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Kinetics Study of Antimicrobial Peptide, Melittin, in Simultaneous Biofilm Degradation and Eradication of Potent Biofilm Producing MDR *Pseudomonas aeruginosa* Isolates

Reyhaneh Shams Khozani[1] · Delavar Shahbazzadeh[2] · Naser Harzandi[1] · Mohammad Mehdi Feizabadi[3] · Kamran Pooshang Bagheri[2]

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Abstract
Biofilm associated *Pseudomonas aeruginosa* infections are of major clinical concern due to treatment failure by conventional antibiotics. Referring to many reports, antimicrobial peptides (AMPs) would be act as a new promising agent to overcome the issue. In this regard, our study was aimed to evaluate the kinetics of melittin as a natural AMP, in simultaneously degrading and killing potent biofilm producing multidrug-resistant (MDR) *P. aeruginosa* isolates. The sensitivity of *P. aeruginosa* clinical isolates against routinely prescribed antibiotics was evaluated using disc diffusion and micro-dilution broth methods. Biofilm formation ability of the isolates was determined by colorimetric method. The biofilm formation kinetics was evaluated in five highly biofilm producer MDR isolates during 48 h. The efficiency of melittin to degradation of biofilm biomass and killing the bacteria within the biofilm were kinetically performed. The degradation activity of melittin on preformed biofilm and also its effect on the morphology of *P. aeruginosa* within the biofilm was investigated by field emission-scanning electron microscopy (FE-SEM). Melittin at the amount of 2 and 4 µg inhibited or killed all the examined strains in planktonic state while at 50 µg degraded the biofilm layer and killed all embedded bacteria after 24 and 48 h, respectively. FE-SEM results confirmed the biofilm removal and killing activities of melittin. Linear regression analysis verified the trend of melittin's activities in a concentration and time dependent manner. In conclusion, it seems plausible that melittin should be further investigated in an animal model of biofilm associated burn infection as a new drug lead.

Keywords Antimicrobial peptides · Biofilm degradation · *Pseudomonas aeruginosa* · Melittin

Introduction

In recent years, the development of multidrug-resistant (MDR) bacteria particularly in burn infections has led to increased interest for new antibiotics from natural sources (Davies and Davies 2010).

Pseudomonas aeruginosa as a highly versatile opportunistic pathogen worldwide, is the major cause of morbidity and mortality in burn patients (Turner et al. 2014). Reference to many documented reports in many countries, the incidence of MDR *P. aeruginosa* is being increased (Falagas et al. 2006; Aloush et al. 2006; Hirsch and Tam 2010). Recent reports on the antibiotic sensitivity patterns of *P. aeruginosa* have highlighted the problem of antibiotic resistance in burn infection strains in comparison with other hospital isolates (Klockgether and Tümmler 2017; DiMuzio et al. 2014).

Pseudomonas aeruginosa persists in a biofilm, which further enhances the high antibiotic tolerance of the organism (Dean et al. 2011; Wood 2017). Heavy prescription of antibiotics has led to the worldwide spread of antibiotic-resistant bacteria in hospitals and communities which poses as a serious danger to human health (Ventola 2015).

✉ Kamran Pooshang Bagheri
k_bagheri@pasteur.ac.ir

[1] Department of Microbiology, Faculty of Sciences, Karaj Branch, Islamic Azad University, Karaj, Iran

[2] Venom and Biotherapeutics Molecules Lab, Medical Biotechnology Department, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran

[3] Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

At the moment, all of the prescribed antibiotics including fluoroquinolones, cephalosporins, aminopenicillins, colistin, carbapenems and tetracyclines, in single or combined therapy cannot guarantee the complete eradication of some *P. aeruginasa* burn infections (Høiby et al. 2010, 2005). This phenomenon is due to the mutations of the antibiotic targets that beside the biofilm barrier can lead to failure in treatment of different kinds of *P. aeruginosa* infections, such as burn and respiratory tract infections (Oliver et al. 2004).

Biofilm is one of the major concerns in the issue of antibiotic resistance in *P. aeruginasa* burn infections (Percival et al. 2015; Costerton et al. 1999). Biofilm can provide a protected niche in which the bacteria enveloped in an exopolysaccharide and extracellular protein secretion. Biofilm is often physically resistant to anti-microbial agents and host immune responses (Wood 2017; Limoli et al. 2015; Verstraeten et al. 2008).

It has been shown that biofilm formation can be a defensive reaction in the presence of antibiotics. While biofilms contain cells with a heterogeneous range of states (Balcázar et al. 2015), on average, bacteria in biofilms have a much higher antibiotic tolerance up to 1000-fold greater than their planktonic counterparts (de la Fuente-Núñez et al. 2013).

Eradication of bacteria within the biofilm is a challenging issue that is corresponded to alginate, as a physical barrier, and remarkable reduction in bacterial metabolism in mature biofilm as well (Ishida et al. 1998; Wood 2016).

These two reasons are the major limitations to the successful implementation of conventional antibiotics. In this vital condition, increasing the dose of prescribed antibiotics, not only fail to address the problem but may also induce significant toxicity. Colistin as a choice to treatment of *P. aeruginosa* burn infection is a good example where nephrotoxicity is a remarkable sequel (Hachem et al. 2007).

Annually, 17 million new biofilm associated infections are reported in the United States, and amongst this figure 550,000 people die (Wood 2017).

In this critical condition, antimicrobial peptides could be new promising agents to overcome this threatening challenge (Mandal et al. 2014). AMPs are evolutionarily conserved key effector molecules of the innate immune system that exist in a wide range of insects, animals, plants, and bacteria (Diamond et al. 2009). The venom of venomous animals also indicates a potential source of AMPs (da Mata et al. 2017).

AMPs usually have common characteristics including small size (12–60 amino acids), an excess positive charge ranging from +2 to +9, around 50% hydrophobicity, and the acquisition of different secondary structures in membranous environments (Jindal et al. 2014).

In comparison to conventional antimicrobial agents, AMPs have some attractive advantages including rapid killing and broad spectrum antimicrobial activity, typically not involving induction of bacterial resistance, and show synergistic activity when combined with classic antibiotics (Chung and Khanum 2017). The AMPs firstly bind to the membrane and subsequently invade it as a detergent or by degradation of the bacterium by a pore forming mechanism. In some cases, the AMPs can transfer themselves into the cytoplasm and interfere with replication, transcription, and translation activities (Gaspar et al. 2013).

Among AMPs, a series of drug candidates derived from antimicrobial peptides (i.e. Locilex, C16G2, Cefilavancin, NVB302, Dalvance, LTX-109, PXL01, and LL-37) which are in the different phases of clinical studies (Felício et al. 2017).

Among antimicrobial peptides, melittin was selected as a potent candidate to prove our hypothesis being that it can degrade biofilm layers and kill the containing bacteria. Melittin ($C_{131}H_{229}N_{39}O_{31}$) is an alpha helical cationic peptide consist of 26 amino acids (Terwilliger and Eisenberg 1982). The first 20 residues (N-terminal) of melittin are predominantly hydrophobic amino acids, whereas the carboxyl-terminal of the peptide is mostly composed of hydrophilic residues. This amphipathic entity allows the peptide to interact with phospholipid membranes (Lee et al. 2013).

Regarding the major concern of biofilm in terms of global and local antibiotic resistance, this study was aimed to evaluate the kinetics of antimicrobial peptide, melittin, in simultaneously degrading and killing potent biofilm producing MDR *P. aeruginosa* isolated from third degree burn patients.

Materials and Methods

Reagents and Media

Antibiotic discs including gentamicin, ceftazidime, ciprofloxacin, piperacillin–tazobactam, doripenem, levofloxacin, cephalothin, doxycycline, and cefepime were purchased from MAST Group Co. (UK). Antibiotic powders including doripenem, colistin, and ceftazidime were obtained from Sigma-Aldrich (St Louis, MO, USA). Muller Hinton Broth (MHB) and Agar (MHA) were purchased from Merck Co. (USA).

All the results are obtained from three independent replicates in three parallel trials otherwise indicated. Error bars represent the mean ± standard deviations.

Peptide Synthesis

The peptide, melittin, was synthesized at Mimotopes Company (Clayton, Victoria, Australia) using Fmoc chemistry with detailed procedures previously described by solid-phase method with C-terminal amidation. The purity of the ordered peptide was roughly greater than 95%. To validate the molecular weight of synthetic melittin, mass

spectrometry was performed in positive ion mode on a Sciex API100 LC/MS instrument (PerkinElmer Co., Norwalk, CT, USA) by the company.

In Vitro Antibacterial Activity of Melittin Against Clinical Isolates

Thirty-three *P. aeruginosa* isolates were collected from third degree burn patients during the period between 2017 and 2018 at Shahid Motahari burn hospital, Tehran-Iran. The samples were collected from burn areas at least 48–72 h after patient hospitalization and detected based on the routine laboratory methods.

Antibiotic susceptibility assay was performed on all isolates using disk diffusion to determine the frequency distribution of antibiotic resistance. Inhibitory and bactericidal activities of melittin were examined on all isolates and compared with some conventional antibiotics in order to show the therapeutic value of melittin against multiple drug resistant bacteria.

Disc Diffusion Assay

Bacterial suspensions were prepared by spectrophotometry at 625 nm. Based on the McFarland method, optical density in the range of 0.08–0.1 is equivalent to 1.5×10^8 CFU/mL. In this study, to increase the accuracy of quantification, the OD of suspension was considered at 0.09. Disc diffusion assay was performed using gentamicin, ceftazidime, ciprofloxacin, piperacillin–tazobactam, doripenem, levofloxacin, cephalothin, doxycycline, and cefepime. Susceptibility testing was performed by the Kirby–Bauer disk diffusion method according to Clinical and Laboratory Standard Institute guidelines (CLSI) (Clinical and Laboratory Standards Institute guideline 2015).

Determination of MIC and MBC

Minimum inhibitory concentrations (MICs) were determined by a standard microtiter dilution method using Muller Hinton Broth (MHB) medium according to CLSI guidelines (Clinical and Laboratory Standards Institute guideline 2015). To compare the antibacterial activity of melittin with the most applied antibiotics, the MIC for colistin, doripenem and ceftazidime were also determined.

Briefly, the bacteria were grown overnight at 37 °C in the MHB. Melittin and the antibiotics were serially diluted in a 96 well polypropylene microplate, 1.5×10^5 bacteria added to each well and then incubated at 37 °C for 24 h. MBCs were determined at the end of the incubation period by subculture of a sample from each well with no visible growth onto MHA. The resultant colonies were counted after overnight incubation at 37 °C. MBC was determined as the lowest concentration of the peptide or antibiotics that kill 100% of the bacteria.

Biofilm Assay

The goal of this assay was tracing for potent biofilm producer strains among MDR isolates. Biofilm assay was performed based on the Segev-Zarko et al. protocol as detailed below (Segev-Zarko et al. 2015). All clinical isolates were cultured in 5 mL of TSB-glucose and incubated at 37 °C for 24 h. The suspensions were washed two times in TSB-glucose medium and their turbidity was adjusted to 0.5 McFarland (OD 0.08–0.1) at 625 nm using a spectrophotometer.

Then, 1.5×10^7 CFU was added to each well that was pre-filled with 100 µL TSB-glucose medium and incubated at 37 °C overnight. After incubation, the upper medium was aspirated entirely and the wells were washed three times with 250 µL of PBS to remove the suspension and the non-attached bacteria. Then 200 µL of methanol (99%) was added to each of the wells and incubated at room temperature (RT) for 15 min let dry.

The wells were stained with 200 µL of crystal violet (1%) for 5 min and washed three times with sterile deionized water. Finally, 200 µL of ethanol (95%) was added to each well and incubated at 37 °C for 30 min in a shaker incubator. The content of each well was transferred to the equivalent well in other microplate and then, the optical density (OD) was measured at 595 nm using a microplate spectrophotometer (EPOCH, BioTek Co., Winooski, VT, USA).

Kinetics of Biofilm Formation

Five MDR *P. aeruginosa* isolates were selected to follow the anti-biofilm activity of melittin. To get more accurate insight into the biofilm formation, kinetics study was performed on the selected strains. Briefly, biofilm assay were performed as aforementioned above but the OD of each sample was measured after 1, 3, 6, 24, and 48 h.

The Kinetics for Multiplication of the Bacteria in Biofilm Environment

Simultaneously, in a parallel group, the selected strains were cultured as detailed before and then the numbers of bacteria were counted in each well after 1, 3, 6, 18, 24, and 48 h. To do this, the upper solution was discarded and the wells were washed three times with saline solution. Afterward, saline (100 µL) was added to each well and the surfaces of the wells were scratched, from which 10 µL was cultured in MHA medium at 37 °C. The resultant colonies were counted after 24 h. The positive control was *P. aeruginosa* (ATCC 27853) in TSB-glucose medium.

Biofilm Degradation Kinetics

This assay was kinetically performed to determine the efficiency of melittin to degradation of biofilm biomass. Different amounts of melittin were examined in different time points to achieve the target amount and time in which the most degradation would be recorded. The bacterial biofilms were produced as aforementioned above. Then, the melittin was serially diluted ranging from 8 to 0.06 µg and added to the wells in a descendent manner. After 1, 3, 6, and 24 h the remaining biofilm was measured as described above.

Time-Kill Kinetics of Melittin Against Bacteria Within Biofilm

In this assay, the potency of melittin for killing bacteria in the surrounding biofilm was kinetically assessed. Different amounts of melittin were examined at different time points to achieve the target amount and time in which the all bacteria would be killed. The biofilms of the bacteria were produced as aforementioned above. Melittin at the amount of 50 µg added to the wells; after 0.5, 1, 3, 6, 24, 48, and 72 h the surface of each well was scratched and 10 µL of the suspension was cultured on MHA, incubated for 24 h, and the possible colonies were counted.

Morphological Assessment for Activity of Melittin by Field Emission Scanning Electron Microscopy

Field emission-scanning electron microscopy was used to visualize the effect of melittin on the biofilm removal of a MDR *P. aeruginosa*. At first, melittin (10 µg) was added to 1.5×10^7 bacteria and incubated at 37 °C for 24 h. Before incubation, sterile slides were aseptically put into the wells. Sample preparation for SEM was performed as previously described (El-Azizi et al. 2015). Briefly, the slides were gently washed three times with sterile distilled water and the sample was fixed in glutaraldehyde (0.1 M in PBS 1×) for 3 h at room temperature. The slides were then rinsed three times in fresh PBS buffer and post-fixed in 1.5% osmium tetroxide for 1 h. They were dehydrated in a series of ethanol solutions (30–100%). The specimens were then mounted on aluminum stubs, allowed to dry for 3 h, coated with gold nanoparticles, and examined in an FE-SEM instrument (MIRA3, TESCAN Co., Czech).

Results

In Vitro Antibacterial Activity

Disc Diffusion

Among 33 strains, 18.18% (6 cases) and 18.18% (6 cases) were resistant and sensitive to all examined antibiotics, respectively. The frequency of strains that were resistant to at least three antibiotics form three antibiotic classes was estimated as 39.4% (13 cases).The lowest resistance was seen against doripenem (Fig. 1).

MIC and MBC for Melittin, Colistin, Doripenem, and Ceftazidime

Melittin at the amounts of 2 and 4 µg inhibited or killed all the examined strains, respectively. The growth of 48% of the strains was inhibited at the concentration of 2.5 µg/mL (0.5 µg) and 31% of them were destroyed in the same amount.

Colistin, doripenem, and ceftazidime at the maximum concentration of 64 µg/mL were not able to inhibit or kill all of the isolates. There was a major difference between MIC and MBC of the antibiotics and melittin (p value < 0.05). The in vitro activitiy of melittin and the examined antibiotics against all of the *P. aeruginosa* isolates are summarized in Figs. 2 and 3.

Comparing the MIC and MBC results for melittin with other examined antibiotics showed that melittin had an eight and fourfold greater inhibitory and killing activities than doripenem, respectively. These values for ceftazidime were

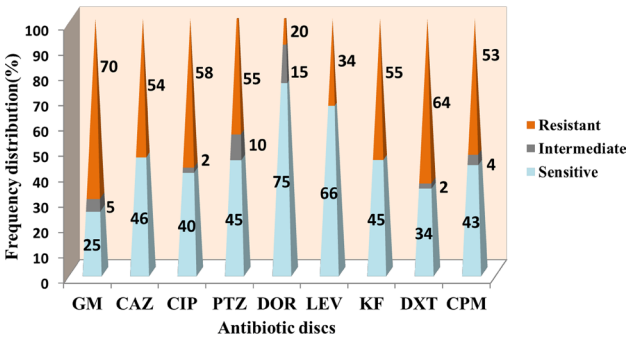

Fig. 1 The frequency distribution of sensitivity in all isolates against the examined antibiotic discs. The results showed that 18.8% of the isolates were resistant to all nine examined antibiotics. The frequency of strains that were resistant to at least three antibiotics form three antibiotic classes was estimated as 39.4% (13 cases). The lowest resistance was seen against doripenem. GM, CAZ, CIP, PTZ, DOR, LEV, KF, DOX, and CPM are abbreviated for gentamicin, ceftazidime, ciprofloxacin, piperacillin–tazobactam, doripenem, levofloxacin, cephalothin, doxycycline, and cefepime, respectively

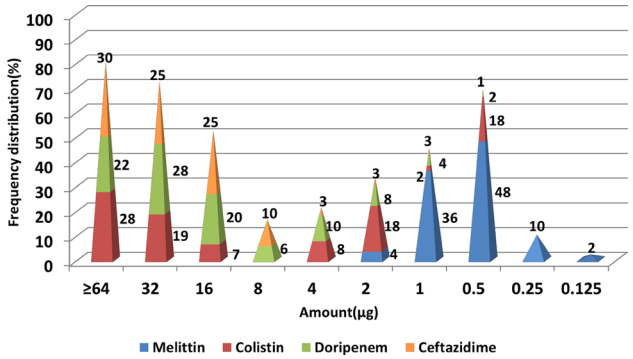

Fig. 2 The frequency distribution of MIC for melittin, colistin, doripenem, and cephtazidime. Reference to the results, the majority of strains was inhibited at lower amounts of melittin in comparison to other examined antibiotics. Melittin at the amounts of 2 μg inhibited the growth of all the examined strains. Colistin, doripenem, and ceftazidime at the maximum concentration of 64 μg/mL were not able to inhibit all of the isolates. There was a major difference between MIC of each of the antibiotics and melittin (p value < 0.05)

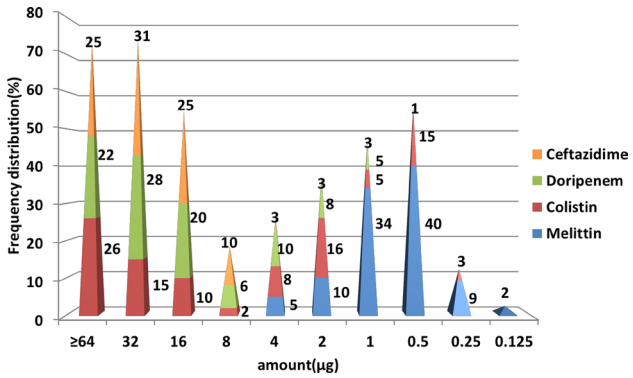

Fig. 3 The frequency distribution of MBC for melittin, colistin, doripenem, and ceftazidime. In reference to the results, the majority of strains were killed at lower amounts of melittin in comparison to other examined antibiotics. Melittin at the amount of 4 μg killed all the examined strains. Colistin, doripenem, and ceftazidime at the maximum concentration of 64 μg/mL were not able to kill all of the isolates. There was a major difference between MBC of each of the antibiotics and melittin (p value < 0.05)

32- and 16-fold whereas for colistin was fourfold for both of MIC and MBC.

The Kinetics of Biofilm Formation

The highest OD for all examined isolates was showed after 24 h that ranged between 0.85 and 2. Biofilm formation was significantly time dependent up to 18 h in all isolates and ATCC strain ($R^2 = 0.92–0.99$) as determined by linear regression assay. Slope of biofilm formation was rapidly elevated between 18 and 24 h followed by a remarkable decrease at 48 h (Fig. 4).

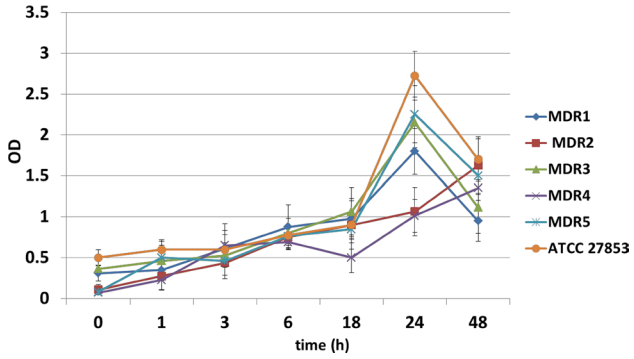

Fig. 4 The kinetics of biofilm formation. The maximum OD was seen at 24 h in all examined bacteria. This data showed that the biofilm biomasses maturated at 24 h. Biofilm formation was significantly time dependent up to 18 h in all isolates and ATCC strain ($R^2 = 0.92–0.99$) as determined by linear regression assay. Slope of biofilm formation was rapidly elevated between 18 and 24 h followed by a remarkable decrease at 48 h

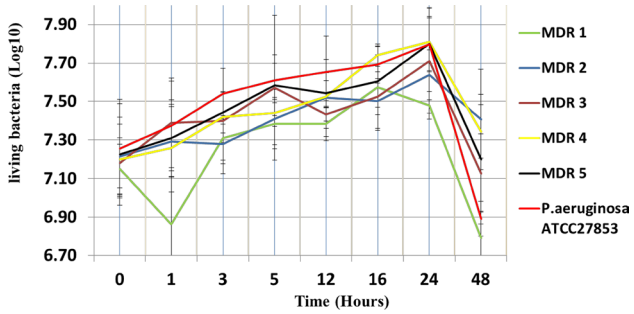

Fig. 5 The kinetics of bacterial multiplication during biofilm formation. The results indicated an increase in the number of bacteria in a gradual manner up to 24 h. Along with the kinetics study of biofilm formation, the numbers of bacteria were counted to determine the velocity of bacterial growth within the biofilm layers. The results showed a time dependent multiplication up to 24 h. The geometric mean of total count of bacteria was at the log of 7.65 after 24 h

The Kinetics of Bacterial Multiplication in Biofilm Environment

During biofilm formation at a determined time point, the numbers of the bacteria were counted. The results indicated that the slope of growth is slow but occurred in a time dependent manner up to 24 h. The numbers of bacteria were decreased from 24 to 48 h (Fig. 5).

Biofilm Degradation Kinetics of Melittin

Different ascending amounts of melittin from 0.06 up to 50 μg were prepared and added to pre-formed biofilm to evaluate the removal activity of melittin in a kinetics trend. The results showed that all the examined doses had removal activity in a time dependent manner. The amount of 50 μg

had the greatest activity to degrade the biofilm layer in comparison to other doses (Fig. 6).

Killing Kinetics Assay of Melittin

Melittin at 50 µg reduced the amount of MDR and ATCC bacteria by a rate of 6 logs after 24 h incubation and eradicated all of them after 48 h (Figs. 6, 7).

Scanning Electron Microscopy

Field emission-scanning electron microscopy was used to examine the biofilm removal activity of melittin. The results demonstrated that melittin at the amount of 10 µg started its biofilm degradation activity so that no biofilm layer was seen on the surface of slides. During this time, melittin could affect the bacterial membrane and cell shape as vesicle and squeezing, respectively. After 60 min, these effects were seen more and more and many of the bacteria disintegrated (Fig. 8).

Discussion

Our current understanding of the players of bacterial biofilm formation has being increased (Batoni et al. 2016) but there is still a great challenge in the development of anti-biofilm drugs. To date, no anti-biofilm drug has been registered for clinical trial yet. Penetration of antimicrobial agents into biofilm layers is a hard challenge (Percival et al. 2011) besides the issue of antibiotic resistance. Another issue for bacterial resistance within biofilm is the slow or inactivated metabolism in persister cells (Wood 2016). These have made the treatment of biofilm-related infections very problematic. In this condition, the need for a new promising anti-biofilm agent is urgently necessitated. In this regard, we tried to examine the anti-biofilm activity of melittin, a potent natural AMP.

The frequency distribution of resistant isolates against the examined antibiotic disks showed that 18.8% of isolates were resistant to nine conventional antibiotics. This issue could lead to devastating outcomes in the burn center that

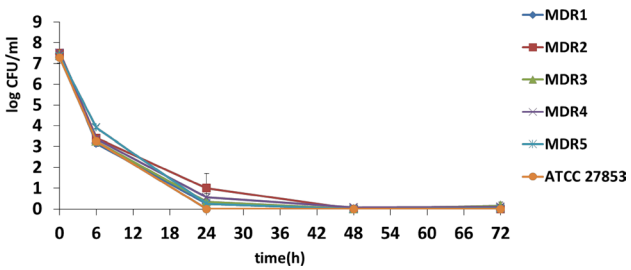

Fig. 7 Killing kinetic curve for the ability of melittin to kill the examined bacteria within the biofilm. The results showed eradication of all bacteria after 48 h. The results indicated that melittin at 50 µg completely eradicated all bacteria after 48 h. This issue indicated that melittin maintains its stability along this duration, penetrates to biofilm layers gradually, and then induces its killing activities

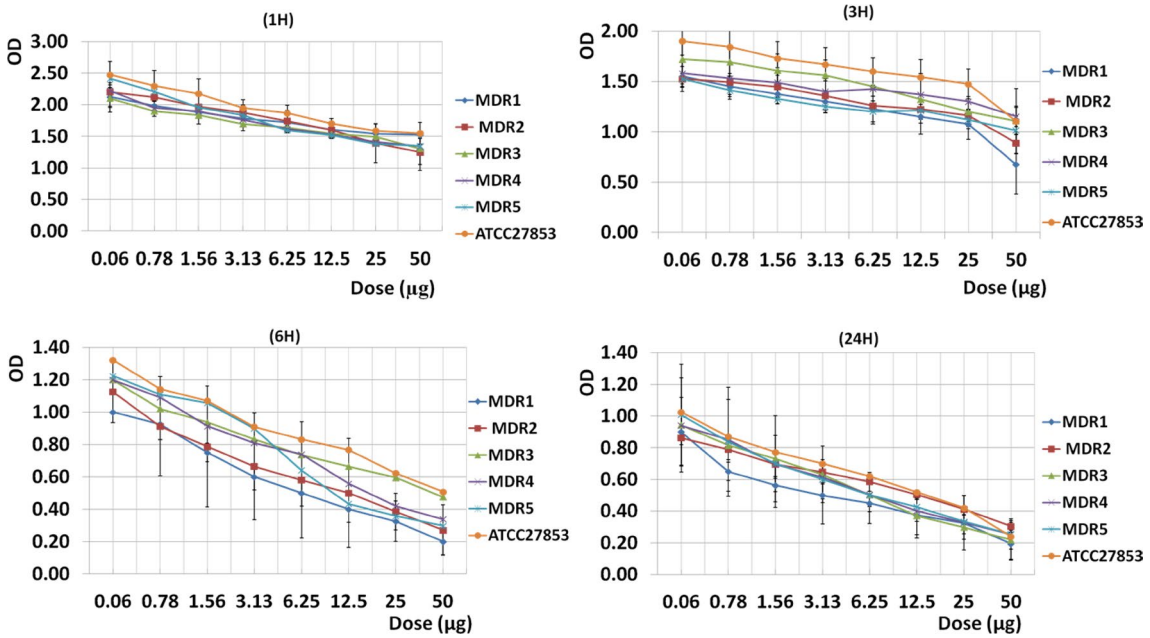

Fig. 6 Biofilm degradation kinetics of melittin. The amount of 50 µg had the greatest activity to degrade the biofilm layer in comparison to other doses. The results showed that all the examined doses had removal activity in a time dependent manner

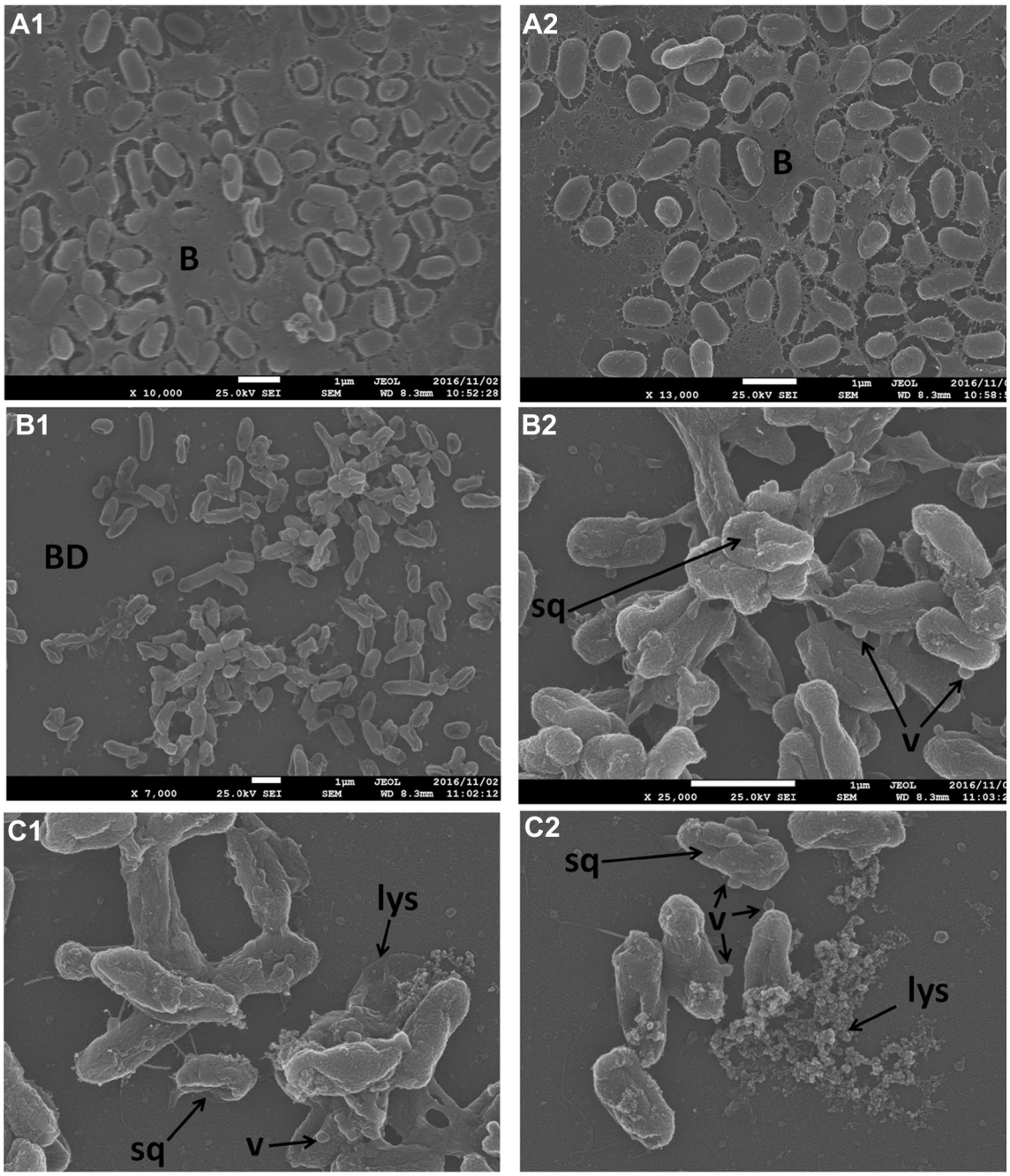

Fig. 8 The morphology of *P. aeruginosa* biofilm was investigated after 30 min treatment with 10 µg melittin by scanning electron microscopy. Analysis of FE-SEM results showed that melittin not only has a significant biofilm degradation activity for removal of biofilm layers but is also able to kill them via invading their membranes. Visualizing many vesicles on bacterial membranes and subsequent death demonstrates this mechanism. *A1 and A2* control, *B1 and B2* Biofilm formation, *C1 and C2 P. aeruginosa* biofilm treated with melittin for 30 and 60 min respectively. *B* biofilm, *BD* biofilm degradation, *lys* lysis, *v* vesicle, *sq* squeezing

the isolates were collected from, in terms of the potential outbreak of XDR bacteria.

The advent of XDR strains in a burn center indicated that an outbreak is being developed. In this condition, treatment of these infections by an alternative antibiotic is very critical to inhibit the spread of XDR strains.

Based on the results, 20% of the isolates were resistant to doripenem. As recently documented, doripenem is a newly presented antibiotic amongst the carbapenem family. The moderate frequency of resistance to this antibiotic is a disappointing issue regarding its future application.

Reference to the results obtained from the MIC and MBC assays, melittin had a more inhibitory and lethal activity against the clinical isolates in comparison to colistin, doripenem, and ceftazidime. According to the MIC value, the most active agents against *P. aeruginosa* were melittin, colistin, doripenem and ceftazidime, respectively.

Our results gathered from disk diffusion, MIC, and MBC assays on the planktonic state of clinical isolates of burn infections suggest that melittin has a precious pharmaceutical value so much so that it is a good candidate for further examination in a mouse model of third degree burn infection.

Pompilo et al. in 2012 obtained the inhibitory activity of natural antimicrobial peptide BMAP-27 and BMAP-28 and also that of an engineered peptide in 25 *P. aeruginosa* isolates of cystic fibrosis patients at the range of 4–16, 4–32, and 4–32 µg/mL, respectively (Pompilio et al. 2012). In comparison, melittin in our study showed greater activity ranging 0.625–10 µg/mL (0.125–2 µg).

In the study of Sánchez-Gómez et al. in 2015, the antibacterial activity of a series of peptides and lipopeptides against planktonic *P. aeruginosa* PAO1 were totally lower than melittin (0.625–10 µg/mL) (Sánchez-Gómez et al. 2015).

In 2015, Beringer et al. showed the MIC and MBC of Rhesus θ-defensin-1 (RTD-1) on 29 *P. aeruginosa* isolates of cystic fibrosis patients at 8 and 64 µg/mL (Beringer et al. 2015). In comparison, melittin had approximately the same inhibitory activity and a 3.2-fold greater killing activity against burn infection *P. aeruginosa* isolates.

Based on the kinetics results for biofilm formation, it could be suggested that the biofilm is being produced in a time dependent manner up to 18 h. After this time, the conditions were ideal for the bacteria to form the maximum amount of biofilm during 6 h so that at the time of 24 h the biofilm reached its maximum maturation. Obtaining this critical time point is necessary to have a precise result in the study of biofilm degradation kinetics and also killing kinetics of melittin. Interestingly, the biomass dropped significantly during the next 24 h. This reduction may be due to accumulation of acidic byproducts and subsequent physical degradation of biofilm layers. It must be noted that this degradation may not happen in clinical conditions.

Production of different biomasses in different bacteria indicates the heterogenicity in biofilm formation ability of the strains. This issue may be due to different expression profiles of the genes involved in biofilm formation. The data reproduced in this manner can improve our insight to develop the experimental design of in vivo studies. From the point of clinical approach, these results indicate that the physicians have only 18 h to start the appropriate antibiotic regimens.

Along with the kinetics study of biofilm formation, the numbers of bacteria were counted to determine the velocity of bacterial growth within the biofilm layers. The results showed a time dependent multiplication up to 24 h. The total count of bacteria was at the log of 7.8 after 24 h.

Knowing this data can be very useful in killing kinetics experiments which can basically inform us how many bacteria exist at the biofilm maturation point.

The results of biofilm degradation and killing kinetics of melittin on the examined bacteria indicated that melittin at 50 µg degraded the biofilm layer remarkably after 24 h and completely eradicated all bacteria after 48 h. This issue indicated that melittin maintains its stability along this duration, penetrates to biofilm layers gradually, and induces its degradation and killing activities.

Hirt and Gorr in 2013 showed that GL13K as an engineered peptide at the amount of 70 µmol killed 99.9% of the bacteria within pre-formed *P. aeruginosa* biofilm during 4 h (Hirt and Gorr 2013) whereas melittin, in our study, eradicates all examined MDR isolates at 0.017 µmol for up to 48 h. Our data indicated that melittin in comparison to GL13K is more promising for eradication of bacteria in the depth of biofilm.

Sánchez-Gómez et al. in 2015 showed that the peptides and peptoids, when tested on biofilms grown under dynamic or static conditions, did not show promising antibacterial activity. Among them, LF11-215 and LF11-227 were the most potent peptides, since, at 10× MIC, they were able to decrease more than 2 logs (i.e. > 99%) of cell viability of biofilms in just 10 min. Furthermore, both peptides caused a 10,000-fold (4 logs) reduction in biofilm cell viability after 1 h of treatment at 10× MIC (Sánchez-Gómez et al. 2015). In comparison to our results, melittin at its MIC dose completely degraded the biofilm in MDR isolates for 6–24 h and also fully eradicated the examined bacteria after 48 h. These comparisons indicate that the stability of the examined peptides and peptoids were being decreased over time whereas melittin induced its activity gradually by maintaining its stability.

Beringer et al. in 2015 showed a modest killing activity of RTD-1 as an engineered peptide on pre-formed biofilm containing *P. aeruginosa* PAO1 strain (Beringer et al. 2015) whereas melittin completely killed the MDR isolates in a time dependent manner. The maximum effect of RTD-1 on decreasing biofilm abundance was obtained at an average of 74.2% whereas melittin gradually degraded the biofilm biomass during 24 h.

Pulido et al. in 2016 examined the anti-biofilm activity of RN3 (5-17P22-36), derived from RNAse 3 antimicrobial peptide, and showed 100% killing activity at 25 µm (Pulido et al. 2016) whereas melittin in our study demonstrated 100% killing activity at 0.017 µm, indicating a more significant activity than the abovementioned engineered peptide.

Melittin degraded about 90–95% of biofilm biomass at 25 MIC (50 µg) during 24 h while Mohamed et al. in 2017

showed that RR-4, an engineered AMP derived from LL-37, reduced 61% of biofilm mass at 32 MIC (Mohamed et al. 2017).

Analysis of FE-SEM results showed that melittin not only has a significant biofilm degradation activity for removal of biofilm layers but is also able to kill them via invading their membranes. Visualizing many vesicles on bacterial membranes and subsequent death demonstrates this mechanism.

Beringer et al. in 2015 demonstrated the effect of RTD-1 on the *P. aeruginosa* PA01 strain as aggregation. RTD-1 failed to kill them during 48 h, shown by SEM (Beringer et al. 2015) while melittin not only degrades the biofilm layer but can induce disintegration of the MDR bacteria, proved by FE-SEM demonstrations.

To the best of our knowledge, in reference to our thorough review on documented papers about natural anti-biofilm or engineered AMPs, there are no promising AMPs which can completely degrade the pre-established biofilm and/or eradicate the bacteria within the biofilm milieu (Batoni et al. 2016; Shang et al. 2017; Mishra and Wang 2017; Grassi et al. 2017; Thamri et al. 2017; Schillaci et al. 2014).

In the serious case of antibiotic resistance, the ability of some bacterial pathogens to produce biofilm aggravates this issue. Recently, AMPs have been made in hopes of not only overcoming the issue of MDR bacteria but dispersing the pre-formed biofilm or killing the internal bacteria.

To eradication of biofilm associated infections, both of biofilm degradation and killing activities are basically needed. A candidate antibiofilm agent must have a complete bactericidal activity against MDR bacteria. Since, melittin had these ideal advantages, this issue encouraged us to examine the in vivo efficacy of it as a topical antimicrobial agent in a mouse model of third degree burn infection in our future studies. Toxicity of melittin on normal cells is a disadvantage for future application of melittin as a drug but it cannot an important issue regarding to its therapeutic effects on third degree burn infections. This suggestion is originated from this fact that in this type of burn, all three layers of skin are destroyed so that the toxicity of melittin is not a limitation factor to its topical application. LL-37, for instance, is a natural toxic human peptide but it has been entered in phase II clinical trials as a topical agent to treatment of leg ulcers (Felício et al. 2017).

In conclusion, melittin successfully degraded all of pre-established biofilm and also eradicated all of *P. aeruginosa* bacteria within the biofilm in a concentration and time dependent manner. Altogether, pharmaceutical value of melittin, as a drug lead, could be examined in a mouse model of burn infection as a topical antimicrobial and antibiofilm agent.

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Compliance with Ethical Standards

Conflict of interest Reyhaneh Shams Khozani, Delavar Shahbazzadeh, Naser Harzandi, Mohammad Mehdi Feizabadi, and Kamran Pooshang Bagheri declare that they have no conflict of interest.

Research involving Human and Animal Participants This article does not contain studies with human participants or animals performed by any of the authors.

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