SHORT COMMUNICATION

Antibacterial, anti-biofilm, and anti-adhesive activities of melittin, a honeybee venom-derived peptide, against quinolone-resistant uropathogenic Escherichia coli (UPEC)

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\textbf{ABSTRACT}

Here, we demonstrated the in vitro and in vivo antibacterial and anti-biofilm activities of melittin, a peptide derived from honeybee venom, against uropathogenic Escherichia coli (UPEC) resistant to quinolones. The minimum inhibitory concentration (MIC) of melittin varied from 0.5 to 8 \( \mu \text{M} \). The bactericidal effect was considered rapid and potent (ranging from 3.0 to 6.0 h after incubation) against a quinolone-resistant and Extended Spectrum Beta-lactamase (ESBL)-producing UPEC strain. Prior exposure to melittin did not reduce the MIC of the quinolones tested, but it decreased the MIC of ceftizoxime by 8-fold due to its ability to form pores in the membrane. Furthermore, melittin disrupted mature biofilms (39.58\% at 32 \( \mu \text{M} \)) and inhibited the adhesion of this uropathogen to the surfaces of urethral catheter. These results show that melittin is a promising molecule that can be incorporated into invasive urethral medical devices to prevent urinary infections caused by multidrug-resistant UPECs.

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1. Introduction

*Escherichia coli*, a member of the *Enterobacteriales* family, is a Gram-negative glucose-fermenting rod that typically inhabits the gastrointestinal tract of humans and animals (Vila et al. 2016). As a commensal, it lives in a mutually beneficial association with hosts. However, some *E. coli* strains are commonly implicated in relevant clinical infections, such as urinary tract infections (UTI) (Kaper et al. 2004). Indeed, community-acquired UTIs (CA-UTI) caused by uropathogenic *E. coli* (UPEC) are the most common bacterial infections, affecting approximately 150 million people annually worldwide (mainly women) (Kucheria et al. 2005). The antibiotics fosfomycin and sulfas are the first choices to treat CA-UTI; however, quinolones have been the most frequently prescribed antibiotic in these cases. This indiscriminate use of quinolones has increased the selective pressure on uropathogens, significantly increasing the incidence of quinolone-resistant UPECs (Lee et al. 2016; da Silva et al. 2017; Vieira et al. 2020).

Infections caused by quinolone-resistant UPECs usually evolve to life-threatening complications, such as pyelonephritis, bacteraemia, and florid urosepsis. These complications are treated with intravenous antibiotic therapy, in which only a few options are available (e.g. polymyxins, aminoglycosides, cephalosporins) (Nicolle et al. 2005; Yamamoto 2016). To make this scenario even more critical, quinolone-resistant UPEC strains typically carry enzymes that encode resistance to other antibiotics, mainly broad-spectrum beta-lactamases (ESBL) associated with resistance against cephalosporins, and the aminoglycosides acetyltransferase that modifies gentamicin, amikacin, and tobramycin (Ali et al. 2016; Halaji et al. 2020). Therefore, the development of new pharmacological agents against quinolone-resistant UPECs is urgently needed. In this context, antimicrobial peptides stand out as a promising source of new anti-UPECs antibiotics. These agents are known to possess a potent bactericidal effect, low capacity to induce resistance, good efficacy in low concentrations, absence of waste
generation after the use of conventional doses, and potent immunomodulatory effect (Bechinger and Gorr 2017; Chen and Lu 2020; Lima et al. 2021). Moreover, antimicrobial peptides are usually highly hydrophilic and thereby captured by the urinary tract after an intravenous administration (Kang et al. 2014; Chen and Lu 2020). The latter is a significant pharmacokinetic advantage in the case of UTIs.

One of the best consolidated sources of antimicrobial peptides is the toxin of venomous animals (Wu et al. 2018), especially arthropods (Samy et al. 2017). Honeybee (*Apis mellifera* L.) venom contains a complex mixture of therapeutic compounds, including antimicrobial peptides, allowing bees to defend their hives against predators and external threats (Leandro et al. 2015; El-Seedi et al. 2020). Several biological and pharmacological studies have examined the peptide melittin, which is the major component of bee venom (40–48%, w/w) (Memariani et al. 2019). This bee venom-derived peptide has been extensively investigated and exhibits potent cytolytic and antimicrobial activities (Choi et al. 2015; Lima et al. 2021). However, little is known about its antibacterial potential against clinically relevant multi-resistant species, such as UPECs (Lima et al. 2021). Therefore, this study aims to evaluate the antibacterial effect of melittin against several planktonic quinolone-resistant UPECs, as well as to investigate its effect on the biofilms formed by these pathogens.

### 2. Results and discussion

Community-acquired urinary tract infections (CA-UTIs) are caused mainly by UPECs (70–90% of all cases) and exhibit a high recurrence rate, which increases morbidity rates worldwide, especially in women (Kucheria et al. 2005). The treatment of CA-UTIs is based on the use of various classes of antimicrobials, of which quinolones are considered the second-line treatment against UPECs. However, due to the indiscriminate use of this class of antibiotics, strains of quinolone-resistant UPEC have been extensively reported in the last decades (Lee et al. 2016; da Silva et al. 2017; Vieira et al. 2020). In this context, the development of new antibacterial agents against quinolone-resistant UPECs is crucial. Antimicrobial peptides derived from animal toxins are a rich source of new biologically active compounds, and the potential of honeybee venom needs to be evaluated (Primon-Barros and Macedo 2017; El-Seedi et al. 2020). Thus, the antibacterial activity of melittin, a peptide derived from honeybee venom, was investigated in this study against 41 clinical isolates of quinolone-resistant UPECs.

Initially, melittin was purified and characterised from honeybee venom. The chromatogram of the apitoxin presented several peaks, in which the retention time of melittin was 42.735 minutes (Figure S1). In addition, this peak was confirmed by MALDI-TOF mass spectrometry and showed a peak of 2,845.88 Da, a value equivalent to the molar mass of melittin (Strohalm et al. 2008). Since more than 90% of the chemical composition of the dry mass of the bee venom is peptides, enzymes, or proteins, the concentration of melittin was estimated in apitoxin as 45%, which is within the expected range and varies between 40% and 60% of the dry weight of apitoxin (Son et al. 2007). The final purification yield was 90%, and its purity was 93%.

Melittin was highly active against clinically relevant quinolone-resistant UPECs, inhibiting the growth of all isolates tested at concentrations ranging from 0.5 to 8 μM.
The concentrations of this peptide required to inhibit 50% (MIC$_{50}$) and 90% (MIC$_{90}$) of the isolates were 4 µM and 8 µM, respectively, while colistin presented an MIC$_{50}$ and MIC$_{90}$ of 1 µM and 1.9 µM, respectively (Table S1). Importantly, melittin kept its antibacterial activity against UPEC strains resistant to several classes of antibiotics, including cephalosporin, amino-penicillin, amino-penicillin $+$ β-lactamase inhibitors, and aminoglycosides, suggesting that cross-resistance between these particular antibiotics and melittin is unlikely to occur (Mohamed et al. 2016). Furthermore, the antibacterial concentration of melittin against UPECs was lower than the cytotoxic concentration against mammalian kidney cells (MDCK cells: CC$_{50}$ 15.8 µg/mL), suggesting a good selectivity for prokaryotic cells in relation to zwitterionic eukaryotic cells (Alsafar et al. 2020).

The MICs reported in this study were similar to those found in previous studies. Stocker and Trayno, in a pioneer study conducted in 1986, showed that melittin was active against Escherichia coli NCIR 9552 at 7 µg/mL ($\sim$2 µM) (Stocker and Traynor 1986). Picoli et al. (2017) found that melittin has MIC of 40–42.5 µg/mL ($\sim$13 µM) and MBC of 64 – 128 µg/mL ($\sim$20–40 µM) against E. coli ATCC 8739 (Picoli et al. 2017). In another study, Han et al. (2009) demonstrated that the MIC of melittin purified from honeybee venom against E. coli ATCC 25922 was 0.125 µg/mL ($\sim$0.04 µM) (Han et al. 2009). However, to the best of our knowledge, this is the first study to point out the effect of melittin on a group of 41 isolates of clinically relevant multidrug-resistant (MDR) Escherichia coli. Melittin has been well explored as an antibacterial agent; however, a gap still exists regarding its spectrum of antimicrobial activity. One of the most relevant question is: Does melittin maintain its potent antibacterial effect against MDR strains? Although a considerable effort has been made to answer this question, most of the studies have focussed on MDR Gram-positive pathogens, such as methicillin-resistant Staphylococcus aureus (MRSA), vancomycin-intermediate S. aureus (VISA), vancomycin-resistant S. aureus (VRSA), and vancomycin-resistant Enterococcus (Choi et al. 2015; Memariani et al. 2019). However, little is known about the antibacterial effect of melittin against Gram-negative multi-resistant pathogens.

Melittin presented a predominantly bactericidal effect (Table S1). This peptide was able to kill quinolone-resistant UPEC isolates at concentrations ranging from 2 to 16 µM, showing MBC$_{50}$ and MBC$_{90}$ of 8 µM and 16 µM, respectively. Cationic AMPs, such as melittin, are known to possess mainly bactericidal action since they can interact electrostatically with the bacterial anionic membrane, forming pores and leading to lysis and microbial death (Pandey et al. 2010; Memariani et al. 2019; Lima et al. 2021).

Moreover, the time-kill curve study confirmed the potent bactericidal effect of melittin (Figure S2). In this assay, for a substance to be considered bactericide a reduction in the number of CFU of 3 log$_{10}$ should be obtained (Alder and Eisenstein 2004). Melittin presented a rapid bactericidal effect, eliminating a high microbial load ($1 \times 10^6$ CFU/mL) of quinolone-resistance and ESBL-producing UPEC strain within 3 h at 5x and 10x MIC. At 2x MIC, melittin took 6 h to sterilise the culture medium, while colistin at 10x MIC has maximum effect after 30 min of incubation (Figure S2). The bactericidal effect of melittin on E. coli was slower and less potent than that presented against methicillin-resistant Staphylococcus aureus in a previous study conducted by
our group (Lima et al. 2021), indicating that the microbicidal action of this peptide is more powerful in Gram-positive bacteria compared to Gram-negative species. However, the fast and efficient elimination of UPEC showed in this work minimises the risk of complications of urinary infection, reduces the antimicrobial concentration required to produce the desired effect, decreases the likelihood of resistance induction during clinical use, and restricts treatment time (Alder and Eisenstein 2004).

The combination of melittin with clinically available drugs is an effective way to reduce its toxic effect since it reduces the concentration of each of the agents during therapy (Tängdén 2014). In this study, we evaluated whether prior exposure to sub-inhibitory concentrations of melittin reduces the MIC of quinolones and beta-lactams against a quinolone-resistant and an ESBL-producing UPEC. As shown in Table S2, while previous exposure to melittin reduces the MIC of ceftizoxime by 8-folds, this effect was not detected for the quinolones tested. The mechanism of resistance to cephalosporin in this isolate is through ESBL production, a saturable system (Rawat and Nair 2010). As melittin forms pores in the bacterial membrane (Pandey et al. 2010), it allows the entry of large amounts of the drug into the cell, which saturates the enzyme, and then the compound can perform its antibacterial action. The mechanism of resistance to quinolone, on the other hand, is by target modification. Indeed, quinolone-resistance in Enterobacteriales was initially attributed to chromosomal mutations in the quinolone-resistance-determining regions (QRDRs) harbouring the genes encoding gyrase (gyrA and gyrB) and topoisomerase IV (parC and parE), which codes for antibiotic-insensitive molecular targets (Vieira et al. 2020). Thus, even though the intracellular concentration of quinolones increases, they will not perform their antimicrobial action since the molecular target is not affected by the compound.

In addition to being the microorganism most frequently involved in CA-UTIs, E. coli is also the most common bacteria in catheter-associated UTIs in hospital settings (Jacobsen et al. 2008). Catheter-associated UTIs are currently recognised as the most common type of nosocomial infection and account for over 1 million cases annually (Jacobsen et al. 2008) and over 40% of all nosocomial infections in hospitals worldwide (Warren 1997). In these cases, the infection is directly associated with the formation of bacterial biofilm on the inert surface of catheters. This bacterial community produces a mucopolysaccharide coating that prevents the penetration of antibiotics and keeps the microorganisms inside it in a stationary phase of growth (i.e. a phase insensitive to the action of most antibiotics, since they are often designed to act on logarithmic growing bacteria) (Mittal et al. 2015). Thus, to assess the potential application of melittin against biofilms, we assessed the ability of this antibacterial peptide to disrupt mature biofilms and inhibit colonisation/biofilm formation on the surface of the urethral catheter. According to the Figure S3A, the bacterial load of urethral catheters treated with melittin at 1 mg/mL (2.02 ± 0.41 Log_{10}CFU/cm) and 10 mg/mL (0.76 ± 0.53 Log_{10}CFU/cm) was significantly lower than that observed in untreated catheters (3.76 ± 0.48 Log_{10}CFU/cm). These results show for the first time that melittin inhibits the adhesion and colonisation of bacteria to the surfaces of invasive medical equipment. As shown in our study, urethral catheter functionalised with melittin has increased resistance to bacterial colonization, which can be associated with a reduced
frequency of latent infections. These infections are often difficult to treat and can be potentially fatal, especially among critically ill patients.

The effect of melittin was also investigated against pre-established biofilms. This peptide significantly reduced the UPEC biofilm biomass at 32 \( \mu \text{M} \) and 8 \( \mu \text{M} \) by 39.58% and 26.78%, respectively. The anti-biofilm effect of melittin was similar to the effect observed for colistin, which reduced the preformed UPEC biofilm by 55.91% and 19.21% at 32 \( \mu \text{M} \) and 8 \( \mu \text{M} \), respectively (Figure S3B). Similarly, other studies have confirmed the activity of melittin against biofilms formed by reference strains of \textit{E. coli} (Han et al. 2009; Picoli et al. 2017). Indeed, antimicrobial peptides are known for their high activity against mature bacterial biofilms because they induce the disruption or degradation of the membrane potential of biofilm embedded cells, inhibit the signalling systems of bacteria by downregulation the genes responsible for biofilm formation and transport of binding proteins, and degrade the polysaccharide and biofilm matrix (Yasir et al. 2018).

3. Experimental

Experimental details are described in the supplementary materials.

4. Conclusion

The results show that melittin has a potent antibacterial effect against UPECs, regardless of their resistance to clinically available antibiotics. Melittin also showed an anti-biofilm effect, breaking mature biofilm and inhibiting the adhesion of bacteria to sensitised urethral catheter. Taken together, these data indicate that melittin is a promising prototype for the development of more effective therapies against urinary tract infections by quinolone-resistant UPECs.

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Disclosure statement

All authors report that they do not have any conflicts of interest.

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