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MINI-REVIEW

Melittin: from honeybees to superbugs

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Abstract

The emergence of antibiotic-resistant bacteria, dubbed superbugs, together with relative stagnation in developing efficient antibiotics has led to enormous health and economic problems, necessitating the need for discovering and developing novel antimicrobial agents. In this respect, animal venoms represent a rich repertoire of pharmacologically active components. As a major component in the venom of European honeybee *Apis mellifera*, melittin has a great potential in medical applications. In this mini-review, we summarize a multitude of studies on anti-bacterial effects of melittin against planktonic and biofilm-embedded bacteria. Several investigations regarding synergistic effects between melittin and antibiotics were also described. On the whole, the properties of melittin can open up new horizons in a range of biomedical areas, from agriculture to veterinary and clinical microbiology.

Keywords Venom · Bee · Melittin · Anti-bacterial activity · Biofilm · Antibiotic

Introduction

Modern medicine has conquered many life-threatening illnesses, but threat of antibiotic-resistant bacteria seems to be a never-ending challenge facing humankind. It is anticipated that annual deaths attributable to anti-microbial resistance will surpass those of cancer by 2050, if we do not take action (O'Neill 2016). The greatest concern is imposed by the ESKAPE pathogens (i.e., *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) which have the ability to "escape" the lethal action of conventional anti-microbials and host immune responses (Rice 2008).

Extensive exposure to antibiotics has rapidly led to emergence and nationwide propagation of multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) bacteria, often dubbed superbugs, making the choice of chemotherapeutics for treatment more problematic (Deslouches et al. 2015; Mohamed et al. 2017). Such fear is even further compounded once pathogens establish biofilms on tissues and medical devices. In this respect, biofilm formation empowers bacteria to abscond from various clearance mechanisms produced by host and synthetic sources (Batoni et al. 2016; Overhage et al. 2008).

While admirable efforts are underway to potentiate current anti-microbial arsenal, the problem of antibiotic-resistance in superbugs is of sufficient importance that effective anti-microbial materials ought to be discovered and evaluated (Deslouches et al. 2015). Among the limited numbers of new anti-microbials in the pipeline, natural peptides from animal venoms have been demonstrated to possess promising biological properties, which warrant their development as efficacious agents against recalcitrant pathogens (Almeida et al. 2018; Memariani et al. 2017; Hale and Hancock 2007). This review summarizes empirical evidences on anti-bacterial and anti-biofilm properties of melittin, a major component of honeybee venom.

Animal venoms

Natural products originated from both plants and animals possess a diverse array of as-yet unidentified substances that

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suggest seemingly limitless possibilities for finding potential leads (Gajski and Garaj-Vrhovac 2013). Animal venoms are poisonous secretions which involved in protection against predators or immobilizing/killing preys (Andreotti et al. 2010). Though the composition of venoms varies from animal to animal, the majority of venoms comprise a myriad of enzymes, peptides, low molecular weight organic molecules, and inorganic salts (Pennington et al. 2018). Animal venoms have been exploited for thousands of years, in many traditional remedies and medicines, to cure a plethora of maladies including atopic dermatitis, arthritis, chronic pain, multiple sclerosis, infectious diseases, cancers, gastrointestinal issues, and cardiovascular diseases (Pennington et al. 2018; Andreotti et al. 2010; Lewis and Garcia 2003).

Recent propitious progresses toward high-throughput screening and characterization of venom components have provided the impetus to search for novel venom-based therapeutics such as those with anti-microbial activity (King 2011). In this context, venom-derived anti-microbial peptides (AMPs) are of particular interest because of their selectivity, broad-spectrum microbicidal activity, and relative safety (Pennington et al. 2018; Memariani et al. 2018a).

Bee venom

Unquestionably, venoms from various animals such as bees, snakes, scorpions, spiders, toads, octopus, and marine cone snails represent a rich source of pharmacologically active components, creating unique avenues for discovering promising biomolecules which can be used per se or as lead compounds in the development of therapeutic drugs (Sabatier 2011; Almeida et al. 2018). Honeybee venom is a complex concoction of biologically active substances, such as melittin, secapin, apamine, hyaluronidase, phospholipase A_2, phospholipids, saccharides, noradrenaline, histamine, and dopamine, with enormous chemical and functional variability (Hider 1988).

Bee venom has been exploited since ancient times to treat several ailments. In oriental traditional medicine, bee venom has been used to treat skin maladies, palliate the back pain, and attenuate chronic inflammation conditions caused by both multiple sclerosis and rheumatoid arthritis (Oršolić 2012; Son et al. 2007). Likewise, honeybee venom can exert anti-atopic dermatitis (An et al. 2018), radioprotective (Varanda and Tavares 1998), anti-mutagenic (Varanda et al. 1999), anticancer (Oršolić 2012), and anti-microbial (AL-Ani et al. 2015) activities, attesting to the therapeutic potential of honeybee venom and its major constituent melittin as well.

Thus far, numerous peptides with anti-microbial activities have been isolated from bee venoms such as melittin (Fennell et al. 1967), melectin (Cerovský et al. 2008), macropin (Monincová et al. 2014), HYL (Nešuta et al. 2016), and Xac-1 (Kawakami et al. 2017), suggesting their potential use as natural antibiotics. Furthermore, there are several reviews in the literature regarding anti-cancer effects (Rady et al. 2017; Gajski and Garaj-Vrhovac 2013), anti-inflammatory properties (Lee and Bae 2016), and anti-diabetic activities (Hossen et al. 2017) of melittin. As far as we know, however, reviews of the anti-bacterial and anti-biofilm activities of melittin are currently not available.

Physiochemical, structural, and biological properties of melittin

Melittin is a major component in the venom of European honeybee *Apis mellifera*. It is a small cationic linear peptide (Fig. 1), comprising at least half of the venom dry weight (Tacón 2016). Melittin is composed of 26 amino acid residues (GIGAVLKVLTTGLPALISWIKRKRQQ-$CONH_2$). At physiological pH, melittin has a net charge of + 6 due to the presence of arginine and lysine residues. The N- and C-terminal regions of melittin are mainly hydrophobic and hydrophilic, respectively (Rady et al. 2017). Polar and nonpolar amino acid residues distribute asymmetrical in melittin, suggesting its amphipathic nature when it is adopted an α-helical conformation. This feature makes the peptide not only water-soluble but also membrane-active (Terwilliger and Eisenberg 1982).

X-ray crystallographic and nuclear magnetic resonance (NMR) studies revealed that each melittin chain has a structure consisting of two α-helical segments, one α-helix containing residues 1–10 and the longer one formed by residues 13–26. These two α-helices are joined by a "hinge" region between residues 11 and 12 to constitute a bent rod (Anderson et al. 1980; Terwilliger and Eisenberg 1982; Bazzo et al. 1988; Lam et al. 2001). Four conformationally identical monomers of melittin are packed together to form a tetramer which is non-lytic and it is predominant at concentrations found in bee's abdominal sack (Tacón 2016; Terwilliger and Eisenberg 1982). It has been shown that melittin exists as a monomer at the minimum concentrations necessary for cell lysis. When the venom is released, dissociation of the tetramer occurs which yields the monomer (Hider 1988; Picoli et al. 2017). It is now apparent that melittin attaches to the membrane surface as monomers but acts on the membrane collectively to produce pores (Lee et al. 2013).

It has been proposed that melittin can form a short-lived pore in the membrane, as evidenced by the release of calcein dye from the liposomes, and the size of pore increases with the peptide-to-lipid molar ratio (P/L) (Matsuzaki et al. 1997). In addition, the peptide induces stable pores in the micromolar concentration range (Lee et al. 2013). Melittin is able to orient either parallel or perpendicular to a lipid bilayer (Smith et al. 1994; Yang et al. 2001). In this context, parallel binding of

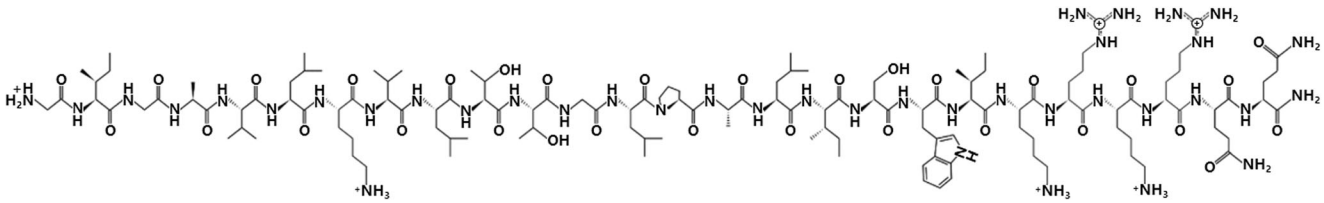

Fig. 1 Structure of melittin

melittin to the membrane prohibits other melittin molecules from incorporating into lipid bilayer and creating pores, thereby protecting the membrane from leakage (van den Bogaart et al. 2008). As P/L exceeds a certain threshold, an increasing fraction of melittin molecules shifts toward the perpendicular orientation (Yang et al. 2001; van den Bogaart et al. 2008). Perpendicular orientation of melittin to the plane of the membrane is required for the formation of transmembrane pores, while the parallel orientation is inactive (Yang et al. 2001; van den Bogaart et al. 2008). When inserted in the membrane bilayers, the attached peptides aggregate, inducing the lipids to bend. It inevitably gives rise to the formation of toroidal pores and the subsequent leakage of cytoplasmic contents (Park et al. 2006). This level of knowledge with regard to membrane-disrupting mechanism of melittin is necessary for developing novel anti-microbial agents with improved therapeutic indices.

Melittin attacks all lipid membranes including those found in the erythrocyte membrane, resulting in hemolysis. Indeed, the release of hemoglobin is succeeded by the formation of ion-permeable pores (Raghuraman and Chattopadhyay 2007). Melittin has also been shown to exert allergenic activity by increasing serum immunoglobulin E (IgE) in nearly one-third of honeybee venom-sensitive individuals (Paull et al. 1977). However, this adverse feature might result from contamination with other bee venom constituents (Lee and Bae 2016). Furthermore, melittin can incorporate into phospholipid bilayers of the cell membranes and induce morphological changes in a dose- and time-dependent manner, thereby leading to cell lysis. Though cytotoxic to normal cells, the toxic effect of melittin against tumor cells is more pronounced (Gajski and Garaj-Vrhovac 2013; Lee and Bae 2016). Thus, possible adverse effects of melittin should be considered before assessing its potential therapeutic applications.

Anti-bacterial effects

In vitro studies

Historically, anti-bacterial activity of bee venom was first reported by Schmidt-Lange (1941). This finding was extended by Ortel and Markwardt (1955) as well as Benton et al. (1963). In the early 1950s, melittin was discovered after electrophoretically separation of direct hemolysin from the indirect hemolysin phospholipase A (Habermann 1972; Neumann et al. 1952). Melittin was proposed as the anti-bacterial constituent in bee venom by Fennell et al. (1967). They demonstrated that anti-bacterial activity of whole bee venom is of the same order of magnitude as that of melittin in vitro. The authors found that both honeybee venom and its melittin fraction had anti-bacterial effects on a penicillin-resistant strain of *S. aureus* (strain 80). It has been also shown that melittin had higher anti-bacterial activity against Gram-positive bacteria in comparison to Gram-negative bacteria (Fennell et al. 1967). Numerous attempts were made to ascertain the susceptibilities of various pathogens to melittin from the 1960s onwards, as evidenced in Table 1.

In vitro anti-mycobacterial activities of melittin were first demonstrated in 1971 by Dorman and Markley (1971). Subsequent surveys during the 1980s and 1990s vividly confirmed that melittin has significant anti-bacterial activities against both reference and clinical strains of bacteria (Steiner et al. 1981; Stocker 1984; Boman et al. 1989; Blondelle and Houghten 1991; Wade et al. 1992; Piers et al. 1994; Oren and Shai 1996). It also showed minimum inhibitory concentration (MIC) values of ≤ 16 μg/mL for a large number of Gram-negative bacteria (Piers et al. 1994). However, there had been major discrepancies between the MIC values reported by previous studies (listed in Table 1), which could be convincingly explained by differences in purities of melittin, bacterial strains, and methodologies.

Melittin has been reported to exhibit an immediate inhibitory activity against *Borrelia burgdorferi*, the etiologic agent of Lyme disease (Lubke and Garon 1997; Socarras et al. 2017). For instance, Lubke and Garon (1997) showed a dramatic decline in the optical density of melittin-treated cultures of *B. burgdorferi* compared to untreated cultures. In this regard, ultrastructural observation of melittin-treated *Borrelia* spirochetes by field emission scanning electron microscopy (FE-SEM) divulged tangible alterations in the surface envelope of bacteria including augmented blebbing of surface components and pore formation. Dark-field microscopy also confirmed that melittin at a concentration equivalent to 100 μg/mL is capable of ceasing spirochete motility within seconds after exposure (Lubke and Garon 1997). Another study indicated that daily administration of melittin could significantly

Table 1 Anti-bacterial effects of melittin against various bacteria in vitro

Year	Method	Active against (anti-bacterial results)	References
1967	Disk diffusion	Gram-negative: Clinical isolates of *Pseudomonas aeruginosa* (D 9.0 mm), *Stenotrophomonas maltophilia* (D 7.9 mm), *Acinetobacter lwoffii* (D 12.0 mm), *Enterobacter aerogenes* (D 7.5 mm), *Enterobacter cloacae* (D 8.0 mm), *Salmonella enterica* serotype Newport (D 8.5 mm). Gram-positive: Clinical isolates of *Enterococcus faecalis* (D 9.5 mm), *Streptococcus liquefaciens* (D 7.8 mm), *Corynebacterium* sp. (D 12.0 mm), and *Staphylococcus aureus* 3A (D 9.3 mm), 53 (D 8.3 mm), and 80 (D 8.3 mm).	Fennell et al. 1967
1971	Agar well diffusion	Gram-negative: *Escherichia coli* and *Salmonella typhi.* Gram-positive: *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Bacillus subtilis.* Acid-fast: *Mycobacterium phlei*.	Dorman and Markley 1971
1981	Agar well diffusion	Gram-negative: *Escherichia coli* K12 strain DR3 (D 7.6 mm) and *Pseudomonas aeruginosa* strain OT97 (D 4.0 mm). Gram-positive: *Bacillus megaterium* (D 8.0 mm).	Steiner et al. 1981
1984	Tube dilution	Gram-negative: *Escherichia coli* NCIR strain 9552 (MIC 0.07 mg/mL and MBC 0.14 mg/mL).	Stocker et al. 1984
1989	Agar well diffusion (Calculation of LC)	Gram-negative: *Escherichia coli* K12 (LC 0.8 μM) and *Pseudomonas aeruginosa* OT97 (LC 3.0 μM). Gram-positive: *Bacillus subtilis* BSl1 (LC 0.2 μM), *Staphylococcus aureus* Cowan 1 (LC 0.2 μM), and *Streptococcus pyogenes* Sp1 (LC 0.5 μM).	Boman et al. 1989
1991	Broth microdilution	Gram-negative: *Pseudomonas aeruginosa* ATCC 27853 (MIC 50 μg/mL). Gram-positive: *Staphylococcus aureus* ATCC 29213 (MIC 25 μg/mL).	Blondelle and Houghten 1991
1992	Agar well diffusion (Calculation of LC)	Gram-negative: *Escherichia coli* K12 (LC 1.5 μM) and *Pseudomonas aeruginosa* OT97 (LC 7 μM). Gram-positive: *Bacillus subtilis* BSl1 (LC 0.4 μM), *Staphylococcus aureus* Cowan 1 (LC 0.4 μM), and *Streptococcus pyogenes* Sp1 (LC 1.5 μM).	Wade et al. 1992
1994	Broth microdilution	Gram-negative: *Pseudomonas aeruginosa* H309, K799, and Z61 (all had MIC of 8 μg/mL), *Escherichia coli* UB1005, SC9251, and SC9252 (all had MIC of 8 μg/mL), *Escherichia coli* DC2 (MIC 4 μg/mL), *Salmonella typhimurium* 14028s (MIC 16 μg/mL) and MS7953s (MIC 8 μg/mL), and *Enterobacter cloacae* 218S and 218R1 (both had MIC of 8 μg/mL).	Piers et al. 1994
1996	Broth microdilution	Gram-negative: *Escherichia coli* DR3 (MIC 5 μM), *Pseudomonas aeruginosa* ATCC 27853 (MIC 25 μM), *Acinetobacter calcoaceticus* Acl1 (MIC 2 μM), and *Salmonella typhimurium* LT2 (MIC 13 μM). Gram-positive: *Bacillus megaterium* Bml 1 (MIC 0.3 μM), *Micrococcus luteus* ATCC 9341 (MIC 0.4 μM), and *Bacillus subtilis* ATCC 6051 (MIC 0.6 μM).	Oren and Shai 1996
1997	Broth microdilution	Mollicutes: *Acholeplasma laidlawii* A-PGS (MIC 0.6 μM), *Mycoplasma gallisepticum* S6 (MIC 5 μM), *Mycoplasma genitalium* G37 (MIC 5 μM), *Mycoplasma mycoides* subsp. *mycoides* KH3J (MIC 10 μM), *Spiroplasma citri* R8A2 (MIC 0.6 μM), and *Spiroplasma melliferum* BC3 (MIC 1.25 μM).	Béven and Wróblewski 1997
1997	Dark-field microscopy, field emission scanning electron microscopy, and optical density measurements.	Gram-negative: *Borrelia burgdorferi* (After treatment with melittin, a dramatic decline in the optical density of *Borrelia burgdorferi* cultures, alterations in surface envelope of bacteria, and inhibition of spirochete motility were observed).	Lubke and Garon 1997
2002	Luminescence microscopy	Gram-negative: *Chlamydia trachomatis* strain E/UW-5/CX (60% and 75% inhibition of infection in HeLa cell line after 24 and 48 h, respectively). Mollicutes: *Mycoplasma hominis* strain H34 (65% inhibition of infection in HeLa cell line after 48 h).	Lazarev et al. 2002
2002	Broth microdilution	Gram-negative: *Escherichia coli* ATCC 25922 (MIC 25 μM), *Escherichia coli* DH5α (MIC 50 μM), and *Klebsiella pneumoniae* ATCC 13833 (MIC 50 μM). Gram-positive: *Bacillus subtilis* ATCC 6051 (MIC 3.1 μM), *Bacillus subtilis* IP 5832 (MIC 12.5 μM), *Enterococcus faecalis* ATCC 19433 (MIC 6.3 μM), *Listeria monocytogenes* NCTC 11994 (MIC 6.3 μM), *Micrococcus luteus* ATCC 9341 (MIC 3.1 μM), *Nocardia asteroides* ATCC 3308 (MIC 6.3 μM), *Streptococcus pneumoniae* ATCC 33400 (MIC 6.3 μM), and *Staphylococcus aureus* ATCC 29213 (MIC 6.3 μM).	Moerman et al. 2002
2003	Broth microdilution	Gram-positive: Twenty clinical isolates of *Acinetobacter baumannii* (MIC range of 0.50–16 μg/mL and MBC range of 0.50–32 μg/mL).	Giacometti et al. 2003

Table 1 (continued)

Year	Method	Active against (anti-bacterial results)	References
2004	Broth microdilution (for determining bactericidal activity)	Gram-negative: Kanamycin-resistant *Escherichia coli* SG 13009 (bactericidal activity at 1 μM).	Bucki et al. 2004
2005	Broth microdilution	Gram-negative and Gram-positive: Thirty nine bacterial strains belonging to seven genera and 12 different species of plant pathogenic bacteria (except for *Dickeya chrysanthemi*, MIC values ranged from 6.5 to 65 μM).	González-Rodríguez et al. 2005
2007	Agar well diffusion	Gram-negative: *Edwardsiella tarda* NUF251 (MIC 0.78 μg/mL), *Escherichia coli* D31 (MIC 0.78 μg/mL), *Enterobacter aerogenes* KCTC 2190 (MIC 1.56 μg/mL), *Klebsiella pneumonia* KCTC 2208 (MIC 0.78 μg/mL), *Pseudomonas aeruginosa* KCTC 2004 (MIC 25 μg/mL), *Salmonella typhimurium* KCTC 1925 (MIC 25 μg/mL), and *Vibrio parahaemolyticus* KTCT 2471 (MIC 1.56 μg/mL). Gram-positive: *Bacillus subtilis* PM125 (MIC 0.75 μg/mL) and *Micrococcus luteus* KCTC 1056 (MIC 1.56 μg/mL).	Kim et al. 2007
2009	Broth microdilution	Gram-negative: *Escherichia coli* ATCC 25922 (MIC 0.125 μg/mL). Gram-positive: *Staphylococcus aureus* ATCC 9144 (MIC 0.06 μg/mL).	Han et al. 2009
2010	Broth microdilution	Gram-negative: *Escherichia coli* ATCC 10536 (MIC 3.8 ± 0.5 μM) and *Escherichia coli* DH5R (MIC 3.8 ± 0.5 μM). Gram-positive: *Bacillus subtilis* ATCC 6633 (MIC 3.9 ± 0.5 μM) and *Staphylococcus aureus* ATCC 9144 (MIC 3.6 ± 0.4 μM).	Pandey et al. 2010
2012	Broth microdilution	Gram-positive: Clinical isolates including 11 methicillin-resistant *Staphyloccous aureus* (MIC range 0.5–4 μg/mL), 15 methicillin-susceptible *Staphyloccous aureus* (MIC range 0.5–4 μg/mL), and 15 *Enterococcus faecalis* isolates (MIC range 1–8 μg/mL).	Dosler and Gerceker 2012
2013	Broth microdilution	Gram-negative: Drug-resistant isolates including 2 *Escherichia coli* (both had MIC of 2 μM), 11 *Pseudomonas aeruginosa* (MIC range 2–16 μM), and 3 ampicillin-resistant *Salmonella typhimurium* isolates (MIC range 8–16 μM). Gram-positive: Sixteen drug-resistant *Staphyloccous aureus* isolates (MIC range 1–8 μM).	Gopal et al. 2013
2015	Broth microdilution	Gram-negative: *Campylobacter jejuni* NCTC 11168 (MIC 2–4 μg/mL), *Escherichia coli* ATCC 25922 (MIC 16 μg/mL), *Salmonella enterica* serovar Typhimurium LT2 (MIC 32–64 μg/mL), and *Yersinia ruckeri* 392/2003 (MIC 16–32 μg/mL). Gram-positive: *Enterococcus faecalis* ATCC 29212 (MIC 2–4 μg/mL), *Listeria monocytogenes* N22–2 (MIC 2–4 μg/mL), and *Staphyloccous aureus* ATCC 29213 (MIC 2–4 μg/mL).	Ebbensgaard et al. 2015
2015	Broth microdilution	Gram-positive: *Streptococcus salivarius* ATCC 25975 (MIC 10 μg/mL), *Streptococcus sobrinus* ATCC 33478 (MIC 10 μg/mL), *Streptococcus mutans* ATCC 25175 (MIC 40 μg/mL), *Streptococcus mitis* ATCC 49452 (MIC 10 μg/mL), *Streptococcus sanguinis* ATCC 10556 (MIC 10 μg/mL), *Lactobacillus casei* ATCC 11578 (MIC 4 μg/mL), and *Enterococcus faecalis* ATCC 4082 (MIC 6 μg/mL).	Leandro et al. 2015
2016	Agar well diffusion	Gram-negative: *Xanthomonas oryzae* pathovar *oryzae* strain ZJ-173 (IC_{50}[a]: 9–10 μM).	Shi et al. 2016
2017	SYBR Green I/propidium iodide (PI) assay	Gram-negative: *Borrelia burgdorferi* strain B31 (Melittin significantly lessened the numbers of both spirochetes and persisters compared to the negative control).	Socarras et al. 2017
2018	Broth microdilution	Gram-negative: Thirty-three *Pseudomonas aeruginosa* strains isolated from patients with third-degree burn (MIC and MBC range 0.125–4 μg/mL).	Khozani et al. 2018

D diameter of inhibition zone, *MIC* minimum inhibitory concentration, *MBC* minimum bactericidal concentration, *LC* lethal concentration, defined as the lowest drug concentration that inhibits growth

[a] IC_{50} The half maximal inhibitory concentration of the peptide as assessed by measuring the optical density (OD) of treated bacteria at 600 nm

diminish the numbers of *Borrelia* persisters (p value ≤ 0.05) in comparison to the negative control (sterile PBS buffer) using SYBR Green I/propidium iodide (PI) assay (Socarras et al. 2017). Compared to doxycycline, melittin significantly lessens the numbers of spirochetes (p value ≤ 0.01) and *B. burgdorferi* persister cells (p value ≤ 0.01) at all concentrations, suggesting melittin as an appropriate candidate to extirpate different forms of *B. burgdorferi* (Socarras et al. 2017).

The first report on anti-bacterial action of melittin against several mollicutes dates back to 1997, when Béven and Wróblewski (1997) investigated effects of ten naturally occurring peptides on viability, morphology, and motility of mollicutes. Mollicutes are distinguished phenotypically from other class of bacteria by the absence of cell wall and their minute size. Melittin was found active against six different genera of mollicutes with MIC values in the range of 0.6–10 μM. Probing mollicute cells through a fluorescent dye (3,3′-dipropylthiodicarbocyanine iodides) revealed that melittin induced depolarization of bacterial membranes (Béven and Wróblewski 1997). Melittin, expressed within plasmid constructs, has been also reported to be effective in intracellular inhibition of urogenital pathogens including *Mycoplasma hominis* and *Chlamydia trachomatis* (Lazarev et al. 2002).

An abundance of evidence with regard to broad-spectrum bactericidal activity of melittin has propelled researchers to use melittin as a positive control AMP for comparison of its anti-microbial activity to other novel-discovered/developed AMPs. For instance, Moerman et al. (2002) used melittin as a positive control and found that it was active against reference strains of both Gram-positive and Gram-negative bacteria (Table 1). In particular, they found that melittin is effective in inhibiting reference strains of *Listeria monocytogenes* and *Nocardia asteroides* (Moerman et al. 2002). When the authors observed MIC increment in the presence of 5-mM extracellular Mg^{2+} ions, they suggested that electrostatic interaction occurs between melittin as a cationic peptide and negatively charged lipopolysaccharide (LPS) in Gram-negative bacterial membrane (Moerman et al. 2002). Furthermore, melittin has proven to be effective in inhibiting *Vibrio parahaemolyticus* KTCT 2471 (MIC of 1.56 μg/mL) and *Edwardsiella tarda* NUF251 (MIC of 0.78 μg/mL), both of which are able to cause disease in aquatic creatures (Kim et al. 2007). Using confocal microscopy technique, Pandey et al. (2010) pointed out that rhodamine-labeled melittin localized onto *E. coli* cells and altered their morphology. It has been also found to create holes of varying sizes onto *Bacillus megaterium*, as revealed by confocal microscopy (Pandey et al. 2010).

Melittin has the ability to inhibit plant-associated bacteria (González-Rodríguez et al. 2005; Shi et al. 2016). In an investigation of the inhibitory effects of melittin on 39 strains belonging to seven genera and 12 different species of plant pathogenic bacteria, the peptide marvelously exhibited 100% growth inhibition in vitro. Except for one strain (*Dickeya chrysanthemi*), the MIC values of melittin against the tested pathogens varied between 6.5 and 65 μM. In another study, using an agar well diffusion assay, Shi et al. (2016) noticed the anti-bacterial potency of melittin against *Xanthomonas oryzae* pathovar *oryzae*, causing agent of rice blight disease (Table 1). SEM revealed that melittin is able to induce surface roughening and shrinking, and pore formation, thereby resulting in rapid bacterial cell death (Shi et al. 2016). Melittin is also capable of binding to bacterial DNA/RNA in vitro, suggesting its probable role in inhibition of intracellular targets (Shi et al. 2016). Thus, these findings open up a range of new applications for melittin in the field of agricultural microbiology.

There are multiple lines of evidence that confirm the anti-bacterial activity of melittin toward antibiotic-resistant bacteria. In a study conducted on 20 MDR nosocomial isolates of *A. baumannii*, MIC and MBC values of melittin were in the range of 0.50–16 and 0.50–32 μg/mL, respectively (Giacometti et al. 2003). Another investigation revealed that melittin has a strong anti-bacterial effect on 41 clinical bacterial strains comprising 11 methicillin-resistant *S. aureus* (MRSA), 15 methicillin-susceptible *S. aureus* (MSSA), and 15 *E. faecalis* isolates (Table 1), with MIC values from 2 to 8 μg/mL (Dosler and Gerceker 2012). One survey also demonstrated that melittin inhibited 32 isolates of antibiotic-resistant bacteria such as *S. aureus*, *P. aeruginosa*, *E. coli*, and *Salmonella typhimurium* strains at concentrations equal to or less than 16 μM (Gopal et al. 2013), suggesting its potential for treating intractable infections caused by nosocomial pathogens.

A study conducted by Leandro et al. (2015) demonstrated in vitro anti-bacterial potency of melittin against prominent etiologic agents of tooth decay with MIC values lying in the 4–40 μg/mL range (Table 1). Considering the adverse impacts of tooth decay on people's health, melittin has potential of inhibiting oral pathogens (Leandro et al. 2015).

In a contemporary study, Khozani et al. (2018) observed that there was a major difference between inhibitory (p value < 0.05) or bactericidal (p value < 0.05) activities of melittin and certain antibiotics including ceftazidime, doripenem, and colistin against 33 *P. aeruginosa* strains from patients who suffered from third-degree burns. The superior in vitro anti-bacterial activity of melittin compared to mentioned antibiotics prompted the authors to suggest melittin as a candidate for evaluating its topical anti-microbial activity in a mouse model of burn infection (Khozani et al. 2018).

Synergism with other anti-microbials

Several investigations have addressed possible synergistic effects between melittin and other anti-microbial agents, in particular conventional antibiotics (Moerman et al. 2002;

Giacometti et al. 2003; Dosler and Gerceker 2012; AL-Ani et al. 2015; Bardbari et al. 2018). For instance, one study indicated that melittin exhibited synergistic activity with erythromycin against *K. pneumoniae*. Moreover, melittin has the ability to act synergistically when combined with amoxicillin, cefuroxime, and erythromycin against *Listeria monocytogenes* (Moerman et al. 2002). Using chequerboard titration method, synergistic effects observed between melittin and β-lactam antibiotics against *A. baumannii* ATCC 19606 and a clinical isolate of *A. baumannii* (04–01) (Giacometti et al. 2003). When exploited either alone or in combination with frequently used antibiotics, melittin exhibited concentration-dependent and rapid bactericidal activity against MRSA, MSSA, and *E. faecalis* isolates according to killing kinetic curves (Dosler and Gerceker 2012). It is noteworthy to mention that fast microbicidal action of AMPs such as melittin not only lessens the duration of treatment but also the possibility of developing anti-microbial resistance among bacterial pathogens (Memariani et al. 2018b). A recent survey assessed conceivable synergistic interactions of melittin in combination with several antibiotics against five clinical isolates of MDR *A. baumannii*. The authors pointed out that melittin exerted significant synergistic behaviors when combined with colistin and imipenem toward MDR *A. baumannii* strains (Bardbari et al. 2018), corroborating previous findings reported by Giacometti et al. (2003).

Aside from antibiotics, plant secondary metabolites combined with melittin were also assessed by chequerboard titration assay to investigate whether their combinations are superior against certain bacteria compared to each agent alone (AL-Ani et al. 2015). In this respect, a predominant synergism was found to occur between melittin and either benzyl isothiocyanate or carvacrol toward both MRSA NCTC 10442 and *E. coli* ATCC 25922, with fractional inhibitory concentration index (FICI) values ranging from 0.24 to 0.5 (AL-Ani et al. 2015).

Synergy of melittin with other anti-microbials might arise from destabilization of outer membrane in Gram-negative bacteria induced by the peptide, facilitating entrance of these anti-microbials into the bacterial cells. In the case of Gram-positive bacteria, it has been suggested that inhibition of peptidoglycan synthesis by β-lactam antibiotics can allow melittin to pass through the altered peptidoglycan layer more easily (Moerman et al. 2002). It should be borne in mind that synergistic activity of melittin with conventional antibiotics decreases the MIC values of both the peptide and antibiotics against tested strains. As a consequence, melittin can be used at non-toxic levels which results in its safe application without cytotoxicity concerns (Bardbari et al. 2018). Based on aforementioned scientific proofs, novel melittin-antibiotic formulations can be applied to eliminate antibiotic-resistant superbugs.

In vivo studies

In addition to in vitro studies, several evidences exist regarding anti-bacterial effectiveness of melittin in animal models. In one of the early attempts to examine the anti-bacterial efficacy of melittin in vivo, Lazarev et al. (2004) observed that aerosolized administration of a plasmid construct expressing melittin gene led to significant inhibition of *Mycoplasma gallisepticum* infection in chicken (Table 2). The authors suggested that melittin has prophylactic and therapeutic potential for mycoplasma infections in poultry farms (Lazarev et al. 2004). The other study in which mice intravaginally infected with *Chlamydia trachomatis*, administration of a plasmid expressing gene for melittin through vaginal route caused 45–80% inhibition of infection, as assessed by direct immunofluorescence with monoclonal antibodies (Lazarev et al. 2007). Furthermore, intradermal injections of living *Propionibactierium acnes* into the mouse ear and subsequent topical treatment with melittin-vaseline mixtures resulted in significant alleviation of swelling and granulomatous response in comparison to mice injected with only living *P. acnes*, suggesting protective effects of melittin in a *P. acnes*-induced in vivo inflammatory model (Lee et al. 2014).

Anti-bacterial effects of melittin on mice skin subcutaneously infected with MRSA USA 300 suspension, containing 10^6 colony forming units (CFUs)/mL, were investigated in a study conducted by Choi et al. (2015). Briefly, in order to examine in vivo efficiency of melittin, each surface lesion was treated with 100 μg of melittin in 80 μL PBS once a day and calipers were applied to gauge lesion dimensions for up to 10 days. The authors demonstrated that treatment of infected zone by melittin for 4 days led to significant decline in diameters of the abscesses in comparison to the PBS-treated group (Table 2). Furthermore, half of the mice intraperitoneally infected with MRSA USA300 were survived after intraperitoneal injection of 5 mg/kg of melittin in 0.1 mL PBS, whereas intraperitoneal injection of either PBS or 2.5 mg/kg melittin failed to survive infected mice after 24 h, as shown by Kaplan-Meier survival curve (Choi et al. 2015). This evidence was the first to confirm significant protective effects of melittin against MRSA infection in vivo.

A new study demonstrated that topical administration of melittin at concentrations of 16 and 32 μg/mL in mice killed 93.3% and 100% of an XDR *A. baumannii* on a third-degree burned area, respectively (Pashaei et al. 2019). Blood samples of mice treated with melittin (32 μg/mL) exhibited no hemolysis, indicating that the peptide is not entered to blood circulation. Moreover, melittin showed no dermal toxicity toward both normal and burned groups. Remarkably, all the examined mice were alive even after 1 month. This finding might create incentives for investigators to re-examine neglected toxic AMPs for at least topical treatment of burned areas.

Table 2 Anti-bacterial effects of melittin in animal models of infection

Animal model	Route of administration	Methods	Active against	Results	References
Ross broiler chicks	Aerosol administration	Pathomorphological, histological, and serological analysis	*Mycoplasma gallisepticum* strain 1226	Compared to infected chickens given no treatment, chickens received a plasmid construct expressing melittin gene had higher mean body mass, fewer lesions of the respiratory tract and different inner organs, thinner mean thickness of mucosal membrane of trachea, lesser numbers of re-isolated bacteria, and lower levels of *Mycoplasma gallisepticum* antibodies.	Lazarev et al. 2004
BALB/c mice	Intravaginal administration	Direct immunofluorescence with monoclonal antibodies	*Chlamydia trachomatis* (strain D/UW-3/Cx, ATCC VR-885)	Mice treated with a plasmid expressing gene for melittin had lower number of chlamydial inclusions per 1 mL compared to mice that had not received the plasmid (2, 6, 9, 13, 16, 20, and 27 days after treatment).	Lazarev et al. 2007
CD1 mice	Topical and intraperitoneal administrations	Measuring the lesion dimensions with calipers and using Kaplan-Meier survival curve.	MRSA USA 300	Diameters of abscesses in mice treated topically with melittin were significantly lower than those of a PBS-treated group. Compared to PBS-treated mice, intraperitoneal injection of melittin (5 mg/kg) led to survival rate of 50% in intraperitoneally infected mice after 24 h.	Choi et al. 2015
BALB/c mice	Topical administration	CFU counting and observing clearance of suspensions prepared from burned tissues	XDR *Acinetobacter baumannii*	Melittin killed 93.3% and 100% of bacteria on a third-degree burned area at concentrations of 16 and 32 μg/mL, respectively. Eradication of bacteria was confirmed after 24 h in Mueller-Hinton medium.	Pashaei et al. 2019

Effects on bacterial biofilms

A biofilm is an organized microbial consortium enclosed in a self-created biopolymer matrix. It adheres irreversibly to biotic or abiotic surfaces (Batoni et al. 2016; Høiby et al. 2010). It has become obvious that biofilm formation is an adaptive mechanism of microbial cells, permitting them to survive harsh growth conditions (George et al. 2005). Owing to the presence of the extracellular matrix barrier and slow growth rate, biofilm-encased bacteria might tolerate up to 1000 times greater concentrations of anti-microbials compared to their planktonic counterparts (Memariani et al. 2016; Macià et al. 2014). Furthermore, detached cells from the biofilm can serve as a steady reservoir of pathogens, giving rise to treatment failure and recurrent infections as well (Haagensen et al. 2015). Thus, there is an imperative need for developing efficient anti-biofilm agents to address concerns about biofilm-related infections.

Over the past few years, there have been several attempts to examine efficacy of melittin on the viability of biofilm-embedded bacteria in vitro, as summarized in Table 3. For example, melittin is effective against clinical isolates of biofilm-producing *P. aeruginosa*, with a minimum biofilm inhibition concentration (MBIC) range of 4–16 μM, which was far more active compared to certain antibiotics including ampicillin, chloramphenicol, and levofloxacin (Gopal et al. 2013). Moreover, melittin has been reported to inhibit either biofilm formation or bacterial surface attachment in a time-dependent manner (Dosler et al. 2016). The peptide was also capable of inhibiting five strong biofilm-producer strains of MDR *A. baumannii* and removing their biofilm formations (Table 3), alone or in combination with colistin and imipenem (Bardbari et al. 2018). Noticeably, melittin lessened both biofilm biomass and viability of biofilm-embedded *B. burgdorferi* strain B31 at different concentrations in comparison to PBS-treated biofilms, which was further confirmed by SYBR Green I/(PI) assay and atomic force microscopy (Socarras et al. 2017). Another study divulged that melittin inhibited biofilm production and destroyed bacterial biofilms (Picoli et al. 2017). A recent survey implied that melittin was able to penetrate into biofilm layers of *P. aeruginosa* gradually and to kill biofilm-residing bacteria kinetically by disrupting bacterial membrane (Khozani et al. 2018). All in all, these evidences suggest that melittin can diminish biofilm formation, biofilm biomass, and viability of bacteria within biofilms in a time- and concentration-dependent manner.

Future prospects

As alluded to above, melittin has strong anti-bacterial and anti-biofilm effects on a broad spectrum of bacterial pathogens, though cytotoxicity of melittin at higher doses may hinder its therapeutic application. Several investigations are

Table 3 Anti-biofilm activity of melittin

Method(s)	Medium for biofilm assays	Biofilm-producing strains	Results	References
Microtitre plate method ($MBIC_{100}$[a] determination)	Müeller-Hinton broth supplemented with 0.2% glucose	Five *Pseudomonas aeruginosa* strains isolated from patients with otitis media	*Pseudomonas aeruginosa* strains 1162 ($MBIC_{100}$: 4 μM), 3547 ($MBIC_{100}$: 16 μM), 4007 ($MBIC_{100}$: 4 μM), 3399 ($MBIC_{100}$: 4 μM), and 1034 ($MBIC_{100}$: 8 μM).	Gopal et al. 2013
Microtitre plate method, biofilm attachment assay, and inhibition of biofilm formation	Tryptic soy broth supplemented with 1% glucose	Three strong biofilm-forming strains of *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae*	At 1/10 × MIC, melittin inhibited bacterial surface attachment for 1, 2, or 4 h. At 1× or 1 × 10 MICs, melittin significantly inhibited the 24-h biofilm formation (p value < 0.001).	Dosler et al. 2016
Microtitre plate method ($MBIC_{90}$[b] determination, biofilm removal assay, and field emission scanning electron microscopy	Tryptic soy broth supplemented with 1% glucose	Five strong biofilm- producer strains of MDR *Acinetobacter baumannii* collected from patients with lung and burn infections	$MBIC_{90}$ range of melittin was 1 to 2 μg/mL. After 6 and 24 h, melittin showed higher biofilm removal activity than that of imipenem and ciprofloxacin (p value < 0.05). Melittin was able to degrade biofilm and to lyse biofilm-embedded bacteria, as assessed by field emission scanning electron microscopy.	Bardbari et al. 2018
Microtitre plate method, inhibition of biofilm formation, and MTT[c] staining	Tryptic soy broth supplemented with 1% glucose	*Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 15442, and *Staphyloccous aureus* ATCC 12600	Melittin reduced biofilm formation of bacteria, particularly at 1× and 1 × 10 MICs. The peptide destroyed bacterial biofilms in a time- and concentration-dependent manner.	Picoli et al. 2017
Microtitre plate method, degradation kinetics, and field emission scanning electron microscopy	Tryptic soy broth supplemented with 0.2% glucose	Five biofilm-producer strains of MDR *Pseudomonas aeruginosa* together with *Pseudomonas aeruginosa* ATCC 27853	Melittin at 50 μg/mL degraded the bio-film layer and eradicated all of the bacteria after 24 and 48 h, respectively. These activities were further confirmed by field emission scanning electron microscopy.	Khozani et al. 2018

[a] $MBIC_{100}$: Minimum biofilm inhibitory concentration was defined as the lowest concentration of melittin that exhibited 100% inhibition in biofilm formation

[b] $MBIC_{90}$: Minimum biofilm inhibitory concentration was defined as the lowest concentration of melittin that exhibited 90% inhibition in biofilm formation

[c] MTT: 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide

ongoing to weaken toxicity of native melittin without influencing its microbicidal activity. Development of drug delivery vehicle by incorporating melittin into nanoparticle represents a safe approach for in vivo application of melittin with favorable pharmacokinetics (Soman et al. 2009). Conjugation of melittin with aptamers is another promising strategy for attenuating hemolytic activity of the peptide (Rajabnejad et al. 2018). Besides, incorporation of AMPs into commercially available hydrogels is rather the other way for an innovative therapy of topical infections, especially those related to burn wounds (Silva et al. 2015; Björn et al. 2015). Development of DNA constructs in which the gene of melittin is under the control of an inducible promoter may hold the potential as future prophylactic and therapeutic approaches. Noticeably, combination of natural melittin and current antibiotics and/or AMPs is another solution to minimize doses of melittin which can lessen both cytotoxicity concern of melittin and the likelihood of developing antibiotic-resistant mutant bacteria. These combinatorial therapies can be useful for future treatment of hard-to-treat MDR, XDR, and PDR pathogens.

Conclusions

Over the past half-century, empirical evidences have expanded our knowledge regarding biological effects of melittin. In this respect, published data suggest that melittin is effective against both planktonic and biofilm-embedded bacteria. Furthermore, the synergism between melittin and antibiotics can be a hopeful solution for treatment of antibiotic-resistant superbugs. The double-edged nature of melittin, as a microbicidal and hemolytic constituent of honeybee venom, should not dissuade scientists to scrutinize its conceivable therapeutic applications. Eventually, anti-infective features of melittin will open up new horizons in a range of biomedical areas, particularly from agriculture to veterinary and clinical microbiology.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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