

In vitro synergistic effects of a short cationic peptide and clinically used antibiotics against drug-resistant isolates of Brucella melitensis

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Abstract

Purpose. In the last few decades, increasing microbial resistance to common antibiotics has attracted researchers’ attention to the development of new classes of antibiotics such as antimicrobial peptides. Accordingly, the aim of the current study was to evaluate antimicrobial effects of the CM11 peptide alone and combined with common antibiotics against drug-resistant isolates of Brucella melitensis.

Methodology. A total of 50 pathogenic samples of B. melitensis were isolated from patients and their antibiotic susceptibility pattern was evaluated by E-test. Then, the synergistic reaction of the peptide with selected antibiotics was evaluated using a chequerboard procedure.

Results. Based on the susceptibility pattern of isolates, ciprofloxacin, rifampin, streptomycin and co-trimoxazole were used for synergistic study. According to the results, synergic effect was observed for streptomycin and co-trimoxazole in combination with the peptide while ciprofloxacin and rifampin showed partial synergy and additive effect, respectively. Consistent with these results, in the time-killing assay, a decrease in colony counts for streptomycin-peptide and co-trimoxazole-peptide was >2 Log_{10} while for ciprofloxacin-peptide and rifampin-peptide it was about 1.5 Log_{10} and <2 Log_{10}, which represents synergy, partial synergy and additive interaction, respectively.

Conclusion. These results showed that by antibiotic-CM11 combination, their effective dose can be reduced particularly for drug-resistant isolates. In conclusion, considering the importance of brucellosis caused by B. melitensis in the Middle East beside reports on antibiotic resistance strains, especially against rifampin, which may literally lead to an increase in resistant strains of Mycobacterium tuberculosis in endemic areas, our findings can be used to develop a suitable alternative treatment for brucellosis, and with less risk.

INTRODUCTION

Infection by Brucella species is a major cause of zoonotic diseases. In humans, brucellosis is usually caused by Brucella melitensis and is acquired mainly through contact with infected animal tissues, ingestion of unpasteurized dairy products, or infectious aerosols [1–3]. Brucellosis is endemic in many parts of the world, including Mediterranean countries, the Middle East, Southwest and Central Asia, Africa and Latin America. Based on WHO reports, more than 500 000 new cases of human brucellosis appear every year, with highest recorded incidence of human brucellosis occurring in the Middle East and Central Asia [4, 5]. Based on recent reports, Iran is also an endemic area for brucellosis. Among the existing Brucella spp., B. melitensis is the most prevalent species in Iran with 34 cases per 100 000 recorded each year [6, 7]. According to WHO guidelines, drugs commonly recommended for the treatment of Brucella infections include doxycycline, rifampin, streptomycin, gentamicin and co-trimoxazole; generally two or three of these are used in combination, especially doxycycline and with other antibiotics. It is noteworthy that since Brucella species are intracellular pathogens, antibiotic therapy with confirmed intracellular activity is the optimal therapeutic regimen in brucellosis, thus effective antibiotics are limited [8]. Therefore, this recommendation is made in order to avoid recurrence and to prevent prolonged use of these...
antibiotics which may lead to the problem of drug resistance arising [9]. Unfortunately, in recent years, the extensive and inappropriate use of antibiotics has led to resistance of many bacterial pathogens to conventional antibiotics, especially in socioeconomically poor countries; hence, in these areas, antibiotic resistance is an important issue in people infected with Brucella or other pathogens [9, 10]. On the other hand, due to the rates of treatment failure or recurrence and the similarity between geographical areas of brucellosis and tuberculosis, as well as the therapeutic regimen of both infections, antibiotic resistance in Brucella species has received much attention in recent years [5, 11]. Based on this issue, research aimed at finding new antimicrobial agents and/or therapeutic strategies is much more important. Among the large number of agents that are currently under evaluation for infection treatment, antimicrobial peptides (AMPs) are promising candidates as novel therapeutic compounds. AMPs, especially cationic peptides, are important members of the host defence system in eukaryotes, with the ability to kill a broad spectrum of microorganisms such as Gram-negative and Gram-positive bacteria, fungi and viruses that are found in almost all forms of life, from bacteria to plants, vertebrates and invertebrates [12]. In nature, most AMPs are cationic with hydrophilic and hydrophobic regions which are the main factors for their antimicrobial activity. However, the practical use of AMPs has limitations, such as toxicity against some eukaryotic cells [12, 13]. In this regard, combinational prescription and synergistic effects can help to decrease the resistance emergence in microorganisms such as bacteria, and also reduce toxicity through using lower doses of both agents [14]. Accordingly, in this study the CM11 peptide (WKLFIKILKVL-NH2) as a cationic peptide was used in combination [14] with antibiotics commonly used to treat brucellosis, to evaluate the synergistic effects of the peptide and related antibiotics.

CM11 is an amphipathic hybrid peptide derived from two to eight cecropin A residues and six to nine residues of melittin with two parts that consist of a highly basic N-terminal domain from cecropin A and a relatively hydrophobic C-terminal domain from melittin [13].

METHODS

Peptide synthesis

For the synthesis of the CM11 peptide, a solid-phase synthesis method was utilized using p-methylbenzhydrolamine resin [15]. The peptide was purified with reversed-phase semi-preparative HPLC on the C18 Tracer column. The peptide purity was more than 95 %. Electrospray ionization mass spectrometry was also used to confirm peptide identity.

Bacterial isolates

A total of 50 B. melitensis isolates were obtained from blood specimens of patients with a clinical diagnosis of brucellosis. Antibody titres ≥1:160 by the Coombs test and titres ≥1:40 by the 2-mercaptoethanol (2-ME) test were considered as a positive serology test. The isolates were retrieved from the Central Laboratory Hospitals in Tehran, Kerman, Hamedan and Arak, Iran, confirmed with standard microbiological laboratory tests. The samples were incubated at 37°C for 48–72 h in Mueller–Hinton (MH) broth plus 5% sheep blood and Brucella agar media. In this study, we also used B. melitensis ATCC 23456 as a control.

Selected antibiotics

According to the Clinical Laboratory Standards Institute (CLSI) guidelines [16] and recommendations, the selected antibiotics were doxycycline (DOX), ciprofloxacin (CIP), rifampin (RIF), streptomycin (STM), tetracycline (TET), gentamicin (GEN) and co-trimoxazole (CTZ), which were purchased as E-test strips (bioMerieux, Marcy L’Etoile, France) and powder (Sigma-Aldrich, USA).

Antimicrobial susceptibility testing and determination of selected antibiotic minimum inhibitory concentrations (MICs)

For antimicrobial susceptibility tests, an initial inoculum of 1.5×10^8 c.f.u. ml^-1 was prepared from Brucella isolates according to the CLSI protocol for measuring in vitro susceptibility of bacteria to antimicrobial agents. Then, bacterial suspension was cultured on Mueller–Hinton agar plates supplemented with 5% blood, and E-test strips were used separately for the cultured plates as recommended by the manufacturer. The plates were incubated at 37°C for 48–72 h under aerobic conditions (5% CO2) [11]. Determination of the MIC was performed in accordance with the recommended reference values of the CLSI guidelines for selected antibiotics. The MIC₅₀ and MIC₉₀ values indicate that the relevant concentration inhibits the growth of 50 or 90% of the bacteria, respectively. The antibiotic resistance rates were determined by measuring the diameter of inhibition zones, then isolates with the highest antibiotic resistance rates were selected among the samples.

Determination of peptide MIC

The peptide was solubilized in phosphate-buffered saline (pH 7.2) to achieve 1 mg ml^-1 concentration. To measure the antibacterial activity of the peptide, the MIC was determined using the broth microdilution method on a Mueller–Hinton broth culture medium. An initial inoculum of 1.5×10^6 c.f.u. ml^-1 was prepared according to the procedures outlined by the CLSI. Bacterial cultures with peptide concentrations ranging from 0.125 to 256 mg ml^-1 were incubated in a shaking bath for 18 h at 37°C. The lowest concentration that inhibited bacterial growth was considered as the MIC. The minimum bactericidal concentration (MBC) was taken as the lowest concentration of each drug that resulted in more than 99.9% reduction in the initial inocula. Experiments were performed in triplicate.

Bacterial sample preparation for scanning electron microscopy (SEM)

Bacteria in the mid-exponential growth phase were diluted with a salt-free medium to the cell density mentioned above and treated with the peptide at concentrations equal to
0.5 × MIC, MIC and 2 × MIC for 2 h at 37 °C. Next, 10 µl of the treated cell suspension was placed onto a 0.45 µm pore membrane filter (Schleicher and Schuell, Dassel, Germany), fixed for 1 h with 3% glutaraldehyde in 0.1 M phosphate buffer pH 7.2, washed and post-fixed with 1% OsO₄. The samples were dehydrated with graded ethanol series and then air-dried. For washing and dilution of fixing reagents, 0.15 M of phosphate-buffered sodium (pH 7.2) was used. A small amount of platinum was sputtered on the samples to avoid charging in the microscope. Microscopy was performed with a Zeiss Supra 55VP (Germany) microscope. Secondary electron images were taken at low electron energies between 2 and 2.5 keV.

**MIC combination assay**

The chequerboard method was used for assessment of the MIC of antibiotics in combination with peptides, based on the broth microdilution method in accordance with the CLSI protocols using cation-adjusted Mueller–Hinton broth [17]. It is noteworthy that for the combination assay we selected isolates with higher MICs and/or resistance to selected antibiotics. A two-dimensional chequerboard with twofold dilutions of each agent was set up according to the protocol described in our previous study [14]. Briefly, the concentration ranges were based on the MICs of the peptide and each antibiotic with values between 4 × MIC and 0.25 × MIC. Each plate contained two controls, including the medium alone and bacterial culture. After 18 h of incubation at 37 °C, the MIC was defined as described above. The interaction of the CM11 peptide (A) with each antibiotic (B) was evaluated by the fractional inhibitory concentration index (FIC). This index is calculated according to the equation: FICI = FIC A + FIC B = (MIC A in combination/MIC A alone) + (MIC B in combination/MIC B alone). FICI was interpreted as follows: FICI < 0.5 synergy, 0.5 < FICI ≤ 1 partial synergy, 1 < FICI ≤ 4 additive effect or indifference, < FICI antagonism. Each test was performed in triplicate [14, 18, 19].

**Bacterial killing assay**

We selected isolates with higher MICs for peptides and antibiotics, and then the bacterial killing assay was performed according to CLSI guidelines. Selected antibiotics and peptides alone and in combination with MICs were tested against bacteria. Test tubes containing Mueller–Hinton broth supplemented with peptides and antibiotics, alone and in combination, were inoculated with fresh 1.5 × 10⁸ c.f.u. ml⁻¹ B. melitensis isolate and incubated at 37 °C. Aliquots were sampled at 0, 0.5, 1, 2, 4, 8, 16 and 24 h, then diluted and cultured on Mueller–Hinton agar plates for cell counting. Synergistic interaction was defined as a ≥ 2 Log₁₀ decrease in c.f.u. ml⁻¹ between the combination and the most active agent at 24 h, while in some studies partial synergy was defined as ≥ 1.5 Log₁₀ decrease in c.f.u. ml⁻¹. Also, additive or indifference effects were defined as a < 2 Log₁₀ c.f.u. ml⁻¹ increase or decrease in colony count at 24 h by the combination compared with the most active single agent. Antagonism was defined as a ≥ 2 Log₁₀ increase in colony count in an overnight period by the combination [14, 20, 21].

**Statistical analysis**

The data in each experiment were representative of three independent experiments expressed as the mean ± standard deviation (SD). The statistical significance of the differences between the control and test values was evaluated using a one-way ANOVA t-test.

**RESULTS**

**Antimicrobial susceptibility and drug-resistant isolates**

The susceptibility of all isolates to selected antibiotics is shown in Table 1. Doxycycline was the most effective antibiotic against isolates with MIC < 0.125 and MIC < 0.19, while streptomycin and rifampin, with MIC = 1, MIC = 3 and MIC = 0.75, MIC = 1, respectively, showed the highest MICs against most of the isolates. Of all samples, a limited number of isolates showed drug resistance to ciprofloxacin, streptomycin, rifampin and co-trimoxazole, with MICs of 1.5, 8, 2 and 2 (mg l⁻¹), respectively. Therefore, these isolates and antibiotics were chosen to investigate peptide and antibiotic synergistic effects.

**MIC peptide determination**

The CM11 peptide showed minimum inhibitory and bactericidal concentration, ranging respectively from 0.25 to 64 and 4 to 128 mg l⁻¹, against clinical isolates of B. melitensis isolates. Also, MIC = 32 and MBC = 64 mg l⁻¹, respectively. The MIC and MBC of peptides against B. melitensis ATCC 23456 were 16 and 64 mg l⁻¹, respectively.

**Scanning electron microscopy**

As shown in Fig. 1, treatment with MIC and 2 × MIC concentrations induced membrane surface disruption in comparison to 0.5 × MIC and control.

**Synergistic effects between the CM11 peptide and antibiotics**

Our results showed positive synergistic effects between the peptide and ciprofloxacin, streptomycin, rifampin and co-

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<th>Antibiotics</th>
<th>Minimum inhibitory concentration (mg l⁻¹)*</th>
<th>Breakpoints for susceptibility [16]</th>
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<td>Range</td>
<td>MIC₅₀</td>
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<tr>
<td>Doxycycline</td>
<td>0.064–0.19</td>
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<td>Ciprofloxacin</td>
<td>0.064–1</td>
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<td>Streptomycin</td>
<td>0.038–8</td>
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<td>Rifampin</td>
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<td>Tetracycline</td>
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<td>Gentamicin</td>
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<td>Co-trimoxazole</td>
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*The data in each column represents three independent experiments (P < 0.05).
trimoxazole. According to FICI, the combination effect of streptomycin and co-trimoxazole with the peptide was synergistic while the combination effect for ciprofloxacin and rifampin was partially synergic and additive, respectively (Table 2).

### Bacterial killing assay

Viable counts of resistant *B. melitensis* isolates to four antibiotics, alone and in combination with the CM11 peptide at MIC concentrations, are shown graphically in Fig. 2. As shown in Table 2, we selected four isolates each of which were resistant to ciprofloxacin (1 mg l\(^{-1}\)), streptomycin (8 mg l\(^{-1}\)), rifampin (1.5 mg l\(^{-1}\)) and co-trimoxazole (2 mg l\(^{-1}\)). The peptide MICs for these isolates were 8, 16, 32 and 32 mg l\(^{-1}\), respectively. Time-killing results showed a decrease in the colony counts of bacteria in combinations of the peptide and antibiotics, so that reduction in colony counts for the streptomycin-peptide and co-trimoxazole-peptide combination was >2 Log\(_{10}\) (synergism), in comparison with the peptide alone as the most active single agent (Fig. 2a, b). Also, the reduction in colony counts for the ciprofloxacin-peptide combination was about 1.5 Log\(_{10}\) which represents a partial synergy reaction (Fig. 2c). Time-killing results for the rifampin-peptide combination showed additive interaction which was the same as the checkerboard result (Fig. 2d).

### DISCUSSION

Brucellosis as an occupational and travellers’ disease is a major global public health problem. The disease has a wide spectrum of clinical manifestations and can affect a variety of organs and systems; therefore, its treatment is considered a major issue in its control [22, 23]. The most recent recommendation by WHO for the treatment of acute brucellosis in adults was published in 1986 [24]; although many studies have been conducted in the past three decades, there is no conclusive evidence on the optimum antibiotic therapy for brucellosis, and disease relapse still represents one of the most important therapeutic problems. On the other hand, current recommended treatment regimens involve the use of two or more antibiotics, which may lead to problems of drug resistance arising due to their long-term use [9]. Considering the importance of brucellosis caused by *B. melitensis*, especially in the Middle East and Iran [4, 11, 25], and reports about antibiotic-resistant strains particularly to rifampin [1, 4], which may lead to an increase in the resistant strains of *M. tuberculosis* in endemic areas [26], the main objective of this study was to provide a new strategy for treatment of *Brucella* infections by focusing particularly on antibiotic resistance. For this purpose, we evaluated the synergistic effect of CM11 as a cationic antimicrobial peptide in combination with antibiotics recommended for brucellosis. According to many studies, AMPs are effective candidates for treatment of bacterial infections, but due to the lack of specific cell targeting, their practical use has its own limitations such as toxicity to eukaryotic cells [27]. To overcome this problem, one solution is to use peptides synergistically with conventional antibiotics.

In this study, the peptide showed a synergistic effect with streptomycin and co-trimoxazole, partial synergy with ciprofloxacin and rifampin, which led to a significant decrease in colony count. Streptomycin is one of the main antibiotics in the treatment regimen of brucellosis; however, it is well known that long-term use of aminoglycosides such as streptomycin increases the risk of nephrotoxicity (if used systemically) or ototoxicity, particularly affecting the vestibulo-cochlear system [28], which limits the therapeutic usage of this class of antibiotics. In our study, we showed that the antibactericidal activity of...
streptomycin was significantly enhanced when combined with the CM11 peptide. On the other hand, because hepatotoxicity and damage to the liver are the most important side effects of rifampin, individuals who received the drug over a long period should be tested for liver function. The synergistic effect between rifampin and the peptide was additive, with a suitable reduction in colony count making it a remarkable potential alternative in the treatment protocol.

Previously, we investigated the in vitro synergistic effect of the CM11 peptide in combination with common antibiotics commonly used against Pseudomonas aeruginosa, Staphylococcus aureus, Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae and Salmonella typhimurium [14]. Our findings showed the synergic effect of the CM11 peptide in combination with penicillin, ceftazidime, norfloxacin and cefotaxime, while the effect of the combination of ciprofloxacin and peptide was partially synergistic, which is inconsistent with the results presented in this study. Similar to our studies, Cirioni et al. [29] investigated a combination of magainin II and cecropin A antimicrobial peptides and rifampin against P. aeruginosa. Their results showed a synergistic effect between the peptides and rifampicin both in vitro and in vivo. They reported that the best results regarding mortality rates and bacteraemia were obtained when these peptide were combined with rifampicin. This combination was also most effective in decreasing the level of some cytokines, which confirmed the capacity of magainin II and cecropin A to neutralize cell-wall components that are the inducers of cytokine activation. According to this report and other studies, cationic AMPs have a key role in anti-inflammatory action which is of crucial importance in decreasing the level of some cytokines, which contributes to the resolution of the inflammatory process and must be able to penetrate the bacterial cell envelope, especially in Gram-negative bacteria where the outer membrane acts as a selective and formidable barrier. Therefore, the permeability properties of this barrier have a major impact on the susceptibility of the microorganism to antibiotics [36]. Studies have also shown that cationic peptides such as the CM11 peptide act as a cell-permeable agent because their positive charge causes electrostatic interactions with the poly-anionic surface of the bacterial cell, including lipopolysaccharide in Gram-negative bacteria and teichoic acid in Gram-positive bacteria. Therefore, their amphiphilic properties enable them to penetrate the hydrophobic region of the cell membrane, which leads to pore formation or voltage-gated channels, resulting in the leakage of essential cellular components or passage of hydrophobic compounds across the cell membrane (Fig. 1) [12, 37, 38]. Accordingly, pores formed by cationic peptides can increase the permeability of the membrane, causing antibiotics to penetrate more quickly. Thus, it seems that permeabilization of the outer membrane might describe positive interactions between the peptide and antibiotics, especially hydrophobic antibiotics such as rifampin and streptomycin. On the other hand, studies have shown that drug influx in Gram-negative bacteria is facilitated by protein channels called porins. These proteins provide a path through the outer membrane for small hydrophilic antibiotics, such as quinolones. In bacteria generally, the cellular concentration of quinolone antibiotics such as ciprofloxacin is regulated by the opposing actions of diffusion-mediated drug uptake and pump-mediated efflux. There is an abundance of antibiotic resistance reports acquired through loss or functional change of porins in a large number of bacteria. In addition, enhanced expression of efflux pumps can also lead to quinolone resistance [36, 39]. As described above, pore formation by cationic peptides in the outer membrane as an alternative route can lead to the availability of these antibiotics inside bacteria.

| Table 2. MICs and FICI for the CM11 peptide and selected antibiotics in combination and alone |
|---------------------------------|---------------------------------|-----------------|-----------------|-------------------|
| Combinations*                  | MICs alone (mg l⁻¹)             | MICs in combinatio (mg l⁻¹) | FICI | Combination effect |
| Streptomycin-peptide           | STM                             | 8                             | 4 | 0.5 | Synergy |
|                                | CM11                            | 16                            | 8 | 0.25 | Synergy |
| Co-trimoxazole-peptide         | CTZ                             | 2                             | 0.25 | 0.25 | Synergy |
|                                | CM11                            | 32                            | 4 | 0.75 | Partial synergy |
| Ciprofloxacin-peptide          | CIP                             | 1                             | 0.625 | 0.75 | Partial synergy |
|                                | CM11                            | 8                             | 1 | 1 | Additive |
| Rifampin–peptide               | Rif                             | 1.5                           | 0.625 | 1.25 | Additive |
|                                | CM11                            | 32                            | 16 | |

*The data in each column represent three independent experiments (P<0.05).

MIC, minimum inhibitory concentration; FICI, fractional inhibitory concentration index.
the bacterial cell or antibiotic recycling to the bacteria cell. Co-trimoxazole, a combination of trimethoprim and sulfamethoxazole that blocks folate synthesis, is another antibiotic showing a synergistic effect in combination with the CM11 peptide. Based on reports, resistance to sulfamethoxazole occurs as a result of either decreased permeability (most common) or increased production of P-amino benzoic acid (PABA), while resistance to trimethoprim develops due either to the synthesis of an enzyme with lower affinity for TMP or by the overexpression of dihydrofolate reductase [40]. Consequently, it seems that synergy between cotrimoxazol and peptides is associated with increased permeability of the outer membrane to sulfamethoxazole.

However, although these results are remarkable, the therapeutic applications of AMPs also have some limitations for drug development, including sensitivity to protease, pH change and also toxicity and the high cost of peptide production [41]. In order to overcome these obstacles, many methods have been proposed, for example using unusual amino acids (D-form amino acids) or modification of the terminal regions, such as amidation and acetylation, that improve the stability of peptides. In this regard, the CM11 peptide was modified in the C-terminal by adding an amide group [12]. Also, using drug delivery systems, such as liposome encapsulation, can be effective for the improvement of stability and reduction of potential toxicity [42]. On the other hand, similar to the results presented in this study, the synergistic interactions of AMPs and common antibiotics can help to reduce the dose of peptides and some antibiotics, which leads to a reduction in toxicity, cost and the risk of resistance.

In the present study, as basic in vitro research, we demonstrated antibacterial activity of the CM11 peptide against drug-resistant B. melitensis and their positive synergistic
effects with selected antibiotics recommended for brucellosis; however, further investigation is recommended. For example, *Brucella* spp. are intracellular pathogens evolved to exist in host macrophages in the presence of a number of host-imposed stresses, such as antimicrobial peptides [43]. In macrophages, production of antimicrobial peptides is one of the defence strategies against bacteria, especially intracellular pathogens, and so the ability of such bacteria to survive within macrophage cells requires resistant mechanisms in order to avoid being killed. Considering this, bacteria, particularly intracellular pathogens, have different strategies for sensing and resisting AMPs. Similarly, in a novel study, it has been shown that the *yej* operon in the *B. melitensis* genome is important for resistance to host AMPs and their persistent survival. Also, resistance to host antimicrobials is a key mechanism of persistent infection for *Brucella*. Thereupon, it is possible that prolonged use of similar peptides against *Brucella* leads to the development of resistance; however, the antibiotic–peptide combination can reduce this risk.

Finally, *in vitro* results showed significant antibacterial activity of the CM11 peptide against *B. melitensis* alone and in combination with selected antibiotics with synergistic reactions. Our findings can be used to develop and provide a suitable alternative treatment for Brucellosis with less risk in comparison to current treatment regimens. However, as described above, for *in vivo* applications, more studies are needed including investigation of the effect of peptides and antibiotic–peptide combinations on *B. melitensis*-infected macrophages.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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