Amino Acids (2014) 46:2759–2766
DOI 10.1007/s00726-014-1833-9

ORIGINAL ARTICLE

Melittin peptides exhibit different activity on different cells and model membranes

Elaheh Jamasbi · Steven Batinovic · Robyn A. Sharples · Marc-Antoine Sani · Roy Michael Robins-Browne · John D. Wade · Frances Separovic · Mohammed Akhter Hossain

Received: 4 July 2014 / Accepted: 26 August 2014 / Published online: 9 September 2014

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 analogs 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.

Keywords Melittin · Antimicrobial peptide · Cytotoxicity · Hemolysis · Dye leakage

E. Jamasbi · M.-A. Sani · J. D. Wade · F. Separovic (✉) · M. A. Hossain (✉)
School of Chemistry, Bio21 Institute, University of Melbourne, Melbourne, VIC 3010, Australia
e-mail: fs@unimelb.edu.au

M. A. Hossain
e-mail: akhter.hossain@unimelb.edu.au

S. Batinovic · R. A. Sharples
Department of Biochemistry and Molecular Biology, Bio21 Institute, University of Melbourne, Melbourne, VIC 3010, Australia

R. M. Robins-Browne
Department of Microbiology and Immunology, Peter Doherty Institute, University of Melbourne, Melbourne, VIC 3010, Australia

R. M. Robins-Browne
Murdoch Childrens Research Institute, Royal Children's Hospital, Parkville, VIC 3052, Australia

J. D. Wade · M. A. Hossain
The Florey Institute of Neuroscience and Mental Health, University of Melbourne, Melbourne, VIC 3010, Australia

Introduction

Antimicrobial peptides (AMP) are found in plants, insects and human cells (Maróti et al. 2011; Reddy et al. 2004; Lee et al. 2014; Gehman et al. 2008; Balla 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). AMP 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 (Fig. 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. 2001, 2002; Hincha and Crowe 1996; Raghuraman and Chattopadhyay 2004, 2007; Sessa et al. 1969; 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 studied a dimeric MLT that contains two fulllength MLT monomers (1–26) and chose 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 in 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 Jähnig 1993) and increased hemolytic activity in comparison to native MLT (John and Jähnig 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 analog MLT-P14C as well as with native MLT (Fig. 1).

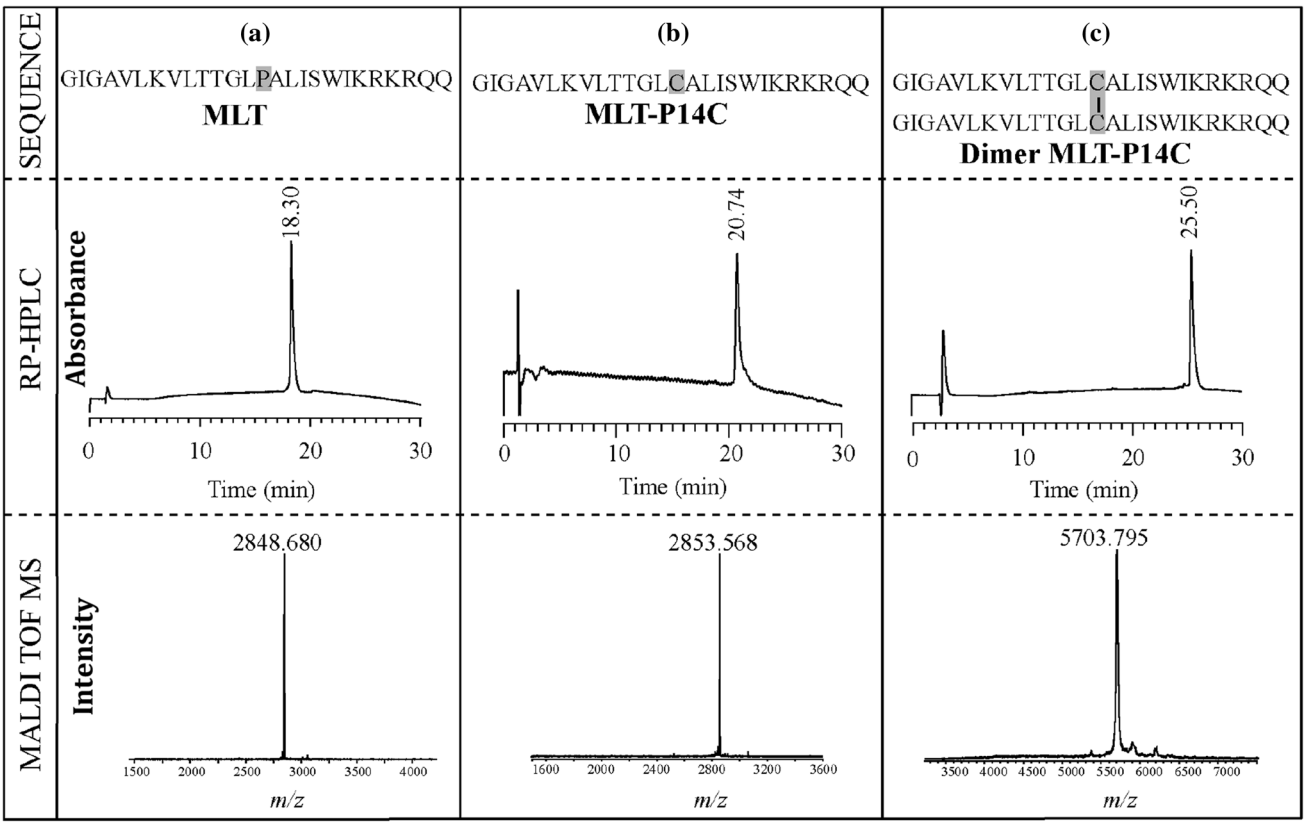

Fig. 1 The amino acid sequences and RP-HPLC and MALDI TOF MS traces of: **a** MLT, **b** MLT-P14C, and **c** dimer-MLT-P14C

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

Melittin and its cysteine analogs, MLT-P14C (Fig. 1), were synthesized using the Fmoc solid phase method (Tosteson et al. 1987; Wade et al. 2012). Crude peptides were analyzed using RP-HPLC on a Phenomenex C4 column (particle size 5 µm, 4.6 × 150 mm), with a gradient of 10–90 % of 0.1 % trifluoroacetic acid (TFA) in acetonitrile (ACN) and 0.1 % TFA in H_2O 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 H_2O 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 h while monitored by analytical RP-HPLC. After 48 h 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 (Fig. 2). The reaction was completed in 30 min with a very high yield. MALDI TOF mass spectra and HPLC profiles for all three purified peptides are shown in Fig. 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 equiv. 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.

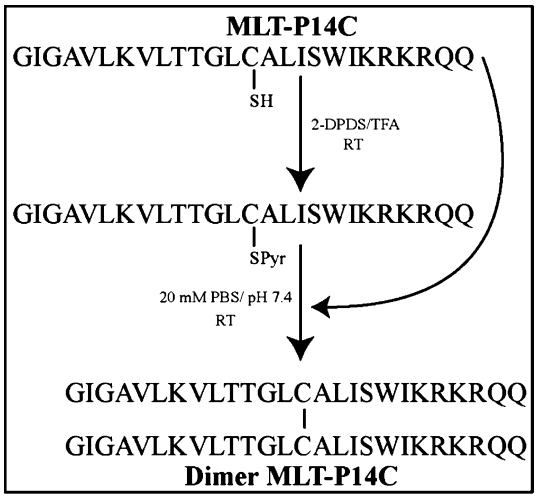

Fig. 2 Schematic representation of the assembly of dimeric MLT-P14C. *DPDS* 2,2-dithiodipyridine, *TFA* trifluoroacetic acid

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-µm pore-size polycarbonate filter to prepare large unilamellar vesicles (LUV). Excess dye was separated by gel filtration on a Sephadex G-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:

$$\% \text{ leakage} = [(I - I_0) \times 100\ \%]/(I_M - I_0)$$

where *I* 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 µl volumes into the wells of 96-well plates. For each test concentration,

triplicate wells were prepared and were then diluted two-fold in cation-adjusted Mueller–Hinton broth (Oxoid, Basingstoke, UK). The final concentrations of each test compound ranged from 125 to 0.24 μM, with the exception of dimer-MLT-P14C, which ranged from 62.5 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 h. 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 h, 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) × 10^8 cells] was 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×*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, 1 U/ml penstrep, 1 % glutamax and maintained in a humidified incubator at 37 °C, 5 % CO_2. HeLa cells were exposed to the MLT analogous peptides at 1, 5 and 10 μM for 16 and 24 h at 37 °C. At each time point, 100 μl of conditioned media was transferred to a 96-well plate and subjected 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

Circular dichroism spectroscopy was used for the structural studies of MLT and analogs, 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 (Khatun and Mukhopadhyay 2013). 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 using the formula $^{\max}[\theta]_{222} = -40{,}000 \times [(1 - 2.5/n)] + (100 \times T)$, 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 (Fig. 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. 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.

Table 1 Purity and yield of MLT analogs

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

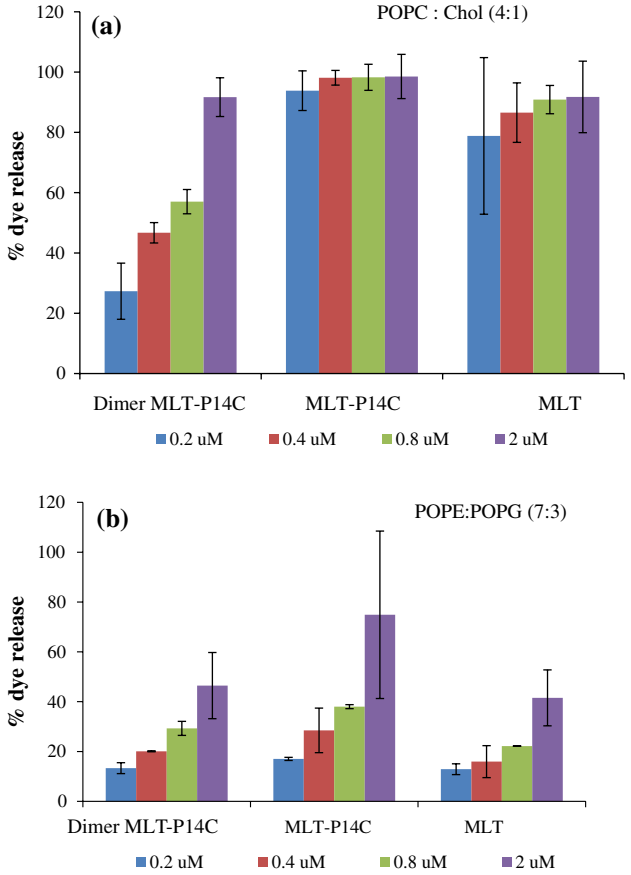

Fig. 3 Dye leakage assay of MLT, MLT-P14C, and dimer-MLT-P14C for **a** POPC/Chol, and **b** POPE/POPG

Table 2 MIC and hemolytic concentration* of peptides

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

* Mean of three separate determinations

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 (Fig. 3a, b). However, induced leakage by native MLT and analogs in POPE/POPG (prokaryotic membrane mimic) was less than in 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 (Fig. 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 analogs 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 1–100 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.

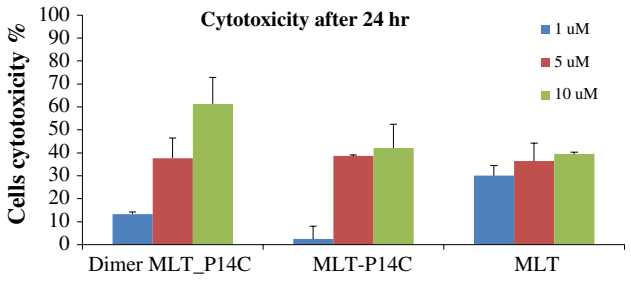

Fig. 4 Cytotoxicity of MLT, MLT-P14C and dimer-MLT-P14C against HeLa cells. Cell survival rates were determined by using the CTB assay after 24 h incubation with each test compound

The cytotoxicity of MLT and analogs for HeLa cells

The cytotoxic effect of MLT and analog peptides was evaluated and compared using the CellTiter-Blue®Reagent assay. As shown in Fig. 4 (24 h), 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 μM whilst MLT was shown to be more cytotoxic than the other peptides at the lowest concentration tested (1 μM).

CD structural analysis

The CD spectra of native MLT and its analogs are shown in Fig. 5 and helix contents are provided in Table 3. The results demonstrated that monomeric MLT and its analog 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, c), 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 analogs, 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 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.

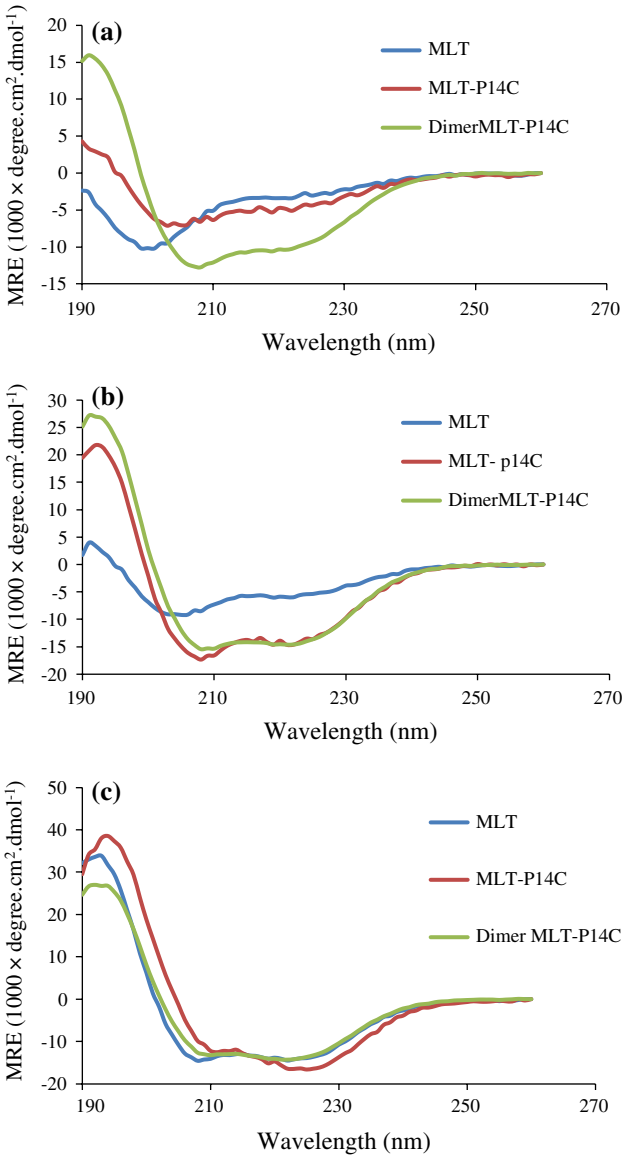

Fig. 5 CD spectra of MLT, MLT-P14C, and dimer-MLT-P14C in: **a** buffer, **b** POPC/Chol, and **c** POPE/POPG with lipid:peptide ratio of 25:1, and peptide concentration of 10 μM. Measurements were recorded at room temperature. *MRE* mean residue ellipticity

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

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 AMP, 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 peptide 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 analogs 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 analogs, 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 "preformed" 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.

Acknowledgments We acknowledge partial support of the studies undertaken in the authors' laboratory by the Australian Research Council (DP150103522) to MAH and JDW. Research at the FNI was supported by the Victorian Government's Operational Infrastructure Support Program. EJ thanks the University of Melbourne for an MIRS.

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. Crit Rev Microbiol 39(2):180–195

Anderson RL, Davis S (1982) An organic phosphorus assay which avoids the use of hazardous perchloric acid. Clin Chim 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. J Biol Chem 279(53):55042–55050

Balla MS, Bowie JH, Separovic F (2004) Solid-state NMR study of antimicrobial peptides from Australian frogs in phospholipid membranes. Eur Biophys J 33(2):109–116

Beveridge TJ (1999) Structures of Gram-negative cell walls and their derived membrane vesicles. J Bacteriol 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 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 Lett 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. Biophys J 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. Biophys J 96(5):1815–1827

Gajski G, Garaj-Vrhovac V (2013) Melittin: a lytic peptide with anticancer properties. Environ Toxicol Pharmacol 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. Bioconjug Chem 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

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. J Mol Recognit 24(1):108–118

Heerklotz H, Seelig J (2007) Leakage and lysis of lipid membranes induced by the lipopeptide surfactin. Eur Biophy J 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. Biochim Biophys 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 Sci 11:20

Izadpanah A, Gallo RL (2005) Antimicrobial peptides. J Am Acad Dermatol 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. Biochem J 403(1):167–175

John E, Jähnig F (1993) A synthetic analogue of melittin aggregates in large oligomers. Biophys J 63(6):1536

Johnson JW, Fisher JF, Mobashery S (2013) Bacterial cell-wall recycling. Ann NY Acad Sci 1277:54–75

Juba M, Porter D, Dean S, Gillmor S, Bishop B (2013) Characterization and performance of short cationic antimicrobial peptide isomers. Pept Sci 100(4):387–401

Khatun UL, Mukhopadhyay C (2013) Interaction of bee venom toxin melittin with ganglioside GM1 bicelle. Biophys Chem 180–181:66–75

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. Biophys J 92(10):3575–3586

Lam YH, Wassall SR, Morton CJ, Smith R, Separovic F (2001) Solid-state NMR structure determination of melittin in a lipid environment. Biophys J 81:2752–2761

Lam YH, Morton J, Separovic F (2002) Solid-state NMR conformational studies of a melittin-inhibitor complex. Eur Biophys J 31(5):383

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. Biochim Biophys Acta Biomembr 1798(10):1977–1986

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. Biochem Biophys Res Commun 443(2):483–488

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. Antimicrob Agents Chemother 56(6):3004–3010

Maróti G, Kereszt A, Kondorosi E, Mergaert P (2011) Natural roles of antimicrobial peptides in microbes, plants and animals. Res Microbiol 162(4):363–374

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 28(4):2217–2224

Popham DL (2013) Visualizing the production and arrangement of peptidoglycan in Gram-positive cells. Mol Microbiol 88(4):645–649

Raghuraman H, Chattopadhyay A (2004) Interaction of melittin with membrane cholesterol: a fluorescence approach. Biophys J 87(4):2419–2432

Raghuraman H, Chattopadhyay A (2007) Orientation and dynamics of melittin in membranes of varying composition utilizing NBD fluorescence. Biophys J 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. Biophys J 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. Biochem J 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. Antimicrob Agents Chemother 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? Biophys J 48(2):327–329

Sessa G, Freer JH, Colacicc G, Weissman G (1969) Interaction of a lytic polypeptide, melittin, with lipid membrane systems. J Biol Chem 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. J Biol Chem 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. Microbiol Rev 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. J Pept Sci 1(2):140–148