

In Vitro Antibacterial Activity of Acyl-Lysyl Oligomers against *Helicobacter pylori*[∇]

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The gastric pathogen *Helicobacter pylori* has developed resistance to virtually all current antibiotics; thus, there is a pressing need to develop new anti-*H. pylori* therapies. The goal of this work was to evaluate the antibacterial effect of oligo-acyl-lysyl (OAK) antimicrobial peptidomimetics to determine if they might represent alternatives to conventional antibiotic treatment of *H. pylori* infection. A total of five OAK sequences were screened for growth-inhibitory and/or bactericidal effects against *H. pylori* strain G27; four of these sequences had growth-inhibitory and bactericidal effects. The peptide with the highest efficacy against strain G27, C₁₂K-2β₁₂, was selected for further characterization against five additional *H. pylori* strains (26695, J99, 7.13, SS1, and HPAG1). C₁₂K-2β₁₂ displayed MIC and minimum bactericidal concentration (MBC) ranges of 6.5 to 26 μM and 14.5 to 90 μM, respectively, across the six strains after 24 h of exposure. G27 was the most sensitive *H. pylori* strain (MIC = 6.5 to 7 μM; MBC = 15 to 20 μM), whereas 26695 was the least susceptible strain (MIC = 25 to 26 μM; MBC = 70 to 90 μM). *H. pylori* was completely killed after 6 to 8 h of incubation in liquid cultures containing two times the MBC of C₁₂K-2β₁₂. The OAK demonstrated strong in vitro stability, since efficacy was maintained after incubation at extreme temperatures (4°C, 37°C, 42°C, 50°C, 55°C, 60°C, and 95°C) and at low pH, although reduced killing kinetics were observed at pH 4.5. Additionally, upon transient exposure to the bacteria, C₁₂K-2β₁₂ showed irreversible and significant antibacterial effects and was also nonhemolytic. Our results show a significant in vitro effect of C₁₂K-2β₁₂ against *H. pylori* and suggest that OAKs may be a valuable resource for the treatment of *H. pylori* infection.

Helicobacter pylori is a microaerophilic gram-negative bacterium that colonizes the gastric mucosa. It is known to be a principal gastric pathogen of humans and is associated with the development of gastritis, gastric ulcers, duodenal ulcers, and gastric cancer (46, 55, 56, 60). Approximately half of the world's population is infected with *H. pylori* (79). Thus, the bacterium poses a significant public health problem, which is further compounded by the fact that *H. pylori* has developed antimicrobial resistance to virtually all current antibiotics, a phenomenon that is hampering efforts to treat the infection (40, 51).

Since the original isolation of *H. pylori* in the early 1980s, treatment of the bacterial infection has undergone a significant evolutionary development from initial monotherapy to dual, triple, and in more recent trials quadruple therapy (8, 18). Current treatment strategies employ combination therapy, since single-antibiotic therapy often results in failure to eradicate the infection (21). The highest *H. pylori* eradication rates have been reported with triple therapy, which involves the utilization of two antibiotics in combination with bismuth or a proton pump inhibitor, PPI (34, 44). Amoxicillin (amoxicilline) with either clarithromycin or metronidazole is often the antibiotic combination of choice as a first- or second-line treatment regimen, respectively. However, in recent years the

efficacy of the standard first-line triple therapy has also been decreasing dramatically, mainly due to development of resistance to the drugs (35, 59). Failure to cure *H. pylori* infection has been noted for more than 20 to 30% of patients (37). In addition, several studies have found an eradication rate lower than 75% (6, 11, 59), and values as low as 25 to 45% have also been recently reported (22, 24). Thus, prolonged standard triple therapy for up to 2 weeks has been recommended (9, 23, 34), and in cases of eradication failure, a quadruple therapy with a proton pump inhibitor, bismuth salt, tetracycline, and metronidazole has been advised as a second-line therapy (8, 13, 44). More recently, sequential therapy (PPI and amoxicillin for 5 days, followed by PPI, clarithromycin, and tinidazole for 5 days) has become very attractive for clinical practice since impressive efficacy was seen (36, 73). However, broad adoption of this strategy as standard first-line therapy for *H. pylori* infection is still debatable because of impending validation in other geographic locations and studies to demonstrate efficacy superior to that of quadruple therapy, which is still considered a simpler regimen than sequential therapy (74). Of note, all the aforementioned therapies including sequential therapies employ multiple drugs and relatively complex regimens for the treatment of *H. pylori* infection, hence the search for new/better antibiotics.

The bactericidal activity of amoxicillin results from interference with the interpeptide linkage of peptidoglycan by binding to penicillin binding proteins and blocking their function as transporters during cell wall synthesis. Clarithromycin, like other macrolides, binds to the 50S subunit of bacterial ribosomes, thus inhibiting translocation of tRNA during translation. Binding of clarithromycin to *H. pylori* ribosomes has been

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shown to be very strong and is irreversible (27). Finally, metronidazole is a 5-nitroimidazole drug whose mode of action is mediated by nitro metabolites, such as the radical anion ($\text{NO}_2 \cdot^-$) and perhaps nitroso (RNO) and hydroxylamine (RNHOH) derivatives (39). Such metabolites have been demonstrated to cause DNA damage that results in cell death.

H. pylori resistance to amoxicillin is very rare, while resistance to clarithromycin varies significantly and may range from 10 to 25% (14). However, in a recent study, it was reported that the first-line anti-*H. pylori* triple therapies containing clarithromycin failed in 7 to 49% of patients (19, 26), indicating the underlying significant increase in antimicrobial resistance and occurrence of refractory *H. pylori* infections (32, 50, 78). Currently, PPI-amoxicillin-metronidazole triple therapy is highly effective as a second-line regimen for the treatment of *H. pylori* infection in patients showing failure of the first-line regimen (PPI-amoxicillin-clarithromycin) (47). However, high rates of resistance have been reported for people with a history of metronidazole treatment (49). Given the immense challenge in rising antimicrobial resistance (38), there is an enormous need for new antibiotics for the treatment of *H. pylori* infection.

One of the pharmacodynamic parameters most studied for antibiotics is the postantibiotic effect (PAE), which describes the suppression of bacterial growth after a short exposure of bacteria to an antimicrobial agent (29). From a clinical standpoint, PAE provides a rationale for the modification of the dosing interval of antimicrobials and could be significant for the optimization of a treatment regimen and the minimization of drug-induced adverse effects. Similarly, the success of intermittent dosing with drugs that exhibit short half-lives has been attributed to the presence of significant PAE. A long and/or positive PAE is considered an attractive characteristic for an effective new antibiotic.

In the last decade, antimicrobial peptides (AMPs) have attracted attention as potential therapeutic agents mainly due to their ability to be promptly synthesized by the host upon induction and their capacity to subsequently lyse cell membranes of pathogens through direct interaction with them. Hence, AMPs are recognized as a cell-free host defense mechanism and are important component of the innate immune systems of living organisms, including plants (76), insects (30), amphibians (75), and mammals (80). These natural membrane-lytic peptides display immense diversity in terms of sequence, secondary structural motifs, charge (cationic and anionic), and/or the abundance of certain specific amino acids (16, 66). Despite the immense diversity, a common feature for cationic AMPs is that they all form amphipathic structures that allow them to bind to the membrane interface of microbes (5, 69). Peptides which are not cationic are known to exhibit less selectivity toward microbes than toward mammalian cells, since electrostatic interactions are critical for initial binding of the peptide to membrane containing anionic lipids (45).

Oligomers of acylated lysines (OAKs) constitute a novel class of synthetic AMP mimics that consists of alternating amino acyl chains and cationic amino acids arranged to create an optimal molecular charge and hydrophobicity for enhanced potency (61, 65). This design has been reported to be advantageous over conventional AMPs by allowing the capacity for fine-tuning of the OAK structure to enhance potency against a broad spectrum of organisms while being devoid of apparent

TABLE 1. Antimicrobial OAK peptide sequences and biophysicochemical characteristics

OAK ^a	Sequence ^b	MW ^c	Q ^d	H ^e
C ₁₂ K-7 α_8	C ₁₂ K-oKoKoKoKoKoKoK	2,212.9	8	47.5
C ₁₂ K-5 α_8	C ₁₂ K-oKoKoKoKoK	1,674.1	6	49.7
C ₁₂ K-2 β_{12}	C ₁₂ K-KIK-KIK	1,234.8	5	51.0
C ₁₂ -2 β_{12}	C ₁₂ -KIK-KIK	1,106.6	4	53.3
2 β_{12}	KIK-KIK	924.3	5	38.1

^a OAK designation, where α_8 and β_{12} represent aminooctanoyl-lysyl and lysyl-aminododecanoyl-lysyl subunits, respectively; C₁₂, dodecanoic acid; K, lysine.

^b o, aminooctanoic acid; l, aminododecanoic acid. For other symbols, see footnote a.

^c Molecular weight.

^d Net charge.

^e Hydrophobicity measured using reverse-phase HPLC.

toxicity against mammalian cells (62, 64). This selective activity has been attributed to a design that lacks the secondary structures present in natural peptides (63) and to a mode of action that appears to target multiple sites, such as membranes and DNA (64). Circular dichroism studies of OAKs have demonstrated that they lack secondary structure in the presence of liposomes or hydrophobic media such as trifluoroethanol and sodium dodecyl sulfate (63). Additional characterization of OAKs with microbial pathogens other than *H. pylori* has demonstrated significant stability in the presence of serum and serum components and has shown no hemolysis of host erythrocytes (64). Two recent *in vivo* studies have also shown that administration of OAKs protected mice from an *Escherichia coli* lethal challenge (63, 64). Therefore, OAKs display characteristic features that are attractive for the development of a potent therapeutic drug.

Given the increasing antibiotic resistance rates of *H. pylori* and the current complicated treatment regime, the need for new potent antibiotics has never been greater. Given the potent effect of OAKs on other pathogens, we investigated the *in vitro* antibacterial activities of five representative OAKs against *H. pylori*. The selected sequences belong to two distinct groups: one group consisted of C₁₂K-7 α_8 and its shorter analog, C₁₂K-5 α_8 , two well characterized compounds (15, 63, 64) both of which are known for potent activity against gram-negative bacteria (25); the second group consisted of the less characterized OAKs C₁₂K-2 β_{12} and two shorter analogs, C₁₂-2 β_{12} and 2 β_{12} , for which a preliminary study (62) predicted broad-spectrum activities at least for the longer analogs. Together, these representative OAKs were anticipated to provide a preliminary structure-activity assessment on the potential activity of OAKs against *H. pylori*. Our results indicate that four of the tested peptides show efficacy against the pathogen. Of these, C₁₂K-2 β_{12} demonstrated the most potent activity, was active against a spectrum of strains, and was remarkably stable at low pH and after exposure to extreme temperatures.

MATERIALS AND METHODS

Peptide synthesis, reagents, and antibiotics. Oligo-acyl lysyls used in this study are listed in Table 1 and were synthesized as described previously (61–63). Briefly, a solid-phase method was used to synthesize peptides applying 9-fluorenylmethoxy carbonyl active ester chemistry. Peptide purity was 98 to 99% on assessment of chromatographic homogeneity by reverse-phase high-performance liquid chromatography (HPLC) (Alliance-Waters). Using a linear gradient of acetonitrile in water (1%/min), with both solvents containing 0.1% trifluoroacetic

TABLE 2. Antimicrobial activity of C₁₂K-2β₁₂

<i>H. pylori</i> strain	Reference	OAK concn (μM) ^a	
		MIC	MBC
G27	3	6.5–7.0	15–20
7.13	20	<9.0	14.5–15
J99	1	10.5–11.0	20–25
HPAG1	57	10.5–11.0	40–45
SS1	43	25.5–26	45–50
26695	72	25–26	70–90

^a MIC (100.1% survival) and MBC (99.9% killing) data represent results from at least three independent experiments.

acid, semipreparative columns (Vydac, C₄, 250 mm by 4.6 mm) were used to perform HPLC runs. Amino acid analysis and electrospray mass spectrometry were used to confirm the composition of purified peptides. Peptides were lyophilized and stored as powder at –20°C. Prior to experimentation, fresh solutions were prepared by two rounds of 2-min vortexing and sonication in a water bath to make a stock solution (1 mg/ml) that was then used in subsequent experiments.

Amoxicillin, ampicillin, vancomycin, and chloramphenicol purchased from Sigma-Aldrich (St. Louis, MO), USB Corporation (Cleveland, OH), and EMD Chemicals (Darmstadt, Germany), respectively, were reconstituted according to the manufacturer's protocol and used at the concentrations indicated throughout this article.

Bacterial strains and growth conditions. *H. pylori* strains used in this study are listed in Table 2. All bacterial strains were maintained as frozen stocks at –80°C in brain heart infusion medium supplemented with 20% glycerol and 10% fetal bovine serum (FBS). Horse blood agar (HBA) plates containing 4% Columbia agar base (Oxoid), 5% defibrinated horse blood (HemoStat Labs, Dixon, CA), 0.2% β-cyclodextrin (Sigma), 10 μg/ml of vancomycin, 5 μg of cefsulodin/ml (Sigma), 2.5 U of polymyxin B/ml (Sigma), 5 μg of trimethoprim /ml (Sigma), and 8 μg of amphotericin B/ml (Sigma), were used to grow bacteria under microaerophilic conditions at 37°C. For liquid culture, *H. pylori* was grown in Brucella broth (Difco) containing 10 μg/ml vancomycin and 10% fetal bovine serum (Gibco-BRL) with shaking in a microaerobic environment. A microaerobic atmosphere with a 5% O₂, 10% CO₂, and 85% N₂ gas mixture was achieved using an Anoxomat instrument (Spiral Biotech, Norwood, MA).

Determination of MIC and MBC. Bacteria harvested from HBA plates were used to inoculate Brucella broth liquid starter cultures containing 10 μg/ml vancomycin and 10% fetal bovine serum (complete Brucella broth medium [CBBM]). These cultures were grown for 16 to 18 h to an optical density at 600 nm (OD₆₀₀) wavelength of between 0.4 and 0.6. The starter culture was then diluted in fresh CBBM to an OD₆₀₀ of 0.05 and aliquoted in sterile 15-mm-diameter glass tubes to make 1-ml cultures containing various concentrations of OAK peptide (range of 0 to 100 μM). The inoculated tubes were incubated at 37°C with shaking at 110 rpm under microaerobic conditions for 24 h. The MICs and minimum bactericidal concentration (MBCs) were determined by enumeration of starting and ending CFU on HBA plates. The lowest concentration of OAK peptide at which there was no evidence of growth (100.1% CFU survival) was recorded as the MIC, whereas the MBC was defined as the lowest concentration of peptide that killed 99.9% of the starting CFU (0.1% CFU survival). MICs and MBCs were determined for six *H. pylori* strains in three to five independent biological assays.

Bactericidal kinetics. The killing kinetics of C₁₂K-2β₁₂ were determined after exposing *H. pylori* strains to twofold the MBC of the peptide. Briefly, 1-ml liquid bacterial cultures were diluted to an OD₆₀₀ of 0.05 in CBBM and exposed to the OAK peptide. After 0, 1, 2, 4, 6, 8, 10, 22, and 24 h of culture in the presence of peptide, 10-fold serial dilutions were plated on HBA plates. The plates were incubated at 37°C for 3 to 4 days, at which point CFU were enumerated.

RBC hemolysis assay. The hemolysis assay was performed basically as described previously (54) with minor modifications. Briefly, red blood cells (RBC) from defibrinated horse blood were washed three times in sterile phosphate-buffered saline (PBS) by centrifugation for 5 min at 2,700 × g and resuspension to a 50% hematocrit value in fresh PBS. Fifty microliters of the RBC suspension was added to microtiter wells containing 200 μl of serial twofold dilutions of OAK peptide solutions in PBS (range, 0.625 to 80 μM), PBS alone (for baseline values), or distilled water (for 100% hemolysis). After incubation for 1, 6, or 10 h with agitation at 37°C, samples were centrifuged. The hemolytic activity was assessed as a function of hemoglobin leakage by measuring the absorbance of 200

μl of supernatant at a 405-nm wavelength. Data were obtained from at least three independent experiments performed in triplicate or quadruplicate. The hemolysis percentage was calculated using the following equation: % hemolysis = [(A₄₀₅ in the peptide solution – A₄₀₅ in PBS)/(A₄₀₅ in water)] × 100, where A₄₀₅ is the absorbance measured at the 405-nm wavelength.

Peptide stability: pH and temperature. The stability of C₁₂K-2β₁₂ at low pH and after incubation at various temperatures was examined using the bactericidal kinetic assay described above. The OAK peptide was first preincubated at 4°C, 37°C, 42°C, 50°C, 55°C, 60°C, or 95°C for 1 h. The bactericidal kinetics were then determined over 24 h of culture at 37°C in a 1-ml liquid culture containing twofold the MBC of peptide (40 μM C₁₂K-2β₁₂ for strain G27). To test OAK peptide for stability at low pH, 4 M HCl was used to adjust the pH of the CBBM to pH 4.5. This CBBM was subsequently filter sterilized to remove precipitates. A bacterial starter culture was grown as described above and then diluted in a 40-ml volume to an OD₆₀₀ of 0.05 and the C₁₂K-2β₁₂ peptide added to a 40 μM final concentration. The liquid bacterial culture was incubated while shaking at 37°C for 24 h. The killing kinetics for both experiments (pH and temperature) were evaluated by enumeration of CFU of samples after plating of serial 10-fold dilutions on HBA plates. Additionally, in order to monitor variations in pH for the pH stability assay during the 24-h culture period, samples were collected to determine the pH at each time point.

Reversibility of OAK peptide effect. The reversibility of the C₁₂K-2β₁₂ effect on *H. pylori* was examined by evaluation of the killing rate following removal of the peptide. One milliliter of bacterial liquid culture with an OD₆₀₀ of 0.05 was exposed to 40 μM C₁₂K-2β₁₂ for 2 h with shaking at 37°C. At this point the bacterial suspension was washed three times in culture medium by repeated centrifugation for 2 min at 2,000 × g and resuspension in Eppendorf tubes. Following resuspension in fresh culture medium, the bacterial culture was incubated with shaking at 37°C for 24 h and bactericidal kinetics determined by enumeration of surviving CFU as described above.

Effect of growth arrest on C₁₂K-2β₁₂ activity. To determine if the activity of C₁₂K-2β₁₂ required active *H. pylori* growth, CFU were enumerated over a 24-h period from 1-ml bacterial liquid cultures containing 40 μM C₁₂K-2β₁₂ and 10 μg/ml chloramphenicol, which stops *H. pylori* growth but does not kill the bacterium. Incubation of *H. pylori* culture with chloramphenicol or C₁₂K-2β₁₂ alone was included as a control.

RESULTS

Screening of OAK peptides against *H. pylori* and determination of MIC and MBC. Given that OAKs have been shown to be effective against a number of bacterial pathogens and given the increasing problem of *H. pylori* antibiotic resistance, we screened five OAK sequences for activity against *H. pylori* strain G27. The names, sequences, molecular weights, net charges, and hydrophobicities of these AMP mimics are given in Table 1. Concentrations of each peptide ranging between 10 and 100 μM were initially screened. Four of the five OAKs examined exhibited growth-inhibitory and/or bactericidal effects against *H. pylori* strain G27 (Fig. 1). Whereas 2β₁₂ did not show any efficacy at the concentrations tested, the remaining four peptides showed various effects that could be divided into two groups: group I, strong-efficacy peptides (C₁₂K-2β₁₂ and C₁₂-2β₁₂) that showed an MBC range of 0 to 50 μM and a MIC of 0 to 25 μM; and group II, weak-efficacy peptides (C₁₂K-7α₈ and C₁₂K-5α₈) that showed an MBC of greater than 100 μM and a MIC of 25 to 50 μM.

The peptide with the strongest efficacy, C₁₂K-2β₁₂, was selected for further in-depth characterization. We chose 2β₁₂ to be used as a control peptide in subsequent experiments since it lacked efficacy against the bacteria. MICs and MBCs were determined against six *H. pylori* strains: G27, 7.13, J99, HPAG1, SS1, and 26695. These strains were chosen based on the availability of a complete genome sequence (G27, J99, HPAG1, and 26695) and the ability to colonize *H. pylori* animal models (7.13 and SS1 infect gerbils and mice, respectively).

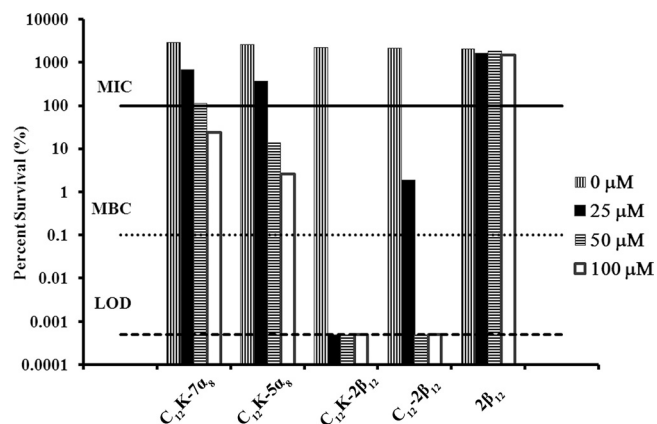


FIG. 1. Screening of five OAK sequences for antibacterial activity. The percent survival of *H. pylori* strain G27 is shown at the indicated peptide concentration. The bacterial CFU/ml was determined by plating following growth for 24 h in liquid culture containing 10 μ M, 25 μ M, 50 μ M, or 100 μ M of each OAK. Percent survival was calculated using the following equation: % survival = $CFU_{24}/CFU_{t_0} \times 100$, where CFU_{t_0} represents CFU at the beginning of the experiment and CFU_{24} represents CFU at 24 h of exposure to the peptide. The three dotted horizontal lines represent the MIC, MBC, and limit of detection (LOD). Data are representative of three independent experiments.

Based on data shown in Fig. 1, tighter OAK doses were designed, with 0.5 μ M and 5 μ M intervals for the MIC and MBC, respectively. Table 2 summarizes the results for determination of the MICs and MBCs for C₁₂K-2 β ₁₂ against the strains tested. All six strains of *H. pylori* tested were sensitive to treatment with C₁₂K-2 β ₁₂. G27 was the most sensitive (MIC = 6.5 to 7 μ M; MBC = 15 to 20 μ M), whereas 26695 was the least susceptible (MIC = 25 to 26 μ M; MBC = 70 to 90 μ M), suggesting that there are strain differences that may affect either the mode of action or the kinetics of interaction of the OAK. Importantly, efficacy of the OAK was dose dependent (Fig. 1 and data not shown), indicating that the peptides interact with a fixed number of targets on the bacteria.

OAK killing kinetics. The rate at which an antibiotic is effective is important due to the ability to maintain suitable concentrations for an affective length of exposure. Therefore, we performed time-kill assays to determine the rate of OAK-dependent killing of strains G27, 7.13, and J99. As shown in Fig. 2, C₁₂K-2 β ₁₂ exhibited rapid killing of strain G27 at both concentrations tested (20 μ M and 40 μ M). At 40 μ M (twofold the MBC of strain G27), no colonies were recovered after 10 h of treatment in liquid cultures containing the peptide. Similar results were obtained with strains 7.13 and J99 at the same peptide concentration (data not shown). It was also observed by determining the slope of the kill curves that C₁₂K-2 β ₁₂ exhibited concentration-dependent killing kinetics (Table 3 and Fig. 2). Following mathematical modeling of the curves, it was demonstrated that at the MBC, the OAK could kill 90% of bacteria in liquid culture two times slower than at concentrations twofold higher than the MBC. Thus, 90% of the bacteria for strains G27, 7.13, and J99 could be killed after incubation with the MBC of peptide in 6, 6, and 4 h, respectively, but the bactericidal rates could be increased to 3, 3.5, and 2 h upon incubation with twofold the MBC of the peptide, respectively

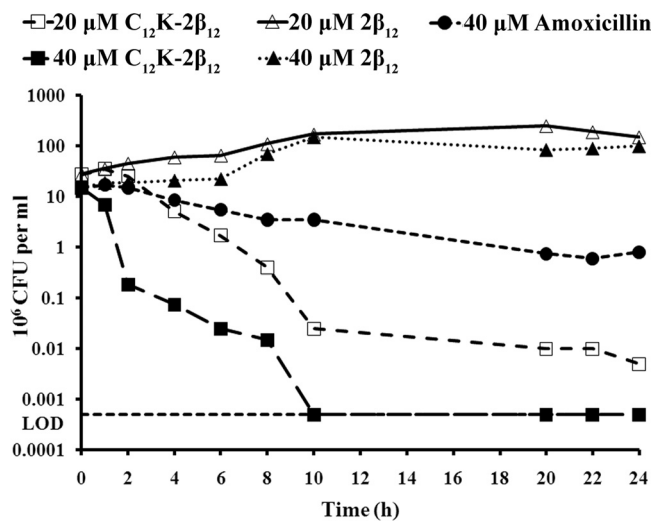


FIG. 2. Bactericidal kinetics of C₁₂K-2 β ₁₂ against *H. pylori*. Bacterial suspensions of *H. pylori* were added to liquid culture medium containing 20 μ M or 40 μ M C₁₂K-2 β ₁₂. The OAK 2 β ₁₂ and amoxicillin were used as negative and positive controls, respectively. Bacterial samples were taken at the indicated time intervals and plated to determine surviving CFU. Plotted values are representative of 11 independent experiments. The horizontal dotted line marks the limit of detection (LOD) (500 bacteria).

(Table 3). A comparison of the killing rates between amoxicillin and C₁₂K-2 β ₁₂ demonstrated superior efficacy by the OAK (Fig. 2). Additionally, testing of the killing rate of C₁₂K-2 β ₁₂ in liquid medium without vancomycin to rule out any potential interaction of the drugs revealed similar efficacy (data not shown). Taken together, these data indicate dose-dependent killing of *H. pylori* by C₁₂K-2 β ₁₂.

Mode of action: reversibility studies and killing kinetics under bacteriostatic conditions. Whether suppression of bacterial growth persists after limited exposure to an antibiotic has been established as an important pharmacodynamic parameter that is usually considered in choosing antibiotic dosing regimens. This is because in a host, there is often a gradual decrease in the antibiotic concentration to subinhibitory levels. Thus, the question of whether the damage to the bacteria is irreversible or not comes into play once antibiotic levels fall below the effective concentration. Therefore, in an attempt to evaluate whether *H. pylori* could recover from the damage imposed by exposure to C₁₂K-2 β ₁₂, the killing kinetics were evaluated under conditions in which *H. pylori* bacteria were exposed to the peptide for 2 h and the peptide withdrawn by washes. The time-kill curves were then compared to those for conditions in which the peptide was not withdrawn. The data

TABLE 3. Dose-dependent killing kinetics of C₁₂K-2 β ₁₂

<i>H. pylori</i> strain	Time-kill curve ^a slope (h for 90% killing)	
	1 MBC	2 MBC
G27	0.163 (6.1)	0.377 (2.7)
7.13	0.164 (6.1)	0.279 (3.5)
J99	0.260 (3.8)	0.567 (1.8)

^a The data represent results from at least three independent experiments.

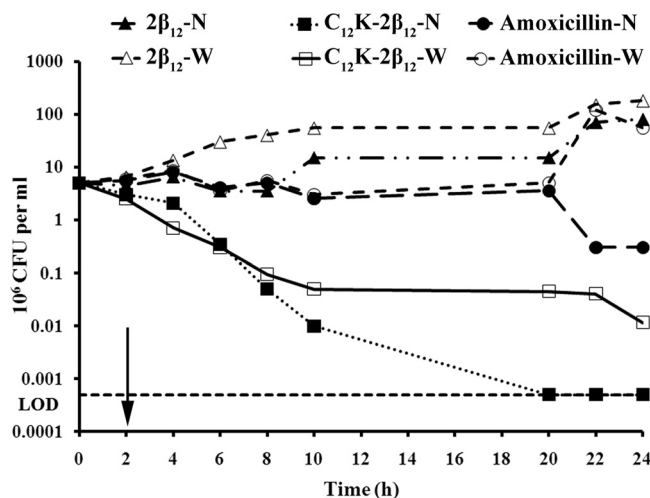


FIG. 3. PAE of the $C_{12}K-2\beta_{12}$ against *H. pylori* strain G27. Bacteria were exposed to $C_{12}K-2\beta_{12}$ at a $40 \mu\text{M}$ concentration for 2 h at 37°C for PAE determination. Equimolar concentrations of $2\beta_{12}$ and amoxicillin were used as negative and positive controls, respectively. Each line indicates the kinetics as determined by enumeration of CFU at the indicated times. The PAE curves in which the peptide was withdrawn (W) were compared with curves in which the peptide was not withdrawn (N). The arrow symbolizes the time point of peptide withdrawal, which was accomplished by three washes with Brucella broth medium. The data presented are representative of three independent experiments. The horizontal dotted line marks the limit of detection (LOD) (500 bacteria).

from these studies are presented in Fig. 3 and demonstrate that although complete killing was abrogated, the killing rate remained similar to that when the bacteria were grown under conditions where the peptide was not withdrawn. These data suggest that the killing effects of $C_{12}K-2\beta_{12}$ are irreversible. In contrast, amoxicillin showed no PAE, an observation that is consistent with previously reported findings (70).

Since some antibiotics are ineffective against slowly growing bacteria and since some bacteria grow slowly at sites of infection (17, 48), we next sought to determine whether the OAK efficacy was maintained when *H. pylori* growth was inhibited. Therefore, the killing profile of the peptide was examined under chloramphenicol-induced bacteriostatic conditions. Notably, the OAK effects were increased and antibacterial activity was sustained under bacteriostatic conditions (Fig. 4). Sequential addition of $C_{12}K-2\beta_{12}$ after 4 h of growth under chloramphenicol-induced bacteriostatic conditions was unable to further increase the activity of $C_{12}K-2\beta_{12}$ (data not shown). Taken together, these data indicate that the $C_{12}K-2\beta_{12}$ peptide does not require active bacterial growth to be effective.

OAK stability after exposure to different ambient temperatures and at low pH. Inactivation by low pH in the gastric environment as well as poor stability in ambient temperatures during transport, storage, and/or after delivery in vivo may be factors that contribute to the limited clinical efficacies of antimicrobial agents that are active in vitro against *H. pylori*. Therefore, we tested the effect of exposure to various temperatures and low pH on the stability of the OAK. As shown in Fig. 5, incubation of $C_{12}K-2\beta_{12}$ at 4°C , 50°C , 55°C , 60°C , or 95°C for 1 h prior to testing had no effect on the efficacy of the peptide against strain G27 (Fig. 5A). Similar data were ob-

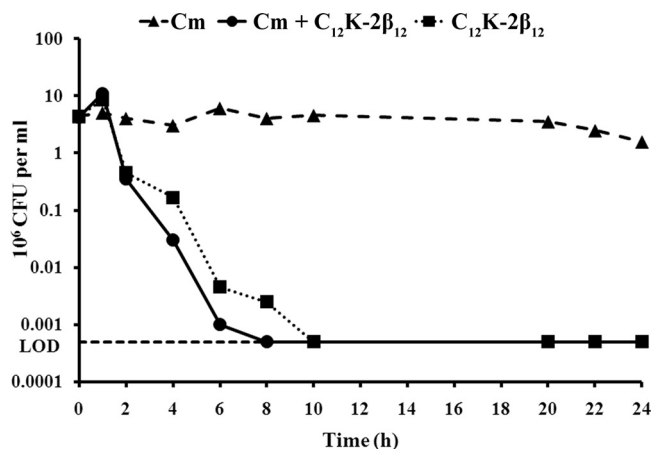


FIG. 4. Determination of OAK efficacy under static and exponential growth conditions. Bactericidal kinetics of $C_{12}K-2\beta_{12}$ against *H. pylori* strain G27 were determined when the bacterial growth was inhibited by the addition of chloramphenicol (Cm) ($C_{12}K-2\beta_{12} + \text{Cm}$) and compared to results under exponential growth conditions ($C_{12}K-2\beta_{12}$ alone). Bacteria cultured with chloramphenicol alone were used as a control. Each line indicates the kinetics as determined by enumeration of CFU at the indicated times. The data presented are representative of seven independent experiments. The horizontal dotted line marks the limit of detection (LOD) (500 bacteria).

tained with peptide preincubated at 37°C or 42°C (data not shown). Similarly, the OAK showed antimicrobial efficacy against strain G27 at pH 4.5 and pH 6.9 despite a reduced rate of killing at pH 4.5 (Fig. 5B). Taken together, these data suggest that $C_{12}K-2\beta_{12}$ is stable at extreme temperatures and shows a reduced rate of killing at low pH.

Hemolytic assay. Most peptides are cationic, and hence their interaction with anionic membrane phospholipids provides a ready explanation for their specificity for bacterial membranes (7, 10). However, some AMPs exhibit hemolytic activities (42), and delivery of such peptides becomes problematic, especially via an intravascular route. Therefore, we tested the hemolytic activity of $C_{12}K-2\beta_{12}$. As shown in Fig. 6, the OAK was non-hemolytic at concentrations from 0.625 to $40 \mu\text{M}$. We observed only minor hemolytic activity (4 to 8%) following incubation of $80 \mu\text{M}$ peptide with erythrocytes for 10 h. These results suggest that $C_{12}K-2\beta_{12}$ is nonhemolytic and may be able to be administered via various routes.

DISCUSSION

Considering the increasing resistance observed among *H. pylori* strains worldwide (38, 81), therapeutic options are becoming significantly limited. Therefore, the aim of the present study was to evaluate the antimicrobial activity of OAKs against *H. pylori*. OAKs are a class of AMPs with a novel design of linear peptidomimetic sequences consisting of alternating acyl chains and cationic amino acids (63). They have previously been tested against several strains of both gram-positive and gram-negative bacteria, including clinically challenging species (*Acinetobacter*, *Klebsiella*, and *Pseudomonas* spp.), but not against *Helicobacter* spp. (64). Here we provide in vitro experimental evidence suggesting that the OAK $C_{12}K-2\beta_{12}$ displays potent antimicrobial activity against *H. pylori*.

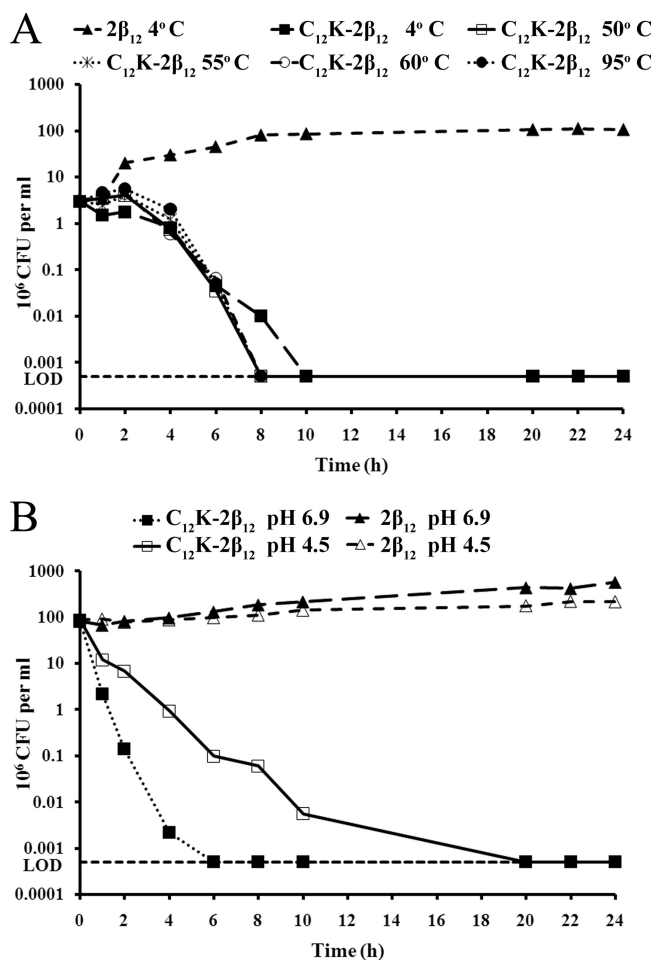


FIG. 5. Effects of temperature and pH on OAK activity and stability. (A) Temperature stability of $C_{12}K-2\beta_{12}$ was evaluated after preincubation of the peptide at 50°C, 55°C, 60°C, and 95°C for 1 h prior to the experiment. Cultures containing $2\beta_{12}$ and $C_{12}K-2\beta_{12}$ preincubated at 4°C were used as negative and positive controls, respectively. The plotted results are representative data from two independent experiments. (B) To evaluate the stability of $C_{12}K-2\beta_{12}$ at low pH, the activity was determined at pH 4.5 and compared to that at pH 6.9. The $2\beta_{12}$ peptide was used as a control. Samples at time t_0 h were plated prior to peptide exposure. The plotted results are representative data from three independent experiments. The horizontal dotted line marks the limit of detection (LOD) (500 bacteria).

We evaluated the in vitro susceptibility of *H. pylori* to OAKs by using time-kill assays. This methodology is superior to measuring the 3-log₁₀ decrease in the number of CFU per milliliter in a 24-h period (71), since it has been shown that the 3-log₁₀ difference method may sometimes leave enough bacteria to establish culture. All *H. pylori* strains tested were sensitive to treatment with $C_{12}K-2\beta_{12}$, which was the most potent of the five OAKs displaying antimicrobial activity. *H. pylori* was unable to resume growth on untreated agar plates after a 6- to 10-h treatment with concentrations equivalent to the MBC. Moreover, in a molar-to-molar comparison, the antimicrobial activity of $C_{12}K-2\beta_{12}$ was superior to that of the commercial antibiotic, amoxicillin. Notably, the six *H. pylori* strains tested exhibited various levels of sensitivity to $C_{12}K-2\beta_{12}$. While the reason for this difference is not completely clear, it is possible

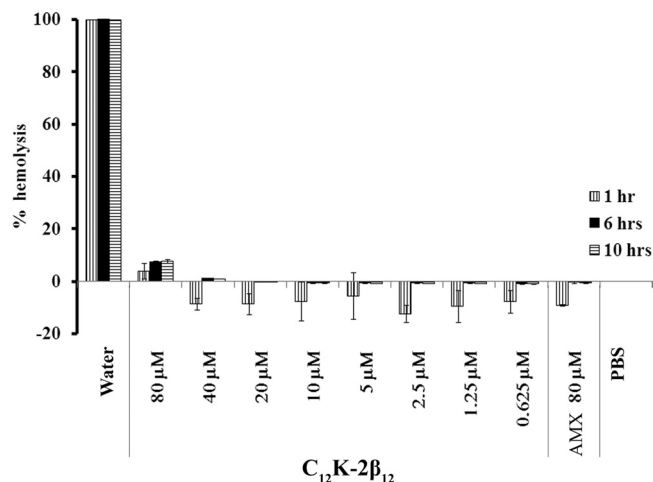


FIG. 6. Hemolytic activity of $C_{12}K-2\beta_{12}$. Erythrocytes at a 10% hematocrit value were exposed for 1 h, 6 h, or 10 h to peptide solution containing serial twofold dilutions of $C_{12}K-2\beta_{12}$ in PBS (range = 0.625 to 80 μM) at 37°C. Erythrocytes incubated in distilled water (100% hemolysis), PBS (no hemolysis), or amoxicillin solution (AMX) (80 μM) were included as controls. Hemoglobin leakage was determined by measurement of absorbance at the 405-nm wavelength. Percent hemolysis was calculated using the following equation: % hemolysis = $[(A_{405} \text{ in the peptide solution} - A_{405} \text{ in PBS}) / (A_{405} \text{ in water})] \times 100$, where A_{405} is the absorbance measured at the 405-nm wavelength. Plotted values represent the means \pm standard deviations obtained from three independent experiments performed in triplicate or quadruplicate.

that the strain-specific sensitivity observed may be related to differential expression of molecules in the anionic phospholipid cell membrane, such as lipopolysaccharides and/or some unknown specific glycoprotein(s) that interacts with $C_{12}K-2\beta_{12}$. Like other cationic peptides, $C_{12}K-2\beta_{12}$ is believed to form pores by interacting with anionic phospholipids. It is therefore expected that the stability and permeability of the cell membrane would play a fundamental role in the adaptation to different peptides/antibiotics and that these properties would be closely related to the lipid and fatty acid content. In keeping with this idea, lipopolysaccharide has been demonstrated to protect *Bordetella bronchiseptica* from the activity of AMPs (4). To our knowledge this has not been tested for *H. pylori*; however, it should be noted that strain-specific differences in sensitivity and resistance to other antibiotics have been demonstrated. Differential sensitivity to metronidazole has been shown to be due to changes in expression of pyruvate oxidoreductase (33) or gene mutations in the NADP (NADPH)-dependent nitroreductase (encoded by *rdxA*) (28) and the NADPH flavin oxidoreductase enzyme (encoded by *frxA*) (41). This suggests that *H. pylori* strain-specific effects can occur with various classes of antibiotics, and future work from our group will seek to understand the nature of these differences for $C_{12}K-2\beta_{12}$.

One of the five peptides evaluated, $2\beta_{12}$, failed to show any antimicrobial activity against *H. pylori*. This OAK also was inactive when tested against a panel of gram-positive and gram-negative bacteria (62). It is therefore possible that this short peptide may not display enough charge and/or hydrophobicity to interact well with the anionic *H. pylori* membrane,

which is consistent with the fact that optimal charge and/or hydrophobicity is critical for potency (2, 53, 62, 64). It is also noteworthy to mention that although the alpha-OAKs C₁₂K-7α₈ and C₁₂K-5α₈ showed only weak efficacy against *H. pylori*, these peptides demonstrated very strong antibacterial activity against various gram-negative bacteria, including *E. coli* (assessed by both in vitro and in vivo studies), *Salmonella* spp., and *Klebsiella pneumoniae* (63, 64). Although differences in cell membrane composition could explain the disparity in these results, the differential antimicrobial activity among the gram-negative bacteria may signal the underlying differences in specificity of peptide-target interaction in different pathogens.

An advantage of OAK peptide design is the possibility to fine-tune the structure to potentiate activity by manipulation of the acyl length and/or lysine residues (63, 64). This is evidenced in our study by comparing the antibacterial effects of C₁₂K-2β₁₂ and C₁₂-2β₁₂; C₁₂K-2β₁₂ showed stronger efficacy. Thus, the differential cationic charge created by the addition of an extra lysine residue results in increased efficacy of C₁₂K-2β₁₂. These results are similar to those obtained by Rotem and colleagues when they compared the effects of C₁₂K-5α₈ and C₁₂K-7α₈ against *Pseudomonas aeruginosa*: the two extra α subunits in C₁₂K-7α₈ were able to enhance potency (64). Taken together, these data confirm the possibility of modifying OAK peptides to optimize potency and enhance activity.

As with most bacteria, doubling times of *H. pylori* at the site of colonization are likely longer than those in vitro. Cozens and colleagues have extensively studied the influence of growth rate on the susceptibilities of members of the family *Enterobacteriaceae* and of *P. aeruginosa* to antimicrobial agents and have shown that the susceptibilities of slowly growing bacteria to antimicrobial agents are greatly reduced (12). This phenomenon may explain the failure of many promising antimicrobial agents when tested in vivo. To address this concern, we asked the following question: is the efficacy of C₁₂K-2β₁₂ against *H. pylori* influenced by the growth rate? Our data demonstrate that C₁₂K-2β₁₂ exhibits antibacterial activity regardless of whether the bacteria are actively growing, indicating that slow growth in the stomach may not influence efficacy of the peptide.

We determined the OAK stability by looking at the effect of extreme temperatures (range, 0 to 95°C) and pHs (4.5 and 6.9) on activity. Our data demonstrated that C₁₂K-2β₁₂ is stable at the afore-mentioned temperatures and at low pH, albeit reduced activity was observed at low pH. pH influences the ionization of charged groups and consequently affects downstream interactions. This fact may explain the slightly reduced kinetics of the C₁₂K-2β₁₂ peptide at low pH. The robust stability suggests slow OAK peptide degradation, sustained antimicrobial activity in the acidic environment of the stomach, and the possibility of overcoming cold-chain storage problems in a clinical setup. Indeed, these results are in accordance with those in studies with the cationic peptide nisin, which have suggested low-pH-induced reduction of peptide net charge and reduced potency (76). In the host, however, *H. pylori* survives strong acidity of the stomach using the enzyme urease to convert gastric urea into ammonia, which then neutralizes the bacterial cytoplasm and microenvironment (52, 58, 67, 68, 77). Thus, the reduced kinetics observed in vitro might not apply in

the host, since the local microenvironment inhabited by the bacteria would have a near-neutral pH.

Persistent suppression of bacterial growth after a short exposure to an antibacterial agent, PAE, was first noted and described more than 65 years ago. Since then, several studies have been undertaken to determine the PAE of numerous antimicrobial agents that have further established the significance of PAE in drug development. Today, it is one of the minimum recommendations in a preclinical evaluation of all new antimicrobial agents that determination of the PAE be performed. This is essentially because PAE is a factor that influences optimal antimicrobial dosing intervals. It is generally an accepted clinical observation that antibiotics without a PAE would usually require more-frequent administration than agents exhibiting PAE. PAE is thus an important pharmacodynamic predictor of clinical application of antibiotic dosage in the drug development process. We performed a PAE assay for C₁₂K-2β₁₂ against *H. pylori* and made a direct comparison of time-kill curves of treated and untreated bacterial cultures using the classical viable-count procedure. Our data demonstrate a significant and irreversible PAE of the OAK against *H. pylori*, a characteristic feature that makes the peptide promising for future drug development. The cellular and molecular events involved in the significant PAE observed here are largely unknown. However, it is reasonable to suggest that the irreversible significant PAE observed might be related to the level of damage done by the peptide to the bacterial cell. Thus, this might indicate that the damage to the bacterial cell is very profound or at least irreparable, a speculation that is consistent with the fact that if the damage is not fixable, then the bacterial cell dies. It is also noteworthy that the amoxicillin data are consistent with the results of a previous study (31) in which other β-lactams were shown to lack PAE against slowly growing *H. pylori*.

In conclusion, our data demonstrate strong in vitro antimicrobial activity of C₁₂K-2β₁₂ against *H. pylori*. Our results suggest that the activity of the OAK is irreversible and sustainable regardless of bacterial growth. Additionally, the peptide is stable at a wide range of temperature and pH conditions. This is the first characterization of synthetic OAK peptides against *H. pylori*, and our results indicate that C₁₂K-2β₁₂ may, in principle by itself, have strong therapeutic potential against *H. pylori*. Future work will investigate the in vivo efficacy of C₁₂K-2β₁₂ using *H. pylori* animal models.

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