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Melittin peptides exhibit different activity on different cells and model membranes

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Abstract

Melittin (MLT) is a lytic peptide with a broad spectrum of activity against both eukaryotic and prokaryotic cells. To understand the role of proline and the thiol group of cysteine in the cytolytic activity of MLT, native MLT and cysteine-containing analogues were prepared using **solid phase peptide synthesis**. The antimicrobial and cytolytic activities of the monomeric and dimeric MLT peptides against different cells and model membranes were investigated. The results indicated that the proline residue was necessary for antimicrobial activity and cytotoxicity and its absence significantly reduced lysis of model membranes and hemolysis. Although lytic activity against model membranes decreased for the MLT dimer, hemolytic activity was increased. The native peptide and the MLT-P14C monomer were mainly unstructured in buffer while the dimer adopted a helical conformation. In the presence of neutral and negatively charged vesicles, the helical content of the three peptides was significantly increased. The lytic activity, therefore, is not correlated to the secondary structure of the peptides and, more particularly, on the propensity to adopt helical conformation.

Key words: melittin, antimicrobial peptide, cytotoxicity, hemolysis, dye leakage.

Introduction

Antimicrobial peptides (AMP) are found in plants, insects and human cells (Gergely et al. ; Reddy et al. 2004; Lee et al 2014; Gehman et al. 2008; Baila et al. 2004) and are typically 10-40 residues in length and positively charged (Henriksen et al. 2014). Although the mechanism of AMP action is unknown and pore formation in cell membranes may be responsible for lytic ability, it is well established that AMP interact directly with the cell membrane surface and are able to enter the lipid bilayer, resulting in cell membrane disruption and leakage of cytoplasmic components which leads to killing of microbes (Lee et al. 2010; Izadpanah and Gallo 2005; Lorenzon et al. 2012; Sani et al. 2013). AMPs have attracted much attention as an alternative to antibiotics because of their effect against bacterial infections and drug resistant bacteria (Juba et al. 2013; Anaya-Lopez et al. 2013).

Melittin (MLT), a lytic and well studied peptide, is the principal active component of bee venom, with high antimicrobial and antiviral activity and cytotoxic properties against mammalian cells (Asthana et al. 2004; Hyun-Ji et al. 2013; Khatun and Mukhopadhyay 2013). MLT is a linear cationic, non-selective, amphipathic peptide of 26 amino acids (Figure 1) in which residues 1-20 are hydrophobic and residues 21-26 are hydrophilic (Raghuraman and Chattopadhyay 2004). MLT can target live cells such as red blood cells and binds to lipid membranes spontaneously leading to lipid bilayer disruption and hemolysis (Lam et al. 2002; Lam et al. 2001; Hinch and Crowe 1996; Raghuraman and Chattopadhyay 2004; Sessa et al. 1969; Raghuraman and Chattopadhyay 2007; Tomoyoshi et al. 2013; Lad et al. 2007; Hall et al. 2011; Zhao et al. 1995). MLT is also known to have anticancer properties (Gajski and Garaj-Vrhovac 2013). As the action mode of MLT depends on membrane composition, different model membranes have been used to study MLT-lipid interaction, with respect to cholesterol content, charge of lipids, and lipid-peptide ratio (Rapson et al. 2011; Ferre et al. 2009; Ningsih et al. 2012; Burton et al. 2013; Klocek and

Seelig 2008). MLT exists as a tetramer in concentrated aqueous solution (Schubert et al. 1985) and two dimers form such tetramer as observed in crystal structures (Terwilliger and Eisenberg 1982). However, results obtained from cross-linking and sedimentation studies suggest that the tetramer dissociates to the monomer and a dimeric state of MLT in solution has not been observed (Takei et al. 1998; Schubert et al. 1985). A synthetic dimeric form of truncated MLT (MLT 1-20) has been reported to exhibit high cytolytic activity (Rivett et al. 1996). The truncated dimer was formed through a disulfide bond at the C-terminus (with cysteine added at the C-terminus). In this work, we undertook to study a dimeric MLT that contains two fulllength MLT monomers (1-26) and choose proline residue at position 14 (Pro-14) as the point of dimerization. Pro-14 plays an important role in the unique conformation as well as the function of MLT. Proline is unique among the common amino acids in that it is unable to form a hydrogen bond in the protein backbone, but instead forms a 'hinge' in a single membrane-spanning helix. Substituting Pro-14 with other amino acids (Lys, Ser or Ala) gave improved helical structures (Rapson et al. 2011; Dempsey et al. 1991; John and Jahnig 1993) and increased hemolytic activity in comparison to native MLT (John and Jahnig 1993). Therefore, we decided to mutate Pro-14 to a cysteine residue by which dimerization was achieved through a disulfide bridge. We compared the activity of this dimeric MLT with its monomeric analogue MLT-P14C as well as with native MLT (Figure 1).

Materials and Methods

Materials

Lipids, palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE) and palmitoyloleoylphosphatidylglycerol (POPG), were purchased from Avanti Polar Lipids (Alabaster, USA). Calcein, Sephadex G-100 gel filtration media

and cholesterol (Chol) were from Sigma-Aldrich (St Louis, USA), Cell Titer-Blue[®] Reagent assay was from Promega (Sydney, Australia), fetal calf serum (FCS), penstrep, 1% glutamax were purchased from Life Technologies.

Peptide synthesis

MLT and its cysteine analogues, MLT-P14C (Figure 1), were synthesized using the Fmoc solid phase method (Tosteson et al. 1987; Wade et al. 2012). Crude peptides were analysed using RP-HPLC on a Phenomenex C4 column (particle size 5 µm, 4.6 x 150 mm), with a gradient of 10-90% of 0.1% trifluoroacetic acid (TFA) in acetonitrile (ACN) and 0.1% TFA in H₂O for 30 min. The peptides were purified using a preparative C4 column with a gradient of 35-65% of 0.1% TFA in ACN and 0.1% TFA in H₂O for 30 min and then lyophilized, freeze-dried and stored at 20°C. The reaction condition for the assembly of dimeric MLT-P14C required optimization. A random oxidation of highly concentrated solution of MLT-P14C at pH 8.5 was tried but no target product was observed within 24 hrs while monitored by analytical RP-HPLC. After 48 hr very little dimer had formed which necessitated an alternative approach. The free thiol group of the peptide was reacted with 2,2-dithiodipyridine (2DPDS, Sigma-Aldrich) and the resulting S-pyridinylated peptide was then reacted with an equivalent amount of free thiol peptide (Figure 2). The reaction was completed in 30 minutes with a very high yield. MALDI TOF mass spectra and HPLC profiles for all three purified peptides are shown in Figure 1.

Dye release in model membranes

The dye release experiments were performed by recording fluorescence using a Cary Eclipse fluorescence spectrophotometer (Varian, Melbourne, Australia). Two different model membranes, POPC/Chol (4:1 mol/mol) and POPE/POPG (7:3), were used to examine the peptide lytic activity. The dye-release assay of native MLT, MLT-P14C and dimerized MLT-

P14C were carried out with the calcein encapsulated in POPC/Chol and POPE/POPG in 20 mM Tris-Cl buffer (pH 7.0 plus 100 mM NaCl). Calcein was dissolved in 4 eq. NaOH and vortexed for complete dissolution. Appropriate volumes of 20 mM Tris buffer (pH 7.0 plus 100 mM NaCl) were added to the calcein solution and then pH adjusted to 7.0 with HCl. Vesicles were prepared by extruding lipid film in buffer containing 80 mM calcein and passing the vesicle suspension through a Sephadex G75 column to separate free calcein.

The vesicles with encapsulated 80 mM calcein, which is a membrane impermeable fluorescent dye (Heerklotz and Seelig 2007), were extruded 10 times through a 0.1- μ m pore size polycarbonate filter to prepare large unilamellar vesicles (LUV). Excess dye was separated by gel filtration on a SephadexG-100 column at a flow rate of 1.5 mL/min. The phospholipid concentration of the most concentrated LUV was determined by phosphorous analysis (Anderson and Davis 1982). The fluorescence intensity (I) was measured after adding peptide to LUV at different lipid to peptide ratios and incubating for 30 min at room temperature. The excitation wavelength was 490 nm and emission wavelength was 512 nm. The maximum fluorescence signal intensity (I_M) was determined by introducing 10% Triton X-100 to the LUV; the minimum, I_0 , was obtained by measuring the fluorescence of LUV in buffer. The leakage fraction was calculated using the following formula:

$$\% \text{ leakage} = [(I - I_0) \times 100\%] / (I_M - I_0) \text{ where } I \text{ is the measured fluorescence.}$$

Determination of the minimum inhibitory concentration (MIC)

The lowest concentration of each peptide that inhibited the growth of selected bacteria was determined in accordance with the Clinical and Laboratory Standards Institute (CLSI). Each test peptide was aliquoted in 50 μ l volumes into the wells of 96-well plates. For each test concentration, triplicate wells were prepared and were then diluted 2-fold in cation-adjusted Mueller-Hinton broth (Oxoid, Basingstoke, United Kingdom). The final concentrations of

each test compound ranged from 125 μM to 0.24 μM , with the exception of dimer-MLT-P14C, which ranged from 62.5 μM to 0.12 μM . For *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA), a 0.5 McFarland standard was prepared by suspending colonies from an overnight HBA plate. For *Escherichia coli* and *Pseudomonas aeruginosa*, three colonies were selected and inoculated into 2.5 ml of heart infusion broth (Oxoid) and incubated at 37°C with shaking for 4 hrs. The optical density of the culture was then adjusted with sterile saline to be the same as that of a 0.5 McFarland standard. The cultures were then diluted 1 in 100 in cation-adjusted Mueller-Hinton broth.

Within 15 min of the inoculum being prepared, each well of a microtiter tray was inoculated with 50 μl of a test bacterial strain. To prevent drying, each tray was sealed with plastic tape before incubation at 37°C for 20 hrs, after which wells were read visually for determination of the MIC.

Control microdilution susceptibility tests were performed with gentamicin and erythromycin for the Gram negative and Gram positive bacteria, respectively. All quality control results were within the acceptable CLSI limits for the test strains used.

Lysis of red blood cells

The hemolytic potency of peptides was evaluated by titration against human red blood cells (RBC) suspended in 70 μl of phosphate buffered saline (PBS). 20 μl of RBC, [(1–2) $\times 10^8$ cells], were incubated with 50 μl of peptides dissolved in PBS in a final volume of 70 μl for 6 min. at room temperature. Cells were centrifuged at 210 $\times g$ for 4 min. Supernatants were transferred to a 96 well plate and diluted 1 in 100 with PBS. Using a spectrophotometer, hemolysis was measured in triplicate at 412 nm. Data were normalized against RBC lysed with sodium dodecyl sulfate (SDS). Distilled water and PBS were used as a positive and negative control respectively. One HU (hemolytic unit) was defined as the minimum amount

of dissolved peptide required to induce hemolysis of 50% of the RBCs after 6 min. (Jackson et al. 2007).

Cytotoxicity of MLT for HeLa cells

The *in vitro* cytotoxicity of the peptide for cancer cells was quantitatively determined using the CellTiter-Blue[®] Reagent (CTB). In this method, lactate dehydrogenase (LDH) is released from damaged membrane cells into the culture medium. The amount of LDH is measured using a coupled enzymatic assay that results in the conversion of resazurin into resorufin, whereby fluorescence is directly related to the amount of LDH released. HeLa cells were seeded at a density of 100,000 cells per well in 12-well plates (Nunc) and cultured in OPTIMEM media supplemented with 10% FCS, 1U/ml penstrep, 1% glutamax and maintained in a humidified incubator at 37°C, 5% CO₂. HeLa cells were exposed to the MLT analogous peptides at 1 μM, 5 μM and 10 μM for 16 and 24 hr at 37°C. At each time point, 100 μl of conditioned media was transferred to a 96 well plate and subject to the CellTiter-Blue assay according to manufacturer's instruction. Fluorescence measurements were performed with an excitation at 560 nm and emission at 590 nm. The level of fluorescence produced is proportional to the number of lysed cells. 2 μl of 9% Triton-X 100 was added to control cells to reach maximum fluorescence intensity as a positive control and phosphate buffered saline (PBS) was used as a negative control.

Circular dichroism (CD) spectroscopy

CD spectroscopy was used for the structural studies of MLT and analogues, and to determine conformational changes upon addition of lipid vesicles. The CD spectra were recorded on a Chirascan-plus instrument (Applied Photophysics, Leatherhead, UK) between 190 and 260 nm using 0.1-cm path-length quartz cuvettes at 25°C as previously described [25]. Spectra were recorded using peptide concentrations of 10 μM in 10 mM Tris buffer (pH 7) at 25 °C.

Helix content of peptide was assumed to be directly proportional to mean residue ellipticity (MRE) at 222 nm $[\theta]_{222}$. One hundred percent helicity was calculated by using the formula $^{max}[\theta]_{222} = -40000 \times [(1-2.5/n)] + (100 \times T)$, where n is number of amino acid residues and T is temperature in °C (Scholtz et al. 1991). Percentage helicity was then calculated as $100 \times [\theta]_{222}/^{max}[\theta]_{222}$.

Results and Discussion

Peptide synthesis

We prepared three MLT peptides for this study (Figure 1). Substituting Pro-14 in MLT with a cysteine residue allowed a disulfide bond to form the dimer MLT. Air oxidation to form the disulfide bridge was slow. By using 2-DPDS and a subsequent thiolysis reaction the dimeric peptide formed rapidly. The free thiol group of the cysteine residue was oxidized by 2-DPDS and the resulting S-S-pyridyl side chain of Cys was attacked by the free -SH group of the MLT-P14C peptide to form an S-S bond (Gali et al. 2002; Maruyama et al. 1999). The peptide purity and yield are summarized in Table 1.

Dye release in model membranes

All three peptides exhibited lytic activity against model membranes, which is consistent with some previous observations that Pro residue can be mutated without altering the lytic activity against model membranes (Rapson et al. 2011). The lytic activity of MLT-P14C was slightly higher than that of native MLT and dimer-MLT-P14C in both model membrane systems (Figure 3A, 3B). However, induced leakage by native MLT and analogues in POPE/POPG (prokaryotic membrane mimic) was less than for POPC/Chol (eukaryotic mimic); the percentage of fluorescence in POPC/Chol reached 100% while in POPE/POPG only 80% was reached. The negative charge of POPE/POPG may have decreased leakage due to a stronger

interaction of peptides with the bilayer surface which consequently reduced the penetration in the bilayer hydrophobic core (Figure 3). The lytic activities of peptides against the two model membrane systems indicated that all three peptides should have lytic activity against erythrocytes and prokaryotic cell membranes.

Inhibition of bacterial growth

Native MLT displayed antimicrobial activity against all four of the bacteria tested. *S. aureus* was the most susceptible with an MIC of 3.9 μM , whilst MRSA was similarly susceptible with an MIC of 7.8 μM . The Gram negative bacteria, *E. coli* and *P. aeruginosa*, exhibited MICs of 15.6 and 31.3 μM , respectively (Table 2). In contrast to the lysis of model membranes, the MLT analogues were less potent and mostly unable to inhibit bacterial growth at the concentrations tested. The antimicrobial activity of the peptides was clearly dependent upon the presence of the native Pro-14 as evidenced by native MLT showing greater activity against bacteria. This result highlights that these model membranes do not reflect those of actual bacterial membranes. Bacteria also possess a cell wall which will affect how bacteria respond when exposed to compounds (Johnson et al. 2013). In Gram negative bacteria, a peptidoglycan layer is sandwiched between the cytoplasmic and outer membrane (Beveridge 1999) while in Gram positive bacteria the peptidoglycan layer is thicker (Popham 2013) and teichoic acid is present in the cell wall (Ward 1981).

Hemolytic activity

The hemolytic activity of peptides for human RBC was determined at a concentration range of 1 μM to 100 μM (Table 2). The dimer-MLT-P14C was more hemolytic than MLT-P14C and native MLT. At concentration of 1 μM , dimer-MLT caused 50% hemolysis, but 5 and 2.5 μM were required for the native and MLT-P14C, respectively.

The cytotoxicity of MLT and analogues for HeLa cells

The cytotoxic effect of MLT and analogue peptides was evaluated and compared using the CellTiter-Blue[®] Reagent assay. As shown in Figure 4 (24 hr), the cytotoxic efficacy of the native MLT, MLT-P14C, and dimer MLT-P14C against HeLa cells increased with increasing concentration of the peptide. The results demonstrate that all peptides were cytotoxic at concentrations of 5 and 10 μ M whilst MLT was shown to be more cytotoxic than the other peptides at the lowest concentration tested (1 μ M).

CD structural analysis

The CD spectra of native MLT and its analogues are shown in Figure 5 and helix contents are provided in Table 3. The results demonstrated that monomeric MLT and its analogue MLT-P14C adopted a random coil conformation as reported for native MLT (Ghosh et al. 1997), while dimer-MLT-P14C showed an α -helical conformation in aqueous solution (Fig. 5A). To our knowledge, this is the first demonstration that a random coil peptide can be assembled into a more structured peptide by chemical modification such as dimerization at an appropriate site. In the presence of POPC/Chol or POPE/POPG LUV (Fig. 5B, 5C), helicity of monomeric peptides increased significantly, indicating a lipid-induced conformational change.

We have examined the role of Pro-14 and thiol group of MLT analogues, MLT-P14C and dimeric MLT-P14C. We have confirmed that native MLT showed greater activity against bacteria compared with a mutant (P14C) monomer and dimer demonstrating the importance of “kink” structure produced by Pro-14. Importance of Pro residues in AMP peptides is demonstrated in the literature including our recent work on maculatin 1.1 (Fernandez et al. 2013). We showed that Pro-15 plays a central role in the membrane interaction of maculatin 1.1 by inducing a significant change in membrane order and affecting the ability of the bilayer to recover from structural changes induced by the binding and insertion of the peptide.

The dimeric peptide has 10 positive charges and yet was less active at bacterial cells and more active on RBC. Similarly, great differences are seen between the native and P14C peptides although they have the same charge and hydrophobicity. The effects of dimerization of an AMP peptide, Ctx-Ha, have recently been reported (Lorenzon et al. 2012). The difference in the biological activity of the monomer and dimeric peptide could not be explained by different mechanisms of action, secondary structure or proximity of the peptide chains and may be sequence dependent. Our result is similar in that dimerization affected the biological activity of the peptide, decreasing the antimicrobial activity and increasing the hemolytic activity.

All three MLT peptides had more lytic activity for neutral vesicles (POPC/Chol) than for anionic vesicles (POPG/POPE), which may be due to the negative charge of anionic model membranes reducing the peptides' lytic activity. Replacing Pro-14 with Cys caused little effect on the lytic activity for POPC/Chol, but dimerization decreased dye release with POPC/Chol considerably. The cytotoxicity of the MLT analogues for HeLa cells was less than that of native MLT at low concentrations. These findings suggest that Pro-14 is important for antimicrobial activity and cytotoxicity, but not for lysis of model membranes or hemolysis.

Conclusion

We have shown that native MLT is more active against bacteria compared with Pro-14 mutated non-native analogues, indicating that antimicrobial activity of the peptides depends on the presence of Pro at position 14. Similarly, Pro-14 was found to be important for cytotoxicity against HeLa cells. In stark contrast, Pro-14 was not essential either for lytic activity of model membranes or RBC membranes. Unlike native MLT and MLT-P14C, our

synthetic dimer MLT-P14C was more structured in the absence or presence of lipids and less active against bacterial membrane suggesting that a “pre-formed” structure of MLT dimer is not favorable for killing bacteria. However, the MLT dimer showed greater activity against RBC cells. We conclude that MLT peptides exhibit different activity on different cells and model membranes.

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Conflict of interest

The authors have no conflict of interest.

References

- Anaya-Lopez JL, Lopez-Meza JE, Ochoa-Zarzosa A (2013) Bacterial resistance to cationic antimicrobial peptides. *Critical Reviews in Microbiology* 39 (2):180-195
- Anderson RL, Davis S (1982) An organic phosphorus assay which avoids the use of hazardous perchloric acid. *Clinica Chimica Acta* 121 (1):111-116
- Asthana N, Yadav SP, Ghosh JK (2004) Dissection of antibacterial and toxic activity of melittin: a leucine zipper motif plays a crucial role in determining its hemolytic activity but not antibacterial activity. *The Journal of Biological Chemistry* 279 (53):55042-55050
- Balla MS, Bowie JH, Separovic F (2004) Solid-state NMR study of antimicrobial peptides from Australian frogs in phospholipid membranes. *European Biophysics Journal* 33 (2):109-116
- Beveridge TJ (1999) Structures of Gram-negative cell walls and their derived membrane vesicles. *Journal of Bacteriology* 181:4725-4733
- Burton MG, Huang QM, Hossain MA, Wade JD, Clayton AHA, Gee ML (2013) Long-Time-Scale Interaction Dynamics between a Model Antimicrobial Peptide and Giant Unilamellar Vesicles. *Langmuir : the ACS journal of surfaces and colloids* 29 (47):14613-14621
- Dempsey CE, Bazzo R, Harvey TS, Syperek I, Boheim G, Campbell ID (1991) Contribution of proline-14 to the structure and actions of melittin. *FEBS letters* 281 (1-2):240-244
- Fernandez DI, Lee T-H, Sani M-A, Aguilar M-I, Separovic F (2013) Proline Facilitates Membrane Insertion of the Antimicrobial Peptide Maculatin 1.1 via Surface Indentation and Subsequent Lipid Disordering. *Biophysical journal* 104 (7):1495-1507
- Ferre R, Melo MN, Correia AD, Feliu L, Bardaji E, Planas M, Castanho M (2009) Synergistic effects of the membrane actions of cecropin-melittin antimicrobial hybrid peptide BP100. *Biophysical journal* 96 (5):1815-1827
- Gajski G, Garaj-Vrhovac V (2013) Melittin: A lytic peptide with anticancer properties. *Environmental toxicology and pharmacology* 36 (2):697-705
- Gali H, Sieckman GL, Hoffman TJ, Owen NK, Mazuru DG, Forte LR, Volkert WA (2002) Chemical Synthesis of Escherichia Coli STh Analogues by Regioselective Disulfide Bond Formation: Biological Evaluation of an ¹¹¹In-DOTA-Phe19-STh Analogue for Specific Targeting of Human Colon Cancers. *Bioconjugate chemistry* 13 (2):224-231
- Gehman J, Luc F, Hall K, Lee T-H, Boland M, Pukala T, Bowie J, Aguilar M-I, Separovic F (2008) Effect of Antimicrobial Peptides from Australian Tree Frogs on Anionic Phospholipid Membranes. *Biochemistry* 47 (33):8557-8565
- Maróti G1, Kereszt A, Kondorosi E, Mergaert P. (2011) Natural roles of antimicrobial peptides in microbes, plants and animals. *Research in Microbiology* 162 (4):363-374
- Ghosh AK, Rukmini R, Chattopadhyay A (1997) Modulation of Tryptophan Environment in Membrane-Bound Melittin by Negatively Charged Phospholipids: Implications in Membrane Organization and Function. *Biochemistry* 36 (47):14291-14305.
- Hall K, Lee TH, Aguilar MI (2011) The role of electrostatic interactions in the membrane binding of melittin. *Journal of Molecular Recognition* 24 (1):108-118
- Heerklotz H, Seelig J (2007) Leakage and lysis of lipid membranes induced by the lipopeptide surfactin. *European Biophysics Journal : EBJ* 36 (4-5):305-314
- Henriksen JR, Etzerodt T, Gjetting T, Andresen TL (2014) Side chain hydrophobicity modulates therapeutic activity and membrane selectivity of antimicrobial peptide mastoparan-X. *PLoS one* 9 (3):e91007
- Hincha DK, Crowe JH (1996) The lytic activity of the bee venom peptide melittin is strongly reduced by the presence of negatively charged phospholipids or chloroplast galactolipids in the membranes of phosphatidylcholine large unilamellar vesicles. *Biochimica et Biophysica Acta* 1284 (2):162-170
- Hyun-Ji C, Jeong-Han K, Kwan-Kyu P, Jung-Yoon C, Yoon-Yub P, Yong-Suk M, Il-Kyung C, Hyeun-Wook C, Cheorl-Ho K, Yung Hyun C, Wun-Jae K, Sung-Kwon M, Young-Chae C (2013) Comparative

- proteome analysis of Tumor necrosis factor α -stimulated human Vascular Smooth Muscle Cells in response to melittin. *Proteome Science* 11:20.
- Izadpanah A, Gallo RL (2005) Antimicrobial peptides. *Journal of the American Academy of Dermatology* 52 (3 Pt 1):381-390
- Jackson KE, Spielmann T, Hanssen E, Adisa A, Separovic F, Dixon MW, Trenholme KR, Hawthorne PL, Gardiner DL, Gilberger T, Tilley L (2007) Selective permeabilization of the host cell membrane of *Plasmodium falciparum*-infected red blood cells with streptolysin O and equinatoxin II. *Biochemical Journal* 403 (1):167-175
- John E, Jähnig F. (1993) A synthetic analogue of melittin aggregates in large oligomers. *Biophysical Journal* 63 (6):1536
- Johnson JW, Fisher JF, Mobashery S (2013) Bacterial cell-wall recycling. *Annals Of The New York Academy Of Sciences* 1277:54-75
- Juba M, Porter D, Dean S, Gillmor S, Bishop B (2013) Characterization and performance of short cationic antimicrobial peptide isomers. *Peptide Science* 100 (4):387-401
- Khatun UL, Mukhopadhyay C (2013) Interaction of bee venom toxin melittin with ganglioside GM1 bicelle. *Biophysical Chemistry* 180–181 (0):66-75
- Klocek G, Seelig J (2008) Melittin Interaction with Sulfated Cell Surface Sugars. *Biochemistry* 47 (9):2841-2849
- Lad MD, Birembaut F, Clifton LA, Frazier RA, Webster JR, Green RJ (2007) Antimicrobial peptide-lipid binding interactions and binding selectivity. *Biophysical journal* 92 (10):3575-3586
- Lam YH, Morton J, Separovic F (2002) Solid-state NMR conformational studies of a melittin-inhibitor complex. *European Biophysics Journal* 31 (5):383
- Lam YH, Wassall SR, Morton CJ, Smith R, Separovic F (2001) Solid-State NMR Structure Determination of Melittin in a Lipid Environment. *Biophysical journal* 81:2752-2761
- Lee J, Lee D, Choi H, Kim HH, Kim H, Hwang JS, Lee DG, Kim JI (2014) Synthesis and antimicrobial activity of cysteine-free coprisin nonapeptides. *Biochemical and biophysical research communications* 443(2):483-8
- Lee TH, Heng C, Swann MJ, Gehman JD, Separovic F, Aguilar MI (2010) Real-time quantitative analysis of lipid disordering by aurein 1.2 during membrane adsorption, destabilisation and lysis. *Biochimica et Biophysica Acta - Biomembranes* 1798 (10):1977-1986
- Lorenzon EN, Cespedes GF, Vicente EF, Nogueira LG, Bauab TM, Castro MS, Cilli EM (2012) Effects of dimerization on the structure and biological activity of antimicrobial peptide Ctx-Ha. *Antimicrobial agents and chemotherapy* 56 (6):3004-3010
- Maruyama K, Nagasawa H, Suzuki A (1999) 2,2'-Bispyridyl disulfide rapidly induces intramolecular disulfide bonds in peptides. *Peptides* 20 (7):881-884
- Ningsih Z, Hossain MA, Wade JD, Clayton AH, Gee ML (2012) Slow insertion kinetics during interaction of a model antimicrobial peptide with unilamellar phospholipid vesicles. *Langmuir : the ACS journal of surfaces and colloids* 28 (4):2217-2224
- Popham DL (2013) Visualizing the production and arrangement of peptidoglycan in Gram-positive cells. *Molecular Microbiology* 88 (4):645-649
- Raghuraman H, Chattopadhyay A (2004) Interaction of melittin with membrane cholesterol: a fluorescence approach. *Biophysical journal* 87 (4):2419-2432
- Raghuraman H, Chattopadhyay A (2007) Orientation and dynamics of melittin in membranes of varying composition utilizing NBD fluorescence. *Biophysical journal* 92 (4):1271-1283
- Rapson AC, Hossain MA, Wade JD, Nice EC, Smith TA, Clayton AH, Gee ML (2011) Structural dynamics of a lytic peptide interacting with a supported lipid bilayer. *Biophysical journal* 100 (5):1353-1361
- Reddy KV, Yedery RD, Aranha C (2004) Antimicrobial peptides: premises and promises. *Int J Antimicrob Agents* 24 (6):536-547
- Rivett DE, Kirkpatrick A, Hewish DR, Reilly W, Werkmeister JA (1996) Dimerization of truncated melittin analogues results in cytolytic peptides. *Biochemical Journal* 316 (Pt 2):525-529

- Sani MA, Whitwell TC, Gehman JD, Robins-Browne RM, Pantarat N, Attard TJ, Reynolds EC, O'Brien-Simpson NM, Separovic F (2013) Maculatin 1.1 Disrupts Staphylococcus aureus Lipid Membranes via a Pore Mechanism. *Antimicrobial agents and chemotherapy* 57 (8):3593-3600
- Scholtz JM, Qian H, York EJ, Stewart JM, Baldwin RL (1991) Parameters of helix-coil transition theory for alanine-based peptides of varying chain lengths in water. *Biopolymers* 31 (13):1463-1470
- Schubert D, Pappert G, Boss K (1985) Does Dimeric Melittin Occur in Aqueous Solutions? *Biophysical journal* 48 (2):327-329
- Sessa G, Freer JH, Colacicco G, Weissman G (1969) INTERACTION OF A LYTIC POLYPEPTIDE, MELITTIN, WITH LIPID MEMBRANE SYSTEMS. *Journal of Biological Chemistry* 244 (13):3575-3582
- Takei J, Reményi A, Clarke AR, Dempsey CE (1998) Self-Association of Disulfide-Dimerized Melittin Analogues†. *Biochemistry* 37 (16):5699-5708
- Terwilliger TC, Eisenberg D (1982) The structure of melittin. II. Interpretation of the structure. *Journal of Biological Chemistry* 257 (11):6016-6022
- Tomoyoshi T, Fumimasa N, Yasunori Y, Yohko T-T, Michio H, Kingo T (2013) Multiple membrane interactions and versatile vesicle deformations elicited by melittin. *Toxins* 5 (4):637-664
- Tosteson MT, Levy JJ, Caporale LH, Rosenblatt M, Tosteson DC (1987) Solid-phase synthesis of melittin: purification and functional characterization. *Biochemistry* 26 (21):6627-6631
- Wade JD, Lin F, Hossain MA, Dawson R (2012) Chemical synthesis and biological evaluation of an antimicrobial peptide gonococcal growth inhibitor. *Amino Acids* 43 (6):2279-2283
- Ward JB (1981) Teichoic and teichuronic acids: biosynthesis, assembly, and location. *Microbiological Reviews* 45 (2):211-243
- Zhao Z, Rolli H, Schneider CH (1995) Immunogenicity of dinitrocarboxyphenylated melittin: the influence of C-terminal chain shortening, N-terminal substitution and prolin insertion at positions 5 and 10. *Journal of Peptide Science* 1 (2):140-148

Table 1. Purity and yield of MLT analogues

Peptide	%Purity	% yield
MLT	96.6	22 ^a
MLT-P14C	97.2	18 ^a
Dimer-MLT-P14C	98.4	53 ^b

^a Yield calculated from the crude starting material

^b Yield calculated from the purified MLT-P14C as starting material

Table 2. MIC and hemolytic concentration* of peptides

Bacterial strain	MIC (μM)		
	MLT	MLT-P14C	Dimer- MLT-P14C
<i>S. aureus</i> ATCC 29213	3.9	62.5	>62.5
MRSA ATCC 43300	7.8	62.5	>62.5
<i>E. coli</i> ATCC 25922	15.6	>125	>62.5
<i>P. aeruginosa</i> ATCC 27853	31.3	>125	>62.5
Hemolytic concentration	5	2.5	1

* Mean of three separate determinations

Table 3. Helical content of peptides

Buffer/Lipid system	% Helicity		
	MLT	MLT-P14C	Dimer-MLT-P14C
Buffer	10	15	27
POPC/Chol	18	44	38
POPE/POPG	43	49	38

Figure Legends

Figure 1. The amino acid sequences and RP-HPLC and MALDI TOF MS traces of: (A) MLT, (B) MLT-P14C, and (C) dimer-MLT-P14C.

Figure 2. Schematic representation of the assembly of dimeric MLT-P14C. 2,2-dithiodipyridine (DPDS), Trifluoroacetic acid (TFA).

Figure 3. Dye leakage assay of MLT, MLT-P14C, and dimer-MLT-P14C for (A) POPC/Chol, and (B) POPE/POPG.

Figure 4. Cytotoxicity of MLT, MLT-P14C and dimer-MLT-P14C against HeLa cells. Cell survival rates were determined by using the CTB assay after 24 hr incubation with each test compound.

Figure 5. CD spectra of MLT, MLT-P14C, and dimer-MLT-P14C in: (A) buffer, (B) POPC/Chol, and (C) POPE/POPG with lipid: peptide ratio of 25:1, and peptide concentration of 10 μ M. Measurements were recorded at room temperature. mean residue ellipticity (MRE).

Figure 1

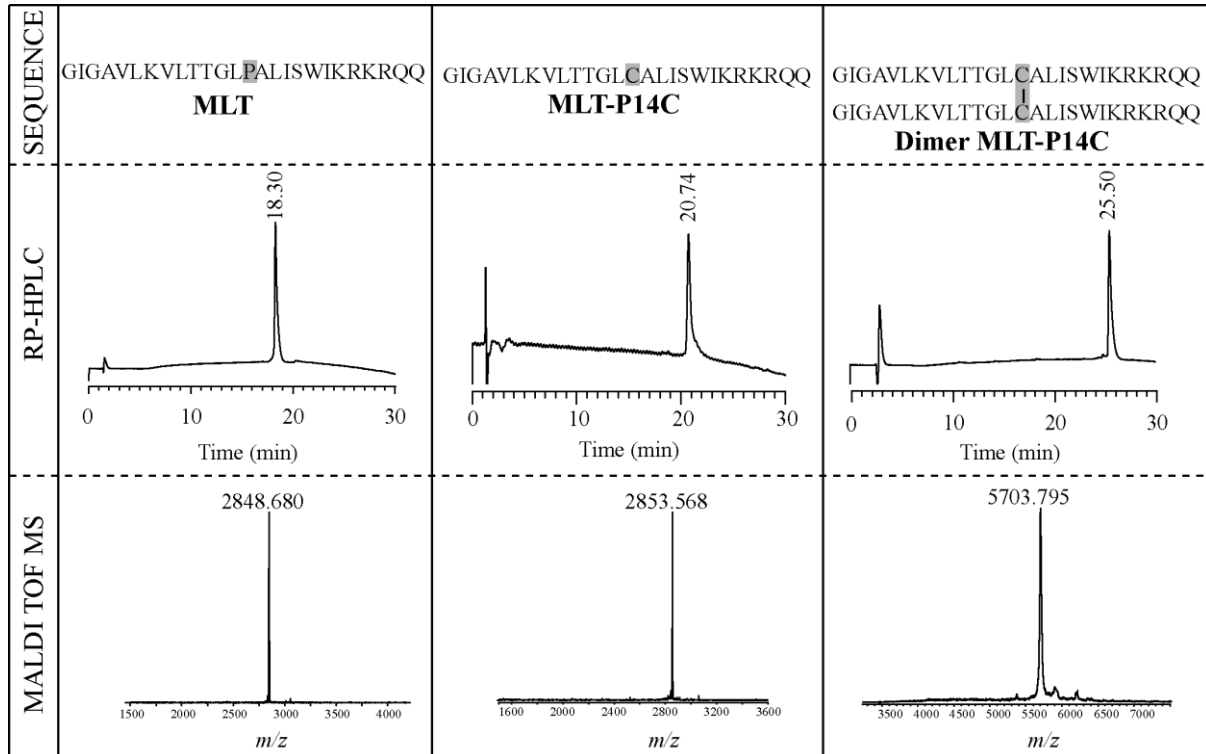


Figure 2

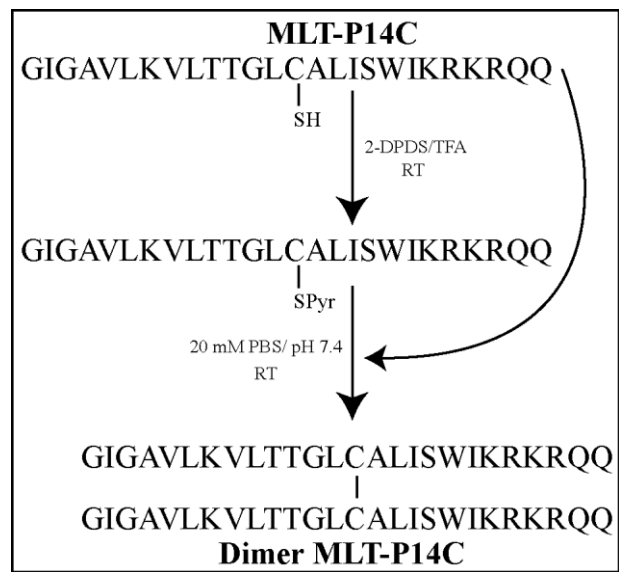


Figure 3

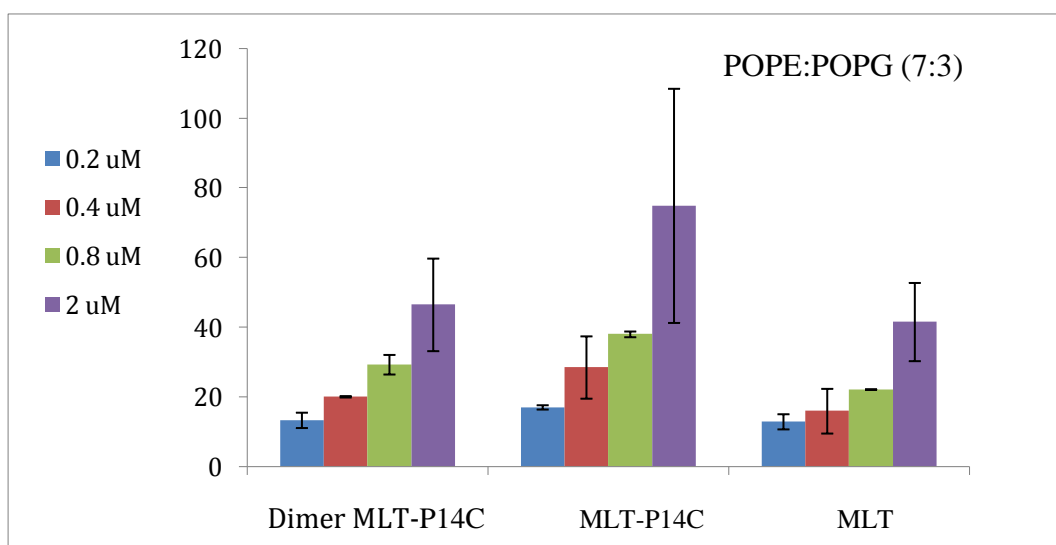
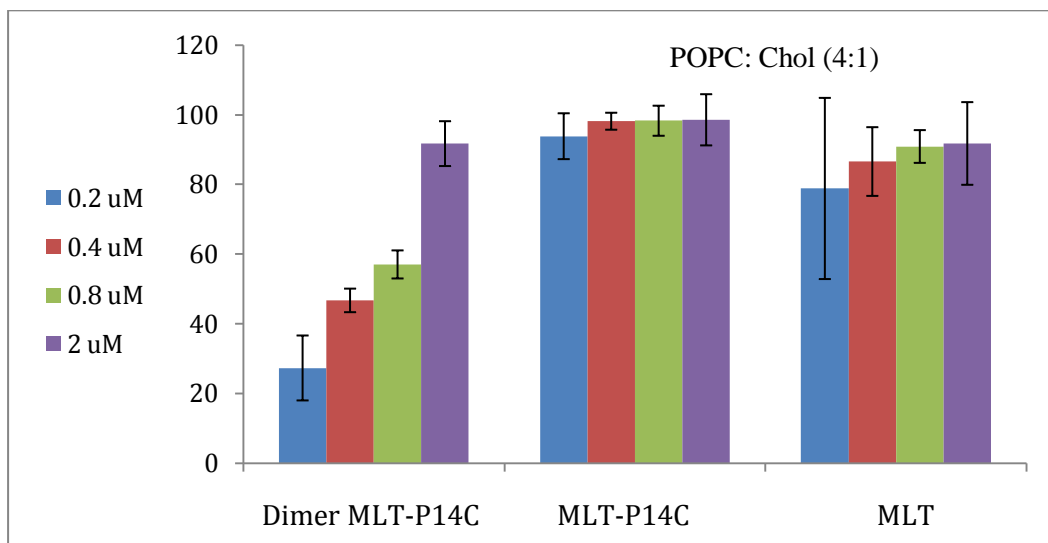


Figure 4

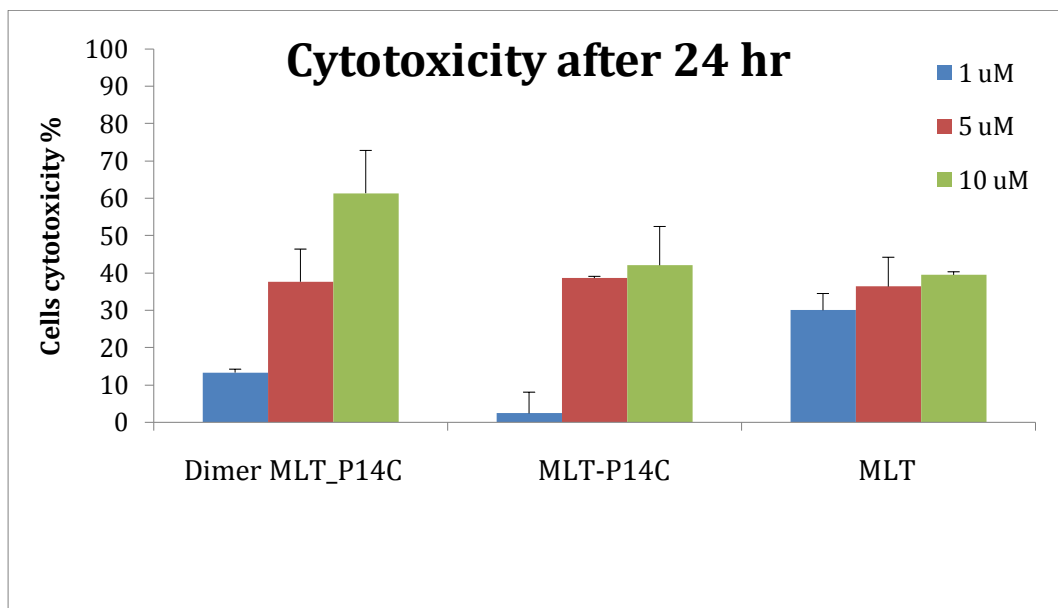


Figure 5

