar to the findings of MTT test, the used concentrations of CPP-PNA did not result in detectable *in vivo* toxicity and immunogenicity in mice. After oral administration of 50 mg/kg CPP-PNA, no changes were observed in weight loss and mortality, and the mice showed a normal growth similar to the negative control group. Additionally, serum ALT and AST levels were not significantly different between the CPP-PNA-treated and untreated mice suggesting the low hepatic toxicity of CPP-PNA (Figs. 7A and 7B). Furthermore, no significant levels of IgG and IgM antibodies were detected in the sera of mice treated with CPP-PNA, indicating the low immunogenicity of this antisense oligomer in mice (Figs. 7C and 7D). These findings verified the safety of the CPP-PNA in mice. The results of this study are consistent with the Zhengyang study in which PNA conjugated to HIV Tat protein was used to inhibit HBV replication. The toxicity assay results in the Zhengyang study showed that PNA concentrations less than 100 µmol/l were not toxic in any of the studied cell lines and had no toxic effects on the cell proliferation, hemolysis and the animal viability and liver tests (41).

In another study by Virendra N.padey, PNA was used to treat HIV. In this study, after PNA administration to the animal, the functions of all organs were normal, and no side effects was seen in the studied mice. The researchers reported PNA as non-toxic at all therapeutic doses (42). Several therapeutic approaches and novel antimicrobial agents are limited due to their potential toxicity and side effects. Consequently, it is absolutely necessary for antisense PNAs to be comprehensively tested for their safety in future clinical trials (43, 44). The low *in vitro* and *in vivo* toxicity of our CPP-PNA suggests that this antisense oligomer can be considered as a potential anti-virulence agent for the treatment of *H. pylori* infections.

Results of this study showed that the apoptosis rate among *H. pylori*-infected macrophage cells, treated with different concentrations of CPP-PNA, increased in a PNA-dose dependent manner (Fig. 3).

Based on the results of several studies, not only in different cell lines but also in animal models, it has been shown that CagA mediates protection against apoptosis and also promotes cell proliferation (10, 45, 46). The results of our study showed that treating *H. pylori* co-cultured macrophage cells with different concentrations of CPP-PNA increased *caspase3* expression level and apoptosis, which is probably suggestive of the successful and specific inhibition of *cagA* by the designed CPP-PNA. On the other hand, this study showed that the expression of IL-6 and IL-8 cytokines significantly reduced due to the treatment with 4 and 8 µM CPP-PNA as compare to the control (Fig. 4). Some previous studies reported the elevated IL-8 expression in cells infected with *H. pylori* strains expressing high levels of CagA virulence factor (11, 12). Therefore, the inhibition of CagA activity by CPP-PNA could efficiently reduce the inflammatory responses in *H. pylori*-infected cells, thereby reducing gastric inflammation which has been associated with an increased risk of developing gastric ulcer disease, duodenal ulcer disease, gastric adenocarcinoma, and gastric lymphoma.

CONCLUSION

We previously designed and synthesized an antisense cell-penetrating peptide-conjugated PNA strand (CPP-PNA), which was complementary to the sequence of *cagA* virulence gene. This CPP-PNA considerably inhibits *cagA* expression in cell culture via a sequence-specific antisense mechanism. In addition, this CPP-PNA exhibited low cytotoxicity and immunogenicity in all *in vitro* and *in vivo* evaluations. To the best of our knowledge, this report is the first attempt to use a *cagA*-targeted PNA as an anti-virulence approach in an experimental investigation. The current study suggests that CPP-PNA as a unique compound with anti-virulence activity against *H. pylori* can be potentially considered in antisense therapy against *H. pylori* infections.

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